Eighth Principal Investigators Meeting

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Innovative Molecular Analysis Technologies (IMAT) Program
July 24-25, 2007
Sofitel San Francisco Bay
Redwood City, CA

Abstracts

Multiplexed Methods for the Study of Chromosomal Aberration in Cancer

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The general aim of our project is to employ highly multiplexed, next-generation genomic sequencing technologies to map chromosomal aberrations in cancer. The proposal aims to develop methods that map rearrangements in pools of bacterial artificial chromosomes (BACs) and then scale the methods to the whole-genome level. This technology development project uses both the commercially available BAC library obtained from the well-established MCF-7 breast cancer cell line as well as genomic DNA from an extant MCF-7 cell line. The BAC library was obtained from the original tumor tissue and thus may lack aberrations that may have accumulated in the cell line over decades.

The next-generation technologies will allow mapping of extensive collections of tags sequenced from ends of clone inserts. Genomic aberrations in pooled BACs and whole genomes will be detected by analyzing patterns of tag mapping. The method will provide an extensive view of chromosomal aberrations across multiple cell lines and primary tumors at an unprecedented level of resolution. A dense set of mapped tags will allow precise delineation of fused genes and the design of PCR-based assays for economical detection of aberrant joins. The biological function of rearrangements will be tested by cloning and expression in both immortalized and transformed breast epithelial cells. Biological assays will depend on the rearrangement or breakpoints of interest but will focus on proliferation, survival, invasion, and transformation.

The developed methods will open new opportunities for understanding recurrent chromosomal rearrangements and molecular mechanisms, identifying drug targets, and developing biomarkers for effective tumor-specific therapy.

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

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Introduction: Histopathology plays a major role in current-day pathological diagnoses in which immunohistochemistry (IHC) is a critical tool for evaluating protein expression at a cellular level where morphological information is preserved. A drawback to IHC is that it has been impossible to predict which proteins or, more specifically, which epitopes can be unmasked for subsequent immunodetection. We present our findings cataloging indepth thousands of proteins and tens of thousands of potential epitopes unmasked under varying antigen retrieval (AR) conditions, including pH of the extraction buffer and application of heat during the AR process.

Methods: Paired fresh-frozen and formalin-fixed human liver samples were analyzed. The fresh-frozen sample was subject to extraction using RIPA buffer. The formalin-fixed samples were subject to AR using 2% SDS in Tris-HCl at varying pH, including 2, 7, 9, and 12. AR was performed by heating the samples at 100 °C 20 minutes then reducing the temperature to 60 °C for 2 hours. AR was performed on an additional pH 7 sample without heat. All samples were dialyzed to remove detergents, then denatured, reduced, alkylated, and digested with trypsin. The resulting peptide mixtures werefractionated by transient capillary isotachophoresis/capillary zone electrophoresis. Approximately 18 fractions were taken of each sample. Each fraction was then analyzed by nanoscale reverse-phase liquid chromatography coupled by electrospray ionization to a linear ion trap mass spectrometer performing tandem mass spectrometry. All samples were analyzed in duplicate. Tandem mass spectra were searched using the OMSSA algorithm and were thresholded to accept hits only below a 1% false discovery rate as determined by the decoyed sequence library search.

Results: Proteins recovered from formalin-fixed liver samples using the AR method compare favorably with those retrieved from fresh tissue using RIPA buffer. The fresh-frozen tissue yielded more than 3,500 proteins, and the pH and pH 9 heated extractions and the pH 7 unheated extraction each retrieved more than 2,500 proteins. The pH 2 and pH 12 extractions yielded fewer proteins, 2,300 and 1,400, respectively. Although clear differences were seen in the physical properties (pI and length) of the peptides extracted by each condition, very little difference was seen in the unique proteins extracted by each condition. That is, proteins from the worst performing extractions, pH 2 and pH 12, were almost wholly contained within the set of proteins extracted from the pH 7 heated and unheated extractions, which in turn were almost wholly contained within the set extracted from fresh-frozen tissue. In each condition, the predicted proteins showed no bias by molecular weight, isoelectric point, or hydrophobicity. Overall, more than 15,000 tryptic peptides, each corresponding to a potential antibody epitope, were identified for more than 3,000 proteins extracted from formalin-fixed human liver. This catalog offers the possibility of predicting which antigen epitopes are available for IHC under varying AR conditions. This is useful for minimizing the trial and error that is currently commonplace when performing IHC and also offers the possibility of raising new antibodies to specific epitopes known to be unmasked by the desired AR condition.

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

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Translational control plays a key role during development, cell cycle control, and mechanisms related to acute drug resistance. In particular, noncoding miRNAs can potentially regulate more than 30% of gene expression at the translational level. Gene expression analysis of actively translated mRNA transcripts provides a unique approach to study posttranscriptional regulation. The current method relies 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. This technology will make investigation of translational control feasible from limited clinical specimens.

Microfluidic Device for Isolating Rare Blood Cells

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The isolation of rare cells from peripheral blood is a difficult and tedious step in the diagnosis and treatment of cancer. Examples include leukocytes, which are of interest in leukemia; circulating endothelial cells, which can serve as a surrogate marker for solid-tumor treatment; and circulating cancer cells, which may provide a tool for diagnosis and prognosis. In most patients, each of these cell types is outnumbered by red blood cells by a factor of at least 1,000. Therefore, isolation of these cells from a sample of whole blood is the required first step of many clinical and basic research assays. We recently described a microfluidic device that takes advantage of plasma skimming and leukocyte margination—intrinsic features of blood flow in the microcirculation—to enrich nucleated cells such as leukocytes directly from whole blood. It consists of a simple network of rectangular microchannels manufactured using standard photolithography and silicone molding techniques and requires only pressure-driven flow to operate. Its initial channel is designed to enhance lateral migration of

spherical cells, which, once near the wall, are easily extracted through small extraction channels. In our preliminary design, a single pass through the device produced a 34-fold enrichment of the leukocyteto- erythrocyte ratio.

We propose to further develop the microfluidics to provide simple, efficient, and inexpensive technology for use as an initial stage in lab-on-a-chip analyses. Its integration into microanalytical devices that require rare cell enrichment will provide less expensive, more reliable clinical assays that are also convenient and portable for point-of-care testing. Specifically, we will maximize the purity and efficiency of nucleated blood cell isolation and compare the performance with traditional separation techniques. When fully developed, this technology will be a necessary and integral component of any microfluidic device analyzing nucleated blood cell populations by eliminating the need for preliminary blood processing steps.

Integrated Microdevice To Capture and Detect Circulating Tumor Cells

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Sensitive detection of earliest metastatic spread of tumor in a minimally invasive and user-friendly manner will revolutionize the clinical management of cancer patients. The current methodology for circulating tumor cell (CTC) capture and identification has significant barriers, including multiple procedural steps, handling of relatively large volumes of blood, substantial human intervention, extremely high cost, and, importantly, lack of reliability and standardization for the detection methods. We report the development and optimization of a novel parylene membrane filter microdevice with a manual syringe injection system for capturing CTCs from undiluted human peripheral blood, which is capable of greater than 80% recovery with high enrichment factor and outperforms most current methods used in the field. Moreover, less than 10 minutes is required for each CTC capture operation compared with current multistep processing requiring more than 1 hour. We have demonstrated a superior recovery rate in comparison with the FDA-approved Cellsearch® system. We have also characterized our filter-based microdevice with a pressure-controlled system in a model system using cultured cancer cells admixed in blood. The effects of changing flow rate, fixative concentration, fixation time, blood dilution factor, and delivery pressure on this novel filter system will be reported. In addition, both off-chip DNA and RNA amplification from captured tumor cells has been demonstrated to work successfully with the potential of on-chip integration. Blood drawn fromcastration-resistant prostate cancer patients have also been studied, and we have demonstrated the feasibility of using our microdevice to work with clinical specimens. This novel filter-based CTC enrichment device will provide a cost-effective method for CTC monitoring, with a higher recovery rate, faster processing time, and more reliable results due to minimal human intervention.

This work was supported by R21-CA123027-01. Funds for the project were made

available on 9/26/2006.

Sample Preparation Methods To Allow Automated Three-Dimensional Analysis of Microvessel Morphology

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We are developing sample preparation techniques that permit high-resolution analysis of tumorassociated microvasculature for the evaluation of vascular change in cancer. Source material will be tissue samples from animal models and fixed archival specimens. It is anticipated that these specimen preparation protocols will produce image data of sufficient quality, and at sufficient tissue depth, to enable automated three-dimensional (3D) computer analysis of vascular morphology within neovascularized peritumoral and viable tumor tissue. Vascular morphology abnormalities are characteristic of cancerous tumors and are associated with a switch from quiescent to aggressively invasive, metastatic behavior. Thus, angiogenic activity, as reflected by morphological microvascular change, is a critical point of assessment in cancer research. Visualization and quantification of microvascular attributes permit monitoring of disease progression and response to therapy. Yet currently available sample preparation methods to produce data at capillary-resolution and suitable for automated 3D quantitative analysis are not well established.

Our goal is to create and refine specimen preparation techniques that allow collection of highresolution 3D vascular image data of tumors and the supporting peritumoral tissue. Simultaneous immunohistochemical labeling of related molecules of interest will advance angiogenesis research in animal model systems. The desired outcome of this project is to develop specimen protocols that produce image data of sufficient quality to allow automated computer analysis of morphological attributes describing the 3D microvessel architecture associated with tumor growth and development.

Our methods will combine protocols used in vascular biology and adapt procedures developed in other fields, such as bioimaging, computer science, and medicine. To accomplish this task, we will undertake perfusion-based preparation methods, combined with optical clearing methods, to produce serial section image data used to generate 3D reconstructions of the microvasculature of whole-mount specimens, while preserving the ability to label other molecules of interest. We will first optimize perfusion-based preparation methods for vessel filling to provide robust imaging of microvascular beds. Specimens prepared in this manner will also furnish comparative data for evaluating the success of our second goal, whole-mount endothelial labeling, which will provide the flexibility to use fixed, archived tissue. To accomplish this aim, we will employ non-perfusion-based, diffusive whole-mount preparations. Methods for labeling other structures of interest will then be incorporated into these two types of protocols. In all cases, image data quality will also be evaluated with respect to its suitability for automated analysis of vascular morphology. Because of the time and expense entailed

in generating tumor tissue, specimen preparation methods will be developed and evaluated in healthy tissue prior to being applied to tumor tissue.

Recovering Proteins and Nucleic Acids From Formalin-Fixed, Paraffin-Embedded Tissues

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The identification of molecular changes such as mutations or altered expression underlying malignancy has led to specific and effective treatments for several forms of cancer. When the clinical course is typically short, it is reasonable to use fresh or frozen tissue for which molecular biologic analyses are relatively straightforward. However, many cancers (such as breast cancer) have a time course in which many years may elapse between treatment of the primary tumor and the appearance of metastasis. In these cases, the time required to obtain clinical correlations could be significantly reduced by performing molecular analyses on formalin-fixed, paraffin-embedded (FFPE) archival tissues for which the time course of the disease is already established. However, this is not currently feasible because both genomic expression and proteomic analyses using this tissue are less than robust, particularly for low-abundance transcripts and proteins.

The long-term goal of our research program is to employ high-throughput proteomic and molecular biologic screening of archival tissue specimens to identify the proteomic and genetic signatures of cancer. To achieve this goal, we are attempting to gain a detailed understanding of the chemical reactions and physical processes that occur from the time a tissue is first exposed to formaldehyde through the dehydration and embedding process and subsequently during efforts to extract molecules for proteomic and genomic analyses. A variety of chemical tools, including gel and liquid chromatography, mass spectrometry, and circular dichroism spectroscopy, have been used to follow these changes. Among these observations are the following:

1. It is possible to create tissue surrogates of one or two proteins that can be used to study in detail the chemical processes that occur during tissue fixation, dehydration, and embedding.
2. The process of high-temperature antigen retrieval, which is used to improve the immunocytochemical reactivity of proteins, results in the reversal of some, but not all, of the crosslinks and methylol adducts formed when proteins react with formaldehyde.
3. Dehydration with alcohol causes additional chemical changes beyond those that occur when formaldehyde reacts with proteins or nucleic acids in water. These changes are less readily reversible than are the changes induced by aqueous fixation.

4. Reaction of formaldehyde with proteins results in increased molecular heterogeneity, which is likely to significantly complicate proteomic analyses under the best of circumstances.
5. Solvent systems that are commonly used for molecular biologic analyses of fresh or frozen tissues are likely to be nonoptimal for recovery of DNA and RNA from formalin-fixed tissues.

To date, this work has enabled identification of some highly effective methods for isolating proteins from FFPE tissue and at the same time has highlighted some difficulties associated with use of these extracts in less than thoughtful proteomic analyses. We expect that our efforts will lead to further improvements in protein recovery for proteomics, as well as improved methods for recovering RNA for gene expression analysis.

Anticell Aptamers for the Diagnosis and Treatment of Cancer

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The Ellington lab has selected aptamers against a wide variety of tumor cells and has shown that these aptamers can be used to specifically label cells. By adapting aptamers to the proximity ligation assay, it has proven possible to identify as few as 10 tumor cells against a background of 100,000 noncognate cells. These methods may therefore prove very useful for the identification of circulating tumor cells in serum samples. In addition, aptamers that bind to cell surfaces have been shown to internalize into cells and can carry cargoes such as toxins and siRNAs. Such internalizing aptamers may prove to be extremely useful for the delivery of antitumor and other siRNAs and RNA therapeutics. We also are developing cell chips that can be used to reproducibly assay reagents, identify novel biomarkers, and help determine strategies for typing and staging tumors.

Boronic Acid-Based Lectin Mimics for Carbohydrate Recognition

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Glycoprofiling is a very important way to study the correlation of glycosylation patterns and pathological states. This is currently done with lectins, which are sugar-binding proteins. However, several issues hinder the application of lectins in glycoprofiling, including limited availability of lectins and their limited structural specificity. Our lab has been working on the development of lectin mimics using both small-molecule sugar sensors and DNA-based fluorescent aptamers. This presentation will discuss the latest results from our lab in developing fluorescent lectin mimics for glycorecognition and glycoprofiling applications. The end products of our platform technologies will be very

useful to glycobiologists interested in studying glycoprofiling and correlation of glycosylation pattern variations with biological and pathological processes.

Prostate Cancer Gene Discovery Using "Sleeping Beauty" Mutagenesis

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Recent large-scale sequencing studies have identified an average of 90 somatic mutations in a typical epithelial tumor. The next step must be to distinguish passenger from driver mutations in carcinogenesis. In this study, we employ a synthetic DNA transposon called Sleeping Beauty (SB) to create random somatic mutations in the prostate of mice, which we hypothesize will result in prostate cancer. This approach utilizes somatic transposition of an SB transposon vector, called T2/Onc, which can induce gain-of-function mutations or loss-of-function mutations upon insertion within or near genes in living mice. Thus, T2/Onc is capable of activating oncogenes or disrupting tumor suppressors. In this study, T2/Onc mutations are directed to the prostate using a CRE-controlled SB transposase knockin allele called Rosa26-LoxP-STOP-LoxP-SB11 (R26-LSL-SB11) in combination with a CRE recombinase transgene driven by the rat probasin promoter (PB-CRE) plus the T2/Onc transposon transgene array. This experiment is being done in otherwise wild-type mice and in mice homozygous for the Ptenflox conditional knockout allele. We hope to determine genes and genetic pathways that can induce prostate cancer in cooperation with loss of PTEN, a common event in human prostate cancer. Thus, the samples generated for this work are mouse prostate tumors. These samples will be analyzed by shotgun cloning and sequencing of T2/Onc transposon insertions. The output is a list of genes that are candidate prostate cancer genes. Proof-of-principle data have been obtained by studying early prostate lesions that develop in T2/Onc mice in which the SB11 transposase protein is expressed ubiquitously from an R26-SB11 transgene. These mice succumb to lymphoma, but lymphomatous mice also show an increase in the frequency of nests of PIN-like, Ki67+ prostate epithelial cells when compared with control prostates. These lesions harbor T2/Onc insertions, a subset of which we have shown to affect the same genes—thus, defining common insertion sites (CISs) and associated cancer gene candidates. Tissue-specific SB mutagenesis studies also are being conducted in other organs of the mouse to attempt to model hepatocellular carcinoma, lung cancer, and gastrointestinal (GI) tract cancer. Current results from the GI tract model are promising. We have combined T2/Onc, R26-LSL-SB11, and the Villin-Cre transgenes. More than 50% of the experimental animals have died between 9 and 15 months compared with 15% of control mice. The majority of experimental mice had GI tract tumors ranging from a few polyps to more than 100 polyps. Several animals also had liver tumors in addition to GI tract neoplasia. We are currently attempting to map transposon insertion sites from

multiple independently generated tumors and identify CISs. These studies are conducted in mice, which may develop some cancer types along genetic pathways not relevant to human cancer. Also, it is possible that some genes cannot be mutated by T2/Onc insertion in a way that can contribute to cancer. Nevertheless, these studies provide the advantage of allowing an in vivo functional screen for potential cancer genes. The results of the in vivo SB-based screens will be useful for scientists searching for cancer-causing events in human samples by exon resequencing, expression microarray, comparative genome hybridization, and other similar strategies.

Folate Receptor-Mediated siRNA Delivery to Cancer Cells

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Posttranscriptional gene silencing by small interfering RNA (siRNA) has evolved into a powerful tool for downregulation of any target gene(s), with both high efficiency and sequence specificity. In principle, siRNA may become the basis for developing the next generation of anticancer agents with high potencies and low side toxicities. However, no therapeutically acceptable delivery methods of siRNA are currently available. Folate receptor (FR) has been shown to be highly expressed by some cancer cells to facilitate the uptake of folate to meet its increased need for biosynthesis, although most normal cells do not express FR. Therefore, FR can be used for targeted drug delivery to certain cancer cells. FR-mediated delivery of a number of chemical agents by endocytosis to cancer cells has been demonstrated to be both efficient and highly specific toward FR-positive cancer cells. In principle, folate can be linked to siRNA to achieve specific siRNA delivery to FR-expressing cancer cells. However, the chemistry of direct coupling between folate and siRNA has not been readily achieved until now. Capitalizing on our recent RNA bioconjugation methods, the current research will develop a novel folate receptor-based siRNA delivery strategy against specific target genes in FR-expressing cancer cells. First, we will chemically synthesize the transcriptional initiator folate-AMP conjugate. Second, a folate-conjugated siRNA against a cancer cell marker, urokinase plasminogen activator receptor (uPAR), will be prepared by our T7 transcription system. Using KB cancer cells (a human nasopharyngeal epidermoid carcinoma cell line) and RNA fluorescent labeling, we will then determine folate-mediated siRNA delivery efficiency and cell specificity (normal cells vs. cancer cells). After successful FR-mediated siRNA delivery, RNAi effects will be assessed by determining the expression of uPAR at both mRNA and protein levels by quantitative RT-PCR and Western blot analysis. Finally, we will test the hypothesis that downregulation of uPAR expression in cancer cells by folate-conjugated siRNA will effectively inhibit the cellular activity of uPAR associated with tumor growth. We expect that the delivery of folate-siRNA against uPAR in KB cells will be more efficient and specific than other currently available methods. As a consequence, tumor growth suppression through uPAR silencing is expected. Results from the proposed research will likely lead to the development of general strategies and methods for FR-mediated delivery of siRNA against specific target genes in FR-expressing cancer cells and, therefore, may lead directly to cancer

therapeutic applications.

Linear Analysis of Genomic DNA in Parallel Nanochannel Arrays

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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.

Techniques that rely on probing chromosomes, such as metaphase fluorescence in situ hybridization, although providing a pangenomic view, cannot resolve structures below the Mb range. By probing uncompressed interphase DNA, resolution can be improved, but spatial organization of the genome is lost, so multiplexed and quantitative information is difficult to obtain. By stretching out DNA using techniques such as “molecular combing” or “optical mapping,” it is possible to probe specific loci in a spatially significant way, with resolutions in the kb range. However, techniques for mechanically fixing DNA on surface are inherently variable, leading to inconsistent stretching of molecules, which often cross over and retract on themselves. This makes it difficult to standardize such techniques as highthroughput methods for the biomedical community.

We have found that an individual dsDNA molecule, essentially a long polymer chain, will elongate and linearize in a consistent manner when streamed into nanofabricated long confining nanochannels with extremely small diameters. In the past 8 months, we have set up a single-molecule imaging lab, developed novel nanofabrication methods to reliably manufacture centimeter-long nanochannels in solid-state material, and demonstrated that megabase-size human genomic DNA can be consistently streamed and analyzed in these nanochannels by direct imaging at the single-molecule level. Conventional genomic DNA sample preparation methods were used, a very small amount of sample is needed, and in vitro cell culturing and/or subsequent PCR amplification are not required steps. This not only reduces cost and time but also, most importantly, allows fast analysis of samples of a scare source, such as needle aspirates, to obtain the most accurate information without introducing artifacts during sample/clonal amplification. Since an intact single molecule is analyzed in its individual channel, this provides the opportunity to interrogate haplotyping information directly from the native patient sample.

Working with our collaborator, we have also obtained preliminary data on imaging locus-specific probes along linearized long genomic DNA molecules as well as short PCR amplicons. This technology does not limit the capability of using PCR products on our chip in case specific target areas from complex or pooled samples are analyzed.

Flexible chip design could allow many samples to be analyzed in parallel in individually registered fluidic compartments on the same chip. DNA region-specific signature probes and multicolor labeling can be incorporated to interrogate the fine details of genomic structural information.

Our ultimate goal is to develop the nanochannel array chip integrated system for the routine and standardized quantitative analysis of genome structure, which will enable archiving and crosslaboratory comparison of data in cancer diagnostics.

Quantification of Human Cancer Markers

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There is great optimism that different cancer disease states will be assessed by quantifying changes in the abundance of human analytes, including posttranslationally modified proteins involved in key signal transduction pathways. We previously developed a technology that measures the abundance of specific analytes in biological samples, such as serum, using protein-DNA fusions known as tadpoles [1]. This technology quantifies multiple analytes over six to eight orders of magnitude, with limits of detection in the hundreds of molecules. Outlined below is our recent progress in accomplishing our NCI-funded goals:

1. We have developed and validated three dsDNA constructs for assembling multiplex assays and two internal amplification control DNAs. We have found that internal calibration templates exceeding 1,000 copies reduce the amplification efficiency of other templates in the same reaction regardless of whether the assay and control templates are amplified by the same primers. Thus, having the number of calibration templates at or below 1,000 copies ensures accurate and precise quantification even in the presence of each other.

2. We have developed a universal antibody-counting tadpole for implementing existing antibodies that bind known cancer markers. Using this tadpole, we measure human interleukin-6 and human prostate specific antigen in human serum as a test system [2].

3. We further extend the use of this universal antibody-counting tadpole to elucidate intracellular signaling state. As a model system, we used this tadpole with specific capture IgY to quantify 1,000 MAPK proteins per cell from as few as 50 yeast cells. Using rabbit antiphospho-p42/44 IgG, we quantify the amount of phosphorylated MAPK produced in response to pheromone exposure for 15 minutes. Our platform will enable the signaling state of primary tumor cells to be detected, mapped, and quantified.

4. We have developed antibody-DNA conjugates by attaching DNA directly to heavy chain carbohydrates, which do not interfere with antigen binding. These reagents serve as alternative detectors for quantifying antigens without the need to develop new affinity proteins, as is necessary for implementing the tadpole. We are optimizing conditions

necessary to achieve multiplex quantification of CA125, prolactin, osteopontin, insulin-like growth factor II, and leptin in human serum.

1. Burbulis I.E., Yamaguchi K., Gordon A., et al. Using Protein-DNA Chimeras To Detect and Count Small Numbers of Molecules. Nat Methods 2(1):31-7, 2005.
2. Burbulis I.E., Yamaguchi K., et al. "Quantifying Small Numbers of Molecules With a Near-Universal Protein-DNA Chimera." Nat Methods, submitted.

Development of an In Vivo Metastasis Screen

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Current metastasis assays tend to evaluate one cell line at a time, resulting in the use of large numbers of nonhuman animals due to the variability in measurements from animal to animal. This project will develop methods for parallel analysis of multiple cell lines for metastatic properties in a single animal, with the goal of evaluating up to 50 genes at a time. The development of such an approach would allow the screening of genes for metastatic effects. Such a screen could indicate the contributions of proteins to the steps of primary tumor growth, intravasation, and lung colonization. The first aim will focus on production of pools of cells expressing or suppressing selected proteins. The second aim will evaluate detection technologies for measuring construct distributions in a pool. The third aim will determine the appropriate formation of pools to be screened. The fourth and fifth aims will perform an initial screen and validate candidates identified in the screen. Sample preparation will involve isolation of viable tumor cells from the primary tumor, blood, and lungs of animals carrying breast cancer tumors. The likely end use of the data is determination of whether specific genes contribute to tumor formation, intravasation, or lung metastasis. Cell lines will be used for production of pools of cells expressing or suppressed for expression of particular proteins. Endogenous variations in cell lines can affect our evaluation of the contributions of the targeted proteins to metastasis.

Cancer Vertical Arrays

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We have developed two technologies for the analysis of tumors. The first is a method we refer to as “vertical arrays,” which are similar to dot blots but use a microarray format and low-complexity representations (LCRs) of total mRNA as the spotted features. These modifications result in improved signal-to-noise ratio, portability, and other advantages of glass slide microarrays. Vertical arrays can be used to assess the expression behavior of a gene in thousands of biological samples. The goal of our research is to prepare vertical arrays from tumor specimens so that the expression of a gene that is implicated in one tumor can be assessed quickly and conveniently in thousands of other tumor specimens, including tumors of the same and different types. Addressing the generality of a molecular marker is an important step in biomarker

validation.

We have recently completed “spike-in” sensitivity and coverage measurements for vertical arrays. “Sensitivity” refers to the number of molecules in a sample for which a change of a certain magnitude can be measured reliably, and “coverage” refers to the number of LCRs needed to measure most of the transcripts in a cell. We find that fourfold changes can be measured very reliably in the neighborhood of less than 1 transcript per cell for 80% of the spike-in transcripts using six LCRs, such that 11 LCRs should cover about 95% of all transcripts in or above this range. The major strengths of this approach are measurement of changes in transcript steady-state levels comparable to quantitative PCR, with the associated conveniences of the microarray format. Specifically, vertical arrays prepared from a common set of tumors could be widely distributed with greater economy than achievable with, for example, microtiter plates for quantitative PCR. Also, all aspects of the preparation of a vertical array are performed robotically, including sample preparation, LCR preparation, and microarray spotting.

One of the obstacles to constructing such a pan-cancer vertical array is assembling a collection of thousands of microdissected cancer samples. For this and other purposes, we have devised a “transverse microtome,” which can tile tumor tissue sections into thousands of well-documented 50 x 50 x 10 micrometer voxels. The transverse microtome can perform about 40,000 cuts per day, and each tissue voxel is mapped to a composite photomicrograph of the entire tissue section. A graphic interface allows the user to select regions of interest from the composite image, and the selections are automatically tabulated in a form that allows a liquid-handling robot to access the corresponding samples. This will allow the user of the eventual pan-cancer vertical array to assess, for example, the ratio of tumor to stroma represented in an LCR spot. We are currently adapting molecular methods, including RNA arbitrarily primed polymerase chain reaction (used to make LCRs) as well as wholegenome amplification, which will be useful for experiments we contemplate to address intratumoral heterogeneity, to samples dissected using the transverse microtome.

Our IMAT project will establish proof of principle through the preparation of a small vertical array focusing initially on prostate cancer. The experiments that we have performed so far on LCR preparation and vertical arrays have used RNA from cells grown in culture and indicate that reproducible LCRs can be prepared from as few as 10 cells. However, we have not yet constructed a vertical array from samples taken from frozen sections of surgically resected tumors. Consequently, we do not yet have an estimate for the smallest amount of tumor tissue that will be needed per LCR.

In an eventual, large pan-cancer vertical array, tumors will be snap frozen as soon as possible after surgical resection. Every LCR will derive from a region of a tumor that is captured in a photomicrograph, and the more information that can be attached to such a sample, particularly the record of presurgical treatment and clinical outcome, the more likely it is that the array will be useful. Frozen tissues have poor morphology relative to fixed tissues, and RNA cannot be recovered from tissue stained with hematoxylin. Still, in many cases, the proportion of cancer to normal tissue can be determined with good

accuracy, and molecular markers (e.g., distinguishing stroma from tumor) may partially solve this problem. Vertical arrays have an inherent self-correcting mechanism, in that mishandled samples would be expected to give rise to outliers. Thus, the high potential throughput of this approach is expected to provide a good buffer of average behavior for occasional mishaps. Nevertheless, good practice in sample handling will allow for the detection of outliers of biological significance.

Characterization of Magnetic Nanoparticles for Cancer Cell Enrichment Using Field-Flow Fractionation and Cell Tracking Velocimetry (CTV)

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Magnetic cell separation as a preenrichment step prior to downstream cellular characterization techniques is becoming more widely accepted. The aim of this project was to develop high-resolution magnetic reagents for use in the quadrupole magnetic cell sorter (QMCS). The QMCS is a flow-through immunomagnetic separation system that can provide sensitive enrichment of circulating tumor cells (CTCs) in blood as well as in other biological fluids and tissues. Defining the criteria to which magnetic reagents must adhere is essential in obtaining successful enrichments using magnetic separation technology. These criteria include high magnetic susceptibility with a well-defined shelf life, narrow particle size distribution, high-density attachment sites for antibodies, and a low level of nonspecific binding to nontargeted cells. CNW has met these criteria through the use of novel techniques, and quantitative specifications of particle manufacturing have been established. Magnetic field-flow fractionation (MgFFF) was used as a means of verifying the mass of iron, including and validating the shelf life of CNW materials. MgFFF is an analytical separation and characterization technique for nanosize and microsize magnetic particles. It is a separative elution technique similar to chromatography in which different components of a small sample elute from the channel at different times. A longer retention time in the MgFFF is identified with particles that interact strongly with the magnetic field and have high magnetic susceptibility. Furthermore, size (or mass) distribution of theinclusions can be determined from the elution profile using a data reduction method. Results from MgFFF indicate that CNW materials have a size distribution of 200 to 400 nm and an average retention time of 15 minutes, indicating high magnetic susceptibility within the magnetic field. MgFFF results also showed that there was no change in the elution profile over time, demonstrating that coating techniques used on CNW materials prevent the iron from oxidizing and losing magnetism.

Nonspecific studies were also performed with CNW particles. Jurkat cells were incubated with varying concentrations of CNW particles for 30 minutes and subsequently washed. Cells were measured by CTV, which measures the movement of cells within a well-defined magnetic energy gradient and generates a value known as

the magnetophoretic mobility of a cell. Cells that moved above 2.5 x 10-5 mm3/T.A.sec were considered magnetic and therefore nonspecifically labeled with CNW nanoparticles. At higher particle concentrations, 48.8% of the cells exhibited a magnetophoretic mobility above 2.5 x 10-5 mm3/T.A.sec, indicating problematic nonspecific binding of CNW particles. When particle concentrations were reduced relative to cell concentration, the nonspecific binding measured by CTV was reduced to 5%. Chemistry modifications were pursued to reduce nonspecific binding of particles at higher concentrations while retaining a high specificity for targeted cells. To reduce nonspecific binding, modified polyethylene glycol (PEG) units were introduced by reductive amination. PEG-modified particles showed a reduction of nonspecific binding. At the higher particle concentrations, less than 1% of cells were determined to be magnetic and nonspecifically bound with the PEG-modified CNW particles.

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

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Signal transduction processes rely on a cascade of posttranslational modifications (PTMs), proteinprotein interactions, modulated catalytic activities, and translocations within a response network of interacting species to generate specific biological outputs. Based on the generation of hydrogen peroxide and other reactive oxygen species (ROS) through the “oxidative burst” that accompanies a number of receptor-mediated signaling processes, we propose that a largely overlooked PTM, cysteine oxidation to sulfenic acid (and subsequent disulfide bond formation), provides the major molecular mechanism through which redox-based modulation of phosphorylation cascades takes place.

Our new technology, a rapidly reacting chemical trap for sulfenic acids (R-SOH) on proteins [Poole et al. 2005], allows us to assess the molecular and spatial location and timing of sulfenic acid formation on proteins in a proteomics-friendly manner. The modifying agents, with fluorescent or biotinylated tags attached to an analog of dimedone, are uniquely reactive toward R-SOH and “lock in” this chemical information in cell-culture- and potentially tissue-derived proteins for later readout by gel and mass spectrometry (MS)-based methods. Proposed improvements to our technology (three-color fluorescent labeling for differential gel electrophoresis and isotope-coded samples for MS) will also allow for enhanced quantitative abilities to assess variations in levels of these modifications among different samples in multiplexed approaches. Bioinformatics tools are also being developed to elucidate “signatures” of reactive sites to better understand the basis for the specificity of given cysteines toward peroxide-mediated oxidation and to predict previously unknown reactive sites across the proteome. Although it is at an early stage, our research using these compounds indicates that an initial “burst” of R-SOH formation is observed within 1 to 2 minutes after addition of TNF-α; to HEK-293 cells. A growing list of oxidized proteins that we have identified

using two-dimensional gel electrophoresis and MS 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.

As indicated above, implementation of our new technology will allow us to capture a new type of information on a redox PTM that can be used to report on the redox status or responsiveness of signaling-relevant proteins in given cell samples under controlled conditions. This novel technology is also likely to have broad applicability in molecular profiling to stratify patients with cancers that are sensitive to ROS-generating therapies and in the development of novel cancer therapies based on the inhibition of ROS-dependent proliferative signaling.

Poole L.B., Zeng B.B., Knagg S.A., et al. Synthesis of Chemical Probe To Map Sulfenic Acid Modifications on Proteins. Bioconjug Chem 16(6):1624-8 2005.

This work is supported by R21-CA112145 (LBP, Principal Investigator; JSF, LWD, SBK, Co-Investigators).

Dynamic Isoelectric Focusing: A New Tool for Proteomics and Fractionation

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Proteomics requires high-resolution separations due to the large number of different proteins in a typical sample. One method that has shown great promise for protein separation is capillary isoelectric focusing (IEF). Capillary IEF allows for a large number of proteins to be separated in a short period of time, but efficiently interfacing it to other separation or analysis methods is difficult and leads to a drastic reduction in resolution and sensitivity. Dynamic IEF is a new technique that overcomes many of the problems of capillary IEF by using a dynamic electric field within the capillary. Manipulation of the electric field using additional high-voltage power supplies permits adjustment of the pH gradient, enabling both the location and width of the focused protein bands to be accurately controlled. Each protein can be migrated to a designated sampling point, while remaining focused, where it can be collected for further analysis. This ability to collect and isolate the protein bands while maintaining a high peak capacity demonstrates the great potential of dynamic IEF for sample separation.

In addition to its utility for proteomics, dynamic IEF can also be used to identify active proteins in complex samples using bioassay-guided fractionation (BGF), which combines analytical separation methods with biological testing to identify compounds responsible for an observed effect. Dynamic IEF has the ability to fractionate a sample into more than 1,000 fractions with little overlap, providing unprecedented potential for BGF. To minimize the number of bioassays performed, successive fractionations will be used. The first round will fractionate a sample into 11 parts, and a Plackett- Burman design will be used to identify which fractions or combinations of fractions are active. Active fractions will be further fractionated using the same experimental design.

Eventually, the active fraction will be greatly simplified compared with the original sample, and liquid chromatography/mass spectrometry (MS) will be used to identify the remaining proteins. The capabilities and benefits of dynamic IEF for both proteomics-guided fractionation and BGF will be demonstrated. Dynamic IEF is able to provide a peak capacity of greater than 1,000, as shown using prostate cancer cell lysates. Samples are then analyzed by MS, giving a total system peak capacity of more than 300,000.

BGF will be demonstrated using culture medium from *Fusarium virguliforme*, the fungus responsible for soybean sudden death syndrome. This fungus produces an unidentified protein toxin causing foliar necrosis. Data will be presented showing the ability of dynamic IEF to perform BGF and to isolate fractions spanning less than 0.01 pH unit.

A Standardized Antibody Microarray-Based System for the Quantitative Measurement of Protein Isoforms Arising From Alternative Gene Splicing

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The genesis of cancer involves several genetic changes that culminate in uncontrolled cellular proliferation. Several key oncogenes and tumor suppressor genes are widely recognized to influence the development and progression of several cancers. This recognition has come in large part from numerous efforts that have defined key mutations in some of these genes associated with altered functions. However, some genes associated with cancer have no apparent mutations that alter their normal function. In these cases, there is evidence that splice variants of a given gene and the ratio of isoforms are significantly changed in the cancerous state. One of the major obstacles in elucidating the significance of cancer-associated splice variants is the lack of a systematic technology that can reliably detect and quantitate variants at the protein level.

Antibody-based methods to measure proteins are powerful, are extensively used in research and diagnostic applications, and fit well in a clinical setting. However, development of immunoassays to discriminate among protein isoforms is challenging due to the large overlap in sequence. Epitome Biosystems, Inc., has developed methodologies that solve this problem through a combination of antibody design and sample treatment aimed to expose differences among protein isoforms. Starting with in silico techniques to identify continuous linear sequences for any protein, unique EpiTags™ are generated. Antibodies are raised against synthetic peptides that make up these unique EpiTag™ sequences rather than against the protein itself. EpiTags™ are made accessible to the antibody by fragmenting proteins in the sample prior to analysis, yielding predictable antibody performance.

For protein isoform detection, a “sandwich” assay format is utilized for unambiguous detection and quantification of each variant form. Specifically, the antibody sandwich is formed by antibodies raised to two different EpiTags™ that exist within a single protein

fragment, liberated by protease digestion of the sample. The protein fragment is selected such that it spans the unique junction region between two exons. Importantly, although individual EpiTag™ sequences are shared between the full-length protein and one or more isoforms, the combination of both EpiTags™ on a single protein fragment liberated by protease digestion is unique. Following sample denaturation and digestion, each individual isoform generates a unique peptide fragment signature that can be quantified based on novel antibody sandwich pair formation. Therefore, Epitome's approach allows for an overall systematic methodology for the detection of virtually any splice variant within the proteome.

Proof of concept has been demonstrated using a model system developed using commercially available peptide-specific antibodies and a set of corresponding synthetic peptides. Assays are being developed to measure the cancer-related splice forms of Bcl-X and CD44. Bcl-X is a member of the Bcl- 2 family of apoptotic regulators, is highly expressed in many lymphomas, and may have a significant role in the genesis of these cancers. This gene has two distinct splice variants (the long and short forms) with opposing apoptotic functions for which EpiTag™ assays are being developed. CD44 has a very large number of potential splice forms. In many human cancers, including carcinomas of the breast, certain CD44 variants are found to predominate. Epitome's initial focus is on these forms, specifically, isoforms Meta-1 and Meta-2.

Chemical Cytometry for Improved Prognostic Accuracy

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The cell is the organizing unit of life, and characterization of single cells provides a level of detail and biological understanding that is lost when cell cultures and tissues are homogenized for analysis. Chemical cytometry employs microscale separation technologies and ultrasensitive laser-induced fluorescence to characterize the composition of single cells. Chemical cytometry reveals details on heterogeneous cell types, such as those found in tumors, neurons, and certain bacterial systems. Cellular heterogeneity may prove to be a fundamental characteristic of life, and understanding that heterogeneity provides insight into disease prognosis.

Microfluidic Platform for Dynamic Cell Microscopy

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We are developing a microfluidic system to allow control of the cell culture environment for longterm, time-lapse microscopy of adherent cells. Building on previous work in microfluidic cell culture [1], the current method integrates a customized digital flow controller to deliver defined fluid inputs to the cultured cells during microscopy.

As the trend toward “systems biology” continues, it will become increasingly important to

study dynamic behavior in individual live cells. The use of fluorescence microscopy offers a promising method to investigate live cell response. However, currently there is no system available to control the delivery of fluids to cultured cells. Current techniques use either static culture plates or bulky flow chambers.

We describe a microfluidic flow chamber that overcomes the major limitations of time-lapse microscopy studies. The microfluidic chamber is designed to have a cell culture region separated from the flow path by an artificial endothelial barrier as previously described [2]. The device is formatted to a standard 96-well plate, allowing liquid and cell samples to be directly pipeted into the appropriate inlet reservoirs. A custom pneumatic flow controller is then used to load the cells into the culture regions as well as to switch between two different exposure solutions. A digital software interface allows the user to program specific inputs (pulses, ramps, etc.) over time to expose the cells to complex functions during time-lapse imaging. Anticipated applications include gene expression kinetics, protein localization, apoptosis, and siRNA silencing.

A key aspect of this work is the potential to standardize live cell experiment data. Since each condition can be well controlled in the microfluidic format (cell type, cell number, temperature, time, flow solutions, exposure profile, etc), the reproducibility of cellular responses from lab to lab should be greatly improved. Toward this goal, we are also working on developing an open-source standard that describes the experimental conditions used to collect any given data. This will make cell biology experiments much more quantitative and portable between research groups.

The format of the microfluidic plate also lends itself to future automation in a screening format. Currently, the prototype plate can run eight independent flow experiments. By adopting this to a 384- well plate and maximizing the usage of each well, it is possible to create 96 independent controllable flow units on a single plate, which can be run using the most current robotic instruments and analyzed via high-content analysis. This can benefit the cancer biology field by enabling a type of analysis not currently available at any scale, much less for medium-throughput screening.

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2 Lee P.J., Hung P.J., Lee L.P. An Artificial Liver Sinusoid With a Microfluidic Endothelial-Like Barrier for Primary Hepatocyte Culture. Biotechnol Bioeng February 7, 2007.

A Combined Atomic Force-Fluorescence Microscopy Technique To Select Aptamers in a Single Cycle From a Pool of Random Oligonucleotides

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We are developing a method that utilizes a combined atomic force microscope (AFM)/fluorescence microscope and small-copy-number PCR to affinity-select individual aptamer species in a single cycle from a small pool of random sequence oligonucleotides. In this method, a library of small beads, each of which is functionalized with fluorescent oligonucleotides of different sequences, is created. This library of oligonucleotide-functionalized beads is flowed over immobilized target molecules on a glass cover slip. High-affinity, target-specific aptamers bind tightly to the target for prolonged periods and resist subsequent washes, resulting in a strong fluorescence signal on the substrate surface. This signal is observed from underneath the sample via fluorescence microscopy. The AFM tip, situated above the sample, is then directed to the coordinates of the fluorescence signal and is used to capture a threedimensional, high-resolution image of the surface-bound bead and to extract the bead (plus attached oligonucleotide). The extracted oligonucleotide is PCR-amplified, sequenced, and may then be subjected to further biochemical analysis.

We describe the underlying principles of this method, the required microscopy instrumentation, and the results of proof-of-principle experiments. In these experiments, we selected aptamers in eight trials from a binary pool containing a 1:1 mixture of thrombin aptamer oligonucleotide and a nonsense oligonucleotide. In each of the eight trials, the positive control aptamer was successfully detected, imaged, extracted, and characterized by PCR amplification and sequencing. In no case was the nonsense oligonucleotide selected, indicating good selectivity at this early stage of technology development.

Enzymatic Luminescence MicroRNA Assay

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A new highly sensitive and cost-effective enzymatic luminescence assay for high-throughput detection and quantification of microRNA in biological samples will be developed in this project. The assay utilizes the same detection concept known from pyrosequencing, yet expands the pyrosequencing detection methodology for an accurate quantification of small RNA molecules. This technique is based on the detection of released inorganic pyrophosphate during reverse transcription of microRNA to cDNA, which is subsequently converted to adenosine 5-triphosphate (ATP) by ATPsulfurylase, and provides energy for luciferase to oxidize luciferin and generate light. Preliminary results show that the proposed method has unique dynamic range and is capable of detecting less than 5 fg of microRNA in a complex RNA mixture. After optimization of reagents and protocols, the assay is expected to outperform real-time PCR for the analysis of small RNA molecules. Compared with current RT-PCR and microarray platforms for RNA quantification, the proposed assay is simpler and faster and requires less expensive reagents. The bioluminescence signals are detected with the widely available luminometer, which has the capability for high-throughput microRNA analysis in a 96-well format but is less expensive than real-time PCR and microarray systems. The accomplishment of the proposed microRNA detection technique will provide a simple, fast, sensitive, and less expensive platform for RNA

detection and quantification to be used in life science research, drug discovery, and clinical diagnosis.

Bioinformatics Tools for IMAT

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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 predict 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.

Diagnostic marker selection and classifier design are two important tasks for molecular classification of multicategory cancers. However, disconnection between marker selection and classifier design exists in two popular scenarios: (1) Classifier-independent marker selection is universally applicable but not optimized for specific classifier designs, and (2) classifier-dependent marker selection may be optimized for specific classifier designs but often is not directly interpretable. For the molecular classification of multicategory cancers, we have developed a biologically guided, joint marker selection and classifier design algorithm called phenotypic-upregulated gene-supported one-versusrest support vector machine (PUG-OVRSVM). To date, we have tested PUG-OVRSVM on four large published and one in-house oligonucleotide microarray data sets. We compared PUG-OVRSVM with four popular benchmark marker selection methods (SNR, t-statistics, BW, SVM-RFE) and two popular benchmark classifiers (K-Nearest Neighbor, Naive Bayes Classifier). PUG-OVRSVM outperforms all other methods on the four cancer data sets in terms of lower error rate, higher performance stability, and least number of genes required for the lowest error rate. PUG-OVRSVM achieved a comparable performance on one muscular dystrophy data set. Furthermore, a two-step gene selection method is also proposed. In the first step, individually discriminatory genes (IDGs) are identified by using onedimensional weighted signal-to-noise ratio. In the second step, jointly discriminatory genes (JDGs) are selected by sequential search methods from the IDGs, based on their joint class separability measured by multidimensional weighted Fisher criterion. By applying the proposed IDG/JDG approach to a microarray study of small round blue cell tumors (SRBCTs), we successfully identified a much smaller yet efficient set of JDGs (nine genes) for diagnosing SRBCTs, with a misclassification error rate of 3.1%.

Multichannel biomedical imaging promises powerful tools for the visualization and

elucidation of important developmental or disease-causing biological processes. Recent research aims to simultaneously assess the spatial-temporal/spectral distributions of multiple biomarkers, where the signals often represent a composite of more than one distinct source independent of spatial resolution. We report a novel blind source separation method for quantitative dissection of mixed yet correlated biomarker distributions. The close-form algebraic solution is based on a linear latent variable model whose parameters are estimated using geodetically principled, nonnegative, leastcorrelated component analysis. We demonstrate the principle of the approach on the mixtures of real cancer images acquired by dual-energy x-ray and dynamic contrast-enhanced magnetic resonance imaging. We observed accurate and robust source separation into component biomarker distributions in agreement with the ground truth or biomedical expectations. With superior performance compared with existing techniques, this method has powerful features that are of considerable widespread applicability.

Effective Mammalian Two Hybrid Screening Approach

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Identification of novel protein-protein interactions is a fundamental step to understanding protein function and signaling networks and allowing efficient implementation of targeted cancer therapy. The majority of protein-protein interactions are currently identified using yeast two hybrid (Y2H), coimmunoprecipitation, and mass spectrometry or protein libraries. Each of these approaches has its own set of major limitations in failing to mimic native physiological conditions (Y2H and protein libraries) or efficiently identify protein interactions on the cytoskeleton or membrane, due either to the location of the interaction (Y2H) or to difficulties in coimmunoprecipitation of cytoskeletal or membrane proteins. Furthermore, conventional Y2H approaches yield false positive signals with transcription factors precluding screening. Therefore, a novel screening method that efficiently identifies biologically relevant protein interactions bypassing the limitations of current screening methods would have wide applicability.

We propose to develop and validate a readily applicable, context-dependent, subcellular localization-, cDNA library-, and cell-type-independent retrovirus-based mammalian two hybrid (ReMTH) screen method for identification of novel protein-protein interactions, including cytoskeletal and membrane proteins, in mammalian cells, allowing native protein folding and posttranslational modifications. In ReMTH, bait protein is fused to one fragment of a rationally dissected fluorescent protein such as GFP. The second, complementary fragment of GFP is fused to an endogenous protein by the retrovirusmediated exon trap vector. An interaction between bait and host protein (prey) can bring the two halves of the GFP molecule into proximity, resulting in reconstitution of fluorescence. The resultant cells will be reagents for the study of the localization and function of the novel protein-protein interaction complex as well as resources for high-content drug or siRNA screening. The fully developed technology will identify functional protein-protein interactions more efficiently than current methods and identify

interactions not discoverable by current methods, particularly in context-dependent mammalian screens. Furthermore, the proposed ReMTH screen has the unique potential to stabilize or trap transient/weak interactions such as enzyme/substrate interactions, allowing identification of components of signaling pathways and networks in previously undetectable cancers. We have completed an initial proof-of-concept screen in HeLa cells for identification of interaction partners of the oncogene AKT1 (Ding et al. 2006). We identified a series of previously known AKT1 interaction partners and substrates, as well as novel interaction partners, including cytoskeleton and membrane proteins. We have confirmed that one novel interaction partner, ACTN4, interacts physically and functionally with AKT1. Thus, the technology will uncover functional proteinprotein interactions not detectable by other approaches and advance our understanding of protein functions and signaling networks in cancer.

Ding Z., Liang J., Lu Y., et al. A Retrovirus-Based Protein Complementation Assay Screen Reveals Functional AKT1-Binding Partners. *Proc Natl Acad Sci U S A* 103(41):15014-9, 2006.

Quantification of Clonal Expansion In Situ Using a Novel Integrated One- and Two- Photon Microscopy Platform

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The clonal expansion of cells with mutations in genes that provide growth and survival advantages is one of the pivotal first steps in carcinogenesis. To understand the earliest stages of cellular transformation, a method to identify and analyze these premalignant cells is needed. We have created a transgenic fluorescent yellow direct repeat (FYDR) mouse in which cells that have undergone a rare DNA sequence rearrangement (via a homologous recombination event) express a fluorescent protein, enabling the labeling of phenotypically normal cells. To measure clonal expansion in situ, we have integrated one- and two-photon microscopy to create a sensitive imaging system that spans four orders of magnitude on the length scale and provides three-dimensional (3D) analysis within intact unfixed tissue. This imaging platform rapidly identifies very rare fluorescent cells within an entire mouse organ (at the cm scale) and subsequently provides 3D images of each fluorescent cell or cluster of cells (at the micron scale). We applied these techniques to study the effect of age on clonal expansion of fluorescent cells in the pancreata of FYDR mice. Results show that as mice age, there is a significant increase in the number of cells within fluorescent cell clusters, indicating that pancreatic cells can clonally expand with age. This combination of mechanico-optical engineering technologies with genetically engineered FYDR mice can be applied to study the effects of genetic and environmental exposures on the risk of clonal expansion.

How Do Colorectal Cancers Arise Despite Surveillance?

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The times required for cancer progression are uncertain. Knowledge of how quickly cancers develop would be useful to guide prevention and screening strategies. However, it is currently impossible to serially observe human cancers because they are removed as soon as they are found. In this study, we seek to develop a method to retrospectively count numbers of divisions during progression by counting numbers of replication errors; that is, the greater the number of divisions or genome replications, the greater the number of replication errors (a “molecular clock” hypothesis). Although somatic mutations are relatively infrequent in most cancers, colorectal cancers deficient in DNA mismatch repair have mutation rates that are 100- to 1,000-fold higher than normal. Nearly all microsatellite (MS) loci are mutated in these cancers. Therefore, it is possible to “count” somatic MS mutations by isolating tumor DNA and then genotyping the sizes of their MS alleles. To “calibrate” our MS molecular clock, “interval” cancers that arise shortly (1-3 years) after a negative clinical examination (colonoscopy) are examined, because visible cancer growth should occur after the negative examination. Preliminary data indicate that sporadic cancers and interval cancers have similar mitotic ages, which suggests that many cancers frequently grow to detectable sizes within a small period of time (years).

A limitation of this study is sample preparation, which uses archival formalin-fixed, paraffin-embedded tissues. Results are confounded by mixtures of tumor and normal DNA, which are currently “filtered” after data collection by various algorithms.

Identification of Areas of Oxidative Damage in Human Genomic DNA

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Our goal is to develop a technique that can determine the relative density of oxidative damage sites in bulk DNA and in specific regions of the genome. A single method that can accomplish all these goals is not yet available, but it is currently possible to determine the overall number of abasic (AP) sites in genomic DNA using slot blots, the presence of hotspots for AP formation in a population of the same DNA fragments using ligation-mediated PCR, and the random or clustered distribution of AP sites (using electron microscopy or atomic force microscopy). We will use DNA combing, a method that generates samples of genomic DNA that are aligned, straightened, and easily visualized. We will then detect the distribution of AP sites by immunofluorescence and refine our methods until we are able to quantitate AP sites within 10% of the measurements made by the slot blot technique, which is the current standard of detection. When this is achieved, our next goal will be to optimize the reaction conditions so that, in addition to quantitation of AP sites, we can employ fluorescence in situ hybridization to identify a specific DNA region and visualize AP sites at that locus. Once we have this ability, our last goal will be to standardize these conditions so that, in cells exposed to different doses of hydrogen peroxide (H2O2), our detection of AP sites

is found to vary with the H2O2 dose at this locus. We expect that the detection of AP sites will fall within 10% of the levels measured by the current standard method of detection of AP sites, with less than 10% variability within repeats of the same sample. The proposed experiments will prove that these methods can be used to investigate complex biological problems.

We believe that a method that could detect the distribution of AP sites, both in the genome as a whole and within a specific region, would be an extremely valuable addition to existing diagnostic tools. It would enable us to determine the extent and distribution of DNA damage in specific regions of the genome, such as origins of replication or promoter regions; lesion formation during normal cell metabolic processes (e.g., replication and transcription); and lesions formed as a consequence of the cells being exposed to carcinogens or to oxidative stress. This method may allow the study of the role of epigenetic changes in DNA damage (e.g., a change in DNA methylation status and/or histone acetylation in active and inactive regions of the genome or in areas of DNA replicated at different times during the S phase). It could also be used for the characterization of similarities and differences in the DNA damage that occurs in different cell types (e.g., normal and breast cancer cells, different breast cancer lines, and cells from different patients affected by the same disease, such as lung cancer, breast cancer, and Alzheimer's disease). Ultimately, these studies might lead to a better understanding of the health risks (with regard to the probability, degree, and distribution of DNA damage) for different genotoxic and epigenetic events. This project seeks to achieve a proof of principle that demonstrates the feasibility of a method that would make the aforementioned studies possible.

Expression of the Notch Ligand Jagged-1 Is Essential for Cell Survival in Ovarian Cancer Cells With Notch3 Signaling Activation

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Interaction between Notch receptor and its ligand activates Notch signaling, which constitutes a molecular circuit in development and tumorigenesis. We have previously reported Notch3 gene amplification in ovarian serous carcinoma and Notch3-dependent growth in cancer cells. In this study, we characterize the roles of the Notch3 ligand Jagged-1 in the tumor progression of ovarian carcinomas. We demonstrated a significant correlation of Notch3 expression and Jagged-1 expression in ovarian cancer based on serial analysis of gene expression (SAGE) data. Quantitative real-time PCR analysis showed significant overexpression of Jagged-1-in ovarian cancer cells compared with ovarian surface epithelial cells and benign cystadenomas. Treatment with Jagged-1-specific shRNA resulted in a significant decrease in cell proliferation and colony formation in OVCAR3 cells and, to a lesser extent, in A2780 and TOVG21 cells. In addition, transwell migration and in vitro wound healing assays revealed that Jagged-1 knockdown reduced cellular motility in OVCAR3, A2780, and TOVG21 cells. These phenotypic changes were accompanied by a decrease in the levels of the active

(intracellular) form of Notch3, which has been demonstrated to be involved in the proliferation and survival in Notch3 overexpressing OVCAR3 and A2780 cells. Furthermore, PBX-1, a potential target gene of Notch3 and a tumor suppressor in ovarian carcinoma, was also downregulated by treating OVCAR3 cells with Jagged-1 shRNA. These data suggest that interaction between Jagged-1 and Notch3 is involved in activating Notch3 signaling in ovarian carcinoma, and concomitant expression of Notch3 and Jagged-1 may contribute to tumor progression.

Glycosylation Profiling of Secreted and Membrane Proteins Associated With Cancer

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Objective: The objective of this proposal was to develop methods to perform glycosylation profiling of cancer-associated proteins. Since most secreted and membrane proteins are glycosylated, they were the targets. The concept was to use immunoprecipitation (IP) on the selected proteins and then analyze them using mass spectrometry and mass gap analysis to find the glycosylation profiles. The use of IP has been modified, since too many proteins are often pulled down with the antibodies or associated with the proteins of interest. There was also considerable concern that these antibodies may selectively enrich certain glycoforms of the proteins and thus alter the profiles. The large amount of antibody in the final sample also makes the glycoanalysis difficult.

Solutions: We have developed a highly efficient method to obtain enriched secreted proteomes from cell lines. This work was published and has recently been applied to the analysis of the MCF10 isogenic breast cancer cell series. Proteins have been detected that are highly associated with aggressiveness (accepted subject to revision). The proteins are fractionated into about 10 subproteomes using liquid chromatography on a reverse-phase column; these are then used as the input samples for glycoanalysis, each containing multiple proteins. Samples are digested and run on a mass spectrometer (MS) using accurate mass data acquisition. As a second part of the solution, we have developed a new analysis strategy and computerized this for the analysis of glycosylation in protein mixtures. Although this still uses the mass gap approach to match together the masses from a single glycosylation site, it uses a subdatabase development and automated glycosylation site prediction routine to identify all possible glycosylated peptides in a sample. The use of a fragmentation strategy also provides information on the likely peptide mass for lookup in the table, but this is not an essential step. To help in the analysis of these complex mixtures, we have also moved the technology to the recently acquired, new, and highly accurate Orbitrap MS instrument, which has significantly enhanced the analysis within these mixtures. As a third step, we are using a property of the glycopeptides to help their enrichment during the MS analysis stage. They consistently have higher m/z (mass/charge) ratios than most normal peptides, and by excluding the low m/z data from the scan, the

interpretation of the data files and the finding of the glycopeptide masses are greatly enhanced. This combination of altered strategies is now being applied to the analysis of site-specific, glycoprotein profiles with the secreted subproteomes from a number of cell lines, and data from some of these will be presented. For membrane proteins, we are using a similar strategy, and we will enrich these following minimal biotinylation of the cell surface using a fusible linker. This work is currently under way, and the results will also be shown. All of these data can now be analyzed using repeat injections and isolations and the variations processed using both commercial software and a set of differential analysis routines separately developed for clinical studies in the facility. Although the work is behind the 2-year schedule, we are surmounting the problems in the original concept and are requesting a no-cost extension to complete the work.

Spinoffs: The techniques we have developed are separately being applied to the analysis of cancer biomarker proteins in body fluids. It has successfully allowed us to analyze prostate specific antigen (PSA) glycosylation in urine, and this is the subject of a grant application. PSA is a secreted protein and is an excellent case of where there are observed differences in glycosylation in cancer, but these cannot be analyzed in clinical samples. Our routines allow the analysis of such changes within clinical trials. In addition, the secreted proteome method has identified at least one glycoprotein biomarker that is common to multiple cancer histiotypes, which will be the subject of another funding request.

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

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We are using head and neck cancer as a model to establish and validate procedures for genome-wide molecular analysis of neoplastic lesions. Samples of head and neck malignancies are being collected prospectively at Yale-New Haven Hospital. DNA is extracted using the Epicenter Master Pure kit, and aliquots are stored before and after whole-genome amplification (Qiagen REPLI-g). All archival DNA samples are assayed for human papillomavirus (HPV) infection. The samples are also processed for analysis of gene losses and gains, using array-comparative genomic hybridization (CGH) in highdensity microarrays. Unamplified DNA from the same tumor DNA archives is analyzed using a custom NimbleGen microarray and an endonuclease-based protocol designed to report changes in DNA methylation status. A unique feature of the method is its ability to report methylation changes for loci associated with repetitive DNA. 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 non-promoter-associated CpG islands,

and more than 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. Analysis of methylation changes in a data set comprising 40 tumors shows striking hypermethylation of a number of candidate tumor suppressor genes and reveals clusters of linked hypermethylated genes within “chromatin neighborhoods.” Abnormal hypomethylation in tumor DNA shows a recurrent, complex component comprising CpG islands associated with interspersed repeat loci. The data underscore for the first time the complexity of the loss of DNA methylation at multiple repetitive DNA loci in tumors and reveals the existence of a genome-wide epigenetic framework that is now accessible to study using relatively small DNA samples. A significant number of DNA methylation abnormalities are observed in “morphologically normal” tissue from the same patients. Given the potential for tumor classification as well as for risk stratification of “morphologically normal” tissue, based on profiles of abnormally methylated genomic loci, this novel approach opens new avenues for a large-scale discovery effort based on non-repeat-masked DNA methylation analysis in any human cancer. The data sets 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 data set 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. However, future application of this technology would benefit from the availability of genetic information for all patient samples, particularly single nucleotide polymorphism haplotypes, which unfortunately are not available in the present study and may limit the power of our tumor morphism classification analysis.

Shared Immunoproteome for Ovarian Cancer Diagnostics and Immunotherapy: Potential Theranostic Approach to Cancer

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Introduction: Elimination of cancer through early detection and treatment is the ultimate goal of cancer research. This is especially critical for ovarian cancer, which is typically diagnosed at very late stages with very poor response rates. Immunoproteomics, which defines the subset of proteins involved in the immune response, holds considerable promise for providing better understanding of early-stage immune response to cancer as well as important insights into antigens that may be suitable for immunotherapy. Early administration of immunotherapeutic vaccines can potentially have profound effects on prevention of metastasis and may potentially cure through efficient and complete tumor elimination.

Method: We developed a mass spectrometry (MS) method to identify novel

autoantibody-based serum biomarkers for the early diagnosis of ovarian cancer. The method uses native tumor-associated proteins immunoprecipitated by autoantibodies from sera obtained from cancer patients and from cancer-free controls to identify autoantibody signatures that occur at high frequency only in cancer patient sera. Antigen-antibody complexes were immunoprecipitated from normal and ovarian cancer patient composites. Autoantibody-reactive antigens were separated from the antibodies and fractionated first by size exclusion chromatography (SEC), then further fractionated by reverse-phase high-performance liquid chromatography (HPLC). Each HPLC fraction was individually treated with trypsin. Major histocompatibility peptide complexes (MHCs) from two ovarian cancer cell lines were isolated by immunoaffinity purification, and the peptide fraction was separated from the protein fraction by boiling, then further fractionated by SEC and HPLC. Tryptic peptide fractions and MHC peptide fractions were analyzed using a liquid chromatography-MS system. The mass spectral data were analyzed using Sequest and Mascot software and the SwissProt human database.

Results: We identified a subset of more than 50 autoantigens that were also processed and presented by MHC class I molecules on the surfaces of ovarian cancer cells and thus common to the two immunological processes of humoral and cell-mediated immunity.

Discussion: These shared autoantigens were highly representative protein families with roles in key processes in carcinogenesis and metastasis, such as cell cycle regulation, cell proliferation, apoptosis, tumor suppression, and cell adhesion. Autoantibodies appearing at the early stages of cancer suggest that this detectable immune response to the developing tumor can be exploited as early-stage biomarkers for the development of ovarian cancer diagnostics. Correspondingly, because the T-cell immune response depends on MHC class I processing and presentation of peptides, the identification of proteins that go through this pathway are potential candidates for the development of immunotherapeutics designed to activate a T-cell immune response to cancer. To the best of our knowledge, this is the first comprehensive study that identifies and categorizes proteins that are involved in both humoral and cell-mediated immunity against ovarian cancer and may have broad implications for the discovery and selection of theranostic molecular targets for cancer therapeutics and diagnostics in general.

Using Synthetic Dosage Lethality To Screen for Novel Antitumor Targets

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We have developed a novel approach to identify new drug targets by uncovering specific genetic interactions involving genes overexpressed in cancer cells. The genetic interaction called synthetic dosage lethality (SDL), which was first described in yeast, occurs when a normally nonessential gene becomes essential due to overexpression of

a second gene. To identify potential therapeutic targets, we define SDL interactions in yeast using orthologs of genes that are overexpressed in different cancers. For example, hRad9 is overexpressed in 33% of cases of non-small cell lung carcinomas. hRad9 and its yeast ortholog Ddc1 are part of the sliding clamp [Rad9/Hus1/Rad1 (9-1-1) in humans and Ddc1/Mec3/Rad24 in yeast], one of the damage sensors recruited to stalled replication forks and DNA breaks. This complex subsequently activates cell-cycle checkpoints in collaboration with ATR, Chk1, and downstream effectors. To search for SDL interactions, we mimicked overexpression of hRAD9 by introducing the yeast Ddc1 gene into every strain in the viable haploid gene disruption library and inducing high-level expression of the Ddc1 protein. We identified a strong SDL interaction in the absence of Cik1, a binding partner of the kinesin motor protein Kar3 that functions to increase the velocity of Kar3 movement affecting microtubule dynamics. Cik1 is important during mitosis for assembly and/or maintenance of the mitotic spindle preventing sister chromatid separation, perhaps by microtubule crosslinking. NuMA, the human counterpart of Cik1, interacts with dynein/dynactin, kinesin motor proteins, and KIF2a, the likely human functional ortholog of yeast Kar3. This target is exciting because anticancer drugs such as taxol are known to function by stabilizing microtubules.

We have also screened for mutations in yeast that are sensitive to overexpression of activated RasG19V in budding yeast, which corresponds to G12V in mammalian cells. We chose Ras since activating mutations in N-Ras and K-Ras are found in a large percentage of human cancers. We have screened 50% of the yeast library, and thus far, no SDL interacting partners have been identified when wild-type Ras is overexpressed. Interestingly, more than 6% of the gene disruptions show slower growth or no growth when the RasG19V allele is overexpressed. Among the genes that we have identified are several in the autophagy pathway. Our experiments support an essential role of autophagy in cancer cell survival and underscore the importance of these genes as targets for therapy.

Finally, to show that the same interactions that we define in yeast are occurring in cancer cells, we will validate our yeast results in mammalian cells. We have established cell lines that overexpress hRad9 and are in the process of validating the Ddc1-Cik1 SDL interaction in these cell lines by reducing expression of the Cik1 homolog NuMA using siRNA and assaying cell survival. The ultimate goal is to show that these same interactions can be found in lung cancer cell lines that exhibit overexpression of hRad9. Similar experiments will be done targeting genes in the autophagy pathway in cells expressing RasG12V. The validation of the interactions that we find in yeast may identify novel therapeutic targets that can selectively kill cancer cells. Such therapies would be a major advance compared with current therapies that often nonselectively target all proliferating cells.

This work is supported by R33-CA125520.

Targeted Antigen Delivery for Cancer Immunotherapy

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The major goal of this project is to establish the proof of principle that topical delivery of tumorassociated antigens can elicit effective antitumor responses and can be used as a novel means for cancer immunotherapy. It is now an accepted view that successful cancer treatment will eventually include a combination of different modalities. Thus, effective cell immunotherapy that is simple and eliminates the need for a specialized laboratory or hospitalization will represent a significant contribution to the pool of available antimelanoma treatments.

As model antigens, we cloned, expressed, and purified a 60-Kd recombinant melanoma protein derived from a shortened sequence of the native gp100 gene (HR-gp100) and a synthetic multiepitope polypeptide. As adjuvants and to enhance transcutaneous delivery, we evaluated two forms of heat-labile enterotoxin and a cell active peptide.

HR-gp100, despite its size, entered the skin without the need of adjuvant. This was demonstrated by (1) production of specific antibodies in mice after topical application of the protein and (2) dosedependent epidermal Langerhans cell (LC) activation following transcutaneous delivery to intact human skin. LC activation was measured using a novel model of human skin transplanted to the highly vascularized chorioallantoic membrane of the chicken egg. Current experiments are analyzing cellular immune responses elicited by transdermal protein delivery.

This project aims at developing tools that will facilitate cell immunotherapy. We expect the data produced to demonstrate the feasibility of transdermal vaccination and to characterize the immune responses elicited by this treatment. We expect the results to be affected by the intrinsic characteristics of the antigenic proteins evaluated.

Widespread Deregulation of Phosphorylation-Based Signaling Pathways in Tumor Cells and Their Microenvironment: Opportunities for Therapeutic Intervention

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Multiple myeloma (MM) is a neoplasm of plasma cell origin that is largely confined to the bone marrow (BM). Chromosomal translocations and other genetic events are known to contribute to deregulation of signaling pathways, which leads to the transformation of plasma cells and progression to malignancy. To provide a more comprehensive molecular analysis of signaling disruptions in this disease, we compared the kinome of normal plasma cells to neoplastic MM plasma cells. The kinome of normal BM stromal

cells and diseased stromal cells were also compared. To do this comparison, we prepared highly purified samples of the above cell populations from the BM of MM patients and normal controls via high-speed cell sorting. The purified cell populations were then lysed in the presence of phosphatase inhibitors and then incubated in the presence of 33P-ATP on PepChip arrays of 1,152 different pseudopeptides representing substrates for most of the kinases present in the mammalian cell. Thus far, we have compared the kinome of four different MM cell samples with that of four different normal plasma cells samples as well as stromal cells from both tumor and normal marrow. These comparisons revealed deregulation of multiple signaling pathways in MM cells but not in the supporting stroma compared with their normal counterparts. The deregulated kinases identified are potential novel molecular targets in this lethal disease.

An Innovative Approach for Profiling Protein Kinase Substrates

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Signal transduction is a central component of nearly all biological processes. Protein kinases play critical roles in signaling pathways by phosphorylating proteins involved in signal amplification and in executing the cellular response to extrinsic or intrinsic stimuli. Aberrant kinase signaling plays a role in the etiology of nearly all cancers and in hundreds of other diseases. Systematic approaches to the elucidation of the signaling pathways driven by kinases have the potential to illuminate new points of therapeutic intervention. The identification of physiologic kinase substrates is an important component of this endeavor. Currently, there is a need for technologies that can be widely deployed to approach this problem. Using protein kinase CK2 as a paradigm, we are developing a simple, straightforward technique for kinase substrate. In this approach, the roles of kinase and substrate in a classic in-gel kinase assay are reversed. In the reverse in-gel kinase assay (RIKA), a kinase is copolymerized in a polyacrylamide gel that is then used to resolve a tissue or cell protein extract. Refolding by buffer exchange to restore kinase activity and substrate structure is followed by a kinase reaction to phosphorylate substrates in situ. We demonstrate that this method can be used to profile true CK2 substrates and show that RIKA can detect as little as 50 femtomoles of a substrate. With further development and validation, this assay has the potential to identify the physiologic substrates of many cancer-relevant serine/threonine kinases.

Ultrasensitive FACTT Assays for Melanoma Serum Biomarkers

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We have developed a new antigen detection and quantification method that we term “fluorescent amplification catalyzed by T7 RNA polymerase technology” (FACTT).

FACTT uses principles similar to enzyme-linked immunosorbent assay (ELISA); however, the detection antibody in FACTT is directly coupled to a double-stranded DNA template that contains a T7 promoter to accommodate the attachment of the T7 RNA polymerase enzyme. The interaction of T7 leads to the production of RNA species that can be monitored by adding a fluorescent RNA intercalating dye. FACTT assay is developed in 96- or 384-well plates. It is an innovative isothermal, quantitative, high-throughput immunoassay platform. Our preliminary data demonstrated that FACTT assays consistently have at least a 1,000-fold higher sensitivity than ELISA.

Malignant melanoma is a deadly disease. Melanoma cells express melanocyte lineage-specific markers, such as Melan-A, tyrosinase, and TRP-1. Melanoma cells secrete soluble tumor markers, such as protein melanoma-inhibitory activity (MIA) and S-100beta. Tumor cell apoptosis and necrosis are common in malignant neoplasms even at an early stage; therefore, lineage-specific markers may be released into the bloodstream.

Our goal is to develop ultrasensitive assays to monitor melanoma progression and response to therapy. FACTT is a versatile platform that also can be used to detect biomarkers for other cancers.

We have set up FACTT assays to detect tyrosinase, TRP-1, and MIA. Pairs of antibodies were purchased from commercial sources. The capture antibody is coated in carbonate-bicarbonate buffer (pH 9.6) to 384-well plates at 5 µg/mL/well and 20 µL/well overnight at 4 °C. 1:100 dilution of serum in the amount of 20 µL per well was added to the coated plate for a 1-hour incubation at room temperature. Twenty µL of diluted biotinylated detection antibody (180 ng/mL, or an optimized concentration for each antibody) was used for each well and incubated at room temperature for 1 hour. Streptavidin and the biotin-DNA template (amplification module [AM]) were added sequentially at 5 µg/mL and 250 ng/mL, respectively, with a 1-hour room temperature incubation for each step. We washed the plate six times with PBST between each binding incubation. After excess AM and proteins were removed by washing, 20 µL of reaction mixture (containing 60 units of T7 RNA polymerase plus [Ambion], 1.25 mM NTP, 1x T7 buffer [Ambion]) was added to each well. RNA amplification was performed at 37 °C for 3 hours. The RNA intercalating dye RiboGreen was added to the reaction mixture (20 µL, 1:200 diluted in the TE buffer supplied by the manufacturer), and the plates were read at Ex 485nm/Em 535 nm in a TECAN SpectraFluor reader. Our preliminary data showed that tyrosinase, TRP-1, and MIA levels were significantly increased in patients with metastatic melanoma.

We plan to optimize the assays to have a concentration curve correlation coefficiency of at least 0.95; coefficient of variation of intra-assay and interassay variance of <10% and a quantification accuracy of >90%. We will establish normal ranges for serum tyrosinase, TRP-1, and MIA in control populations using FACTT assays. We have already started to collect sera and plasma from patients with history of melanoma. We will use the assays to measure the serum protein levels in these samples to see whether these serum proteins can be used as biomarkers for prognosis and response to

therapies.

Identification and Validation of Two Types of Novel Posttranslational Modifications: Lysine Propionylation and Butyrylation

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The positively charged lysine residue plays an important role in protein folding and functions. Neutralization of the charge often has a profound impact on the substrate proteins. Accordingly, all of the known posttranslational modifications at lysine have pivotal roles in cell physiology and pathology. We report the discovery of two novel in vivo lysine modifications in histones—lysine propionylation and butyrylation. We confirmed, by in vitro labeling and peptide mapping by mass spectrometry, that two previously known acetyltransferases, p300 and CBP, could catalyze lysine propionylation and lysine butyrylation in histones. Finally, p300 and CBP could carry out autopropionylation and autobutyrylation in vitro. Taken together, our results conclusively establish that lysine propionylation and lysine butyrylation are novel posttranslational modifications. Given the unique roles of propionyl-CoA and butyryl-CoA in energy metabolism and the significant structural changes induced by the modifications, the two modifications are likely to have important, distinct functions in the regulation of biological processes. The modifications are highly likely to link with cancer because lysine aceytlation has an important role in cancer.

Imaging Transcriptional Activation of Gliomas

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Malignant gliomas are characterized by heterogeneous regions of necrosis, apoptosis, proliferation, invasion, and angiogenesis. Understanding differences in gene expression between normal brain and malignant glioma tissue is useful for diagnosing and treating gliomas. Recently, serial analysis of gene expression data for malignant gliomas has become available through the Cancer Genome Anatomy Project (CGAP), and several genes have been identified that are overexpressed in gliomas but not in normal brain. Some have been postulated to correlate with a particular glioma phenotype, such as invasion or angiogenesis. We propose to use a set of technologies to translate knowledge obtained from CGAP in imaging the transcriptional activation of the glioma-expressed genes and to correlate such activation with the phenotypic heterogeneity observed in in vitro and in vivo glioma models. We have devised a simple and efficient methodology to convert any BAC or PAC library clone into a herpes simplex virus (HSV)-based amplicon vector and have demonstrated that genomic loci of up to 150 kb can be delivered intact into and expressed in target cells. We plan to combine this technology, designated infectious BAC (iBAC), with bioluminescence imaging and/or magnetic resonance imaging to monitor and visualize transcriptional activation of glioma-expressed genes in glioma models.

Among the genes upregulated in human malignant gliomas, we selected SPARC (secreted protein, acidic and rich in cysteine) as a model gene to study. SPARC is known to be involved in remodeling of the extracellular matrix and is associated with tumor invasion. To monitor transcriptional activity of the SPARC gene, we first identified a BAC clone that contains the 120 kb of 5' flanking region and the first exon of this gene. By homologous recombination in Escherichia coli, we inserted into the BAC clone a bicistronic reporter cassette that contained firefly luciferase and internal ribosome entry site green fluorescent protein within the first exon of the SPARC gene. Both reporter genes are thus expressed under the control of the SPARC promoter and its native regulatory sequences. To deliver the indicator BAC efficiently into glioma cells, the BAC clone was retrofitted with HSV amplicon elements (oriS and pac) using the Cre-loxP system. The engineered SPARC-indicator iBAC (iiBAC) clone was then packaged into HSV virions using the helper virus-free HSV amplicon packaging system. When the packaged SPARC-iiBAC was inoculated into human glioma cell lines, we observed levels of luciferase expression that increased in an MOI-dependent manner. This result confirmed that the HSV amplicon-delivered 5' flanking region and promoter of the SPARC gene are functional in human glioma cells. We are investigating whether the levels of reporter expression from the SPARC-iiBAC recapitulate endogenous levels of SPARC expression in various cell lines or culturing conditions.

Development of Enhanced Peptide Amide Hydrogen/Deuterium Exchange Mass Spectrometry Analysis of Integral Membrane Proteins: A Gateway to the High-Throughput Structural Analysis of Membrane Proteins

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Our overarching aim in this new R21 IMAT grant is to develop and implement methods that will allow enhanced peptide amide hydrogen/deuterium exchange mass spectrometry (DXMS) analysis of integral membrane proteins. With the support of our prior IMAT R33 grant, we have had considerable success in using DXMS to improve the quality of soluble protein crystallographic constructs for structure determination efforts. Furthermore, we are developing a revolutionary approach to protein structure determination in which high-resolution DXMS data obtained on small amounts of soluble protein are used to identify the correct structure for a protein from within a large set of computationally predicted structures for the protein (DXMS-COREX-Rosetta), bypassing the need for x-ray or nuclear magnetic resonance analysis. Development of integral membrane protein DXMS will allow us to use these two approaches to robustly address the critical need for high-resolution, threedimensional structures of membrane proteins. Our developmental studies are focusing on integrins and G-protein-coupled receptors (GPCRs).

One of the most important issues regarding integrin functioning is the molecular mechanisms that underlie "inside-out" and “outside-in" signaling. We have succeeded in preparing complete highdensity probe maps of the alpha-1 beta-1 integrin "I" domain, an integrin substructure that recapitulates much of the binding and activation activity

expressed by the intact integrin. We are now analyzing the data obtained from our first complete DXMS study of the I domain in the resting state (with bound magnesium ion) versus the activated state (with bound manganese ion) as well as with control conditions of calcium or no divalent cation (EDTA) conditions. We will next be mapping the binding epitopes of integrin-neutralizing monoclonal antibodies with this integrin.

Regarding GPCRs, we deem the development of the ability to robustly perform DXMS analysis of these seven-membrane-spanning proteins to be a rigorous criterion for the success of our methods. We are simultaneously attacking three different GPCRs: (1) bacteriorhodopsin, the prototypic GPCR for which a high-resolution crystal structure has been obtained; (2) chemokine receptors, GPCRs that constitute a major potential therapeutic target for antinflammation and anticancer therapeutics. With our collaborators, we have prepared one of the receptors in quantities sufficient for DXMS study and have succeeded in preparing very high-quality probe maps of its chemokine ligand. (3) The GPCR beta adrenergic receptor is one of those under study by the NIH-funded Joint Center for Innovative Membrane Protein Technologies (JCIMPT) in efforts to improve the design of protein constructs suitable for structural studies. We are now preparing fragmentation maps of the purified protein as a preamble to DXMS analysis of the JCIMPT's present constructs for this protein.

SPEAKER ABSTRACTS

Application of a Sensitive Double-Clad Optical Fiber (DCF) for Two-Photon Fluorescence Measurement in Tissues

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Quantification of tissue fluorescence using conventional techniques is difficult due to the absorption and scattering of light in tissues. Whole-body fluorescence imaging techniques do not provide accurate quantitative information on the distribution of a fluorescently tagged molecule in tissues. Owing to the limited tissue penetration of light, these methods also lack sensitivity for detection of low concentrations of tissue fluorescence. Previously, we developed a two-photon optical fiber fluorescence (TPOFF) probe as a minimally invasive technique for quantifying fluorescence on solid tumors in live mice on a real-time basis [1]. In those studies, we used a single-mode optical fiber (SMF) through which femtosecond laser pulses were delivered into the tumor, which enabled us to measure low micromolar concentrations of targeted fluorescent nanoparticles. It is essential that a more sensitive TPOFF device is developed for quantification of lower levels of a targeted fluorescent agent. We demonstrate the biological application of a DCF that can keep high excitation rate by propagating ultrashort laser pulses down an inner single-mode core, while improving the collection efficiency by using a high-NA multimode outer core confined with a second clad.

Since the DCF does not have holes, any capillary suction of biological fluids is prevented, which is a problem for the biological application of a previously described double-clad photonic crystal fiber (DCPCF) [2]. The solid DCF used has a numeric aperture of 0.46, which is smaller than that of a DCPCF (Figure 1).

Although the DCF's collection efficiency is not as high as that of the DCPCF, it provides significant improvement over traditional single-clad fibers. Initially, we compared the two-photon fluorescence detection efficiency of using the DCF versus the SMF with standard solutions of the dye 6-TAMRA (6T) and the generation 5 dendrimer (G5) nanoparticles G5-6T and G5-6T-folic acid (G5-6T-FA). We have observed about a fivefold increase in the detection efficiency of these fluorescent agents.

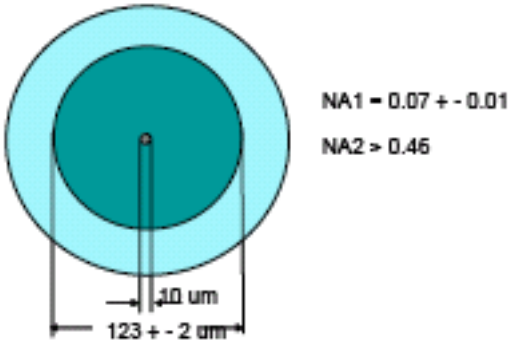

Figure 1. Schematic representation of the cross section of the DCF used in our studies.

We then compared the targeting of G5-6T-FA in FA receptor (FAR)-expressing cells in vitro. The results showed a fivefold higher detection efficiency in the bound G5-6T-FA conjugate (Figure 2). Finally, we demonstrated the applicability of the DCF fiber to quantify the in vivo targeted uptake of G5-6T-FA in mice tumor expressing FAR. We observed a threefold increase in the background-subtracted counts obtained by the DCF fiber over the SMF fiber and a tenfold increase in the tumor uptake of the targeted G5-6T-FA conjugate versus the control conjugate G5-6T (Figure 3).

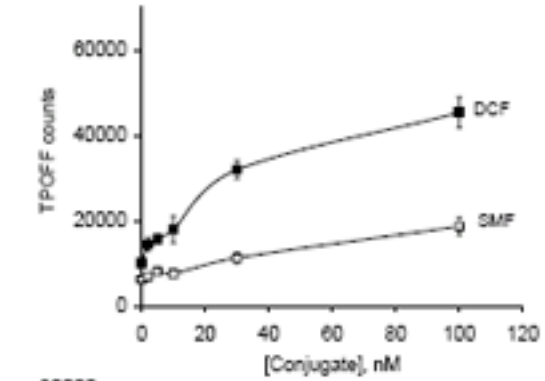

Figure 2. Comparison of the targeting of G5-6T-FA in KB cells determined by SMF and DCF TPOFF measurements. KB cells in suspension were incubated with different concentrations of the conjugate for 1 h in the cell pellet. All data shown are the mean ±SE of 4-5 counts taken in different regions of each cell pellet.

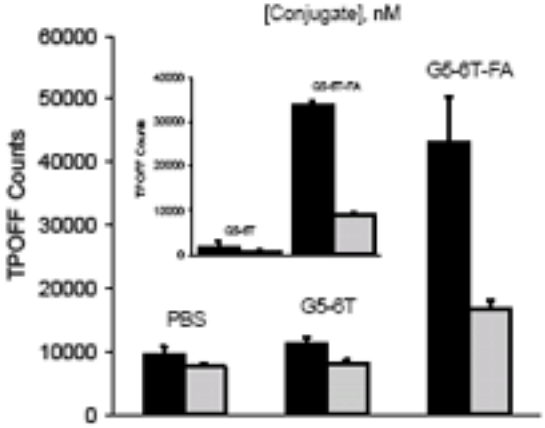

Figure 3. In vivo tumor targeting of G5-6T-FA, determined by TPOFF. KB cell tumors were developed in SCID mice and were intravenously injected with 15 nmol each of the targeted conjugate G5-6T-FA, the control conjugate G5-6T, or their vehicle PBS. After 15 h, the tumors were isolated and the TPOFF counts were taken in different internal regions of the tumor using the DCF (solid bars) or SMF (shaded bars) fiber probe. *Inset*: Counts corrected for the background fluorescence of the PBS-tumor.

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Background: Knowledge of the role of key signaling proteins in cancer has prompted the development of anticancer agents directed against targets such as EGFR. Considerable effort has centered on antagonizing EGFR tyrosine kinase activity, and EGFR mutations have been identified that correlate with tumor shrinkage following treatment with kinase inhibitors. However, clinical trials in non-small cell lung cancer (NSCLC) have revealed highly variable levels of effectiveness to EGFR antagonists in cancer patients. Moreover, little is known about the precise mechanism of these anticancer agents in human tumors, often because of the difficulty of performing relevant molecular assays on limited tissue material. Uncertainty about how these agents affect EGFR status/signaling in human tumors has made the development of these drugs extremely difficult. Thus, a key goal of the present research is to improve quantitative methodologies based on liquid crystals (LCs) for reporting the expression and activation (phosphorylation) of EGFR and certain tumor-associated EGFR mutants. This method of LC-based analysis is termed the “torque balance method” and offers the potential to provide (1) improved quantitative approaches for the detection of EGFR and its phosphorylation status, (2) the exquisite sensitivity needed for small sample sizes, and (3) the basis of a method that can quantify analytes in small surface areas (10 x 10 mm), thus making it suitable for highly multiplexed assays (microarrays). Information about EGFR levels, mutational status, and activation state in cell extracts and tumor biopsies should facilitate the choice of therapeutic options that target EGFR and/or its

mutants and the evaluation of experimental therapeutics with respect to their ability to target EGFR expression/activation in vivo.

Method: The anchoring of LCs is a process wherein the orientation and appearance of an LC is dictated by its interaction with a surface. The design and fabrication of surfaces with topographies matched to the sizes of proteins make possible the amplification and transduction of subnanometerscale processes associated with molecular recognition into measurable optical outputs using LCs. Since molecules within LCs can communicate across regions of the fluid that are up to 100 micrometers away, protein binding to nanostructured surfaces possessing specific antibodies or other capture molecules can be amplified into changes in the orientations of micrometer-thick films of LCs. The change in LC orientation triggered by the presence of a targeted protein binding to the surfaceassociated antibody leads to a striking change in the optical appearance of the LC and in the torque balance method. The protein binding-induced change in LC orientation at the surface in response to an applied torque is measured as a change in the anchoring energy. Compared with other techniques, a photonics approach based on the exquisite sensitivity of LCs to nanometer-scale differences in surface topography possesses many noteworthy features, including estimated detection limits of 1,000 to 10,000 copies of a protein (e.g. three to four orders of magnitude more sensitive than gel electrophoresis). This sensitivity opens the way to the possibility of single-cell analyses, thus greatly reducing the tissue sample sizes required and opening up the possibility of analyzing cells obtained by aspiration.

Current Results and Directions: Our data show that we can fabricate surfaces with nanometer-scale topographies to identify EGFR expression and phosphorylation status in cell preparations using LCs and anti-EGFR antibodies and detect inhibition of EGFR phosphorylation and kinase activity in cell extracts following exposure to EGFR antagonists. Our goals are to refine, validate, and implement this methodology to allow high-throughput screening on limited clinical specimens and to ultimately use this technology to assess which tumors are most likely to respond to EGFR antagonists. The aims are to (1) optimize the torque balance method for utilizing LC-based assays to report EGFR expression and phosphorylation status in cell preparations in a highly sensitive reproducible and quantitative manner and (2) evaluate and refine the concept that small numbers of cells are sufficient to report the expression, phosphorylation status, and tyrosine kinase activity of EGFR. Currently, the technology utilizes samples that include purified receptor and cell lysates/membrane preparations generated from human epidermoid cancer cells, human lung cancer cell lines, and murine fibroblasts expressing wild-type and various mutant human EGFRs. Ultimately, we wish to test the hypothesis that this LC technology can used to reproducibly detect the expression and activation of the EGFR pathway in human NSCLC tumor biopsies (including needle aspirates and paraffin-embedded samples) as well as in normal surrounding tissue and in skin biopsies.

Ultrasound Tissue Fixation and Processing Achieve Superior Morphology and Macromolecule Integrity

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Formalin fixing/paraffin embedding (FFPE) has been a standard tissue preservation method employed in more than 90% of cases for clinical histology diagnosis. However, this method has three disadvantages: (1) slow fixation and processing of tissue (more than 24 hours), (2) slow quenching of enzymatic activity causing macromolecule (such as RNA) degradation, and (3) extensive molecular modification affecting protein antigenicity.

We have developed the ultrasound-accelerated FFPE (US-FFPE) technology for rapid and multipletissue preparation. The US can achieve tissue fixation and processing within 1 hour. In downstream assays, US-FFPE achieves comparable tissue morphology and superior molecular integrity using highdefinition H&E, the latest two-color HER2 silver in situ hybridization (SISH), and immmunohistochemistry (IHC) such as ER, PR, Ki67,EGFR, HER2neu, Claudin 1, p16, and MCM7.

To further take advantage of this technology, solvable protein and nucleic acid are extracted from sections of fresh, FFPE, and US-FFPE tissues to evaluate protein and RNA recovery efficiency with Western blot and PCR. We have found that US-FFPE achieved better recovery of protein and RNA, even from long-term storage of tissue sections. In addition, through rapid US-FFPE, phosphorylated proteins such as pERK1/ERK3, pAKT, and pSTAT3 can be effectively preserved and examined for downstream quantitative analysis with high-throughput tools.

We also found that, during fixation, tissue displays physical changes that can be monitored and reflected as changes in transmission US signals. Further study of this phenomenon may provide a method to control and monitor the standardization of tissue fixation and processing.

Identification of Immune-Selected Breast Cancer Antigens

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Several methods have been employed to attempt to identify tumor antigens from patient samples. These include antibody SELEX technologies as well as ScFv-phage display library synthesis and selection against tumor proteins. The innovative technology we have developed takes advantage of the immune responses to breast cancer in the

tumor-draining lymph nodes. These lymph nodes are then used to isolate cDNAs that encode the variable region of antibodies, and those with antigendriven mutations are then expressed as recombinant proteins and used to identify endogenous tumor antigens.

The critical steps in this technology are (1) rapid storage of sentinel lymph node and primary tumor surgical samples using direct freezing in OCT media, (2) immunohistological analysis of lymph nodes for reactive and proliferative germinal centers, (3) isolation of B-cell mRNAs and amplification of heavy chain variable region (VH) antibody cDNAs, (4) sequencing multiple VH clones and analyzing for V-D-J recombination and somatic hypermutation events, (5) expression of recombinant VH monobodies with epitope and purification tags in insect cells with baculovirus vectors, and (6) utilization of VH affinity binding to breast cancer cell extracts to identify novel tumor antigens with LC-MS/MS proteomic analysis.

The hallmarks of immune activation in tumor-draining lymph nodes demonstrated that only 1 out of 42 B-cell zones in each sentinel lymph node was “activated” and proliferative. Thus, selection of these defined areas for the preparation of VH cDNA libaries represents a significant reduction of complexity within the library of potential antitumor antibodies. Further elimination of nonproductive cDNAs was provided by sequence analysis for B-cell amplification and somatic hypermutation. Only those VH clones that possess a complete open reading frame and somatic hypermutation sequence were used to produce recombinant monobodies.

The selection of potential antigens was provided by immunoprecipitation from breast cancer cell lysates using magnetic bead separation and analysis of coprecipitating proteins with proteomic identification of silver-stained bands. The next iteration of the technology will employ direct elution and proteomic identification directly from the magnetic selected immunoprecipitations. Medium-throughput screening of monobodies indicates that approximately 2 to 3 of 10 recombinant VH monobodies show specific protein binding capacity, and several potential antigens have been identified. One of these, neuroplastin, has been shown to be expressed in invasive breast cancer and promotes tumor growth and angiogenesis in vivo using xenograft implants of human breast cancer cell, which overexpress this antigen.

In summary, this technology can be applied to human cancers to identify novel tumor antigens, provide reagents to identify these antigens in patient samples, and potentially be useful as therapeutic reagents to target metastatic disease.

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

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We are continuing work on our RNA sensor platform, which involves formation of a "hybridization sandwich," consisting of two antisense oligonucleotides (ASOs) targeted to optimized sites in a preselected target RNA for a particular cancer type. One of the ASOs is attached to a nanowire (NW), and the other is attached to a gold particle for added mass. Binding of target RNA+Au-ASO to the NW induces a shift in the resonance frequency of the NW that is proportional to the number of binding events, allowing quantitative evaluation of target RNA molecules. The derivatized NWs were electrofluidically deposited onto predetermined chip locations for multiplexing, and binding to selected target RNAs was initially detected optically.

This project is directed toward detection of circulating tumor cells (CTCs) in low concentrations. We have library-selected optimal binding sites for DD3 and AMCAR for prostate cancer and tyrosinase and Melan-A/MART-1 for melanoma and have quantified the average number of transcripts per cancer cell. Sample preparation involves fractionating peripheral blood using a porous-membrane, densitygradient centrifugation device, which affords essentially quantitative recovery of tumor cells with an approximate 300 to 400x enrichment, followed by extraction of RNA. We have initiated a small clinical trial to quantify CTC levels in newly presenting melanoma patients and are quantifying levels using QPCR for benchmarking. Samples will be banked and examined using the RNA sensor when a prototype is ready.

Using DD3 as a target, we have used spotted arrays to develop binding conditions that allow single mismatch discrimination. With optical detection of NW frequency shifts, we have now obtained initial results for specificity of detection of complementary strands of DNA. Resonance frequency shifts are proportional to the visually determined number of bound particles, with a variation of about 5 to 10%. Control experiments with noncomplementary DNA have yielded near-zero frequency shifts. In addition, we have made resonance measurements of NWs under varying chamber conditions, from atmospheric pressure to high vacuum, to aid in selection of the minimal vacuum equipment that will enable point-of-contact sensing in practical versions of these nanoscale sensing devices. Work is also proceeding on chip design features and optimization of NW properties for electrofluidic deposition (including use of modified ASOs).

The input samples will be RNA from peripheral blood fractionated using the simple-gradient column devices, and standardization of protocols should be straightforward. It is anticipated that samples from patients with late-stage disease will contain increasingly higher levels of CTCs and that earlystage patients (e.g., no breast cancer patients or radial growth phase melanoma patients) will also exhibit detectable levels of CTCs.

We ultimately envision this device as a noninvasive screen for a battery of common

cancers, including prostate, breast, melanoma, etc. Our basic premise is that there is an early window of opportunity in which cancer cells appear in peripheral blood but before metastases have developed. We therefore anticipate that our device will ultimately afford highly sensitive detection of multiple cancer types at very early (curable) stages of diseases.

Tissue Print Micropeels for the Molecular Profiling of Cancer

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To realize the full potential of a wealth of new tumor biomarker information, it is essential to develop strategies for profiling human tissue and tumor specimens that are workable in a clinical setting. We have developed a set of novel “tissue print micropeel” technologies that allow us to profile the molecular markers over extended areas of human tissue and tumor samples without damaging the specimen [Gaston et al. 2005]. “Tissue printing” transfers cells and extracellular matrix components form a tissue surface onto nitrocellulose membranes, generating a two-dimensional anatomical image on which molecular markers can be visualized by specific protein and RNA/DNA detection techniques. The resulting marker maps can then be superimposed directly onto histopathological and radiological images of the specimen, permitting molecular identification and classification of individual malignant lesions. By design, the front-end collection of tissue print micropeel samples is simple and straightforward; non-laboratory-trained personnel master the technique very quickly. For biomarker discovery and multianalyte profiling, we have developed interfaces that couple our sample collection platform with downstream analytical technologies; these allow us to generate multilayer maps of the specimen that can incorporate RNA-, DNA-, and protein-based biomarkers. As with conventional sample collection techniques, preventing degradation of labile analytes is key to the success of the method. Endogenous proteases and nucleases vary from one type of tissue to another, and the tissue of origin is the most important “pedigree” variable that must be managed during specific applications of our tissue print sample collection technology.

Two clinically relevant proof-of-principle applications illustrate the utility of our tissue print micropeel approach to the collection and analysis of high-quality human tissue samples that are difficult or; impossible to obtain using conventional sample collection techniques. We have used prostate wholemount tissue print biomarker maps to identify and characterize patterns of gene expression that correspond to magnetic resonance imaging/magnetic resonance spectroscopy (MRI/MRS) and dynamic contrast-enahanced (DCE) MRI visible phenotypes in human prostate cancers. Because we can obtain high-quality RNA with our tissue printing techniques, our biomarker maps include detailed gene expression profiles from both Affymetrix GeneChip® microarrays and quantitative reverse transcriptase PCR (qrt-PCR) analysis. High-quality DNA is also

prepared from the tissue prints in parallel with the RNA, adding another layer of biomarker data. One major advantage of our tissue print technologies, illustrated by this MRI-pathology-biomarker application, is the ease with which the entire surface of a specimen slice can be captured on tissue prints; colocalization of the tissue print whole-mount gene expression maps with MRI and histopathology is thus straightforward. We have used this approach to identify several previously unrecognized patterns of prostate cancer gene expression that are potentially important in the clinical interpretation of MRI/MRS and DCE MRI.

A second application focuses on molecular biomarker analysis of prostate needle biopsies. Prostate biopsies are central to the diagnosis of prostate cancer, but because the cores must be submitted in their entirety for surgical pathology, these samples are rarely available for molecular biomarker analysis. We have demonstrated that our tissue printing techniques do not compromise prostate needle biopsy cores for pathology diagnosis. In addition, in collaboration with Dr. Gary Latham (also an IMAT principal investigator), we have shown that our biopsy tissue print protocols yield highquality RNA samples that produce global gene expression profiles with excellent print-core concordance. In January 2006 we opened an IRB-approved protocol for the prospective collection of tissue prints from diagnostic prostate biopsies obtained as part of a standard clinical evaluation for prostate cancer; we have now collected more than 200 sets of biopsy prints. It should be noted that only 10 to 15% of men who undergo prostate needle biopsy will ever undergo radical prostatectomy; thus, our biopsy tissue print samples include important patient subgroups that are not captured in conventional prostate cancer tissue banks. The NCI Early Detection Research Network has recognized this unique resource and provided support for a pilot study in which our cohort is being used to evaluate the prevalence of a newly identified set of prostate cancer biomarkers (gene fusions arising from chromosome 21 rearrangements) in a clinically representative prostate biopsy population. This real-world translational application, in which tissue prints provide high-quality gene expression data that are often difficult or impossible to obtain from formalin-fixed, paraffin-embedded samples, demonstrates how our innovative tissue print sample preparation technologies expand the pedigree of patient samples available for biomarker discovery and for the next generation of clinical tests for cancer.

Gaston S.M., Soares M.A., Siddiqui M.M., et al. Tissue-Print and Print-Phoresis as Platform Technologies for the Molecular Analysis of Human Surgical Specimens: Mapping Tumor Invasion of the Prostate Capsule. Nat Med 11(1):95-101, 2005.

Phosphoprotein Profiling for Quantitative Analysis of Protein Phosphorylation Patterns

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The primary goal of this research is to develop quantitative assays to measure BCR-ABL tyrosine kinase activity and inhibitor sensitivity in cell extracts. This effort is stimulated by the recent development of imatinib (STI571) and newer, more potent ABL kinase inhibitors such as dasatinib and nilotinib. These ATP analogs target BCR-ABL, an oncogenic kinase that results from the t(9;22) reciprocal translocation characteristic of chronic myelogenous leukemia (CML) and some acute lymphoblastic leukemia. Typically, imatinib induces remission, but patients may develop resistance, leading to recurrence. Our efforts have been directed toward delivering robust clinical assays for BCR-ABL activity and inhibition to guide therapy and develop assays amenable to high-throughput screening to identify novel BCRABL inhibitors. 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. Year 3 of the development phase has led to validation of our assays in formats amenable to analysis of patient material and/or high-throughput screening. The Kron lab has adapted acrylamide hydrogelcoated multiwell plates for high-throughput screening for new BCR-ABL inhibitors. Kron, Kent, and Palecek have continued to develop acrylamide copolymerization methods with photocleavable peptides to develop a solid-phase detector with MALDI-TOF MS read-off. For this, Kron and Kent developed novel methods for quantitation of phosphorylation in MALDI-TOF. Hoping to increase assay sensitivity, Palecek investigated the effects of polymer network structure and composition on macromolecular diffusion rates into the gels. Using unsteady-state diffusion transport models, we calculated the diffusivity of labeled dextrans into polyethylene glycol acrylamide (PEGA) hydrogels. At minimal monomer concentration for mechanical stability, both monoacrylate and diacrylate PEGA gels permit substantially greater diffusivities than polyacrylamide materials. We are simplifying our methods to make them compatible with clinical labs. Kron has recapitulated our approach as a Luminex Corporation bead array assay, and Stock has developed a novel homogeneous antibody beacon assay in which real-time detection of enhanced fluorescence as a ligand is displaced from antiphosphotyrosine antibody by the binding of newly phosphorylated peptide substrates. BCR-ABL kinase activity and its inhibition were readily detected in whole-cell extracts of CML cell lines and patients. Finally, to enhance molecular detection of mutant BCR-ABL prior to biochemical resistance, Strauss has pursued detection of mutants by PCR with suppression of wild-type background with LNA oligos. We anticipate that whole-cell lysates of leukocytes purified from blood obtained by venipuncture during regular visits by CML patients will be the typical sample. Our experience suggests that standard phlebotomy procedures are sufficient to maintain BCR-ABL activity, facilitating use in the clinic. Assays will reliably determine the presence of BCR-ABL activity and IC50 of inhibition by imatinib and other drugs. Physicians can then use these data to make optimal choices for a first-line drug in newly diagnosed patients and to optimize response to acquired resistance.

This work was supported by CA103235

Automated, Whole-Slide-Based, Multiplexed Molecular Marker Assessment in Formalin- Fixed, Paraffin-Embedded Tissues

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The purpose of this work is to provide reliable, cellularly resolved molecular pathway assessment in cancer biopsies to assist pharmaceutical drug development and provision of patient-specific prognosis and therapy guidance ("personalized medicine"). The organizing theme is that the appropriate unit of analysis should be the individual cell as opposed to averaged tumor extracts that are typically used for genomic, gene expression, and proteomics analysis. To this end, we have combined multispectral imaging techniques with automated slide scanning and powerful tissue segmentation-based software. Spectral imaging and advanced image analysis tools permit multitarget immunohistochemical and/or immunofluorescence detection at the cellular and subcellular levels in intact, formalin-fixed, paraffin-embedded (FFPE) tissue sections. CRI-developed image-processing and machine-learning tools provide automation and hands-off quantitation options and can be combined with multiplexed staining protocols that yield independent, potentially stoichiometric labeling.

Multispectral imaging, using either modulation of the light source to provide wavelength-specific illuminants or detection of emitted or transmitted light using a liquid-crystal, tunable filter-based system (Nuance™), was used to resolve multiple molecular markers in FFPE tissues. Coexpression of nuclear markers, such as estrogen receptor (ER) and progesterone receptor (PR), using chromogenic stains is easily quantified and can be combined with membrane markers and counterstain in the same sample. In fluorescence, we have been able to image five quantum-dot-labeled antigens and a DAPI nuclear stain using a single spectrally resolved acquisition (requiring no switching of filter wheels or filter cubes). Autofluorescence, which degrades sensitivity and clarity, can be spectrally removed, providing dramatic improvements in signal-to-background ratio for low-abundance targets.

Whole-slide scanning with multispectral capabilities has been developed at CRI, permitting automated slide handling and imaging. The final piece of the puzzle is the ability to detect appropriate regions for extraction and quantitation of molecular data. For example, ER and PR scores are usually of interest only from cancer deposits, and data of normal breast epithelium and stroma should be excluded. We have developed a machine-learning, neural-net-based capability that can be readily trained to segment tissues into appropriate classes. We have combined this with nuclear segmentation tools and can therefore extract molecular data from appropriate nuclei. Preliminary comparisons with human scoring (so far of single molecular markers) indicate high concordance. Future work will include investigation of coordinated subcellular location of "pathway" molecules, with simultaneous assessment of cell-surface receptors (e.g., EGFR, VEGF, HER2neu), downstream signaling proteins and phosphoproteins (e.g., pAKT, pERK), nuclear proteins (e.g., ER, Ki67), and novel players such as protein-folding mediators (e.g., BIP1).

The sum of these parts is a system that can scan whole slides, isolate regions containing only cancer cells, spectrally unmix multiple molecular markers, and provide quantitative metrics, including cell-bycell colocalization indices. This combination of capabilities should prove to be of value both for all tissue-based molecular studies—for basic science, preclinical studies, and ultimately, for patient-based molecular medicine in the clinic.

Exon-Specific Sequencing of Whole Genomic DNA

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Despite recent progress in high-throughput genome sequencing technologies, whole-genome sequencing of multiple neoplasms per patient is not yet economically feasible. Targeted sequencing of specific exons is more practical, but high-level multiplex amplification incurs significant technical risks. RDT proposes to utilize microfluidic-based technologies for manipulating droplets of fluids to enable ultrafast, de facto, multiplex amplification and sequencing of up to thousands of exons from preemulsified primer libraries and individual DNA samples.

RDT has developed a microfluidics instrument capable of encapsulating individual exon-specific primer pairs from thousands of different chromosomal regions into a library composed of individual, discrete, subnanoliter droplets. Each droplet in this library will contain both an exon-specific pair of PCR primers and a DNA capture bead on which reverse primers are immobilized. The RDT instrument is also able to combine purified genomic DNA and PCR reagents with the individual primer-containing droplets and directly amplify the merged droplets via high-speed microfluidic PCR, with the amplified exon immobilized on the DNA capture bead for subsequent sequencing via 454 sequencing technology.

RDT has successfully demonstrated a high-speed, high-throughput microfluidic NanoReactor PCR Chip, generating more than 3,000 droplets per second and amplifying more than 1Kb products in less than 12 minutes; this is more than 10 million separate PCR reactions in an hour. Primer libraries containing five distinct primer pairs have been generated and merged with DNA-containing droplets, with subsequent amplification of all products. Fluorescence-based droplet sorting has also been implemented and verified, providing the potential to sort droplets based on the yield of the PCR amplification.

Starting Material Degradation Test Is Tied to Success in Whole-Genome Amplification From Diverse Clinical Samples

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Archival specimens represent a vast resource for discovery and evaluation of prognostic DNA markers. In the United States alone, there are more than 300 million archived tissue samples, with approximately 20 million samples added annually. These archived samples contain a wealth of genetic information and offer great potential for the discovery and analysis of biomarkers with diagnostic and therapeutic significance. Laser capture microdissection (LCM) samples, tissue print micropeels, and plasma-circulating DNA are additional sources of valuable clinical material with potential for discovery and evaluation of cancer biomarkers. However, the need to apply modern technologies to analyze DNA from old and diverse clinical samples often stumbles on suboptimal sample quality. The quantity of DNA trapped in these samples has also proven to be a significant barrier, leading to the frequent need for whole-genome amplification prior to applying downstream assays. Finally, knowledge of DNA quality is important to determine the types of techniques that the material can support. For example, the quality of archived specimens is dependent on fixation and storage conditions and can vary highly from sample to sample. Because specimen yield is often a limiting factor in studying nonrenewable clinical samples, the ability to assess DNA quality with a minimal amount of material prior to investing time and resources for sample analysis is of paramount importance.

The principal aims of this sample preparation project are to (1) develop and validate a novel methodology, RCA-RCA, that is tolerable to sample degradation and enables faithful whole-genome amplification of DNA from formalin-fixed, paraffin-embedded (FFPE) and other clinical specimens and successful downstream genetic screening and (2) provide researchers with methods to assess sample quality before investing time in testing clinical samples of uncertain status, thereby saving substantial effort and resources that can be utilized for screening samples of optimal quality.

We have adapted the originally published RCA-RCA protocol to correspond closely to the starting DNA quality of the material that is to be amplified. We evaluated the effect of a variety of commonly used conditions for formalin fixation of surgical samples on the ability to (1) produce uniform wholegenome amplification across the genome, (2) identify polymorphic sites and mutations in diverse chromosomes, and (3) apply array-CGH to identify genome-wide copy number changes. To enable rapid assessment of starting DNA quality, we developed a simple approach to assess DNA fragmentation in minute clinical samples of widely different origins and the likelihood of success of RCA-RCA and subsequent assays. A multiplex PCR amplification of four GAPDH amplicons of varying sizes is performed using 1 ng genomic DNA, followed by size discrimination on fluorescent dHPLC. Even minimal quantities of longer PCR fragments (~300-400 bp), visible via high-sensitivity fluorescent dHPLC, was essential for the success of RCA-RCA and subsequent assays. Multiplex-PCR-dHPLC correctly predicted the likelihood of assay success in FFPE samples fixed under controlled conditions and of different ages (0-12 years old) in LCM samples in tissue print micropeels and plasma-circulating DNA. The assay is rapid and convenient and can be used widely to characterize DNA from any clinical sample of unknown quality. Assessing DNA quality prior to whole-genome amplification enables adaptation of the whole-genome amplification protocol to correspond to the condition of the starting material and enables

extraction of information from older specimens. We anticipate a major impact of this technology on the identification of biomarkers from clinical samples and bodily fluids.

This work is supported by sample preparation project 1R21-CA111994

Clinical Application of Multispectral Imaging Flow Cytometry

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The ImageStream® multispectral imaging flow cytometer has been developed to produce highresolution brightfield, darkfield, and fluorescence images of cells prepared in suspension at rates of up to 100 cells per second. Its analysis software quantifies more than 200 morphometric and photometric parameters for each cell. The ImageStream® is particularly suited to analyze cells that are naturally in suspension (suspension cell lines, hematological malignancies), and like standard flow cytometry, successful analysis of solid tissue is dependent on how well the sample lends itself to preparation of single-cell suspensions. Our studies will focus on the quantitative study of intracellular localization of molecular targets, which traditionally has been studied using molecular techniques or (confocal) microscopy, with the disadvantage of lacking specificity for target cell populations or the ability to evaluate the large cell populations required for statistically robust analyses. This application has particular relevance to cancer research because, in cancer, aberrant signal transduction, often associated with abnormal intracellular distribution of specific signaling intermediaries, is commonly observed, and an increasing number of novel therapeutics targeting these signaling cascades are being developed and studied. The goal of our studies is to evaluate the potential of the ImageStream® to measure target-cell-specific, therapy-induced changes in nuclear cytoplasmic distribution of specific signaling pathway intermediaries as response parameters in clinical samples. The proposed studies will ultimately (R33 phase) be conducted on peripheral blood samples from patients with acute myeloid leukemia who are participating in a Phase I clinical trial with the macrolide rapamycin (sirolimus), which is known to affect the function (and cellular localization) of nuclear factor-?B (NF-?B). During the R21 phase of this proposal, system hardware, software, and sample preparation conditions will be optimized to efficiently and accurately determine NF-?B translocation events using cell-line model systems. In the R21 phase, ImageStream® hardware and software improvements will be pursued to (1) increase data collection speed and sample throughput capability, (2) develop a fluorescence-independent focus parameter, (3) develop an analysis method for probes for which the subcellular redistribution is less robust, and (4) increase flexibility in probe selection by adding additional laser sources. From the cell biology aspect, the effect of drug-induced changes on cell morphology will be studied as well as concentration and time kinetics of drug effect on the target molecule to predict optimal timing for sampling. Sample preparation is of paramount importance since many signal transduction pathways, including NF-?B, are activated or deactivated in response to cell stress. Since the

ultimate application of our studies is to use the ImageStream® in the evaluation of intracellular distribution of signaling pathway intermediaries as a parameter of treatment response, it is important to verify that variations in pathway activity detected by the ImageStream® are truly associated with a drug response rather than an artificial effect from sample collection/preparation procedures.

Expression Profiling in Paraffin-Embedded Tissues and Patient Cell Lines Reveals Predictive Markers in Intestinal Tumorigenesis and Colorectal Cancer Treatment

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Identifying markers of 5-FU response in human colorectal cancer: A major obstacle in the treatment of colorectal cancer patients is resistance to 5-fluorouracil (5-FU)-based therapy. By combining in vitro 5-FU sensitivity data with single-cell gene expression profiles of human colorectal cancer cell lines, we have developed a gene expression signature that is predictive of 5-FU resistance. Using fluorescence in situ hybridization (FISH) with probes against target nascent mRNAs, the number of individual cells with transcription sites for a panel of candidate genes was measured. We then used logistic regression to build a model that predicts the response of an individual cell line to 5-FU based on its gene expression profile. Our model was tested for predictive accuracy and robustness using leave-one-out cross-validation on a training set of cell lines that represented the extremes of sensitivity or resistance. The model with the highest predictive accuracy was then used to classify a set of blinded, independent test cell lines. Currently, we plan to test our predictive model on patient tissues in which response to 5-FU is known in order to correlate our gene expression signature with clinical outcomes.

Examining the spatial-temporal distribution of transcription of Notch and Wnt signaling targets in the mouse duodenum: To characterize the initial events in colorectal cancer progression, we analyzed the spatial-temporal distribution of key proliferation and differentiation genes in the intestinal epithelium. Notch signaling plays an important role in intestinal differentiation, and alterations in this pathway have been implicated in the development of colorectal cancer. Linking gene expression with spatial and temporal information is particularly important in investigating the interaction of signaling pathways as cells migrate along the crypt-villus axis. By defining transcriptional profiles for individual cells along the crypt-villus axis within the mouse small intestine, we can determine the pattern of expression of genes involved in lineage-specific differentiation. Using FISH in paraffin-embedded mouse duodenum tissue, we have identified the spatial-temporal pattern of gene expression as cells migrate along the crypt-villus axis. FISH for Notch1, Hes1, Math1, c-myc, cyclin D1, and Sox9 was performed on serial sections of normal mouse duodenum tissue. From this analysis, we can define the position along the crypt-villus axis where Notch and Wnt

target genes become transcriptionally active in normal intestinal epithelium.

Mutations in the adenomatous poliposis coli (APC) gene affect the expression of Wnt signaling targets in the intestine; however, it is not clear whether deficiency in APC has an effect on the spatialtemporal distribution of Notch signaling. Therefore, we examined the expression of Notch target genes along the crypt-villus axis in paraffin-embedded duodenum tissue from APC +/- mouse mutants using FISH. The spatial-temporal expression of both Wnt and Notch target genes was disrupted in APC mutants, providing further evidence of an interaction between Notch and Wnt signaling in intestinal lineage-specific differentiation and tumorigenesis. Analysis of APC mutants indicates that Notch and Wnt may interact in the development of intestinal tumors and suggests that the spatial-temporal distribution of key genes plays an important role in colorectal cancer development.

This work is supported by CA083208.

Developing Proteomic Technologies for Rapid, Real-Time, Label-Free Detection of Protein Interactions

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Key cell signaling events occur through the interactions of proteins; however, very little is known about “who” they interact with and “how” they interact. Large-scale studies using technologies such as yeast two hybrid (Y2H) and immunopreciptiation with mass spectrometric (IP/MS) detection have revealed thousands of interactions in various organisms. These approaches have uncovered proteinprotein interactions for only a fraction of what is expected biologically. Our goal is to develop novel high-throughput approaches to identify who the proteins interact with as well as measure the kinetics to understand how they interact. We would like to extend this not only for protein-protein interactions but also for interactions with other biomolecules such as small drug molecules, nucleic acids, lipids, etc., without the need for labeling. To achieve this, we couple the multiplexing capabilities of planar protein arrays to a real-time, label-free sensing device based on surface plasmon resonance.

To generate the protein array, we rely on a unique method of producing proteins in situ. We use a selfassembling antigen array, nucleic acid programmable protein array (NAPPA), where cDNAs encoding potential antigens are printed onto glass slides and then translated into target proteins with a mammalian reticulocyte lysate. This robust method obviates the need to purify antigens, avoids stability problems during storage, and provides sufficient protein for testing. NAPPA technology is capable of producing thousands of different proteins, including transmembrane proteins, in a single step. Moreover, proteins produced by this method have been shown to be functional by maintaining the appropriate interactions and/or enzyme activity. We are now adapting

this method to a prototype surface plasmon resonance (SPR) imaging device from Lumera Corporation called the ProteomicProcessorTM. This allows for the detection of ~1,000 binding events simultaneously without the need for labeling and in real time by using a rapid scanning microelectromechanical system to rapidly raster the laser light over the entire SPR surface.

We will demonstrate the use of the NAPPA technology in detecting protein interactions, enzyme activity, small-molecule binding and various other applications involving identifying immunodominant antigens for infectious diseases as well as discovering potential biomarkers in cancer and other diseases. Furthermore, we will discuss our ongoing efforts in adapting NAPPA to SPR toward building a next-generation tool for functional proteomics.

Mapping the Transcriptional Regulatory Networks and Epigenome of Cancer Cells: A ChIP-chip Approach

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Cancer cells can be distinguished from normal, untransformed cells by their characteristic gene expression patterns. Understanding how such gene expression patterns are generated in cancer cells will help uncover the mechanisms of tumorigenesis and develop novel targeted therapeutic strategies. Transcription of eukaryotic genes is regulated by complex interactions between transcription factors and the cis-regulatory sequences and is also subject to control by the specific epigenetic status of DNA, including methylation and nucleosome modifications. We have developed a high-throughput, experimental approach for mapping in vivo protein/DNA interactions and epigenetic states along the genome of cancer cells. This method, known as ChIP-chip, involves immunoprecipitation of transcription-factor-bound DNA from the chromatin of crosslinked cancer cells, followed by parallel detection of enriched DNA species using high-density oligonucleotide arrays. We have demonstrated high sensitivity (82-95%) and low false detection rates (<5%) of this method in identifying the binding sites of transcription factors and chromatin proteins along the genome in human cells. With this technology, we are able to identify transcriptional promoters, enhancers, and insulators in the genome, which serve to regulate transcription of genes [1,2]. We are also able to generate highresolution maps of histone modifications along the human genome, revealing distinct and predictive chromatin modification patterns associated with human promoters and enhancers [3]. Using this technology, we have begun to systematically identify genes directly regulated by some oncogenic transcription factors. For example, we have recently defined nearly 2,000 binding sites of the transcription factor TCF4 (also known as TCF7L2) in the genome of human colon cancer cells. These binding sites not only occur near promoters but also frequently reside in distal regulatory sequences. We are able to confirm known target genes of TCF4 and the Wnt signaling pathway and uncover a large number of novel targets. Some of these target genes are involved in pancreatic development, thus providing an unexpected link between the Wnt signaling pathway and diabetes.

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Activity-Based Probes for Profiling Histone Deacetylase Complexes in Proteomes

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Activity-based protein profiling (ABPP) is a chemical proteomic method that uses active site-directed probes to report on the functional state of enzymes in complex biological systems. The approach differs from conventional methods in that it offers a powerful means to measure dynamics in the activity of enzymes that may occur in the absence of changes in the abundance of their corresponding transcript and/or protein. We have previously applied ABPP to identify several serine protease activities upregulated in human cancer cells and primary tumors. To further expand the utility of this approach, we have extended both the scope of the probes (to include those that target metalloproteases [MPs] and histone deacetylase [HDAC] complexes) and the potential applications, moving from a gel-based approach for detection of misregulated enzymes to gel-free platforms that allow identification of targeted enzymes via liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) and enzyme-specific high-throughput sample profiling via antibody microarrays.

Using the suite of MP probes developed under this grant, we can detect the activities of enzymes from nearly all branches of the diverse metalloprotease family using ABPP-LC-MS/MS. This discovery technique has high sensitivity (0.1 -1 µg of MPs in a complex background of 1 mg of protein can be detected) and resolution (scores of MPs can be identified and quantitated in a single run) but suffers in throughput (only 2 samples/day per instrument). However, once important misregulated MPs are identified, microarray-compatible antibodies can be developed against these proteins, which can then be applied to the ABPP microarrays. This translational platform offers superior sensitivity—as little as 0.002 to 0.008 ng/mL PSA and 0.12 to 0.25 ng/mL MMP9 can be detected—and the high throughput commonly associated with microarrays. The limitation of the approach is that sensitivity is antibody dependent. To address this issue, we have developed a trilevel assay for identification of microarraycompatible antibodies to provide optimal sensitivity of enzyme targets.

Most recently, we have developed probes to identify the components of HDAC complexes. This advancement will allow us to not only profile HDACs, which are targets for FDA-approved cancer therapies but also study the associated proteins that modulate

HDAC function and specificity. Thus far, we have profiled HDAC activities in a variety of cancer cell lines via LC-MS/MS and gel-based approaches and demonstrated that these probes can accurately report on changes in HDAC complexes in proteomes and in live cells. Development of optimized a-HDAC antibodies should allow us to profile samples in our translational microarray system.

For all of the probes we have developed, we anticipate two major uses of the data by downstream users. First, we expect the proteins identified as misregulated in our discovery efforts to become focal points in the search for new therapeutic targets and/or diagnostic markers. Second, since these probes report the activity state of enzymes, they can also be used to screen (and counterscreen) inhibitors, reporting on both the potency and specificity of compounds. Ultimately, we see the flexibility of the ABPP approach impacting fields as diverse as systems biology, diagnostics, and drug discovery.

Evaluation of the Value of Frozen Tissue Section Used as the Gold Standard for Immunohistochemistry

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Since the application of immunohistochemistry (IHC) half century ago, frozen cell/tissue sections have been used as the gold standard, based on the longstanding belief that this approach provides the best method for preservation of antigenicity, albeit that morphologic detail is compromised. Recently, this viewpoint has been challenged by us and others. To examine the traditional concept that acetone or ethanol-fixed frozen tissue sections represent the best that can be achieved—the gold standard for IHC—we have conducted a comparative study evaluating frozen sections together with various conditions of fixation and antigen retrieval (AR). Fresh human tissues from breast, colon, adrenal gland, bladder cancers, melanoma, and lymph node were frozen at optimal cutting temperature. In every case, an adjacent block of tissue was fixed “routinely” in 10% neutral buffered formalin (NBF) and formalin-fixed, paraffin-embedded (FFPE). Preparations of a human cell line were also processed into frozen and FFPE cell blocks in parallel to confirm the IHC results obtained by tissues, with the additional capability for performing Western blots of the cell preparations. Tissue sections from the frozen blocks were fixed by six protocols prior to IHC staining: acetone 10 min; ethanol 10 min; NBF 30 min; NBF 24 hrs; NBF + CaCl2 30 min; NBF + CaCl2 24 hrs. Sections also were prepared from the routine FFPE blocks. The AR technique was used for all NBF-fixed frozen sections and the FFPE tissue sections. A total of 26 antibodies, including nuclear, cytoplasmic, and cell surface markers, were tested. The VECTASTAIN Elite ABC kit with DAB was used to generate the IHC signal. Results showed that more than half of the antibodies (16/26, 61.5%) showed identical IHC staining results among acetone-fixed sections, NBF-fixed frozen tissue sections, and FFPE sections. Among the remaining antibodies, eight (30.8%) showed better IHC signals following NBF and AR. Remarkably, only two antibodies gave better IHC

staining results for acetone-fixed frozen tissue sections. It appears that most cytoplasmic proteins (10/13) showed comparable IHC signal between acetone and NBF-fixed tissue sections. For nuclear proteins, NBF-fixed tissue sections gave better IHC signals than obtained by acetone-fixed sections. For cell surface proteins (CD markers), acetone-fixed frozen tissue sections showed slightly better results. In most cases, NBF yielded a stronger signal, with less background staining noise than the corresponding frozen section and better morphology. We conclude that the data do not support the use of acetone-fixed frozen tissue sections as the gold standard for IHC. In examining any "new antigen," it would be prudent to employ a combination of both acetone-fixed frozen sections and NBF fixation, and FFPE tissue sections may serve as the standard for most antigens for IHC.

This project is supported by 1 R33-CA103455.

Integrated Polymer-Based Microfluidic Systems for the Efficient Capture and Enumeration of Circulating Tumor Cells (CTCs) From Peripheral Blood

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Specific capture of circulating tumor cells (CTCs) and their enumeration can be used as diagnostic or prognostic markers for the management of many cancer-related diseases. The challenge with using CTCs is the low frequency of these targets in most clinical samples (1-10 CTCs/mL of peripheral blood). The typical modalities by which CTCs are selectively captured include immunomagnetics or sizeselective filtering followed by enumeration via microscopic inspection of fluorescently stained cells, which can provide challenges in terms of sample transfer from one system to another (sample loss), staining steps, and the required sophisticated hardware. In this presentation, a general description will be provided of the use of polymer-based microfluidics for building highly integrated systems for capturing CTCs from a variety of clinical samples and enumerating the captured cells using an electrical readout modality that does not require staining of CTCs. The integrated system is fabricated from a polymethyl methacrylate (PMMA) wafer using microreplication and nanoreplication technologies and consists of a sampling unit and an electrical detector for CTC enumeration—all packaged into a device measuring 1 by 3 inches. The sampling unit contains a series of 190-nL high-aspect ratio (depth = 200 μm; width = 30 μm) fluidic vias hot-embossed into the PMMA (Figure 1). The walls of the vias are decorated with anti-EpCAM antibodies used to recognize and select epithelial-based CTCs from peripheral blood. The system can exhaustively process 1 mL of whole blood in ~10 minutes, with a capture efficiency of cells overexpressing EpCAM of 97%. The captured cells are subsequently released from the surface-immobilized antibodies using trypsin and then enumerated by measuring the bulk solution conductance change when single cells traverse through a pair of platinum wires (76 μm diameter) with a counting efficiency of 100%. RBCs or WBCs could not be "transduced" with the current detector design and, as such, do not interfere with the measurement of CTCs (Figure 1). Future efforts using this polymer-

based microsampling device will focus on (1) the analysis of other clinical samples such as urine (bladder cancer) or saliva (oral cancers), (2) the use of aptamers for the molecular recognition of cells to provide robust systems for potential point-of-care applications, and (3) a singlecell analysis system integrated with this sampling unit for providing molecular profiles of the captured cells to determine their organ of origin.

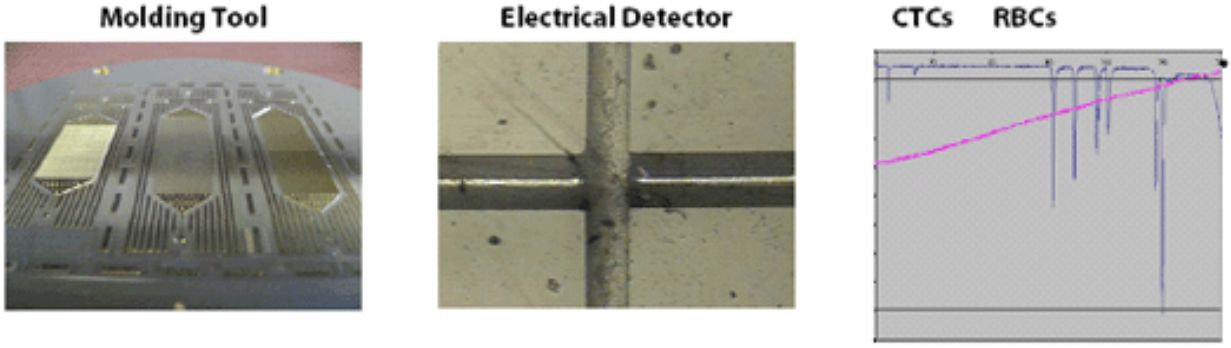

Figure 1. Molding tool used to prepare the sampling unit; Pt wires integrated into the packaged device and a trace showing electrical response of CTCs released from the sampling unit and also RBCs.