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Accelerating Cancer Research With Single Cell Arrays

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Most solid tumor cells encountered in the onological practice appear quite 'cell-smart', being a product of a long evolutionary period which seems to have equipped them with very sophisticated ways of escaping common allogenenic rejection. So, a sensitive detection of single neoplastic or tumor stem cells, an accurate characterization of abnormal or mutant cells vs. hyperplastic entities and the isolation of mutant cells from small neoplastic lesions or metastases still remains a major challenge in tumor cell diagnosis. Promising a tremendeous impact on patient management and anti-tumor regimens [treatments], or even a paradigm change in occupational medicine, this project addresses the sensitive detection and to the extend possible, accurate delineation of chromosomal changes such as small translocations, rearrangements or genomic imbalances in apparently normal individuals, benign neoplasia, premalignant lesions and cancer. Current techniques for full karyotype analysis of individual cells require metaphase cells; cells in interphase or non-viable cells can not be analyzed. The objective of the proposed research is the development of technologies to support the cytogenetic analysis of extremely small amounts of fresh [swabs], fixed or archival tissues regardless of the cells' proliferative stage. A highly sensitive, fluorescence in situ hybridization (FISH)-based technology platform termed 'Single Cell Arrays (SCAs)' will the allow the detection of small chromosomal rearrange¬ments or deletions incl, viral integration in interphase and metaphase cells by combining the high-resolution DNA in situ analysis with sensitivity in the kb range. This has been achieved by immobilizing cell nuclei on glass slides and controlled stretching of chromatin in specially design micro-chambers followed by cytogenetic analysis using FISH. The aims of this R21 feasibility study are 1. Demonstrate the feasibility that interphase cell nuclei can be immobilized in defined pattern and reproducibly extended for subsequent cytogenetic analysis. We will demonstrate the feasibility to prepare SCAs comprised of individual cell nuclei arranged in defined pattern inside microscopic reaction chambers and elongated/stretched by a constant force. Importantly, the extent of chromatin stretching will be controlled by cell fixation and adjusting environmental parameters such as buffer, chamber temperature and humidity, and the force applied to pull the chromatin. 2. Develop an optimized assay for the sensitive, high-resolution cytogenetic analysis of SCAs. We will develop a protocol for a FISH-based multi-locus cytogenetic analysis of SCAs. The assay is expected to provide near kilobase sensitivity for the detection of single copy nucleic acids with a resolution in the order of 10-20 kb, while minimizing the overall loss of DNA. The assay will be tested by analyzing SCAs prepared from different breast or thyroid cancer cell lines. Additional collaborators are sought after. SCAs will become powerful tools in basic and applied/clinical research, where chromosomal changes often affect a cell's phenotype and the fate of its progeny. In clinical practice, for example, such a sensitive assay may support cell classifications, thereby benefiting patients with de novo translocations or premalignant lesions as well as cancer patients. Furthermore, SCAs will allow the analysis of very small samples regardless of their integrity or cell cycle stage. This will open new avenues for the analysis of small samples like those obtained by fine needle biopsies as well as the analysis of circulating or exfoliated tumor cells.

Analysis of Tumorigenic Signaling Pathways With PROTACS

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The transformation of quiescent, somatic cells into proliferating tumor cells often results from the oncogenic activation of one of the many growth factor signaling pathways. In response to this, small molecule inhibitors of receptor tyrosine kinases (RTK) have been added to the established arsenal of anti-cancer drugs. In an effort to better match chemotherapeutics with those tumors against which they will be most effective, we are developing a peptide-based approach which will identify of the transforming RTK signaling pathway in tumor samples from cancer patients. This novel technology centers around the ability these peptides to knockdown levels of a downstream effector of a RTK pathway in response to overactivation of that pathway.

The first stage of this project consisted of the proof-of-principle that knockdown of a target protein could be made conditional upon activation of a specific RTK pathway. We synthesized a cell-permeable chimeric peptide, which targeted the signaling molecule FRS2α; for degradation only when the NGF receptor, TrkA, was activated. Binding of the peptide to FRS2α; was dependent upon phosphorylation of the former by TrkA; the peptide-bound; would then be targeted to the 20S proteasome for degradation by way of an E3 ubiquitin ligase-recognition domain also in the peptide. Using PC12 cells as our model system, we showed that this peptide could inhibit both NGF-triggered Erk1/activation as well as the acquisition of a neuronal phenotype in response to NGF. Because this peptide functions as a phosphorylation-dependent proteolysis-targeting chimera, we refer to it as a "PhosphoPROTAC."

The current phase of the project focuses on the development of a panel of such PhosphoPROTACs, each of which incorporates a trans-autophosphorylation site of a different oncogenic tyrosine kinase. While deriving from different receptor tyrosine kinases, each of these phosphorylated sequences will bind the common signaling effector phosphatidylinositol-3-kinase (PI3K), a crucial pro-survival signaling protein in many tumors. Accordingly, each of these PhosphoPROTACs will be capable of binding to and knocking down PI3K only following phosphorylation by its activated cognate RTK. Knockdown of PI3K will result in cell death, but only when the appropriate PhosphoPROTAC is paired with the appropriate oncogenic RTK. Validation of the effectiveness and selectivity of the PhosphoPROTACs will be accomplished through testing for their phosphorylation-dependent cytotoxicity against a collection of fibroblast-derived cell lines, each having been transformed through overexpression of an oncogenic RTK.

The ultimate goal is to employ the panel of peptides to test tumor biopsies from cancer patients in order to determine the identity of the oncogenic signaling pathway, which drives the tumor to grow. With this information in hand, clinicians would be able to make more informed decisions regarding chemotherapeutic options.

Application of Multiplexed BIOCD Assays for Cancer Diagnostics and Prognostics

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A human diagnostic/prognostic BioCD assays for potential serum markers for endothelial ovarian cancer and markers with modified expression in acute lymphocytic leukemia (ALL). Purified CA125, human epididymus protein 4 (HE4), and osteopontin proteins in phosphate-buffered saline/0.5% Tween 20 (PBST) were used during the materials and technology validation stage. This was followed by marker proteins spiked into a 50% dilution of fetal bovine serum with PBST. Preliminary tests were conducted using a real-time binding (RTB) assay on the

BioCD, with 50% PBST/ human cancer patient serum vs. 50% PBST/healthy human serum with respect to endogenous CA125. Each assay began with an equilibrium wash with running buffer, followed by a switch to antigen-spiked running buffer or diluted patient sample, followed by a second running buffer wash and, finally, running buffer spiked with secondary antibody for a sandwich assay. The results of a series of CA125 experiments starting with spiked PBST on to diluted clinical human sera will be presented, including results from an ovarian cancer patient, and the 2 new markers OP and HE4.

Following similar protocols, we have generated real-time binding curves for acute lymphocytic leukemia (ALL) markers p15, p57 and p73. We will present the results of an experiment using a mouse monoclonal anti-p15 antibody (Novus Biologicals) as capture antibody against a purified p15-GST fusion protein (Novus Biologicals) spiked into PBST at 2 μg/mL plus 2 μg/mL bulk rabbit IgG (Pierce) positive control antigen. The results are positive in the sandwich assay vs. the negative control. The real-time binding results for a positive spiked sample of p57 have also been obtained.

The relevance of this research to public health is to establish the BioCD as a novel high-capacity resource for diagnostic and prognostic applications for cancer, including the first application to clinically relevant markers in human patients.

Application of Next-Generation Sequencing to Cancer Epigenomics

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The two major processes that contribute to the epigenome of a cell are DNA methylation and histone modifications. Methylation of cytosine residues at CpG dinucleotides is known to regulate gene expression in different normal and disease tissues. Aberrant promoter hypermethylation has been associated with transcriptional silencing of tumor suppressor genes in cancer. Although a number of methods are available for DNA methylation analyses, few of them enable quantitative, large-scale, and single-base resolution mapping of DNA methylation states in cancer genome. The power of next-generation DNA sequencing technology such as Roche/454 GS FLX, Illumina Genome Analyzer and ABI SOLiD, is transforming the landscape of epigenomic research. New epigenomic applications such as ChIP-Seq and genome-wide bisulfite sequencing (BS-Seq) have emerged. However, to take full advantage of the throughput of next-generation DNA sequencers, technology developments have to be made in converting the samples of interest to DNA sequencing libraries more efficiently. In this study, we present an approach that combines solution-based hybrid selection and massively parallel bisulfite sequencing to profile the DNA methylation in a large number of CpG islands (CGIs) across cancer genome. 55,000 single strand DNA oligonucleotides were synthesized and each oligonucleotide consists of 160-mer sequence that targets one of the CGIs in human genome. The long oligonucleotides were converted into biotin-labeled RNA bait for capture of target DNA. This approach increased the specificity of the hybridization capture, and made the capture process highly flexible. Since all CGIs were captured and sequenced as a pool through a series of single-tube reactions, the method can be easily scaled up to deal with a large number of samples. The targeted bisulfite sequencing strategy will generate digital profiles of aberrant DNA methylation for individual human cancer and provide a robust method for epigenetic classification of cancer subtypes.

Application of a Novel Nanotechnology for Molecular Profiling of Tumor Cellular Elements

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A fundamental hurdle to the detailed study of complex tissues is the lack of tools to be able to study the various cellular subsets present without substantial perturbation. These sorts of studies are further complicated by the fact that most tissues contain predominantly adherent cells. We are developing a novel technology, the micropallet array, for the direct analysis and recovery of individual adherent cells from a heterogeneous cellular population. This work, supported by 1R21CA132039-01, comprises a transdisciplinary effort combining expertise in micro-/nano-technology engineering, advanced optical imaging, and cellular biology to establish a technology that enables the efficient identification, selection, and recovery of single adherent cells derived from small biopsies of complex tissues.

Micropallet arrays consist of microscale polymer pedestals ("micropallets"), uniformly arrayed on a glass microscope slide, made from a high aspect, optically transparent, low autofluorescence, negative photoresist using photolithographic methods. Post-production modifications allow applied cells to settle out of suspension and fall stochastically upon the surface of the array, with single cells adhering to individual micropallets. Cells can then be analyzed *in situ*, see below, with single cells selected and collected from the array by releasing the underlying micropallets using a focused pulsed laser without damaging the adherent cell (Salazar, et al. Anal. Chem. 2007;79(2):682). The original proof of concept work was performed with robust, adherent, immortalized cell lines. The application of this technology to primary tissue samples requires several improvements to the base micropallet array platform, described below, in order to meet the overall research objective and enable the molecular evaluation of cells obtained from complex primary tissue fine needle or cutting needle biopsies.

Extracellular matrix (ECM) micropallet array coatings: We have established conditions and methodologies that enable the efficient and uniform coating of the micropallet arrays with various ECM components. In order to maintain the integrity of the microarray platform for recovery of single cells, methods for assuring that the ECM coatings were restricted to the top surface of the micropallet and that there were no bridging ECM structures, were critical. We have demonstrated this capacity for basement membrane extract (Matrigel®), collagen, fibronectin, and laminin. This advance is required for adequate adherence of primary cells.

Rare cell detection: Integrating large-field immunofluorescent imaging we have established the capacity to identify rare cells in mixed cellular populations. Mixtures of known percentages of two or more cell types with different cell surface molecule expression, the targets for immunofluorescent detection, were used as a model system. These cells included, mouse fibroblastic 3T3 cells, both wild type and genetically engineered to express the neu gene product, and various human tumor cell lines. We have demonstrated detection thresholds of nearly 0.01%, due in part to very low background signal. This is a level that is unattainable using competing technologies such as fluorescent-activated cell sorting (FACS), which cannot be effectively employed with small samples, is ill-suited for use with adherent cells, and because of inherent background signal issues has an accepted detection threshold of 0.02%.

Advanced micropallet recovery technology: The original, proof of concept, method for the recovery of micropallets with adherent cells was inherently inefficient. In order to provide higher throughput capability, efficient micropallet recovery technologies need to be developed. We have developed methods to confer magnetic properties to the micropallets by incorporating ferromagnetic nanoparticles into the photoresist material, while not compromising the capacity to coat the micropallets with ECM components or visualize adherent cells, as above. We have also demonstrated proof of concept for recovery of released micropallets using magnetic probes. Refinement of the design of these recovery probes is ongoing.

These important improvements lay the groundwork for testing of the micropallet technology in the investigation of cellular profiles and evaluation of molecular signatures of representative individual cells from tissue-specific cellular subsets identified and isolated using the micropallet array, e.g., via single cell rtPCR. Future work is aimed at expanding immunofluorescent cell identification, with an aim for use of 6 channels, and establishing proof of concept for the determination of selected molecular signatures from individual single cells. These marked improvements to the base micropallet array platform will, enable higher throughput analyses as well as shaping the technology into a potentially extremely useful clinical tool, particularly when sample size is limited to a few thousand cells. In the case of tumor tissues, these analyses will have the capacity to direct the design of individualized treatment as well as shedding important light on the biology of various rare cell populations/subsets in tumors.

Applying the Reverse In-gel Kinase Assay to Vet Kinase Activity Biomarkers

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There is currently an urgent need for biomarkers to determine whether investigational kinase inhibitors are truly affecting their intended targets in vivo. Ideal biomarkers would respond rapidly, robustly, specifically, and reproducibly to inhibitor treatment. For most kinases, our knowledge of physiologic substrates is woefully inadequate. Consequently, choices for biomarkers of kinase inhibitor activity are extremely limited, and those in use have typically been chosen based on availability rather than for specific attributes. Moreover, there are no currently available assays that can systematically quantify changes in phosphorylation state across a broad spectrum of true substrates in a cell or tissue lysate. Using the Reverse In-gel Kinase Assay as a platform, we have developed a novel approach to systematically profile, identify, and validate kinase substrates that respond to small molecule kinase inhibitors in vivo. Using cultured cells as a model, Protein Kinase CK2 substrates that respond to a novel inhibitor were profiled. Proteins that became hypo-phosphorylated in the presence of inhibitor were identified. Eukaryotic Translation Elongation Factor α; was shown to undergo a rapid and profound decrease in phosphorylation index in response to CK2 inhibition. These data demonstrate that the Reverse In-gel Kinase Assay can be applied to identify and validate kinase substrates that are robust biomarkers of kinase activity in vivo. This technology, coupled with development of specific anti-phosphosite antibodies could be a powerful new system to determine the efficacy of kinase inhibitors in patient samples.

Architectural Proteomics for Early Risk Assessment in Breast Cancer

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One of the biggest public health challenges in cancer prevention is the development of technologies that can identify risk factors and enable precise, early-stage, identification of lesions that will advance in aggressive ways. Proteomic research will play an essential role in solving these problems by providing an understanding of the basic subcellular machinery involved in malignant transformation. The Lelièvre and Knowles Laboratories are contributing to this effort by developing methods for turning high resolution fluorescence images of human mammary epithelial tissue into quantitative morphology and feature maps which will allow better detection of non-neoplastic, premalignant and malignant phenotypes at cellular resolution. We have pioneered methods that have showed that measurements of the distribution of a single nuclear protein can be used as a biomarker to distinguish functionally normal, non-neoplastic proliferating, preinvasive neoplastic and invasive neoplastic breast epithelial cells. The success of this work strengthens our driving hypothesis that the nuclear organization of proteins is tightly linked to the regulation and expression of genes that are involved in cancer development and progression. Vast improvements in the

ability to understand and classify tissue behaviors will be possible once we are able to understand the cellular heterogeneity of a tissue within the context of its morphology, and link these to the emergence of a particular phenotype.

The quantitative analysis of protein distribution, which we have termed architectural proteomics will enable better understanding and diagnosis of neoplastic diseases. Two of our immediate goals are to provide tools that can be used to measure the effects of exogenous factors on mammary gland homeostasis

BAC-FISH Assays for Sensitive Karyotyping of Cancer Cells

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Frequently, a detailed cytogenetic analysis of human cancer cells or tissues revealed the presence of structural chromosome abnormalities. These changes are often specific for the type of tumor or cancer and the cells' altered phenotype. Furthermore, investigators could demonstrate a correlation between the type or the extent of chromosome changes, disease progression and outcome. However, many of the laboratory techniques applied in the screening for structural abnormalities are very limited with respect to the detection of small, 'cryptic' translocations (CTs). For example, Giemsa (G) - banding of metaphase chromosomes or the fluorescence in situ hybridization (FISH)-based techniques of whole chromosome painting (WCP) and Spectral Karyotyping (SKY) analysis typically miss translocations that involve segments of less than 10 megabasepairs (Mbp), i.e., about the size of a chromosome band.

We postulate that CTs exist undetected in the genomes of individuals with a normal phenotype or diseases such as mental retardation, impaired fertility, precancerous lesions or early stage tumors. Knowledge about such structural alterations and chromosomal imbalances might help clinicians to make more accurate predictions regarding the onset and course of a disease. This R21 project investigates the feasibility to rapidly and inexpensively screen the entire human genome for the presence of cryptic translocations. Specifically, we develop and test FISH assays using collections of validated bacterial artificial chromosomes (BACs) for the detection of CTs in human cancer cells. With BAC probes spaced on average 0.8 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 to WCP or banding tests. Our innovative assay for sensitive genome-wide screening for translocations is presently developed with breast and thyroid cancer cell lines for which limited information about structural abnormalities is available. At the end of this project, we will be well positioned to conduct a larger study of the frequency of CTs in the normal population as well as familial tumor cases and to offer BAC-FISH screening and reagents to research and clinical laboratories.

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Capillary Electrophoresis for Analysis of Barrett's Esophagus Biopsies

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The study of the protein expression has shown promise to predict disease progression. In order to exploit the potential of protein expression as a prognostic tool, the proteins must be quickly separated and detected at very low concentrations. We performed Capillary Isoelectric Focusing (cIEF) on a post-column sheath flow cuvette system with ultrasensitive laser-induced fluorescence detection. Proteins were labeled with Chromeo P503, and we obtained detection limits in the low attomole (10-18 mol) range. A standard solution

of four proteins was separated with high resolution; we achieved a correlation coefficient of 0.9 for the relationship of pI and molecular weight. A protein homogenate from a Barrett's esophagus biopsy was also analyzed; over 150 components were resolved within 35 minutes. However, due to background fluorescence produced by trace impurities within the ampholytes used in the separation, detection limits for cIEF were four orders of magnitude poorer thenCE methods. In order to improve detection limits, we explored different method to decrease the fluorescent background such as photobleaching, oxidation reaction, and carbon treatment. Nearly two orders of magnitude improvement in detection limits resulted from shifting the excitation and emission wavelengths to 532/ 580 nm and by photobleaching the ampholytes before use. Detection limits are 150 zeptomole (1 zmol = 10-21 mole) for standard proteins.

Capture of In Vivo Assembled RNA/Protein Complexes for Mass Spectrometry Analysis

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Goal: The goal of the project is to develop an RNA-tagging method for rapid isolation of bona fide in vivo-assembled RNA/protein complexes. RNA/protein complexes mediate processes of post-transcriptional regulation and these processes are often disrupted or altered in cancer. Identifying the protein components of these complexes will aid in the development of diagnostics for cancer and increase our understanding of the mechanisms that cause cell transformation. However, isolation of authentic, in vivo assembled RNA complexes is difficult because of their dynamic nature and because there are limited tools for rapid capture. Our strategy addresses these challenges with the following features 1) the RNA is synthesized in vivo by RNA polymerase II as a tagged RNA bait, 2) rapid capture of the complex is performed under physiological or denaturing conditions from whole cells, cytoplasm or nuclei, 3) the RNA bait is captured through specific, high affinity interactions with a biotinylated protein (MS2) that recognizes the RNA tag, 4) mass spectrometry analysis of the purified RNA/protein complex is semi-quantitative (SILAC) and sensitive, and 5) the strategy is designed to be broadly useful for many different RNA elements and classes of RNAs.

Preliminary Results: To develop this method we have used the 5' untranslated region of LEF1 mRNA. This region contains two internal ribosome entry sites (IRES), a class of RNA regulatory element that is poorly characterized in cellular mRNAs, and is often found in cancer-relevant mRNAs. We demonstrate that our method of affinity purification can rapidly isolate the LEF1 bait RNA, as well as associated protein complexes. Currently we capture >90% of the biotinylated MS2 protein and up to 60% of the tagged RNA. Capture is performed with streptavidin beads specific for biotinylated MS2. Background binding of non-specific proteins is 20% and elution of specific proteins is >95%. From 25 mg of whole cell lysate, we reproducibly identify a set of proteins with known associations to RNA processes, i.e., 20-30% of the IRES-enriched proteins have known functions related to RNA processes or translation regulation. Included in this set are ribosomal proteins and translation factors implying that mainly cytoplasmic RNA-protein complexes were captured. In addition, we isolated several known IRES trans-acting factors and other RNA associated proteins that have the potential to be IRES regulatory factors (helicases, hnRNPs, and translation factors). Importantly, many abundant cytoplasmic and RNA binding proteins were discounted as non-specific binders through the use of SILAC analyses. These preliminary results suggest that IRES motifs may not be regulated by a subset of unique factors but rather an assortment of canonical RNA regulatory factors. This method is a versatile approach and adaptable to any RNA-protein interaction.

Clinical Applications of Quantifying Nuclear Translocation Events by Multispectral Imaging Flow Cytometry

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The ImageStream platform combines the throughput power of flow cytometry with high image content analysis of microscopy by acquiring spectrally separated but spatially correlated images from cells in a flow suspension. The first year of the R21 phase was dedicated to optimizing the ImageStream's performance by increasing sample throughput, improving focus quality of collected images, and adding 405nm, 561nm and 658nm excitation sources to the standard 488nm single laser set-up. The second year was dedicated to standardizing and verifying data acquisition and analysis for quantifying nuclear translocation events with the ImageStream.

Cells respond to extracellular stimuli through receptor-mediated signal transduction and many cancers, including AML, have been associated with aberrant activities of signal transduction pathways. Signal transducer and activator of transcription-3 (STAT3) and nuclear factor-κB (NF-κB) are downstream targets at the convergence of many signaling cascades and the leukemia-specific nature of their anomalies make them attractive therapeutic targets in AML. Nuclear factor of activated T-cells (NFAT) is a family of transcription factors involved in regulating the immune response. The STAT3, NF-κB and NFAT pathways have in common that during the inactive state, intermediaries of these transcription factors are sequestered to the cytoplasm and upon activation are translocated to the nucleus where they bind to relevant promoter sites and activate transcription of associated genes. Thus, the nuclear versus cytoplasmic localization of these transcription factors correlates with their signaling activity.

Data are presented to demonstrate the applicability of the ImageStream platform to quantify nuclear versus cytoplasmic localization of NF-κB, NFAT and STAT-3 in immunophenotypically defined (target) cells. Dose- and time-kinetics of NF-κB and STAT-3 nuclear localization following in vitro cytokine exposure was studied in AML cell lines and leucocytes from AML patients and healthy volunteers. The ability to translocate NFAT following in vitro exposure to anti-CD3/anti-CD28 was studied in tumor infiltrating lymphocytes in ovarian carcinomas. Following the desired treatment, cells were stained with combinations of cell lineage specific antibodies, anti-p65, anti-NFAT1, or anti-STAT3 antibodies and a nuclear DNA stain (DRAQ5). Multi-spectral images of at least 5,000 cells were then acquired with the ImageStream platform. Following hierarchical gating strategies to identify single, in-focus and labeled cells, image analysis algorithms were applied to each cell to determine the 'similarity' of its nuclear image (defined by DRAQ5) and its p65, NFAT or STAT3 image. The correlation between STAT3, NFAT orNF-κB images and DRAQ5 images was then quantified for each individual cell by a 'similarity score', a log transformed Pearson's correlation between corresponding pixel values of each image. As such the similarity score is a measure of nuclear translocation that is measured as a continuous variable from –infinity to +infinity. The higher the score the higher the degree of nuclear translocation. In all model systems tested, dose- and time-kinetic changes of similarity scores corresponded well with expected nuclear translocations. The data illustrate the applicability of this approach to study nuclear translocation events as a parameter of response in immunophenotypically defined target cells in patients undergoing therapy with agents targeted against these signaling pathways. Supported by NIH 1R21 CA126667.

Defining Epigenetic Proteomes Using Novel Crosslinking Agents

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Epigenetic changes alter chromatin structure, thereby regulating gene transcription. A major limitation to making significant advances in diagnosing and treating cancer based on altered DNA methylation within tumors is the fact that we do not have a thorough understanding of the mechanisms leading to abnormal DNA methylation in cancer cells. New techniques are needed to identify the DNA methyltransferase (DNMT) enzymes and their accessory proteins that mediate the DNA methylation changes of particular promoters in cancer cells. Our goal is to develop a unique method of chemical crosslinking and protein complex identification to define a quantitative molecular signature for DNA methylating complexes in cancer, using breast cancer as a model system.

We have synthesized a variety of compounds that we are exploiting for crosslinking DNMTs and accessory proteins to derivatized oligonucleotides whose sequence corresponds to promoters regulated by DNA methylation in cancer cells. Our crosslinking reagents include: a cytosine with a disulfide tether on the N4-position (C*), diazirine-base derivatives that can be incorporated into the major or minor grooves of DNA to promote photo-crosslinking, and disulfide-linked deoxyribose (DLD). To date, we have achieved the most efficient crosslinking with disulfide-modified oligonucleotides containing C* and the catalytic domain of DNMT3A with a point mutation, C710S, as well as with the DNMT3B catalytic domain with an equivalent point mutation, C651S. The presence of the point mutation facilitates high-efficiency crosslinking through a cysteine located in the base-flipping pocket. In the next phase of our work, we plan to use the purified catalytic domains crosslinked to oligonucleotides of specified sequence (e.g., BRCA1 or CDH1 promoters) to affinity purify associated proteins from breast cancer cell extracts and identify them using mass spectrometry. After optimizing our approach using cultured cell lines, we will perform similar experiments using nuclear protein extracts from primary breast tumors.

Our new strategy has significant advantages over other approaches in that it assembles a protein complex directly on a specific biologically relevant DNA, which may help stabilize a protein complex that might not form otherwise. This project has the potential to transform the way we study individual genes that are regulated by DNA methylation in cancer and in other conditions in which epigenetic changes regulate gene expression. Because changes in DNA methylation represent some of the earliest molecularly defined alterations observed in cancer cells, our findings could provide the basis for new diagnostic tools to identify transformed cells based on the presence of quantitative protein levels present within DNA methylating complexes. Cancer cells could be distinguished from normal cells based on the identification of characteristic protein levels, as a signal of altered DNA methylating activity. In addition, if we are able to identify the factors involved in promoter hypermethylation in breast cancer, we could then consider developing new agents that could alter DNA methylation in a sequence-specific manner. Moreover, by stabilizing DNMT-DNA complexes through covalent crosslinking, we could facilitate structural determination of these interactions. In principle as a platform technology, our crosslinking approach can be applied to any gene of interest in any cancer. We are also developing new crosslinking reagents that can be incorporated directly into proteins that will allow the isolation of proteins that come into direct contact with the DNMTs.

Deregulation of Phosphorylation-Based Signaling Pathways in Myeloma Plasma Cells and Their Microenvironment: Opportunities for Clinical Intervention

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Multiple myeloma (MM) is a malignant hematological disorder characterized by the accumulation of mature plasma cells (PC) within the bone marrow, resulting in renal failure due to excessive immunoglobulin production and bone lesions as a result of local bone destruction by malignant myeloma cells. Despite the emergence of promising new drugs such as the proteosome inhibitor bortezomib and the thalidomide derivative lenalidomide, MM remains an incurable disease with an almost 100% relapse incidence and an overall survival of 5 years. Aberrant expression and activity of numerous kinases have been described in a variety of cancers, and several specific kinase inhibitors have been developed for clinical use in the treatment of these illnesses. In this study, we used a novel peptide array (PepChip) based approach to analyse the phosphoproteome of bone marrow-derived FACS-sorted $CD38^{++}CD138^{+}$ PC from MM patients (n=10) and their healthy bone marrow counterpart (NBM, n=10). Our results indicate an increased signalling towards the ribosomal S6 pathway in MM PC, which was confirmed by demonstrating increased phosphorylation of mTOR, p70Sk and S6 by conventional Western blotting techniques. In addition, we observed an increased nitric oxide and reduced Casein Kinase 2 (CK2) signalling in MM PC by PepChip analysis. Interestingly, as orally tolerated small molecule inhibitors against the mTOR, NO and CK2 pathways are available, our findings suggest that these pathways might provide clinical potential for mono- or combination therapy.

MM cells have been shown to be engaged in a reciprocal interaction with their microniche, affecting BM composition and thereby modulating their own environment. Analysis of $CD38^{dim/-}CD138^{-}$ cells by flow cytometry revealed a reduced percentage of lymphoid ($CD3^{+}$) and myeloid ($CD11b^{+}$ and $CD33^{+}$) cells in MM BM aspirates (n=7 for MM, n=6 for NBM). In contrast, an increased percentage of $CD41^{+}$ megakaryocytic cells was observed, indicating that normal hematopoiesis is indeed disturbed in MM BM. Next, we investigated the kinase activity of FACS-sorted $CD38^{-}CD138^{-}$ bone marrow stromal elements from MM patients and healthy controls (n=10). This fraction, consisting mostly of cells from the erythroid lineage ($CD36^{+}$,$CD71^{+}$, GPA^{+}), showed a reduced pyruvate kinase signaling in MM BM, possibly contributing to the anaemia often observed in MM. A reduced cytoskeletal/adhesion signaling profile was found in the MM non-PC fraction, and confirmed by western blotting of phospho-Pak and Focal Adhesion Kinase. In addition, decreased S6-kinase activity was observed and confirmed in $CD38^{-}CD138^{-}$ MM BM cells. These results suggest a disturbed constitution and signalling in the MM PC bone marrow environment.

In conclusion, our data demonstrate the potential of whole kinome profiling for the identification of aberrantly activated signaling pathways in MM PC, providing putative targets for treatment.

Detection and Identification of Oxidative Cysteine Modifications 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. Based on the generation of hydrogen peroxide and other reactive oxygen species (ROS) through the activation of NADPH oxidases 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 approach for detecting and identifying H_2O_2-sensitive sites in signaling relevant proteins is to introduce detectable labels into the initial protein oxidation product, cysteine sulfenic acid, using trapping agents directed toward these chemically distinct modifications. The modifying agents, with fluorescent or biotinylated tags attached to an analogue 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. Our research using these compounds indicates that the probes are specific for cysteine sulfenic acid and that an initial "burst" of R-SOH formation is observed within the first 10 minutes after addition of TNF-α to HEK-293 cells and is sustained for at least 30 minutes. Both cytokine and growth factor signaling are associated with ROS bursts and R-SOH generation, and we have recently shown that inhibition of endogenous catalase with 3-aminotriazole, which specifically increases H_2O_2 levels, further increases R-SOH modifications as assessed by biotin incorporation into total cellular protein (after incubation with our biotinylated reagent, DCP-Bio1, in cell lysis buffer). DCP-Bio1 labeling has also confirmed the intermediacy of sulfenic acid formation in the transient oxidative inactivation of PTEN and SHP-1 during signaling. To demonstrate the utility of our new technology to detect novel oxidation sites that regulate protein function, we also investigated more deeply whether a few of our newly identified oxidized proteins are functionally changed by oxidation; we showed that the activity of topoisomerase I, a protein which was identified as a sulfenic acid modified protein in cells treated with either TNFα or PDGF, was strongly inhibited by [H_2O_2] as low as 50 µM. Full proteomic analyses to identify oxidation-sensitive proteins and map the specific reactive cysteinyl residues within them are currently underway, and suggest that oxidative PTMs involved in signal transduction occur within cellular proteins involved in phosphorylation cascades (kinases as well as phosphatases) and in calcium-regulated processes.

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. Supported by R33 CA126659 (L.B.P., PI; L.W.D., C.M.F. and S.B.K., Co-Is).

The Development of an Aerodynamic Focusing Device, the Air Amplifier, for Improved Biomarker Analyses by Mass Spectrometry

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Efforts directed towards an improved air amplifier design that will provide a routine improvement in detection limits for electrospray ionization mass spectrometry has involved a combination of aerodynamic simulations using computer modeling, precision machining, bench testing, and mass spectrometry experiments. To this end, the Aerospace Engineering Computational Fluid Dynamics Lab, the Precision Engineering Center and the W.M. Keck FT-ICR Mass Spectrometry laboratory have been working together to design, fabricate and evaluate the performance of candidate air amplifier designs.

This combination of computational fluid dynamics simulations, precision engineering, and systematic testing of the air amplifier will facilitate a fundamental understanding of how the device serves to improve electrospray ionization as it is applied in targeted and discovery biomarker experiments. The numerical tool to be used in the computational fluid dynamics symulations is a general-purpose Navier-Stokes solver

(termed REACTMB-MP) for multi-phase, multi-component, reactive flows that has been developed in the AECFDL at NCSU over the last several years. Air amplifier designs that have been considered to have good potential are then precision machined using single-point diamond turning (SPDT).

We have designed a more robust positioning mechanism for the air amplifier by employing piezo-electric stacks to vary the annular gap. By precisely controlling this annular gap (within microns) we can properly simulate the actual conditions of the device. Furthermore, air amplifier conditions can be reproduced. This device has been fabricated and has demonstrated an improvement in terms of the aerodynamic performance. This was measured by monitoring the static pressure (i.e., vacuum generated by the device in the axial direction) and the stagnation pressure (i.e. gas flow out of the device nozzle). The static pressure measured in our current design shows a greater vacuum was generated in the device. Additionally, the stagnation pressure was more symmetric than the previous device. These characteristics should lead to a more robust and reproducible method when using the air amplifier. Preliminary measurements on Fourier transform ion cyclotron resonance and triple quadrupole mass spectrometers have indicated that the device offers improvements in signal even for nano-flow ESI. Continued exploration with designs and applications with LC-MS will result in further improvements and robustness of the device.

The Development of a Chip-Scale Nano-Calorimeter

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The study of binding interactions is central to basic biology research and pharmaceutical R&D and there are numerous analytical methods available to study various aspects of these interactions. Each has strengths and weaknesses. Calorimetry provides detailed information on the nature of binding reactions by measuring the energy released/absorbed by a reaction over a range of reactant concentrations, and uses these data to determine the relative contributions (to a binding interaction) of enthalpically driven processes (related to the number and types of bonds) and entropically driven processes (related to the shapes of the binding site and the ligand). Unfortunately, the need for a large amount of protein (up to 5mg) and its low throughput limit its usage. Additionally, there are some reactions where the amount of heat is too small for the current generation of calorimeters to measure.

In this project we propose to develop a chip scale calorimeter based on extraordinary optical transmission (EOT) through an array of nanometric apertures. Stark et al. and Brolo et al. have shown that these nanohole array devices can be used as affinity sensors where one of the binding partners is immobilized on the surface of the nanohole array device. It is well known in the surface plasmon resonance sensing field that the sensor signal is temperature dependent due to the dielectric function of the buffer/sample changing the plasmon excitation conditions. This is also true with nanohole array sensing. Holding the concentration constant in an approximately 100nm thick layer of dielectric directly above the nanohole array surface enables the use of EOT as a fast and sensitive (minimum $\Delta T = 0.001C$) temperature sensor to measure the heat of reaction (enthalpy, ΔH) from binding events. The ability to multiplex many nanohole array sensor devices on a single chip enables the simultaneous measurement of confounding effects (e.g., buffer dilution, mixing, DMSO in the buffer, heat transfer effects) and deconvolution of these effects to determine the true measured heat of reaction.

The basic measurement principle is: (1) the heat of reaction changes the temperature of a dielectric medium in direct contact with the gold surface of the nanohole array device, (2) the dielectric function of this medium is temperature dependent so the resulting temperature change alters the dielectric function which changes the EOT conditions, (3) which in turn, changes the amount of light emitted via EOT which is measured and related to the heat of reaction.

The objectives for this project are:

1. to create a chip-scale calorimeter that addresses the critical limitations (amount of protein required, sensitivity and throughput) with current calorimeters and

2. to show proof of principle for this approach.

If successful, we expect calorimetry to become a standard method in the study and evaluation of binding interactions.

Development of a Sensor Platform for Detection of Cancer Marker RNAs and Proteins

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We have continued development of our sensor platform which utilizes elecric-field assisted assembly of functionalized nanowires to detect cancer markers. The initial development used a "sandwich" hybridization assay to detect epithelial cancer selective RNA markers in circulating tumor cells (CTCs). We have now extended the utility of the platform, by substituting antisense oligonucleotides to marker RNAs, for aptamers which recognize distinct epitopes of circulating cancer marker proteins. The initial target chosen was S100b protein as a plasma marker for melanoma. We cloned human S100b, expressed it in vitro, and used library selection technology to identify a number of aptamers which bind to distinct epitopes on S100b. We are collecting clinical blood samples to quantify S100b levels in early-stage melanoma patients using conventional ELISA assays, and banking the balance of the specimens for testing with the sensor platform. With regard to the sensor platform: 1) We used spatially confined electric fields to assemble different populations of DNA-derivatized nanowires to desired position with accuracy that enabled post-assembly fabrication of contacts to each individual nanowire, with high yield and without loss of function; 2) We demonstrated selective functionalization of Au tips on Rh nanowires. DNA sandwich hybridization assays were performed on the Au segments; this is an attractive as a means to limit AuNP binding to the tips of nanowire cantilevers; 3) We demonstrated on-chip integration of axially-doped silicon nanowire field effect devices, and showed stable and reproducible changes in electrical conductance of devices with pH; and 4) Directed assembly of integrated arrays of individual nanowire devices with a functioning multiplexed silicon MOS transistor array, and showed that it is possible to read out the electrical state of thousands of devices in parallel. We plan on continuing platform development, with a goal of developing electrical readout strategies, and to begin preliminary sample analyses.

Developing a Single Cell Growth Assay Platform for Monitoring Response to Cancer Therapies

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Changes in cell growth kinetics are a hallmark of cancer cells. Anti-cancer therapies often attempt to non-specifically inhibit the growth of rapidly dividing cells by anticipating a greater impact on cancerous versus normal cells. Mechanistically, cancer cells' changes in growth patterns and rates may originate with alterations in growth signaling networks (e.g., mutations in constituent proteins, changes in protein abundances, etc.). Numerous 'targeted' therapeutics are designed to halt the growth of a tumor via specific disruptions in these networks. However, an obstacle to the widespread use of targeted therapeutic approaches is their lack of generality; while highly effective in some patients, targeted agents may have little or no benefit to other patients with seemingly the same gross tumor type. For example, geftinib produces an objective response in approximately 10-15% of patients with advanced non-small cell lung

cancer. Our studies here will be focused on developing an assay platform for measuring single cell growth with the aim of rapidly assessing the efficacy of therapeutic agents on a patient-by-patient basis. We are currently developing methods that will allow mass accumulation, volume and fluorescence to be measured simultaneously in real-time from a single cell. Therapeutic agents will be either delivered to a single cell during the measurements, or to a population of cells that will subsequently be measured. Once validated, we will determine if this measurement approach can be used to classify the response of cancer cells to pathway-directed therapeutic agents. Our approach for single cell analysis is based on a sensing technology known as the suspended microchannel resonator (SMR) that can achieve femtograms precision (1Hz measurement bandwidth) in fluid which is over a million-fold more sensitive than previous methods. This will ultimately enable the growth rate of a mammalian cell that divides in 10 hours to be measured in just a few minutes. In SMR detection, a cell is weighed in real-time as they flow through a vibrating suspended microchannel. What separates the SMR from other resonant mass sensors is that the solution environment is confined within the resonator itself; this allows it to oscillate in a vacuum environment where there is minimal loss due to viscous damping. Elucidating the molecular basis of the growth response (here defined as changes in mass, volume and/or density over time) will be an important factor in clinical decision-making. For example, it may be necessary to differentiate non-specific cytotoxicity from alterations in pathway activation.

Consequently, we believe the integrated measurement of cell growth and cell-surface protein expression may lead to extremely sensitive tools for allowing both determination of cancer therapeutic efficacy and the molecular mechanism of that response. Proteomics studies have discovered sets of proteins known to change abundance in response to geftinib intervention. In addition, the basal levels of these proteins differentiate geftinib responsive tumors from non-responsive tumors and thus reveal molecular differences between cell types. The relationship between changes in protein markers and changes in physiological properties of cells has not been previously possible to characterize. As our method will have the capability to simultaneously monitor single cell fluorescence, it will be possible to correlate growth measurements to the expression of cell surface receptors.

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 the 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. Considering numerous very effective therapies, including cisplatin, induce DNA damage, one pathway that is directly related to how individuals respond to certain therapeutic treatments is DNA repair. In the context of cisplatin based cancer chemotherapy, reduced DNA repair capacity is associated with increased sensitivity, while increased repair activity is associated with resistance. The goal of the research is to develop an ELISA-based assay to accurately determine DNA repair capacity in cancer tissue, focusing on the nucleotide excision repair (NER) pathway. 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 we are developing novel methodologies to determine the extent of specific posttranslational modifications of key NER proteins and actual repair activity. Our initial experiments involved detection of RPA and XPA in an ELISA format with immobilized DNA. In these experiments we are able to Detect 10 ng of purified XPA with a signal 5-fold above background. ELISA detection of DNA repair protein complex formation was performed and titrations

of XPA and RPA binding assessed to a double strand cisplatin-damaged DNA substrate. Detection of XPA at 10 ng of protein is greater than 10-fold above background. We also assessed purified XPA to determine selectivity for cisplatin-damaged DNA. Using a 60-bp duplex DNA substrate with a single cisplatin lesion we are able to obtain three-fold selectivity for the cisplatin damaged DNA compared to undamaged DNA. In conjunction with RPA the selectivity of XPA is increased to greater than 3.4-fold. NER complex formation was first detected with RPA on duplex DNA containing a single cisplatin lesion in comparison with undamaged DNA. The result of this analysis demonstrate that RPA can be detected in a damage specific fashion with a greater than 5-fold selectivity and a signal to noise ratio greater than 20 at 10 μg and at a ratio of 50 with 100 μg of total protein. The results are significant in the demonstration that a solid phase assay is sufficient to allow detection of DNA repair proteins at the levels desired. The signal to noise ratio allow the detection of these protein at levels present in extracts obtained from cultured cells and likely tumor biopsies. The specificity of the assay is also significant in that the antibodies employed thus far do not cross react with cellular proteins under the conditions we have established. Thus we have decreased the potential for identifying false positives in terms of DNA repair proteins capacity and expression.

Diagnostic Analyses of Protein Interactions: Interaction Network Profiling by Proximity Ligation With Dual-Tag Microarray Read-Out

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Biological processes are regulated and executed by cellular networks of proteins and other macromolecules. Monitoring the interaction networks in clinical specimens could therefore provide important information to diagnose disease and follow disease progression or responses to drug treatment. However, methods have been lacking for high-throughput profiling of endogenous protein-protein interaction networks in clinical samples and in primary tumor cultures.

The proximity ligation assay (PLA) is a technique that enables sensitive detection of individual or sets of interacting proteins (Fredriksson et al., 2002). In PLA two or more antibodies conjugated to DNA oligonucleotides (Gullberg et al., 2004) bind individual target molecules or different members in molecular complexes. Upon co-localization of two antibodies due to proximal binding to the same or interacting protein molecules, the two oligonucleotides will be brought near each other, allowing them to be joined by ligation. The ligation products serve as reporters that can be amplified and detected using standard nucleic acid methods. PLA has been applied for detection of multiple protein biomarkers in patient plasma samples (Fredriksson et al., 2007) to diagnose single pathogen agents (Gustafsdottir et al., 2006) and to reveal protein interactions (Schallmeiner et al., 2007) and their inhibition by low molecular weight compounds (Gustafsdottir et al., 2008). In situ PLA is a variant of the technique that allows single molecular interaction events to be visualized in cell cultures and tissue sections (Söderberg et al., 2006).

By integrating PLA with a dual tag microarray (DTM) readout technology (Ericsson et al., 2008), we are now developing a strategy for profiling multiple endogenous protein-protein interactions. The method can enable parallel measurement of sets of binary protein-protein interactions in genetically unmodified cells as well as in clinical samples like serum or tissue sections. We are demonstrating the use of the platform for interrogating interaction network dynamics among members of the NF-κB protein family. The method exhibits excellent specificity and limits of detection as we are currently detecting two-fold changes in numbers of interaction in less than 100 cells. With our broad repertoire of proximity ligation-based assays, interactions of interest found in this multiplex assay can be further studied and validated, with the final goal of gaining new knowledge about the mechanisms behind cancer and developing new diagnostic tests.

Differential Protein Expression Analysis of Early-Stage, Recurrent/Non-Recurrent Breast Cancer

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Expression Pathology Inc., Rockville, Maryland

This Phase 1 SBIR grant seeks to discover proteins that consistently correlate with specific stages of early stage breast cancer and that can be exploited to give 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.

Expression Pathology Inc. (EPI), in collaboration with the Clinical Proteomics Facility-University of Pittsburgh School of Medicine, has applied mass spectrometry-based global protein expression analysis directly to formalin fixed breast cancer tissue in order to identify candidate proteins that can molecularly distinguish between early stage, recurrent breast cancer and early stage, non-recurrent breast cancer. Multiple candidate biomarkers were discovered that could provide for advanced diagnostic, prognostic, and therapeutic protein biomarkers for breast cancer.

This was accomplished by mass spec proteomic profiling of Liquid Tissue® lysates from microdissected cancerous epithelium collected directly from 26 formalin fixed breast cancer tissue blocks. One set of 9 samples was from patients whose primary breast cancers demonstrated metastatic disease at time of presentation (Stage 3). A second set of 8 primary breast cancer tissue samples were from patients whose primary cancers showed no spread to the surrounding lymph nodes at time of presentation and who, after long term follow-up, remain cancer free (Stage 1). A third set of 9 samples were from patients whose primary tumors showed no surrounding lymph node involvement at time of presentation (Stage 2), which were then further subdivided into 2 sets where one set (5 samples) is from patients that showed recurrent disease and the other set (4 samples) where patients remained disease-free for at least 2 years. This last set of 9 is the test set whereby data collected from analysis of Stage 3 and Stage 1 primary tumors might be able to predict the propensity for recurrent disease in these Stage 2 patients.

Each of the 29 Liquid Tissue® protein lysates was analyzed on a ThermoFisher LTQ-Orbitrap linear ion trap instrument and peptides identified by searching against the 10_08 human database. A range of 4,000 to 7,000 peptides were confidently identified across all tissue samples. Total protein IDs showed a range of approximately 2,000 to >3,500 across all samples, with a range of 400 to 700 proteins identified by 2 or more peptides across all samples. Non-label, quantitative protein expression data was developed using the spectral count method. Differential protein expression data was developed by subtractive analysis and clustering. Results indicate identification of multiple candidate proteins that define each stage of breast cancer as well as providing for protein biomarkers than can potentially determine the aggressive behavior of early stage breast cancer. In addition to differences in protein quantitation, differential phosphorylation of specific peptides was found. A range of 300 to 480 phosphorylated peptides were identified per sample across the entire tissue set and specific phosphorpeptides were found to correlate with specific stages of breast cancer. These results provide for additional candidate biomarkers of breast cancer. The most significant protein differences are currently being confirmed by IHC and SRM analysis of the same tissues.

The use of formalin fixed tissue was critical to this project because specific, defined cancer tissues were more easily assembled from formalin fixed tissue banks than could have been collected from frozen tissue banks, and the combination of Expression Pathology's novel, patented technology with high-resolution mass spectrometry from the Clinical Proteomics Facility-University of Pittsburgh School of Medicine provided for highly valuable protein expression data about breast cancer staging. Differential quantitative and qualitative protein expression analysis and candidate protein biomarkers of early stage, late stage, and recurrent breast cancer will be discussed.

DNA-Templated Semiconductor Nanocrystals: One-Step Synthesis of Cancer Cell Specific Lumiphores

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Bright, photostable luminescent labels are powerful tools for the imaging of biological events *in vitro* and *in vivo*. Semiconductor nanocrystals have emerged as attractive alternatives to commonly used organic lumophores due to their high quantum yields and the spectral tunability that can be achieved through synthetic control. While conventional synthetic methods generally yield high-quality nanocrystals with excellent properties for biological imaging, ligand exchange and biological conjugation are necessary to make nanocrystals biocompatible and biospecific. These steps can result in substantial deterioration of optical characteristic of these nanocrystals. Moreover, the complexity of multistep nanocrystal synthesis, typically requiring inert and anhydrous conditions, prohibits many end users of these lumiphores from generating their own custom materials. We sought to streamline semiconductor nanocrystal synthesis and develop synthetic routes that would be accessible to scientists from all disciplines. In search of such an approach we turned to nucleic acids as a programmable and versatile ligand set,[1-3] and found that these biomolecules are indeed appropriate for biocompatible semiconductor nanocrystals preparation. In this presentation we present a summary of our work on nucleic acids-programmed nanocrystal synthesis that has resulted in the successful development of a one-step synthesis of biofunctionalized nanocrystals in aqueous solution.[4] We show that, according to the design of the biorecognition domain of each DNA ligand, the nanocrystals can specifically bind DNA, proteins, or specific cancer cell types having known surface recognition markers.

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Edgetic "Perturbation Models of Human Disease"

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Cellular functions are mediated through complex systems of macromolecules and metabolites linked through biochemical and physical interactions, represented in interactome models as “nodes” and “edges”, respectively. Better understanding of genotype-to-phenotype relationships in human disease will require modeling of how disease-causing mutations affect systems or interactome properties. We investigated how perturbations of interactome networks may differ between complete loss of gene products (“node removal”) and interaction-specific or edge-specific (“edgetic”) alterations. Global computational analyses of ~50,000 known causative mutations in human Mendelian disorders revealed clear separations of mutations likely corresponding to node removal versus edgetic perturbations. Experimental characterization of mutant alleles in various disorders identified diverse edgetic interaction profiles of mutant proteins that correlated with distinct structural properties of disease proteins and disease mechanisms. Edgetic perturbations seem to

confer distinct functional consequences from node removal since a large fraction of cases where a single gene is linked to multiple disorders can be modeled by distinguishing edgetic network perturbations. Edgetic network perturbation models might improve understanding of dissemination of disease alleles in human populations and development of molecular therapeutic strategies.

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 to understand 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, either due to the location of the interaction (Y2H) or due to 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 biological-relevant protein interactions bypassing the limitations of present 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) screen method for identification of novel protein-protein interactions, including cytoskeletal and membrane proteins, in mammalian cells allowing native protein folding and post translational modifications. In ReMTH, bait protein is fused to one fragment of a rationally-dissected fluorescent protein, such as GFP. The second, complementary fragment of GFP is fused to an endogenous protein by the 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 screens 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 have confirmed multiple novel interactions by Co-IP. We also estimated the efficiency of the ReMTH screen technology and will improve the technology to reduce the false positives rate. The fully developed technology will identify functional protein-protein interactions more efficiently than present methods and 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/weak interactions such as enzyme/substrate interactions allowing identification of components of signaling pathways and networks in cancers previously undetectable. Thus the technology will uncover functional protein-protein interactions not detectable by other approaches and advance our understanding to protein functions and signaling networks in cancer.

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Elevated Hydrostatic Pressure Promotes Protein Recovery From Formalin-Fixed, Paraffin-Embedded Tissue Surrogates

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Introduction: High-throughput genomic and proteomic methods hold great promise for developing knowledge of the molecular characteristics of cancer, which can be translated into practical interventions for the diagnosis, treatment, and prevention of this disease. When fresh or frozen tissue is used for proteomic analyses, the results cannot be related directly to the clinical course of diseases. If routinely fixed and embedded archival tissues could be used for standard proteomic methods such as 2-D gel electrophoresis and mass spectrometry (MS) these powerful proteomic techniques could be used to both qualitatively and quantitatively analyze large numbers of tissues for which the clinical course has been established. However, the analysis of archival FFPE tissues by high-throughput proteomic methods has been hampered by the adverse effects of formalin fixation, specifically formaldehyde-induced protein adducts and cross-links that are formed during tissue fixation and subsequent histological processing. Age and condition of the archival samples may also affect the protein extraction efficiency and the quality and reproducibility of the proteomic analyses.

Methodology: We have recently demonstrated the use of high hydrostatic pressure as a method for efficient protein recovery from FFPE tissue surrogates, which are model tissue plugs formed from one or more cytoplasmic proteins. High-pressure experiments were conducted at 65–100°C under a pressure of 45,000 psi in a 3-ml capacity stainless steel reaction vessel coupled to a manually operated HiP High Pressure Generator (High Pressure Equipment Company, Erie, PA, USA). The sample incubation temperature was regulated with a Eurotherm 2132 temperature controller (Leesburg, VA, USA) connected to an aluminum heating collar surrounding the reaction vessel. An inline Gilson model 303 HPLC pump (Middleton, WI, USA) supplied the buffer to be pressurized. After extraction at elevated pressure for 2-18 hours, the tissue surrogate extracts were separated by SDS-PAGE and analyzed. In-gel digestion of individual bands and mass spectrometry were also performed.

Results: Reversal of formaldehyde-induced protein adducts and cross-links was observed by SDS-PAGE when lysozyme tissue surrogates were extracted at 45,000 psi and 80–100°C in Tris buffers containing 2% sodium dodecyl sulfate and 0.2 M glycine at pH 4. These conditions also produced peptides resulting from acid-catalyzed aspartic acid cleavage. Additives such as trimethylamine N-oxide or copper (II) chloride decreased the total percentage of these aspartic acid cleavage products, while maintaining efficient reversal of inter-molecular cross-links in the FFPE tissue surrogates. Mass spectrometry analysis of the recovered lysozyme yielded 70% sequence coverage, correctly identified all formaldehyde-reactive amino acids, and demonstrated hydrolysis at all of the expected tryptic cleavage sites.

Future Directions: This study demonstrates that elevated hydrostatic pressure treatment is a promising approach for improving the recovery of proteins from FFPE tissues for proteomic analysis. The strengths of this process are improved overall protein recovery, improved protein identification and the potential to recover whole proteins from FFPE tissues. Further studies with high pressure extraction will utilize 2-D gel electrophoresis and MS to compare protein extracts from fresh-frozen breast cancer tumors with those recovered by high pressure treatment of FFPE tissue. Proteomic analyses will be conducted comparing (1) a fresh tissue lysate with (2) a matched FFPE breast tissue extracted under elevated pressure or at atmospheric pressure and (3) the protein difference map obtained using fresh tissue lysates compared to that obtained from matched FFPE tissue. This technology will ultimately be used for MS and immunologically based biomarker discovery.

Enhanced Stain Penetration in Tumor-Bearing Tissue for Microscopic Analysis

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We are investigating chemical and biophysical methods to enhance penetration of fluorescent labeled molecules and antibodies in biospecimens. The goal of this project is to develop and refine specimen preparation protocols to facilitate extended depth interrogation of stem cells and microvessel features in tumor-bearing tissue. Stem cells are characterized by their ability to self renew and to give rise to cells that differentiate along a specific pathway. Cancer stem cell theory hypothesizes that only a small subpopulation of tumor cells, termed cancer stem cells or tumor initiating cells, have the ability to continuously self renew, divide asymmetrically and give rise to the bulk of tumor tissue.

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. Melanoma B-16 tumor cells were implanted into a GFP transgenic mice where TRITC-dextran or tomato-lectin administered by tail vein injection was used for microvessel decoration. Extended stain penetration protocols were applied to tumor-bearing tissue pieces 3-5 mm in thickness to probe for the distribution of stromal stem cells.

Immunodecoration of subcutaneous GFP positive B16 melanoma produce microscopic image data useful to assess the nature and distribution of stromal stem cells in context with microvessels in the tumor margin.

The extended depth penetration protocol produce promising results, however success remains limited due to the duration (days) required. Therefore, we are investigating biophysical parameters using tuned radiofrequency energy to shorten time of staining, improve specificity and reduce background in tumor-bearing tissues. This approach has the potential to advance biospecimen sample preparation methods used in cancer and stem cell biology.

Evolution of Histone-Specific Aptamers for Recognition Imaging Microscopy

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Aptamers, pieces of single-stranded DNA or RNA that fold into structures with binding sites that are complementary in shape and charge to target antigens, provide an attractive alternative to traditional antibodies as possible affinity reagents for large-scale proteomics research. Because these molecules can be produced *in vitro* using test-tube evolution methods, their recognition properties can be tailored to bind almost any type of molecular target. Aptamers have been selected to bind ions, small molecules, drugs, peptides, proteins, and even whole cells. Despite these advances, very few aptamers have been selected to bind protein post-translational modifications (PTMs). Here we describe the evolution of aptamers selected to bind specific histone PTMs and explore the use of these affinity reagents in the atomic force microscopy technique known as recognition imaging microscopy.

Exploring Stromal-Carcinoma Cell Interactions in Breast Cancer via Microscale Three-Dimensional Co-Culture Systems

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Reciprocal interaction between carcinoma and stromal cells contributes to tumor initiation and progression. However, tumor heterogeneity between patients suggests the need for improved tools that can examine stromal-carcinoma interactions using a screening approach. We have developed microscale three-dimensional (3D) co-culture systems to study patient-specific stromal-carcinoma interactions in breast cancer. The systems require small numbers of cells (1,800 cells per channel) enabling primary cell-based assays with multiple treatments per patient sample, and device design can be tailored (e.g. mixed co-culture, compartmentalized co-culture) facilitating higher content assays.

We first optimized a 3D collagen culture platform (an array of microchannels with one input/one output) to establish a robust system by controlling polymerization parameters, and immortalized human mammary fibroblast (HMF) cells were tested. Sample loading and media changes were accomplished via surface-tension driven pumping. Second, HMF cells and T47D breast carcinoma cells are co-cultured in the system to validate existing biology. The growth of T47D breast carcinoma cells was stimulated by the presence of HMF, consistent with previous conventional studies. This mixed co-culture system is used to examine candidate molecules using cancer-associated fibroblasts (CAF) and normal fibroblasts (NF) from individual patient samples to explore the heterogeneity of breast cancer. Last, the microchannel design was modified (an array of microchannels with three inputs/one output) to enable compartmentalized culture - loading T47D cells in a center compartment and CAF and NF cells in each adjacent side compartment via pumping them through three different inlet ports. Using this system, we observed differences in migration toward the center T47D region between CAF and NF, and increased T47D growth was observed at the CAF interface compared to NF interface, which showed patient-dependent.

In conclusion, we have demonstrated two co-culture systems (for mixed and compartmentalized culture) to study stromal-carcinoma cell interactions. The systems lay the foundation for high content high throughput 3D cellular screening by incorporating an array-based approach and surface-tension driven pumping that is compatible with existing automated liquid dispensing systems.

Fabrication of Miniature Electrodes for Monitoring Extracellular Metabolites

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The goal of our project is to integrate miniature biosensors for glucose and lactate detection with cancer cells in order to monitor how cellular consumption/release of these metabolites changes in response to therapeutic agents. Standard semiconductor fabrication techniques were combined with novel biomaterial fabrication approach to integrate arrays of gold electrodes with enzyme-carrying hydrogel microstructures. Hydrogel microstructures were composed of poly(ethylene glycol) (PEG) and contained glucose oxidase (GOX) or lactate oxidase (LOX) enzymes as well as molecules that made the hydrogel conductive. Importantly, microfabrication approaches allowed encapsulating GOX and LOX in distinct hydrogel structures and positioning these structures on adjacent miniature electrodes separated by tens of micrometers. Electrochemistry was used to determine sensitivity, response time and stability of the enzyme electrodes. The hybrid biomaterial/goal electrodes fabricated by us have several advantages: 1) PEG hydrogel is a non-fouling material that can be used to control/guide cell attachment on the surface. This ensures that cells attach in pre-defined locations right next to the electrodes, obviating the need to manipulate the position of

an electrode. 2) PEG hydrogel is also an excellent matrix for encapsulation of functional enzymes. 3) The number of recognition elements incorporated into the same electrode array may be expanded to detect multiple analytes from the same small group of cells. Currently, experiments are under way to integrate these miniature biosensors with cancer cells inside microfluidic devices to monitor in real-time changes in glucose/lactate fluxes in response to stimulation with model anti-cancer therapeutics.

Free Solution Conjugate Microchip Electrophoresis for Mutation Detection and Scanning Assays

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Colorectal cancer (CRC) represents the third-leading killer amongst all cancer-related diseases in the US. While a number of biomarkers and technologies have been extensively evaluated for the management of this disease, few have emerged for use by clinicians in diagnosing/prognosing CRC with the predominant screening methods still consisting of monitoring blood in the stool and/or colonoscopy. In this R21 application, research is focusing on developing electrophoresis platforms that can monitor the absence/presence of molecular biomarkers originating from nuclear DNA that provide diagnostic/prognostic information. The molecular assays being investigated require high-resolution electrophoresis for reading out the results. DNA is typically separated by electrophoresis in a viscous sieving matrix using fused-silica capillaries or microchips (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 free from contamination issues and 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 these problems, the gel must be reloaded between every run and these gels can be expensive. The elimination of sieving matrices and the development of free-solution electrophoresis to sort DNA would dramatically decrease the cost associated with electrophoresis-based molecular assays as well as simplify system set-up and reduce run time. Since both the charge and the friction scale linearly with DNA 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 to negate the need for a sieving matrix, 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 using replication technology will be used for several molecular diagnostic assays, including the Ligase Detection Reaction (LDR) for scoring the presence of known point mutations in *K-ras* oncogenes and an EndoV/LDR assay, a mutation scanning assay to score the presence of sporadic p53 mutations. In addition, microsatellite instability (MSI) will also be evaluated using μ-CE. The analysis of MSI is undertaken using a panel of markers from nuclear DNA that are PCR amplified and subsequently analyzed via electrophoresis. Comparisons of electrophoretic mobilities of diseased tissue versus normal tissue provide an indication of MSI status, which can be used as a genetic prognosticator for determining effective therapies for treating CRC patients.

A Generic Microfluidic Platform for Ultrafast Genotyping: Sample-In/Answer-Out Capabilities That Revolutionize Clinical Diagnostic Analysis

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A Microfluidic Genetic Analysis (MGA) system capable of genetic analysis with sample-in/answer-out capabilities must be able to accept real-world samples, execute multiple, sequential sample preparation steps, and then provide an interpretable read-out following separation and detection. These processes involve chromatographic separation of sample components for isolation of DNA, the enzyme-mediated amplification of target DNA sequences in a temperature-dependent manner, the electrophoretic separation

of the products of amplification, and detection by fluorescence. In order to accomplish the tasks within the nanospace of the microfluidic architecture, there has to be exquisite nanoliter volume fluidic control. This is accomplished using nothing more than a single nanoliter-flow syringe pump, a series of elastomeric valves, and restrictive flow built into the microchannel architecture which, collectively, allow for precise control of fluid flow through the sample preparation domains and into the separation domain. The effectiveness of the MGA system for the detection of select infectious disease agents (*B. Pertussis*; *B. Anthracis*) from multiple sample types (nasal swab, blood, nasal aspirate) in microliter (and sub-microliter) volume samples has been shown. In addition, the potential for interrogation of human genomic DNA for mutations associated with the diagnosis of T-cell lymphoma will be discussed. Together these represent a microchip capable of sample-in/answer-out analysis - a *bona fide* micro-total analysis system – and a technology ripe for translation in the clinical medicine sector. However, much work remains to be done with respect to simple valving solutions for accurate fluidic control, minimizing the external hardware for packaging into a portable system and improved detection.

Genome Sequencing Technology for Studying Melanoma

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We have made significant progress on further developing polony sequencing technology during the past year. Namely, we have currently sequenced ~24 invasive Group A Streptococcus (GAS) clinical isolates. The GAS strains we have sequenced served as a test system for optimizing the sequencing library construction, sequencing biochemistry, basecalling algorithms, and data analysis pipeline. Our preliminary efforts have proved very successful and our major accomplishments are summarized below. (1) We have developed an optimal genome sequencing library construction protocol. Our original protocols were barely adequate for sequencing bacterial genomes due to stochastic bias in the construction, which resulted in only ~70% genome coverage. We have identified the inefficient steps and optimized the entire protocol, now we are able to generate libraries that are sufficient for human genome sequencing. (2) We have optimized the sequencing biochemistries for high quality sequencing. The optimal biochemistry allows for 30 base reads. We are now moving toward 50 base reads. (3) Our basecalling algorithms have been optimized to maximize the amount of sequence obtained from a sequencing run. Currently we are able to identify and call bases from ~70 million beads per lane in a sequencing run. We have the potential to run 16 lanes simultaneously.(4) We have now established a data analysis pipeline that takes the raw data from the polonator and maps the raw reads to a similar known genome and makes final assembled base calls. The final assembly calls every base with a probability, or P-value. Therefore, in the end we can call SNPs or somatic mutations with a defined P-value. Specifically, at this point in time, we have obtained patient blood samples, and generated mate-paired genome sequencing libraries. We have initiated sequencing of the libraries and are on schedule to complete the sequencing within the next 2-3 months.

High-Resolution DNA Methylation Profiling Using Methylated CpG Island Amplification and Massively Parallel Sequencing

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Progress in understanding epigenomic dysregulation in human disease will require rapid and affordable analysis and comparison of a large number of samples. It is currently unrealistic to perform whole-genome DNA methylation analysis at single CpG resolution on a large number of human samples. Methylated CpG island amplification (MCA) is a DNA library construction technique that permits amplification of 146,148

methylated CpG rich regions throughout the whole genome. Briefly, genomic DNA is digested with a methylation sensitive restriction enzyme (SmaI) that digests unmethylated CCCGGG sites but leaves methylated sites intact. The DNA is then digested with a SmaI isoschizomer (XmaI) that is not methylation sensitive (i.e., cutting is not blocked by methylation). Importantly, unlike SmaI, XmaI digestion leaves a 5' overhang (sticky ends). Subsequent ligation-mediated PCR results in amplification of the genomic fraction corresponding to methylated DNA. We have demonstrated that coupling MCA with microarrays (MCAM) enables excellent sensitivity and specificity in genome-wide DNA methylation analysis, making it very powerful for the high throughput analysis of samples of limited quantity 1, 2. As shown by our computational analysis, an MCA-library presents unique properties in markedly reducing genome complexity (targeting 3% of the genome) while maintaining reasonable good coverage of genes (76% of all genes and 39% of all promoters) and CpG islands (70%). In addition, our detailed annotations of the human MCA-library allows us to compare DNA methylation with known gene transcription start sites, CpG islands and repeats to better understand the biological significance of our results.

We have recently coupled MCA with Illumina Solexa 1G sequencing (MCA-Seq) on three samples: one from normal female PBL, one from a leukemia cancer cell line (Raji), and one from fully methylated DNA after in vitro methylase (SssI) treatment as positive control. We obtained ~2 million sequencing reads that match to genomic location (unique or multiple) from one sequencing run on a single lane. As expected, about 60% of the reads match to multiple genomic locations, indicating that most fragments were derived from methylated repetitive regions across the genome. Among the reads that could match uniquely to genomic locations, >90% align to our in-silico MCA library (based on SmaI/XmaI genomic intervals), indicating the specificity of this method. When we compared non-CpG island DNA methylation across the normal (PBL) and cancer (Raji) genomes, we found no significant differences; a high level of methylation was found in both samples, comparable to that of the fully methylated control (SssI). In contrast, the mapping patterns of CpG island methylation were remarkably different in normal and cancer cells. The global methylation of CpG islands is significantly increased in cancer cells relative to that in normal blood (with the exception of the X chromosome). This was anticipated, since this normal blood sample is from a female individual in which X-chromosomal CpG islands are methylated due to X-inactivation. Finally, to evaluate the quantitative ability of MCA-Seq we compared our MCA-Seq data with bisulfite-pyrosequencing for 20 genes in Raji and PBL. We found an excellent correlation (R2=0.76), demonstrating the highly quantitative ability of the method.

Clearly, combining MCA with Illumina Solexa sequencing performs well in terms of specificity (few false positives and low background of non-targeted sequences); however, we recognize several limitations. Currently we are applying various strategies to optimize MCA-Seq to improve coverage and minimize the quantity of initial DNA required. Additionally, we will build quality controls for MCA-Seq and develop optimized algorithms for data analysis. The goal of this project is to develop a simple, robust, and reliable genome-wide DNA methylation method, which will have broad utility for epigenomic studies.

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High-Throughput High-Content Cell Screening in a Microfluidic Device

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A comprehensive, systems level understanding of cell signaling networks requires methods to efficiently assay multiple signaling species, at the level of single cells, responding to a variety of stimulation protocols. Here we describe a microfluidic device that enables quantitative interrogation of signaling networks in thousands of individual cells using immunofluorescence-based readouts. The device is especially useful for measuring the signaling activity of kinases, transcription factors, and/or target genes in a high throughput, high content manner. We demonstrate how the device may be used to measure detailed time courses of signaling responses to one or more soluble stimuli and/or chemical inhibitors as well as responses to a complex temporal pattern of multiple stimuli. Furthermore we show how the throughput and resolution of the device may be exploited in investigating the differences, if any, of signaling at the level of a single cell versus at the level of the population. We also outline our current efforts to use this technology to develop diagnostic and prognostic tests of human cancers.

High-Throughput Intracellular Microrheology: A New Tool for Cancer Research

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Cancer mortality and morbidity are critically related to tumor invasion and metastasis in which the molecular mechanisms are poorly understood. Until their etiology is better revealed, attempts to develop new cancer therapeutics would remain empirical. Cell motility, which drives cancer metastasis, involves dynamic and regulated re-arrangements of the cytoskeleton. Our work and that of several other groups have shown that cytoskeleton phenotypes are typically accompanied by drastic changes in the viscoelastic properties of the cytoskeleton, which in turn modulate the ability of the cytoskeleton to generate net pushing forces at the leading edge and allow the cell to change its shape.

Changes in cell mechanical properties have long been predicted to correlate with metastatic potential. However, current cell-mechanics approaches suffer from serious drawbacks - including time of measurement, lack of multiplexing, ambiguity of measurements - which prevent a direct test of this important hypothesis. The objective of this study is to: develop a highly-optimized high-throughput ballistic injection nanorheology (htBIN) technological platform to measure the micromechanical properties in cancer cells rapidly (< 30 s per cell) and reliably, and to assess these biophysical properties as a function of cell migration and invasion by comparing ovarian cancer cells of low and high invasive nature to normal cells, all obtained from patients at the Johns Hopkins Hospital. The proposed instrument, which is based on multiple-particle microrheology, presents key advantages over current approaches to cell mechanics. Our device will serve as a new tool for cancer research to study cell mechanics in the context of cancer cell migration and adhesion and may ultimately serve as a diagnostic tool for patients who are at high risk for ovarian cancer, complementing more conventional biomolecular markers of cancer in a clinical setting.

While our proposed approach to cell mechanics is a priori applicable to detect intracellular mechanical differences in any type of cancer cells, a primary focus of this project is ovarian cancer. Ovarian cancer was selected as the disease model in this study because it represents one of the most aggressive cancers in women.

Identification of Areas of Oxidative Damage in Human Genomic DNA

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This project seeks to develop a technique to determine the distribution and relative density of oxidative damage that yields abasic (AP) sites in specific regions of the genome. Current methods permit the determination of the overall number of abasic (AP) sites in genomic DNA using slot blots, the presence of hotspots for AP sites in DNA fragments using ligation mediated PCR, and the random or clustered distribution of AP sites (using 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 electron microscopy (EM) or using a fluorescently tagged secondary antibody to visualize AP sites by confocal laser scanning microscopy (CLSM). The EM studies showed the distribution of AP sites to be non-random with about 50% of AP sites clustered in a small fraction of DNA fibers. These observations of AP site clusters suggest that there are locations in the genome that are preferentially vulnerable to AP site formation. In originating this project we set the following milestones to be achieved to prove the feasibility of our approach: 1) to shown that we could quantify fluorescently tagged AP sites on extended DNA fibers to within 10% of those detected by the slot blot technique that is the current standard for detection of AP sites, and 2) to show that the number of AP sites detected would increase when AP sites were induced by exposure of cells to hydrogen peroxide (H_2O_2) in cell culture.

To quantify the number of AP sites within genomic DNA using the methods we proposed we lysed cells directly onto siliconized glass slides, straighten and align the genomic DNA using a method called fiber spreading, identified the AP sites with biotin labeled aldehyde-reactive probe with a red fluorescent tag, and stained genomic DNA with a green fluorescent DNA-specific dye. Initially images of the labeled extended DNA fibers were analyzed manually in areas of the slide that contained green fluorescence (DNA). Image analysis determined the lengths of DNA scored and the number of AP sites contained in that DNA. We improved the quality of the image analysis by determining algorithms for suppression of background and validating the images of AP sites. The low signal thresholds levels were adjusted to minimize the background while retaining the signal for valid AP sites. Using this approach, we analyzed over $1x10^9$ nucleotides (nt) of DNA and determined that this control DNA contained 5.4 AP sites per 10^6 nt (which is within the range found by slot blot and EM analysis). When we analyzed $7x10^8$ nt from cells exposed to 20 μM H_2O_2, we found the number of AP sites to have risen to about 7 AP sites per 10^6 nt (which is also consistent with slot blot analysis). In both untreated and treated cells, we observed many AP sites to be clustered. We then labeled sites of DNA replication and we were able to determine that newly replicated DNA contained 8.2 AP sites per 10^6 nt under normal conditions, whereas in cells exposed to 20 μM H_2O_2, the number of AP sites in newly replicated DNA increased to 16.6 AP sites per 10^6 nt. The accomplishment of our proposed goals require us to use fluorescence in situ hybridization (FISH) to identify specific DNA regions and visualize AP sites at those locations. Recent FISH studies have allowed us to discover six adjacent origins of replication in a 340 kb segment of DNA in 1p36. Focusing our analysis of AP sites in this genomic region identified by FISH would enable us to determine the extent and distribution of AP site formation in relation to the origins of DNA replication or transcriptional promoters already mapped to this region. Such an approach would allow us to determine DNA lesion formation during normal cell metabolic processes (e.g., replication); and those formed following exposure of cells to carcinogens or to oxidative stress. In addition, studies are ongoing to automate the processes of fluorescent staining, slide imaging and fiber image analysis to increase throughput of our studies.

Identification of FADD-Kinase Inhibitors Using a Live Cell Reporter

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FADD expression has been linked to poor clinical outcome in a number of cancers. A causal relationship between the phosphorylation of FADD and NF-κB activation, a hallmark of an aggressive and therapy resistant cancer phenotype has also been demonstrated. Therefore, we hypothesized that inhibiting FADD phosphorylation in tumor cells may reverse the transformed phenotype. To aid in experimentation of this hypothesis, we have developed a FADD kinase reporter (FKR). The sensitivity and specificity of FKR has been demonstrated. We have also performed a live cell based high throughput screen to identify small molecules that target FADD phosphorylation. These lead compounds validate FADD kinase activity as a novel target for anticancer therapies and provide novel insight into the molecular basis of FADD phosphorylation and overexpression in cancer.

Imaging Tumor Stroma Associated Fibroblast Activation Protein

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Activated tumor stromal fibroblasts are needed for tumor growth since the very early phase. On the outer surface of these activated tumor stromal fibroblasts, 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 exo-dipeptidyl peptidase activity which is extremely similar to the activity of dipeptidyl peptidase-IV (DPP-IV). DPP-IV, unfortunately, also circulated in blood stream, making in vivo imaging of FAP challenging. Recently we have found that endo-peptidase activity of FAP is a better choice for probe design, because DPP-IV lacks endo-peptidase activity. Screening a series of peptide substrates against FAP has found Gly-Pro is an excellent substrate for endo-peptidase cleavage. Proteolytical activation of imaging probes with various repeats of Gly-Pro suggested that single Gly-Pro is as good as four Gly-Pro repeats, but with slower kinetics. In vivo experiment with xenographic tumor model indicated that FAP activity could be clearly imaged in less than 2 hours. Treatment effect could also be imaged in real rime. Further optimization, evaluation and application of the developed molecular probes are in progress.

In-Depth Characterization of Cancer Genomes Using Paired-End-Tag (PET) Technologies

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Human cancers frequently carry somatically acquired mutations that can be implicated in the development of the disease. Beyond point mutations, large-scale genomic rearrangements are also common in cancer and can have drastic implications such as the generation of fusion transcripts that result in fusion oncoproteins. Unfortunately, conventional strategies for characterizing such rearrangements are either low-throughput or have low resolution. We have developed a number of paired-end-tag (PET) sequencing strategies that can provide a global map of all such structural changes at both the RNA and DNA level in a high throughput and cost effective manner. By applying these technologies and reaching up to 100X physical coverage, we have constructed comprehensive karyo-genomic maps of 2 normal samples, 5 breast cancer tumors and 2 breast cancer cell lines. The comparative analysis of these data sets revealed markedly different patterns of mutations distinguishing germline structural variants from the somatic rearrangements observed in tumor samples and from the ones observed in cell lines which were characterized by regions of complex amplification. The results demonstrate the feasibility of in-depth comprehensive characterization of rearrangements in cancer.

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

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Receptor tyrosine kinases (RTKs) process extracellular cues by activating a broad array of signaling proteins. Paradoxically, they often use the same proteins to elicit diverse and even opposing phenotypic responses. Binary, "on-off" wiring diagrams are therefore inadequate for explaining their differences. Here, we show that when six diverse RTKs are placed in the same cellular background, they activate many of the same proteins, but to different quantitative degrees. Additionally, by measuring interaction affinities between RTK-derived phosphopeptides and SH2/PTB domains using protein microarray technology, we find that the relative phosphorylation levels of upstream signaling proteins can be accurately predicted using linear models that rely on combinations of receptor-docking affinities and that the docking sites for PI3K and Shc1 provide much of the predictive information. In contrast, the phosphorylation levels of downstream proteins cannot be predicted using linear models. Together, these results show that information processing by RTKs can be segmented into discrete upstream and downstream steps, suggesting that the challenging task of constructing mathematical models of signaling can be reduced to separate, more manageable layers.

Mapping Chromosomal Aberrations in Cancer by Long-Insert Paired-End Massively Parallel Sequencing

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We are developing a method that that maps chromosomal aberrations in cancer using long-insert (40Kbp) paired-end sequencing. The method produces 54bp ditags that can be read on any massively parallel sequencing platform. We have developed a validation benchmark by mapping chromosomal aberrations in the MCF-7 breast cancer cell line using independent technologies at a resolution that allows PCR across rearrangement-induced breakpoint joins [1]. We are currently optimizing a second-generation of the method that is compatible with any next-generation sequencing technology, and is fully supported by turnkey informatics. Our benchmark experiments have already incresed our understanding of the structural instability of the MCF-7 breast cancer sublines, allowing us to reconstruct the sequence of rearrangements in specific widely used sublines and to reconstruct the phylogenetic tree of major MCF-7 sublines. A number of gene fusions of potential sigificance for cancer progression have been discovered and some have been validated in functional assays. We plan to further validate the method biologically by applying it to multiple cancer genomes and by identifying recurrent chromosomal rearrangements relevant for the progression of cancer.

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Mapping DNA in Tissues: A Spatially Resolved Array Technique With Integrated Tissue Transfer, DNA Extraction, Amplification, and Detection

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We have developed a new technique for mapping DNA in tissue sections by using a single-vial tissue transfer, DNA extraction, amplification, and detection process and by performing this process in a parallel array of vials. Our initial results showed that a genomic DNA sequence can be mapped across a prostate tissue section, and that it is possible to adapt macro-scale DNA detection methods to a miniaturized device by consolidating off-chip DNA pretreatment protocols and PCR detection into a streamlined, single vial process. To create the DNA detection map, cells were first transferred from a prostate tissue section into the array of vials, preserving the 2D architecture of the tissue. A new DNA extraction process was then used to break down and immobilize cellular fragments, establishing a clean environment for subsequent PCR amplification in each vial. The amplified DNA in each vial was then detected by staining with a fluorescent dye. In the future this technique could be adapted to map multiple genes at once, to study methylated DNA or mRNA, and miniaturized further to provide pathologists with unprecedented resolution of data regarding molecular heterogeneity in tissues.

Mesoporous Metal Oxide Nanomaterials for Mass Spectrometry-Based Phosphoproteomics for the Molecular Analysis of Cancers

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Reversible protein phosphorylation is a ubiquitous post-translational modification playing a vital role in the control of many biological processes. Aberrant phosphorylation is believed to be one of the underlying mechanisms for cancer. While mass spectrometry (MS) techniques have been successfully applied to determine the phosphorylation state of single proteins/peptides, proteome-wide MS analysis of phosphorylation still poses substantial challenges due to the low abundance of phosphoproteins and substoichiometric phosphorylation. Therefore, isolation and enrichment of the phosphoproteins/peptides are essential for MS based phosphoproteomics. Immobilized metal ion affinity chromatography (IMAC) and microparticles of titanium dioxide (TiO2) and zirconium dioxide (ZrO2) have demonstrated specificity for trapping phosphate. Here we describe the synthesis of large surface area mesoporous ZrO2 and hafnium dioxide (HfO2) nanomaterials for highly selective enrichment of phosphoproteins/peptides for MS-based phosphoproteomics.

The synthesized mesoporous nanostructured materials have small pores on the order of 6 nm with a periodicity of about 10 nm and display small ordered domains of 2D hexagonal structure and a large surface area of 72 m^2/g. Materials were first tested for their ability to enrich peptides from a tryptic digest of a standard phosphoprotein, α-casein. The enrichment using mesoporous metal oxides are extremely effective as shown by the high resolution ESI/FT mass spectra of the protein digest acquired before and after the enrichment. Only 6 phosphopeptides were detected without enrichment, all of which are low abundance peaks owing to ion suppression from abundant non phosphopeptides. In contrast, 18 and 20 phosphopeptides, respectively, corresponding to 20 unique phosphorylation sites were detected in a single mass spectrum with mesoporous ZrO2 and HfO2 enrichment with much higher signal-to-noise ratios. Furthermore, we evaluated the use of these materials with a more complex tryptic digest of a 6-protein mixture (bovine serum albumin, α-casein, troponin C, ubiquitin, ribonuclease B, and β-lactoglobulin). Without enrichment many non phosphopeptides in this mixture dominate the MS spectrum so that phosphopeptides are hardly observable. A single enrichment with mesoporous ZrO2 or HfO2 identified 18 or 20 phosphopeptides, respectively, while both mesoporous HfO2 and ZrO2 materials show highly effective and specific enrichment of phosphopeptides (almost like purification).

Microfluidic Three-Dimensional Scaffold Assay for Cancer Cell Migration and Intravasation

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Migration through extra-cellular matrix (ECM) and intravasation across a cellular barrier comprise the initial, rate-limiting steps of cancer metastasis. Physiologically relevant and well-controlled models that mimic the in vivo tumor microenvironment will enable better understanding of the initial steps of metastasis and evaluation of potential therapy efficacy. In vivo models have physiological relevancy, yet inherently lack a high level of control. In vitro cancer migration models have high levels of control, yet lack critical components of the tumor microenvironment. We propose a new technology, a microfluidic migration and intravasation assay (μMIA). The μMIA replicates essential components of the in vivo tumor microenvironment, including a 3D ECM and a vasculature, while providing tight control of biochemical and biophysical parameters. To further establish the μMIA, we propose to use it to investigate a specific biophysical factor - interstitial flow - which has not previously been studied in the context of metastatic

disease. The objective of the proposed work is to evaluate the metastatic potential of cancerous cells by developing the μMIA and identifying novel extent of invasion metrics (Specific Aim 1), and applying them to study the influence of interstitial flow on cancer cell metastasis (Specific Aim 2). The μMIA will have an input channel for the cancer cells, a 3D collagen gel to simulate native ECM, and an endothelial cell (EC) layer adherent to the gel in a second channel. Sample inputs may range from simple cell models in suspension through biopsy specimens, with sources ranging from known libraries of cells to patient samples. The configuration will permit migration of cancer cells either from the input channel or within the gel towards the second channel. Optimized gel parameters will present appropriate chemotactic gradients and physical parameters simulating a tumor microenvironment and inducing cancer cell migration. The EC layer will mimic the in vivo vascular barrier allowing observation of cancer cell intravasation. Optical access from two vantage points will permit real time observation of cancer cell migration and intravasation. The optical access combined with image processing techniques will quantify cancer cell morphological and migratory parameters, leading to identification of novel extent of invasion metrics that will quantify the metastatic potential of cancer cells. Finally, we will leverage the microfluidic capability of the μMIA to induce interstitial flow across the gel, and quantify the effects of this biophysical parameter on cancer cell invasion. Initial development and application of the μMIA indicates a promising platform to observe aspects of cell migration. A characterized gradient of vascular endothelial growth factor (VEGF) resulted in migration of endothelial cells into the collagen gel forming vascular-like structures. In addition, interstitial flow induced angiogenic sprouting from an endothelial cell layer. More specifically, the μMIA has allowed observation of glioblastoma cells migrating through the gel and interacting with angiogenic sprouts, illustrating the ability to demonstrate tumor-endothelial cell interaction in a controlled, yet physiologically representative, 3D in vitro microenvironment. Taken together, the two aims establish the μMIA as an excellent platform for quantitative research of molecular mechanisms governing cancer cell invasion. For example, therapies capitalizing on altered vascular morphology near tumors would clearly benefit from using the μMIA as a development platform, as the system provides a characterized EC layer in conjunction with a well-controlled system. End use of the data will include diagnosis of invasion potential of cells and development of treatments targeting combinations of angiogenesis, cancer cell migration, and intravasation. As patient-specific specimens are valid inputs to the μMIA, the opportunity exists for development and characterization of custom therapies. Future development will enable the μMIA to serve as a cancer cell diagnostic device and a high throughput drug development tool.

A Microfluidic System for High-Throughput Evaluation of T Cell Functionality With High Resolution in Time

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Adoptive transfer of T cells is a promising clinical cancer therapy that relies on enhancing the adaptive immune response to target tumor cells in vivo. Widespread application of this therapy, however, has been hindered by the necessary expansion of large populations of T cells for each patient (often selected for tumor antigen specificity) and loss of functionality of the T cells post-transfer. The ex vivo expansion step requires many population doublings that ultimately results a fraction of the cells reaching senescence pre-transfer. A standardized method for quickly evaluating multiple characteristics of T cell functionality without sacrificing many cells would enhance the implementation of cell-based therapies. Microfluidic chips are ideal for high-throughput parallel experimentation and automation. In addition, microfluidics also provides the relevant length scales (~microns) and unique physical phenomena (e.g. laminar flow) to handle cells. The objective of our R21 project is to engineer a multiplex microfluidic assay to quantify T cell activation on a small population of cells with high temporal resolution. The hypothesis is that capturing the early dynamics of T cell activation of ex vivo expanded clones would improve upon current measures of T cell functionality.

The first component of this project has been the development of a high-throughput microfluidic system for multiple time-point stimulation and lysis of cells. This chip is engineered into two modules. The first module provides rapid mixing and controlled anti-CD3 stimulation of T cells for 8 precise timepoints. The second module has parallel branches for the stimulated cells to be simultaneously formalin-fixed or detergent lysed from the same population of cells. After confirming fluid flow properties of the device and the lack of shear stress on the cells, we characterized the reproducibility of signaling dynamics of Jurkat cells using a commercially available 7-plex xMAP bead assay. The precision of the microfluidic device-based stimulation and lysing resulted in markedly reduced error compared to conventional benchtop methods. Furthermore, 48 signaling measurements were made from only 10 million cells, roughly 5% of the amount needed in conventional methods.

The second component of the project is evaluation of primary T cells with the device. Signaling trends for phosphorylated Lck, ERK, CREB, Zap70, CD3 and LAT are conserved across primary T cells populations isolated from different donors. The microfluidic assay is being used to characterize ex vivo expanded CD8+ T cells over longer culture times to distinguish senescent/anergic versus responsive behavior. Using CD28 as a biomarker of time in culture, we are constructing a dataset to train a partial least squares regression model for relating signaling dynamics to the fraction of senescent cells in a population. The approach is innovative because the technology developed here dramatically increases the capabilities and throughput of existing assays in evaluating T cells for adoptive transfer. The modularity of the chip allows flexibility in generating high-throughput signaling dynamics for any soluble stimuli with suspension cells. This work tests a new paradigm in T cell evaluation using multiplex quantitative means. We believe the technology will expand the toolbox of cancer therapy and possibly other related quantitative biosciences and medical technologies.

MMPA: A Novel Multiplexing Methylation Analysis Technology

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This project is to develop a novel MMPA technology for methylation analysis of DNA derived from clinic specimen. Specifically, we will develop the MMPA assays (one for each type of cancer) for analysis of DNA methylation in colorectal, lung, and breast cancer, three deadliest cancers, respectively. Each assay can SIMULTANEOUSLY determine the methylation status of 20 genes in a 10,000 folds more excess of unmethylated DNA. The assays can also determine the degree of methylation and the relative abundance of methylated genes in clinic specimen. In addition, the assays will be cost-effective and easy to operate.

Aim of Phase I is to determine if the MMPA assay can accurately reveal methylation in DNA derived from clinic samples. Experimentally, we will use our existing MMPA assay to profile the methylation status of 8 genes in DNA derived from stools, the most complex specimen. The specific milestone is to demonstrate that this assay can achieve the detection sensitivity and specificity of 90% or better, respectively. Phase II is to develop the MMPA assays for analysis of DNA methylation in colorectal, lung, and breast cancers, respectively. Each MMPA assay fires one shot to kill four birds. First, the assay can determine the methylation status of an individual gene; second, the assay profiles DNA methylation among a number of genes; third, the assay has the capability of determining the degree of methylation of methylated genes; and fourth, the assay provides insights into the abundance of methylated genes in clinic specimen. Clearly, these unique features will make MMPA the method of choice for methylation analysis and thus success of this project will have a profound impact on both cancer screening and basic cancer research.

Molecular Analysis of the EGF Receptor Using Liquid Crystal Technology, Nanostructured Surfaces, and the Torque Balance Method

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Background: The epidermal growth factor receptor (EGFR) is a receptor protein-tyrosine kinase that is often over-expressed or mutated in human cancers, including non-small cell lung cancers and glioblastomas. Given its contribution to aberrant growth control, many studies have utilized the EGFR as a target for the development of cancer therapeutics. However, a lack of understanding about how these agents affect EGFR-mediated events in human tumors, or which EGFR mutations (or over-expression) are most clinically relevant, has made the development of these drugs challenging. In this regard, we have established a new class of highly sensitive tools that use nanostructured surfaces and liquid crystals (LCs) to amplify and image molecular interactions so as to assess EGFR expression, phosphorylation and mutation in cancer cell extracts. Our long-term goals have been to refine, validate, and implement this methodology to allow high throughput screening on limited clinical specimens.

Approaches and Outcomes: This LC-based approach for assessing EGFR expression/activity is termed the torque balance method and involves measuring a change in the orientation of an LC at a surface in response to an applied torque. Changes in the anchoring energy of the LC in the presence of captured biomolecules (e.g., via their direct surface binding or via their binding to a capture molecule immobilized on the surface) can be measured optically. We have used this technique to analyze surfaces patterned with biochemical functionalities relevant to the development of surface-based analytical methods and found that the approach exhibits i) a sensitivity in the range of pg/mm^2, ii) a compatibility with automated data acquisition/analyses, thereby allowing for the mapping of surfaces with a resolution of 10 mm x 10 mm, iii) a capability of characterizing chemical transformations on surfaces, and iv) an ability to quantify protein binding events over 4 orders of magnitude (10 pM to 100 nM). In these studies, we established methods for EGFR capture to polydimethylsiloxane (PDMS) surfaces presenting covalently immobilized antibodies directed against EGFR, and found that we can detect both total EGFR and phosphorylated (activated) EGFR using either purified EGFR or extracts of human epidermoid carcinoma cell lines and EGFR-transfected murine fibroblasts. The signal-to-noise obtained with PDMS surfaces was 82:1, exceeding that measured with ELISA plates (<48:1), supporting the concept that LC-based approaches can be used to assess EGFR status in complex solutions such as cell lysates. The approach is in the early stages of being adapted for the screening of clinical specimens, with the goal of using the technology to determine which EGFR alterations and/or mutations are relevant to specific tumor biopsies so as to aid in determining which cases are most likely to respond to EGFR antagonists. Also, this approach should be readily adaptable to other molecular markers of relevance to the detection and treatment of various cancers.

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 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 are often 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, detectible before measurable amounts of the antigens themselves 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, 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. Conditions for immobilization of the antigens of interest (antigen-expressing phage particles, and selected purified proteins) in MSD Multi-Array panels are being optimized. These panels will be screened with positive and negative control sera for ability to detect specific humoral responses in prostate cancer patient samples, allowing multiple simultaneous determinations per well. This academic/industry collaboration addresses NCI goals of translational medicine, by advancing potential biomarkers from discovery towards 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.

Nanoporous Silica Chip Technology for the Identification of Circulating Proteomic Markers for the Monitoring of Fever-Range Thermal Therapy

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In the post-genome era, the major challenge yet to be addressed is the sensitive and selective detection of circulating biomarkers to improve diagnosis, assess treatment efficacy, and design personalized therapies with limited invasiveness. The Low Molecular Weight (LMW) region of the blood proteome provides an unprecedented opportunity for clinical diagnosis or prognosis, and for monitoring response to therapy [1, 2]. Proteins and peptide are degraded by proteases in the tumor stromal environment and shed into the circulation from leaky vessels, therefore, LMW peptidome presents an attractive opportunity to capture pathological changes occurring in the tumor [3]. However, despite such promise, successful translation of this technology to routine clinical application is limited due to; a) the large dynamic range of blood proteins limiting the detection of low abundance biomarkers; b) the rapid degradation by endogenous and exogenous proteases [4]. To overcome the vast complexity and the relative instability of serum samples, we have developed a high throughput and reproducible fractionation system based on Nanoporous Silica Chips (NSC) for the depletion of most abundant High Molecular Weight (HMW) proteins and the enrichment and stabilization of LMW species present in the human circulating proteome. We have designed and engineered the NSC with defined nanopore size and physico-chemical properties allowing substantial control over the molecular cut-off, the specific harvesting and stabilization of proteins and peptides. This NSC technology in combination with mass spectrometry will provide a fast, efficient, and reliable fractionation system for high throughput enrichment, stabilization and detection of LMW biomarkers present in the human circulating proteome. In the clinically relevant MTLn3 rat mammary adenocarcinoma model, whole body fever-range thermal therapy enhances the anti-cancer activity of oxaliplatin chemotherapy resulting in 50% of cured animals [5]. Successful combination of thermal and chemotherapy initiates cell death and immune response pathways that cause specific associated proteins to circulate in the blood. Detection of these markers using specific NSC enrichment and mass spectrometry allowed us to identify proteomic patterns associated with early response to thermochemotherapy.

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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 the microPET are capable of quantifying the spatial distribution of radiolabeled probes, but have detection limit on the order of 1-10 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, while 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, imaging as well as quantification of trace amounts of radioactivity on the microfluidic chip. This device will employ direct detection of b-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 silicon device, manufactured by us at RMD, that operates at high gain (~1000) with low noise (~120 e--rms for 14x14 mm^2 size) 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.

A Novel Magnetic Bead-Based Assay for Tyrosine Kinase Activity and Inhibition in Cancer Cells and Tumors

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Deregulated activity of tyrosine kinases has been identified in a wide range of malignancies and selective inhibition of protein tyrosine kinases is an effective therapeutic approach for the treatment of a wide range of human cancers. The remarkable clinical success of the Bcr-Abl inhibitor imatinib in treatment of chronic myelogenous leukemia (CML) has stimulated the rapid discovery of kinase inhibitors targeting a number of oncogenic kinases. Thus, detection of the activation of oncogenic signaling can serve as a valuable molecular marker for cancer diagnosis and as a predictive tool for selection of therapy. Histology and tumor architecture provide complementary and critical information about cancer stage and grade. By adapting and extending emerging technologies for kinase sensor biochips, we intend to develop the capability to image the distribution of cancer signaling in tumor tissue obtained by biopsy or surgical excision.

We have previously developed robust, sensitive and specific bead-based and biochip-based assays for kinase activity in cellular lysates, using immunodetection or MALDI-TOF MS analysis as a read-out. In order to address the challenge of measuring kinase activity in solid tumor tissue, we are developing a novel kinase assay geometry that combines the advantages of bead and chip-based assays. Here, we are using peptides immobilized on magnetic beads as substrates in kinase assays. Our plan is to apply the beads to samples to allow their phosphorylation and then use magnetic surfaces to corral the beads and maintain spatial distributions.

Initially, we have immobilized biotinylated peptides onto biocompatible streptavidin magnetic beads. The nonocovalent binding facilitates direct detection of the phosphorylated and unphosphorylated peptides (proteins) by MALDI techniques. In turn, phosphorylated peptides can be also analyzed by chemifluorescence using an anti-phosphotyrosine and a horseradish peroxidase (HRP)-conjugated secondary antibody. These two detection modes are compatible with detection in wells or imaging on surfaces.

We validated the new kinase assay technique using c-Abl kinase and the peptide Abltide as a substrate. Two Bcr-Abl kinase inhibitors, imatinib and dasatinib, were evaluated using the magnetic bead-based kinase assay and MALDI and chemifluorescence detection. IC_{50} values determined by the two detection techniques are consistent and close to the values reported in literature. We also tested the high throughput capability of the magnetic bead-based kinase assays by screening a panel of candidate Bcr-Abl inhibitors. Both MALDI and chemifluorescence techniques gave consistent screening results. We also tested multiplexing detection by immobilizing multiple peptides on each bead and then detecting them and their phosphorylation by MALDI. This approach makes the magnetic bead-based assays particularly valuable for quantitative evaluation of multiple kinase activities and comparing the potencies of inhibitors toward different kinases.

We are examining reversible chemistries to tether peptides to beads that will allow a reversible covalent attachment of unmodified peptides. We anticipate being able to adapt this new method to serve as a high throughput screening assay to examine kinase inhibitors in multiwell plates and to be easily adapted to measure distributions of kinase activity by taking full advantage of the properties of magnetic particles.

Occult Tumor Burden in Lymph Nodes Predicts Time to Recurrence and Disease-Free Survival in pN0 Colorectal Cancer

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Context. The established relationship between lymph node metastasis and prognosis in colorectal cancer suggests that recurrence in 25% of patients with lymph nodes free of tumor cells by histopathology (pN0) reflects the presence of occult metastases. GUCY2C is a marker expressed by colorectal tumor cells that identifies occult tumor cells in lymph nodes associated with recurrence risk. **Objective.** To examine the association of occult tumor burden, estimated by quantifying GUCY2C mRNA with the reverse transcriptase-polymerase chain reaction (RT-PCR), with recurrence and survival in patients with colorectal cancer. We define occult tumor burden using recursive partition analysis Patients and Methods. Prospective enrollment of 257 patients with pN0 colorectal cancer yielded 85 patients with complete (>12) collections of lymph nodes (range: 12-149) for histopathology and GUCY2C RT-PCR. Patients were followed for a median of 24 months (range: 2-63) for disease recurrence or death. Time to recurrence and disease-free survival served as primary and secondary clinical outcomes, respectively. The association of prognostic markers, including occult tumor burden, with clinical outcomes was estimated by recursive partition analysis. Results. For time to recurrence, the only prognostic markers selected by recursive partition analysis to stratify recurrence risk in pN0 patients were measures of occult tumor burden quantified by GUCY2C RT-PCR. Thirty-four (40.0%) patients exhibited low tumor burden [pN0(molLow)], and all but 1 remained free of disease during follow-up (recurrence rate 2.6% [95%CI 0.8-20.8%]); 40 (47%) patients exhibited intermediate tumor burden [pN0(molInt)], and 10 (25.0% [15.8-26.8%]) developed recurrent disease (p=0.001); and 11 (13.0%) patients exhibited high tumor burden [pN0(molHigh)], and 9 (81.8% [15.8-26.8%]) developed recurrent disease (p=0.001). Multivariable analyses revealed that occult tumor burden in lymph nodes was an independent marker of prognosis. Patients who were pN0(molInt) and pN0(molHigh) exhibited a graded risk of earlier time to recurrence (pN0(molInt), 13.59 [2.44, 86.49]; p=0.030; pN0(molHigh), adjusted hazard ratio 103.54 [20.28, 8518.54]; p<0.001) and reduced disease-free survival (pN0(molInt), adjusted hazard ratio 4.71 [0.79, 19.88]; p=0.205); pN0(molHigh), adjusted hazard ratio 38.09 [8.33, 214.86]; p<0.001). **Conclusions.** Sophisticated quantification in qRT-PCR paired with novel algorithms provides a measurement technique that may identify occult tumor burden in lymph nodes in colorectal cancer. Occult tumor burden in lymph nodes quantified by GUCY2C qRT-PCR and defined by recursive partition analysis is independently associated with time to recurrence and disease-free survival in patients with pN0 colorectal cancer. These combined quantitative and laboratory techniques may identify early stage patients who would benefit from adjuvant therapy.

Optimization of Collection and Characterization of Expressed Prostatic Secretions for MiRNA and Protein Biomarkers of Prostate Cancers

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Prostate-specific antigen (PSA) is widely used as a serum diagnostic marker for prostate cancer and as a predictor of treatment outcome. However, PSA is tissue specific but not prostate cancer specific and there is a need to develop more accurate and specific biomarkers that could be used in the early detection of prostate cancer.

Recent evidence has indicated that an abundant class of small non-coding RNAs of ~22 nucleotides in length, referred to as microRNAs (miRNAs), might also be useful diagnostic biomarkers for disorders such as prostate cancer. MiRNAs constitute a newly discovered class of regulatory molecules that repress gene expression post-transcriptionally by binding to complementary sequences in their target protein-coding messenger RNAs (mRNAs). MiRNAs have gained considerable attention in recent years because these genes are often misexpressed in various human cancers and implicated to function as tumor suppressor genes and oncogenes. Expressed prostate secretions (EPS) are collected during digital rectal exam and gentle massage of each side of the prostate gland, thus stimulating release and movement of prostate fluids and detached epithelial cells into the urethra. These prostate fluids and cells are collected when the patient voids urine following the massage, and the fluid is largely devoid of seminal vesicle derived proteins or sperm. Collection of prostatic fluid undiluted in urine can also be obtained from patients under anesthesia. This collection is not disruptive to a standard urological exam, nor does it add excessive time to the visit. Our objectives for clinical diagnostic application of these EPS samples are two-fold: 1) characterize the secreted prostatic proteins and miRNA species present in these fluids, and 2) standardize reproducible and portable EPS collection protocols. For proteomic characterization, representative EPS urines from non-diseased and prostatic diseases have been concentrated, desalted and prepared for use with one of three front-end separation methods: glycoprotein binding lectins, 2D gels, or liquid chromatography. Following tandem mass spectrometry identification of proteins within these fractions, over 400 individual proteins have been cumulatively identified, most functionally associated with extracellular and cell surface processes. Determining prostate specific glycoprotein species that can be used as internal standards of sample quality are in progress. Initial post-collection stability and temperature variables indicate that EPS urine proteins are stable for at least 5 hours. For miRNA, protocols using TaqMan based quantitative real-time PCR have been used to detect sentinel miRNA species. Determining the minimal amounts of EPS urine sample required for detection and of different miRNA species in combination with different front-end enrichment strategies are ongoing.

Partial-Wave Spectroscopic Microscopy for Surveillance and Diagnosis of Gastrointestinal Malignancies

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Our group is interested in integrating novel optical techniques in surveillance and diagnostic strategies for gastrointestinal malignancies. Our approach is based on both the concept of a field effect of carcinogenesis, the proposition that a neoplastic lesion in a particular tissue site should be detectable farther away from this location due to the similar genetic or environmental milieu, and the recognition regarding the limitations of conventional microscopy in detecting subcellular alterations. Our strategy builds upon the growing experience of developing and applying light scattering spectroscopy techniques to gastrointestinal malignancies. Partial-wave spectroscopic microscopy (PWS) is an emerging technology that offers a novel approach to access the unique statistical properties of refractive index variation of nano-structural molecules within a single cell, arising from the changes in the concentration of intracellular solids (DNA, RNA and proteins, etc.) at a single cell level, well beyond what standard light microscopy can reveal. Specifically, a PWS microscope simultaneously obtains microscopic image at both reflectance and transmission mode and records the scattering spectrum (scattering intensity vs. wavelength) from each pixel. Unlike conventional microscopy, in which an image is formed by integrating the reflected or transmitted intensity over an incident white-light spectrum, PWS measures spectral variations in light scattered from single cells or smaller particles.

PWS can be applied on cytologic specimens obtained by fine-needle aspiration or brushing to improve diagnostic accuracy. This is based on the premise that PWS analysis of those epithelial cells, which appear normal by conventional light microscopy, will have subcellular changes that can distinguish between patients with a neoplastic or benign lesion. Results of a recent pilot study have demonstrated the promise of combining conventional cytology with PWS to improve the diagnostic accuracy of endoscopic ultrasound-guided fine needle aspiration for pancreatic adenocarcinoma. Our proposed approach of initial evaluation by a cytologist followed by subsequent PWS analysis of non-diagnostic (indeterminate or benign) cases is clinically feasible. Further studies aimed at expanding our tumor types including cholangiocarcinoma are being conducted.

Prior optical studies have demonstrated the ability to predict the presence of neoplastic processes based on the examination of normal appearing mucosa. Our group is interested in studying whether optical changes detected from normal appearing duodenal cells obtained adjacent to the major ampulla (opening of the bile and pancreatic ducts into the small intestine) can be used in a surveillance strategy for biliary and pancreatic cancers. The need to establish a standard operating procedure is an important first step in adapting this technology into a clinical setting. We recruited 10 patients during the performance of a scheduled routine upper endoscopic procedure. Duodenal cells were obtained by cytologic brushing of endoscopically normal appearing mucosa adjacent to the ampulla. A variety of cytology preparation methods were tested on 10 patients (5 benign and 5 pancreatic cancer patients), including two different fixatives (95% alcohol and Cytolyt®), three different specimen processing methods (Thinprep®, cytospin and smear) and two different staining (unstained and Papanicolaou staining) with and without applying a coverslip. The variation of optical signals at different time points was also investigated. We found that Cytolyt® fixation and Thinprep® processing is the ideal sample preparation technique. We selected two methods that appear to best distinguish pancreaticobiliary cancer patients: unstained/no coverslip and Papanicolaou staining/coverslip. We determined that all the specimens needed to be processed within the first 48 hours following the endoscopic procedure. Presently we are expanding our patient numbers in a variety of pancreaticobiliary disease conditions (e.g., cholangiocarcinoma, primary sclerosing cholangitis, chronic pancreatitis, pancreatic cysts, etc.). We are planning to investigate the role of new optical parameters in combination with previously reported parameters can develop better prediction rules for different neoplastic processes. The technique will be cross-validated on an independent patient population in a double-blinded manner. If successful, a prospective multi-center trial is warranted.

A Perturbation-Based Approach to Measuring and Modeling the EGF Signaling Network Using Lysate Microarray Technology

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Mammalian signaling networks comprise biochemical pathways with shared components, common inputs, and overlapping outputs. Understanding how information flows through these pathways requires information on signaling networks as a whole, rather than on one or two components. To study signaling at a systems level, we need ways to measure the abundances and post-translational modification states of many proteins in a parallel, quantitative, and reliable manner. In addition, since an understanding of signaling requires the frequent temporal sampling of many proteins under many conditions, these methods must be high throughput in nature. Over the past several years, we have been developing lysate microarray technology to be quantitative, reliable, and high throughput. We can now culture cells in 96-well microtiter plates and subject them to a variety of targeted perturbations in an automated fashion (stimulation with epidermal growth factor in the presence of carefully validated shRNAs). Once the perturbation has been effected, the cells are lysed in situ and the resulting lysates are spotted at high spatial density onto a series of glass-supported nitrocellulose membranes. By probing each membrane with a different antibody, the multidimensional 'state' of the signaling network can be assessed under thousands of different conditions.

As an initial experiment, we perturbed the EGF signaling network in A431 cells with 21 different shRNAs directed at intracellular signaling proteins. We then measured the phosphorylation status of 20 different proteins at 11 different time points in quadruplicate following EGF stimulation. The resulting data revealed many unexpected influences in the network. For example, we found that knocking down members of the MAP kinase signaling cascade led to increased phosphorylation of Akt (a previously unrecognized feedback loop), and that knocking down EGFR led to increased levels of phospho-Rsk3. This latter result has important medical implications since EGFR is a recognized drug target and Rsk3 promotes cellular proliferation. We also found that knocking down upstream signaling proteins often does not inhibit downstream components. We submit that feed-forward loops play a crucial role in mediating many of these unexpected influences and provide small-molecule evidence for a putative feed-forward inhibition between c-Raf and Erk1/2. Finally, we found that protein phosphatases account for much of the observed inter-pathway crosstalk and intra-pathway feed-forward inhibition. We are now using our systematic perturbation data, in collaboration with Doug Lauffenburger at MIT, to build predictive models of EGF signaling. These models provide a way to rapidly investigate thousands of different combinations of perturbations in silico and thus provide a way to rationally predict new strategies for combination chemotherapy.

Preanalytical Fluctuations in Tissue Phosphoproteins: Tissue Is Alive and Reactive in the Post Excision Hypoxic Environment

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Little is known about the pre-analytical fluctuations of phosphoproteins during tissue procurement for molecular profiling. This information is crucial to establish guidelines for the reliable measurement of these analytes. Tissue proteomic biomarkers contain information about the ongoing functional molecules driving the pathologic process. Emerging molecular targeted anti-cancer therapeutics are designed to normalize or reverse defective kinase signaling networks. Proteomic biomarkers provide a means to develop a profile of the activated state of signal pathways or receptors that contain the molecular targets of this new class of therapeutics. Thus tissue protein biomarkers offer a means for individualizing therapy by revealing how a individual patient's tumor cells are driven by an activated or amplified proteomic signal pathway. If the revealed pathway contains the drug target of a specific therapeutic, then this information could be used by the physician to select the optimal therapy for this patient. In the past, the discovery and clinical implementation of tissue biomarkers has been delayed by several hurdles including tissue heterogeneity and the lack of sensitive technology to identify and measure proteins in small volumes of human biopsy tissue obtained for research. The problem of protein biomarker instability and perishability within the tissue aspirate or biopsy is a very serious issue that has not been adequately studied or solved. If this problem is not resolved then the future promise of tissue biomarkers will never be realized. To develop phosphoprotein profiles as tissue reacted to the trauma of post excision, we measured the fidelity of 60 signal pathway phosphoproteins over time in tissue specimens procured in a community clinical practice. This information provides strategies for potential surrogate markers of stability, and the design of phosphoprotein preservative/fixation solutions. Eleven different specimen collection time course experiments revealed augmentation (±20% from the time zero sample) of signal pathway phosphoprotein levels, as well as decreases over time, independent of tissue type, post-translational modification, and protein sub-cellular location (tissues: breast, colon, lung, ovary, uterus (endometrium/myometrium, metastatic melanoma)). Uterine tissue (endometrium/myometrium) showed >20% decrease of AKT S473 and MARCKS S152/156 within the first 60 minutes post excision. Proteins in apoptotic (Bad S112, Cleaved Caspase 3 D175), hypoxia (p38T180/Y182), proliferation/survival (AKT T308, EGFR Y1148, ERK T202/Y204, GSK3αβ S21/9), and transcription factor pathways (STAT1 Y701, CREB S133) showed >20% increases within thirty minutes post

procurement. eNOS S1177 did not change over the time period evaluated with breast or leiomyoma tissue. Treatment with phosphatase inhibitors revealed that tissue kinase pathways are active ex vivo. Combinations of kinase and phosphatase inhibitors appeared to stabilize proteins that exhibited increases in the presence of phosphatase inhibitors alone (ATF-2 T71, SAPK/JNK T183/Y185, STAT1 Y701, JAK1 Y1022/1023, and Pak1/Pak 2 S199/204/192/197). This timecourse study 1) establishes the dynamic nature, of specific phosphoproteins in excised tissue, 2) demonstrates augmented phosphorylation in the presence of phosphatase inhibitors, 3) shows that kinase inhibitors block the upsurge in phosphorylation of phosphoproteins, 4) provides a rational strategy for room temperature preservation of proteins, and 5) constitutes a foundation for developing evidence based tissue procurement guidelines. Living tissue ex vivo goes through a defined stage of reactive changes that begin with oxidative, hypoxic and metabolic stress, and culminate in apoptosis. Depending on the delay time ex vivo, and the reactive stage, protein biomarkers, such as signal pathway phosphoproteins will be elevated or suppressed in a manner which does not represent the biomarker levels at the time of excision. Our proteomic data documenting reactive protein changes in tissue post collection indicates the need to recognize and address tissue stability, preservation of post-translational modifications, and preservation of histologic and morphologic features for molecular analysis. The results support the conclusion that kinase pathways remain active and reactive during the immediate ex vivo period. These data have profound implications for tissue collection protocols, tissue banking, and development of preservative chemistries for molecular profiling of proteins. We propose technical solutions for a) assessing specimen quality via identification of a panel of natural proteins as a means to qualify the state of protein analyte preservation (surrogate stability markers), and b) the use of a multi-purpose fixative solution designed to stabilize, preserve and maintain proteins, nucleic acids and tissue architecture.

The Prevalence and Nature of Glycan Alterations on Specific Proteins in Pancreatic Cancer Patients Revealed Using Antibody-Lectin Sandwich 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. Here we report the use of a previously-developed antibody-lectin sandwich array method to characterize both the protein and glycan levels of specific mucins and CEA-related proteins captured from the sera of pancreatic cancer patients (n = 23) and control subjects (n = 23). The MUC16 protein was frequently elevated in the cancer patients (65% of the patients) but showed no glycan alterations, while the MUC1 and MUC5AC proteins were less frequently elevated (30% and 35%, respectively) and showed highly-prevalent (up to 65%) and distinct glycan alterations. The most frequent glycan elevations involved the TF antigen, fucose, and Lewis antigens. An unexpected increase in the exposure of alpha-linked mannose also was observed on MUC1 and MUC5ac, indicating possible N-glycan modifications. Because glycan alterations occurred independently from the protein levels, improved identification of the cancer samples was achieved using glycan measurements on specific proteins, relative to using the core protein measurements. The most significant elevation was the CA 19-9 antigen on MUC1, occurring in 19/23 (87%) of the cancer patients and 1/23 (4%) of the control subjects. Follow-up studies using blinded samples from patients with benign and malignant pancreatic disease also have been performed and will be discussed. This work gives insight into the prevalence and protein carriers of glycan alterations in pancreatic cancer and points to the potential of using glycan measurements on specific proteins for highly effective biomarkers.

Recombinant Affinity Reagents for Molecular Analysis of Cancer

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Posttranslational modification of short peptide motifs and their interactions with modular interaction domains play central roles in signal transduction and in cancer biology. Therefore, quantitative detection, functional assessment and isolation of such motifs and their interaction partners are critically important for molecular analysis of cancer. However, there is a paucity of high-quality affinity reagents to this class of high-value biomarkers that can be reproduced and distributed economically, which has now been recognized as a major bottleneck in cancer analysis. The goal of this project has been to develop a technology platform for generating recombinant affinity reagents that recognize these biomarkers with high-affinity and high-specificity. Our specific focus is on phospho-Tyr containing peptides and their interaction partners, the SH2 domains. To this end, we have established fully recombinant protein-engineering strategies by combining structure-guided design and high-throughput library selection. Our innovative protein engineering strategy is termed "Affinity Clamping."

Rehybridization of Promoter DNA Targets in Chromatin to Discover Transcription Factors

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Identification of transcription factors (TF) assembled at promoter regions of oncogenes is vital in understanding how gene expression is altered in cancers. Most TF identification methods are based on one-on-one DNA/protein interactions in vitro, which may not reflect DNA/protein interactions in vivo and may overlook protein complex information. We are pursuing a new approach, chromatin rehybridization (CR), which preserves and detects DNA/protein and protein/protein interactions that occur in live cells. Specific promoter regions are then targeted for study through rehybridization with appropriate oligonucleotide probes. The DNA target along with any associated proteins is then captured. The proteins are then dissociated from the DNA and may be detected using immuno-assays for known proteins or analyzed using mass spectrometry for protein identification. The G-quadruplex forming Pu27-mer located upstream of the c-myc oncogene and associated TFs including cellular nucleic acid binding protein (CNBP) serves as the model system for development of CR. Preliminary results demonstrate that CNBP is pulled down with the targeted G-rich strand of the Pu27-mer in MCF-7 cells using the newly developed CR assays. At least one unreported TF associated with c-myc was also detected and will be identified using mass spectrometry. The CR method will then be applied to detection and discovery of TFs associated with other oncogenes (e.g. HER2). The CR method may create a new paradigm in TF discovery. Identification of the full complement of proteins associated with a particular DNA target of interest in chromatin will lead to a better understanding of gene regulation and to the discovery of new biomarkers and drug targets. This new technique may also be utilized to explore unknown genes.

Quantum Dots-Based Methods for Highly Sensitive Detection of Cancer Markers

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The unique photophysical properties of semiconductor quantum dots (QDs) have made them ideal for use as spectral labels and luminescent probes. In recent years, there have been several QD applications that utilize these nanocrystals as scaffolds and active participants in biosensing, wherein biological specificity within

hybrid inorganic/organic assemblies results in capture and detection of molecular disease markers. The high surface area to volume ratio, and well-documented conjugation chemistries for QDs allow attachment of biomolecular probes, thus transforming the nanocrsytals into scaffolds for molecular interactions. QDs also make excellent donors to pair with organic dyes in the fluorescence resonance energy transfer (FRET) process due to the features of narrow emission spectra and small Stokes shift. This enables FRET with minimal direct acceptor excitation and donor-acceptor crosstalk, thereby permitting the design of FRET molecular sensors with extremely low intrinsic fluorescence backgrounds necessary for detecting biomolecular targets at low abundance. We have demonstrated the use of QDs in developing molecular assays for detecting biomarkers at both the genetic and epigenetic levels. A point mutation assay is developed by incorporating QDs into DNA ligation reactions, facilitating highly sensitive and specific mutation detection in a simplified homogeneous format. This mutation nanoassay has been exemplified with detection of Kras point mutations in clinical samples from patients with ovarian serous borderline tumors (SBTs). In addition, a DNA methylation assay called MS-qFRET is developed based on the above QD-FRET technique. This approach detects as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles and allows for multiplexed analyses. The high sensitivity of MS-qFRET enables one-step detection of methylation at ASC/TMS-1 gene in patient sputum samples that contain low concentrations of methylated DNA, which normally would require a nested PCR approach. The direct application of QD nanoassays on clinical samples offers great promise for its translational use in early cancer diagnosis, prognostic assessment of tumor behavior, as well as monitoring response to therapeutic agents.

Replacing PCR With COLD-PCR Technology Enriches Variant DNA Sequences and Redefines the Sensitivity of Mutational Profiling in Cancer

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We are gradually moving towards an era of personalized medicine, in which genetic testing prior to radio/chemotherapy may affect prognosis or optimal choice of treatment regiment. The Polymerase Chain Reaction (PCR) has become the cornerstone of genetic testing, with almost every assay aiming to identify DNA sequence-variation incorporating PCR. A commonly encountered problem with PCR is that variant DNA sequences exist in the presence of a large majority of wild-type alleles such as when DNA is obtained from heterogeneous cancer biopsies. As a result, downstream assays are limited in their ability to identify subtle somatic genetic changes that can have profound impact in clinical decision-making and outcome.

We recently described Co-amplification at Lower Denaturation temperature (COLD-PCR), a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence (1). In COLD-PCR, an intermediate annealing temperature is used during PCR-cycling to allow cross-hybridization of mutant and wild type alleles; hetero-duplexes, which melt at lower temperatures than homo-duplexes, are then selectively denatured and amplified at Critical Denaturation Temperature, while homo-duplexes remain double-stranded and do not amplify efficiently.

By replacing regular PCR with COLD-PCR prior to application of downstream mutation detection assays (Sanger sequencing; Pyrosequencing; MALDI-TOF; dHPLC; RFLP; Taqman; HRM) we improved mutation detection sensitivity up to 100-fold. Thereby we identified several additional p53/Kras/EGFR mutations in heterogeneous cancer samples that were not detected when preceded by regular-PCR, but that were detectable following COLD-PCR. When followed by High Resolution Melting, COLD-PCR enables identification of low-level (~0.1-1%) variants that can be directly sequenced. COLD-PCR in real time 'Taqman' format increases detection sensitivity for the resistance-causing EGFR mutation T790M in lung cancer by 250-fold (2). By COLD-PCR-based deep-sequencing of 48 snap-frozen lung cancer specimens for

p53 alterations, we identified a novel spectrum of low-level of unknown missense somatic mutations some of which were not detectable by any previous technologies (3). DNA from FFPE specimens leads to reduction of COLD-PCR detection sensitivity depending on specimen 'age', however, in all cases the sensitivity is highly improved compared to conventional PCR, irrespective of the downstream method used. COLD-PCR performed from whole genome-amplified DNA retains the ability to identify low-level mutations. Finally, combination of COLD-PCR with second generation sequencing enables both 'deep-sequencing' and 'high-throughput performance', thereby allowing the application of new sequencing to 'real' patient samples that invariably involves stromal cells or tumor heterogeneity.

In summary, COLD-PCR is a new platform that provides a universal boost to most mutation detection technologies and enables them to be used with the required confidence in routine screening of cancer specimens for traces of somatic mutations. Supported by IMAT grant R21 CA 138280.

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Sample Preparation From Fixed Cellular Biospecimens for Measurements of Protein Phosphorylation

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Objective: The objective of the project is to optimize tissue fixation and protein extraction conditions for preparation of samples for measurements of protein phosphorylation.

Methods: PC-3 prostate cancer and IGROV-1 ovarian cancer cells (obtained from ATCC) are adapted to growth in suspension culture on HEMA (Poly(2-hydroxyethyl Methacrylate) - coated plates. Cells are washed once with PBS/1mM Na-Vanadate/20 mM β-glycerphosphate/100 nM staurospaurin. Five million cells per tube are frozen or fixed for 48 hours with formalin (10% Neutral Buffered Formalin, VWR cat# BDH0502), or with the non-crosslinking fixative, UMFIX (Sakura Finetek). Fixed cells are extracted with RIPA buffer (1% Triton X-100, 0.5% Deoxycholate, 0.1% SDS 20 mM Tris pH 7.5, 100mM PBS, 1mM EDTA, protease inhibitors (Roche), phosphatase inhibitors (Phosphatase inhibitor Cocktail Set-II, CALBIOCHEM), kinase inhibitors (Staurosporine, Sigma) or 8M Urea/2%SDS (protease inhibitors, phosphatase inhibitors, kinase inhibitors), or digested with RIPA buffer/Trypsin (2.5 Units per 10 microgram protein, Promega V511A 260233705) or 2M Urea/2%SDS/Trypsin. The protein concentration is measured with the 2D Quant assay system (2D Quant kit, GE Healthcare). For measurements of protein phosphorylation samples are analyzed by Western blotting or applied onto nitrocellulose membranes in a 2-fold dilution series. Blots are probed with the 4G10 phosphotyrosine antibody (Millipore) and total protein phosphoryaltion is determined with the pro Q Diamond dye system (Molecular Probes).

Results: Our initial attempts to use prostate cancer xenografts directly from patients cancers was not successful, because we were unable to identify a xenograft from the 29 xenografts that we tested that possessed a sufficient amount of protein phosphorylation for the project. To establish a robust system that is amenable to the development of a protocol for the preservation of protein phosphorylation, we resorted to cells grown as clusters in suspension. The three-dimensional growth pattern of cancer cells in suspension

mimics patient cancers more accurately than tumor cells, which adhere to tissue culture plastic. The phosphorylation of proteins on tyrosine amino acids was increased in suspended, compared to adherent cells. Both RIPA buffer and 8M urea/2% SDS were unable to release protein or protein fragments. Pre-treatments with heat and trypsin are being tested to improve protein extraction efficiencies for measurement of protein phosphorylation.

Strengths and limitations: protein extraction from formalin-fixed samples is poorly efficient. Hydrolyzing formalin bonds and trypsin digestion are needed for preparation of samples for measurement of protein phosphorylation.

Serum Biomarkers for Colorectal Cancer Detection

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When cancer is identified at the earliest stages, cancer survival rates dramatically increase and therefore diagnostic screening tests that can detect early-stage cancer are crucial. The overall goal of our research is to develop such an early-detection screening test.

We intend to prospectively accrue a large, well-defined independent cohort of colorectal cancer (CRC) cases where we collect extensive data on this cohort at the time of diagnosis. We will generate a comprehensive database that includes data on demographics, personal and family history, tumor characteristics, and comorbidities.

Our preliminary research efforts have been to develop a detection assay utilizing the sera of our discovery cohort of CRC patients and healthy controls. We have developed a high throughput method to isolated cDNA clones of antigens which can be used to identify cancer cases by detecting the presence of auto-antibodies to tumor proteins in the serum of the test subject. Our first aim is to identify the minimal number of antigen clones that are critical in distinguishing sera from patients with colorectal cancer from healthy controls utilizing our initial discovery cohort. We will eliminate antigen clones from our discovery set of 3800 clones that react with sera from patients with other cancers, benign gastrointestinal conditions, are duplicates or do not react with any CRC sera.

Our second aim is to determine the test characteristics (sensitivity, specificity, accuracy) on these newly selected antigen markers for distinguishing colorectal cancer cases using newly acquired sera samples not previously used in the development of the marker set.

Lastly, we intend to determine the test characteristics of these antigen markers on a large, independent, well-defined cohort of colorectal cancer patients and healthy controls. In addition, due to the size, racial/ethnic makeup of the study population, and captured patient data, we will be able to evaluate the expression of these markers in relationship to important subgroups.

Singlet Oxygen-Mediated Protein Proximity Assay

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Dissection of the human genome has revealed a large number of important genes and corresponding proteins, the vast majority for which the function is unknown. Gene arrays and RNAi methodology has discovered a large number of gene products that are essential in determining disease processes or

susceptibility to medications, but what these proteins actually do within the cell is largely a mystery. Most proteins function in the context of binding to other proteins in polymeric protein complexes and these complexes have evolved to perform related biochemical functions within the cell. Currently there are no efficient ways to identify what the partners for a given protein are, so as to get clues to their function. Most methodologies attempt to identify protein-protein interactions in a lysed cell or are capable of only identify protein binding partners in direct contact with the protein of interest. We are developing a singlet oxygen-mediated protein proximity assay. The hallmarks of this assay are that a known protein of interest is modified so that when it is expressed in a mammalian cell, any protein that is associated with the protein of interest (within ~100-200 nm as part of a macromolecular complex) can be tagged in a manner which allows for its easy identification by mass spectroscopy and also enables the tagged protein to be readily purified. The tagging process does not require that the associated proteins are known a priori but allows for their identification from the total mix of cellular proteins. Furthermore, the tagging process is readily controlled to start and stop time so that the tagging the interacting partners can be instituted immediately after changing a parameter such as the addition of a growth factor. This technology has a myriad of potential applications from functional proteomics to drug discovery.

Smart Nanoparticles Overcome Basic Roadblocks to Biomarker Discovery and Validation

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The focus is novel nanotechnology that directly overcomes the fundamental physiologic roadblocks to cancer biomarker discovery, measurement and validation. Cancer-associated blood biomarkers exist in exceedingly low concentrations within complex mixtures of high-abundance proteins such as albumin and immunoglobulins. For this reason, existing methods for biomarker discovery and measurement may not have the sensitivity to detect important biomarkers of early stage cancer. A further roadblock to biomarker development is biomarker instability. Biomarkers in the blood may be highly labile and subjected to degradation during blood collection, transportation, and storage. Biomarker instability is a significant source of bias for validation, and can prohibit practical routine clinical use. The nanotechnology developed under this proposal overcomes these serious roadblocks that prevent promising biomarkers from being discovered, or hinder biomarker utility for patient benefit. We have created N-isopropylacrylamide (NIPAm) hydrogel porous sieving nanoparticles containing an internal affinity bait. The nanoparticles perform three independent functions within minutes, in one step, in solution (serum, plasma, or urine): a) molecular size sieving, b) affinity capture of all solution phase low abundance target analyte molecules, and c) complete protection of harvested proteins from enzymatic degradation. The captured analytes can be readily eluted for analysis. We have developed a variety of novel high affinity chemical bait chemistries that appear to have the capability to sequester six major classes of biomarkers. Six bait categories with fourteen different chemistries, will extend analyte targets to the following major categories of body fluid cancer biomarkers: 1) basic proteins and peptides, 2) acidic proteins and peptides, 3) glycoproteins, 4) hormone metabolites, 5) phosphoproteins, and 6) micro RNA. These novel baits now offer the opportunity to create a family of nanoparticle "flavors" that can be mixed together in one sample collection vessel. The nanoparticle family will capture, in one step, in the same fluid volume, a wide variety of proteins, peptides, metabolites and nucleic acids that constitute potential cancer biomarkers for both proteomic and genomic applications. The envisioned technology is a panel of dry lyophilized, sub-micron sized harvesting particles that carry specific affinity baits for known classes of biomarkers. Following introduction of the blood or body fluid, the respective particle populations will remove all of their target molecules, in one step, in solution, from the entire volume of the sample and concentrate the sequestered analytes inside the particles. Analytes can then be eluted from the particles in a small volume to yield a much higher concentration and purification

compared to the starting sample. Depending on the starting volume, this technology can concentrate a biomarker many hundred fold, and fully prevent biomarker degradation, within minutes. We propose the development of two classes of novel technology to be used at the bedside or in the field: a) blood collection tubes that fully stabilize biomarkers and concentrate them 100 fold, at the time of venipuncture collection, and b) a diagnostic skin patch which harvests, concentrates, and stabilizes a panel of biomarkers derived from skin transudate or sweat. Our published studies supported under this funded study document a) Sequestration of greater than 99% of the target low abundance biomarker in less than 5 minutes in serum, with complete exclusion of albumin; b) Full protection (>95%) against degradation of labile biomarkers at 37 °C for at least 24 hrs; and c) Amplification of the effective concentration of low abundance endogenous or spiked biomarkers such that biomarkers at femptomole concentrations well below the lower detection limits of clinical grade 3rd generation immunoassays and mass spectrometry, could now be measured in the linear range of detection with high precision.

Spatial Multiplexing of Immunoassays on a Digital Microfluidic Platform

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Immunoassays are routinely performed in clinical laboratories using macrofluidic equipment utilizing large liquid volumes with mechanically complex robots at high cost and comparatively low speed. Last year, we demonstrated the feasibility of performing a magnetic bead immunoassay on a digital microfluidic system [1]. Here, we demonstrate the capability of spatial multiplexing of immunoassays by dividing each assay into a separate and independent droplet. Figure 1 shows a schematic of a digital microfluidic multiwell plate that has 8 reagent reservoirs and 12 sample reservoirs. All the steps involved in an immunoassay, including sample and reagent aliquoting, incubation with antibodies, bead washing and enzymatic detection, are fully automated on the chip.

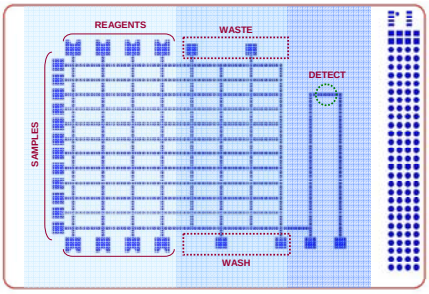

Figure1- Schematic of a digital microdluidic cartridge

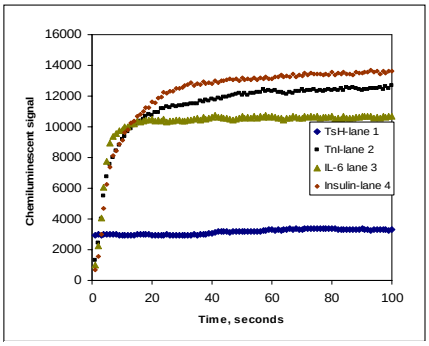

Figure 2- Multiplex immunoassays

In addition to core lab clinical diagnostic applications, where a single assay is run on several samples, the digital microfluidic multiwell plate has also been demonstrated to perform several-fold multiplexing on a single sample. 8-plex assays were demonstrated on our model targets as a proof of feasibility by performing duplicate immunoassays on a spiked human serum sample for TnI, Insulin, IL-6 and TSH by splitting a single sample from a reservoir into multiple droplets. The chemiluminescence signal thus obtained was fitted into respective standard curves, which were also generated on the chip, to calculate spike recoveries. Kinetic curves of the chemiluminescent signal for all the 4 assays are shown in Figure 2.

We are currently developing immunoassays to determine the phosphorylated and non-phosphorylated status of proteins. We employ a "spatial multiplexing" strategy where a sample droplet is divided into multiple droplets and an immunoassay is performed on each one of the droplets separately. The main advantage in this method is that cross reactivity between antibody pairs, which is a problem with other multiplexing approaches, is altogether avoided. The cell supernatant containing a mixture of analytes (EGFR, ERK-1 and Akt-1) is dispensed into six droplets and transported to different reaction areas on the cartridge where each sample droplet is mixed with another droplet containing magnetic beads labeled with capture antibodies

which bind to the specific phosphorylated or non-phosphorylated sites of the antigen. Incubation is performed by shuttling the droplet mixture over a set of electrodes to facilitate resuspension of magnetic beads. After primary capture, another droplet containing secondary antibody labeled with alkaline phosphatase is mixed with the magnetic bead suspension and incubated using the same protocol. The magnetic beads are immobilized using a permanent magnet placed under the cartridge and the unbound material is washed away using a dilution-based washing protocol. After washing, each droplet containing the magnetic beads with the immuno-complex is presented to a droplet containing chemiluminescent substrate. All the droplets are marched to a detection spot where the chemiluminescence is detected, the intensity of which is directly proportional to the concentration of the antigen. The versatility and programmability of the digital microfluidic multiwell plate lends itself readily adaptable to high throughput research environments as well as single-use point-of-care applications.

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Standardized Nanoarray PCR for Gene Expression Profiling of Lung Cancer

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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 absolute transcript abundance for 21 genes from routine clinical samples such as FFPE or FNA tissue blocks. SNAP assays are designed to work with highly degraded RNA as they use short (<80 length) PCR products. The use of internal standards ensures that each absolute abundance measurement will be consistent from lab-to-lab. SNAP accuracy is less sensitive to PCR inhibitors, often present in fixed clinical samples, as measurements arise from competitive PCR. Lastly, SNAP multiplex PCR enables low sample input typically isolated from fix clinical samples. The poster will present the initial analytic evaluation of SNAP dynamic range, precision, accuracy and sensitivity. SNAP and real-time qPCR measurements of lung tumor samples will be compared.

Study of Serum Biomarkers Using an Assay With Improved Specificity

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In a typical sandwich ELISA, the presence of human anti-animal immunoglobulin antibodies (HAIA) will interfere the antibody-antigen binding and lead to errors in the assay. If the existing HAIA recognize the Fc region of the capture and detection antibody, it will crosslink these two antibodies and indicate a 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 (MBB) to dilute the serum samples. As HAIA 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 with conventional BSA- based buffer. Using this assay system, we showed that significantly elevated serum levels of tyrosinase, cathepsin B and IL-8 in melanoma patients

are associated with poor prognosis; and the levels of nine cytokines are statistically increased in melanoma patients.

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 pre-clinical and clinical testing. Analysis of rodent and human tumor samples have been compared to focus on clinical meaningful targets and to understand model relevance to human disease. Tissue and tumor microenvironmental influences on endothelial cell expression are extensive. We have developed quantitative proteomic analysis using a new spectral intensity index to identify proteins specific to tumor vs. normal endothelium as well as concentrated in caveolae; many of which are confirmed by immuno-electron 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 function to 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 with in 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 deep and 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 hrs and after most of the injected antibody has been pumped inside the tumor where it is now trapped to kill not only tumor cells but also stromal cells surrounding the tumor and constituting the critical tumor microenvironment. This tumor penetration and treatment efficacy requires the presence of caveolae on the tumor endothelium and only occurs 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 the rodent models. We are testing different radionuclides to evaluate which one is most effective. Toxicology studies are ongoing. Our antibody appears 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.

Targeted Delivery of Folate-Conjugated Polymer/siRNA Complexes to Cancer Cells and Subsequent Gene Down-Regulation

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Folate receptors (FR) are over-expressed in some cancers, such as ovarian, lung, kidney, and brain cancer. Therefore folic acid (FA) and folate conjugates have been frequently used to deliver diagnostic and therapeutic agents to cancer cells through FR-assisted endocytosis. Towards the goal of developing efficient and cancer specific siRNA delivery reagents, we have developed a series of multivalent, biocompatible FA-conjugated polymers that complex with small interfering (siRNA) and protect it from enzymatic degradation. Herein, we demonstrate the cell-specific delivery of FA-polymer/siRNA complexes and subsequent gene suppression. Firstly, using a fluorophore-labeled siRNA (against human survivin) and fluorescence microscopy, we show the internalization of the FA-polymer/siRNA by Z-axis scanning and time-lapse analysis in KB cells (human nasopharyngeal cancer cells). Two other cancer cell lines have also been tested, including SKOV3 cells (Human ovarian cancer cells), and Hela cells (Human cervical cancer cells). Lyso-tracker, a specific endosome/lysosome fluorescence probe, is used to study colocalization of the fluorophore-labeled siRNA and endosome in KB cells, which indicates the FR-mediated siRNA delivery via endocytosis. Secondly, siRNA accumulates in cytoplasm at 36 hr post incubation, further indicating internalization of polymer/siRNA complexes. Additionally, KB cells are transfected with FA-polymer/siRNA complexes to examine the induction of gene silencing. We have observed significant gene suppression starting at 24 hr to 72 hr post transfection. A reduction of 60% in surviving mRNA has been achieved at 48 hr post incubation with 200nM siRNA and no cytotoxicity has been observed under the conditions. Furthermore, cell-specific targeting has been established by the following experiments: i) polymers without FA conjugation can complex siRNA, but do not deliver siRNA; ii) free FA competitively blocks the uptake of complexes and gene knockdown; and iii) FR-negative cancer cell line A549 (Human lung cancer cell) does not show uptake of FA-polymer/siRNA complexes. Taking together, we have illustrated a cell-specific siRNA delivery system that can achieve significant gene down regulation with low cytotoxicity. The system does not display some common drawbacks of conventional transfection reagents, and may lead to the development of therapeutic siRNA delivery systems for cancer therapy.

Using New Optical Techniques to Study Cell Signaling in Three-Dimensional Matrices

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Despite much progress in understanding cancer, additional information would be gained by the ability to image signaling events during carcinoma progression in vivo. Nonlinear optical imaging techniques such as multiphoton laser scanning microscopy (MPLSM) and second harmonic generation microscopy (SHG) used in conjunction with novel signal detection techniques such as spectral and fluorescence lifetime imaging (FLIM) hold great promise in both in vitro and in vivo cellular studies. As one approach, we are making use of endogenous fluorophores to better image stages in cancer progression. Cancer cells have altered metabolism marked by increased rates of glycolysis compared to normal cells. There are two ubiquitous metabolic cofactors, NAD(P)H and FAD, that play a key role in glycolysis and whose rates are dramatically changed as a result of tumor progression. These molecules are autofluorescent, and therefore can be exploited to image the metabolic state of the cell. Using FLIM, we find striking differences between tumor and normal cells in the intensity and lifetime of these metabolic intermediates, suggesting they may be useful optical biomarkers for tumor progression. As a second approach, we are using fluorescently-tagged exogenous probes to detect activation of the small GTPase, Rho, during tumor cell invasion into 3D and in vivo matrices. Visualization of signaling events in 3D matrices has not been widely performed, as imaging

under these conditions bears additional challenges. However, the use of FLIM and MPLSM allows us to observe signaling events in live cells in a 3D context, and detect FRET interactions by FLIM. Moreover, we can make use of SHG to image collagen fibers within the matrix. We find that Rho is activated along regions of cell contact with the collagen matrix in regions of contractility. Collectively, these observations will provide novel insights regarding the molecular mechanisms of carcinoma progression.

Frozen Sample Aliquotter

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Freezing biological specimens is a ubiquitous method of maintaining their original biochemical composition during extended storage in repositories. RNA and some proteins are known to degrade at liquid temperatures or with freeze-thaw cycling and cell life is extended by freezing. Repositories are faced with a critical choice when new samples are added to the collection: either freeze in aliquot sized volumes and suffer an increase in storage volume and the associated costs; or freeze in larger volumes reducing intitial processing and storage costs at the expense of freeze-thaw cycling when aliquots are requested from the repository.

We report on a robotic system that offers a compromise between these two positions, the reduced storage volume associated with larger initial storage volumes, and the avoidance of freeze thaw cycling associated with storing the sample in aliquot sized volumes which is combined with the low operating costs that accrue from an automated system. This system was described last year at ISBER, and this year we report on the automated design and more extensive biochemical testing of this system. The robotic system and the biochemical performance data will be presented, showing that the system achieves sample processing throughput and uses a protocol that is compatible with use in repositories, and assay results for five different analytes in serum that support the use of the aliquots for research purposes. The five analytes were selected because they represent the range of what is typically requested from clinical labs.