7th Principal Investigators Meeting

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Abstracts

Proteomics of Hepatic Neoplasia

Ruth Angeletti Albert Einstein College of Medicine, Bronx, New York

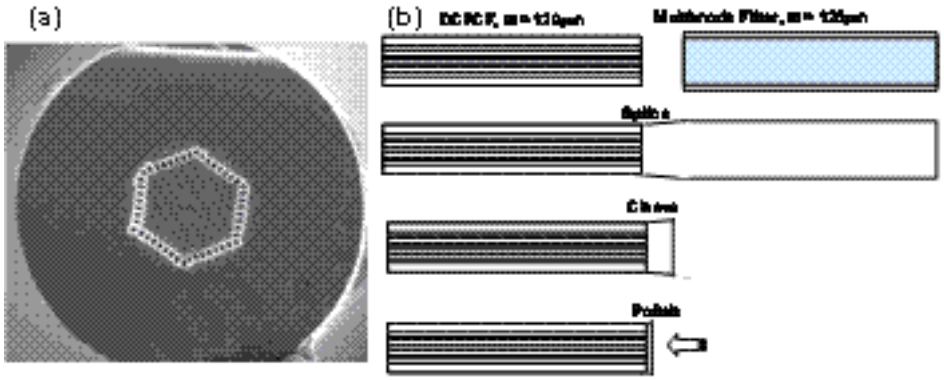
This applications project focuses on discovery of targets and biomarkers in three stages of the Solt-Farber rat model of hepatic neoplasia, which has well-characterized, synchronously developed stages. For analysis of tissue, laser-capture microdissection is being used to afford discrimination among hyperplastic cells and nodules, and surrounding stromal tissues. Both a global analysis of proteins and a targeted, tubulin proteomics approach are being used for the studies of laser-captured tissue. While the amount of protein for analysis of the serum from these animals is much larger, the challenge of dynamic range is an issue for each subproject. Tubulin isotypes may change during the neoplastic process, and the proteins on their cytoskeletal architecture (signaling, motor, etc.) may also be altered and provide mechanistic insights. Separation of the tubulin polypeptides from the associated proteins by a modified procedure permits the direct analysis of the tubulin isotypes by nano LC-MS, and the associated proteins, after tryptic digestion, by nano-LC-MALDI-TOF/TOF. Global analysis of the proteins in the tissue samples is limited by the presence of abundant proteins and by the losses incurred during fractionations of small amounts of protein. A selective approach to analyze only one-two peptides from each protein limits the impact of the most abundant proteins. Novel, open-source software has also been developed to analyze the MS signals from these experiments.

New Fiberoptic Technology for the Detection of Biomarkers in Tissue

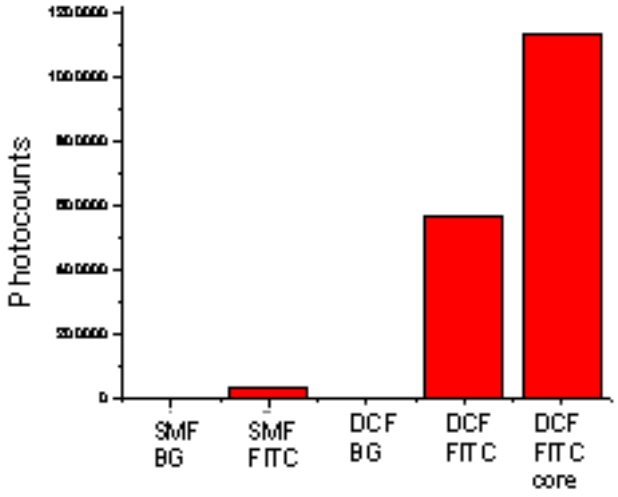
James Baker, Jr., Jing Yong Yee, Thommy Thomas, Ted Norris Michigan Nanotechnology Institute for Biology and the Medical Sciences and Michigan Center for Ultrafast Optics, University of Michigan, Ann Arbor, Michigan

Conventional fluorescence quantification in tissues and whole body fluorescence imaging techniques do not provide accurate quantitative information on the distribution of fluorescently tagged molecules in tissues. Owing to the limited tissue penetration of light, these methods also lack sensitivity for detection of biomarkers using fluorescence labels. We have developed a two-photon optical fiber fluorescence (TPOFF) probe as a minimally invasive technique for quantifying fluorescence in solid tumors in vitro and in vivo on a real-time basis [1,2,3]. In these studies, we used a single mode optical fiber

(SMF) through which femtosecond laser pulses were delivered into a tumor, which enabled us to measure low micromolar concentrations of targeted fluorescent nanoparticles. For detection of nanomolar levels of fluorescently labeled biomarkers, it is essential that a more sensitive TPOFF device be developed. We now are attempting to adapt double-clad optical fibers that keep high excitation rates by propagating ultrashort laser pulses down an inner single mode core, while improving the collection efficiency by using a high-aperture multimode outer core confined with a second clad.



The first fiber we investigated is a double-clad photonic crystal fiber (DCPCF, the end surface shown in Figure 1a). Air holes provide the cladding and need to be sealed before this fiber can be used for in vivo measurements to avoid entry of liquid from biological samples. This would reduce the light confinement capability of the fiber and introduce artifacts in the measurements. We developed a solution to this by splicing a multimode fiber to the end of the DCPCF, then cleaving off all but a thin piece of the multimode fiber (Figure 1b). It is critical to keep the thickness of the multimode fiber tip down to a few microns to optimize the fluorescence collection efficiency.



We also used a solid, double-clad fiber (DCF) to avoid the air hole problems. The solid DCF used has an aperture of 0.46, which is smaller than that of a DCPCF. Although it is not as efficient as the DCPCF, it still provides more than an order of magnitude improvement over traditional single-clad fibers. We have tested the two-photon

fluorescence detection efficiency of using this DCF using FITC dye solutions. In comparison with the best commercially available single-mode optical fiber (SMF), the detection efficiency has been improved by a factor of about 12, if the total output power from the DCF is considered as the excitation power, and more than 20 if the inner core power of the DCF is considered as useful excitation power for two-photon fluorescence excitation (Figure 2). The efficiency of this DCF is therefore about two times lower when compared to the 40 times enhancement achieved with the DCPCF, but lacks the technical probes of the holes. In summary, we have developed two options for optical detection of biomarkers that should have detection capabilities within the range needed for biological samples.

1 Ye J.Y., Myaing M.T., Norris T.B., et al. Biosensing Based on Two-Photon Fluorescence Measurements Through Optical Fibers. *Optical Letters* 27:1412, 2002.
2 Thomas T.P., Myaing M.T., Ye J.Y., et al. Detection and Analysis of Tumor Fluorescence Using a Two-Photon Optical Fiber Probe. *Biophys* J 86:3959, 2004.
3 Thomas T.P., Ye J.Y., Yang C., et al. Tissue Distribution and Real-Time Fluorescence Measurement of a Tumor-Targeted Nanodevice by a Two Photon Optical Fiber Fluorescence Probe. Proceedings of the SPIE 6095:60950Q, 2006.
4 Ye et al., Optical Letters 27:1412, 2002; Thomas et al., *Biophys* J 86:3959, 2004; Thomas et al., Proceedings of the SPIE, Vol 6095, 2006.

Integrated Top-Down/Bottom-Up Comprehensive Proteomics

Brian Balgley Calibrant Biosystems, Gaithersburg, Maryland

We have developed a prototype proteome instrument that is capable of capillary electrophoresis-based separation, multiplexed capillary reversed-phase liquid chromatography and interfacing between chromatography fraction deposition and matrix-assisted laser desorption/ionization (MALDI) target preparation. The first implementation of the instrument is focused on improving the degree of automation for interfacing multiplexed liquid chromatography to MALDI-MS without sacrificing the throughput and resolution achieved during chromatography separations. In addition to interfacing multiplexed liquid chromatography with MALDI-MS in an offline approach, we have further implemented an automated column-switching scheme for online coupling of dual chromatography separations with electrospray ionization (ESI)-MS. The position of the dual columns with respect to the orifice of MS is controlled by a stepping motor for directly analyzing eluted peptides and proteins using MS measurements.

Besides the development of an automated and high-throughput prototype proteome instrument, our collaborative research efforts with Professor Eric H. Baehrecke's laboratory at the Center for Biosystems Research, University of Maryland Biotechnology Institute, have contributed to further understanding of the regulation of programmed cell death at the proteome-wide level using the fruit fly Drosophila melanogaster as an

experimental model system. A total of 5,661 proteins are identified from stages just before and after the onset of cell death. Analyses of these data enable us to identify proteins from a number of interesting categories, including regulators of transcription, the apoptosis, autophagy, lysosomal, and ubiquitin proteasome degradation pathways and proteins involved in growth control. Several of the identified proteins, including the serine/threonine kinases, are not detected using whole-genome microarrays, providing support for the importance of such high-throughput proteomic technology. These kinases regulate cell cycle arrest and apoptosis, and, significantly, mutations in kinases prevent destruction of salivary glands [1].

In addition to analyzing the soluble fraction of cell lysates, we have further expanded and demonstrated our bioanalytical capability toward the analysis of complex membrane proteomes. Although proteomics technologies have made significant progress in the analysis of soluble proteins in recent years, membrane proteins have lagged behind and are typically underrepresented in datasets. However, the importance of membrane proteins in drug discovery cannot be overemphasized—membrane proteins currently account for approximately 70% of all known pharmaceutical drug targets. In this study, the membrane protein fraction of yeast cell lysates is extracted from pellets using a 2% sodium dodecyl sulfate (SDS) solution. A total of 10,088 distinct peptides are identified, leading to the identification of 2,084 distinct yeast proteins in the membrane fraction of yeast cell lysates using the combined top-down/bottom-up proteome platform. By using the trans-membrane hidden Markov model (http://www.cbs.dtu.dk/services/TMHMM), 624 yeast proteins are predicted to contain 3 or more transmembrane domains. We have identified 258 proteins with 3 or more predicted integral membrane domains from the pellets treated with SDS, corresponding to 40% membrane proteome coverage. It should be emphasized that these membrane proteins are identified with high confidence with a false-positive rate of less than 1%. By combining with the proteome results obtained from the soluble fraction, the collective analysis yields the identification of 3,523 distinct proteins, with an average of 4.5 fully tryptic peptides per protein. By comparison with the reported yeast proteome studies, our results present the most comprehensive analysis of the yeast proteome, including low-abundance membrane proteins [2].

1 Martin D.N., Balgley B.M., Chen J., et al. Proteomic Analysis of Steroid-Triggered Autophagic Programmed Cell Death in Drosophila. *Curr Biol*, in press.
2 Wang W., Guo T., Rudnick P.A., et al. Comprehensive Yeast Proteome Analysis Including the Identification of Low Abundance Membrane Proteins, manuscript in preparation.

Development of a High-Throughput Diagnostic Assay for Lung Cancer

Kathlynn Brown University of Texas Southwestern Medical Center, Dallas, Texas

Lung cancer is the largest cancer killer of both men and women. The correct histopathological diagnosis of a tumor is critical in determining the appropriate

treatment. However, precise classification of tumors remains a significant biomedical challenge. Furthermore, tumors of similar histology can have different clinical outcomes, stressing the need for more detailed molecular classifications. Our overall goal is to generate a panel of cell-specific molecules that can be used to classify tumor types and utilize these cancer-specific reagents in a high-throughput diagnostic assay. Using phage display technologies, my laboratory has developed methodologies to isolate peptides that bind to specific cell lines, even in the absence of knowledge of the cell surface receptor profile. By this method, we have identified cell-specific targeting peptides for five lung cancer cell lines and have determined their binding profile to a large panel of lung cancer cell lines. The isolated peptides display remarkable cell specificities and are able to discriminate between normal and cancerous cells, as well as different lung tumor cells. Furthermore, these peptides can be conjugated to fluorescent nanoparticles (quantum dots, Qdots) in order to address cellular binding. This fluorescent-based assay can be multiplexed so that multiple binding events can be examined on a single sample and is amenable to the use of fresh or fixed cells. Our approach solves two problems in the use of molecular markers for cancer diagnosis. First, we can generate cell-specific ligands rapidly with no prior information about the cell surface. In effect, ligand binding can be thought of as a unique biomarker. Second, we are developing a multiplexed binding assay that allows multiple binding events to be monitored simultaneously on a small number of cells. At the end of this project, we will have developed a novel diagnostic platform that can be expanded to clinical samples.

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

Han Cao BioNanomatrix, Philadelphia, Pennsylvania

We are developing a nanochip device for manipulating long genomic DNA for high-resolution (kilobase), whole-genome analysis of cancer biomarkers, such as gene amplifications, deletions, and translocations. These chromosome structural aberrations are strongly implicated in the process of malignant transformation and are important diagnostic, prognostic, and therapeutic indicators for many types of cancer. Although sequencing and PCR offer the ultimate (single-base) resolution for detecting and analyzing these anomalies, it is impractical for scanning the entire genome in a comprehensive, linear fashion. Techniques that rely on probing chromosomes, such as metaphase FISH, while providing a pan-genomic view, cannot resolve structures below the Mb range. By probing uncompressed interphase DNA, resolution can be improved, but spatial organization of the genome is lost, so multiplexed and quantitative information is difficult to obtain. By stretching out (linearizing) interphase DNA, using techniques such as “molecular combing” or “optical mapping,” it is possible to probe specific loci in a spatially significant way, and with resolutions in the kb range. However, techniques for mechanically linearizing DNA are inherently variable, leading to inconsistent stretching of molecules, which often cross over and retract upon themselves. This makes it difficult to standardize such techniques as high-throughput methods for the biomedical community.

We are developing an innovative alternative to mechanical stretching of DNA. We have

found that individual DNA molecules, because of the self-avoiding nature of the DNA polymer, will elongate and straighten in a consistent manner when streamed into confining nanometer-scale channels (nanochannels). We have used a novel nanoimprint lithography technique to reliably manufacture nanochannel structures in silicon chips and have demonstrated that DNA in these nanochannels can be visualized and their dimensions measured. We now ask the question, can we quantitatively interrogate this linearized DNA with locus-specific probes for the detection of chromosome structural aberrations associated with cancer? Our product, the nanochannel array chip, will comprise part of an integrated platform for the routine and standardized quantitative analysis of DNA structure that will enable archiving and cross-laboratory comparison of data.

Longitudinal Cancer-Specific Serum Protein Signatures

Songming Chen[1], Lin Li[2], Richard Hay[1], William Catalona[4], Ziding Feng[2], Robert Vessella[3], Brian B. Haab[1]

[1]Van Andel Research Institute, Grand Rapids, Michigan; [2F]red Hutchinson Cancer Research Center, Seattle, Washington; [3]University of Washington Medical Center, Seattle, Washington; [4]Northwestern University, Chicago, Illinois

Angiogenic factors in serum, such as VEGF, IL-8, are strongly related to cancer development and metastasis; however, they showed poor sensitivity and specificity as a biomarker used in cancer diagnosis or prognosis. This is mainly the result of the heterogeneity in the baseline levels of the marker proteins and heterogeneity in the tumors and patients. We have proposed that a biomarker discovery strategy using individualized thresholds, based on longitudinal measurements, would more precisely define abnormal levels for each individual. Multiplexed, specific detection of serum markers using antibody arrays makes a systematic exploration of this hypothesis possible. We are using the detection of prostate cancer recurrence as a model system. Serum samples have been assembled from 17 patients who had recurrence and 17 who did not, with up to 5 samples from each patient, from a pre-surgery sample up to the time of recurrence or no recurrence. We have developed array-based sandwich assays for 13 potential markers, representing pro- and anti-angiogenesis factors. The detection limit of the assay can be as low as 1 ng/mL with a CV less than 20%, and 500 pg/mL for PSA with a CV less than 20%. We are also using a two-color rolling-circle amplification method to measure the relative levels of 75 different cancer-associated proteins, and we are using a novel glycan-detection assay to measure the levels of specific glycans on multiple glycoproteins. Several approaches toward modeling the longitudinal changes of the markers are being pursued. We are comparing the sensitivity and specificity of cancer detection between the conventional single-timepoint method and the longitudinal strategies. By testing many markers, we can evaluate the generality and range of marker improvement using longitudinal information.

Baculoviral Production of Recombinant Human Variable Domain Antibodies for the Identification of Breast Cancer Antigens

Kevin Claffey University of Connecticut Health Center, Farmington, Connecticut

Immune-dependent responses are selective in defining non-self or aberrant antigen presentation. Unfortunately, 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 breast tumor antigens. This novel approach involves the construction of antibody variable region cDNA libraries, synthesis of recombinant VH monobodies, and identification of tumor antigens with a sensitive “antigen-trap” assay followed by LC-MS/MS protein identification. One key element of this process is to produce expression-ready cDNA libraries that can be shuttled into baculoviral vectors for efficient protein synthesis. These libraries are screened for orientation by multiplex PCR and in-frame by direct sequencing. The utility of this process provides correctly folded recombinant VH monobodies with a V5 epitope tag as well as a 6x histidine C-terminal domain for metal affinity purification. The purified recombinant VH proteins can identify tumor antigens from primary tumor extracts as well as on histological slides. The library synthesis and baculovirus shuttling platform is an effective way to shuttle cDNA sequences into a protein synthesis method suitable for biochemical, diagnostic, and therapeutic applications. Future directions will include screening additional antibody libraries, development of highly sensitive cancer antigen diagnostic screens, and plans for therapeutic assessment of human VH monobodies targeted to tumor antigens in vivo.

Targeted Antigen Delivery for Cancer Immunotherapy

Shoshana Frankenburg Sharett Institute of Oncology, Hadassah Hebrew University Medical Center, Jerusalem, Israel

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

The study will be based on two antigenic proteins derived from melanoma: the first is a hydrophilic recombinant gp100 protein, and the second is a multiepitope polypeptide that comprises three repeats of 4 HLA-A2 melanoma peptides derived from three different melanoma proteins. In order to allow and to improve topical transdermal delivery, the antigens will be genetically fused to potential carrier molecules. One of these is E. coli heat-labile enterotoxin, a molecule recently shown to act as carrier and

adjuvant. Another is a novel haptotactic C-terminal fibrinopeptide (Haptide). During the first phase of the project, the new antigenic entities will be cloned, expressed, and purified. Novel in vitro models using human skin will be used to evaluate transcutaneous passage of molecules, activation and mobilization of Langerhans' cells, and stimulation of specific cytotoxic T cells. During the first phase of the project, we expect to demonstrate efficacy of antigen delivered transcutaneously to stimulate the immune system in human in vitro models and will allow for the selection of the molecules that will be further evaluated in depth in in vivo models. During the second phase, specific immune responses of splenic T cells from vaccinated mice will be evaluated, tumor models will be established in mice, and the response to vaccination will be determined. Finally, the most effective molecule/s will be produced under GMP or GMP-like conditions for Phase I/II clinical trials in a subsequent study.

The success of this project would allow topical application of an immunostimulant for treatment of melanoma and other cancers and would thus significantly simplify treatment, eliminating the need for hospitalization and even day care and the need for a specialized laboratory. As a result, one could treat a much larger number of patients, with the potential to clinically evaluate new antigens and immunotherapeutic modalities, improving the life quality and expectancy of metastatic melanoma patients.

NanoSelection® – A Single-Cycle Method To Select Individual Aptamer Species From a Small Pool of Random Oligonucleotides

Martin Guthold Wake Forest University, Winston-Salem, North Carolina

We are developing a novel, single-cycle method to affinity-select individual aptamer species from a small pool of random oligonucleotides (oligos) using combined fluorescence and atomic force microscopy (AFM). Target molecules immobilized on a cover slip are exposed to a library of fluorescently labeled oligos. High-affinity, target-specific aptamers bind tightly to the target for prolonged periods and resist subsequent washes, resulting in a strong fluorescence signal. The AFM tip is then directed to the coordinates of the fluorescence signal for high-resolution aptamer imaging and extraction. The extracted aptamer species is PCR-amplified, analyzed, and sequenced. Our method is designed to identify individual target-binding oligos in a single cycle of detection, isolation, amplification, and sequencing. Here, we demonstrate proof of principle of all steps of our method, namely instrumentation development, sample preparation, and selection of the known thrombin aptamer GGTTGGTGTGGTTGG from a binary pool of random oligonucleotides.

Molecular Beacons for Protein Detection: Development of Homogeneous and Heterogeneous Assay for NF-kB

Tomasz Heyduk Saint Louis University, Saint Louis, Missouri

The overall 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. In the next step, we generalized our molecular beacon design to include detection of proteins lacking natural DNA-binding activity. In this design, a pair of aptamers recognizing two distinct epitopes of the protein were used to prepare the beacon. Here, we present the data illustrating development of molecular beacons detecting NF-kB, a transcription factor involved in regulation of genes important for inflammatory response, cancer cell invasion, metastasis, angiogenesis, and resistance to chemotherapy. Two versions of the assay (homogeneous and heterogeneous) were developed. Performance of the assay was tested using purified NF-kB and cellular extracts spiked with various amounts of purified protein. Both assays were able to detect NF-kB with desired specificity. Heterogeneous assay provides an opportunity for large-scale multiplexing by adapting it to a microarray format. Proof-of-principle experiments demonstrated the feasibility of this concept.

Surface Proteome Signatures To Classify Breast Cancer

Daniel Jay

Tufts University School of Medicine, Boston, Massachusetts

Customized optimal treatment based on a precise classification of each patient's tumor would have a marked health benefit. For example, if a diagnostic set of biomarkers applied to biopsy could predict whether a patient's cancer was susceptible to a particular chemotherapy, this would facilitate treatment and improve prognosis. It remains a major challenge to classify cancers presented by patients, which are remarkably diverse due to individual differences in both tumor and patient. Molecular classification using expression microarrays shows promise, but much of the complexity is engendered by proteomic differences. To begin to address proteomic complexity, we have developed surface proteome signatures (SPS) as a means of molecular classification of cancer and identification of potential biomarkers. This approach is based on differential immunocytochemistry using a library of single-chain antibodies (scFvs) derived from phage display that recognize the surface proteome of HT-1080 fibrosarcoma cells. We have developed and tested SPS doxorubicin using human breast cancer lines that are either sensitive or resistant to doxorubicin: MCF7, MCF7-Adr, which is resistant because of overexpression of the multidrug resistance P glycoprotein, and MCF7-2R, a spontaneous mutation from the parent MCF7 line for which proteome changes are not known.

SPS using 70 scFvs was not able to distinguish between two near identical cell types derived from the same parent line (MCF7 vs. MCF7-2R) but could distinguish between MCF7-Adr and its parent line and clearly differentiated between breast cancer and fibrosarcoma cells. Importantly, SPS was able to identify a small subset of putative biomarkers that best distinguished between cell types. These scFvs are candidates for developing a diagnostic for breast cancer classification. The first biomarker analyzed was shown to be CD44, and we have implicated this protein in a key role in MCF7-Adr

resistance to doxorubicin. We developed human breast tumors from these lines in a xenograft model and developed SPS from resected tumor cells. Based on these SPS, we can predict the efficacy of doxorubicin treatment in tumor progression in these mice. We are able to acquire SPS rapidly with small numbers of cells (derived from passaged tumors [500 per assay] and predict whether doxorubicin would be effective or not.

Together, our findings establish a proof of principle of the SPS technology. This new technology has several important applications. First, it provides a molecular classification of tumors that is complementary to expression microarray and SAGE analysis and has the potential to address the additional complexity engendered by the proteome. For example, SPS analysis could be applied to identify class descriptors for tumor aggressiveness or homing to other tissues during metastasis. Second, the analysis also provides a rapid identification of potential biomarkers that may also be tested for functional importance by using the scFv for knockdown of the antigen bound by that scFv using fluorophore-assisted light inactivation (FALI). The limited patient material required (500 cells/assay) means that SPS may be compatible with analysis of resected tumors or large biopsies from patients. Third and finally, the technology can be generalized and applied to other health-relevant questions such as identifying new and better stem cell biomarkers.

Affinity Capture Method To Isolate Actively Translated mRNAs for Gene Expression Analysis

Jingfang Ju University of South Alabama Mitchell Cancer Institute, Mobile, Alabama

Translational control plays a key role during development, cell cycle control, and mechanisms related to acute drug resistance. In particular, non-coding miRNAs can potentially regulate over 30% of gene expression at the translational level. Gene expression analysis on actively translated mRNA transcripts provides a unique approach to study posttranscriptional regulation. Current method relied on a traditional sucrose gradient ultracentrifugation procedure to isolate polysome complexes and requires a large number of cells (up to 500 million cells). As a result, this still remains a major bottleneck for the investigation of posttranscriptional regulation with limited quantities of clinical samples. Therefore, there is an urgent need to develop a novel approach to isolate actively translated polysomes from a small number of cells. The new approach will allow us to systematically study translational regulation with limited clinical samples. It has been shown that actively translated mRNAs are associated with multiple units of ribosomes and the newly synthesized polypeptides are closely associated with molecular chaperones such as hsp73. These molecular chaperones assist in the proper folding of nascent polypeptides into higher ordered structures. These chaperones will provide the anchor to separate actively translated mRNAs associated with polysomes from free mRNAs. Affinity antibody capture beads will be developed to capture hsp73 chaperones associated with the polysome complexes so that all polysomes can be separated from monosomes and free mRNAs. The isolated actively translated mRNAs will be used for high-throughput gene expression analysis.

Improved Methods of Insertional Mutagenesis for Forward Genetics in Mammalian Cells

Eugene S. Kandel, Tao Lu, Youzhong Wan, Maupali Dasgupta, Mukesh K. Agarwal, Mark W. Jackson, Patrick Varley, George R. Stark Cleveland Clinic Foundation, Cleveland, Ohio

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 showed 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. Previously, we have used reversible insertional mutagenesis by modified retroviral vectors to search for factors that may cause hyperactivity of k;B, as frequently seen in cancerous cells. Reversible mutants with hyperactive NF-kB were readily obtained and the relevant integration targets were unambiguously identified, even when multiple inserts were found within the same cell. The regulatable nature of the mutant phenotype allowed us to observe new properties even for well-known components of NF-kB pathway. For example, overexpression of such components, caused by promoter insertion into the 5' end of the corresponding genes, leads to k;B-dependent secretion of factors that further activate k;B through cell-surface receptors, establishing an autocrine loop.

Vectors based on Sleeping Beauty transposon also were successful in delivering the mutagenic promoter cassette. Similar to retroviral vectors, transposons generated mutants with constitutive NF-kB activation that were useful for investigating the functions of the targeted genes. For example, an insert in the middle of the RIPK1 gene resulted in the production of an apparently hyperactive C-terminal fragment of the classical RIPK1 protein. Bioinformatic analysis predicted the presence of a natural promoter in the vicinity of the integration site. This promoter should produce a shorter form of RIPK1 identical to the one generated in the mutant. Initial experimental data are consistent with the presence of the shorter form of RIPK1 in some cancer cells.

Relative simplicity and robust target validation make reversible insertion of a promoter suitable for a broad range of applications. Taking advantage of the cell-autonomous and regulatable nature of transposition, we are currently adopting our mutagenesis strategy for use in animal models of cancer.

Nanoscale Electrocatalytic Protein Detection

Shana Kelley Boston College, Chestnut Hill, Massachusetts

We have developed a platform for electronic protein detection. The assay relies on a robust electrocatalytic process that reports on biomolecular complexation events and is executed on a nanostructured electrode platform that provides high sensitivity. The electrocatalytic method was originally developed by the Kelley laboratory for the sequence-specific analysis of nucleic acids and was used to detect bacterial genes with single-base resolution [1] and attomole sensitivity [2,3]. Current efforts to implement this system with protein targets have produced a new system for the detection of DNA repair factors and other cancer-related proteins. The eventual outcome of this project will be multiplexed detection of panels of different biomolecular targets on an electronic chip.

1 Lapierre M.A., O'Keefe M., Taft B.J., et al. Electrocatalytic Detection of Pathogenic DNA Sequences and Antibiotic Resistance Markers. *Anal Chem* 15;75(22):6327-33, 2003.
2 Gasparac R., Taft B.J., Lazareck A.D., et al. Ultrasensitive DNA Detection at 2D and 3D Nanoelectrodes. *J Am Chem Soc* 126(39):12270-1, 2004.
3 Lapierre-Devlin M.A., Asher C., Gasparac R., et al. Amplified Electrocatalysis at DNA-Modified Nanowires. *Nano Let* 5(6):1051-5, 2005.

Absolute Quantification of HER2 Protein Expression in Formalin-Fixed Archival Breast Cancer Tissue

David Krizman Expression Pathology, Inc., Gaithersburg, Maryland

In the United States, breast cancer is the second leading cause of cancer-related deaths, in women. HER2 is an important oncoprotein, the expression level of which has been shown to be directly indicative of aggressive breast cancer, and is the protein target of immuno-based therapy. Diagnostic detection and quantification of HER2 in cancerous tissue are routinely carried out by standard immunohistochemistry. However, this methodology lacks sensitivity and relies on subjective data analysis. We have developed and evaluated a method for direct detection and absolute quantification by selected reaction monitoring (SRM) of HER2 directly in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue using a stable isotope standard (SIS) peptide derived from HER2. A tryptic peptide from HER2 was synthesized with 13C to serve as an internal standard for quantification. The linear range and limits of detection and quantitation were evaluated by nanoflow reversed-phase liquid chromatography (nanoRPLC) coupled online with a linear ion trap mass spectrometer (MS) operation in tandem MS mode. Breast cancer cells were procured from formalin-fixed tissue culture cells expressing known amounts of HER2 protein and from standard fixed breast cancer tissue sections showing a broad range of HER2 expression (by IHC). Soluble protein extracts were prepared using Liquid Tissue® protocol/reagents and spiked with known concentrations of the HER2 SIS peptide followed by nanoRPLC-MS/MS SRM analysis. Quantitation was accomplished by comparison of peak areas generated from reconstructed ion chromatograms. Results indicate the ability to quantitate HER2 expression in Liquid Tissue® extracts from fixed tissue sections and demonstrate the ability to quantify HER2 in IHC negative cells. Results will be discussed within the

context of a novel, highly sensitive cancer diagnostic technology designed to impart absolute quantification of cancer biomarkers in formalin-fixed cancer tissue.

Development of a Frozen Sample Aliquotter

Dale Larson Harvard Medical School, Boston, Massachusetts

A system is being developed to enable the extraction of aliquots from frozen serum samples without thawing the samples. Currently, the samples experience at least two freeze-thaw cycles before being analyzed. This system will require only one freeze-thaw cycle before analysis, and, because it is automated, it will increase sample processing throughput thereby reducing the lead time associated with acquisition of these samples from biobanks such as the Nurses' Health Study. The ability to extract frozen cores has been demonstrated, and the force profile and needle geometry have been optimized.

Protein Localization: Multiaperture Near-Field Optics

Dale Larson Harvard Medical School, Boston, Massachusetts

Surface plasmon-enhanced illumination (SPEI) is a technology that enables the creation of an array of nanoscale beams of semi-collimated propagating light. A scanning probe microscope based on the scanning of an SPEI device across a sample and recording the fluorescence excited in the sample has been created. The geometric characteristics of these beams have been determined by exposing photoresist and measuring the resulting patterns in the photoresist. These beam characteristics and initial scanning results will be presented.

Multispectral Imaging Enables Double-Nuclear and Double-Membrane Immunohistochemistry

Richard Levenson Cambridge Research and Instrumentation, Inc., Woburn, Massachusetts

Background: Multi-analyte immunohistochemistry has potential applications in drug development, tumor biology, and clinical assessments, including (1) detection of target colocalization, (2) evaluation of spatial relationships between cell types, (3) signal transduction studies, (4) reduced sample depletion, and (5) decreased reagent utilization. However, analysis of two or more colocalized antigens by brightfield (nonfluorescence-based) microscopy is difficult because overlapping absorbing colored dyes (chromogens) are hard to resolve visually. Multispectral imaging, however, can “unmix” such signals to generate quantitative images of individual analytes. This has immediate application in the study of estrogen-receptor (ER) and progesterone (PR) expression and colocalization in breast cancer, as well as in double-immunophenotyping in hematopathology.

Design: Staining and imaging parameters were evaluated to determine optimal

procedures. These factors included spectral analyses and comparisons of commercially available chromogen combinations, order of chromogen development, and chromogen development time. Imaging was accomplished using CRi-developed spectral imaging systems and software that can easily be integrated into any microscope. Automated software tools were developed to quantitate nuclear percent-positivity and degree of colocalization of dual nuclear markers, and assessment of immunophenotype for multiple membrane markers. Additional tools that allowed linking of nuclear to membrane phenotype were also developed and applied. Implementation of these tools on an automated, whole slide-scanning microscope system is under way, with application to tissue-microarray samples.

Results: Once staining protocols were optimized, quantitative spectral unmixing of multiple chromogens from the hematoxylin counterstain was achieved and automated. Colocalization of ER and PR in breast cancer was notably higher in breast cancer lesions compared to corresponding normal breast tissue. Identifying MIB1-(+) B-cells by linking nuclear and membrane markers automatically was also accomplished.

Conclusions: Automated spectral tools can easily separate and quantitate multiple chromogens and counterstain. Colocalization and associations between specific membrane and nuclear markers are readily determined without the use of fluorescence-based techniques. These multispectral microscopy approaches will allow for multiplexed molecular markers to be assessed on a cell-by-cell basis in brightfield with conventional chromogenic stains and will be useful for further characterizing and subtyping cancers and other pathological processes.

Ultrasound-Accelerated Tissue Fixation/Processing Achieves Superior Morphology and Macromolecule Integrity

Qi Liang[1], Zhenqing Zhu1,[2], Minqi Wei[1], Randy King[1], Jo-Ann Andriko[3], Wei-Sing Chu1,[2]

[1]Department of Scientific Laboratories, Armed Forces Institute of Pathology, Washington, D.C.; [2]Bio-Quick, Inc., Silver Spring, Maryland; [3]Department of Pathology, Walter Reed Army Medical Center, Washington, D.C.

The first crucial step in cancer management is to ensure timely and accurate pathological diagnoses. Formalin fixation and paraffin embedding (FFPE) has been a standard tissue preservation method employed in over 90% of the cases for clinical histology diagnosis. However, FFPE has three disadvantages: (1) slow fixation (16-24 hours) hinders intra-operative decision-making, (2) slow quenching of enzymatic activity causes RNA degradation, and (3) extensive molecule modification affects protein antigenicity.

We have developed the ultrasound-accelerated FFPE (US-FFPE) technology for rapid and multiple-tissue fixation and processing. Applying high-frequency and high-intensity ultrasound to formalin and other reagents cuts fixation and processing time from over 16 hours to within 1 hour. Compared to conventional FFPE, US-FFPE provides similar

preservation in tissue morphology with similar long-term storage stability, improved preservation of protein structure, antigen properties, and mRNA integrity. US-FFPE tissues present less alteration in protein antigenicity, so rapid immunohistochemical reactions occur with higher sensitivity and intensity, reducing the need for antigen retrieval pretreatment. Stronger and more uniformed signals in in situ hybridization suggest better RNA preservation. In addition, US-FFPE tissues better support downstream molecular analyses because proteins and nucleic acids extracted from US-FFPE tissues are of greater integrity and quantity compared to those from conventional FFPE tissues.

We also found that during fixation, tissue displays physical changes that can be monitored and reflected as changes in transmission ultrasound signals. To our knowledge, this is the first effort to monitor tissue physical changes during fixation. Further study of this phenomenon may provide a method to control and monitor the level of fixation for quality controls.

Overall, ultrasound-facilitated tissue preservation can provide rapid and improved morphological and molecular preservation to better accommodate both traditional and molecular diagnoses.

Whole-Genome Amplification of Degraded DNA Using Rolling-Circle Amplification

Mike Makrigiorgos Dana-Farber Cancer Institute/Brigham and Women's Cancer Center, Boston, Massachusetts

New technologies for analyzing gene expression, genomic, and epigenetic DNA changes are expected to lead to profound changes in the detection, prognosis, and therapy of cancer. However, prospective clinical studies to validate the new molecular knowledge take several years to yield their conclusions and can be very expensive. Retrospective studies on existing formalin-fixed, paraffin-embedded (FFPE) specimens for which complete clinical data are available are a useful alternative. However, the efficient use of FFPE specimens for research is hindered by technical issues. Because cancer specimens are heterogeneous and often contain substantial amounts of normal tissue, identification of cancer-specific genetic abnormalities from FFPE samples requires microdissection and whole-genome amplification before screening. Major hurdles to this process are the introduction of amplification bias and the inhibitory effects of formalin fixation on DNA/RNA amplification.

The aim of this sample preparation project is to enable screening of FFPE specimens via advanced genome analysis technologies, thus accelerating discovery of genes and clinical biomarkers relevant to early detection, prognosis, and therapy of cancer. We continue evaluating RCA-RCA, a rolling-circle amplification-based technology for whole-genome amplification that is tolerant to sample degradation, for recovery of molecular information from degraded FFPE samples.

In the first year of the R21, we have evaluated the effect of a variety of commonly used

conditions for formalin fixation of surgical samples on the ability to (a) produce efficient whole-genome amplification across the genome, (b) identify polymorphic sites and mutations in diverse chromosomes, and (c) apply array-CGH to identify genome-wide copy number changes. FFPE samples at least 10 years old have been successfully recovered using RCA-RCA. A simple and sensitive molecular test was developed (Index of Sample Degradation) that can be used before performing molecular assays in order to predict with confidence the probability of successful outcome of downstream assays depending on sample condition. Finally, the RCA-RCA methodology was shown to be very useful in the amplification of DNA collected from bodily fluids (e.g., plasma), which is often enzymatically degraded at the time of collection. We anticipate a major impact of this technology in the identification of biomarkers from clinical samples and bodily fluids.

Novel Therapy for Glioma Using Combination FasL RNA Interference and Cytokine Delivery

Timothy Jansen, John Laterra, Alessandro Olivi Johns Hopkins University, Baltimore, Maryland

Recent advances in surgery, radiotherapy, and chemotherapy have made the clinical management of malignant gliomas more successful. However, the prognosis in patients diagnosed with these tumors remains poor. Malignant gliomas appear to proliferate in the brain without significant inhibition from the immune system. The immune privilege demonstrated by these tumors is partly attributed to tumor expression of immune-suppressive agents, such as the Fas ligand (FasL). Immunotherapy using interleukins, particularly IL-2 and IL-12, has been shown to be a promising strategy for the treatment of experimental gliomas but is impeded by T cell apoptosis. We propose that experimental gliomas impair the T cell-mediated antitumor responses by expressing FasL, which induces apoptosis of peritumoral T cells and decreases tumor cell killing by cytokine-activated T cells.

The hypothesis of this work is that immunotherapy of experimental gliomas can be enhanced by inhibiting FasL expression by glioma cells using RNA interference. We have provided ample evidence that the FasL counterattack exists in malignant gliomas using the 9L rat model. We first characterized the expression of FasL in the rat glioma cell lines 9L, C6, and F98 using immunoblot analysis and the real-time polymerase chain reaction. Immunohistochemistry was used to show the presence of apoptotic tumor-infiltrating lymphocytes (TILs). Inhibition of FasL expression in 9L, C6, and F98 glioma cell lines was also accomplished in vitro using siRNA. These data suggest that by using RNA interference techniques, FasL expression by glioma cells can be inhibited and the subsequent effects on survival and function of TILs determined. To further test our hypothesis in vivo, stable FasL knockdown and overexpressing glioma clones have been created using the rat 9L, C6, and F98 cell lines and are being implanted into immune-competent rats. This approach has the potential to improve existing and future immunotherapy strategies utilizing interleukins or vaccines.

University of South Alabama Mitchell Cancer Institute, Mobile, Alabama

Profiling Glycosylation in Secreted Proteomes Lewis Pannell

Glycoproteins are becoming of increasing interest as better biomarkers of disease. The analysis of the glycosylation pattern on a single glycosylation site in a protein can be a time-consuming process requiring highly purified proteins. We have developed a method for the computer-assisted characterization of glycosylation structures using accurate mass gaps between the glycoforms [1]. An MS-only data file of the protein digest is searched for sets of these gaps, which correlate in both the mass and time domains. While this works well for the single proteins, we have made major improvements for the analysis of protein mixtures, making the isolation of proteins a much less stringent requirement [2]. We have also optimized the methods for obtaining secreted proteomes and can now obtain highly enriched samples [3]. The method for the secreted proteome will be discussed along with the approaches used for the isolation and analysis of glycosylation profiles. Our approach to analyzing mixtures of glycoproteins using sub-database development of glycol-predictive software will be elaborated. Using these approaches, the glycosylation profile of galectin 3 binding protein, highly associated with metastatic cancer cells, will be presented.

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Identification of Autoantigens in Ovarian Cancer

Ramila Philip[1], Sid Murthysup>1, Jonathan Krakoversup>1, Sinnathamby Gomathinayagamsup>1, Lorraine Keller[2], Kim Lyerly[2]

[1]Immunotope, Inc., Doylestown, Pennsylvania; [2]Duke University, Durham, North Carolina

The goal of this project is to identify autoantibody-reactive tumor-associated antigens for the development of early-stage diagnostics and immunotherapeutics for ovarian cancer. The serum of cancer patients frequently contains circulating levels of antibodies that recognize autoantigens present in the patients' tumor tissues. We have analyzed 5 composite samples consisting of sera from 20 different ovarian cancer patients and 3 composites representing 12 normal donor serum samples.

Method: Tumor antigens were prepared from extracts of two ovarian cell lines, which yielded consistent, homogeneous and high-concentration protein preparations. Normal and ovarian cancer patient serum composites were mixed with tumor lysate, antigen-antibody complexes were immunoprecipitated, and immunoreactive proteins were isolated and subjected to tryptic digestion. Autoantibody-reactive antigens were separated from the antibodies, fractionated first by SEC, and then further fractionated by reverse-phase HPLC. Each HPLC fraction was individually treated with trypsin. Trypsin-digested samples were analyzed using an LC/MS/MS system. The mass spectral data were analyzed using two different human protein databases, PIR/UniProt and SwissProt, using Sequest and Mascot software. The search results were analyzed to identify autoantigens found in all five cancer composites and not in normal serum.

Results: Differential analysis identified more than 30 candidate autoantibody-specific autoantigens associated with cell growth, structure, DNA replication, and cell adhesion that were present in all five ovarian cancer serum composites and not in normal serum composites. Additional proteins associated with various tumor functions and known tumor markers were immunoprecipitated by both normal and tumor sera in four or fewer composites.

Discussion: We have developed and validated a high-sensitivity immunoproteomics technology for the identification of autoantibody-reactive proteins. The study has revealed a number of interesting candidates involved in cell division, cell cycle regulation, DNA synthesis and repair, and other essential tumor functions. Several novel proteins were also identified in the screen. Additional patient and normal serum sample analyses are under way to confirm the data. In addition, identification of a specific epitope recognized by the autoantibody is in progress with the goal of developing a sandwich ELISA-based early-stage diagnostic test for ovarian cancer.

Nanoparticles for Efficient Delivery to Solid Tumors

Suzie Pun University of Washington, Seattle, Washington

Nanoparticulate delivery vehicles offer distinct advantages to anticancer treatment approaches, including selective accumulation in tumor areas due to the enhanced retention and permeability effect and delivery of macromolecules, such as nucleic acids and proteins. In order to effectively treat a solid tumor, it is necessary for the nanoparticles to reach as many cells within the tumor as possible, but significant penetration into tumor tissue is not seen for relatively large particles such as liposomes and viruses that are directly injected in solid tumors.

The major goal of this project is to develop a noninvasive approach to overcome transport barriers that prevent nanoparticle penetration of solid tumors. We are investigating transcellular methods used by bacterial pathogens to invade tissues. In addition, we are evaluating methods to increase paracellular transport of nanoparticles. In this study, we systematically investigate the effects of nanoparticle size and extracellular matrix integrity on nanoparticle penetration in a 3D, multicellular spheroid

tumor model. The penetration of nanoparticles ranging from 20 to 200 nm in MCS was assessed. In addition, the effect of ECM-modulating enzymes on nanoparticle penetration was investigated.

Applications of Self-Assembling Protein Microarrays in Functional Proteomics and Biomarker Discovery

Niroshan Ramachandran Harvard Medical School, Cambridge, Massachusetts

Protein microarrays empower investigation into a broad range of biochemical properties and activities of the target proteins on the array. The strength of this approach is that by spotting many proteins on a single array, it is possible to test the functions of all of the proteins simultaneously. Without protein microarrays, these applications are tedious and expensive. They require high-throughput methods to produce proteins, automation using large liquid-handling robots, and highly trained personnel. Yet, despite their immense potential and strong demonstrations of feasibility, protein microarrays are not widely used. In large part, this is due to the labor and technical issues associated with producing the arrays. Traditionally, arrays are assembled by producing, purifying, and printing proteins onto the array surface.

We developed a novel approach to generate protein microarrays by printing cDNAs onto glass slides and then translating target proteins with mammalian reticulocyte lysate. This robust method obviates the need to purify proteins, avoids protein stability problems during storage, and captures sufficient protein for functional studies. This technology, nucleic acid programmable protein array (NAPPA), is capable of producing thousands of different proteins including transmembrane proteins all in a single step. NAPPA has already been demonstrated to be robust for the detection of protein-protein interactions by fluorescence detection. As we previously reported, in protein interaction studies, NAPPA recapitulated 85% of the known protein interactions, demonstrating that proteins produced on NAPPA are highly functional.

We are now adapting NAPPA to several more complex functional-proteomics applications, to assess the binding selectivity of small molecules to a family of related proteins (e.g., kinases) or to a mutant series of a single protein, or to screen for substrates for an active enzyme. Current efforts are focused on adapting emerging technologies to facilitate real-time, label-free detection of biological interactions using high-density surface plasmon resonance (SPR). The amalgamation of the two HT technologies, NAPPA and SPR, promises to provide high information content regarding protein interactions.

Apart from functional proteomics, NAPPA is being used in several projects to screen for immune responses to a large panel of antigens. The goal is to discover vaccine candidates in infectious diseases (cholera and tularemia) and to discover biomarkers in various cancers and autoimmune diseases. For example, patients with cancer are known to produce antibodies against tumor antigens. In some cases, immune response to the tumor antigen has been detected before the disease clinically presents itself.

Although the specificity for these responses is high, typically only 5% to 20% of patients demonstrate a response to any given antigen, limiting the usefulness of single-antigen responses as biomarkers and demanding high-throughput techniques like NAPPA.

Nanobeads for Cancer Cell Selection

Arfaan Rampersaud[1], Kristie Melnik[1], Jeffrey Chalmers[2]

[1]Columbus NanoWorks, Columbus, Ohio; [2]Department of Chemical and Biomolecular Engineering, Ohio State University, Columbus, Ohio

Cell separation is becoming increasingly commonplace for researchers and clinicians, who isolate rare cells from complex cell populations to understand how cells function in different environments or to diagnose disease occurrence, recurrence, or progression. Complex mixtures and searches for small variations in cell samples require new techniques that provide exquisite sensitivity with high throughput; therefore, advances in cell separation technology must allow the researcher to find a low percentage of cells of interest as well as purify them to allow for accurate assessment of biological relevance. The aim of this project was 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 too large, lack size uniformity, or have low magnetic susceptibility. As part of the Phase I goals, Columbus NanoWorks (CNW) demonstrated the manufacturing capability of magnetic nanoparticles that were uniform in size within a range of 50 to 70 nm, had a ligand density of 2 to 5 μg/mL Fe particles, and elicited a magnetophoretic mobility of immunomagnetically labeled cells an order of magnitude greater than commercially available magnetic reagents. The aforementioned criteria, defining magnetic particles achieved in the Phase I proposal, allowed the enrichment of 1 ovarian tumor cell in 107 total nucleated blood cells with an average recovery of 74.1% of spiked tumor samples, as well as an average 3.21 log10 depletion of contaminating cells in the QMS. The Phase II research will call for the design and commercialization of magnetic reagent kits specific for QMS applications involving cancer cell enrichment. CNW will (1) scale up processing efforts to obtain a fiftyfold increase over current process techniques, (2) establish procedures and practices that assure consistent control of nanoparticle size and magnetic properties, (3) develop protocols for CNW cell enrichment kits for use in the commercial prototype QMS, and (4) beta testing of protocols using breast cancer patients and head and neck cancer patients. The proposed Phase II end items are customized magnetic reagent kits specific for the QMS system that will allow the enrichment of cancer cells from various blood sources such as bone marrow and peripheral blood. Specifications of the particles will provide a greater than 70% recovery of desired cells, as well as a 4 log10 depletion of contaminating cells. The resultant enriched fraction using CNW nanoparticles in the QMS system will provide a quality-purified product for further downstream processing

and subsequent molecular analysis. The goal is for CNW-customized reagents to become an integral part of gold standard testing in the current pathology repertoire.

Genome-Wide Analysis of CTCF Binding Reveals Widespread Activities of Insulators in the Human Genome

Bing Ren Ludwig Institute for Cancer Research, La Jolla, California

Insulators, also known as boundary elements, play crucial roles in establishing the regulatory domains in the genome by preventing the encroachment of heterochromatin and restricting the action of transcriptional enhancers. To date, our understanding of insulators' role in global gene regulation is still limited because of a lack of comprehensive knowledge of the locations of these elements in the genome. In mammalian cells, virtually all the known insulators interact with the zinc-finger protein CTCF, and loss of CTCF binding to these regions has been implicated in loss of insulator function that results in human diseases. In order to systematically define the insulators in the human genome, we have identified the CTCF binding sites in the genome of primary human fibroblasts using chromatin immunoprecipitation combined with high-resolution genome-tiling arrays analysis. Our study identified 13,804 potential insulators and an insulator motif that is highly conserved in vertebrate species. Using this motif, we predicted over 4,000 additional potential insulators in the human genome. Together, these CTCF sites defined more than 7,300 gene domains, some of which contain as many as 50 genes. Our results provide the first genome-wide map of insulators, reveal cell type-dependent usage of these elements, and will aid the systematic analysis of transcriptional regulation in human cells.

Advanced Microarray Platform for Profiling Enzyme Activities in Complex Proteomes

Cleo Salisbury The Scripps Research Institute, La Jolla, California

Proteases are suspected of playing major roles in cancer, including the activation/inactivation of growth factors and the degradation of extracellular matrix components to promote cancer cell migration and invasion. Consistent with this premise, transcript and protein levels for many proteases are up-regulated in cancer cell lines and tumors. However, whether these changes in protease expression correlate with changes in protease activity remains a critical, but largely unanswered, question. Indeed, proteases are regulated in a complex series of posttranslational events, meaning that their expression levels, as measured by conventional techniques, fail to accurately report on the activity of these enzymes. To address this problem, we have introduced a chemical proteomics technology, referred to as activity-based protein profiling (ABPP), which utilizes active site-directed probes to determine the functional state of large numbers of proteases directly in whole cell, tissue, and fluid samples. We have applied ABPP to identify several serine protease activities up-regulated in human cancer cells and primary tumors. Recently, we have created an advanced antibody-based platform for ABPP that enables profiling of protease activities while requiring only

minute amounts of proteome. We are now developing methods to further increase the sensitivity of the microarray platform by developing signal enhancement tools and generating optimized micorarray-compatible antibodies for key cancer proteases. Further, we have developed new probes for the sensitive detection of metalloproteases and related enzymes, which are known to be misregulated in cancer. By combining the new probes with the enhanced-sensitivity microarray platform, we are in a unique position to study the misregulation of protease activities in cancer samples, with an unprecedented combination of sensitivity, resolution, and throughput.

Standardization of Immunohistochemistry for Formalin-Fixed, Paraffin-Embedded Tissue Sections Based on the Antigen Retrieval Technique: From Hypothesis to Experiments

Shan-Rong Shi, Cheng Liu, Clive R. Taylor Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, California

Standardization of immunohistochemistry (IHC) for archival formalin-fixed, paraffin-embedded (FFPE) tissue sections has become increasingly important as a result of the emergence of a new field of pathology that requires demonstration of the differential expression of various prognostic markers for individualized cancer treatment. From a practical point of view, one of the most difficult issues in the standardization of IHC for FFPE tissue is the adverse influence of formalin upon antigenicity, and the great variation in fixation/processing procedures. Our previous studies have demonstrated a potential approach to standardization of IHC for FFPE tissue based on optimal antigen retrieval (AR), to achieve a maximal degree of retrieval that provides a comparable level of IHC staining among various FFPE tissue sections that have been fixed in formalin for 4 hours to 7 days. On this basis, the following hypothesis is proposed: “The use of optimized AR protocols permits retrieval of specific proteins (antigens) from FFPE tissues to a defined and reproducible degree (expressed as R%), with reference to the amount of protein present in the original fresh/unfixed tissue.” This hypothesis may be explained mathematically. Suppose the amount of a protein in a fresh cell/tissue = Pf, and that Pf produces an IHC signal in fresh tissue of ò (Pf). When the IHC signal of FFPE is ò (Pffpe), then the retrieved rate of AR (R%) is calculated as AR rate (R%) = ò (Pffpe)/ò (Pf) 100%, the amount of protein in the FFPE tissue of Pffpe = Pf x R%. In a situation where optimized AR is 100% effective, then Pffpe = Pf if the IHC signal is of equal strength in fresh tissue and FFPE tissue. It is proposed that this calculation applied to selected ubiquitous antigens (analytes) may form the basis for developing a system if there are quantifiable internal reference standards.

Further studies are required to test this hypothesis. A research design using cell/tissue models is presented to test limitations of this hypothesis, based on correlation of accurate quantitative biochemical measurements with IHC staining results.

How Do Colorectal Cancers Arise Despite Surveillance?

Darryl Shibata University of Southern California Keck School of Medicine, Los Angeles,

California

The time between initiation and tumor removal is uncertain for most human cancers because serial observations are impractical. In this study, cancer mitotic ages (numbers of divisions) are retrospectively estimated by counting numbers of somatic microsatellite mutations in colorectal cancers deficient in DNA mismatch repair. The greater the numbers of divisions, the greater the numbers of mutations (molecular clock hypothesis). Preliminary data indicate sporadic cancers, and “interval” cancers that arise within years after a negative clinical examination, have similar mitotic ages, which suggests many cancers frequently physically grow to detectable sizes within a small period of time (years). It appears that cancers' histories are surreptitiously written within their genomes.

Characterization of Gene Expression Profiles in Paraffin-Embedded Tissues and Patient Cell Lines Lead to Predictive Markers

Robert H. Singer[1], Rossanna Pezo[1], Melissa Lopez-Jones[1], Shailesh Shenoy[1], Paola Capodieci[2]

[1]Albert Einstein College of Medicine, Bronx, New York; [2]Aureon Laboratories, Yonkers, New York

We have adapted the application of fluorescence in situ hybridization (FISH) to investigate gene expression profiles in paraffin-embedded tissue samples. We have used this approach to evaluate prostatectomy material from patients. The characterization of the gene expression profiles indicates that a prognostic or diagnostic test is feasible using FISH on paraffin-embedded samples using five informative genes. One of the important results is that archival samples (22 years) were as easily profiled as new samples. Currently,we are analyzing the expression profiles from patients where outcomes are known using tissue microarrays and have found a number of predictive genes.

This technology has also been utilized to investigate the spatial coordination of mouse cyclin D1 transcription in the intestinal mouse mucosa as cells migrate along the crypt/villus axis. The majority of human colon tumors are associated with the elevation of ƒÒ-catenin/TCF signaling due to the loss of APC. It has been well established that ƒÒ-catenin/TCF activity is linked to several genes associated with cell cycle regulation, including the positive regulation of c-myc and cyclin D1. In the intestinal mucosa, these events are modulated along the crypt/villus axis. Using p27 +/+ APC +/+ paraffin-embedded mouse duodenum tissue samples, we can determine a transcriptional profile of cyclin D1 and c-myc in normal intestinal mouse mucosa. To evaluate percent transcription in the crypt and the villus, experiments were performed using probes labeled with spectrally distinctive fluorophores recognizing the cyclin D1 transcript. Preliminary results indicate that there is a 40% reduction in cells expressing cyclin D1 in the villus (1.98%) versus the crypt (3.29%). Using this powerful technology, we can further elucidate specific developmental and signaling pathways important in the

intestinal homeostasis and potential perturbations leading to tumorigenesis.

Using Single-Cell Expression Profiling To Identify Markers of 5-FU Resistance in Colorectal Cancer

We are examining the transcriptional profile of individual colorectal tumor cells to better define the indications for chemotherapy using 5-fluorouracil (5-FU). Our goal is to develop a biomarker composed of several key genes that would be predictive of response to 5-FU-based chemotherapy. This approach would also enable us to identify novel genes involved in mediating sensitivity to 5-FU and to further elucidate the mechanisms by which tumor cells acquire resistance to 5-FU. Preliminary results in human colorectal tumor cell lines suggest that a set of four genes can be used to predict resistance to 5-FU-based chemotherapy in colorectal cancer. Transcription sites for specific genes are identified using probes directly labeled with multiple fluorophores for FISH. This method enabled us to simultaneously visualize many nascent mRNA transcripts and provided a gene expression profile for each single cell. Application of this technique to clinical samples allows us to preserve tissue architecture while circumventing the issue of tissue heterogeneity. Gene expression profiles can then be applied to clinical samples and correlated with outcomes. Single cell expression profiling of tumors would allow us to identify subpopulations of cells which may only represent a minor percentage of the sample but may have important phenotypes such as chemosensitivity or metastatic potential. These techniques will provide valuable data for correlating the transcriptional profile of cells with tumorigenesis, metastasis, and drug response. Using this information, chemotherapy treatments can be tailored to the individual tumor and patient.

Protein Extraction From Formalin-Fixed, Paraffin-Embedded Tissue Sections Under the Influence of Heating and pH

Clive R. Taylor, Shan-Rong Shi, Cheng Liu Department of Pathology, University of Southern California Keck School of Medicine, Los Angeles, California

Protein extraction from formalin-fixed, paraffin-embedded (FFPE) tissue sections is critical for the application of advanced proteomics-oriented technology in cell biology and medical research, paralleling the use of the heat-induced antigen retrieval (AR) technique that has revolutionized routine immunohistochemistry (IHC) for FFPE tissue sections worldwide. We have previously documented a protocol by boiling FFPE tissue sections in Tris-HCl containing 2% sodium dodecyl sulfate (SDS), which provides promise as a method to extract protein from FFPE tissue. To improve the efficiency of protein extraction from FFPE tissue, it is necessary to analyze each potential factor that may influence the effect of heat-induced retrieval procedure, including the pH value of retrieval solution, the heating condition (temperature and the time of heating treatment), and the chemicals used in the retrieval solution. In addition to the primary purpose of establishing a practical efficient protocol for protein extraction from FFPE tissue, we intend to test our previous conclusion concerning the AR basic principle: heating under the influence of pH.

Liver tissues taken from seven white mice (strain CD-1) were processed routinely by formalin fixation and paraffin-embedding. One rodent liver tissue was frozen and embedded in OCT for control. For each batch of experiments, 50 tissue sections (10 microns each) were used to extract a sufficient quantity of total protein for 12 to 15 different AR conditions. Protein extraction was performed based on the heat-induced AR principle, using a universal buffer of Britton and Robinson solution, and Tris-HCl buffer solution. Both buffer solutions were prepared at pH values ranging from 1 to 12 (1 to 10 for Tris-HCl buffer). Three heating times, 2, 10, and 30 minutes, were used for boiling FFPE tissue sections in each retrieval solution. SDS-PAGE was used to display the total proteins extracted from FFPE and from frozen tissue sections to compare the efficiency of extraction under different heating conditions and pH values. Results show the following: (1) Proteins extracted from FFPE and frozen tissue sections showed comparable SDS-PAGE patterns, albeit the former showing much weaker bands (due in part to lack of 2% SDS in the retrieval solutions tested in this experiment), (2) higher pH values combined with longer time of heating provided better results, and (3) low pH solutions (at 1.0) showed much poorer results when heating for longer times. Evaluation is ongoing to determine whether different categories of proteins respond differently at various pH values, as demonstrated previously for AR-IHC on FFPE sections.

The Origin of Tumor-Specific Serum Peptidome Patterns

Paul Tempst Memorial Sloan-Kettering Cancer Center, New York, New York

As cancer involves transformation and proliferation of altered cell types that produce high levels of specific proteins and enzymes such as proteases, it will modify not only the array of existing serum proteins (serum “proteome”) but also their metabolic products, i.e., peptides (serum “peptidome”). However, it was unclear whether this complex peptidome may provide a correlate of biological events occurring in the entire organism.

We have developed an automated procedure for the simultaneous measurement of peptides in serum that utilizes magnetic, reversed-phase beads for analyte capture and a MALDI-TOF MS readout. This system is more sensitive than surface capture on chips as spherical particles have larger combined surface areas, and therefore higher binding capacity, than small-diameter spots. Coupled to high-resolution MS and MS/MS, hundreds of peptides can be detected in a single droplet of serum, many of which can be readily identified without further fractionation. Automation facilitates throughput and ensures reproducibility. We then use a minimal entropy-based algorithm that simplifies and improves alignment of spectra and subsequent statistical analysis.

MS-based sequence characterization indicated that a large part of the human serum “peptidome,” as detected by MALDI-TOF MS, is produced ex vivo (i.e., after the blood sample collection) by degradation of abundant substrates by endogenous proteases. Polypeptide fragments are generated during the proteolytic cascades that take place in the intrinsic pathway of coagulation and complement activation. Once generated, the “founder peptides” are pared down by exoproteases into ladder-like clusters. By

correlating identified proteolytic patterns with disease groups and controls, we have shown that exoprotease activities, superimposed on the ex vivo coagulation and complement-degradation pathways, contribute to generation of not only cancer-specific but also “cancer type”-specific serum peptides. None of the signature peptides were derived from cancer cells, which implies that different tumor types secrete and/or shed distinct sets of proteases that, through their catalytic activity, generate unique serum peptide profiles. The small number of blood proteins that are the source of nearly all the peptides in the prostate, bladder, and breast cancer signatures are not biomarkers in a strict sense but simply serve as an endogenous substrate pool for the real ones, i.e., tumor-derived proteases. However, there is no actual relationship between the precursor substrate concentrations and the MS-ion intensities of many of the degradation products. For instance, highly abundant serum proteins such as albumin and immunoglobulins were not represented.

Direct MALDI-TOF MS-based serum peptide profiling is thus a form of activity-based proteomics, monitoring proteome “metabolomic” products. It makes this approach particularly well suited for detection of cancer as proteases are well-established components of cancer progression and invasiveness. Focused mass spectrometric analysis of key peptides, derived from endogenous or custom-synthetic substrates, and utilization of isotopically labeled standards to quantify all pattern-contributing peptides, may lead to functional assays and facilitate future introduction of this technology into clinical practice. Identification and characterization of the protease panels could also lead to direct immunoassay-based, quantitative diagnostic tests that may be better suited to a clinical environment.

Piezoelectric Pipetting Technology for DNA Analysis

Derek Thirstrup[1], Peter Wiktor[1], Jim Mcgill[1], Rob Sullivan[2], Helmut Zarbll,[2]
[1]Engineering-Arts, Mercer, Washington; [2]Fred Hutchinson Cancer Research Center, Seattle, Washington

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 (FHCRC). 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 GE Healthcare to develop piezoelectric pipetting technology to construct their whole genome expression arrays.

Our current focus is shRNA loss-of-function arrays, which 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 between adjacent spots. We are also working on enhancing the transfection efficiency of RNAi complexes using recombinant Ad5 penton capsid as a facilitator of receptor-mediated endocytosis. We will subsequently generate an apoptosis microarray screen by targeting two oncogenes: Bcl-2 and HPV E7 oncogenes associated with colorectal and cervical cancer, respectively. Our surface transfection array will result in an RNAi-dependent apoptosis screen of two human epithelial cancer cell lines with apoptosis index quantitation using a commercial apoptosis screening assay.

Dynamic Isoelectric Focusing for Cancer Proteomics

Luke Tolley Southern Illinois University, Carbondale, Illinois

Dynamic isoelectric focusing is a new technique for protein separation. It is related to capillary isoelectric focusing, but uses additional high-voltage power supplies to provide control over the shape of the electric field within the capillary. Manipulation of the electric field changes the pH gradient, enabling both the location and width of the focused protein bands to be controlled. The proteins can be migrated to a designated sampling point, while remaining focused, where they can be collected for further analysis. This ability to collect and isolate the protein bands while maintaining a high peak capacity demonstrates that dynamic isoelectric focusing has great potential for proteomic analyses. Dynamic isoelectric focusing is demonstrated to achieve a peak capacity of over 1,000 in less than half an hour, as shown by both mass spectrometry analysis and direct imaging.

Drug Response Indicators Test for Personalized Anticancer Chemotherapy for Breast Cancer Patients

Paul O. Ts'o[1], Stephen A. Lesko[1], Zhao-Ping Lum[1], Scott Deamond[1], Enzhi Shan[1], David Zhou[1], John Daniel1, Katherine Tkaczuk[2], Nancy S. Tait2, Olga Ioffe[2], David Van Echo[3]

[1]CCC Diagnostics, LLC, Baltimore, Maryland; [2]University of Maryland Greenebaum Cancer Center, Baltimore, Maryland; [3]Harbor View Cancer Center, Baltimore, Maryland

Drug response indicators test (DRIT) is a diagnostic assay performed on fixed and embedded breast tumor sections or circulating cancer cells in order to quantitate the chemoresistance of tumors in individual patients. The DRIT may be used to provide information for predicting patient response to seven U.S. Food and Drug Administration-approved, National Comprehensive Cancer Network-recommended anticancer drugs based on the expression of seven DRIs in the tumor. The results are expressed in a probability percentage of chemoresistance according to the correlation of clinical response with drug response indicator (biomarker) expression.

DRIT involves three components: (1) Quantitative measurement of several DRIs simultaneously in the tumor. The expression of DRIs in tumor cells can be evaluated by immunofluorescence utilizing labeled monoclonal antibodies and a computerized fluorescence microscope. Numerical values can be derived and normalized to a fluorescent reference standard for comparison. (2) Establishment of a statistically significant correlation of DRI expression with the cytotoxic response of tumor cell lines to a related drug. An in vitro indexing system was established to correlate the cytotoxic effect of each drug to the corresponding DRI measurements. Seven breast cancer cell lines with different sensitivities to various anticancer drugs were utilized. The effect of tamoxifen, paclitaxel, trastuzumab, and doxorubicin was correlated with the DRI expression of each drug in these cell lines. The DRIs are estrogen receptor, beta tubulin III, HER2/neu, and topoisomerase II, respectively. (3) Clinical studies designed to correlate the level of DRI expression in tumor cells of individual patients with the clinical response [complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD)] to mechanistically related drugs. Initially, retrospective studies will be conducted on the basis of patients' archival tumor tissues and related records of treatment. This retrospective clinical study will be carried out with 200 patients, in conjunction with a consortium of five hospitals in the Baltimore-Washington, D.C., area. In this correlation study, a probability percentage of chemoresistance (leading to PD) will be established for each breast cancer patient's tumor, providing information as to which drug is likely not to be effective.

The utilization of DRIT to provide advisory information to physicians can lead to cost savings, reduction in side effects, and better utilization of the time allowed in the treatment window. Thus, the DRIT can result in a greater efficacy of current drugs through personalized anticancer chemotherapy.

Interactome Networks

Marc Vidal Center for Cancer Systems Biology, Dana-Farber Cancer Institute, Boston, Massachusetts

For over half a century, it has been conjectured that macromolecules form complex networks of functionally interacting components and the molecular mechanisms underlying most biological processes correspond to particular steady states adopted by such cellular networks. However, until recently, systems-level theoretical conjectures remained largely unappreciated, mainly because of lack of supporting experimental data.

To generate the information necessary to eventually address how complex cellular networks relate to biology, we initiated, at the scale of the whole proteome, an integrated approach for modeling protein-protein interaction or “interactome” networks. Our main questions are the following: How are interactome networks organized at the scale of the whole cell? How can we uncover local and global features underlying this organization? How are interactome networks modified in human disease, such as cancer?

Incorporation of a Boronic Acid Moiety Into DNA for Aptamer Selection

Binghe Wang Georgia State University, Atlanta, Georgia

The boronic acid moiety is a versatile functional group useful in carbohydrate recognition, glycoprotein pull-down, inhibition of hydrolytic enzymes, and boron neutron capture therapy. The incorporation of the boronic acid group into DNA could lead to molecules of various biological functions. We have successfully synthesized a boronic acid-modified thymidine triphosphate (B-TTP) and incorporated it into DNA. The synthesis was achieved using the Huisgen cycloaddition as the key reaction. We have demonstrated that DNA polymerase can effectively recognize the boronic acid-modified DNA (BA-DNA) as the template for DNA polymerization, which allows PCR amplification of boronic acid-modified DNA. DNA polymerase recognition of the B-TTP as a substrate and the boronic acid-modified DNA as a template are critical issues for the development of DNA-based lectin mimics via in vitro selection.

Development of a Bioinformatics Tool Suite Using Machine Learning

Yue Wang Virginia Tech, Arlington, Virginia

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 significantly 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 predicting clinical heterogeneity in cancer treatment and potentially discover new diagnostic and therapeutic targets. This IMAT project aims to develop a bioinformatics tool suite for data modeling and analysis consisting of most major computational tasks in cancer research.

This presentation will first report our experience in developing caBIGTM Silver-Maturity data clustering software, VISDA. The software has been applied to sample and gene, as well as unsupervised and supervised data clustering, with rigorous comparisons to major existing software. Second, we will report our recent effort in developing adaptive hierarchical subspace expert (AHSE) software. Different from most existing predictive classification algorithms, AHSE provides the ability to learn the tree of phenotype and hierarchical subspaces and perform adaptive multicategory cancer classifications. Third, we will report our preliminary study on decomposing the spatial-temporal images of tumor angiogenic vasculature into permeability factor images and associated perfusion time activity curves. We conducted clustered-component analysis (CCA) on dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI). The objective is to characterize the response of tumor angiogenic vasculature to antiangiogenesis therapies. Last, we will summarize the algorithm development for data normalization, biomarker discovery and validation, and tissue heterogeneity correction.

Diverse Novel Approaches for Proteomics of Posttranslational Modifications

Yingming Zhao University of Texas Southwestern Medical Center, Dallas, Texas

More than 200 known posttranslational modifications have been reported, most of which have been shown to be intimately associated with cancer. However, only a few of them have been extensively investigated. The chemical nature of the protein modifications offers very limited insights into their roles in biochemical pathways. The vast number of uncharacterized protein modifications suggests that new technologies remain to be developed for their functional studies. Largely because of recent funding from the IMAT Program, our laboratory was able to develop a series of novel technologies to address these challenges. In this presentation, we will highlight four approaches, which we developed in the last few years, for detection, isolation, and proteomics of posttranslational modifications. These methods take advantage of highly sensitive mass spectrometry and efficient isolation of posttranslationally modified proteins/peptides, which is based on immunoaffinity purification, tagged substrates, ionic interactions, and pI difference. We applied these technologies to global analysis of several posttranslational modifications, including lysine acetylation, sumoylation, prenylation, O-GlcNAc modification, and phosphorylation. Our proteomics analysis of lysine acetylation found that acetyllysine is present in diverse non-nuclear proteins, therefore revealing previously unappreciated roles for lysine acetylation in the regulation of diverse cellular pathways outside the nucleus. Many of the identified acetyllysine substrates are involved in the regulation of cell growth, survival, and apoptosis, shedding new light into its roles in cancer. Integration of these methods with isotope labeling promises dynamic studies of protein modifications under diverse cellular environments involved in the progression of diverse cancer.

Prostate Cancer Gene Discovery Using Transposon-Based Somatic Mutagenesis

Lara S. Collier, Eric P. Rahrmann, Laura E. Green, Paul C. Marker, David A. Largaespada University of Minnesota, Minneapolis, Minnesota

Human prostate cancer progresses through a series of pathologically defined stages: prostatic intra-epithelial neoplasia, carcinoma in situ, invasive adenocarcinoma, and metastatic disease. These pathological changes are hypothesized to be accompanied by the accumulation of genetic changes that allow adult prostatic epithelial cells to first proliferate and then invade surrounding tissues, and to metastasize to distant sites. Clinically, advanced prostate cancer is usually treated by hormone ablation therapies. Although tumors usually initially respond to this treatment, many eventually relapse due to androgen insensitivity. The specific genetic pathways that are disregulated in each step of prostate cancer progression vary from tumor to tumor. In principle, these differences could allow medical intervention for prostate cancer to be tailored to the genetic makeup of a patient's particular cancer. To elucidate the genetic changes that accompany each step in prostate cancer progression, we are using Sleeping Beauty transposon-mediated insertional mutagenesis [1-3] to identify new prostatic oncogenes and tumor suppressors in mouse models of prostate cancer initiation and progression.

Mobilization of highly mutagenic transposons via ubiquitous expression of transposase leads to highly penetrant lymphocytic leukemia [2]. Cloning transposon insertion sites from a limited number of leukemias revealed the presence of common integration sites (CISs) at both known and candidate cancer genes. Closer examination of 4- to 6-month old male mice that succumbed to leukemia has revealed abnormal proliferative lesions in the prostatic epithelium. Work on methods to clone transposon integration sites from laser-captured lesions identified by immunohistochemical staining for cell cycle markers is progressing. We are also developing new transgenic lines that express transposase specifically in the luminal epithelial cells of the prostate so that mutagenesis will be restricted to the proper cell type. We will use this tissue-specific transposon mobilization to accelerate prostate tumor progression. Specifically, we propose to screen for genes involved in prostate cancer metastasis and progression by mobilizing transposons in a murine prostate cancer model induced by prostate-specific loss of the PTEN tumor suppressor gene. PTEN-deficient murine prostate tumors are adenocarcinomas but rarely, if ever, metastasize [4,5]. In addition, the tumors are responsive to androgen ablation therapy [5]. We hypothesize that transposon mobilization in these tumors will "tag" and mutate genes involved in prostate cancer progression, leading to widespread tumor metastasis. Furthermore, we hypothesize that in animals receiving hormone ablation therapy, transposon-mediated mutagenesis will identify genes responsible for progression to a hormone refractory state. In conclusion, our data suggest that Sleeping Beauty is a useful tool for cancer gene discovery in epithelial tumors, such as prostate cancer.

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Mapping Regulatory Pathways in Cancer

Roland Green[1], Nan Jiang[1], Tae Hoon Kim[2], Bing Ren[2]
[1]NimbleGen Systems, Inc., Madison, Wisconsin; [2]Ludwig Institute for Cancer Research, Department of Cellular and Molecular Medicine, University of California, San Diego, School of Medicine, La Jolla, California

We have performed genome-wide ChIP chip experiments to map the binding sites of TCF4 in a colorectal carcinoma cell line, HT29. TCF4 is a transcription factor in the Wnt/beta-catenin signaling pathway, and mutations that inappropriately activate this pathway have been implicated in approximately 90% of colorectal carcinoma cases. This study determined a set of target genes that are regulated by Wnt/beta-catenin signaling. This set of target genes should lead to a better understanding of the cellular pathways controlled by the oncogenes and help in the development of therapeutic strategies. In addition to the genome-wide ChIP chip study, we have several projects under way to reduce the cost of genome-wide microarray studies. We have developed a new microarray substrate that allows for robust array reuse. This new substrate provides superior reuse properties and will greatly reduce the cost of arrays in genome-wide studies. We are also building a new microarray-manufacturing tool that will produce microarrays with 2.1 million features. This is compared to our current arrays, which contain 385,000 features. These new higher density arrays will allow us to cover the entire human genome on 7 arrays compared with 38 arrays. This alone will produce a fivefold reduction in array costs for a genome-wide study. These reusable, higher density arrays will make genome-wide microarray studies economically feasible for any laboratory interested in these types of experiments.

Replacing the Human Observer/Operator in a System for Autonomous Microscopy and Multiparameter Single-Cell-Level Analysis

William Cleveland St. Luke's-Roosevelt Hospital Center, University Hospital of Columbia University, New York, New York

The Cleveland and Yao laboratories are developing an autonomous system for single-cell-level studies that will combine multidimensional optical microscopy with massively parallel multiparameter molecular methods such as microarrays, mass spectrometry, and RT-PCR. A critical requirement for this system has been the development of computer algorithms that facilitate the elimination of a human observer/operator during the routine operation of the system. Progress toward this goal has led to the development of binary classification algorithms that recognize and localize unstained viable cells in microscope images obtained with brightfield illumination. Even without the use of a viability stain, these algorithms can distinguish between viable and nonviable cells.

In many practical culture systems, there are often multiple types of cells. In order to use our robotic system with these cultures, multiclass classification algorithms will be needed to distinguish between the different types of cells in the cultures. Our recent progress has led to a multiclass classification strategy that uses composite images formed by combining images of the same field that are acquired with brightfield contrast, phase contrast, and Hoffman Modulation Contrast. This strategy takes advantage of the fact that cell types that look similar with one contrast technique may look different with another. We find that multicontrast composite images yield better results than monocontrast images and provide a multiclass classification accuracy that is sufficient for many practical applications.

The success of our binary and multiclass cell recognition algorithms raises the possibility of a highly autonomous robotic system for microscopy, manipulation, and molecular analysis of single living cells.

Integrated Systems for Multiplexed Hybrid Molecular Analysis Using Modular Polymer-Based Devices

Steven A. Soper, Robin L. McCarley, Jost Goettert, Michael C. Murphy, David Spivak, Robert P. Hammer, Feng Xu, Andre Adams, Suying Wei, Proyag Datta, Subramanian Balamurugen, Matsuez L. Hupert Departments of Chemistry and Mechanical Engineering, Center for BioModular Multi-Scale Systems, Louisiana State University, Baton Rouge, Louisiana

We are developing polymer-based, modular microfluidic systems that can be produced in a high-production mode and at low cost using replication micro- and nanotechnologies and assembled into 3D architectures to carry out multistep bioassays for clinical diagnostics. Because of the low cost production of these systems, they can be configured into disposable formats appropriate for point-of-care (POC) testing and, when fabricated into multichannel formats, high-throughput applications in clinical laboratories. In this presentation, a general description will be offered concerning the use of polymer-based microfluidics for building highly integrated molecular diagnostic systems for monitoring the presence of a variety of biomarkers, such as rare point mutations, proteins, and mRNAs. In addition, transport phenomena, both electrokinetically and hydrodynamically actuated flows, in these polymer-based systems will be presented. The individual modules we have developed consist of high-speed thermal cyclers (for PCR and allele-specific ligation assays [LDRs]), solid-phase extraction chips (for nucleic acids and proteins), and microarrays assembled into polymer microfluidic chips. The system was evaluated by analyzing point mutations in oncogenes, which provided high diagnostic value for breast cancer. The construction of the fully integrated flow-through bioprocessor consisted of a module to isolate and extract genomic DNA from whole cell lysates, PCR amplify the genomic DNA, score the presence of point mutations using LDR, and monitor the presence of the LDR products using a universal array microchip. The microsystem comprised three different modules containing passive elements: a polycarbonate (PC) chip for cell lysis and DNA preconcentration/isolation from a crude whole cell lysate, a PC chip for performing thermally driven PCRs and LDRs, and a poly(methyl methacrylate) chip for the detection of the LDR products using a medium density DNA universal microarray. The microarray was patterned on a polymer planar waveguide to allow imaging the entire array onto a CCD camera. With this fluidic system, a molecular assay could be completed in less than 10 minutes starting from whole cell inputs.

Microfluidic Isolation and Molecular Analysis of Tumor Cells Award CA88364

Peter Gascoyne Department of Molecular Pathology, University of Texas M.D. Anderson Cancer Center, Houston, Texas

To isolate rare tumor cells from peripheral blood and cancer cell subpopulations from tumor biopsies, we developed and refined a chamber system for dielectric flow fractionation (DFF) by combining dielectrophoresis and field-flow fractionation. This approach harnesses the sensitive discrimination of field-flow fractionation to exploit cell dielectric differences (which sensitively reflect cell morphological properties) and cell density. Sorting by these intrinsic cell properties does not require the tumor or blood cells to be labeled. Using DFF for rare cell isolation experiments, tumor cells salted into peripheral blood were recovered with 80% efficiency regardless of the starting tumor cell concentration. Ten milliliters of peripheral blood specimens (containing approximately 2.5 x 107 PBMNCs) were seeded with tumor cells at ratios from 103:1 to 105:1. After fractionation, the tumor cell-bearing fractions contained approximately 103 residual PBMNCs mixed with 80% of the starting tumor cells from the specimen. With different settings, the DFF was able to fractionate tumor cells from trypsin-digested tumors and cultures to provide isolates of tumor cell subpopulation having distinct cell morphologies. The isolated tumor subpopulations ranged from fractions having small, putative tumor stem morphology, through modal tumor cells, to tumor cells having large, aberrant morphology and nuclei. We also developed an enhanced form of the fractionation method that incorporates a periodic magnet to allow trapping of magnetic antibodies in addition to the cell morphological and density discrimination capabilities of the basic device. This capability allowed residual PBMNCs to be removed from rare tumor cell isolates via CD45 pan-leukocyte antibodies and might be used to target tumor cell-specific or other cell antibodies if needed. An important feature of our method is that it has the capacity to sort specimens ranging from very small numbers to tens of millions of cells within a single chamber in the same timespan and provide viable fractions for analysis and, under sterile conditions, for culture. In this presentation, the theory and implementation of the technology will be shown together with results for rare cell isolations and tumor cell subpopulation analysis.

Alternative Inclusion of FGFR2 Exon IIIc in Dunning Prostate Tumors Reveals Unexpected Epithelial Mesenchymal Plasticity

Sebastian Oltean[1,4], Brian S. Sorg[2], Todd Albrecht1,[4], Vivian I. Bonano1,[4,5], Robert M. Brazas1,[4], Mark W. Dewhirst[2], Mariano A. Garcia-Blanco1,[3,4]

[1]Department of Molecular Genetics and Microbiology, [2]Department of Radiation Oncology, [3]Department of Medicine, [4]Center for RNA Biology, 5University Program in Genetics and Genomics, Duke University Medical Center, Durham, North Carolina

In epithelial cells, alternative splicing of FGFR2 transcripts leads to the expression of the FGFR2(IIIb) isoform, whereas in mesenchymal cells, the same process results in the synthesis of FGFR2(IIIc). Expression of the FGFR2(IIIc) isoform during prostate

tumor progression suggests a disruption of the epithelial character of these tumors. To visualize the use of FGFR2 exon IIIc in prostate AT3 tumors in syngeneic rats, we constructed minigene constructs that report on alternative splicing. Imaging these alternative splicing decisions revealed unexpected mesenchymal-epithelial transitions in these primary tumors. These transitions were observed more frequently where tumor cells were in contact with stroma. These transitions were frequently observed among lung micrometastases in the organ parenchyma and immediately adjacent to blood vessels. Our data suggest an unforeseen relationship between epithelial mesenchymal plasticity and malignant fitness.

Phosphoprotein Proteome Profiling by PA-GeLC-MS/MS

Kolbrun Kristjansdottir[1], Don Wolfgeher[1], Tamara Nyberg[2], Brian J. Agnew[2], Stephen J. Kron[1]

[1]Center for Molecular Oncology, University of Chicago, Chicago, Illinois; [2]Molecular Probes-Invitrogen, Eugene, Oregon

While individual phosphoprotein biomarkers of cell proliferation have provided useful reporters for cancer diagnostics, therapeutic monitoring, and drug discovery, interrogating global protein phosphorylation would provide richer information about cell signaling. Our goal is to develop a direct method to catalog global phosphorylation events and their patterns via an unbiased and label-free approach, amenable to use in the clinical setting. Two-dimensional gel electrophoresis provides patterns, but identification of phosphoproteins is problematic. Phosphospecific antibodies are highly specific and sensitive reagents but detect only a fraction of known phosphorylation sites. To take advantage of the power of mass spectrometry for phosphoproteomic profiling, we have developed a streamlined protocol dubbed PA-GeLC-MS/MS, combining phosphoaffinity protein purification, 1D SDS PAGE gel electrophoresis, and proteolysis before reverse-phase liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS).

In most mass spectrometry phosphoproteomic studies, phosphopeptides are purified from tryptic digests of whole cell extracts and subjected to LC-MS/MS analysis to generate lists of phosphorylation sites. However, enrichment of the native phosphorylated proteins before digestion and LC-MS/MS analysis can provide greater confidence in protein identification as well as the identities of high-affinity binding partners. To develop phosphoproteome profiling, we exploited a commercially available phosphoprotein affinity matrix, Pro-Q Diamond resin (Molecular Probes-Invitrogen), to quantitatively retain phosphoproteins and binding partners from cell extracts. PAGE separation of the eluates followed by proteolysis and ESI LC-MS/MS analysis allowed straightforward identification of phosphoproteins and a subset of their phosphorylation sites. Importantly, as proteins were often represented by two to five different peptides, we could apply stringent criteria for identification. Requiring at least two peptides with >95% confidence, over 100 proteins were identified with >99% confidence from one gel lane derived from Pro-Q purification of a yeast cell extract. The majority were known

phosphoproteins or their binding partners. Current challenges include improving quantitation of phosphoprotein abundance, detecting lower abundance proteins, and developing tools for data analysis and pattern recognition. By combining PA-GeLC-MS/MS phosphoprotein profiling and cell synchronization with stable isotopic labeling (SILAC), we are defining the cell cycle-regulated phosphoproteome. Extending this analysis to cancer cell lines will allow detection of phosphoprotein signatures associated with deregulated cell proliferation, activation of oncogenic signaling, and the response to signal transduction inhibitor drugs.

Automated Cell Preparation in Tubes for 3D Microscopy

Mark R. Holl1, Thomas Neumann[3], Melissa W. Crawford[3], Shawn McGuire[1], Mark Fauver[3], Michael G. Meyer[3], Florence W. Patten[3], Alan C. Nelson[3], Eric Seibel[2], Deirdre R. Meldrum (PI)[1]*

[1]Genomation Laboratory, Department of Electrical Engineering, University of Washington, Seattle, Washington; [2]Department of Mechanical Engineering, University of Washington, Seattle, Washington; [3]VisionGate, Inc., Gig Harbor, Washington

We present progress toward the development of an automated instrument that when completed will process human samples (e.g., body fluids, fine-needle biopsies) for imaging with the Optical Projection Tomography Microscope (OPTM) with high quality, high repeatability, and high throughput. The method differs from traditional (slide-based) microscopy by having the cells embedded in an optical gel and injecting in a rotating microcapillary tube that allows for imaging of the cell from many angles. The sample preparation method is planned for use in lung cancer screening using sputum and also for the detection of precancerous conditions in cells isolated from sputum samples of high-risk patients. To date, lung cancer can be cured only when detected in very early stages.

Methods and materials that can be transferred to the design of the automated preparation instrument and disposable components were examined. Specific emphasis was given to preparing human sputum samples. Using experimental setups designed to characterize automation feasibility within the methods and materials constraints, we examined each step of the automation protocol from specimen debulking, fixing, staining, eliminating debris and contaminating cells, embedding of the cells of interest into optical gel, and cell type classification. While sputum samples can be difficult to process, in our filtration experiments with simulated sputa, we were able to capture between 62% and 92% of epithelial cells, which represented the target cell count for lung cancer screening. In parallel with the validation experiments, feasible conceptual designs for disposable integrated sputum sample processing cassettes were developed. The results and designs illustrate the potential for automated processing of complex body fluid samples for analysis with the emerging OPTM technology.

Solid-Phase Assays of Oncogenic Tyrosine Kinase Activity for Use in Diagnostics and Drug Discovery

Stephen Kron[1], Stephen Kent[2], Wendy Stock[3], Sean Palecek[4], Ding Wu1, Laurie Parker[3], Jennifer Campbell[1], Amanda Hauser[1], Dorie Sher[2], Matthew Myers[2], Shariska Petersen[1], Britton Walker[1], Chantel Johnson[1]*

[1]Center for Molecular Oncology and Departments of [2]Biochemistry and Molecular Biology and [3]Medicine, University of Chicago, Chicago, Illinois; [4]Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, Wisconsin

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 mesylate (IM, 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, IM induces remission, but patients may develop resistance, leading to recurrence.

Our work is directed toward delivering a robust clinical assay for Bcr-Abl activity and inhibition to guide therapy. Our approach has been to incubate immobilized peptide Bcr-Abl substrates with cell extracts and ATP, with or without inhibitors, and quantitate specific tyrosine phosphorylation. Progress to date has contributed to four papers published and four patent applications.

Year 2 of the development phase has led to further analysis of the determinants of sensitivity and specificity of Bcr-Abl substrates. We have developed novel methods for acrylate labeling of thiols to activate peptides for immobilization. Exploiting this chemistry, we have adapted hydrogel-coated multiwell plates for high-throughput screening for new Bcr-Abl inhibitors. We have also combined acrylate labeling and co-polymerization methods with photocleavable peptides to develop a solid phase detector with MALDI-TOF MS read-off. For this application, we developed novel methods for quantitation of phosphorylation in MALDI-TOF MS. Current work is directed at adapting our methods to other formats, such as Luminex Bead Array assays.

Microfluidic Platform for Live Cell Screening

Philip Lee CellASIC Corporation, Richmond, California*

We have developed an innovative microfluidic chip for multiplexed cell culture and high-content analysis. The current chip enables a researcher to control 16 independent perfusion experiments on adherent cells over time periods of days to weeks. The design of the microfluidic culture regions and nutrient flow paths resemble the physiological microcirculation and ensure uniform culture conditions while eliminating cross-

contamination. Our microfabrication process enables reliable production of features down to 1 micron, with a cost per data point over 10 times lower than that of current flow cell systems. This chip has been used to demonstrate cytotoxicity of anticancer drugs on cultured cell lines. An alternate geometry has also been used to create and maintain well-defined multicellular tumor models in a multiplexed microfluidic format. Because of the small volume requirements for the microfluidic chip, multiplexed screening of primary cell cancer models can be readily implemented. The continued development of microfluidic tissue culture technologies will enable more accurate in vitro results for cancer cell screening experiments.

Facile Characterization of Protein Structure/Function With Enhanced Peptide Amide Hydrogen-Deuterium Exchange Mass Spectrometry

Virgil Woods University of California, San Diego, La Jolla, California*

Our efforts focused on the development and refinement of enhanced peptide amide hydrogen-deuterium exchange mass spectrometry (DXMS) methodology into a robust, high-throughput tool for the characterization of protein structure and function. This presentation will review the principles that underlie DXMS and present exciting applications, including the facilitation of protein crystallization for structural studies, definition of protein dynamics and conformational changes, and characterization of protein-protein and protein-small molecule interfaces.

DXMS analysis of proteins can rapidly identify the subset of peptide amides within a protein that are highly solvated, both those present in disordered regions of the protein and those in structured, but surface-disposed regions. Attempts at the crystallization of proteins for 3D structure determination often fail, and the presence of disordered regions within otherwise well-structured proteins is thought to play a role in many of these failures. We have found that DXMS can be used to identify disordered regions in proteins and that this information is an effective guide to redesigning protein constructs, selectively depleted of disorder, that crystallize well.

The increasing DXMS throughput afforded by our methodological improvements allowed us to provide access to DXMS analysis for more than 25 collaborating laboratories in the past year. The result to date has been truly remarkable productivity in using DXMS analysis to address critical structure/function issues in a number of proteins important to our understanding of a variety of diseases, including cancer. In just the past year, we have published 10 reports describing the results of these studies. Furthermore, our methods for DXMS-guided crystallographic construct design are now in successful use by other groups. Three U.S. Patent applications have been filed and prosecuted by the University of California, and one of these has been licensed to the pharmaceutical industry (see http://www.exsar.com/construct_optimization.shtml).

We will also discuss the status of our most ambitious application to date, to employ DXMS data alone to determine the high-resolution 3D structures of proteins, including membrane proteins.

Age-Dependent Accumulation of Recombinant Cells in the Mouse Pancreas Revealed In Situ by Fluorescence Imaging

Bevin Engleward Massachusetts Institute of Technology, Cambridge, Massachusetts*

Mitotic homologous recombination (HR) is critical for the repair of double-strand breaks. Conditions that stimulate homologous recombination are associated with an increased risk of deleterious sequence rearrangements that can promote cancer. Because of the difficulty of assessing HR in mammals, little is known about HR activity in mammalian tissues or about the effects of cancer risk factors on HR in vivo.

To study HR in vivo, we have used Fluorescent Yellow Direct Repeat mice, in which an HR event at a transgene yields a fluorescent phenotype. Results show that HR is an active pathway in the pancreas throughout life, that HR is induced in vivo by exposure to a cancer chemotherapeutic, and that recombinant cells accumulate with age in pancreatic tissue. Furthermore, we developed a novel in situ imaging approach that reveals an increase in both frequency and size of isolated recombinant cell clusters with age, indicating that both de novo recombination events and clonal expansion contribute to the accumulation of recombinant cells with age.

This work demonstrates that aging and exposure to a cancer chemotherapeutic increase the frequency of recombinant cells in the pancreas and provide a rapid method for revealing additional factors that modulate HR and clonal expansion in vivo.

An Instrument for High-Throughput Microdissection

John Welsh Sidney Kimmel Cancer Center, San Diego, California

We have devised an instrument for high-speed microdissection. This instrument takes, as its input, a thin section mounted on a plastic slide. Photomicrographs of the entire thin section are collected by moving it in a rectangular grid pattern beneath the objective of an autofocusing microscope with a camera. These are then stitched together into a composite image. The thin section and the plastic slide upon which it is mounted are then moved under a punch-like mechanical knife, which is beveled to one corner. The slide rests upon a flat plastic receiving plate, which has the length and width of a standard microtiter plate. As the knife descends, the lowest corner presses the thin section and plastic slide against the receiving plate, and as it continues to descend, it cuts off a piece of the slide and drives it into the receiving plate, forming a well in the process. The slide and the receiving plate are incremented appropriately, and the cycle repeats. This process is very fast. With our prototype instrument, we can approach 80,000 cuts per day, and 50-micron square cuts are easy to achieve. Each receiving plate can hold 96, 384, or 1,536 samples in the standard configurations, and when one plate is full, it is automatically exchanged with a fresh plate. The instrument has software facilities that allow one to draw polygons around features of interest in the composite image and translate these pixel coordinates into the plate and well

coordinates that hold the corresponding tissue voxels. These coordinates can then direct a liquid handling workstation for sample collection. This device should be useful for studying intratumoral heterogeneity, as well as macromolecule distributions in developing embryos.

Tissue Print Micropeels for Molecular Profiling of Cancer: Application to Prostate Needle Biopsies

Sandra Gaston Beth Israel Deaconess Medical Center/Harvard Medical School, Boston, Massachusetts

Sample preparation technologies serve as a foundation for the translation of state-of-the-art molecular profiling from the basic research “discovery” laboratories into multicenter clinical trials and ultimately into general clinical applications. With support from the NCI Innovative Molecular Analysis Technologies (IMAT) program, the Gaston laboratory has developed a set of novel “tissue print micropeel” technologies that allow us to profile multiple molecular markers from human tissue and tumor samples without damaging the specimen. The basic “tissue print” technique is straightforward and easily mastered: Cells and extracellular matrix components are transferred from a tissue surface onto a nitrocellulose membrane, forming a “molecular Xerox” of the specimen. Our technologies couple this basic tissue sampling concept with a series of advanced biomarker detection techniques that allow us to generate a series of spatially oriented molecular maps of the surgical specimen or tissue biopsy. These marker maps can be superimposed directly onto histopathology and radiological images, permitting molecular identification and classification of individual malignant lesions.

As a proof-of-principle application, we are formatting our tissue print micropeel techniques as a platform technology for multi-analyte molecular biomarker analysis of prostate needle biopsies. Prostate biopsies represent a class of clinical specimens that are rarely available for molecular profiling studies; each tissue core must be submitted in its entirety for review by the surgical pathologist. The prostate biopsy population is significantly different from the smaller subgroup of patients who undergo radical prostatectomy, and this has important implications for the clinical application of prostate cancer biomarker research. Specifically, both the “no cancer” and the “advanced cancer” populations are missing from the most common source of tissue bank samples used in prostate cancer research: radical prostatectomy specimens. In order to support the translation of our IMAT sample preparation technologies into a format that will satisfy the requirements of this important and technically challenging clinical application, we have recently opened a protocol for prospective collection of tissue prints from prostate biopsies; this study enrolls a clinically representative patient population in an outpatient office setting. In the first phase of this study, each of the biopsy print micropeel samples is processed for simultaneous extraction of protein, RNA and DNA biomarkers; these fractions are used to evaluate a series of protocols suitable for biomarker discovery and/or sample banking.

This “multi-analyte” approach has proven particularly useful in the evaluation of a newly

identified class of prostate cancer biomarkers that arise from gene fusions associated with tumor-specific chromosome 21 rearrangements. Specifically, our tissue print micropeel technologies have allowed us to perform detailed molecular analyses of chromosome 21 gene fusion markers in our biopsy cohort; importantly, this group represents a patient population of high interest that would ordinarily be missing from this type of tissue-based molecular biomarker study. The IMAT Program has played a significant role in the formation of important collaborations that have advanced our vision for the tissue print micropeel technologies. Because the collection and processing of samples suitable for analysis of RNA-based tumor markers present a particular challenge in most clinical settings, the Gaston and Latham IMAT research teams formed a collaboration with the ultimate goal of combining two innovative technologies as a semi-automated sample preparation platform that is well suited for clinical trials and for routine applications of RNA-based diagnostics. In studies preliminary to a proposed R33 phase project, we have demonstrated that RNA and DNA can be purified from tissue print micropeel samples using a modified protocol built around the Ambion/Asuragen MELTTM (Multi-Enzyme Liquefaction of Tissue) reagent and magnetic RNA capture technologies. 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 the need for simple, effective methods to stabilize samples at the site of collection. Our proposed R33 objectives for this collaboration focus on the tasks required to bring our innovative sample preparation platform into the larger cancer research community, using the prostate needle biopsy application to demonstrate the unique capabilities of these technologies for cancer biomarker research and clinical applications.

Recovery of Protein From Formalin-Fixed Paraffin-Embedded Tissue

Timothy O'Leary Veterans Health Administration, Armed Forces Institute of Pathology and American Registry of Pathology, Washington, D.C.*

The overall goal of this project is to improve our understanding of how protein fixation and defixation are affected by the dehydration and embedding steps of tissue processing. The first of the specific aims is to use tissue surrogates and mass spectrometry to determine the specific formalde-induced chemical modifications that occur during processing, while the second is to use biophysical, functional, electrophoretic, and immunologic methods to characterize recovered proteins.

Two approaches have been used in these investigations. First, we have investigated proteins (ribonuclease A and lysozyme) that have been reacted with formaldehyde in aqueous solutions. Second, we have utilized proteins that have been fixed at high concentrations, which form plugs that can be dehydrated and embedded under conditions similar to those used in typical histologic processing. Using the first approach, we have obtained MS/MS data that demonstrate unequivocally that prolonged fixation in formaldehyde results in formation of more adducts than does short-

duration fixation. We have further shown that room-temperature antigen retrieval processes result in removal of formaldehyde from fixed proteins. These findings are confirmed by electrophoresis experiments that show the formation of protein multimers after low-concentration ribonuclease A is reacted with formaldehyde, which would be expected as a result of cross-linking. These cross-links are readily reversed by incubating the proteins at elevated temperatures, which is a technique that is widely used to restore immunogenicity of antigens for immunohistochemical analysis.

Using the tissue surrogate approach, we have established that the process of dehydration with alcohols and embedding results in additional chemical changes that greatly increase the stability of the cross-links formed during fixation. The cross-links that follow dehydration are not so readily reversed as those that are formed during aqueous fixation. We have explored a variety of techniques for recovering proteins from these tissue surrogates. Although incubation at very high (121 °C) temperatures in Tris/SDS solutions results in considerable recovery, electrophoresis shows significant protein degradation. Lower temperature incubations reduce degradation, but at the cost of significantly lower recovery. Mass spectrometric experiments that further characterize the cross-links formed during tissue dehydration are currently under way.
* Speaker

NCI's Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) Programs

Michael Weingarten, Andrew J. Kurtz, Sheri Schully, Alok Doshi Office of Technology and Industrial Relations, National Cancer Institute, Bethesda, Maryland

The goal of the National Cancer Institute (NCI) is to eliminate the suffering and death due to cancer. The Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) programs address this goal by serving as NCI's engine of innovation for developing and commercializing novel technologies and products to prevent, diagnose, and treat cancer. The SBIR and STTR programs both seek to increase the participation of small businesses in Federal research and development; however, the unique feature of the STTR program is the requirement for the small business to formally collaborate with a research institution.

These programs support cancer research and technology development across several broad topics including anticancer agents, biomarkers, informatics, medical devices, nanotechnology, proteomics, health communications, and many other biotechnologies and programs. Companies are invited to apply first for a Phase I award of up to $100,000 to test the scientific, technical, and commercial merit and feasibility of a particular concept. If Phase I proves successful, the company may apply for a 2-year Phase II award of up to $750,000 to further develop the concept, usually to the prototype stage. All proposals are judged competitively on the basis of scientific, technical, and commercial merit. Following completion of the Phase II award, small businesses are expected to obtain funding from the private sector and/or non-SBIR or STTR government sources to develop the concept into a product for sale in the private

sector.

SPEAKER ABSTRACTS

Imaging Transcriptional Activation in Gliomas

E.A. Chiocca, Sean Lawler, Yoshinaga Saeki, Jakub Godlewski, Hiroshi Nakashima Dardinger Laboratory for Neuro-Oncology and Neurosciences, Department of Neurosurgery, Comprehensive Cancer Center, Ohio State University Medical Center, Columbus, Ohio*

Imaging of transcriptional activity of genes involved in brain tumor invasion could become a useful tool to assay gene-phenotype correlations in this process. SAGE (serial analysis of gene expression) has provided data related to genes that are up-regulated in glioma. Additionally, we have used a quantitative RT-PCR screen to validate genes up-regulated in a spheroid model of invasion. As a first molecular imaging target, we have selected the gene for secreted protein acidic and rich in cysteine (SPARC). To confirm that SPARC is made by gliomas, we confirmed that SPARC is secreted at various levels in glioma cells, fresh glioma tissue, and astrocytes. We also show up-regulation of SPARC transcription in human glioma specimens grown as tumor spheroids during the process of invasion. We have cloned the SPARC 5' transcriptional responsive element in our iBAC genomic vector upstream of imaging reporter genes for GFP, RFP, and/or luciferase. We will employ this vector to image transcriptional activation of the SPARC gene during gliomagenesis.

A Chip-Based RNA Sensor Platform for Detection of Circulating Tumor Cells

Gary A. Clawson[1], Rustom Bhiladvala[2], James Sioss[3], Weihua Pan1, Mingwei Li[2], Ping Xin, Susan Patrick[1], Susan Trolier-McKinstry[4], Chris Keating[3], Theresa Mayer[2]*

[1]Department of Pathology and Gittlen Cancer Research Foundation, Penn State University, Hershey Medical Center, Hershey, Pennsylvania; [2]Electrical Engineering and Materials Research Institute, Penn State University, State College, Pennsylvania; [3]Chemistry Department, Penn State University, State College, Pennsylvania; [4]Ceramic Science and Engineering and Materials Research, Penn State University, State College, Pennsylvania

We are developing a chip-based sensor platform, with an initial application to detect tissue/tumor-specific RNAs isolated from circulating tumor cells (CTCs) in blood. A porous barrier density gradient centrifugation technique is used to isolate a population of cells greatly enriched for CTCs, and RNA is extracted. Target RNAs have been selected for various cancers (prostate, melanoma, and breast initially), cloned and expressed, and optimally accessible sites on the full-length target RNAs have been defined by SELEX-based library selection protocols. We are currently quantifying transcript numbers/cells, recovery of spiked tumor cells from blood, and detection limits

using QPCR.

The format for the detection platform consists of a “sandwich” hybridization assay. One antisense oligonucleotide (ASO1) targeted to a library selected site is covalently attached to a silicon nanowire (SiNW), and a second ASO2, targeted to another library selected site on the target RNA, is covalently attached to a 12-nm Au particle. Following hybridization, a sandwich is formed, consisting of the SiNW-ASO1:target RNA:ASO2-Au particle, the latter of which greatly increases the mass change upon binding while requiring a second specific hybridization. Using microarrays in an analogous approach “off-chip,” we are defining many parameters, including hybridization conditions, which provide for excellent (single nucleotide) specificity.

We are initially working with an optical detection strategy; specifically, the SiNW resonate with a characteristic resonance frequency. Upon binding of the target RNA to the ASO1-SiNWs, this resonance frequency shifts. The shift in resonance frequency is proportional to the number of binding events and is greatly amplified by the coupled binding of the ASO2-Au particle. We have achieved excellent quality (Q) factors for the resonance peaks in high and modest vacuums, which remain very good up to essentially atmospheric conditions, and can easily measure the resonance shifts induced by the mass changes. We are also beginning to work on piezoelectric detection methods, which will utilize PZT-coated SiNWs and multi-layer core shell structures. These materials possess large piezoelectric response, so that an electrical charge is developed across the oxide, which is proportional to the strain induced on the thin film, which can be directly converted into a voltage signal (which could provide mV level signals). The modified SiNWs are being electrofluidically deposited on CMOS chips using a “bottoms-up” integration strategy, which will allow us to multiplex for many different cancer RNA markers as desired. Using microarrays off-chip, we have demonstrated that the modified SiNWs survive the conditions necessary for chip manufacture (photoresist removal, etc.), retaining their hybridization properties intact.

Development of High-Throughput Methods for the In Vitro Selection of RNA Aptamers That Can Distinguish Between Tumor Cell Types

Andrew Ellington University of Texas, Austin, Texas*

Since 1998, our laboratory has advanced methods for the automated selection of RNA aptamers that bind to protein targets with high specificity and subnanomolar affinity. We now routinely use our Biomek 2000 liquid handling robots to simultaneously select aptamers against multiple protein targets within 2 to 3 days. A bottleneck, however, in applying our automated in vitro selection technology for the high-throughput production of aptamers for cancer diagnosis has been the difficulty and expense in obtaining a steady stream of purified cancer biomarker target proteins. Accordingly, we have used our National Cancer Institute funding to directly select aptamers against prostate cancer biomarker molecules on the surfaces of cultured mammalian cells. We have employed this scheme in the selection of two sets of aptamers that bind specifically to one of two

different prostate tumor lines, LNCaP and PC3 cells. LNCaP cells overexpress this surface marker, whereas PC3 cells have very little PSMA on their surfaces. Based on our success in selecting aptamers directly against PSMA-expressing mammalian cells, we have begun selections against three lung tumor cell lines that differ in their epidermal growth factor receptor (EGFR) expression. We are now adapting our automated methods to establish a pipeline for the direct selection of RNA aptamers against various cancer cell surfaces with the goal of developing highly multiplexed assays for cancer in vitro diagnostics.
* Speaker

Proximity Ligation Assays for the Specific Detection of PSMA-Expressing LNCaP Prostate Cancer Cells

We have developed proximity ligation assays that employ aptamers to identify PSMA-expressing cells. Anti-PSMA aptamer A9 was extended during chemical synthesis with new sequences at either its 5' or 3' end, and DNA oligonucleotides that were complementary to these extensions were hybridized to the constructs. The adjacent binding of the two aptamer:DNA conjugates on a cell surface enabled the ligation (using a splint oligonucleotide and T4 DNA ligase) and subsequent generation of a signal amplicon that could be detected by real-time PCR. PLA assays were carried out using the anti-PSMA aptamer:DNA conjugate and both PSMA-positive LNCaP cells versus PSMA-negative PC3 cells. In order to optimize the assays, three different splint concentrations of 400 pM, 40 pM, and 4 pM were used along with an aptamer-probe concentration gradient ranging from 1 nM to 0.1 pM. For almost all splint and aptamer:DNA concentrations, LNCaP cells were detected by PLA and real-time PCR earlier than the PC3 cells. Positive signals were analyzed as a function of cycle threshold (C[T]) values; delta C(T) values were calculated by subtracting C(T) values obtained from reactions without cells from C(T) values obtained from reactions with cells. PSMA-overexpressing LNCaP cells showed a delta C(T) of up to 10 cycles, which is an extremely large shift relative to most signals seen in real-time PCR assays with small analyte concentrations. We then used PLA to detect 102 LNCaP cells in a mixture with 105 HeLa cells. The sensitivity of detection with samples containing mixtures of cells was not only comparable to that of samples containing only LNCaP cells, with a delta C(T) of 7. Non-cognate HeLa cells on their own were not detected by PLA. These results demonstrate the utility of aptamers for PLA, and the sensitivity and specificity of PLA reactions in general.

Specific, Label-Free Detection of Proteins Using DNA Aptamers With Rolling-Circle Amplification Fluorescent Signal Generation

We have adapted rolling-circle amplification (RCA) fluorescent amplicon reagents to function with DNA aptamer probes for the specific detection of platelet-derived growth factor (PDGF). An existing anti-PDGF aptamer was synthesized with a 5' monophosphate, and with an original 13-nt sequence appended to the 3' end. In the

absence of PDGF, the 13-nt sequence region anneals to a complementary sequence within the aptamer domain, thereby stabilizing it in an inactive hairpin structure. In the presence of PDGF, the engineered aptamer undergoes conformational change from a hairpin structure to its native conformation and displaces the 3' complementary sequence region. As a result, the 3' and 5' ends are brought into close proximity and then ligated by T4 DNA ligase. The two designed linker regions connecting the 5' substrate and the 13-nt sequence to the aptamer form a ring structure that serves as a template for RCA amplification. The resulting double-stranded DNA product from RCA was stained with SYBR Green in the reaction mixture to generate a fluorescent signal that can be read in our ABI 7300 RT-PCR machine. This approach yielded aptamer-RCA sensors that could linearly detect PDGF across a range of 1.6 to 80 nM in solution.

Analysis of Genetic and Epigenetic Alterations in DNA Archives Generated From Tumors of the Head and Neck

*Sebastian Szpakowski1, José M. Lage1, Jill Rubinstein1, Xueguang Sun2, Andrew Dyer1, Katie Crist1, Diane Kowalski1, Michel Krauthammer1, David Tuck1, Perry Miller4, Hongyu Zhao2, Janet Brandsma1, Clarence Sasaki3, Jose Costa1, Paul M. Lizardi1**

Departments of 1Pathology, 2Epidemiology and Public Health, 3Surgery, and 4Medical Informatics, Yale University School of Medicine, New Haven, Connecticut

We are using head and neck cancer as a model to establish and validate procedures for DNA archiving and molecular analysis of neoplastic lesions. Samples of head and neck malignancies are being collected prospectively. Archival DNA is stored before and after whole genome amplification. The amplified, archival DNA samples have been assayed for human papillomavirus (HPV) infection. The samples have also been processed for analysis of gene losses and gains, using array-CGH in high-density NimbleGen microarrays. Unamplified DNA from the same tumor DNA archives has been analyzed using a custom NimbleGen microarray and a protocol designed to report changes in DNA methylation status. A unique feature of the method is its ability to report methylation changes for loci that map within repetitive DNA domains. Such loci represent nearly 50% of the human genome and have formerly resisted analysis using microarray approaches. The microarray profiles comprise relative methylation levels at 25,000 promoter CpG islands, 46,000 nonpromoter-associated CpG islands, and over 200,000 CpG islands mapping to interspersed repeats or tandem repeats. A subset of the DNA methylation data has been validated by bisulfite PCR analysis of CpG islands at gene promoters, at LINE-1 elements, at Alu repeats, and at LTR elements. Methylation changes include striking hypermethylation of a number of candidate tumor suppressor genes and often comprise clusters of several linked genes within a chromatin neighborhood. Abnormal hypomethylation in tumor DNA shows a recurrent, highly prevalent, and complex component comprising CpG islands associated with interspersed repeat loci. To our knowledge, the data underscore for the first time the complexity of the loss of DNA methylation at multiple repetitive DNA loci in tumors and

reveal the existence of a highly complex epigenetic framework that is now accessible to study using relatively small DNA samples. Given the potential for tumor classification based on the pattern of abnormally methylated repeat loci, this novel approach opens new avenues for a large-scale discovery effort based on nonrepeat-masked DNA methylation analysis in any human cancer. The datasets showing DNA methylation abnormalities in head and neck squamous cell carcinoma tumors are being combined with the gene locus gain/loss information obtained by array-CGH, to enable improved tumor class comparison, class discovery, and class prediction. Computational and statistical tools are being used to construct classification schemes based on distance-based trees, as well as different clustering algorithms, utilizing the complete dataset of array-CGH, DNA methylation, and HPV infection status observations. One of our goals is to derive a conditional risk model identifying those patients who are most likely to develop additional head and neck cancers in the future.

Detection and Identification of Cysteine Sulfenic Acids in Proteins Involved in Signal Transduction Pathways

Leslie Poole Wake Forest University School of Medicine, Winston-Salem, North Carolina*

With this new era of biological discovery introduced by the availability of a molecularly detailed view of an increasing number of genomes, new technologies, which offer us the ability to leverage this sequence-based knowledge with information about actual functional states of proteins and the intricacies of their regulatory networks in cells, are the next major thrust of biomedical research. Within the realm of cancer research, the largest impacts are likely to be felt through a better understanding of both the biological regulatory networks that underlie signal transduction, and of the processes that lead to the progressively developing disease states in individuals with cancer. Toward these goals, microarray and proteomic analyses are being used to assess mRNA and protein levels in various manipulated cell lines or patient samples. Complementary to those efforts, “functional proteomics” approaches are being developed. These evaluate not only the levels of proteins, but aspects of the proteins that report on their functional status, such as activity-based profiling or the presence and location of posttranslational modifications. Our premise for this project is that the evaluation of the “redox status” of proteins, particularly as it changes during the course of the transduction of an external signal through the cell's response network, provides critical “missing” information that can be used to construct a higher order understanding of the interplay between such regulatory (and transient) phenomena as phosphorylation and oxidation.

It is now widely accepted that reactive oxygen species are generated in response to growth factor or cytokine stimulation of cells through activation of mitogenic NADPH oxidases. Because cysteine is a highly sensitive and specific target for peroxide-mediated oxidation in some proteins, and the first metastable product of cysteine oxidation by hydrogen peroxide or peroxynitrite is cysteine sulfenic acid (Cys-SOH), this species represents an excellent target for discovering and identifying peroxide-

modifiable cysteine residues in cellular proteins. Our laboratories have been developing proteomics-friendly methodologies for detecting and identifying Cys-SOH-forming proteins using fluorescent or biotinylated tags attached to a dimedone analogue that is uniquely reactive toward Cys-SOH. In other efforts, bioinformatics tools are being developed to elucidate “signatures” of reactive sites in order to better understand the basis for specificity of given cysteines toward peroxide-mediated oxidation and to predict previously unknown reactive sites across the proteome. Although at an early stage, our research using these compounds indicates that an initial “burst” of Cys-SOH formation is observed within 1-2 minutes after addition of TNF-a to HEK-293 cells. A growing list of oxidized proteins that we have identified using 2D gel electrophoresis and mass spectrometry methods includes a number of signaling-relevant proteins (including both phosphatases and kinases) as well as proteins with other functions; the majority of the proteins identified so far have not previously been known to be redox regulated. Proteomics experiments such as these 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.

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Selective Profiling of Proteins in Cancer Cells From Fine-Needle Aspirates by MALDI-TOF Mass Spectrometry

Michelle Reyzer Vanderbilt University, Nashville, Tennessee*

Lung cancer diagnosis and classification is typically accomplished by examining hematoxylin and eosin (H&E) stained tissue biopsies or needle aspirates using light microscopy. This current classification system is of limited use in predicting patient outcome or individualizing therapy. We have in the past shown the potential utility of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) performed directly on tissue sections from surgical frozen tissue specimens in classifying tumors on the basis of their protein expression profiles. However, fine-needle aspirates are frequently the only samples available for making a clinical diagnosis, and these are usually heavily contaminated with blood and normal cells, making blind proteomic analysis impractical.

To further develop MS for quickly and accurately diagnosing and classifying clinical samples, we have developed a method to acquire mass spectrometric data selectively from clusters of tumor cells contained within fine-needle aspirates. After depleting samples of contaminating red blood cells (erythrocyte lysis buffer), cytocentrifugation onto MALDI time-of-flight (TOF) MS compatible metal-coated transparent glass slides and ethanol-based cell fixation, we can stain the sample with MALDI-compatible dyes to directly visualize clusters or tumor cells. Manual or automated application of a sinapinic acid matrix solution specifically to the identified clusters of cancer cells followed by targeted MALDI-TOF MS analyses results in the production of high-quality, cancer cell-specific protein profiles from these samples.

* Speaker

First, we have demonstrated a high correspondence between the protein signals detected from cancer cells within fine needle aspirates and those observed from the direct analysis of tissue sections from the corresponding tumor biopsies. Second, we have also demonstrated that the profiles recovered from fine-needle aspirates are highly reproducible when comparing results obtained from identical tumor types. Finally, the specificity of the approach was demonstrated by comparing the profiles obtained from two different tumor types. In this case, after a supervised hierarchical cluster analysis carried out on the top statistically significant markers, a clear separation between differing groups of samples was achieved with 96% accuracy.

This highly reproducible technique, although developed for quickly profiling proteins from cancer cell clusters in fine-needle aspirates from lung tumors, could be applied to many other types of cancer and is foreseen to be very useful for clinical diagnosis, classification, and potentially, the individualized treatment of cancer patients.

Sensing and Imaging Activated Fibroblasts in Tumor Stroma

*Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts Ching Tung**

Activated tumor stromal fibroblasts are needed for tumor growth since the very early phase. On the outer surface of these activated tumor stromal fibroblasts, a high level of fibroblast activation protein (FAP) was found, but not on epithelial carcinoma cells, normal fibroblasts, or other normal cells. Expression of FAP in transfected cell lines has been shown to enhance tumor growth in animals. Thus, FAP has been suggested as a unique molecular target for tumor detection and therapy. Inhibitors and antibodies against FAP are currently under clinical trials for cancer treatment and imaging. The goal of our research is to develop novel fluorescence probes to image FAP activity and apply the developed probes in tumor detection, tumor classification, and treatment evaluation.

FAP is a type 2 membrane-bound glycoprotein belonging to the serine protease gene family and is known to have exo-dipeptidyl peptidase activity. Its exo-peptidase activity is extremely similar to dipeptidyl peptidase-IV (DPP-IV), which is, unfortunately, circulated in the bloodstream. Thus, for in vivo imaging of FAP activity, substrate specificity is an essential requirement. Recently, in addition to the previously known exo-peptidase activity, endo-peptidase activity of FAP was also reported. Based on these unique endo and exo properties, novel near-infrared fluorescence probes with specific switches have been designed and synthesized. These probes emit minimum fluorescence in their initial intact states, but become brightly fluorescent after specific proteolysis by FAP. In vitro, the synthesized FAP probes can be activated only by FAP, not by DPP-IV. Greater than sevenfold signal enhancement was observed after FAP interaction. Initial in vivo experiments with a xenographic tumor model indicated that FAP activity could be clearly imaged in less than 2 hours. To our knowledge, this is the

first time that tumor stromal marker was imaged in vivo. Further optimization, evaluation, and application of the developed molecular probes are in progress.
* Speaker