Ninth Principal Investigators Meeting

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Innovative Molecular Analysis Technologies (IMAT) Program
October 26-28, 2008
Hyatt Regency Cambridge
President's Ballroom

Abstracts

Development of a Membrane Microfilter Device for Capture and Characterization of Circulating Tumor Cells in Blood

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Isolation of circulating tumor cells (CTCs) from human blood currently poses significant challenges to the evaluation of tumor metastasis. Although a number of technologies have been developed to improve the ability to identify and monitor CTCs in the blood of cancer patients, difficulties with sensitivity, specificity, efficiency, and high costs of materials and reagents continue to limit these efforts. We present a novel membrane microfilter device for isolation of CTCs in blood by exploiting size differences between tumor cells and normal blood cells. We evaluated the sensitivity and efficiency of CTC capture in a model system and compared the membrane microfilter device with the CellSearch platform, which is recognized as the gold standard for the isolation of CTCs, in blood samples from patients with cancer.

For the model system, five cultured human cancer cells were directly micropipetted into 7.5 mL of whole blood from healthy, cancer-free donors and were processed by the membrane microfilter device. Fifty-eight trials of this experiment were performed—29 in which the J82 bladder cancer cell line (known to have a characteristically small diameter relative to other epithelial cancer cell lines) was used and 29 in which tumor cells from a mixture of six human cancer cell lines (J82 and T24 bladder; MCF-7, SK-BR-3, and MDA-MB-231 breast; and LNCaP prostate) were used to simulate maximal size heterogeneity. In addition, the membrane microfilter device was used to analyze 7.5-mL blood samples from 49 patients with prostate cancer—21 with confirmed metastasis from Memorial Sloan-Kettering Cancer Center (MSKCC) and 28 with undisclosed status of metastasis from The University of Chicago Medical Center (UC). A 7.5-mL amount of blood from all patients at MSKCC was also processed by the CellSearch platform. The membrane microfilter device successfully recovered 1 tumor cell in 96.5% (28 of 29) and 93.1% (27 of 29) of trials in which five J82 cells or six tumor cell types were used, respectively, and recovered 3 or more cells in 64% of these trials. Statistical analysis confirms that the true chance of recovering at least one tumor cell when five are seeded

from 7.5 mL of blood is 95% or greater. In clinical samples, the membrane microfilter device successfully recovered CTCs from blood in 100% (21 of 21, 14-182 cells recovered) of patients with metastatic prostate cancer from MSKCC. In contrast, the CellSearch platform recovered CTCs in 57% (12 of 21, 0-140 cells recovered) of corresponding blood samples. When CTCs were detected by both methods, greater numbers of cells were recovered by the membrane microfilter device in all but three patients. Of the 28 samples analyzed from UC, the microfilter device detected CTCs in 74% (17 of 23) of patients with confirmed metastasis. CTCs were detected in 20% (1 of 5) of prostate cancer patients with no evidence of metastasis. Multimarker immunofluorescence (IF) can be performed and evaluated directly on the membrane microfilter device, and up to four IF markers can be simultaneously assessed by utilizing quantum dots as labels. We have successfully developed a quadruplexed assay for CD44, CD24, ALDH-1, and pancytokeratin in breast cancer cell lines captured by the device.

Our data demonstrate that the sensitivity and efficiency of CTC isolation by the membrane microfilter device compare favorably with the current gold standard CellSearch platform. The membrane microfilter device has transformative potential to provide a cheaper, faster, and better alternative to current approaches to CTC isolation and does not depend on affinity-based capture of cells as do other platforms. A major advantage is that cell characterization can take place directly on the device. Additional efforts using the membrane microfilter device will focus on the enumeration of CTCs as an early indicator of therapeutic efficacy and on the biological characterization of CTCs, including identification of therapeutic targets, using multimarker immunohistochemistry/IF, fluorescence in situ hybridization, polymerase chain reaction, and other techniques directly on the device. Further uses of the membrane microfilter device include improved diagnosis of bladder cancer by microfiltration of urine, an idea we are currently testing.

Development of an RNA Sensor Platform for Detection of Circulating Tumor Cells

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We are developing a chip-based RNA sensor with an initial application for the detection of circulating tumor cells (CTCs). CTCs are harvested from blood using a simple porous, membrane-gradient centrifugation device, and RNA is extracted. Targeted RNAs from specific cancers are selected from the literature, and library selection is performed to optimize antisense oligonucleotide (ASO) binding sites. Detection is based on a "hybridization sandwich" in which one ASO1 is covalently attached to silicon or rhodium nanowires and the other ASO2 is covalently attached to a 40-nm Au particle. Formation of the ASO1-RNA-ASO2 sandwich induces a shift in the resonance frequency of a nanowire cantilever, which is measured optically. We have optimized

ASO sites for a number of targeted RNAs for prostate, breast, and melanoma cancers; developed conditions that allow single nucleotide mismatch discrimination; shown that the ASO-derivatized nanowires remain functional following assembly and on-chip integration; developed the ability to assemble derivatized nanowires at predetermined chip locations with high yield; and shown that the formation of each target RNA sandwich complex induces a shift in resonance frequency that can be easily detected. The platform is currently being extended to allow quantification of proteins by replacing the ASOs with aptamers, and the next phase of development will include transitioning to direct electrical readout of binding events. We have initiated clinical trials with melanoma patients; samples will be analyzed using conventional techniques (quantitative polymerase chain reaction, enzyme-linked immunosorbent assay) for benchmarking, and the balance of the samples will be analyzed using the RNA sensor platform when it is available.

Isolation of Rare Cells From Whole Blood Using Microfluidics

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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 surrogate markers 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 with standard photolithography and silicone molding techniques and requires only pressure-driven flow to operate. Its initial channel is designed to enhance the 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 produces 34-fold enrichment of the leukocyte-to-erythrocyte ratio.

We are now further developing this device 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 and more reliable clinical assays that are also convenient and portable for point-of-care testing. When fully developed, this technology will be a necessary and integral component of any microfluidic device used to analyze nucleated blood cell populations by eliminating the need for preliminary blood processing steps.

Performance Characteristics of a New CTC Enrichment Method Using a Negative Selection Strategy

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Developing an assay to reliably identify, in a sensitive and specific manner, tumor cells shed into peripheral blood has historically been a challenging goal. The task is primarily complicated by the fact that the handful of shed solid tumor cells are dispersed amid billions of (analytically irrelevant) red and white blood cells. Conventional methods of removing erythrocytes have resulted in relatively low tumor cell recoveries. Consequently, recent efforts have tended to avoid that step altogether. Methods that positively select circulating tumor cells (CTCs) out of whole blood are constrained by the degree of tumor cell marker expression, which is often downregulated on shed tumor cells. Methods that analyze all blood cells (without removing erythrocytes) are constrained by the level of background signal from the billions of red and millions of white blood cells.

With Phase I funding, we developed a new method that overcomes these pitfalls by using a negative selection (depletion) method. The method kit comprises three separate monoclonal antibodies, which bind to and deplete erythrocytes, leukocytes, and platelets. During Phase I, we developed a new antibody to erythrocytes with unique characteristics, which was not available from academic or commercial sources. This method depletes >99.99% of erythrocytes, approximately 90% of leukocytes, and 70% of platelets in 15 minutes. Tumor cell recoveries were measured by spiking HeLa cells into blood samples at 8, 16, 32, and 64 cells per blood sample. For each data point, tumor cell recovery was measured from five replicate samples. The average tumor cell recovery was 69%, with a range of 62% to 75%.

Our sample preparation process involves neither centrifugation nor erythrocyte lysis, both of which lowered tumor cell recoveries to unacceptably low levels. Rather, the antibodies cause agglutination of erythrocytes, platelets, and leukocytes in a manner that does not entrap shed tumor cells. The entire process takes 15 minutes. The agglutinate precipitates at 1xg and the CTCs are collected in the plasma. The simplicity and high tumor cell recoveries make this sample preparation process ideal as the upstream sample preparation step for a clinical laboratory CTC assay. Moreover, since the sample preparation method does not require expression of any particular tumor cell-surface marker, it is adaptable to the isolation of many different types of shed tumor cells, such as carcinomas, melanomas, and sarcomas. The Phase II effort will focus on evaluating different downstream analytical methods in the context of pilot clinical trials.

NCI Resources for Investigators Developing Technologies That Use Human Specimens Quality Matters

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The application of analytical chemistry to biologic specimens is not new, but the resolution of these measurements has changed with the addition of novel technologies. A major emphasis of translational medicine is on the measurement of biomacromolecules and how their presence and modifications predict clinical behavior rather than measure simple and stable analytes as measures of disease. These new approaches have also brought new headaches. Sample sizes have escalated, and the use of archived and multicenter acquired specimens for validation requires a new paradigm of considerations of the biospecimens being analyzed. There is also considerable heterogeneity in the way human specimens (tissues and biofluids) are collected, processed, stored, and disseminated, with limited scientific evidence available relating to how this diversity of specimen handling affects the quality and reproducibility of data from cancer research. It is inevitable that emerging technologies that use biospecimens will encounter these issues.

The NCI recently established the Office of Biorepositories and Biospecimen Research (OBBR) to guide, coordinate, and develop the NCI's biospecimen resources and capabilities, with the overall goal of increasing access to high-quality biospecimens for research. The Biospecimen Research Network (BRN) was formed by the OBBR to perform analytical studies to inform the development of appropriate data-driven, evidence-based practices and protocols for specific specimen types and molecular analysis platforms. In addition to its intramural and extramural support, the BRN also hosts an annual symposium to fuel advances in personalized medicine through the dissemination of scientific data and community building. The symposium focuses on addressing the significant impact of preanalytical biospecimen variability on cancer research and molecular medicine, with the goal of developing solutions to these issues. Information about the upcoming symposium and archived webcasts of the 2008 BRN Symposium "Advancing Cancer Research Through Biospecimen Science" can be found at http://www.brnsymposium.com.

This poster discusses the different resources available from the NCI and presents the need and opportunities for novel technologies in the biospecimen sciences.

High-Resolution Karyotype Analysis of Single Cells Using BAC-FISH Assays

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This project addresses the sensitive detection of chromosomal changes such as small translocations, inversions, or genomic imbalances in apparently normal individuals; benign neoplasia; premalignant lesions; and cancer. Current techniques for full-karyotype analysis of individual cells such as G-banding require cells in metaphase. Cells in interphase or nonviable cells such as tumor cells isolated from peripheral blood cannot be analyzed. The objective of the proposed research is twofold: (1) development of technologies to support the cytogenetic analysis of small amounts of fresh, fixed, or archival tissues regardless of the cells' proliferative stage and (2) investigation of the feasibility to rapidly and inexpensively screen the human genome for the presence of occult cryptic trans-locations (OCTs).

A highly sensitive, fluorescence in situ hybridization (FISH)-based technology platform termed "single cell arrays" (SCAs) will allow the detection of small rearrange-ments in interphase and metaphase cells by combining high-resolution DNA in situ analysis with sensitivity in the kb range. This will be achieved by immobilizing cell nuclei on glass slides, swelling them in buffer containing one or several detergents, controlled stretching of chromatin in specially design microchambers, and followed by cytogenetic analysis using FISH. The aims of this R21 feasibility study are to (1) demonstrate the feasibility that interphase cell nuclei can be immobilized in a defined pattern and reproducibly extended for subsequent cytogenetic analysis and (2) develop an optimized assay for the sensitive, high-resolution cytogenetic analysis of SCAs.

We will demonstrate the feasibility of preparing SCAs that make up individual or bulk preparations of cell nuclei arranged in a defined pattern inside microscopic reaction chambers and elongated/stretched by a constant force. A protocol for a FISH-based multilocus cytogenetic analysis of SCAs is expected to provide near kilobase single-copy detection sensitivity with a resolution in the order of 10-20 kb, while minimizing the overall loss of DNA. The assay allowing the analysis of very small samples, regardless of their integrity or cell cycle stage, is currently tested by analyzing SCAs prepared from breast and thyroid cancer cell lines. Successful completion of the project will open new avenues for the analysis of small samples such as those obtained by fine-needle biopsies as well as the analysis of circulating or exfoliated tumor cells.

To support the development of genome-wide screening assays for OCTs in human cancer cells or oncogenes activated by chromosomal rearrangement, we develop FISH assays using collections of validated chromosome-specific bacterial artificial chromosomes (BACs). With BAC probes spaced on average 0.9 Mbp apart and covering the entire euchromatic part of the human genome, we expect our "BAC-FISH" assay to lead to greatly increased sensitivity compared with existing tests. Our assay for sensitive genome-wide screening for translocations is currently developed with cancer

cell lines for which limited information about structural abnormalities is available. In parallel, we use pools of contiguous DNA probes ("BAC-contigs") to investigate cytogenetic changes at selected loci in interphase cell nuclei, which can be applied immediately to the before-mentioned SCAs. At the end of this project, we will be well positioned to conduct a larger study of the frequency of OCTs in the normal population as well as tumor cells and to offer BAC-FISH screening and reagents to research and clinical laboratories.

This work is supported by CA080792, CA123370, and CA132815.

Magnetically Patterned Co-Cultures for Cancer Studies

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The objective of this project is to establish a novel technique that can provide new insight into the mechanisms by which cancer cells respond to heterotypic cell-cell interactions that naturally occur in the tumor microenvironment. This technique is based on microfabricated and nanofabricated systems and involves the trapping of two cell types into large populations of heterotypic pairs arrayed in microwells on a substrate. The microwells are defined by ellipsoidally shaped holes etched in a magnetic thin film covering the substrate. The resulting magnetic poles at the ends of the holes are used in conjunction with external magnetic fields so that cells tagged with magnetic particles are selectively directed and trapped into specific locations on the substrate. This allows for high-precision construction of cell pairs. Instead of using magnetic beads or other common biomagnetic particles, which require inhomogeneous fields with exceedingly strong gradients to generate large forces, we employ nanoengineered high-aspect-ratio, rod-shaped magnetic particles termed "magnetic nanowires." These nanowires, in the presence of the magnetic field gradients of the traps, generate large forces that pull suspended cells to the traps.

Parallel with the work of developing the magnetics of these devices, we have recently demonstrated the scale-up of the fabrication methods to arrays containing upward of 10,000 traps. This has allowed us to test our methodology using nonmagnetic traps and carry out experiments to establish the ability of the trap arrays to measure effects of cell-cell interactions, using an endothelial cell (EC)/smooth muscle cell (SMC) model system. This study has allowed us to test many aspects of our technology, including automated analysis software for processing multichannel fluorescence data taken from the arrays. Using a BrdU-based proliferation assay, we find a clear enhancement of the SMC proliferation rate in heterotypic pairs compared with homotypic pairs on the same array, suggesting that EC-SMC contact can alter rates of proliferation. These experiments have established that biologically meaningful results can be obtained from our cell trap arrays and highlight the potential for detailed measurements of cell-cell interactions using microfabricated systems.

Microfluidic Channels for High-Density, High-Performance Culture Assays

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Cancer research relies heavily on biological models. In general, as one moves through the list from biochemical to animal models, increased biological relevance is achieved. Increased relevance, however, goes hand in hand with increases in cost, labor, and experiment duration. The practical compromises that must be made between cost and relevance limit the rate of research progress. We propose to utilize a microscale culture platform to improve relevance while lowering the cost/effort per data point, providing a step forward in the molecular analysis of cancer.

Although microfluidics holds tremendous potential, the complexity and requirement for specialized setup and equipment of many microfluidic approaches to cell-based assays have tended to prevent their adoption by biologists in everyday use. We are developing a "tubeless" approach that integrates with existing methods and tools common to life sciences labs (e.g., manual or automated pipettor).

Recent work has focused on validating the operational robustness of the platform. We have achieved robustness of 97.23% of 810 channels and 99.92% of the 28,350 operations. Cell seeding consistency to within less than 5% variance has been achieved. We compared image-based assays (fluorescent immunocytochemistry and phase contrast) between microchannel and well formats. We have also performed culture and staining of primary cells in microchannels. We have initiated an increased throughput experiment that will produce 1,800 data points requiring over 60,000 fluid manipulations.

To enable cost-effective readouts, we have adapted In Cell Western (ICW) techniques to microfluidic culture and compared to macroscale immunocytochemistry, ICWs and traditional Western blots. This demonstration used epithelial to mesenchymal transition (EMT) phenomena, where the response of NMuMGs to TGF-β were performed in both macroscale and microscale cultures at the same surface density. E-cadherin expression in NMuMGs was compared between platforms (microchannel ICW, 96-well plate ICW, immunocytochemisty and traditional Western).

Finally, we have begun to develop a panel of "stress" assays to understand molecular changes in cell behavior caused by the culture platform (micro vs. macro) laying the foundation for a more comprehensive understanding and interpretation of microscale culture data.

Sample Preparation Methods for Microvessel Analysis in Tumors

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We are investigating chemical and biophysical methods to enhance the rate of penetration of fluorescence-labeled molecules and antibodies in tissue. Confocal and two-photon microscopes record subsurface image data from tissues to interrogate innately expressed molecules. Specimen preparation protocols limit the detection of exogenously applied probes to depths far shorter than instrumentation capabilities allow. The goal of this project is to develop and refine specimen preparation protocols to facilitate extended-depth interrogation in tumors and supporting peritumoral tissue for high-resolution vascular analysis.

Preparation of whole-mount specimens of 3- to 5-mm subcutaneous tumors from mouse models bearing B16 melanoma, stomach cancer, and Lewis lung cell carcinoma (LLC) produces image data with morphologic attributes sufficient to describe the three-dimensional (3D) microvessel architecture associated with tumor growth and development. Nonperfusion-based, diffusive whole-mount protocols immunolabel molecules of interest, such as nuclei and endothelial cells, to simultaneously place the complex geometry of microvascular beds in context with these tumors. We have applied perfusion-based preparations combined with optical clearing methods to produce specimens suitable for recording extended-view stacks of serial section image data. TRITC-dextran administered by tail vein injection tested the replicability of the method, resulting in acceptable microvessel decoration for detailed analyses of vascular metrics.

Immunodecoration of subcutaneous GFP-positive LLC (n=4) and B16 melanoma (n=5) produces data for vascular segment analyses of branch, interval, tortuosity, and density per unit volume of tissue. Tortuosity measurements recorded from image data in LLC tumor-bearing tissue show 40% of microvessels to be low amplitude-low frequency; 47% to be low frequency-high amplitude; 10% to be high frequency-high amplitude; and 3% exhibit abrupt changes in lumen diameter. Fifty percent of the microvessels in melanoma-bearing tissue exhibit low amplitude-low frequency features, 22% appear as low frequency-high amplitude vessel segments; high frequency-high amplitude vessels were absent, and 28% exhibited abrupt changes in lumen diameter.

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.

Interaction-Specific Network Perturbation of Disease Proteins

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Cellular functions are mediated through complex systems of macromolecules and metabolites, linked through biochemical and physical interactions, and represented in interactome models as "nodes" and "edges," respectively. A better understanding of genotype-to-phenotype relationships in human genetic disorders will require modeling of how disease mutations affect interactome properties. We investigate the extent to which genetic disease perturbations of interactome networks result from complete loss of gene products ("node removal") or, more precisely, from interaction-specific ("edge-specific" or "edgetic") perturbations. Node removal versus edgetic network perturbations could result in vastly different phenotypic outcomes of disease and health. By examining ~50,000 known causative alleles in a total of ~1,200 genes involved collectively in ~2,500 human Mendelian disorders, we find that distinct modes of inheritance and complex gene-disease relationships correlate with node removal or edgetic protein interaction defects. Experimental investigation of allele-specific network perturbation in various diseases revealed diverse edgetic interaction profiles of mutant proteins that correlate with known structural or functional protein properties. Edgetic network perturbation models might contribute to the understanding of the global evolution of disease alleles in human populations and improve molecular therapeutic strategies.

Impact of q-RT-PCR Analytical Methods on a Multicenter Biomarker Trial in Colorectal Cancer

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Guanylyl cyclase C (GCC), an emergent intestinal tumor suppressor, is the receptor for the paracrine hormones guanylin and uroguanylin, gene products frequently lost early in colon carcinogenesis [1,2]. Lymph nodes and tumor specimens were dissected from colon, and rectal resections were performed in the surgical departments of seven academic medical centers and two community hospitals in the United States and Canada between January 2003 and June 2007. Followup, based on periodic evaluations, was confirmed for all patients through December 2007. GCC and beta-actin expression were measured based on standard curves formed from serial dilutions. Gene expression was also estimated by logistic regression analysis of amplification profiles from individual q-RT-PCR reactions, providing an efficiency-adjusted relative quantification based on parameter estimates from the fitted models [3]. We show that

the measurement techniques developed impact the analysis and interpretation of this large multicenter prospective trial by reducing the measurement error in q-RT-PCR. In multivariable Cox models of early-stage colorectal cancer patients (n=259), controlling for T stage, tumor location, lymphovascular invasion, and tumor differentiation, GCC q-RT-PCR remains an independent predictor of recurrence (adjusted hazard ratio [AHR]=4.51, p=0.039, 95% CI=1.07, 18.90). Moreover, GCC q-RT-PCR is an independent predictor of disease-free survival (AHR=3.08, p=.035, 95% CI=1.08, 8.75).

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Sequence Enrichment Using Droplet-Based Microfluidics

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High-throughput sequencing of genomic DNA has gained increasing acceptance as a means to identify the genetic origins of disease. However, whole-genome sequencing of thousands of patients in large cancer studies is not yet practical. Targeted sequencing of specific exons is more practical, but there is a lack of good tools to selectively enrich target DNA sequence from a preponderance of off-target DNA. Current tools for enriching target DNA sequence results in the absence of some target regions, the inclusion of off-target sequence, and the introduction of a bias in the way target loci are enriched. These shortcomings limit the amount of information that can be obtained from sequencing samples prepared with current tools and largely offset the anticipated cost savings.

RainDance Technologies is developing a microfluidic tool for sequence enrichment that enables highly uniform amplification of target loci without the introduction of off-target sequence. The key to this technology is a library of PCR primer droplets that encapsulate individual primer pairs in 10 picoliter droplets. These droplets are then combined with 15 picoliter template droplets to amplify a unique target in a 25 picoliter reaction volume using conventional laboratory thermocyclers. With processing rates of thousands of droplets per second, large numbers of individual reactions are used to amplify target loci in a simple, robust manner that is uniform across multiple loci and provides a practical, economic solution for sequence enrichment.

Novel Biomarkers for Assessing Epigenetic Instability and Risk of Head and Neck Cancer

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In published work, we have shown that metrics based on variational statistics for a large set of somatically mutated alleles can be of utility for the risk stratification of patients with pancreatitis, since some of these individuals progress to pancreatic cancer. We reasoned that DNA methylation metrics could also be useful for cancer risk assessment, provided we could identify a large number of genomic compartments associated with informative epigenetic markers of genome instability in preneoplastic stages. Using high-density microarrays, we investigated DNA methylation abnormalities genome-wide in samples from patients with head and neck cancer and in morphologically normal margins from the same patients. Importantly, the analysis included the measurement of DNA methylation changes in noncoding DNA and repetitive DNA elements in addition to all gene promoter CpG islands. After analysis of 35 tumors, 18 paired samples of "normal" adjacent mucosa, and buccal mucosa from 10 healthy adults, we performed statistical analysis to choose those probes, among more than 300,000 used for microarray analysis, that best distinguish tumors from adjacent nontumor mucosa. Surprisingly, among the most informative probes, we found a dramatic enrichment for probes interrogating the methylation status of certain full-length, primate-specific, Line-1 retroelements. Using the methylation status of specific probes comprising the most informative subsets of several different retroelement families, we can generate a classifier that stratifies the adjacent nontumor tissue on the basis of retrotransposon-silencing abnormalities. It will be important to find out whether these retrotransposon-derived epigenetic metrics are predictive of incipient structural instability in the genome. Additional studies on a larger set of samples will allow us to evaluate the potential utility of these novel epigenetic instability metrics as molecular tools for the assessment of cancer risk in individuals without cancer and for prediction of the likelihood of tumor recurrence in cancer patients. Notably, the high copy number of abnormally methylated retroelements elements may facilitate their detection in the body fluids.

Digital Transcriptome Subtraction To Discover New Human Tumor Virus

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Digital transcript subtraction (DTS) was developed to subtract known human sequences from expression library data sets in silico, leaving candidate nonhuman sequences for

further analysis. This approach requires precise discrimination between human and nonhuman cDNA sequences. Database comparisons show a high likelihood that small viral sequences can be successfully distinguished from human sequences. Using this approach, we excluded with 95% confidence that viral mRNAs are expressed in four squamous cell conjunctival carcinoma tumors at levels greater than 20 transcripts per million (~4 transcripts per cell). We next examined Merkel cell carcinoma (MCC), an aggressive, immunodeficiency-related skin cancer. Performing DTS on four MCC tumors, we identified one transcript human-virus fusion transcript belonging to a novel polyomavirus similar to but distinct from known polyomaviruses. We refer to this virus as Merkel cell polyomavirus (MCV). MCV sequences were detected in 8 of 10 (80%) MCC tumors but in only 5 of 59 (8%) control tissues from various body sites and in 4 of 25 (16%) control skin tissues. In six of eight MCV-positive MCCs, viral DNA was integrated within the tumor genome in a clonal pattern, suggesting that MCV infection and integration preceded clonal expansion of the tumor cells. DTS is a useful approach to both discover and rule out certain classes of human tumor viruses.

Going After the "Sweet Spot" in Selecting Aptamers for Glycoproteins

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Posttranslational modifications, such as glycosylation, profoundly affect the activities of many proteins. However, detecting and differentiating various such modifications are not trivial issues. We describe a strategy to select aptamers for glycoproteins with the ability to recognize differences in glycosylation states. This was achieved through the incorporation into the DNA aptamer of a boronic acid moiety, which is known to have strong interactions with carbohydrates and hydroxyl groups, to bias the selection toward the glycosylation site and therefore for the recognition of differences in glycosylation. Using fibrinogen as a model, we have selected boronic acid-modified DNA aptamers that have high affinities (low nM Kd) and the ability to recognize changes in glycosylation states. Control experiments with unmodified DNA aptamers, modified DNA without the boronic acid moiety, and deglycosylated and glycan-modified fibrinogen strongly suggest that the selected boronic acid-modified aptamers recognize fibrinogen through boronic acid interaction with the glyosylation site. Further control experiments through cleavage of the boronic acid moiety of the selected aptamer with H_2O_2 provide additional evidence for the same. The method developed should also be applicable in the selection of aptamers for the recognition of other glycoproducts, such as glycolipids and glycopeptides.

Real-Time PCR Quantification of Precursor and Mature MicroRNAs as a Means To Study Posttranscriptional Regulation of MicroRNAs

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MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate translation following binding of the mature miRNA to conserved sequences within the target messenger RNAs. The ~21 nt mature miRNA is processed from longer precursor molecules in two subsequent steps: (1) processing of the primary precursor miRNA (pri-miRNA) to the precursor miRNA (pre-miRNA) by the nuclear Drosha and (2) processing of the pre-miRNA to the mature miRNA by the cytoplasmic Dicer. Using real-time PCR assays developed in our lab to the pri-miRNA and the pri-/pre-miRNAs and commercially available assays to the mature miRNA, we were able to assay each of the miRNA species. This allowed us to determine whether miRNA processing is regulated and at which step. By profiling over 200 precursor and mature miRNAs in HL60 cells induced to differentiate with 12-*O*-tetradecanoylphorbol-13-acetate, it was possible to identify miRNAs whose processing is regulated during differentiation. The precursor and mature miRNA from 22 different human tissues, 37 human cancer cell lines, and 16 pancreas and liver tissues/tumors was profiled. Pearson correlation between the precursor and mature miRNA expression was closer to 1 for the tissues and pancreas tumors/tissues but closer to zero for the cell lines and liver tumors/tissues, suggesting that processing of miRNA precursors is reduced in cancer cell lines and in liver cancer. Our results demonstrate that the miRNA precursors do not always predict the expression of the active, mature miRNA [1] and that quantitative real-time PCR is an effective method to study posttranscriptional regulation of miRNA. Several recent reports have demonstrated the importance of the regulation of miRNA processing during development and in cancer [2,3]. As the importance of posttranscriptional regulation of miRNA processing is appreciated, quantitative PCR assays to quantify individual miRNA species will become more valuable.

This work was supported by CA114304.

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One-Step Synthesis of Biofunctionalized Quantum Dots Using DNA Ligands

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Colloidal semiconductor nanocrystals are typically synthesized with the aid of passivating organic ligands. When biofunctionalized nanocrystals are desired for imaging applications, biomolecules are conjugated postsynthetically to provide specific-binding functionality. We show that a single designer ligand—a DNA molecule—can controllably program not only luminescent nanocrystal growth but also biofunctionalization. Previous work illustrated that stable, nontoxic nanocrystals can be made with DNA as a ligand [1-3]; we show that these materials can be made to bind to specific nucleic acid sequences, proteins, or cell types. One portion of the DNA sequence used in the nanocrystal synthesis controls nanocrystal size and passivation; another is displayed on the resultant nanocrystal surface and controls biorecognition. The outcome of the synthesis is programmed by the bifunctional ligand design. The synthetic protocol is straightforward and produces a homogeneous dispersion of biofunctionalized nanocrystal lumiphores. The nanocrystals exhibit strong optical emission in the visible spectrum and possess ~6-nm hydrodynamic diameters, which are compatible with applications in circulating systems. We show that, according to the design of the biorecognition domain of each DNA ligand, nanocrystals can specifically bind DNA, proteins, or specific cancer cell types having known surface recognition markers.

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Technology To Optimize scFvs for Targeting Therapeutics

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Antibodies provide superior targeting capabilities to a variety of therapeutic agents. Several technologies have greatly facilitated the initial identification of a variety of antibody reagents, including scFv and Fab antibodies, with virtually any possible specificity (e.g., phage display). However, lead antibodies often require further optimization to maximize their therapeutic performance-optimization of antibody expression and folding in eukaryotic cells and optimization of the affinity of the antibody for the target antigen of a particular application. The development of promising targeting

antibodies against cancer often languishes at this bottleneck. Therefore, technologies to facilitate antibody adaptation and optimization are urgently needed. An scFv display technology that employs the efficient protein synthesis and quality control system of eukaryotic cells, combined with a viral display platform, would best optimize the therapeutic parameters of targeting antibodies destined for use in humans. We have recently demonstrated the feasibility of a retrovirus, avian sarcoma leukosis virus (ASLV), as a viral platform for the display of a variety of polypeptides, including scFvs, in eukaryotic cells. The scFv polypeptide is expressed on infectious viral particles as N-terminal extensions of the ASLV envelope glycoproteins.

The main goal of our R33 application was to demonstrate the efficiency of using the ASLV display technology for the optimization of the scFv scaffold for efficient folding and expression in eukaryotic cells and for generating a panel of scFvs with a range of affinities for their target antigen with an optimized scaffold. We set out to optimize an scFv with known specificity for tumor neovasculature: an antilaminin scFv (L36) capable of inhibiting angiogenesis in a variety of assays in vitro, presumably due to the exposure of laminin in the extracellular matrix during tumor neovessel formation. The ASLV scFv display system allowed efficient construction of a variety of ASLV mutant L36 scFv libraries by both random and site-specific mutagenesis. Several screening methods were evaluated to select mutant L36 scFv virions with improved binding affinity for laminin. By far the most stringent screening protocol employed biotin-labeled laminin and selection of mutant L36 scFv displaying virions in solution. Selection of scFv mutants with higher affinity for laminin by coating laminin on plates was ineffective due to the high avidity of the display of scFv on virions. ASLV vectors also have been constructed that can deliver (1) a gene encoding a membrane-bound form of a selected scFv and (2) a gene encoding a secreted form of a selected scFv for protein production. We will present data demonstrating that the ASLV scFv display system is an outstanding technology for optimizing scFv expression and binding affinity.

An Integrated Programmatic Environment To Support Indepth Proteomics and Phosphoproteomics Profiling and Studies of Gas Phase Chemistry of Peptides and Phosphopeptides

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A major part of our work is exploiting information about physicochemical properties of peptides, including use of simulation of the MS/MS spectra by the program MassAnalyzer, and extending these to phosphopeptides. As part of that overall goal, we evaluated the simulated spectra in spectra/spectra matching for identifying the peptide sequences, utilizing the program X!Hunter. Good performance was found in analysis of correct vs incorrect with a 50 protein standard mixture. However, analysis of complex samples was compromised by poor discrimination for a subset of the data. Use of a consensus approach with Mascot showed similar results to those for Mascot/Sequest

consensus, but the X!Hunter search was 80 times faster than the Sequest search.

Next, we want to extend the MS/MS simulator to phosphopeptides; to do this, we are using small libraries of phosphopeptides to identify mechanisms to incorporate into the model and reliable data for fitting model parameters. Using our first ten libraries, we tested whether the Hunt model for the parent-H_3PO_4 product behaves as predicted in MS3 data. This revealed expected reduction on the C-terminal side of pSer or pThr, but little change on the N-terminal side, indicating that either the structure is not correctly predicted, or it is more labile than thought.

The other major effort in our laboratory is development of methods for analysis of large datasets collected over many experimental variables. To do this it is critical that the protein inference have good reproducibility. In evaluating six protein profiling programs, including our in-house IsoformResolver, we found that different protein isoforms were reported in up to 6% of the proteins, including high spectral count cases. This also impacted quantification by spectral counting, with artifactual differences of up to 3-fold observed. By processing all data through parsimony together, and considering the data by protein groups, this problem is minimized.

Nanoscale Detection of the Disease Proteome

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Work in our lab focuses on the development of nanocapillary assays to detect and quantify the amounts of multiple cancer-specific splice variants and posttranslational modifications (e.g., glycosylation, phosphorylation, and proteolysis) in breast cancer cell lines and conditioned media using a novel, highly sensitive, fully automated nanocapillary electrophoresis platform.

This technology will enable detection of protein isoforms and posttranslational modifications using a single detection reagent that will produce more complete protein profiles than can be achieved with standard protocols. We expect that the sensitivity and quantitative nature of the nanocapillary technology will be advantageous in this effort. The ultimate goal is to develop a technology to propel cancer biomarker discovery and enable early detection of breast cancer.

Initial work focused on the development of assays to detect CD44 protein variants resulting from differential phosphorylation, glycosylation, or alternate splicing in protein lysates from breast cancer cell lines, which are known to express these variants. We are using this model system to develop and optimize the nanocapillary immunoassay to reach optimal sensitivity (fg/μL), accuracy, and reproducibility. This part of the project entails engineering improvements in protein capture chemistry, analyte detection, background reduction, and temperature control to increase sensitivity. Additionally, in

collaboration with CellBiosciences, Inc., we are in the process of upgrading and developing new computer applications to control the instrument and analyze assay data as well as optimizing instrumentation to maximize usable data points. We also plan to develop a new detection system to enable multiplexed analysis of several analytes in a single assay. Standardized protocols are being developed for optimal protein extraction from a range of biological samples coupled with full automation, resulting in high throughput and reproducibility. These approaches will yield a systematic and quantitative approach to protein analysis.

Our next efforts will focus on the analysis of serum-free conditioned media to detect the secreted forms of CD44, and finally, optimized assays will be applied to serum samples from breast cancer patients whose tumors have been shown to produce variant proteins for which the assays have been developed.

CD44 is an ideal model protein that, if we are successful, will demonstrate the potential of the technology to identify multiple cancer-specific splice variants and posttranslational modifications (e.g., glycosylation, phosphorylation, and proteolysis) in cancer cell lines, tumor samples, and blood serum. We anticipate that these studies will demonstrate proof of principle for nanocapillary-based early cancer detection.

A Universal Technology for Profiling the Dynamics of Normal and Oncogenic Signaling

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Receptor tyrosine kinases (RTKs) are critical in the initiation of many protein signaling pathways. Ligand binding and activation of membrane-associated RTKs, followed by autophosphorylation of key tyrosine residues, serve as initial events in the response of cells to many different extracellular growth factors. Upon activation, autophosphorylation generates new sets of binding sites that serve to recruit different arrays of downstream signaling proteins to properly initiate complex signal transduction relays, which ultimately control multiple fundamental cellular processes, including cell survival, proliferation, and differentiation. With most RTKs exhibiting multiple phosphorylation sites, it is now believed that the dynamics of phosphorylation and dephosphorylation, which result in unique transient sets of phosphorylation sites, may control and regulate the subsequent recruitment of different sets of downstream proteins to effectively modulate different signal transduction pathways. Thus, dynamic control of multiple phosphorylation modifications of a single RTK can manifest critical control on multiple signal transduction pathways. Alterations of this sensitive dynamic in growth factor

networks and aberrant activities of RTKs often have severe biological consequences and are linked to oncogenic processes in many human cancers. Indeed, the success of tyrosine kinase inhibitors such as Iressa® and Gleevec® heralds a recent strategy of precisely targeted cancer therapy.

Ligand stimulation and autophosphorylation of RTKs occur on the timescale of subseconds to seconds. These autophosphorylation events are not random but rather are under precise temporal control that allows for the kinetic control and ordered orchestration of tyrosine phosphorylation and subsequent protein complex formation. Understanding the molecular mechanisms of these early dynamic events may hold the key to understanding and predicting the nature of oncogenic behavior and predicting the effects of RTK targeted therapy. An increased understanding of the early temporally regulated states will enable more precise strategies for selectively targeting downstream pathways and may offer a unique approach to cancer therapy based on dynamic rather than static intervention. Our work is directed toward developing an integrated platform of novel technologies and approaches to provide temporal resolution of these rapid, early events at a molecular level both in vitro as well as in cell culture. This involves creating a validated general set of innovative and established technologies, including rapid reaction methodologies, a novel time-resolved electrospray ionization mass spectrometry (ESI-MS) technique, nanospray ESI-TOF, phosphopeptide mapping using ESI-MS LC/MS/MS, and site-specific phosphotyrosine antibody detection, that will be applicable for the analysis of a wide range of RTKs, their autophosphorylation patterns, and downstream signaling events in the critical subsecond to multisecond time domain.

We have chosen a prototypical RTK, epidermal growth factor receptor (EGFR) tyrosine kinase, to develop this platform. The EGFR tyrosine kinase pathway has been chosen because it is (1) a well-characterized RTK, (2) has many available protein constructs and can be precisely activated by EGFR and subsequent dimer formation in cellular expression systems, and (3) an important molecular target for targeted cancer therapeutics such as the small-molecule ATP mimetic Iressa® (gefinitib), which has been approved by the FDA and underscores the generation of specific kinase targeting as a new paradigm for cancer therapy. The ability to probe the molecular and temporal details of the earliest events of autophosphorylation will reveal signature patterns that will provide a new understanding of the differences between normal and oncogenic forms of EGFR and an expanded functional understanding of the emerging therapeutic class of targeted kinase inhibitors. We believe that a profile of the temporal dynamics of phosphorylation in signaling provides a unique molecular fingerprint or signature for distinguishing normal and cancer cells and the responsiveness to targeted inhibitors.

New Technology of Selective Delivery of PNAs in Cancer Cells In Vitro and In Vivo

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Many cancers arise from the gradual accumulation of genetic changes in cells. Gene therapy approaches, as well as techniques for recognizing cancer cells with abnormal genes or elevated level of certain mRNAs, include the design and delivery into cancerous cells of antisense and antigene oligonucleotides or their synthetic mimics such as peptide nucleic acids (PNAs). PNAs are highly stable and resistant to nucleases and proteases and bind RNA and DNA targets in a sequence-specific manner with high affinity. One of the main obstacles for gene therapy is a lack of technology for selective delivery of gene agents into cancer cells in vivo. We propose a new technology for selective delivery into cancer cells of PNAs targeting mRNAs involved in tumor growth and metastasis. It is well established that tumors develop hypoxia and an acidic extracellular environment, especially in the earlier stages.

We designed a short peptide, which is soluble in water and able to insert into membrane as a transmembrane alpha-helix at low pH (<6.5) but not at normal pH (7.4). The peptide acts as a nanosyringe: It is inserted into the membrane at low pH, translocates, and releases in cytoplasm various molecules, including dyes, toxins, and PNAs [Reshetnyak et al.]. The fluorescent PNAs were translocated into cells and stained cytoplasm and nuclei. The mechanism of translocation by the pH low insertion peptide (pHLIP) is based on a protonation of two Asp residues in the transmembrane domain and is fundamentally different from all reported peptide delivery agents. Whole-body imaging revealed that fluorescent pHLIP is accumulated in tumor in mice. The accumulation in tumor occurs because pHLIP inserts into the membrane at low pH, whereas it interacts only weakly with the surface of cells in tissue with normal pH. The replacement of two Asp residues by Lys or Asn residue eliminates the ability of pHLIP to accumulate in tumor, which confirms the proposed mechanism of insertion of pHLIP into cells.

We synthesized several pHLIP-S-S-PNA constructs and demonstrated the ability of pHLIP to deliver PNA to HeLa pLuc705 cells (carrying the silent gene of luciferase) and turn on the luciferase expression in vitro and in vivo. Several other delivery peptides were tested for comparison. pHLIP showed higher efficiency in vivo, whereas some peptides such as (D-Arg)8-Lys(Deca) (developed by Dr. Nielsen, Denmark) showed better performance in vitro. We showed that pHLIP targets more efficiently on metastatic rather than nonmetastatic tumors because metastatic tumors are more acidic than nonmetastatic tumors. We demonstrated that pHLIP was able to target the metastatic lesions in lungs.

Our biophysical studies evaluated the amount of energy that is released during insertion/folding of pHLIP into lipid bilayer of membrane and that could be used to move cargo molecules through the membrane. By calculating the partition coefficient of a cargo, we can predict the probability of translocation of this cargo inside the cell. Currently, we are investigating the therapeutic effect of pHLIP-S-S-PNA constructs targeting expression of cyclin B1, which plays an important role in cell proliferation. We are using metastatic (M4A4) cancer cells to validate the therapeutic efficiency of pHLIP technology in vitro and in vivo. We are using fluorescently labeled PNA to demonstrate its translocation by pHLIP in vitro and vivo. The biological effect of translocation is

studied by measuring the amount of expressed cyclin B1, the cell proliferation rate, and the changing of tumor sizes. Our preliminary data show the feasibility of using pHLIP technology to specifically deliver gene-regulating agents into cancer cells in vitro and in vivo.

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Molecular Analysis of Epidermal Growth Factor Receptor Expression and Activation With Nanostructured Surfaces and Liquid Crystal-Based Technologies

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Insight into the role of key signaling proteins in cancer has led to the generation of anticancer agents directed against cell signaling targets such as epidermal growth factor receptor (EGFR). The lack of understanding of how various therapeutic agents affect EGFR-mediated events in human tumors and which EGFR mutations are most clinically relevant has created challenges in the development of these drugs. The current project seeks to further establish a technology that entails a new class of sensitive tools that use nanostructured surfaces and liquid crystals (LCs) to amplify and image molecular interactions. Our goals are to refine, validate, and implement this methodology to allow high-throughput screening of limited clinical specimens and to use this technology to determine which regulatory pathways and/or EGFR mutations are most likely to respond to certain antagonists. This approach is readily adaptable to other molecular markers that are of relevance to the detection and treatment of various cancers. Our work has focused on (1) optimizing LC-based assays to report EGFR expression and phosphorylation status in cell preparations in a highly sensitive, reproducible, and quantitative manner and (2) evaluating the concept that small numbers of cells are sufficient for this technology to report the expression, phosphorylation status, and tyrosine kinase activity of EGFR.

Use of LC anchoring energies to quantify proteins. We have developed a simple optical method that employs the measurement of the interaction energy of an LC with a surface (the anchoring energy) to report proteins captured on surfaces via their interactions with immobilized binding groups. To define the sensitivity and dynamic range of the response of the LC, we covalently immobilized a tyrosine-containing, 13-residue peptide encompassing EGFR Y1173 (an autophosphorylation site to which the adapter protein Shc binds). Using peptide-decorated surfaces containing Y1173 or pY1173 (the corresponding phosphopeptide), we determined that only pY1173 surfaces reacted with antiphosphotyrosine to yield a change in the LC anchoring energy (i.e., it decreased systematically from 4.4 to 1.4 μJ/m2 as the antibody concentration increased from 10 pM to 100 nM). These results provide the first demonstration of the use of

anchoring energy measurements of LCs to report proteins captured by immobilized ligands on surfaces. The sensitivity and dynamic range of the method suggest that it may offer the basis for a simple yet broadly useful principle for reporting the interactions between proteins and other biomolecules that underlie complex and poorly understood chemical and biological events.

Engineering poly(dimethylsiloxane) surfaces to capture complex and clinically important proteins. Elastomers based on poly(dimethylsiloxane) (PDMS) are promising materials for the fabrication of a wide range of microanalytical systems, given their mechanical and optical properties and their ease of processing. We describe (1) the reproducible capture of EGFR on PDMS surfaces presenting covalently immobilized anti-EGFR antibodies and (2) the subsequent isolation of the captured receptor by its mechanical transfer onto a chemically functionalized surface of gold film for detection. We benchmarked the performance of anti-EGFR antibodies that had been covalently immobilized on the PDMS against the performance of the same antibodies physisorbed to conventional surfaces used in enzyme-linked immunosorbent assays (ELISAs) by ^{32}P-radiolabeling the EGFR. These results revealed that panreactive anti-EGFR antibodies (H11 and 111.6) and a phospho-EGFR-specific antibody (pY1068) captured the receptor on both PDMS and ELISA plates. When H11 antibody was used to capture EGFR and its subsequent treatment with a stripping buffer (NaOH and sodium dodecyl sulfate) to isolate the receptor, the signal-to-noise ratio obtained with the PDMS surface was 82:1, well exceeding that measured on the ELISA plate (<48:1). We also characterized the isolation of captured EGFR by mechanical contact of the PDMS surface with a chemically functionalized gold film. The efficiency of mechanical transfer of the transmembrane protein from the PDMS surface was found to be 75% to 81%. Interestingly, the transfer of nonspecifically bound protein was substantially less, leading to the important finding that mechanical transfer of EGFR leads to an additional fourfold increase in signal-to-noise ratio, from 20:1 to 88:1. Currently, the technology uses samples that include purified receptor and cell lysates or 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 be used to reproducibly detect the expression and activation of the EGFR pathway in human non-small cell lung carcinoma tumor biopsies (including needle aspirates and paraffin-embedded samples) as well as in normal surrounding tissue and skin biopsies.

In summary, EGFR is a clinically important protein and the target of numerous anticancer agents. These results provide guidance for the design of PDMS- and LC-based microanalytical systems for the capture, isolation, and quantification of complex and clinically important transmembrane proteins.

Using the Reverse In-Gel Kinase Assay To Monitor Kinase Inhibitor Efficacy In Vivo

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The reverse in-gel kinase assay (RIKA) is a powerful method to facilitate discovery of new biomarkers of kinase inhibition and to measure response to kinase inhibitor treatment. The RIKA was developed to profile physiologic kinase substrates in complex protein extracts. To perform the assay, a purified kinase is cast in a denaturing acrylamide gel that is then used to resolve proteins. After in-gel refolding of the kinase and resolved proteins, a kinase reaction is performed in the presence of ^{32}P-ATP. Positions of substrates are revealed by autoradiography, and protein identification is accomplished by subsequent mass spectrometry. In the RIKA, only phosphoacceptor sites that are not occupied in vivo can be labeled in the gel, and global dephosphorylation of protein extracts leads to a dramatic increase in the RIKA signal. Likewise, in vivo kinase inhibition leads to a specific, quantifiable increase in true substrate labeling in the RIKA. Using protein kinase CK2 as a paradigm, we demonstrate the use of the RIKA to monitor kinase inhibitor activity in cultured cells. Dose-dependent changes in the RIKA signal were observed on exposure of HeLa cells to the highly selective pharmacologic CK2 inhibitor 4,5,6,7 tetrabromobenzotriazole. Extending from these observations, we are also exploring the possibility that the RIKA can be used to globally profile and quantify the phosphorylation index of kinase substrates. These two applications of the RIKA technology provide new approaches to measure the efficacy of kinase inhibitors by monitoring changes in phosphorylation in multiple substrates simultaneously. This capability could expand the options available to determine patient response to kinase inhibitor treatment.

Studies in Peptide Identification and Protein Inference in Support of Signaling Studies in Human Cells

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A vexing problem in shotgun proteomics is the detection of phosphopepides in complex mixtures. Development of protocols to test hypotheses about this problem is hindered by the fact that the phosphopeptides in complex mixtures are largely unknown. We have generated 20 libraries with ~70 to 350 phosphopeptides in each case, including peptides with up to four phosphates. The total number of peptide MS/MS characterized to date is ~6,000. Analysis of the MS/MS in a 4000QTrap versus an LTQ/Orbitrap revealed unexpected differences in the cases identified in each instrument. In general, better fragmentation was obtained with the 4000QTrap, making the identification rate

better than that seen with the LTQ/Orbitrap, even when using MS3. However, many internal fragment ions were also observed in the QTrap. Using these spectra, we found improved performance of Mascot when allowing the internal fragment ions in the search. Currently, we are testing models of phosphopeptide fragmentation, as well as using the libraries to test ETD versus CID. In addition, the libraries and data sets will be made available to other researchers to test protocols for phosphopeptide analysis.

Linear Barcoding Analysis of Genomic DNA in a Nanochannel Array

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We have developed a nanochannel array device that is able to massively stream long, linearized genomic DNA for direct imaging analysis at the single-molecule level. A single-molecule barcode labeling method was developed to mark regions of individual long genomic DNA, and the unique pattern signatures were imaged and analyzed to reflect the structural aberrations responsible for malignant cell transformation.

Past technology, such as fiber-fluorescence in situ hybridization (FISH) and surface combing, which have mostly been used in research environments, suffers from inconsistent DNA linearization and protocols that are difficult to implement and hence hard to scale up as a reliable, commercial-grade tool. Mature microfabrication and nanofabrication technology derived from the semiconductor industry has very high inherent quality in creating ultrasmall nanofluidic channel array structures. The advantages of this platform are as follows: (1) Long DNA can be reliably and consistently linearized by nanoscale physical confinement without external force; (2) high-quality quantitative measurement of the barcode labeling along linear DNA is possible and comparable between experiments; (3) flexibility is provided in loci-specific or pangenomic interrogation; (4) continuous sample streaming allows high-throughput analysis at the single-molecule level; (5) an extremely small (to approximately the femtoliter level) sample detection volume is provided; and (6) the potential for massive nanofabrication of the chips makes it scalable and very inexpensive for molecular diagnostics.

During the Phase I study, the device we developed typically had 100 to 1,000 nanochannels per field of view (120 by 120 microns) using a standard fluorescent microscope and hundreds of thousands of channels per chip at the size of one-half centimeter by one-half centimeter. DNA molecules with a size of 10 kilobases (kb) to 10 megabases can be analyzed directly on this chip. Linear imaging resolution of barcoded markers is typically 1 kb and better, which would cover most typical copy number variations and structural variations. The analyzed molecules are typically very long and contextual, and haplotyping information is preserved, which automatically alleviates the assembly issues encountered by short read methods. Since data extraction was based

on individual molecules, subset data of the heterogeneous population of sample will not be lost or diluted in the typical averaged sample analysis in bulk solution assay. Sample preparation could be done in conventional solution with clones such as bacterial artificial chromosome DNAs, long-range polymerase chain reaction amplicons, and native genomic DNA for streaming analysis. Because each channel has its own fluidic environment, channel array could be compartmentalized or grouped to accommodate many samples from many patients and pedigrees. With further development, this could be interfaced with an upstream sample handling automation system for high-throughput analysis. We envision that end-users will be the biomedical and clinical research community and the high-throughput molecular diagnostics industry.

In a future phase design, the nanochannel array chip and single-molecule structural barcoding method will be part of an integrated system for the routine and standardized quantitative analysis of cancer genomes that will enable data archiving and cross-laboratory comparison.

Identification of Areas of Oxidative Damage in Human Genomic DNA

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The goal of this project is to develop a technique that can determine the relative density of oxidative damage sites in specific regions of the genome. A single method that can accomplish all of these goals is not yet available, but it is currently possible to determine the overall number of abasic (AP) sites in genomic DNA by using slot blots, the presence of hotspots for AP sites in a population of the same DNA fragments by using ligation-mediated polymerase chain reaction, and the random or clustered distribution of AP sites by using electron microscopy (EM) or atomic force microscopy. Previously, we developed a technique to detect AP sites in isolated genomic DNA by tagging AP sites with biotin and then using a streptavidin-coated gold particle to enable visualization by EM or secondary antibody to facilitate visualization by confocal laser scanning microscopy. With EM, we showed that the distribution of AP sites by immunofluorescence was not random but included a large fraction of clustered AP sites. These AP site clusters suggest that there are locations in the genome that are preferentially vulnerable to AP site formation. We set as the milestone for this project the demonstration that we could refine our ability to fluorescently tag AP sites so that we could quantitate AP sites to within 10% of the measurements made by the slot blot technique, which is the current standard of detection for AP sites. We also proposed to show that the number of AP sites detected would increase when AP sites were induced by exposure to different doses of hydrogen peroxide (H_20_2).

To quantify the number of AP sites within genomic DNA, we lyse cells directly onto siliconized glass slides, straighten and align the genomic DNA with a method called fiber spreading, identify the AP sites with a red fluorescent tag, and stain genomic DNA

with a green fluorescent DNA-specific dye. Images are taken of areas of the slide that contain green fluorescence (DNA), and those images are analyzed to determine the amount of DNA per image as well as the number of AP sites contained within that DNA. The resulting images can be assessed with fully or partially automated image analysis, and we have been refining this process to achieve quantifiable and reproducible results. The image analysis threshold levels were adjusted to minimize the background and to define the range within which detected AP signals remained stable. Using this approach, we analyzed over 1x109 nucleotides (nt) of DNA and determined that DNA contained 5.4 AP sites per 106 nt (within the range found by slot blot and EM analysis). Furthermore, when we analyzed 7x108 nt from cells exposed to 20 μM H^2O^2, we found that the number of AP sites increased to about 7 AP sites per 106 nt (also consistent with slot blot analysis). In both the untreated and treated cells, we observed AP sites occurring as clusters. The accomplishment of further goals now requires us to use fluorescence in situ hybridization to identify specific DNA regions and to visualize AP sites at those locations. Achievement of this capability will allow us to detect the distribution of AP sites in a specific genomic region. This would enable us to determine the extent and distribution of DNA damage in specific regions of the genome, including functionally defined locations such as origins of replication or promoter regions, lesion formation during normal cell metabolic processes (e.g., replication), and lesions formed as a consequence of the cells being exposed to carcinogens or to oxidative stress.

Ultrasound-Facilitated Fast Tissue Specimen Preparation

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Ultrasound (US)-facilitated, formalin-fixed, paraffin-embedded (FFPE) tissue specimen preparation technology (US-FFPE) is developed from the widely accepted FFPE technology by introducing US in each step of the process. The US-FFPE does not change the gold-standard FFPE tissue morphology. However, it greatly reduces tissue sample preparation time. The whole procedure of FFPE tissue specimen preparation is reduced from 16 to 48 hours to 20 to 60 minutes [1,2].

The final diagnosis of cancers can be produced in a few hours after surgery or biopsy instead of a few days. This increase in turnaround rate will considerably save patient care costs and greatly relieve patients' psychological burdens in waiting for results.

The US-FFPE technology changes the mechanism of biomolecule modification and cross-linking caused by formalin fixation. Biomolecules are much more accessible for detection and extraction. The fact that tissue specimens are "frozen in time" at the point of US-FFPE fixation can greatly improve the integrity of biomolecules in tissue specimens, especially for mRNA and phosphoproteins.

We have constructed a prototypical, multisample, investigational US-FFPE device. Morphological and molecular biology assay results on tissue specimens produced by the prototype US-FFPE device were highly comparable to those on tissue specimens produced by the conventional FFPE method.

We also tested the US-FFPE device with the nonformalin-based fixative Z7 and produced highly satisfactory morphological and molecular assay results, indicating that the US-FFPE device can also be used with other fixatives.

Time Required for US-FFPE Tissue Preservation					
Tissue thickness	Formalin	Alcohol 1	Alcohol 2	Xylene	Paraffin
1 mm	2-3 min	2-4 min	2-4 min	2-4 min	3-5 min
2 mm	2-4 min	3-7 min	3-7 min	3-6 min	4-7 min
3 mm	3-7 min	6-12 min	6-12 min	6-8 min	6-10 min
4 mm	5-10 min	8-17 min	8-17 min	7-12 min	8-12 min

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Analysis of Tumorigenic Signaling Pathways With PROTACs

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A key part of determining the course of treatment for a specific cancer is the identification of the specific activated signaling pathways that cause malignant growth. In fact, the treatment for a given cancer can be dependent on the activated signaling pathway; for example, HER2/neu-positive and -negative breast cancers are treated differently. This personalized medicine approach is best exemplified by the development of the Abl tyrosine kinase inhibitor imatinib (Gleevec®), which has revolutionized the treatment of chronic myelogenous leukemia. As more drugs that target specific signaling pathways are developed, it will be important to identify those oncogenic signaling pathways that are activated in a given tumor biopsy. Toward this end, our long-term goal is the development of a library of small molecules for use as diagnostic tools in assessing primary cancer tissue samples.

Using a multidisciplinary team of chemists, biochemists, and cell biologists, we have developed a new technology, known as **PRO**teolysis-**TA**rgeting **C**himera molecules (PROTACs), that can selectively knock down a specific protein in vivo. These cell-permeable, heterobifunctional molecules utilize the cell's own ubiquitin-proteasome

protein degradation pathway to selectively destroy a chosen target protein. We propose to adapt this technology so that proteins required for continued tumor growth are degraded only in those cells with a particular activated tyrosine kinase pathway. In this way, it will be possible to identify those signaling pathways that are upregulated in a particular tumor cell and that are required for its growth.

Our recent preliminary data have validated this approach in a series of proof-of-concept experiments in which we can use a "phosphodependent" PROTAC to selectively induce the degradation of the TrkA downstream signaling component FRS2 in response to nerve growth factor.

Development of Two-Dimensional Capillary Isoelectric Focusing/Capillary Sieving Electrophoresis With Laser-Induced Fluorescence for the Analysis of Barrett's Esophagus Tissues

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Two-dimensional (2D) capillary electrophoresis (2D-CE) with laser-induced fluorescence (LIF) detection is used for separation of complex biological samples. Previous 2D-CE separations from our lab employed a sieving dimension and a micellar electrokinetic chromatography (MEKC) dimension. Although we have shown that smaller biomolecules are separated in the MEKC dimension with high efficiency, studies have suggested that bigger protein molecules are not well resolved in the MEKC dimension. We have been developing a 2D-CE system that would greatly improve the resolution by coupling capillary isoelectric focusing (cIEF) and capillary sieving electrophoresis (CSE). There are several challenges involved in coupling cIEF with CSE. The focused pI zones must be mobilized from the first capillary to the second. We are investigating various buffer conditions that will facilitate mobilization and that will also be compatible with the second-dimension sieving separation. 2D cIEF/CSE has many advantages over traditional 2D-gel electrophoresis, including higher sensitivity, faster analysis time, less labor, and potential for automation. Biogenic amines are labeled by chromeo-P503, a pyrilium compound that reacts with primary amines to produce a positively charged pyridinium ion. Fluorescently labeled biomolecules are separated by 2D-CE and detected inside a sheath-flow cuvette with a 473-nm solid-state laser and fiber-coupled avalanche photodiode (APD). 2D-CE-LIF is capable of detecting zeptomole (10-21) quantities of P503-labeled proteins. In 2D-CE, two capillaries, with i.d.s of 50 and 30 μm, are aligned at a buffer-filled interface. The proteins are separated by cIEF in the first dimension. Fractions are electrokinetically transferred to the second capillary and are separated by CSE. This technology is used to study protein expression in Barrett's esophagus (BE), a condition caused by complications in gastroesophageal reflux disease. The squamous epithelium is replaced with the specialized metaplasia BE. Patients with BE have a thirtyfold to fortyfold increased risk of progression to adenocarcinoma. BE is the only known precursor to

esophageal adenocarcinoma.

Effective Mammalian Two Hybrid Screening Approach

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Discovery of cancer drug targets and efficient implementation of targeted therapy are dependent on the understanding of protein functions and signaling networks in cancer. A fundamental step in understanding the function of a protein is to identify its interaction partners. Currently, the majority of protein-protein interactions are identified using yeast two hybrid (Y2H), co-immunoprecipitation and mass spectrometry, or protein libraries. Each of these approaches has its own set of major limitations, failing to mimic native physiological conditions (Y2H and protein libraries) or to efficiently identify protein interactions on the cytoskeleton or membrane, due to either the location of the interaction (Y2H) or difficulties in co-immunoprecipitation 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 developed and validated a readily applicable, context-dependent, subcellular localization-, cDNA library-, and cell type-independent retrovirus-based mammalian two hybrid (ReMTH) screening method for the identification of novel protein-protein interactions, including cytoskeletal and membrane proteins, in mammalian cells, allowing native protein folding and posttranslational modifications [Ding et al., 2006]. In ReMTH, bait protein is fused to one fragment of a rationally dissected fluorescent protein such as green fluorescent protein (GFP). The second, complementary fragment of GFP is fused to an endogenous protein by the retrovirus-mediated 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.

We have performed multiple rounds of ReMTH screening for AKT and p85 binding partners. Multiple previously known and likely interaction partners of AKT1 and p85 were identified, which validated the screening technology. We also estimated the efficiency of the ReMTH screen technology and will improve the technology to reduce the false-positive rate. The fully developed technology will identify functional protein-protein interactions more efficiently than present methods and will identify interactions not discoverable by present methods, particularly in context-dependent mammalian screens. Furthermore, the ReMTH screen has the unique potential to stabilize or trap transient or weak interactions, such as enzyme-substrate interactions, allowing

identification of components of signaling pathways and networks in previously undetectable cancers. Thus, the technology will uncover functional protein-protein interactions not detectable by other approaches and advance our understanding of protein functions and signaling networks in cancer.

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Measuring c-Abl Activity in CML Using a High-Affinity Nanosensor

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The ability to monitor and measure protein kinase activity in tumorigenesis and cancer can be indicative of and critical to the transformation process and therefore represents an attractive diagnostic strategy, with a multibillion dollar market opportunity. Given the fact that ~400 disorders such as cancer have been associated with protein kinases, the development of a family of sensitive nanosensors will facilitate the diagnosis of these diseases sensitively and selectively and therefore becomes of paramount importance and will find immense utility in all the various facets of our health care—from drug discovery to patient health and point-of-care diagnostics.

Recent Modifications to the ImageStream Technology Improving Its Applicability To Study NF-κB Activation in a Clinical Research Setting

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During the first year of funding, the emphasis of this work has been to increase data collection speed, sample throughput, focus quality of collected images, and flexibility of excitation sources and to optimize analysis algorithms.

Data collection speed/sample throughput. In the original architecture of the ImageStream, 5,000 events of a 1x106-cell standard sample took 6 minutes to acquire, and the in-between sample wash cycle took 8 minutes, providing an overall sample throughput of 4 samples per hour. Hardware and fluidic script changes now collect 5,000 events of a 1x106-cell sample and wash in 5 minutes, providing a throughput of 12 samples per hour.

Focus quality. The image focus quality has been improved by installation of an

extended depth of field (EDF) optical element, which extends the depth of focus from 2 µm to 16 µm. The EDF principle is based on the ability to develop an optical point spread function (PSF) that is invariant to focus over a range equivalent to the diameter of a large cell (~15 µm). If the PSF remains invariant, it can be mathematically deconvolved, thereby producing an image of the entire cell in focus. The EDF option uses a Wavefront CodingTM element developed by CDM Optics, Inc., which substantially changes the size and nature of the PSF.

Since the initial beta launch, changes to hardware, calibration routines, and deconvolution algorithms have substantially improved the EDF element's PSF invariance to focus. When combined with the newly developed and greatly improved deconvolution techniques, the resulting imagery has noticeably fewer artifacts, maintains a natural appearance, and leads to an overall improved image quality.

Excitation sources. The basic commercial release of the ImageStream has a single 488-nm excitation source. Corrections for the blue-channel image size difference (associated with a 405-nm laser excitation) and the red laser scatter leakage into channel 5 of the ImageStream (associated with a 658-nm red laser) have now been solved. Combined, the above modifications improved the overall effectiveness and applicability of the ImageStream, not only with regard to assessment of NF-κB translocation but also for all applications of the system.

Analysis algorithms. The current analysis algorithm uses a "similarity score," which is a log-transformed Pearson's correlation coefficient of the pixel values of the DRAQ5 (nuclear) and NF-κB images. If NF-κB is localized to the nucleus, the two images are similar, and a large positive value is generated. If NF-κB is cytoplasmic, the two images are antisimilar, and a large negative value is generated. Changes in the distributions can be reported as a percentage shift into a rigorously set "translocated" gate—which requires a decision on how much nuclear NF-κB is considered baseline—or as an Rd metric, which is a measure of the shift of two distributions (taking into consideration their respective median and standard deviations). Alternatively, the nuclear translocation of NF-κB can be measured as the relative nuclear concentration compared with the whole-cell concentration. The effects of data acquisition (with or without EDF), nuclear segmentation algorithms (how the nuclear area is defined), and accuracy and reproducibility of these analysis approaches are currently being studied.

EpiTag™ Peptide-Based Immunoassay Platform for the Measurement of Protein Splice Variance in Complex Mixtures

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The genesis of cancer involves genetic changes that culminate in uncontrolled cellular proliferation. Indeed, key oncogenes and tumor suppressors are widely recognized to

have a major impact on the development and progression of cancers. This recognition has come in large part from numerous efforts that have defined critical mutations in the genetic sequences of genes that alter their respective functions. However, there are cases in which genes are associated with cancer but have no apparent mutations that alter their normal function. In a number of cases, there is evidence that splice variants of a given gene and their respective ratios at both the RNA and protein levels are significantly altered 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 different protein isoforms.

Antibody-based methods to measure proteins are powerful and are extensively used in research and diagnostic applications in clinical settings. However, development of immunoassays to discriminate among protein isoforms is challenging due to significant shared sequence identity in protein isoforms. Epitome Biosystems, Inc., has developed methodologies that solve this problem through a combination of antibody design and sample treatment aimed to exploit differences between protein isoforms. Starting with genomic databases, in silico techniques are used to identify continuous linear sequences for any protein (EpiTags™) that are unique relative to linear sequences found in all other proteins within a proteome. Antibodies are raised against synthetic peptides that make up these unique selected sequences. The corresponding selected peptide sequences (EpiTags™) derived from protein samples are rendered accessible to the antibody by proteolytically fragmenting the sample prior to analysis, resulting in predictable antibody performance.

A "sandwich" assay format is utilized for unambiguous detection and quantification of each protein isoform. 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, whereas the 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. After sample denaturation and digestion, each individual isoform generates a unique peptide fragment signature that can be quantified on the basis of novel antibody sandwich pair formation. Therefore, Epitome Biosystems' approach allows for an overall systematic methodology for the detection of virtually any splice variant within a proteome.

We initially demonstrated proof of concept by using a contrived model system that was developed with commercially available peptide-specific antibodies and a set of corresponding synthetic peptides. Subsequently, we developed assays to measure the cancer-related splice forms of Bcl-X, a member of the Bcl-2 family of apoptotic regulators. Because it is highly expressed in many lymphomas, Bcl-X may play a significant role in the genesis of these cancers. This protein has two distinct splice variant forms (long and short) with opposing apoptotic functions. Assays have been developed and demonstrate quantitative measurements of the long, short, and full-length forms of the Bcl-X protein in a number of cell lines.

Folate-Receptor-Mediated siRNA Delivery to Cancer Cells

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Posttranscriptional gene silencing by small interfering RNA (siRNA) is a powerful tool for downregulation of target genes, with both high efficiency and sequence specificity. In principle, siRNAs may become the next-generation therapeutic agents with high potencies and low side toxicities. However, target-specific delivery of siRNA represents a great challenge. 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, whereas 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 to cancer cells by endocytosis has been demonstrated to be both efficient and highly specific toward FR-positive cancer cells. In searching for siRNA delivery agents based on FR, we are developing novel polymer-siRNA complexes that contain multiple folic acids, a hydrophilic component, and a charged moiety. The complexes can drastically enhance the hydrolytic stability of siRNA against RNA nucleases by in vitro analysis. The linked folic acid of the complex can bind cell-surface FR so that cellular internalization of the complex occurs via FR-assisted endocytosis. By using KB cancer cells (a human nasopharyngeal epidermoid carcinoma cell line), fluorescently labeled siRNAs have been shown to penetrate the KB cell membrane within 30 minutes in the absence of other delivery agents. On the other hand, the polymer-siRNA complex cannot internalize into human umbilical vein endothelial cells, which do not express FR. Our results indicate that the siRNA delivery system is effective and can specifically deliver siRNA into FR-expressing cells. Therefore, the system may be used to develop therapeutic agents that direct siRNA into FR-expressing cancer cells.

Innovative Technology Development Directed at the Elucidation of Biomarkers for the Detection of Early-Stage Ovarian Cancer

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Epithelial ovarian cancer (EOC), the fifth leading cause of cancer deaths among women in the United States, is a very lethal malignancy owing to metastatic capacity and resistance to chemotherapeutic drugs. Indeed, the poor survival-to-incidence ratio of this neoplasm is largely a consequence of inadequate diagnostic capabilities in EOC's preliminary stages of development. Early-stage carcinogenesis presents with few, if any, symptoms, and disease detection seldom occurs prior to metastasis, when survival rates decline sharply. Only 18% of women diagnosed with late-stage EOC are able to

live for 5 years, whereas 88% of those detected with early-stage disease can reach this survival mark. The mucin glycoprotein CA-125 is the only FDA-approved EOC biomarker in use today. Unfortunately, lack of sensitivity and specificity renders this diagnostic test ineffective as a population screening marker, and other tests currently being pursued are similarly deficient [1].

The molecular analysis of EOC biospecimens has greatly benefited in recent years from novel mass spectrometric platforms, which may provide more robust markers for early detection in the foreseeable future. We have developed and applied Fourier transform mass spectrometry (FT-MS) in our quest to elucidate predictive biomarkers for the early detection of EOC. These developments are in the form of plasma processing strategies [2], separation science [3], understanding cocrystallization in the MALDI process [4], and the development of new mass calibration laws for FT-ICR-MS [5]. These and other developments will be presented.

Previous reports have described differential blood protein glycosylations between EOC and control subjects. We present a detailed study of EOC-induced O-glycan changes in plasma glycoproteins. We explore 118 plasma samples originating from cancer patients across all stages of the disease, as well as age- and menopause-matched controls consisting of healthy individuals and those diagnosed with benign gynecologic tumors. Complementary glycan information between the dual analytical approach of MALDI-FT-MS and LC-LTQ-Orbitrap-MS is observed in preliminary results. Metrics including area under the receiver operator characteristic curve, relative sensitivities and specificities, and positive predictive values are applied to evaluate the accuracy of potential glycan markers.

We have also found preliminary evidence using 2D-PAGE that C-reactive protein is more abundant in the plasma of EOC patients relative to controls. To enable the analysis of 118 patient samples, we developed a protein-cleavage isotope dilution mass spectrometry method. These data clearly demonstrate that candidate biomarkers elucidated in a global proteomics inquiry yield intriguing results; however, semivalidation using targeted approaches often reveals poor specificity and sensitivity.

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Translational Control Analysis by Novel Transip-Chip Approach

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Translational control plays key roles in development, cancer stem cell development/ differentiation, and acute chemoresistance. In particular, noncoding miRNAs can potentially regulate over one-third of gene expression at the translational level. Gene expression analysis on actively translated mRNA transcripts provides a unique approach to study posttranscriptional regulation. The current method relies on the traditional sucrose gradient ultracentrifugation procedure to isolate polyribosome complexes, which requires up to 500 million cells. As a result, this remains a major bottleneck for the investigation of posttranscriptional regulation with limited quantities of clinical samples. We have developed a new technology to systematically study translational regulation from a small number of cells. This approach is based on the fact that actively translated mRNAs are associated with multiple units of ribosomes and that newly synthesized polypeptides are closely associated with molecular chaperones such as hsp70s. 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 polyribosomes from free mRNAs. Affinity antibody capture beads were developed to capture hsp73 chaperones associated with the polyribosome complexes so that polyribosomes could be separated from monosomes and free mRNAs. The isolated, actively translated mRNAs were used for high-throughput gene expression analysis. The feasibility was demonstrated using a rabbit reticulocyte lysate in vitro translational system with known translational regulated mRNA transcripts such as thymidylate synthase (TS) and p53. We further developed the approach using HCT-116 colon cancer cells with both TS and p53 as positive controls. Our results showed that the levels of both TS and p53 mRNAs were not altered after 5-fluorouracil treatment by real-time qRT-PCR analysis based on steady-state total RNA. By contrast, the protein expression levels of both genes were increased. The differences in the translational rates of these two mRNAs can be captured with our new approach from as low as 500 cells. High-throughput gene expression analysis reveals that additional translational regulated genes (>800) can be identified (e.g., PP2A). This technology will have a strong potential to make investigation of translational control feasible from limited clinical specimens.

Epigenetic Image-Based Screen for Mammary Tissue

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We are developing methods for turning high-resolution fluorescence images of human mammary epithelial tissue into quantitative morphology and feature maps that will allow better detection of nonneoplastic, premalignant, and malignant phenotypes at cellular resolution. The hypothesis is that malignant transformation of cells is accompanied by changes in gene expression, which alter the nuclear organization of chromatin-related proteins. This in turn will permit epigenetic, imaging-based screening of nuclei, providing macromolecular information about a tissue, which will enable the recognition of subtle differences in tissue morphology and behavior and better detection of benign and malignant lesions. We have previously established the ability to link nuclear organization with tissue phenotype in a cell culture model of mammary epithelial tissue [1] and have demonstrated the sensitivity of our technique by predicting nonneoplastic and malignant epithelial cells with high accuracy [2].

However, the goal is not only to provide epigenetic, macromolecular information about neoplastic diseases but also to provide tools to investigate breast cancer at early stages of the disease before invasion occurs. In our current work, we are focusing not only on cultured epithelial models but also on archival human tissue. Hyperplasia, characterized by multiple layers of epithelial cells, is a possible precursor of a form of invasive neoplasia. By developing fluorescence-based immunostaining methods that work with fixed, deparaffinated human tissue sections, we show that hyperplastic epithelial cells can be subclassified into multiple phenotypes based on the distribution of NuMA, a chromatin-related protein. The results not only reveal the presence of different phenotypes of epithelial cells in the earliest stages of histologically reportable premalignant lesions but also show how these phenotypes are organized spatially within the tissue.

The goal is to bring together multiple quantitative features describing the organization of several nuclear proteins and create a morphology and feature atlas describing various pathologies of human tissue. This atlas will provide the basis for studying correlations between macromolecular organization and tissue phenotype.

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Affinity Clamps: High-Performance Affinity Reagents for Molecular Analysis of Cancer

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Short peptide motifs within proteins and posttranslational modification thereof play central roles in signal transduction regulation and in cancer biology. Therefore, quantitative detection, functional assessment, and isolation of such motifs are critically important for the molecular analysis of cancer. However, there is a paucity of high-quality affinity reagents (reagents that bind to a target with high affininty and specificity) to this class of high-value epitopes, which has now been recognized as a major bottleneck in cancer analysis. The goal of this project is to develop a powerful technology platform for generating high-affinity and high-specificity affinity reagents for short peptide epitopes, with a specific focus on phosphorylated peptides. To this end, we have established a fully recombinant protein-engineering strategy termed "affinity clamping. " Our strategy is unique in that we aim to develop distinct types of affinity reagents.

Quantitative Proteomics of Metastasis

David Burke Krizman, Marlene Darfler, Brian Hood, Thomas Conrads

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Expression Pathology, Inc. (EPI) has developed mass spectrometry-based protein expression data directly from formalin-fixed (FF) tissue, strongly suggesting that specific proteins known to be involved in tumor cell attachment, invasion, and locomotion are upregulated in epithelium microdissected from primary tumors that had already shown the metastatic phenotype versus epithelium microdissected from primary tumors that had not metastasized at the time of collection. In collaboration with the Clinical Proteomics Facility at the University of Pittsburgh School of Medicine, EPI expects to apply the emerging technical capabilities of global quantitative protein expression analysis, with specific emphasis on these proteins, to firmly establish that increased expression of multiple proteins from entire families of proteins that mediate the metastatic phenotype correlate directly with the metastatic status of a primary breast tumor.

This objective will be accomplished by utilizing Liquid Tissue® reagents and a protocol to prepare soluble tissue protein lysates of microdissected epithelium directly from 36 FF breast cancer tissue samples. The use of FF tissues is critical to this project because precise collections of specific cancer samples can be more easily assembled from FF tissue collections. The first set of 12 tissue samples will be collected from patients whose primary breast cancers demonstrated metastatic disease at the time of

presentation (Stage III). A second set of 12 primary breast cancer tissue samples will be collected from patients whose primary cancers showed no spread to the surrounding lymph nodes at the time of presentation and who, after long-term followup, remain cancer free (Stage 0). A third set of 12 samples will be collected from patients whose primary tumors showed no surrounding lymph node involvement at the time of presentation (Stage II). These 12 tissue samples will be further subdivided into two sets of 6 samples each, in which 1 set is from patients who showed recurrent disease and the other is from patients who remained disease free for at least 2 years. This last set of 12 samples is the test set, whereby data collected from the analysis of Stage III and Stage 0 primary tumors might be able to predict the course of disease in these 12 patients.

Each of the 36 Liquid Tissue® protein lysates will be analyzed by mass spectrometry-based global proteomic profiling and quantitative protein expression data developed with the spectral count method and Seive software. Differential protein expression by mass spectrometry will be validated by immunohistochemical methods for the proteins that correlate most closely with metastatic disease. The capability to quantify multiple metastasis-associated proteins simultaneously directly in FF breast tissue is important to this application because it allows for interrogation of tissue collections with extensive clinical data, which cannot be duplicated with frozen tissue collections. This fact becomes even more prominent during a proposed Phase II study, in which much larger tissue collections with extensive clinical data and followup will need to be interrogated. Technological capabilities that have been codeveloped and already demonstrated by collaboration between these groups will form the foundation for the success of this proposal.

This Phase I Small Business Innovation Research grant seeks to establish whether metastasis-associated proteins are upregulated in primary breast cancers that have shown the ability to become mobile and invade nonbreast tissue and whether this phenomenon can be exploited to provide an indication of the aggressiveness and/or metastatic potential of earlier stage breast cancers. Predicting the clinical aggressiveness of a tumor through molecular pathology indications could have far-reaching ramifications for personalized patient management. Successful achievement of the stated milestones will provide for a subsequent Phase II proposed study to explore and develop large-scale quantitative protein expression applications of these metastasis-associated proteins to provide for clinical diagnostic and prognostic assays of FF pathologic tissue. This will be achieved by the mass spectrometry-based quantitative proteomic technology of multiple reaction monitoring through an expanded collaboration between EPI and the Clinical Proteomics Facility at the University of Pittsburgh School of Medicine, involving large collections of breast cancer tissue.

Diagnostic Analyses of Endogenous Protein Interactions Using Proximity Ligation

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We have developed a proximity ligation technique that allows detected proteins to be represented as DNA strands that can then be analyzed using DNA tools such as real-time polymerase chain reaction, DNA sequencing, oligonucleotide arrays, or rolling-circle replication for localized amplification in microscopy. The method provides extremely sensitive detection of individual or large sets of proteins in, for example, serum or sample lysates [1,2] and permits visualization of the distribution of individual protein molecules and their modifications or interaction partners during microscopy [3,4].

In the current project, we will extend the assay to also permit simultaneous assessment of multiple interactions among a set of investigated proteins in a tumor sample. We believe that such assays will have important diagnostic value and can permit monitoring of therapeutic effects during drug development or as companion diagnostics.

The assay starts from lysates of cell lines, but later we shift to using tissue samples obtained by clinical collaborators. Next, we add sets of antibodies with specificity for proteins of interest, each carrying an oligonucleotide with a unique tag sequence. Oligonucleotides on antibody pairs that bind interacting protein molecules are brought into proximity and can be joined by ligation and amplified to easily detected levels. By decoding which pairs of tag sequences have been joined, it is possible to reveal interactions in the investigated protein set.

This technique depends on high-quality antibodies to minimize the risk of nonspecific detection and close attention to requirements for sample preservation. As our model system in this work, we have taken the signaling cascade activated by transforming growth factor beta binding to its surface receptor. We recently described a dual-tag microarray platform that is suitable to monitor large sets of potential interactions [5], but parallel DNA sequencing approaches can also be used to identify ligation products.

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Microfluidic Matrix-Assisted Laser Desorption Ionization—Mass Spectrometry Device for High-Throughput Proteomics and Biomarker Discovery

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Cancer is a leading cause of death in the United States; more than 1 million new cases are diagnosed every year. As a result of the high levels of sensitivity and specificity that are required to justify large-scale population screening, only a very few single-protein biomarkers are routinely used today in the clinical setting. It is a critical priority to develop novel technologies that will enable the rapid detection of a plethora of biomarkers that are relevant to early diagnosis, prognosis, staging, and treatment response.

The goal of our research is to combine the emerging technology of microfluidics with state-of-the-art mass spectrometry (MS) detection to enhance our capacity for analyzing molecular structure and function in biological systems. Our proposal capitalizes on the distinguishing capabilities of microfluidic architectures that enable process integration, multiplexing, fast and high-throughput processing of minute amounts of sample, and the power of MS detection that provides the sensitivity, specificity, and resolving power necessary for unambiguous detection of trace-level components. In particular, we plan to develop a compact, low-cost, and disposable microfluidic analysis platform with matrix-assisted laser desorption ionization (MALDI)-MS/MS detection for high-throughput proteomics that will enable the study of protein coexpression patterns and biomarker discovery. The microfluidic device will make it possible to carry out parallel liquid chromatography separations and will integrate a novel microchip-MS interface to enable sensitive MALDI-MS/MS detection directly from the chip. We will demonstrate the effectiveness of the microfluidic MALDI-MS/MS platform for the detection of multiple cancer biomarkers in cellular extracts. We will analyze cellular fractions from the MCF7 breast cancer cell line for identifying target proteins that are involved in the essential cellular processes leading to the onset and development of cancer (cell proliferation, cell cycle regulation, DNA repair, apoptosis, and invasion/metastasis).

The proposed microfluidic-MS technology will advance a novel and versatile strategy for proteomic investigations that will enable the biomedical research community to perform high-throughput discovery and screening of prognostic/diagnostic biomarker components. The unique architecture of these devices, together with the miniature, disposable, and contamination-free format, will enable the possible future implementation of this technology in the clinical setting for high-throughput population

screening.

Microfluidic Platform for Dynamic Live Cell Imaging

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The study of living cells in vitro provides critical insight into the behaviors, signaling pathways, and molecular mechanisms of cancerous tissues. Recent advances in microscopy technology and intracellular probes enable researchers to visualize and quantify cellular processes with very fine spatial and temporal resolution. However, only limited technology has been developed to improve the physical cell culture environment during cell imaging studies. This is becoming increasingly important as it is now known that that the cell microenvironment is a critical aspect of cancer progression. In this work, we have developed a microfluidics-based system for continuous-perfusion cell culture and dynamic solution exchange during live cell imaging studies. The basic platform consists of a flow control unit and consumable microfluidic plates. The flow control system enables computer-controlled programming of perfusion flows, and the microfluidic plates house all the cells, exposure solutions, and reagents. An add-on module to the flow controller enables precise temperature and CO_2 regulation to the microfluidic plates. The microfluidic plates contain application-specific designs to accommodate a wide variety of cell culture experiments. In the simplest case, cells can be seeded and observed during time-lapse imaging in response to defined medium changes. More complex microfluidic chambers allow features such as continuous concentration mixing, spatial gradients, extracellular matrix deposition, "tumor-like" culture models, co-culture, etc. We believe that this current platform will provide a straightforward method for cell biologists to observe dynamic events in live cells. Additionally, the flexibility of the platforms enables easy adaptation to more specialized and complex cell environments.

Elucidating Signaling Networks in Clinical Tissues With Multiparameter Automated Molecular Imaging

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A common goal in pharmaceutically oriented research is to discover protein expression patterns that correlate with clinical outcomes and/or drug responses. Once these patterns are uncovered, they can be used to inform target validation, trial design, patient selection, response assessment, and if trials are successful, the diagnostic component of personalized therapy.

Detecting multiple, often weakly expressed targets in clinical tissue sections is challenging. Critical capabilities include multilabel staining methods that are quantitative, independent, and specific; a way to isolate label signals from one another (e.g., from autofluorescence and other labels); fully automated image analysis algorithms for segmenting cellular regions (cancer versus normal, stroma, necrosis, etc.) and for segmenting cellular compartments (nuclei, cytoplasm, membrane); and a slide-scanning platform capable of sampling a sufficient area across a tissue section to capture and quantify its heterogeneity.

The goal of our IMAT project was to develop and validate an imaging platform to provide these capabilities, with an immediate application to signal transduction pathway research in tissue sections. The platform is intended to support several phases of research and implementation, including:

- Conducting exploratory work employing quantum-dot (QDot) labels and multispectral imaging in tissue microarrays (TMAs) of samples from patients with known clinical outcomes to elucidate protein expression patterns
- Developing and deploying multicolor chromogenic immunohistochemical staining protocols to support investigations involving conventional clinical biopsy material
- Performing drug-response assessment in clinical studies by using automated multispectral slide-scanning and machine-learning-based image analysis

Multispectral imaging, using a CRi Nuance spectral scanning imaging system, was performed on two triple-stained TMAs: the first stained with QDots targeting pMEK and pAKT and the second with QDots targeting p53 and stathmin, both counterstained with a nuclear dye. QDot labeling methods were chosen because they lend themselves well to multiplexed assays and resist photobleaching. QDot signals were spectrally unmixed and isolated from each other, tissue autofluorescence, and nuclear stain. Machine-learning-based automated image analysis was performed to locate cancer cells, segment subcellular compartments, and extract QDots signals on a per cell basis, and the data were analyzed and displayed using flow cytometry analysis software.

Protein expression levels resulted in relatively weak but specific QDot signals that were tenfold lower than the nuclear label and twofold lower than tissue autofluorescence. Multispectral imaging successfully detected and separated QDot-labeled target signals from other fluorescent emissions. The automated image analysis tools employing machine learning and other proprietary approaches proved to be robust at segmenting cancer-containing regions and extracting signals from relevant cell compartments. Together, the innovative multispectral platform and software capabilities provided "flow-on-a-slide" functionality, sensitively capturing cellular heterogeneity in an intact tissue architectural context.

Advanced Technology for Assaying Cancer-Drug Resistance

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Significant advances have been made toward the development of a new generation of molecularlytargeted cancer drugs, many of which are only now emerging from the pipeline. This project aims to develop a new, highly sensitive technology for detecting drug-resistance mutations in proteins, which preexist prior to treatment or are acquired due to the selective pressure exerted by treatment with molecularly targeted anticancer drugs (ACDs). This problem is exemplified by drug resistance developed in patients treated for chronic myeloid leukemia (CML) with the small-molecule drug imatinib (Gleevec®/Glivec/STI571). It is well documented that this resistance arises from mutations in the BCR-ABL tyrosine kinase, the target for imatinib. Drug resistance also occurs in both Philadelphia chromosome positive (Ph+) acute lymphatic leukemia (Ph+ ALL) and gastrointestinal stromal tumor patients who are treated with imatinib. Although direct DNA sequencing can be used to scan for previously characterized and uncharacterized drug-resistance mutations, sensitivity is limited to a 20% mutant population, impairing the early detection of developing drug resistance. In this project, these limitations are overcome by using a novel technology developed by AmberGen Inc., for isolating highly purified, cell-free-expressed polypeptide fragments of the drug-targeted protein(s) and detecting characteristic drug-resistance mutations using a matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) scanning technique. The new approach, termed "drug-resistance assay for mutations against anticancer drugs (DRAMA-ACD)," allows low-cost scanning for mutations (even those previously undiscovered) with high sensitivity and high throughput. During Phase I, we demonstrated that DRAMA-ACD could detect mutant BCR-ABL tyrosine kinase at a sensitivity of at least 5% in a background of 95% wild-type. Furthermore, PC-SNAG, a method of capturing and photoreleasing cell-free expressed fragments of the BCR-ABL tyrosine kinase, significantly reduces background in the MALDI-MS-based assay. Finally, the throughput of DRAMA-ACD can be significantly increased using multiplexed, solid-phase PCR coupled with multiplexed cell-free protein expression. During Phase II, we will continue to focus on the development and application of DRAMA-ACD to detect mutations in the BCR-ABL tyrosine kinase. An important milestone will be a demonstration of the ability to detect these mutations in CML patients with a sensitivity of 1%. The research will be carried out in collaboration with Dr. Adam Lerner, Associate Professor of Medicine and Pathology, Boston University School of Medicine, a leading expert in the area of hematologic malignancies, who will provide us with samples for analysis from CML and Ph+ ALL patients undergoing imatinib treatment. Overall, the high-sensitivity mutation scanning capabilities of this novel approach will have widespread applications in the molecular analysis of cancer.

Single-Molecule Imaging of Nucleosome Acetylation With a DNA Aptamer

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Posttranslational modifications found on the tails of histone proteins constitute an elaborate epigenetic code that regulates access to DNA by mediating synergistic or antagonistic interactions with chromatin-associating proteins. We have used in vitro selection to generate a DNA aptamer with high specificity to histone H4 proteins containing an acetyl group on lysine 16 (H4-K16Ac) and have demonstrated the utility of this aptamer as an affinity reagent in the single-molecule detection technique known as recognition imaging microscopy. When assayed as a recognition element on the cantilever tip of an atomic force microscope, the DNA aptamer recognized almost exclusively histone H4-K16 acetylated nucleosomes, whereas a commercial anti-H4-K16Ac antibody detected acetylated and nonacetylated nucleosomes equally. This level of selectivity demonstrates the ability of DNA aptamers to distinguish key epigenetic modifications involved in gene activation and silencing and provides new tools for elucidating the histone code.

Early Signaling Events in T-Cell Activation by Parallel Multi-Timepoint Stimulation and Lysis On-Chip

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Success in implementing adoptive transfer as a widespread cancer therapeutic tool requires understanding how T-cell activation is dampened in vivo by the tumor milieu and the ability to evaluate the responsiveness of ex vivo expanded T cells accurately and systematically. Our objective is to engineer a multiplex microfluidic assay to quantify T-cell activation on a small population of cells with high temporal resolution. We hypothesize that capturing the early dynamics of T-cell activation of ex vivo expanded clones would improve on current measures of T-cell functionality. This measurement would be optimal for evaluating whether transferred T cells have retained their response to antigenic stimulation under culture conditions. The rationale for this research is that the microfluidic format could be applied to monitoring T-cell specificity and functionality both pretransfer and posttransfer because of its small sample requirements and the high-throughput nature of the experiments, in turn, providing the much needed feedback on the quality of T-cell therapy during cancer treatment.

Toward these goals, we present a two-module microfluidic platform for simultaneous multi-timepoint stimulation and lysis of T cells for early timepoint signaling activation with a resolution down to 20 seconds using only small amounts of cells and reagents.

The key design features are rapid mixing of reagents and uniform splitting into eight channels for simultaneous collection of multi-timepoint data. Chaotic mixing was investigated via computational fluid dynamic modeling and was used to achieve rapid and complete mixing. This modular device is flexible; with easy adjustment of the setup, a wide range of timepoints can be achieved. We show that treatment in the device does not elicit adverse cellular stress in Jurkat cells. The activation of six important proteins in the signaling cascade was quantified on stimulation with a soluble form of α-CD3. The dynamics from device and conventional methods are similar, but the microdevice exhibits significantly less error between experiments. We envision this high-throughput format to enable simple and fast generation of large sets of quantitative data, with consistent sample handling, for evaluating the properties of T-cell populations.

Linear Combinations of Docking Affinities Explain Quantitative Differences in Receptor Tyrosine Kinase Signaling

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Receptor tyrosine kinases (RTKs) receive and process extracellular cues by activating a broad array of intracellular signaling proteins. Paradoxically, they often use a common set of transducer proteins to elicit diverse and even opposing phenotypic responses ranging from adhesion to migration, differentiation to proliferation, and survival to apoptosis [1,2]. Although cellular context largely determines how receptor activation is interpreted [3-5], different RTKs have been shown to elicit different outcomes when expressed in the same cell [6,7]. How, then, are their intrinsic differences manifested, where does the information reside that defines these differences, and how is that information processed? Using quantitative immunoblotting, we found that when six diverse RTKs are placed in the same cellular background, they activate many of the same proteins but to different quantitative degrees. To determine what defines these differences, we used protein microarrays comprising almost every SH2 and PTB domain encoded in the human genome to measure binding affinities between these domains and phosphopeptides representing physiological sites of tyrosine phosphorylation on the RTKs [8,9]. Using partial least-squares regression, we found that the relative phosphorylation levels of upstream signaling proteins can be accurately predicted using linear models that rely on combinations of docking affinities and that the number and affinity of PI3K and Shc1 docking sites provide much of the predictive information. In contrast, we found that the phosphorylation levels of downstream proteins cannot be predicted using linear models. Taken together, these results show that information processing by RTK signaling networks can be segmented into an upstream layer comprising proteins that are activated in a linear fashion via combinations of receptor-docking affinities and a downstream layer comprising proteins that are activated in a nonlinear fashion. This suggests that the challenging task of constructing mathematical models of RTK signaling can be parsed into discrete and

more manageable layers.

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Whole-Genome Amplification of Laser Capture Microdissected DNA From Archived Samples Using WGA of Circular Templates (RCA-RCA)

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Retrospective studies on existing formalin-fixed, paraffin-embedded (FFPE) specimens for which complete clinical data are available are a useful alternative to prospective studies that may take years to complete. However, the efficient use of FFPE specimens for research is hindered by technical issues. Because cancer specimens are heterogeneous and often contain substantial amounts of normal tissue, identification of cancer-specific genetic abnormalities from FFPE samples requires microdissection and whole-genome amplification prior to screening. Major hurdles to this process are the introduction of amplification bias and the inhibitory effects of formalin fixation on DNA/RNA amplification.

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

molecular information from degraded FFPE samples.

Over the past year, we applied and tested technology developed in the R21 phase in laser capture microdissected archived samples. Five previously characterized colon cancer surgical specimens, in the presence or absence of laser capture microdissection (LCM), were employed for this validation. Following LCM, DNA was extracted from these specimens using the methods we recently reported. DNA extracted from FFPE specimens underwent real-time polymerase chain reaction (PCR)-based assessment of DNA quality using Multiplex-PCR on ~1 ng DNA (Molecular Index of Degradation [MID]). Samples were then amplified via RCA-RCA and characterized via real-time PCR. In agreement with MID results, the data demonstrated that two of five LCM samples were successfully amplified, whereas three samples could not be amplified via RCA-RCA due to the high degree of DNA degradation. However, one of the RCA-RCA properties is that the amplification can be adapted to match the degree of sample degradation by changing the restriction enzyme used. Thus, we are currently evaluating the adaptation of RCA-RCA to also achieve successful amplification for the three samples that underwent high degradation due to the harsh formalin fixation conditions applied to them. Finally, whole-genome amplification was also shown to be useful in the amplification of DNA that was collected from body fluids (e.g., plasma) and that is often enzymatically degraded at the time of collection. We anticipate that RCA-RCA also will be useful in the identification of biomarkers from clinical samples and body fluids.

This work was supported by CA111994 (sample preparation RFA).

Multiplexed Biomarker Panels for Early Detection of Prostate Cancer

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The goal of this SBIR project is development of sensitive diagnostic serological assay panels for the detection of early-stage prostate cancer using Meso Scale Diagnostics (MSD) electrochemiluminescence-based Multi-Array® technology. Currently, the only biomarker used in prostate cancer diagnosis is the measurement of prostate-specific antigen (PSA) in patient serum. PSA levels can be elevated by various conditions and often are not at the diagnostically critical levels when cancer develops. There is a need for alternative biomarkers to more efficiently diagnose prostate cancer, particularly for early detection when aggressive treatments would be most effective. Approaches that detect autoimmune responses to cancer-related antigens are being developed as an effective means of early cancer detection, before measurable amounts of antigens accumulate in serum. Prostate-cancer-specific immune responses are being identified using several approaches, including screening with prostate-cancer-specific cDNA expression systems as well as purified prostate-cancer-related proteins. Great value is seen in the ability of these antigens used in multiplex to specifically and sensitively detect cancer, which are superior to PSA-based determinations. MSD's sensitive Multi-

Array® technology is being combined with the extensive prostate cancer biomarker expertise and resources of Dr. Arul Chinnaiyan and Dr. George Wang (University of Michigan) to develop highly specific and sensitive multiplex serological screening panels for early detection of prostate cancer. The antigens of interest (antigen-expressing phage particles, or purified proteins) are being immobilized in MSD Multi-Array® panels, which can accommodate 1 to 25 assays per well of a 96-well plate. These panels will be optimized to detect specific humoral responses in prostate cancer patient samples, allowing multiple simultaneous determinations per well. MSD multiplex panels for serum-based measurements have shown exquisite assay sensitivities (as low as 0.1 pg/mL in direct detection of protein analytes), three to five orders of magnitude dynamic range, rapid throughput, and minimal sample usage, factors that are critical in developing successful serological screening panels. This academic/industrial collaboration addresses the NCI's goals of translational medicine by advancing potential biomarkers from discovery toward clinical applications using a versatile and robust assay platform and the IMAT goal of evaluating technologies that are ready for initial clinical or laboratory application in cancer research.

Development of Parallel Capillary Perfused Bioreactors for Leukocyte Transendothelial Migration Analysis

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The tumor microenvironment, in particular tumor-associated inflammation, is a driving force of tumor progression. Immune cell (i.e., leukocyte) infiltration into sites of inflammation requires the coordinate regulation of multiple steps, including arrest on endothelium, migration through the endothelial barrier, and directed migration through connective tissue. Our studies suggest that matrix proteases enhance leukocyte extravasation. Direct examination of the roles of key molecules during leukocyte extravasation is possible through the analysis of primary cells isolated from mice with genetic ablation or targeted overexpression. However, current technologies to assay leukocyte extravasation either do not recapitulate key physiological parameters such as the microfluidic shear and apical-basolateral organization of endothelium or require extensive tissue samples, which excludes their use with primary cells isolated from mice. Our goal is to exploit the expertise of our collaborative team of cancer biologists and engineers in applying soft-lithography microfabrication technology to the biological challenge of the study of tumor-associated leukocyte infiltration ex vivo. We are using a multidisciplinary approach in the development of planar and multilayer parallel capillary perfused bioreactors (PCPBs) that (1) better approximates the spatial constraints and architecture of blood vasculature (2) can provide regulated shear force, and (3) is high throughput in design, requiring minimal cell samples for assay conditions. As a first step

toward developing the multilayer PCPB capable of transendothelial migration analysis, we have begun device validation with a simplified planar version of the PCPB. Capillary surfaces coated with L-selectin, V-cam, and I-cam receptors—together with murine primary neutrophils—were used for reactor validation. By directly observing cell position in the capillary and accurately measuring differences in rolling velocities between the control and the receptor-coated surfaces at different flow rates, we were able to estimate the magnitude of interaction forces between the activated leukocytes and surface-bound receptors. Moreover, we noticed an increase in the interaction forces in the presence of higher shear stresses, an observation also supported by the literature. Additionally, we have employed COMSOL Multiphysics® to predict the distribution of shear stresses in the PCPB and the effect of already-adhered leukocytes on the fluid flow and cell rolling. We are building up in a modular approach from the foundation of the planar PCPB to develop a multilayer PCPB that incorporates a functional polarized endothelial monolayer. We have begun testing inclusion of an endothelial monolayer and assaying leukocyte attachment under target physiological flows. Our long-term objective is to build a bioreactor within which primary cells from various murine genetic models can be used to reconstitute an artificial endothelium under shear flow and to monitor the transendothelial migration of leukocytes and other cell types. These small-scale migration bioreactors will provide independent control of growth factor gradients, shear forces, tissue perfusion, and permeability of physical barriers to cellular migration, permitting meaningful control and manipulation of parameters relevant to the in vivo process when circulating cells attach and invade across the endothelium in response to cytochemical signals. In addition, the design of these devices will allow detailed optical observation of cell migration, attachment, and transendothelial migration.

Mapping Chromosomal Aberrations in Cancer by Combining Long-Insert Cloning and Next-Generation Sequencing

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We have developed the first version of a method that maps chromosomal aberrations in cancer by combining next-generation genomic sequencing technologies and efficient large-insert cloning (fosmid and bacterial artificial chromosomes [BACs]). We have applied the first version of the method to obtain a comprehensive map of chromosomal aberrations in the genome of the MCF-7 breast cancer cell line at a resolution that allows polymerase chain reaction to be performed across rearrangement-induced breakpoint joins. A total of 157 distinct somatic breakpoint joins were identified, providing insights into errors in double-strand repair mechanisms responsible for genomic instability in MCF-7. A total of 79 genes are involved in rearrangement events, including 10 fusions of intact coding exons from different genes and 77 other aberrant breakpoints involving known or predicted genes. Functional validation of gene fusions is

in progress. In collaboration with Dr. Pieter J. de Jong's laboratory at Children's Hospital of Oakland Research Institute, we are in the process of developing the second version of the method that is scaleable, works on a small amount of input material (<1,000 cells), is compatible with any next-generation sequencing technology, and is fully supported by turnkey informatics. We anticipate validating the second version of the method by early fall 2008. By applying the method to multiple breast cancer genomes, we aim to identify recurrent chromosomal rearrangements that are relevant for the progression of cancer.

Standardized Nanoarray PCR for Gene Expression Profiling of Lung

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Clinical deployment of multivariate transcript abundance assays used for cancer management is prevented by lack of qPCR standardization. Standardized NanoArray PCR (SNAP) combines the rigorous QA/QC of Start-PCR competitive internal standards with the simplified nanofluidic PCR workflow of the BioTrove OpenArray™. The lung prognostic assay being developed will measure 21 gene transcripts in cDNA synthesized from FPET isolated RNA. The poster will present the initial PCR primer pair development and a highly accurate method for measuring the molar ratios between native template and competitive internal standards. A proof-of-principle demonstration of SNAP work flow suggests that the method will exceed expectations for accuracy and precision.

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), protein-protein interactions, modulated catalytic activities, and translocations within a response network of interacting species to generate specific biological outputs. On the basis of 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, now allows us to assess the 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 tissue-derived proteins for later readout by gel and mass spectrometry (MS)-based methods. Proposed improvements to our technology (isotope-coded samples for MS) will also allow for enhanced quantitative abilities to assess variations in levels of these modifications between different samples in multiplexed approaches. Our research with these compounds indicates that the probes are specific for cysteine sulfenic acid; an initial "burst" of R-SOH formation is observed within the first 10 minutes after addition of tumor necrosis factor alpha (TNF-α) to HEK-293 cells and is sustained for at least 30 minutes. Two approaches have been explored to identify labeled proteins in different systems, including HEK-293 cells stimulated with TNF-α and NIH3T3 cells stimulated with platelet-derived growth factor. Both of these approaches utilize a version of our probe containing a biotin tag and purification of the biotinylated proteins by affinity enrichment with streptavidin beads and stringent washing. The first, more targeted approach probes the affinity-enriched samples for the presence of specific proteins using Western blots, whereas the second approach yields proteome-level information and the identity of the sensitive cysteine(s) on each protein with traditional liquid chromatography MS/MS analyses. A growing list of oxidized proteins that we have identified includes a number of signaling-relevant proteins, including phosphatases, kinases, and proteins involved in calcium-dependent signaling; 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.

This work was supported by CA112145 (L.B.P., Principal Investigator; J.S.F, L.W.D, and S.B.K., Co-Investigators).

IMAGEtags (Intracellular Multiaptamer Genetic Tags) for Real-Time Imaging of Gene Expression

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Current reporter systems for imaging gene expression and for tracking cells in vivo

involve the expression of proteins such as GFP or luciferase. These reporter systems have been the basis of a revolution in cellular imaging capabilities. However, they have several disadvantages, including very slow response times and the high energy cost for synthesizing and maintaining reporter protein levels. These limitations prevent the use of reporter systems for detecting changes in gene expression in real time and can deplete the energy reserves of normal cells in vivo, such as stem cells that need to proliferate and populate a niche in damaged tissue where oxygen and nutrient concentrations are likely minimal. The purpose of this study is to establish an alternative reporter system that can be used for a real-time, in vivo imaging method for tracking stem cells. This reporter system involves the use of **I**ntracellular **M**ulti**A**ptamer **GE**netic tags (IMAGEtags), which are strings of aptamers that are expressed from a promoter of choice. To establish this reporter system, the available RNA aptamers for tobramycin, neomycin, and theophylline are being used, and their targets are labeled with fluorescent molecules for imaging. Vectors that express multiple repeated aptamer sequences have been developed to increase the aptamer concentration in the cell and thus also increase signal-to-noise for imaging. To further increase signal-to-noise, aptamer targets are separately labeled with fluorescent FRET pairs to decrease the background noise. The IMAGEtag system is currently being developed for reporting gene expression changes in yeast and cultured mammalian cells. Once optimized for imaging in cell culture, the IMAGEtag reporters will have many possible imaging applications, including real-time detection of changes in gene expression and mRNA processing, tracking cell movements in vivo, and tracking RNA movements in cells.

Application of Multiplexed BioCD Assays for Acute Lymphocytic Leukemia

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The BioCD is an emerging label-free assay technology with the potential for high multiplexing and high throughput to screen for many analytes across many samples simultaneously [Wang et al. 2007]. The basis of the BioCD technology is rapid optical interferometric scanning. Interferometry is the most sensitive and quantitative means of direct optical detection, is faster than fluorescence, has better signal-to-noise, and requires no labels, which is essential for multiple analyte detection. We are currently applying the BioCD for the prognosis of cancer. The goal is to assay multiple biomarkers across a large cohort of patients to predict patient outcome in response to chemotherapy.

Acute lymphocytic leukemia (ALL) comprises a heterogeneous group of lymphoid malignancies with distinct molecular and phenotypic characteristics. The incidence in adults is 1/100,000 persons per year, and the prognosis worsens gradually with age. At the present time, therapy for adult ALL patients is stratified based only on the presence of the Philadelphia chromosome (Ph), a phenomenon that occurs in 15% to 25% of

patients. The rest of patients (Ph negative) are generally treated with standard intensive chemotherapy programs that do not account for the patient heterogeneity. Although initial complete remission rates are very high with these chemotherapy programs, overall survival (OS) is only 35%, with some subgroups having excellent long-term outcomes (OS ~70%) and others rarely surviving for more than 1 year. From these data, it is obvious that risk-adapted therapies, and the molecular tools to define them, are needed for adult ALL patients.

Recently, a lack of expression has been observed in a triad of cell cycle regulatory genes, composed of p73, p15, and p57KIP2, that occurs in 20% to 30% of ALL patients and confers poor prognosis independently of other variables to patients carrying this abnormality. Many other candidate markers may also correlate with disease, but screening and validation of these markers will require hundreds of assays. This scale is outstripping conventional assays, demanding the high-throughput, automated approach of the BioCD.

The relevance of this research to public health is the expansion of the marker and sample base to establish stronger clinical confirmation of the prognostic value of inactivation of a molecular pathway in patients with standard-risk ALL and to establish the BioCD as a novel, high-capacity resource for diagnostic and prognostic applications for cancer.

Wang X., Zhao M., Nolte D.D. Common-Path Interferometric Detection of Protein on the BioCD. *Appl Opt* 46:7836-49, 2007.

Characterization and Cell Binding Properties of Novel Magnetic Nanoparticles for High Throughput Magnetic Sorting

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The aims of this project were to develop high-resolution magnetic reagents for use in the quadrupole magnetic sorter (QMS), a flow-through immunomagnetic separation system that can be used for the enrichment of circulating tumor cells (CTCs) in blood as well as in other biological fluids and tissues. The criteria by which magnetic reagents achieve successful rare cell enrichment include high magnetic susceptibility with narrow particle size distribution, high-density attachment sites for antibodies, and a low level of nonspecific binding to nontargeted cells. Columbus NanoWorks (CNW) has focused on producing a reliable product with the aforementioned specifications in a scaled-up manner.

Our approach has included the development of a novel (patented) self-assembly reaction that uses magnetic iron cores coated with a fatty acid to retain a high magnetic

susceptibility. Self-assembly uses hydrophobic dextrans to coat the cores by a process that may involve interdigitation of fatty acid groups associated with the magnetic cores. As one of its milestones, CNW has shown that greater than 99% of the self-assembled particles were dispersable in water, with fatty acids of chain lengths less than 12 carbons giving rise to materials with shelf lives of less than 6 months. Particles made with long chain (C18) hydrocarbons had shelf-life stabilities of greater than 6 months. This is based on studies using magnetic field-flow fractionation (MgFFF) and inductively coupled plasma-optical emission spectroscopy (ICP-OES), which we have shown previously to be useful tools for evaluating magnetic nanoparticles. Neutravidin was then attached to the surface of the dextran coat via carboxyl groups that had been derivitized with EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride]. Conjugation efficiency was measured by standard HABA binding assays as well as by a novel technique developed by CNW using microarray technology.

Results from these studies indicated a coupling efficiency of approximately 100 umoles protein per mg Fe. Cell binding assays were also performed with peripheral blood mononuclear cells (MNCs) to show that particles had a high affinity for the antigen targeted, while maintaining a low nonspecific binding to cells not targeted. MNCs were obtained after a Ficoll-Hypaque gradient enrichment and were resuspended in buffer containing phosphate-buffered saline supplemented with 0.5% HSA and 2mM EDTA. A portion of MNCs were incubated with a biotinylated primary antibody directed toward the CD45 antigen. After a washing step, the MNCs were incubated with neutravidin coupled with CNW magnetic nanoparticles. The remaining MNCs were incubated with neutravidin-coupled particles to determine the percentage of nonspecific binding of the particles to the cells without a primary antibody present on the surface of the cells. Both cell suspensions were run through Miltenyi MS+ columns to determine the percentage of cells magnetically bound for both specific and nonspecific binding assays. Cell counts were performed on both the negative and positive fractions that were eluted from the MS+ columns and showed that 100% of the cells that were specifically labeled were retained in the magnetic fraction. Nonspecific binding studies indicate that roughly 6.7% of cells were retained within the magnetic fraction. Additional steps are being pursued to reduce the amount of nonspecific binding of particles to nontargeted cells by adding a blocking step in the incubation phase of the labeling process and optimizing the blocking strategy of reactive functional groups on the surface of the particles that have not bound protein in the conjugation process.

Using Synthetic Dosage Lethality To Find Novel Anticancer Targets

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A significant challenge in cancer therapy is selectively killing cancer cells while not harming normal cells. We have taken a novel approach to identify genes that are

essential only in cancer cells. Our scheme is based on synthetic dosage lethality (SDL), which defines a genetic interaction where a nonessential gene becomes essential only when a second gene is overexpressed. This normally nonessential gene becomes a target for drugs to inactivate it. Such drugs should selectively kill cancer cells and not cells from normal tissue. Since it is commonly found that cancers often increase expression of specific genes due to translocations or gene amplifications, uncovering SDL interactions may identify specific targets for selective drug therapy. Since many essential functions that are deregulated in cancer are conserved between yeast and humans, yeast can be used as a surrogate to identify SDL interactions. By creating yeast strains that overexpress the yeast orthologs of genes overexpressed in tumors, we have been searching for SDL interactions among the 4,827 nonessential yeast genes. We are using an innovative new method that we developed called ploidy ablation.

Rad9 is overexpressed in 33% of non-small cell lung carcinomas (NSCLC) and plays a role in cell cycle checkpoint control, DNA repair, and genome stability. We performed an SDL screen with the yeast ortholog of human Rad9 (*DDC1*) by transferring a copper-inducible *DDC1* expression plasmid into every strain of the yeast gene disruption library. *DDC1* showed a strong SDL interaction with *cik1*. Cik1 is a binding partner of the kinesin motor protein Kar3 and functions in the assembly and/or maintenance of the mitotic spindle. We identified NuMA as the functional human ortholog of Cik1. Like Cik1, NuMA is important for proper mitotic spindle assembly and regulation of microtubule dynamics and cross-linking. To show that the *DDC1-cik1* SDL interaction is conserved in human cells, we exogenously induced overexpression of human Rad9 in Hela cells in which NuMA was downregulated by shRNA. Our results indicate that cells with reduced levels of NuMA are highly sensitive to increased Rad9 levels. In contrast, control cells were unaffected by increased Rad9. We next tested the effect of NuMA downregulation in lung cancer cells by transducing two different NSCLC cell lines (A549 and SKLU) with a lentivirus containing a GFP reporter or a GFP reporter + NuMA shRNA. The A549 cells were sensitive to NuMA downregulation, whereas the SKLU cells were not. We went on to test four breast cancer cell lines and five prostate cancer cell lines typed for increased *RAD9* expression. However, they do not show decreased viability upon NuMA knockdown. Despite this result, we believe that our fundamental approach is sound and that we can still identify conserved genetic interactions in human cells.

We performed a second SDL screen using a constitutively active oncogenic mutation of *ras* (G12V) constructed at the conserved position in yeast *RAS1* (G19V). Expression of *ras1V19* in every strain of the yeast gene disruption library using a copper-inducible promoter resulted in 230 deletion strains showing significantly repressed growth. The list includes many genes previously identified as having genetic interactions with *ras1* or *ras2*. In addition, the list is enriched for GTPases and kinases, suggesting that components of signaling cascades can partially compensate for expression of the oncogenic allele. Finally, the list is enriched for components of the vesicle trafficking pathways, including genes involved in autophagy, which is downregulated in cells expressing activated ras.

Developing an Integrated Cell Culture Platform for In Vitro Monitoring of Cancer Cell Metabolism

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The physiological or pathophysiological state of cells and organs is reflected in their energy metabolism. The energy metabolism of cancer cells differs strikingly from that of normal tissue; glycolysis and subsequent lactate production are much more prominent in cancer cells, even under aerobic conditions. Increasingly, therapeutic strategies for cancer treatment are designed to target metabolic deviations, making levels of energy metabolites such as glucose or lactate potential correlates of drug efficacy. The goal of this project is to develop a lab-on-chip platform for in vitro monitoring of the effects of drug candidates on the energy metabolism of cancer cells. The proposed platform will intimately integrate small groups of glioma (brain cancer) cells with miniature glucose biosensors in a microfabricated device in which the cellular microenvironment can be precisely defined and easily modulated. This platform will be used to (1) establish tumor-mimicking microenvironment conditions (e.g., hypoxic, acidic, nutrient-limiting) in glioma cell cultures, (2) challenge the cells with pharmacological inhibitors of kinase signal transduction pathways central in energy metabolism, and (3) monitor local extracellular glucose levels. Therefore, the cell culture/biosensor platform will connect the tumor microenvironment, energy metabolism, and anticancer drug efficacy and will help delineate the conditions that make cancer cells more susceptible to therapy. Because work on the proposed project has commenced only recently, this poster presentation will highlight some of the novel approaches developed in the PI's laboratory for miniaturizing cell culture systems and integrating biosensors with small groups of cells. Extracellular detection of model analytes secreted by small groups of cells or single cells will be demonstrated.

Targeting Vascular Endothelial Caveolae To Pump Antibodies Into Tumors for Enhanced Specific Imaging and Therapy

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Cancer and other disease biomarkers and targets may provide key diagnostic, prognostic, and therapeutic opportunities, including clinical trial surrogates and screens for patient treatment assignment. Drugs, gene vectors, and nanoparticles may benefit greatly from improved in vivo delivery through homing to specific disease biomarkers. Yet in vivo barriers limit access to most disease targets, including cancer. We have developed novel systems biology approaches that integrate nanotechnology-based subcellular fractionation, quantitative organellar and subtractive proteomics,

bioinformatic interrogation, antibody generation, expression profiling, and various in vivo imaging modalities to quickly identify and validate target candidates for preclinical and clinical testing.

Analysis of rodent and human tumor samples has been compared to focus on clinically meaningful targets to understand model relevance to human disease. Tissue and tumor microenvironmental influences on endothelial cell expression are extensive. We have developed a quantitative proteomic analysis using a new spectral intensity index to identify proteins specific to tumor versus normal endothelium as well as concentrated in caveolae, many of which are confirmed by immunoelectron microscopy. Novel targets in caveolae enable antibodies to penetrate deep into solid tumors and single organs and were utilized to improve tissue-specific imaging and treatment.

Our recent findings reveal that caveolae not only express tissue-specific proteins but also rapidly and actively pump specifically targeted antibodies and nanoparticles across the endothelial cell barrier and into the tissue interstitium. This targeted penetration of the antibody into the tissue (transcytosis) occurs within seconds to minutes in normal tissues and within minutes to a few hours in various tumor models tested. Such pervasive access inside the tumor improves the efficacy of radioimmunotherapy in destroying both stromal and tumor cells and in treating a wide variety of solid tumors. Various rodent tumors are imaged rapidly and specifically after intravenous injection of specific monoclonal antibodies. Because the antibody not only targets the tumor endothelium but also crosses it to penetrate deeply throughout the tumor tissue, this radioimmunotherapy effectively destroys tumors in rodent models to increase survival and even apparently cure the disease. Blood flow stops selectively within the tumor within 24 hours and after most of the injected antibody has been pumped inside the tumor, where it is then trapped to kill not only tumor cells but also stromal cells that surround the tumor and that constitute the critical tumor microenvironment. This tumor penetration and treatment efficacy require the presence of caveolae on the tumor endothelium and occurs only when the antibodies deliver their attached radionuclides directly and rapidly into the tumors.

So far, we have tested breast, lung, ovarian, prostate, and liver tumors with similar success. We have antibodies that recognize this target in humans. A wide variety of human tumors express this novel accessible endothelial cell surface target in a pattern quite similar to that of the rodent models. We are testing different radionuclides to evaluate which one is most effective. Toxicology studies are ongoing. Our antibody appears to be useful in tumor-specific imaging as well as in treating a wide variety of solid tumors.

This work represents a novel discovery, validation, and delivery strategy that so far provides promising and unprecedented results. Testing in humans is now necessary to understand limitations and possibilities for clinical translation to imaging and treating human disease.

Single-Molecule Genome Analysis of Oligodendroglioma

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Technological advances broaden our understanding of genomes, and new approaches employing single-molecule analytes offer unique advantages for the discovery and characterization of genomic alterations complementing discernment of single nucleotide polymorphisms (SNPs) and copy number polymorphisms (CNPs). CNPs commonly represent genomic events (polymorphic or aberrant), such as amplifications and deletions usually found by DNA hybridization to either chip-synthesized oligonucleotide or spotted arrays. Although such measures of genomic alteration are relatively comprehensive, CNPs effectively flag broad classes of genomic alterations but do not readily discern genomic structural alterations embodied as translocations, gene-fusion events, insertions, or rearrangements—both large- and small-scale (subgenic). Consequently, physical mapping by fluorescence in situ hybridization or PCR laboriously characterizes a subset of findings providing structural detail on a per CNP basis. The optical mapping system we have developed exploits the detection range afforded by restriction fragment length polymorphism analysis but with high throughput and single-DNA molecule precision engendered by automated fluorescence microscopy. Optical mapping enables the construction of genome-wide physical maps (consensus maps) from ensembles of *ordered*, single-DNA molecule restriction maps developed from genomic sources, obviating clone libraries, PCR, and hybridization. Comparison of an optical consensus map against a reference map reveals structural alterations as "differences," in the form of novel restriction sites (missing cuts or extra cuts [MCs or ECs]) or "indels" (insertions or deletions), which are statistically assessed in part based on the number of single-DNA molecule optical maps collectively represented by the consensus map. Since high-resolution restriction maps intrinsically reveal genome structure, elusive differences such as indels, inversions, and balanced translocations are economically discoverable and physically characterized.

The Laboratory for Molecular and Computational Genomics has used the optical mapping system for the discovery and high-resolution characterization of genomic aberrations in oligodendroglioma tumor samples and several commonly investigated breast cancer cell lines. This work has identified a number of novel candidate genomic loci associated with tumor formation and establishes a new paradigm for the elucidation of genomic aberrations in cancer genomes.

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 animals due to 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 screening technique could indicate the contributions of proteins to the steps of primary tumor growth, intravasation, and lung colonization. The first aim of this project is to produce pools of cells that express or suppress selected proteins. The second aim is to evaluate detection technologies for measuring construct distributions in a pool. The third aim is to determine the appropriate formation of pools to be screened. The fourth and fifth aims are to perform an initial screen and to validate candidates identified in the screen. Samples will be prepared by isolating viable tumor cells from the primary tumor, blood, and lungs of animals carrying breast cancer tumors. The likely end use of the data is the determination of whether specific genes contribute to tumor formation, intravasation, or lung metastasis. Cell lines will be used to produce pools of cells that express or are suppressed for expression of particular proteins. Endogenous variations in the cell lines can affect evaluation of the contributions of the targeted proteins to metastasis. Preliminary tests of both protein overexpression and suppression have been performed with pools of 10 transductants, as reported below.

To evaluate overexpression of selected proteins, we evaluated rat MTLn3-ErbB3 and human MDA-MB-231 tumor cells. Eight specific open reading frames (ORFs) were subcloned into retroviral expression vectors, retrovirus was produced, and transductants were generated. Detection of individual constructs using quantitative polymerase chain reaction (qPCR) with primers for the vector and the ORF was found to be adequate for evaluation of relative proportions. The transductants were pooled (together with two control empty-vector transductants), the MTLn3-ErbB3 pool was injected into the mammary fat pads of five mice, and the MDA-MB-231 pool was injected into the tail veins of four mice. Two mice carrying MTLn3-ErbB3 tumors showed tumor cells in the blood and lungs and were further analyzed. However, there were no significant differences in the proportions of transductants in the primary tumor, blood, or lungs. Two of the animals injected with MDA-MB-231 showed significant numbers of lung metastases and were further analyzed. Transductants expressing epiregulin, a ligand for epidermal growth factor receptor, showed a relative increase in proportion in lung metastases. This result is consistent with published data indicating that epiregulin contributes to the metastatic efficiency of MDA-MB-231 tumor cells.

To evaluate the suppression of selected proteins, pGIPZ lentiviral short-hairpin RNA constructs have been used. Because the selection of constructs targeting human genes was much greater than that targeting rat genes (MTLn3 is derived from a rat mammary adenocarcinoma), only MDA-MB-231 was evaluated. Each construct has a unique 60-base-pair barcode sequence, which was used for detection with qPCR. MDA-MB-231 cells were transduced, and pools of 10 transductants were generated. The pools were injected into both mammary fat pads and tail veins. No differences in proportions of cells injected via tail vein were detected. However, in cells growing in mammary fat pads, a dramatic enhancement of the proportions of cells in which glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) is suppressed was found. This result is consistent with published data indicating that GAPDH can enhance apoptosis. The GAPDH result is being pursued further by using additional suppression constructs.

In summary, preliminary tests with pools of 10 transductants confirmed that methods for the generation and detection of transductions have been established. Current work is focusing on generating and testing pools of 25 transductants.

Genome-Wide DNA Methylation Maps of Follicular Lymphoma Cells

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Follicular lymphoma (FL) is a common type of non-Hodgkin's lymphoma (NHL). Recent molecular studies have established that transcriptional silencing of tumor suppressor genes, associated with DNA methylation, is a common epigenetic event in FL. However, our knowledge of genome-wide distribution of DNA methylation and how it relates to histone modifications and gene expression in FL remains limited. In this study, we developed a large-scale bisulfite sequencing approach for analyzing genome-wide DNA methylation patterns by combining bisulfite treatment of genomic DNA with single-molecule-based parallel sequencing. The methylated DNA fragments from lymphoma cells were isolated and enriched using the methylated CpG islands (CPGIs) recovery assay (MIRA), which is based on the high affinity of the MBD2b and MBD3L1 protein complex for methylated DNA. The methylation-enriched genomic DNA was treated with bisulfite and amplified by polymerase chain reaction (PCR) with primers designed to amplify DNA molecules carrying bisulfite-modified adapter sequences at both ends. The PCR amplicons were sequenced with the Roche-454 GS FLX sequencer. We generated 516K mappable bisulfite sequencing reads (approximately 100 Mb of data) with an average read length of 143 base pairs (bp) (range, 20-444 bp). Among the 516K bisulfite sequences, approximately 436K reads (85 percent) were uniquely mapped to the human genome (NCBI build 36.1). The total number of bases covered on the genome was 18.6 million, including 5.4 million cytosines and 739,260 CpGIs. We identified 13,524 clustered genomic regions that were mapped by multiple bisulfite sequencing reads (>5). These methylation hot spots were associated with 4,508 CpGIs, including CPGIs associated with several large gene clusters, such as Hox gene clusters and protocadherin gene clusters. The genome-wide DNA methylation patterns correlated well with transcriptome data from Illumina BeadArray and ChIP-on-chip analyses of genome-wide histone modifications such as trimethyl-H3K27, dimethyl-H3K9, and trimethyl-H3K4. In addition, using a modified ChIP-on-chip assay (ChIP-chop-DMH), the methylated promoters directly associated with specific histone modifications were identified. These integrated approaches have led to the discovery of novel targets for aberrant DNA methylation in the lymphoma epigenome and provide a comprehensive analysis of the DNA methylation sequence composition and distribution.

The new generation of high-throughput bisulfite sequencing will provide digital profiles of aberrant DNA methylation for individual human cancers and offers a robust method for the epigenetic classification of tumor subtypes.

Free-Solution Conjugate Electrophoresis Using Polyamide "Drag Tags" and Microchip Electrophoresis for Mutation Detection and Scanning Assays

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Colorectal cancer (CRC) is the third leading killer among all cancer-related diseases in the United States. Although a number of biomarkers and technologies have been evaluated for the management of this disease, few have emerged for use by clinicians in battling CRC, and the predominant screening methods still consist of monitoring blood in the stool and/or colonoscopy. Our efforts are focused on developing a microchip electrophoresis platform that can monitor the absence or presence of molecular biomarkers that originate from nuclear DNA and that provide diagnostic and/or prognostic information about CRC.

The molecular assays being investigated require high-resolution electrophoresis for reading out the results. DNA is conventionally separated by electrophoresis in a viscous sieving matrix using fused silica capillaries or microchips (microchip-based electrophoresis [μ-CE]). μ-CE is particularly attractive because it can be integrated to front-end processing steps to provide automated sample processing in a closed architecture that is free from contamination issues and is envisioned for potential point-of-care testing applications. The matrix is loaded into the device using high pressure, which can be time and energy intensive. To compound the problem, the gel must be reloaded between every run, which is expensive. The elimination of sieving matrices and the development of free-solution electrophoresis to sort DNA would drastically decrease the cost associated with molecular assays, simplify system setup, and reduce run time. Because both the charge and the friction scale linearly with chain length, the electrophoretic mobility of DNA in free solution does not change with increased chain length.

In order to run free-solution electrophoresis of DNA, the DNA must be conjugated to an uncharged perturbing entity or "drag-tag," producing free-solution conjugate electrophoresis (FSCE). In this presentation, FSCE with μ-CE devices, fabricated in polymers with replication technology, is being used for several molecular diagnostic assays, including ligase detection reaction (LDR), for scoring the presence of known point mutations in *K-ras* oncogenes and an Endo V/LDR assay, a mutation scanning-based assay to score the presence of sporadic p53 mutations. In addition, microsatellite instability (MSI) is also being evaluated using μ-CE FSCE. The analysis of MSI is undertaken with a panel of markers from nuclear DNA, which are amplified by polymerase chain reaction and subsequently analyzed via FSCE. Comparisons of

electrophoretic mobilities of diseased tissue versus normal tissue provide an indication of MSI status and can be used as a genetic prognosticator for determining effective therapies for treating CRC patients. The drag-tags are a series of poly-N-substituted glycines (peptoids) with *N*-methoxyethylglycine (NMEG) side groups of various lengths. Linear NMEGs are the most economical drag-tags because they can be chemically synthesized by solid-phase synthesis and easily purified to complete monodispersity (a requirement for FSCE applications) using reverse-phase high-pressure liquid chromatography. Linear NMEG drag-tags apply an appropriate amount of added drag to the DNA molecules to resolve the genotyping fragments in polymethylmethacrylate-based microchips, which possess low electro-osmotic flows. A heterobifunctional, maleimide-based linker is used to conjugate the drag-tags to thiolated DNA primers used for the mutation scanning and detection assays [1].

We have successfully demonstrated the conjugation of polyamide drag-tags onto a set of four ligase detection reaction (LDR) primers designed to probe the *K-ras* oncogene for mutations that are highly associated with CRC and the simultaneous generation of fluorescently labeled LDR/drag-tagged products in a multiplexed, single-tube format with mutant:wild-type ratios as low as 1:100, respectively [2].

1 Meagher R.J., Coyne J.A., Hestekin C.N., et al. Multiplexed p53 Mutation Detection by Free-Solution Conjugate Microchannel Electrophoresis With Polyamide Drag-Tags. *Anal Chem* 79:1848-54, 2007.
2 Sinville R., Coyne J.A., Meagher R.J., et al. Ligase Detection Reaction for the Analysis of Point Mutations Using Free Solution Conjugate Electrophoresis in a Polymer Microfluidic Device. *Electrophoresis*, in press.

Novel Detector for Molecular Imaging Studies

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Microfluidic microreactor chips are a promising, emerging technology that can be used for the synthesis and study of new molecular imaging probes and biological processes. They can contain a variety of microcircuitry and microwells and are capable of manipulating with excellent precision nanoliter samples of reagents, solvents, and biological specimens. Adding the ability to quantify and image low amounts of radioactivity on a microfluidic chip can provide researchers with a tool to study new imaging probes as well as molecular processes with radiolabeled probes in a controlled in vitro environment.

Current in vivo biological imaging systems such as microPET are capable of quantifying the spatial distribution of radiolabeled probes but have detection limits on the order of 1 to 10 nCi or higher and spatial resolution, at best, on the order of 1 mm^3. Well counters

in turn can detect radioactivity at low picoCurie levels; however, samples must be prepared inside a test tube and counted sequentially. In addition, cell uptake quantitation by well counters typically requires terminal experiments in which the cells are destroyed by lysis. As a result, although well counters can be used in combination with traditional methods (96 well plates) for terminal in vitro studies, a technology that could repeatedly quantitate uptake of radiolabeled probes in intact cells in the microfluidic culturing environment would greatly facilitate the study of new molecular imaging probes as well as biological processes at the cellular level.

The goal of our effort is to create a new integrated microfluidic device that includes a charged particle imaging detector coupled to the microfluidic chip for detection and imaging as well as quantification of trace amounts of radioactivity on the microfluidic chip. This device will employ direct detection of beta-particles emitted from the fluid inside the microfluidic chip with a position-sensitive avalanche photodiode (PSAPD). This approach will allow quantitative detection of low levels of radioactivity along with spatial as well as temporal count-rate distribution. The PSAPD is a 14 mm x 14 mm silicon device, manufactured by us at RMD, that operates at a gain of about 1,000, with low noise and high intrinsic spatial resolution (~0.3 mm-FWHM). The effort is being carried out in collaboration with Imaging Sciences Group at the Crump Institute for Molecular Imaging at UCLA.

Nucleosomal Biosensor for Nuclear Hormone Receptor Ligands

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We propose an innovative approach to identify functional ligands of nuclear hormone receptors (NHRs). NHRs modulate transcription by binding small lipophilic ligands and have a profound impact on normal cellular function and development. Aberrant NHR function occurs frequently in cancers, which has prompted the development of therapeutic ligands targeting NHRs (e.g., tamoxifen in breast cancer, ATRA in promyelocytic leukemia). New natural or synthetic ligands for NHRs are likely to include useful anticancer agents. Most current screening strategies for NHR ligands either analyze binding to receptors or use cellular models to screen for function over hours to days. Our project seeks to develop a platform that measures binding and function of NHR ligands concurrently in a span of seconds.

The focus of our work is the construction of a nucleosomal biosensor for estrogenic ligands. A key feature of this biosensor is a nucleosome in which fluorophore-tagged DNA containing an estrogen response element (ERE) is wrapped around histones and serves as a fluorescent sensor of initial steps of the in vitro transcriptional response. The fluorophores are positioned so that in the (tightly wrapped), transcriptionally inactive state, energy is transferred from the donor fluorophore (Cy3) to the acceptor fluorophore (Cy5) (fluorescence resonance energy transfer [FRET]). Activation of bound estrogen

receptor by ligand is predicted to initiate unwrapping of nucleosomal DNA from core histones leading to loss of FRET. Our preliminary work has demonstrated success in monitoring nucleosomal integrity in real time via FRET changes on either a single molecule or population basis under varying salt conditions. To adapt this system to serve as a biosensor of estrogenic ligands, nucleosomal DNA was constructed based on GUB [1], 601 [2] and on 612 [3] nucleosomal positioning elements. The substitution of wild-type ERE (5'-GGTCAAGGTGACC-3') or mutant (5'-TCCCTAGTCTCCA-3') estrogen receptor response motifs in these nucleosomal DNAs was optimized according to published models of AA/TT phasing in nucleosomes. The wild-type (but not the mutant) motif-bearing DNAs were shown to bind to estrogen receptor in electrophoretic mobility shift assays, and gel shift assays also confirmed the ability of each of these DNA constructs to combine with histones to form nucleosomes.

Although initial placement of fluorophores in positions 1 and 75 led to fluorophore approximation within the Forster radius and successful FRET signal, this configuration proved to be an inconsistent indicator of nucleosomal formation. This was most likely because the fluorophore positioned at the 5'-end of the DNA was more susceptible to nonspecific DNA fluctuation and unwinding. Optimized positioning is under way using incorporation of alternating 2-laser excitation of nucleosomal DNA to identify and subtract fluorescence "blinking" artifacts. Successful completion of this project will make it possible to visualize the action of estrogen or estrogen-like drugs at the levels of single and pooled nucleosomal DNA. This methodology could be adapted for screening biologically relevant molecules in chemical libraries to facilitate the identification of new drugs that mimic or block estrogen. Validation of this proof of principle for biosensor function will set the groundwork for rapid in vitro functional screening of libraries of putative nuclear hormone receptor ligands.

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2. Gansen A., Hauger F., Toth K., Langowski J. Single-Pair Fluorescence Resonance Energy Transfer of Nucleosomes in Free Diffusion: Optimizing Stability and Resolution of Subpopulations. *Anal Biochem* 368:193-204, 2007.
3. Ibid.

Enzymatic Bioluminescence Assay for MicroRNA Quantification

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The goal of this project is to develop a novel bioluminescent enzymatic assay for high-throughput detection and quantification of microRNA (miRNA). This approach is based on the detection of inorganic pyrophosphate (PPi) released during DNA polymerase

reaction, which utilizes a specially designed long oligonucleotide template with sequence complementary to the microRNA target of interest. The hybridization of miRNA as a primer to the template and the subsequent primer strand extension give rise to the nucleotide incorporation by polymerase and the generation of PPi molecules, with the amount proportional to the miRNA target concentration. The luminescence detection of PPi is performed by a multienzyme reaction system. The assay is designed to convert PPi to ATP by ATP-sulfurylase, which provides energy for luciferase to oxidize luciferin and produce light. In this study, the characteristics of different DNA polymerases were investigated for their specificities and efficiencies of utilizing miRNA as a primer. Experimental results show that thermophilic DNA polymerase has a high specificity for miRNA target recognition and can discriminate miRNAs with two to three mismatched bases. The assay has a high dynamic range and provides linear readout over different miRNA concentrations, with detection sensitivity at the femtomolar range. The signal detection is performed by a commercial luminometer, either in a tube format for fewer samples or in a 96-well microplate for simultaneous analysis of multiple samples. The advantages of the assay are that it uses miRNA directly without ligation or reverse transcription to cDNA, which greatly simplifies the procedure, and that it can be accomplished in real time at room temperature without the thermal cycling of polymerase chain reaction (PCR). Compared with current RT-PCR, microarray, and hybridization-based miRNA detection methods, this assay provides a simpler, faster, and less expensive platform to be used in life science research, drug discovery, and clinical diagnosis.

Quantitative Analysis of Protein Phosphorylation Patterns

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Our work toward IMAT R33 CA103235 is directed at developing quantitative assays for profiling tyrosine kinase activities and inhibitor sensitivities that are compatible with patient material in a clinical setting. This effort was stimulated by the recent development of potent inhibitors, such as the ATP analogs imatinib, dasatinib, and nilotinib, that target the onocogenic kinase BCR-ABL, expressed as a result of the t(9; 22) reciprocal translocation characteristic of chronic myelogenous leukemia and some acute lympohoblastic leukemias Typically, imatinib induces remission, but patients may develop resistance, leading to recurrence. Our efforts are concerned with delivering a robust assay, amenable to high-throughput screening of novel BCR-ABL inhibitors, to guide therapy and monitor patient response.

Our general approach has been to incubate cell extracts with immobilized peptide substrates in the presence of ATP, with and without kinase inhibitors. Specific tyrosine phosphorylation is quantitated by one of three methods: (1) internal standard-corrected antibody labeling using a novel application of Luminex beads, (2) chemiluminescence

and chemifluorescence detection in optimized acrylamide hydrogel formats, and (3) label-free solid-phase detection by MALDI-TOF of acid and photocleavable substrates in hydrogels. In addition, isotope-labeled phosphoproteomic profiling of whole-cell extracts was performed to drive the discovery and potential development of novel targets.

With the aim of improving the resolution of diagnostic tests by providing more than one marker of patient progress, most methods are multiplexed to measure multiple kinase activities in a single experiment. Assays reliably measure BCR-ABL and Src activity and determine the IC_{50} of inhibition by imatinib and other drugs. Physicians can use data from these assays to make optimal choices for a first-line drug in newly diagnosed patients and optimize response to acquired resistance.

Imaging Gene Expression in Single Living Cells With Quantitative Molecular Beacons

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Variations in gene expression are commonly considered the major determinants for dictating cell behavior. Accordingly, methods to measure gene expression, such as reverse-transcriptase polymerase chain reaction (RT-PCR) and DNA microarrays, have proven to be invaluable in understanding cell regulatory processes and disease mechanisms. However, these methods generally provide only the relative change in gene expression for a population of cells. Under many circumstances, it is the aberrant behavior of only a few cells or the stochasticity of RNA expression within a population that leads to disease evolution. Although laborious techniques such as single-cell RT-PCR can provide a closer look at RNA transcripts within single cells, the RNA must still be extracted from the cells and processed prior to analysis. The shortcomings associated with RNA handling have been highlighted in several recent studies, which have shown that up to 90% of transcripts can be lost during RNA purification, cDNA synthesis, and other steps required for PCR. Therefore, there clearly remains a need to develop new methods that can be used to measure gene expression in single living cells more efficiently and more accurately.

Recently, we developed a novel molecular imaging probe, termed "quantitative molecular beacons" (QMBs), that can be used to image RNA expression in single living cells. QMBs were synthesized by conjugating molecular beacons (hairpin-forming antisense oligonucleotide probes that are labeled with a reporter fluorophore on one end and a quencher on the other) to an optically distinct "reference" dye (e.g., fluorescent nanoparticle/macromolecule), which remains unquenched regardless of the conformation of the probe [1]. The inclusion of a reference dye provides several important advantages compared with conventional (i.e., nonconjugated, molecular beacons). For example, we have shown that the ability of the reference dye to prevent

nuclear localization (via size exclusion) results in significant improvement (approximately threefold) in signal-to-background and sensitivity. Supporting experiments showed that this is because nuclear localization is associated with protein-MB interactions that lead to false-positive signals. Another significant benefit of the unquenched reference dye is that it allows transfection efficiency to be monitored. This significantly reduced false-negatives by allowing for the differentiation between untransfected cells and cells with low gene expression levels, which was not previously possible.

In recent work, we found that QMBs could be efficiently delivered into live cells through microporation (>90% transfection, >85% viability) and that the high-throughput analysis of RNA expression was possible with flow cytometry [2]. We are now continuing to develop our QMB technology such that RNA expression can be rapidly measured in cells isolated from biopsies, fine-needle aspirations, and effusion samples with high sensitivity and specificity even at low cellularity. Our goal is to develop a simple assay that can be adopted for rapid onsite cytological evaluation to assist with therapeutic and surgical decisions.

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Developing Imaging Probes for Tumor Stroma-Associated Fibroblast Activation Protein

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

FAP is a type II membrane-bound glycoprotein belonging to the serine protease gene family and is known to have exodipeptidyl peptidase activity that is extremely similar to

the activity of dipeptidyl peptidase-IV (DPP-IV). DPP-IV, unfortunately, circulates in the bloodstream, making in vivo imaging of FAP challenging. Recently, we found that endopeptidase activity of FAP is a better choice for probe design, because DPP-IV lacks endopeptidase activity. Screening a series of peptide substrates against FAP has shown that Gly-Pro is an excellent substrate for endopeptidase cleavage. Proteolytical activation of imaging probes with various repeats of Gly-Pro suggests that single Gly-Pro is as good as four Gly-Pro repeats but with slower kinetics. Initial in vivo experiments with a xenographic tumor model indicated that FAP activity could be clearly imaged in less than 2 hours. Treatment effect could also be imaged in real time. Further optimization, evaluation, and application of the developed molecular probes are in progress.

Development of Methodologies for the Analysis of DNA Repair Capacity To Predict the Response to Platinum-Based Therapies

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Following a cancer diagnosis, determining the best course of treatment is of paramount importance. Along with recent advances in understanding the biology and pathways involved in the initiation and progression of certain cancers have come advances in individualizing treatment based on molecular analyses of these pathways. The most convincing case involves analysis of breast cancer to determine which individuals will most likely require and benefit from adjuvant therapy. Expanding this type of analysis to other cancers holds the promise of similarly impacting cancer therapy. As numerous, very effective therapies including cisplatin induce DNA damage, DNA repair directly impacts how individuals respond to these therapies. In the context of cisplatin-based cancer chemotherapy, reduced DNA repair capacity is associated with increased sensitivity, whereas increased repair activity is associated with resistance. The goal of this research is to develop methodologies to accurately determine DNA repair capacity in cancer tissue, focusing on the nucleotide excision repair (NER) pathway. The NER pathway is also responsible for removing DNA damage resulting from exposure to a variety of insults, including cigarette smoke. Our hypothesis is that reduced DNA repair capacity increases the risk of smoking-induced carcinogenesis and also contributes to the dramatic initial tumor regression often observed on administration of cisplatin-based therapies for treating lung cancers. The relatively short-lived response and subsequent resistance severely limit the utility of platinum-based therapies. Our hypothesis is that the observed resistance is impacted by increased repair in the resistant tumors. To further test these hypotheses, an accurate measure of DNA repair activity is required. Measuring gene expression or protein expression, while useful, does not always correlate with bona fide NER repair activity. Numerous NER proteins are regulated not only at the level of mRNA or protein expression but also by posttranslational

modification and protein-protein interactions. Therefore, this application focuses on the development of novel methodologies to determine the extent of specific posttranslational modifications of key NER proteins and actual repair activity.

A Nanobiosensing System for Point Mutation Detection of Cancer

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Detection of point mutations in tissues and body fluid DNA has widespread implications in studying the molecular etiology of cancer as well as in developing new technologies for future clinical applications. We are developing a clinically relevant genetic analysis technology that enables multiplex detection of point mutations in unamplified genomic DNA using limited amounts of clinical samples. This amplification-free detection technology has been developed using a combination of two innovative technologies, single-molecule detection (SMD) and quantum dot (QD)-mediated fluorescence resonance energy transfer (qFRET). We have demonstrated that this integrative SMD-qFRET technology is able to detect DNA targets at extremely low concentrations (~5 fM), obviating the need for target amplification. When incorporated with allele-specific oligonucleotide ligation, this technology can enable detection of low-abundance point mutations in unamplified genomic DNA. Although the SMD technology has the ultimate optical sensitivity to resolve single-molecule fluorescence, its practical application for molecular detection can be complicated by the low mass detection efficiency (~1%) due to its minute detection volume. We have recently developed a new SMD spectroscopy using a 1-D beam expansion technique to substantially increase the mass detection efficiency to ~100%. We are integrating this new spectroscope in the mutation detection system to improve the analytical sensitivity and reliability. We will further improve the resolution of the system by optimizing both the design of the qFRET system and the ligation reaction conditions. The analysis throughput and mass detection efficiency of the assays will be increased by implementing this new detection method in a multiplex, microfluidic format. We will evaluate the system by analyzing four representative point mutations in the KRAS gene (at codon 12 and codon 13) and one commonly occurring mutation in the BRAF gene (at codon 599) in unamplified genomic DNA from ovarian serous tumors.

Chemical Proteomic Approaches To Profile Enzyme Activities in Complex Biological Systems

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Researchers in the postgenomic era are challenged with the task of assigning cellular

function to the myriad of predicted gene products. Toward this goal, technologies have emerged to globally analyze the collective protein repertoire of a cell, but these approaches monitor changes in protein abundance, providing an indirect measure of activity changes. To overcome this limitation, we have developed a chemical proteomic strategy termed "activity-based protein profiling (ABPP)" which utilizes active-site-directed probes to profile the functional state of enzymes in native proteomes. Current activity-based probes are derived from well-characterized affinity labels known to covalently modify the enzyme-active site, exemplified by probes for the serine hydrolase and cysteine protease families. However, many enzymes do not contain such cognate affinity labels, and the design of activity-based probes for these protein classes has posed a challenge. We introduce approaches toward converting noncovalent enzyme inhibitors into covalent activity-based probes by incorporation of benzophenone photoaffinity labels and carbon electrophiles with promiscuous reactivity. These studies have resulted in the development of probes for the metalloprotease and histone-deacetylase families. Additionally, progress in the arena of probe development has been accompanied by technological advances that serve to maximize the applications, throughput, and information content obtained from ABPP experiments. Advances in click chemistry techniques allow for *in vivo* profiling of biological systems. Microarray and gel-based platforms allow for high-throughput screening of multiple probe-labeled proteomes in parallel, and high-content mass spectrometry platforms provide identification and quantitation of probe-labeled proteins and sites of modification. All of these chemical proteomic tools and analytical platforms accelerate the assignment of protein function and streamline the discovery of novel biomarkers and protein targets for the diagnosis and treatment of human disease.

Collection, Stabilization, and Storage of Saliva Samples for Cancer Research

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Saliva is a biofluid that offers the emerging potential to detect oral and systemic cancers in a completely noninvasive manner. This research project focuses on the development of optimal sample processing for the clinical use of salivary messenger RNA for the early detection of cancer. The ability to diagnose cancer at an early stage will greatly enhance chances of treatment success and reduce mortality and morbidity. Currently, there is no molecular test that can diagnose or screen for oral cancer noninvasively. We have identified a seven-gene signature that can predict the presence of an oral cancer with an accuracy of 82% [1]. Since 2004 the characterization and diagnostic use of human salivary RNA have been actively pursued in our laboratory, including an independent validation by the Early Detection Research Network's Biomarker Reference Lab [2]. In addition to oral cancer, we have identified and validated discriminatory salivary biomarkers for Sjögren syndrome [3]. On the technology front, we have advanced the salivary transcriptome technology to the exon level for disease biomarker discovery [4].

Our proposal aims to develop optimal and standardized collection, RNA stabilization, storage, and processing protocols to perform gene expression analysis of salivary mRNA. Three specific aims are in place to address the multifaceted nature of this RFA. Aim 1 will establish optimal extraction methods for mRNA from saliva. Aim 2 will determine the intraindividual fluctuation of mRNA levels, define adequate endogenous transcripts for normalization, and validate our oral cancer markers and additional new candidates in an independent cohort. Aim 3 will determine the stability of saliva and expression patterns over periods of up to 6 months with different storage conditions and with stabilizing reagent.

At this juncture we have completed the first of the three milestones. Our data support the notion that the magnetic bead-based automated RNA extraction system from King Fisher/Thermos is best for saliva RNA extract, allowing for >80% of RNA recovery and reproducibility of RNA extraction with average CV <10%. We have also quantified the amount of genomic DNA in the saliva samples and found that it was nondetectable after DNase I treatment.

For the next year, our major objective is to obtain data for milestones 2 and 3, which are to quantify/normalize oral cancer biomarkers and develop saliva collection and processing protocols to preserve samples for immediate and long-term storage, respectively.

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High-Throughput, High-Resolution Structure Determination of Membrane Proteins With Enhanced Peptide Amide Hydrogen/Deuterium Exchange Mass Spectrometry

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We are developing a revolutionary approach to protein structure determination in which high-resolution deuterium exchange mass spectometry (DXMS) data obtained from small amounts of protein are used to identify the correct structure for a protein from within a large set of computationally predicted structures for the protein, bypassing the need for x-ray or nuclear magnetic resonance analysis. With the support of our IMAT R33 grant, we have developed methods of DXMS data acquisition and analysis that

allow us to quickly determine the precise rates of hydrogen exchange of each peptide amide within a protein—rates that are exquisitely dependent on the structure and dynamics of the protein. We have also developed computational methods that allow us to calculate hydrogen exchange rates for each amide in a protein from knowledge of the protein's precise 3D structure. In the DXMS-based structure determination method, the experimentally determined DXMS exchange-rate "fingerprint" for a protein is obtained and compared with the "virtual rate fingerprints" calculated for each of a large number of predictions for the protein's structure, and the correct structure is thereby identified.

Our R21 IMAT grant is allowing us to develop methods by which the needed high-resolution DXMS data can be obtained for integral membrane proteins while they are still in their native state, resident in the plasma membrane. The combination of these two breakthrough technologies will allow us to robustly address the critical need for high-resolution, 3D structures of membrane proteins.

Identification of Soluble Proteins in the Breast Tumor Microenvironment Using a Novel Approach Combining Microdialysis and Proteomics

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The complex tumor microenvironment is virtually impossible to reproduce in vitro. A sampling technique that can continually collect proteins from tumor interstitial fluid in live animals is highly desirable. Protein levels found in this fluid reflect changes in the tumor cells and the tumor microenvironment. Comprehensive identification of protein expression changes is needed to understand the complex signaling pathways in the tumor microenvironment. In this project, in vivo microdialysis has been successfully combined with proteomics techniques to identify the soluble proteins in the breast tumor microenvironment. Hundreds of proteins were obtained in vivo from breast tumor and normal mammary gland using microdialysis and proteomics. The differential protein expressions, including various cytokines, were quantitatively and reproducibly measured in different microdialysis perfusates. Among the proteins overexpressed in the mammary tumors, osteopontin was further studied to understand its roles in the breast tumor microenvironment. Osteopontin was found to be expressed in tumor cells and the tumor-associated stroma in both mouse and human breast tumors. It could directly stimulate breast tumor cell proliferation and migration. The expression of osteopontin was shown to be positively regulated by the transforming growth factor-beta signaling pathway in mammary fibroblasts. Furthermore, osteopontin was found to be overexpressed in the peripheral blood in mice bearing breast tumors, which is consistent with previous reports that osteopontin was a prognostic plasma protein biomarker for cancer metastasis and patient survival. In addition to osteopontin, exploration of many other identified extracellular proteins with previously unknown biological roles will further increase our understanding of the signaling pathways in the breast tumor microenvironment. Overall, microdialysis combined with proteomics is an

attractive approach to study soluble protein changes in tumor microenvironment in vivo.

Study of Serum Biomarkers Using an Innovative Assay With Reduced Human Anti Animal Immunoglobulin Antibody Interference

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High levels of heterophilic human anti-animal immunoglobulin antibodies (HAIAs) have been reported in certain patient populations. In a typical sandwich ELISA, the presence of HAIAs will interfere with antibody-antigen binding and lead to errors in the assay. If the existing HAIA recognizes the Fc region of the capture and detection antibody, it will cross-link these two antibodies and indicate false-positive signals in the absence of the true antigen. On the other hand, if the existing HAIA binds to the Fv regions of the capture or detection antibodies, it will prevent the true antigen from binding to the detection or capture antibody and indicate a false-negative signal.

To solve the HAIA problem in ELISA, we have developed a special buffer to dilute the serum samples. Since HAIAs are generally less specific and their binding to capture/detection antibodies is not as strong as the specific binding between capture/detection antibodies and antigens, our special diluent is optimized to reduce HAIA interference and eliminate the false-positives that are easily observed under standard conditions. Using this assay, we are able to study several biomarkers (IL-8, MIA, and tyrosinase) in the serum of melanoma and breast cancer patients.

Two-Photon Fluorescence Biosensing Through a Dual-Clad Optical Fiber

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Methods using fluorescent probes to identify cancer signatures and biological activities of cancer cells hold great promise. However, one of the major limitations of fluorescence analysis is the penetration depth for in vivo detection due to tissue scattering and absorption. Most ex vivo analyses using a flow cytometer or a confocal microscope require that tissue be removed from the body and often be disrupted into cells, then fixed and analyzed in a static manner. Although whole-body imaging has been used to visualize fluorophores concentrated in organs, such analysis is not quantitative and requires large fluorescent signals from a high concentration of fluorophores for penetration through tissue barriers. To address this limitation, we have developed a unique two-photon optical fiber fluorescence (TPOFF) probe that can bypass tissue scattering and absorption, thus providing quantitative results from deep

tissues of interest.

We coupled femtosecond laser pulses into an optical fiber for two-photon excitation in the close vicinity of the fiber output end and collected the emitted fluorescence back through the same fiber. This compact configuration allows localized detection of desired regions in deep tissues. However, there is a tradeoff in detection sensitivity for two-photon fluorescence measurements through commercially available single-mode or multimode optical fibers. We have demonstrated that the use of a unique dual-clad optical fiber ultimately solved the tradeoff problem and optimized the TPOFF probe sensitivity.

We utilized this dual-clad TPOFF probe and analyzed various biological samples. For example, we measured the biological activity of epidermal growth factor (EGF) molecules coupled to a fluorescein-labeled polyamidoamine dendrimer. We observed that the conjugate bound and internalized into cells with overexpression of EGF receptors. The conjugate effectively induced EGF receptor phosphorylation and acted as a superagonist by stimulating cell growth to a greater degree than free EGF. We further used this probe to quantify the targeted uptake of a dendrimer-fluorophore conjugate with folic acid (FA) as the targeting agent in mouse tumors that overexpress FA receptors. In addition, we also demonstrated the specific uptake of dendrimer conjugates in HER2 tumors.

We further demonstrated the application of the dual-clad TPOFF probe for time-resolved fluorescence detection and two-photon excited fluorescence correlation spectroscopy (FCS). We showed that the utilization of a dual-clad fiber is critical for sensitive two-photon fiber FCS. Our system has the ability to detect fluorescent nanospheres as small as 12 nm in radius and quantum dots with a radius of 7 nm. In a traditional FCS setup, a confocal or multiphoton microscope is employed. The use of bulky optical instrumentation limits the use of FCS in situ or in vivo deep inside the body. The use of a fiberoptic probe as a miniaturized setup for FCS can provide dynamic information for analyzing quantities such as diffusion coefficient, binding dynamics, and flow velocity of the fluorescent species in deep tissues. In addition, we utilized the dual-clad TPOFF probe to measure flowing cells in whole blood and showed that blood absorption and tissue scattering can be minimized in this detection configuration, thus opening up the possibility for in vivo, real-time flow cytometry for long-term monitoring of multiple populations of circulating cells.

Nonlinear Optical Imaging of Cellular Processes in Breast Cancer

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Changes in the collagen-rich stroma surrounding breast carcinomas aid in the

progression and invasion of breast tumor cells. We have found that signaling through the small GTPase, Rhois one key mechanism by which breast cells recognize and respond to changes in stromal collagen. Moreover, the physical properties of 3D collagen matrices regulate Rho activity. To understand the molecular basis for these interactions, it is necessary to observe the relationship between signaling molecules and subcellular structures, such as small GTPases, actin, focal adhesions, and components of the extracellular matrix within a relevant 3D environment. We are using nonlinear optical imaging techniques such as multiphoton and second harmonic generation microscopy used in conjunction with novel signal analysis techniques such as spectroscopic and fluorescence excited state lifetime detection for in vitro and in vivo cellular studies. Compared to other optical methods, these modalities provide superior depth penetration and viability and have the additional advantage that they are compatible technologies that can be applied simultaneously. Application of these nonlinear optical approaches to the study of breast cancer holds particular promise as these techniques can be used to image exogenous fluorophores such as GFP-Rho reporters, as well as intrinsic signals such as second harmonic generation from collagen and endogenous fluorescence from the metabolic intermediates, NADH or FAD. In this poster we discuss the application of multiphoton excitation, second harmonic generation, and fluorescence lifetime imaging microscopy techniques to investigate tumor-stromal interactions, cellular metabolism, and cell signaling in breast cancer. The ability to record and monitor the intrinsic fluorescence and second harmonic generation signals simultaneously with exogenous fluorescence reporters provides a unique tool for researchers to understand key events in cancer progression in its natural context.

Development of Rapid Nanodroplet Real-time PCR With All-Optical Control

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Aqueous solutions dispersed in an oil phase (inverted emulsions) have been suggested to be one of the most attractive approaches for high-throughput lab-on-a-chip applications [1] because they are stable and can be easily generated and spatially arrayed on a solid substrate. Droplets as small as few picoliters in volume have been shown to work as independent polymerase chain reaction (PCR) microreactors [2]. Moreover, our recently developed technique for optical manipulation of individual droplets [3], (optical microfluidics), sets the stage for the direct application of a droplet-based approach to the development of a rapid PCR assay in a highly parallel array-based manner.

For the fast and high-throughput PCR assay, a large number of miniaturized PCR chips have been fabricated. But the temperature cycling, the driving force of PCR assay, requires an contact heater and/or a temperature sensor to be incorporated in the substrate, a step requiring complex and expensive fabrication, which is at odds with the

desire to use a contamination-free single-use reaction substrate.

We present a new optical heating method to run a fast real-time PCR assay with an infrared laser. Our method makes use of non-contact heating of droplets dispersed in an oil phase using a 1.46 μm laser (intended for telecommunications) and low-cost disposable plastic substrates. Temperature is measured using fluorescence of LDS 698 dye added to the droplets. Real-time Taqman amplification of the purified human 18S RNA gene was also monitored with fluorescence, through the intensity of FAM. The laser heating was used for temperature cycling between the annealing/extension and melting steps with an optically transparent and resistively heated plane surface that was used to maintain the oil at the annealing/extension temperature. We have also shown that a laser can do all of the heating from room temperature without help from the heater. The raw fluorescence images collected during the experiment were converted to the conventional PCR amplification curves after simple image processing steps so that the analysis of the end data is straightforward. Monitoring the amplification kinetics allowed us to run a highly quantitative assay of the target concentration through a conventional Taqman assay. The total reaction time for 40 cycles including the 10 seconds of an enzyme activation was less than 7 minutes, which matches the fastest reaction time reported in the literature [4].

Current manual deposition of droplets will be replaced by automated array printing using a spotting method we have developed. Based on this array technology, we plan to execute a cell array assay by trapping a single live cell in each droplet, then lysing the cell and running realtime PCR in a highly parallel manner without transporting the sample. In doing so, we expect to save assay time as well as minimize use of expensive reagents. Moreover, the assay uses only a plain piece of plastic as a reaction substrate to complete the full assay, eliminating any risk of cross contamination.

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The Prevalence, Nature, and Diagnostic Value of Glycan Alterations in Pancreatic Cancer Revealed Using Antibody-Lectin Arrays

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Changes to the glycan structures of proteins secreted by cancer cells are known to be functionally important and to have potential diagnostic value. However, an exploration of the population variation and prevalence of glycan alterations on specific proteins has been lacking due to limitations in conventional glycobiology methods. We apply a previously developed antibody-lectin array method to characterize the nature and prevalence of glycan alterations in the circulation of pancreatic cancer patients and to investigate the diagnostic usefulness of those measurements. Twenty-one different lectins and glycan-binding antibodies were used to probe the glycan structures on specific mucin and CEA-related proteins captured from the sera of pancreatic cancer patients (n=23) and control subjects (n=23). High-prevalence glycan alterations were observed on MUC1 and MUC5ac, showing both truncated O-glycan chains and increased Lewis antigen presentation. An unexpected increase in the exposure of alpha-linked mannose also was observed on MUC1 and MUC5ac, indicating possible N-glycan modifications. Improved identification of the cancer samples was achieved using the glycan measurements relative to the core protein measurements. We also examined the variation between disease states (pancreatitis, early-stage cancer, and late-stage cancer) in the protein carriers of a specific cancer-associated glycan, the CA 19-9 antigen. We found that the disease states, and possibly subclasses of patients, can be distinguished by the protein isoforms that carry CA 19-9. Accordingly, the measurement of CA 19-9 on particular proteins, instead of the measurement of total CA 19-9, led to the improved discrimination of certain patient classes. This work gives insight into the prevalence and protein carriers of glycan alterations in pancreatic cancer and shows that measurements of glycans on specific proteins can yield improved diagnostic performance relative to measurements of proteins alone.