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1. **2D-PCR: A Method of Mapping DNA in Tissue Sections**

*Benjamin Shapiro, Michael Armani, Jaime Rodriguez-Canales, John Gillespie, Michael Tang, Heidi Erickson, Michael R. Emmert-Buck, Elisabeth Smela*

*University of Maryland, College Park, Maryland*

A novel approach was developed for mapping the location of target DNA in tissue sections. The method combines a high-density, multi-well plate with an innovative single-tube procedure to directly extract, amplify, and detect the DNA in parallel while maintaining the two-dimensional (2D) architecture of the tissue. A 2D map of the gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was created from a tissue section and shown to correlate with the spatial area of the sample. It is anticipated that this approach may be easily adapted to assess the status of multiple genes within tissue sections, yielding a molecular map that directly correlates with the histology of the sample. This will provide investigators with a new tool to interrogate the molecular heterogeneity of tissue specimens.

2. **A High-Throughput Platform to Characterize Interaction-Specific Network Perturbations by Cancer-Associated Mutant Proteins**

*Quan Zhong, Yun Shen, Changyu Fan, Dawit Balcha, Venus Swearingen, Tong Hao, Kourosh Salehi-Ashtiani, Wenchao Wang, Rani George, Michael E. Cusick, David E. Hill, Marc Vidal*

*Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts*

Cellular functions are mediated through complex systems of biochemical and physical interactions among macromolecules and metabolites. Better understanding of genotype-to-phenotype relationships in human diseases will require modeling how disease-causing mutations affect systems and interactome properties. We previously uncovered two distinct classes of interactome perturbations by causative mutations in human inherited disorders: gene loss versus interaction-specific perturbation. Interaction-specific perturbation confers distinct phenotypic consequence from gene loss (see reference). To begin to understand complex changes of cellular networks in cancer and to start assessing genotype-to-phenotype relationships in patients, we assembled a high-throughput platform to capture interaction-specific changes resulting from cancer-associated mutations. Following a Gateway compatible site-directed mutagenesis method we are generating ~300 mutant alleles in 67 cancer-causing genes. Each mutation confers a single amino acid change to the corresponding wild-type protein. Mutations include both germline and somatic variations and are associated with either cancer or non-cancer phenotypes. For simultaneous sequence verification of large numbers of mutant clones, we adapted our cloning pipeline to next-generation sequencing. Using a highly stringent yeast two-hybrid (Y2H) platform, we are determining interaction profiles for each cloned mutant protein and the corresponding wild type. Y2H interaction pairs will be validated with orthogonal protein interaction assays. A proof-of-principle profiling of a few selected cancer-associated mutant proteins revealed allele-specific loss or gain of specific protein-protein interactions, which appear to be relevant for the development of distinct forms of cancer.

**Reference**

3. An Integrated Microfluidic Device for Enrichment of Circulating Tumor Cells

Hsian-Rong Tseng

University of California, Los Angeles, Los Angeles, California

Over the past decade, circulating tumor cells (CTCs) has become an emerging biomarker for detecting early-stage cancer metastasis, predicting patient prognosis, as well as monitoring disease progression and therapeutic outcomes. However, isolation of CTCs has been technically challenging due to the extremely low abundance (a few to hundreds per ml) of CTCs among a high number of hematologic cells (109 per mL) in the blood. Our joint research team at UCLA has developed a new cell enrichment technology for quantification of CTCs in whole blood samples. Similar to most of the existing approaches, epithelial cell adhesion molecule antibody (anti-EpCAM) was grafted onto the surfaces to distinguish CTCs from the surrounding hematologic cells. The uniqueness of our technology is that it leakages the powers of (i) a high-affinity cell capture substrates based on nanostructured surfaces [1] and (ii) enhanced cell collision generated by a chaotic mixer [2]. The uniqueness of high-affinity cell capture substrates is the use of nanostructured surfaces, which facilitates local topographical interactions between CTCs and substrates at the very first cell/substrate contacting time point. In conjunction with the We demonstrated the ability of these nanostructured substrates to capture CTCs in whole blood samples with significantly improved efficiency and selectivity. The successful demonstration of this cell capture technology using brain, breast and prostate cancer cell lines encouraged us to test this approach in clinical setting. We have been able to bond our first validation study with a commercialized technology based on the use of immunomagnetic beads (i.e., CellSearchTM Assay). A group of clinically well-characterized prostate cancer patients at UCLA hospital have been recruited and tested in parallel by these two technologies.

Reference

4. A Novel Multiplex Assay Combining Autoantibodies Plus PSA Improves Classification of Prostate Cancer From Nonmalignant Cases

Gang Zeng¹, Chong Xie¹, Grace Kim², Gang Li², Allan Pantuck², Hideki Mukouyama³

¹Fudan University, Shanghai, China; ²University of California, Los Angeles, Los Angeles, California; ³Okinawa Nanbu Tokushukai Hospital, Yaese, Okinawa, Japan

Circulating autoantibodies (autoAb) against clinically relevant tumor-associated antigens (TAA) in cancer patients provide useful information regarding the status of cancer. However, current approaches to autoAb detection require preparation of phage lysates or purification of recombinant proteins. To circumvent these difficult procedures, we identified B cell epitopes from a number of previously defined prostate cancer-associated antigens (PCAA) and confirmed that autoAb against these peptide epitopes reacted specifically with the full-length protein. Peptide epitopes from cancer/testis antigen NY-ESO-1, XAGE-1b, SSX-2,4, as well as prostate cancer overexpressed antigen AMACR, p90 autoantigen, and LEDGF were conjugated with seroMAP microspheres to allow multiplex measurement of autoAb in serum samples. Furthermore, simultaneous quantification of autoAb plus total PSA was achieved in one reaction, and termed the A+PSA index. A logistic regression-based A+PSA index enhanced sensitivities and specificities over PSA alone in distinguishing prostate cancer from nonmalignant cases in a pilot study involving pre-surgery sera from 131 biopsy-confirmed prostate cancer patients, 121 benign prostatic hyperplasia/prostatitis patients, and 124 healthy donors. The A+PSA index also reduced false positive rate and improved the area under a
receiver operating characteristic curve, which was particularly pronounced for patients with intermediate levels of PSA. The A+PSA assay represents a novel platform that integrates autoAb signatures with a conventional biomarker, which may aid in the diagnosis and prognosis of prostate cancer and others.

5. A Novel Nanoparticle-Coupled Mass Spectrometry Approach for High-Throughput Low Molecular Weight Biomarker Enrichment, Isolation, Preservation, and Discovery

Lance Liotta1, Alessandra Luchini1, Davide Tamburro1, Claudia Fredolini1, Emanuel Petricoin1, Virginia Espina1, Benjamin Espina2

1George Mason University, Manassas, Virginia; 2Ceres Nanoscience, Manassas, Virginia

Mass spectrometry (MS) is a powerful tool for biomarker discovery, has intrinsic low sensitivity, and must be coupled to an appropriate sample enrichment method. Many of the widely accepted upstream protocols such as depletion columns, magnetic beads, and surface enrichment strategies have limitations. These include low binding capacity (sensitivity), user to user variability (reproducibility), susceptibility to protease degradation, prohibitive cost, and labor intensive protocols. Furthermore, many investigators are turning to the low molecular weight (LMW) and peptidomic information archive for biomarker discovery because this information traverses the endothelial barrier from tissue interstitium to circulation. A novel nanotechnology reagent, the Nanotrap, a core-shell hydrogel nanoparticle, was developed to address these issues in one rapid step. The nanoparticles simultaneously harvest LMW biomolecules through a molecular sieve shell from a complex biological matrix, and trap them by a tunable affinity bait covalently bound to the interior of the nanoparticles. This sequestration protects the labile biomarkers from degradation and dramatically concentrates them. The larger, abundant proteins are excluded from the nanoparticles and washed away. The purified and concentrated biomarkers are eluted for mass spectrometry analysis. The nanoparticles have been developed and optimized for a highly reproducible biomarker discovery platform combining Nanotrap biomarker harvesting, matrix assisted laser desorption/ionization time of flight (MALDI-TOF) or ESI MS analysis and rapid bioinformatics screening. As a proof of principle, this technology was applied to sequester a highly labile and low abundant molecule, platelet derived growth factor (PDGF) from its carrier protein, albumin. The nanoparticle sequestered proteins exhibited a remarkable 1000-fold increase in PDGF peptide signal in comparison to non-trapped samples. We have used this nanoparticle platform to identify hundreds of low abundance cellular proteins that have not previously been described in the HUPO plasma proteome database.

6. Advanced Technology for Assaying Cancer-Drug Resistance

Mark Lim, Vladislav Bergo, Ziyiing Liu, Karen Braunschweiger, Kenneth Rothschild

AmberGen Inc., Watertown, Massachusetts

Significant advances have been made toward the development of a new generation of molecularly targeted cancer drugs, many of which are only now emerging from the pipeline. This project aims to develop a new, highly sensitive technology for detecting drug-resistance mutations which preexist prior to treatment or are acquired due to the selective pressure exerted by treatment with molecularly targeted anti-cancer drugs (ACDs). This problem is exemplified by drug resistance developed in patients treated for chronic myeloid leukemia (CML) with the small molecule drug Imatinib (Gleevec/Glivec/STI571), by way of mutations in the BCR-ABL tyrosine kinase, the target for Imatinib. While direct DNA sequencing can be used to scan for previously characterized and uncharacterized drug-resistance mutations, sensitivity is limited to a 20% mutant population, impairing the early detection of developing drug resistance. More sensitive techniques generally require a priori knowledge of the mutation. In this project, these limitations are overcome by using novel technology developed by AmberGen for isolating highly purified cell-free
expressed polypeptide fragments of the drug-targeted protein(s) and detecting drug resistance mutations using a matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) scanning technique. The new approach, termed drug resistance assay for mutations against anti-cancer drugs (DRAMA-ACD), has the advantage that it allows low-cost scanning for mutations (even those previously undiscovered) with high sensitivity and high throughput. During Phase II we have shown detection of drug-resistance mutations in BCR-ABL at a sensitivity of 1% in a background of 99% wild-type. Furthermore, we have developed methods of confirming the mutations using the TOF/TOF capabilities of mass spectrometry to perform in-line sequencing of the test peptides. Finally, we have progressed towards a highly automatable, high throughput and multiplexed approach to DRAMA-ACD that will facilitate the broad applicability of this technology to the ultra-high sensitivity mutation scanning of cancers, across a multitude of target genes. Towards this end, we have developed an innovative approach termed Single-Bead DRAMA-ACD (sbDRAMA-ACD). sbDRAMA-ACD utilizes multiplexed solid-phase PCR to amplify the test gene segments, each sorted on their respective micro-beads. The entire DNA-bead population is then subjected to a single cell-free expression reaction whereby proteins are captured back onto the cognate (parent) DNA-beads from which they were made. Lastly, the beads are deposited on a surface to create an array, and the entire bead-array is scanned using an innovative MALDI-MS mass-imaging approach capable of spatially resolving individual 35 micron beads. This streamlined workflow reduces what would be hundreds of poorly automatable reactions into a few multiplexed and automatable reactions. Overall, the high-sensitivity and multiplexed mutation scanning capabilities of this novel approach will have broad-reaching applications in the molecular analysis of cancer.

### 7. Analysis of Tumorigenic Signaling Pathways With PhosphoPROTACs

*Craig Crews, John Hines, Peter Gareiss*

*Yale University, New Haven, Connecticut*

We have developed a strategy whereby the expression level of downstream effector proteins in mitogenic/oncogenic signaling pathways can be reduced or eliminated (“knocked down”) depending on the activation state of the upstream receptor tyrosine kinase (RTK). More specifically, through the use of a cell-permeable, chimeric peptide ligand, the target protein can be recruited to be ubiquitinated and degraded by the 26S proteasome. The peptide works by incorporating one of more tyrosines that, upon phosphorylation by RTKs, become ligands for PTB- or SH2-domain-containing proteins. By varying the sequence of the peptide, we have successfully knocked down different effector proteins. Since the activity of the peptide ligand is dependent upon its first becoming phosphorylated by the cognate RTK, we refer to it as a phosphorylation-dependent proteolysis targeting chimeric peptide, or “PhosphoPROTAC.”

We have previously demonstrated the effectiveness of the PhosphoPROTAC approach by knocking down the PTB domain-containing protein FRS2α upon activation of the NGF receptor, TrkA. Knockdown was selective for FRS2α and dependent on tyrosine phosphorylation of the PhosphoPROTAC by TrkA. The resultant inhibition of TrkA signaling blocked both activation of erk1/2 and NGF-induced differentiation of PC12 cells.

Our long-standing goal has been to develop a panel of PhosphoPROTACs that would (i) all target the critical survival-promoting effector, phosphatidylinositol-3 kinase (“PI3-K”), for knockdown, and (ii) each be activated by a different RTK. In this manner, the identity of the causative oncogenic signal in an unknown tumor sample could be determined by screening it with the panel of PhosphoPROTACs: only the PhosphoPROTAC corresponding to the causative RTK would be activated, resulting in growth inhibition and/or cell death due to PI3-K knockdown. We have succeeded in developing a PI3-K-targeting PhosphoPROTAC which is activated by the receptor tyrosine kinase ErbB2, which is frequently over-activated in human breast and prostate tumors. Tested in MCF-7 cells, the PI3-K-targeting PhosphoPROTAC dose-dependently and selectively knocks down its target upon neuregulin-stimulation of ErbB2/ErbB3 signaling, but does not reduce levels of non-target proteins like CD54 or SOCS3. Furthermore, knockdown of PI3-K leads to inhibition of Akt phosphorylation, and ultimately inhibits cell proliferation and/or
viability. Our PI3-K-targeting PhosphoPROTAC is even more potently active in MDA-MB-175 breast cancer cells, which secrete their own neuregulin and are highly dependent on its autocrine effects for their survival.

In addition to targeting PI3-K through an ErbB2-activated PhosphoPROTAC, we have created another PhosphoPROTAC that is activated by Flk-1, the VEGF receptor, which is also overexpressed in breast cancers. We are currently evaluating the effectiveness of this Flk-1-activated PhosphoPROTAC in both breast cancer cells and in primary endothelial cells. The ultimate goal is to use this technology to better match chemotherapeutics (e.g., kinase inhibitors) with those tumors against which they will be most effective by identifying the transforming RTK signaling pathway in primary tumor samples taken from patients.

8. Antibody Detection of Translocations (Adot) in Ewing’s Sarcoma

Stephen Lessnick, Wen Luo, Brett Milash, Brian Dalley, Richard Smith, Natalie Dutrow, Brad Cairns

Huntsman Cancer Institute, Salt Lake City, Utah

Objective
The detection and identification of chromosomal translocations have important implications in the diagnosis, prognosis, and treatment of patients with cancer. Current approaches to translocation detection often have significant shortcomings, including limited sensitivity and/or specificity, and can be difficult to apply to formalin-fixed paraffin-embedded (FFPE) clinical samples. We developed a new approach, that we refer to as antibody detection of translocations (ADOT), that avoids many of the shortcomings of current techniques.

Methods
ADOT combines a transcriptional microarray-based approach with a novel antibody-based detection method. The details of the methodology will be presented.

Results
ADOT allows for the accurate identification of translocations and also provides exon-level information about the specific fusion transcript produced. Furthermore, the technique is readily generalizable to any potential fusion transcript, and can also detect previously undescribed fusions. We demonstrate the feasibility of ADOT by examples in which both known, and unknown, translocation fusion transcripts are identified from cell lines, tumor xenografts, and primary tumor samples (including FFPE samples), even in cases where RT-PCR fails.

Conclusion
These results demonstrate that ADOT may be an effective approach for translocation analysis in clinical specimens with significant RNA degradation. This suggests that ADOT may be used as a diagnostic tool for pediatric sarcoma or other translocation-based cancers.
9. Bead Array-Mass Spectrometer: Conception, Challenges, and Realization of the Prototype

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At the heart of the project is a transformational technology that enables highly parallel, high-throughput, and highly multiparametric cell assays which permit extraction of larger amounts of data from smaller samples at increasing cost-effectiveness. The new technology takes advantage of the high resolution and sensitivity of Inductively Coupled Plasma Mass Spectrometry (ICP-MS), combined with the many available stable isotopes of elements of the periodic table, to simultaneously determine many proteins and gene transcripts in samples through the quantification of stable isotope tags bound to a wide variety of bioaffinity molecules. Our group has built and tested the Bead Array-Mass Spectrometer (BA-MS) which is at the center of this integrated massively multiplexed technology. Issues that remain to be addressed in this project, such as the complexity and expense of the BA-MS system; the uniformity of metal-encoded microbeads; and the development of sophisticated software for multiplex assays will be discussed in this poster.

We describe this novel bioanalytical platform, discuss its figures of merit, and present demonstrative applications. The approach we proposed and employed is based on the use of stable isotopes as “bar-codes” incorporated into the beads, with BA-MS instrumentation including Inductively Coupled Plasma ion source and Time of Flight (TOF) detection system. The instrument ion source employs a high temperature plasma, created through the coupling of radiofrequency (rf) energy into a flowing inert gas (argon). Challenges and solutions of design and implementation of the detection and sample introduction systems will be discussed.

The performance will be demonstrated using a prototype of the matching reagent support/encoding platform which is based upon metal-encoded functionalized polymer microbeads with the potential for creating more than a million distinguishable beads. These beads, in combination with a metal-chelating polymer reporter tag will provide researchers and clinicians with massively multiplexed analytical capabilities. Precepts that are being addressed include optimal functionalization to attach affinity reagents, uniformity of bead size and composition, and reproducible and narrow concentration of encoding atoms. These characteristics are preferable, if not essential, for quantitative massively-multiplexed bioassays, which is the larger goal of this research project.

Currently, our research group is fully involved in achieving the final goal of this project which is to demonstrate that BA-MS offers many advantages over fluorescence based assays in terms of the vast increase in magnitude of multiplexing that is accessible at similar or enhanced levels of sensitivity. Although the BA-MS instrument is still evolving, it is a fully functional prototype and some demonstrative examples will be presented. We will also describe experiments that will allow us to develop the sample preparation methods needed to make these massively multiplexed assays a reality. We already demonstrated that our technology is able to recognize many thousands of distinguishable analytical beads created by the incorporation of various concentrations and ratios of metal ions and results will be provided.

Our group also achieved significant progress in the development of algorithmic methods of bead recognition and presentation of multidimensional data in a format that the user can directly interpret and report. For the BA-MS application of many-dimensional measurements, the principal need is for recognition of specific multivariate bead signatures. Our achievements in solving this problem will be discussed.
10. CITP-Based Selective Tissue Proteome Enrichment

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In contrast to universally enriching all analytes by a similar degree, the result of the capillary isotachophoresis (CITP) stacking process is that major components may be diluted, but trace compounds are concentrated. Such selective enhancement toward low abundance proteins will drastically reduce the range of relative protein abundances within complex tissue proteomes, and greatly enhance the proteome coverage using the CITP-based proteomic technology. Our proposed research efforts therefore aim to fully characterize, develop, and exploit the use of this differential concentration effect to achieve comprehensive proteome analysis of clinical specimens with limited sample availability.

The laser capture microdissection (LCM) process provides a rapid and straightforward method for isolating selected subpopulations of cells for downstream biochemical and molecular analyses. On the basis of cell enrichment, LCM also serves as a targeted sample fractionation approach toward the reduction in protein complexity and relative abundance. The proposed coupling of tissue microdissection for diseased cell enrichment with CITP-based selective analyte concentration not only presents a synergistic strategy for the detection and characterization of low abundance proteins, but also offers a novel biomarker discovery paradigm for enabling the identification of tumor-associated markers, exploration of molecular relationships among different tumor states and phenotypes, and a deeper understanding of molecular mechanisms that drive cancer progression.

11. 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 domains of the de novo DNA methyltransferases DNMT3A and DNMT3B. The most efficient crosslinking is obtained using DNMTs with point mutation of a cysteine located in the base-flipping pocket: C710S, in the case of DNMT3A, and C651S, DNMT3B. We have achieved high efficiency crosslinking with oligonucleotides specific for the BRCA1 promoter, and are in the process of purifying the DNMT-oligonucleotide complexes and performing affinity purification of associated proteins from breast cancer cell extracts. 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.

12. 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$_2$O$_2$-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, mass spectrometry (MS), and imaging based methods. We have shown that these probes are specific for cysteine sulfenic acid and that an initial “burst” of R-SOH formation is observed within the first 2-30 minutes after stimulation of cells with various cytokines and growth factors. Moreover, inhibition of endogenous catalase with 3-aminotriazole, which specifically increases H$_2$O$_2$ 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). 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$_2$O$_2$] 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 occur within cellular proteins involved in phosphorylation cascades (kinases as well as phosphatases) and in calcium-regulated processes. Another of our probes, the fluorescent DCP-Rho1, can be used to label sites of protein oxidation in situ in cells; subsequent immunochemical staining demonstrated that foci of protein oxidation are observed around
regions of endocytosed receptor complexes after treatment of prostate cancer (PC-3) cells with lysophosphatidic acid, an endogenously produced growth factor.

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 the development of novel cancer therapies based on the inhibition of ROS-dependent proliferative signaling.

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We previously reported novel nucleoside linker chemistry designed for positioning covalently bound ligands between any nucleotides in a DNA or RNA duplexes without interfering with Watson-Crick base paring [1]. The goal of current study was to investigate interactions of NF-kB p50/p65 transcription factor complex with near-infrared FRET and quenched DNA duplex sensors based on the above chemistry by applying a time domain multiexponential analysis for resolving discrete fluorescence lifetimes (FL-TD) present within the sample mixture [2]. We used hybrid phosphorothioate-phosphodiester DNA duplexes for creating efficient trans-duplex FRET sensor (FRET efficiency ~ 82%, Cy5.5 - donor/Cy7 or 800CW - acceptor) carrying the dyes oppositely linked to complementary strands. In such DNA duplex sensor the dye pair is non-interacting and sterically constrained from quenching. We assayed the sensitivity of FL-TD to the presence of FRET and non-FRET Cy5.5 populations, which showed good correlation of FL and data obtained in phantoms consisting of a mixture of FRET (Cy5.5/Cy7) and non-FRET (Cy5.5) duplexes, where FL of Cy5.5 was short in the case of FRET. These experiments suggested that FL-TD can be used for detecting minor fractions of non-FRET duplexes in mixtures of fluorescent probes. The addition of p50 and p65 (3 fold molar excess over DNA) to the solution of FRET probe (100 nM) resulted in a measurable increase of Cy5.5 dye lifetime, which depended on acceptor (Cy7- 1.05 to 1.13 ns and 800CW - 1.1 to 1.25 ns). These results suggest interference of protein-DNA interactions with NIR FRET. In view of (1) applicability of NIR fluorochromes for in vivo imaging and (2) inherent advantages of the asymptotic FL-TD approach to fluorescence tomography when deconvoluting discrete lifetimes [2], we anticipate further development of chemically stabilized NIR FRET sensors for direct imaging of transcription factor interactions in living systems using fluorescence lifetime techniques.

References
14. Development of Endogenous Epitope Tagging (EET) Technology for Identification and Analysis of Protein/Protein Interactions in Human Cancer Cells

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Epitope tagging is a powerful and commonly used approach for studying the physical properties of proteins and their functions and localization in eukaryotic cells. In the case of Saccharomyces cerevisiae, it has been possible to exploit the high efficiency of homologous recombination to tag proteins by modifying their endogenous genes, making it possible to tag virtually every endogenous gene and perform genome-wide proteomics experiments. However, due to the relative inefficiency of homologous recombination in cultured human cells, epitope-tagging approaches have been limited to ectopically expressed transgenes, with the attendant limitations of their nonphysiological transcriptional regulation and levels of expression. To overcome this limitation, we are developing a modification and extension of adeno-associated virus-mediated human somatic cell gene targeting technology that makes it possible to simply and easily create an endogenous epitope tag in the same way that it is possible to knock out a gene. Using this approach, we have created and validated human cell lines with epitope-tagged alleles of a variety of cancer-related genes (e.g., PTEN, p53) in several untransformed and transformed human cell lines. Mass spectrometry analysis of immunoprecipitates from these tagged cell lines has identified a variety of known and novel interacting proteins. Current work is focused on optimizing the tag(s) for this approach and for generating a complete interactome of a cancer signaling pathway. This straightforward approach makes it possible to study the physical and biological properties of endogenous proteins in human cells without the need for specialized antibodies for individual proteins of interest.

15. Development of a Hand-Held Cancer Biomarker Monitor

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The project develop a Hand-Held cancer biomarker monitor based on munochromatographic technology and nanoparticle based electrochemical immunoassay for rapid, sensitive, and lowcost detection of cancer biomarkers in human blood samples. Breast cancer biomarkers including carcinoenbryonic antigen (CEA), CA15-3 and human mammaglobin will be used as model biomarkers to demonstrate the proof of principle. The principle of the proposed device is based on nanoparticle-powered multiplex bioelectrochemical immunodetection and immunochromatographic separation technique. The multiplex capabilities and significant signal amplification of electrochemical immunoassay are realized conveniently by the use of multiple metallic phosphate nanoparticle labels. Integrating with immunochromatographic separation technology, the new biosensor microanalysis device will provide a portable, sensitive, simple, and low-cost tool for the rapid detection of multiple breast cancer biomarkers. The complete assay time will be less than 10 minutes and the detection limits are 10 times lower than the cutoff values. The device will be used to detect the samples from breast cancer patients and the results will be validated with traditional Enzyme-Linked ImmunoSorbent Assay. If the project is successful, the proposed device will be an effective and innovative tool in aiding early cancer diagnosis, monitoring response to therapy and providing real-time prognostic information in patients with cancers. The developed device can also be applied for the detection of other cancer biomarkers.
16. Development of Imaging Reagents to Monitor GTP Levels and GTP/GDP Ratios In Vivo

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Cellular processes intimately tied to cancer, and to a variety of other human pathologies, are globally regulated or coordinated by GTP and ATP, molecules that represent the energy currency of the cell and which are also the most common allosteric modulators of protein function. Methods to measure the levels of these molecules in vivo, with high temporal and spatial resolution, would be invaluable in understanding how variations in GTP and ATP concentrations regulate and coordinate cellular metabolic processes, and in elucidating the role played by disruption of cellular metabolism in cancer and other diseases. Such methods luminescence by insect luciferase and fluorescence from GFP ATP binding protein fusions exist for ATP. The luciferase assay is widely used and has led to important recent discoveries regarding the role of variations in ATP levels in the cell cycle and human disease, including cancer. However, no equivalent methods exist for GTP, even though GTP, through its action on numerous G-proteins, arguably plays a larger regulatory role in the cell than ATP. To address the need for such technology we will (1) engineer insect luciferase so that it will specifically use GTP, rather than ATP, to generate light and (2) engineer GFP-G-protein fusions that will exhibit altered excitation spectra upon binding GTP. These genetically encoded sensors will provide two complementary methods for monitoring GTP levels and GTP/GDP ratios inside living cells with high temporal and spatial resolution.

17. Dominant Factors Promote Genome Demethylation in Mammary Carcinoma

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Genomic methylation patterns have long been known to undergo alterations during carcinogenesis, but the scale and nature of the changes are controversial. We examined whole-genome primary mammary carcinoma methylation patterns with a novel method that profiles methylated sites over the entire assembled genome. We show that demethylation is a common event in breast cancer, and that both single copy and repetitive sequences are affected. Cell hybrids made from a severely demethylated breast cancer cell line and a methylated cell line showed a net loss of methylation, which indicates that the general demethylation seen in tumors is caused by a dominant factor that induces demethylation rather than loss of a factor required for maintenance methylation. The data lead to a reevaluation of the role of abnormalities of genomic methylation patterns in breast cancer and suggest that methylation abnormalities are likely to be a manifestation of a protective system that kills incipient cancer cells.
18. Enhanced Sensitivity of Pancreatic Cancer Detection by Measuring the CA 19-9 Antigen on Selected Protein Carriers

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We report here progress on the development of a blood test that can detect a higher percentage of pancreatic cancer patients than the tumor marker CA 19-9. We tested the hypothesis that the measurement of the CA 19-9 antigen (an oligosaccharide found on multiple proteins) on individual proteins could contribute to improved performance over the standard CA 19-9 assay, which measures the CA 19-9 antigen on all proteins. Serum or plasma samples were incubated on microarrays containing antibodies against the mucin proteins MUC1, MUC5AC, and MUC16. After the proteins were captured by the immobilized antibodies, the levels of the CA 19-9 antigen on the captured proteins were measured by incubation of the CA 19-9 monoclonal antibody. Four sample sets from three different institutions were examined, with a total of 333 individual samples from patients with pancreatic adenocarcinoma or pancreatitis. The CA 19-9 marker distinguished cancer from benign disease with 84%-87% sensitivity at 75% specificity. The measurements of CA 19-9 on individual protein carriers contributed complementary information to the standard CA 19-9 marker. Thresholds could be set to detect elevations in a subset of the patients that showed no CA 19-9 elevations, while not increasing the false positive rate. In all sample sets, the panel showed improved sensitivity (85%-100%) over total CA19-9 alone (79%-90%). Among all false negatives classified by total CA19-9, the additional three markers in the panel picked up from 25% to 100% of them. The consistent performance over independent sample sets supports the generality and reliability of the novel marker panel as a tool for improving the accuracy of diagnoses of pancreatic cancer.

19. FRET-Based Biosensors to Monitor Redox in Cell Cycle Regulation

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Cancer can be viewed as a state in which the balance between cell proliferation and cell death aberrantly favors the former. We and others have discovered that the intracellular redox environment exerts a profound influence on the normal cellular processes that regulate the balance between proliferation and cell death, including DNA synthesis, enzyme activation, cell cycle progression, proliferation, differentiation, and apoptosis. In fact, it could be argued that redox homeostasis is central to the governance of cell fate. Unfortunately, molecular mechanisms mediating redox sensitivity and regulation within cells are still poorly defined. Current pharmacological methods to alter intracellular redox state are limited by (i) their inability to operate independent of global biochemical alterations and cellular toxicity, and (ii) the required significant manipulation of culture conditions that perturb intracellular homeostasis. Our genetic constructs overcome these limitations as they enable real-time and extended assessment of alterations in intracellular redox without cellular disruption. These constructs use fluorescence resonance energy transfer (FRET), a distance- and orientation-dependent energy transfer process between donor and acceptor fluorophores. In these biosensors a change in redox induces a conformational change in the redox-sensitive switch that links the donor and acceptor, changing their distance, which in turn causes a detectable change in FRET efficiency. Here we propose to further define the sensitivity and dynamic range of our FRET biosensors relative to changes in the intracellular redox environment that appear to dictate cell fate. Advantages of this approach include: (1) the ability to quantify the change in redox state; (2) independence of sensor concentration; and (3) the ability to precisely tune the redox sensitivity and range by exchange of the switch or the fluorophore modules in the construct.
Aim 1
Define the sensitivity and dynamic range of genetically engineered FRET redox biosensors during proliferation by comparison of nontransformed fibroblasts and isogenic porcine tumor cell lines with respect to the presence or absence of contact inhibition. Specifically, detection of physiologically relevant changes during successive stages of cell growth is proposed.

Aim 2
Determine the extent to which the FRET biosensors are sensitive to changes in the intracellular redox environment of isogenic HCT116 p53+/+ and p53-/- cells treated with the chemotherapeutic drugs fluorouracil and doxorubicin in combination with perturbations in glutathione homeostasis. Specifically, the intracellular redox environment will be visualized in response to common chemotherapeutic drugs in combination with agents that modulate biosynthesis or metabolism of glutathione.

Aim 3
Create second generation FRET biosensors that permit visual monitoring and dissection of intraorganellar local redox potentials. Specifically, we intend to quantify differences in redox potentials within subcellular organelles that are at a nonequilibrium steady-state with respect to each other in living cells.

In sum, the proposed work will provide novel molecular tools that enable in depth examination of the role of redox signaling at the intracellular and intraorganellar level in cancer development.

20. Functional Metabolomics and Metabolic Flux Analysis in Cancer

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The mechanistic and transformative role of metabolism in tumor initiation and progression is a research topic of increasing importance. Isotopomer-based functional metabolomics and metabolic flux analysis are the most direct and informative approaches with which to study cellular metabolic processes. However, for a number of reasons, the robust isotopomer methodologies developed in bacterial systems are not commonly used in mammalian cancer research. This research proposal aims to evaluate and address the major issues confronting the application of isotopomer tracing in mammalian cells, including the use of non-glucose substrates, sample size and throughput, and the relevance of key tumor model systems. We will build on our recent development of a glucose-based metabolic model for human tumor cells to improve our detection capabilities, expand our isotopomer model and determine the extent of conservation between in vitro and in vivo models, all with the overall goal of facilitating the broader use of functional metabolomics in cancer research. This will directly support both the NCI’s goal of funding research with a high potential for positive patient impact and the IMAT program’s goal of supporting the development of transformative technologies.

Specifically, we are asking three interrelated questions. (1) How can we conserve the isotopic and chemical information provided by isotopic labeling while significantly decreasing sample size and increasing throughput? (2) Which metabolic fluxes can be quantified by tracking various metabolic substrates, and how is this information best incorporated into a comprehensive flux model? (3) To what extent does tumor cell metabolism in two-dimensional tissue culture reflect tumor metabolism in vivo?

The results presented here will focus on (1) our improvements in sample size requirements and throughput, and (2) expanding the metabolic fluxes that we are able to quantify. In regard to goal (1), we have decreased the sample size required for mapping central carbon metabolic fluxes 100-fold and are nearing the 1 million cell threshold. In goal (2), we have nearly doubled the number of metabolites that we are able to identify by GCMS while conserving all stable
isotope-derived information. Additionally, we have integrated both metabolite pool size data and $^{13}$C labeling data from media components into our isotopomer model. This allows us to calculate cellular import, export and exchange rates of key metabolites in addition to intracellular fluxes. These technical advances have been developed in human tumor cells and are generally applicable to all mammalian cell systems.

21. Gas-Phase Ion-Electron Reactions for Carbohydrate Structural Determination

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Cell surface carbohydrates have been shown to be altered in several types of cancer. Methods for analyzing carbohydrates include lectin affinity and tandem mass spectrometry involving collision activated dissociation (CAD). The former technique combined with fluorescent labels is very sensitive but does not provide detailed overall structural information. The latter technique generates carbohydrate composition but often fails to yield detailed structural information, including specific linkage and branching. CAD can be combined with permethylation or other types of chemical derivatization to improve glycan structural characterization but such reactions are challenging for low sample amounts. Complete structural information is essential, e.g., when synthesis of a cancer vaccine candidate is to be performed.

The work funded by our NCI grant involves the development of alternative electron-based gas-phase fragmentation techniques for generation of structurally informative cross-ring fragments, complementary to those generated by CAD. Further, we are applying metal oxide-based enrichment to specifically target acidic glycans, i.e., sulfated and sialylated species, which are known to be particularly important in cancer progression and metastasis. In collaboration with Prof. Diane Simeone, we are combining gas-phase ion-electron reactions and metal oxide-based enrichment to target the acidic cell surface glycome of pancreatic cancer stem cells. A majority of cell surface markers indicative of cancer stem cells are glycoproteins. Our hypothesis is that cell surface glycosylation differs between cancer non-stem and stem cells and that this difference may be exploited to specifically target cancer stem cells.

A major challenge for analyzing the cell surface glycosylation of pancreatic cancer stem cells is their low occurrence in a tumor: only ~0.5% of cells carry the surface markers CD24, CD44 and ESA, which are indicative of pancreatic cancer stem cells. Further, acidic glycans are particularly challenging to analyze with mass spectrometry because they ionize poorly in positive ion mode and they are labile in the gas phase. We will implement on- and off-line normal phase nano-scale liquid chromatography with metal oxide enrichment on a high resolution FT-ICR mass spectrometer to reach the desired detection limit along with several improvements to current glycan release procedures.

Work since the grant was awarded includes a demonstration that electron-induced dissociation (EID) yields significantly more product ions compared to CAD for protonated glycans. We have also studied the effect of carbohydrate derivatization and found that fluorescent labels (9-aminofluorene, 2-aminobenzamide (2-AB), and 2-aminobenzoic acid (2-AA)) increase structural information derived from EID, presumably due to introduction of aromatic electrons that can more easily undergo electronic excitation upon electron irradiation. These labels are commonly used for detection via UV absorbance or fluorescence. In electron detachment dissociation (EDD) we found that labeling can decrease the amount of generated glycan structural information. We hypothesize that this reduction in cross-ring fragmentation is due to altered charge location in the gas-phase glycan ions.

Overall, these approaches will allow more detailed glycan structural information to be generated at the sensitivity compatible with sorted cells from a single xenograft tumor.
22. Global Peptide Microarray Analysis of Tyrosine Kinases Deregulated in Cancer

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Protein tyrosine kinases (PTKs) play pivotal roles in human cancer and are the targets of a major class of emerging anti-cancer drugs. In a single tumor, multiple PTKs are active and a substantial number are essential for maintaining the transformed phenotype. Though large numbers of phosphorylation sites have been mapped in cancer cells through mass spectrometry, the identity of the specific kinases that phosphorylate these sites are with very few exceptions unknown. To more thoroughly establish direct kinase-substrate relationships, we are using peptide microarrays to identify consensus phosphorylation sequences for the entire set of human PTKs. Microarrays of biotinylated peptides were printed on avidin-coated glass slides. Immobilized peptides are treated with kinase in the presence of radiolabeled ATP, and the extent of phosphorylation is quantified through exposure to a phosphor screen. We are using a set of 171 peptide substrates in which the amino acid located at each of 9 positions surrounding a central tyrosine residue is systematically substituted for each of the 20 amino acids (excluding cysteine). The microarrays therefore indicate those residues that are preferred or disfavored by the kinase at each position. This method produced qualitatively similar results, though with a higher background, when compared to a previous “macroarray” approach. We found however, that much smaller quantities of kinase were required to phosphorylated the microarrays, thus enabling larger scale analysis of kinase specificity. We have thus far generated a set of 30 mammalian expression vectors corresponding to almost all human non-receptor tyrosine kinases (NRTKs) that produces each one as glutathione S-transferase fusion protein. Each kinase was expressed and purified from HEK293T cells and subjected to peptide microarray screening. This analysis has revealed specific sequences preferred by 24 kinases at phosphorylation sites in their target substrates, while the remaining kinases either provided insufficient signal above background. We observe some features common to all NRTKs, including a strong preference for aliphatic residues at the position immediately upstream of the phosphorylation site. In addition, the various subfamilies of NRTKs can be distinguished based on their preferred phosphorylation site sequences. Correlation of residues found in the kinase catalytic domains with those selected in substrates revealed structural features controlling substrate specificity, which allow us to predict the behavior of kinases that were intractable to microarray analysis. We are currently expanding these efforts to cover the full complement of human tyrosine kinases. We will use this data to mine phosphoproteomics data from cancer cells to connect known sites of phosphorylation to their respective kinases. These studies will enrich our understanding of the basic mechanisms of cellular transformation and tumor maintenance, provide insight into the mechanisms of action of kinase-targeted therapeutics, and suggest new targets for therapeutic intervention.

23. High-Definition Clonal Analyses of Archival Pancreatic Adenocarcinoma Sample

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A fundamental challenge for the application of high definition genomic technologies to the study of human cancers is the non tumor cell admixtures in clinical samples. Furthermore multiple tumor cell populations which cannot be distinguished by morphology may be present even in small biopsies. In order to address this heterogeneity we have applied DNA content based flow cytometry to identify and subsequently purify distinct sets of aneuploid and diploid cell populations directly from clinical samples of interest. These include frozen resection samples, needle biopsies, pleural effusions, and archived formalin fixed paraffin embedded (FFPE) samples. In each case tissues are disaggregated, and then the nuclei are extracted and resuspended as single particles in the presence of the DNA stain DAPI. For single parameter DNA content assays samples are then analyzed with an Influx Cytoperia Influx
cytometer (Becton-Dickinson, San Jose CA), with ultraviolet excitation and DAPI emission collected at >450nm. To advance this technology for the study of pancreatic ductal adenocarcinoma (PDA) we selected a series of samples from the Johns Hopkins Rapid Medical Donation Program. These include primary and metastatic lesions from individual patients who died from advanced PDA. In each case we profiled matching fresh frozen and FFPE samples from each site. We optimized the extraction of nuclei from these samples and their preparation for flow cytometry. The histograms of the major populations detected in these samples have coefficients of variation of less than 10% making it possible to distinguish and isolate diploid, aneuploid and 4N (G2/M) populations from each tissue of interest. We have optimized the preparation and use of genomic DNA for whole genome oligonucleotide based aCGH studies extracted from these sorted samples. Quality metrics include the purity and specific activities of labeled DNA, the distribution of signal intensities in the sample (Cy-5) and reference (Cy-3) channels, and the width of the distribution of the background subtracted dye normalized ratios for the probes on the array. In each case we compared these metrics in matched fresh frozen and FFPE samples using the diploid and aneuploid populations sorted from each tissue. Presently we have obtained high definition whole genome oligonucleotide based aCGH results from FFPE materials using genomic DNA extracted from 25,000 flow sorted nuclei. We have also shown that these methods work with tissues from other indications including glioblastoma and melanoma using both single parameter and multiparameter assays. The definition of these clonal analyses in FFPE samples include the detection of homozygous deletions, the discrimination of single copy losses, and the mapping of the boundaries of amplicons and breakpoints.

24. High Pressure-Assisted Extraction for the Improved Proteomic Analysis of FFPE Tissue

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Background and Significance
Proteomic methods hold great promise for developing knowledge of the molecular characteristics of disease. Formaldehyde-fixed, paraffin-embedded (FFPE) tissue repositories represent an invaluable resource for the retrospective study of disease progression and response to therapy. However, the analysis of FFPE tissues by proteomic methods has been hampered by formaldehyde-induced protein adducts and cross-links.

Sample Preparation Technology
We have previously shown that heat augmented with high pressure reverses inter-molecular cross-links in a single-protein tissue surrogate, a model FFPE tissue formed by treating cytoplasmic proteins with formaldehyde (Fowler, et. al. Lab Invest 88, 185). In this study, FFPE mouse liver sections were cleared of paraffin and homogenized in 50 mM Tris/2% SDS, pH 4, 7, or 9. The tissue was incubated 100 °C for 30 min followed by 80 °C for 2 h at 1 atmosphere, or 40,000 psi. Extracts from matched fresh tissue and the FFPE tissue were separated by SDS-PAGE. The tryptic digests of each gel lane were analyzed by mass spectrometry (MS). A multi-protein tissue surrogate was also heated with or without elevated pressure and the quality of the recovered proteins was evaluated by MS.

Results
The protein extraction efficiency was 4-fold greater for pressure-extracted tissue surrogates, than for surrogates extracted at atmospheric pressure. MS of the high pressure-retrieved FFPE surrogates also showed that the low and high-abundance component proteins were identified with sequence coverage comparable to the unfixed protein mixture. Non-pressure-extracted surrogates yielded few peptide identifications and a high number of false peptide identifications by MS (42%-100%). When the FFPE mouse liver was extracted with heat and elevated pressure, there was an almost 2-fold increase in the number of unique protein identifications by MS compared to FFPE tissue.
extracted without elevated pressure. More importantly, the number of unique peptides and proteins identified from FPPE mouse liver were virtually identical to matched fresh tissue.

**Conclusions**

In this study, we show dramatically improved identification of proteins by MS in FFPE tissue and tissue surrogates. Accordingly, these experiments establish that elevated hydrostatic pressure treatment is a promising approach for improving the recovery of proteins from archival tissue for proteomic analysis.

### 25. 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. 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, covering 76% of all genes and 70% of all bona fide CpG islands. When coupled with microarrays, this technique has proven to be highly specific and very useful for the high-throughput analysis of genome-wide methylation in normal and cancer cells. Problems such as non-uniform probe performance, cross-reactivity, difficulties of normalization, and issues of relevant controls, however, prevent the full quantitative potential of MCA from being realized. We have developed and validated a high-resolution tool for DNA methylation profiling by coupling MCA with “next generation” Solexa1G sequencing technology (MCA-Seq). Here we describe various strategies to optimize MCA-Seq to improve coverage and minimize the quantity of initial DNA required. Additionally, we have established quality controls for MCA-Seq and developed optimized algorithms for data analysis. We also outline our current efforts to evaluate the sensitivity, specificity, and quantitative accuracy of MCA-Seq using an innovative approach that simulates biological variation in methylation. The development and validation of MCA-Seq will result in the development of a simple, robust and reliable genome-wide assay for DNA methylation which will have broad utility in cancer research.

### 26. High-Sensitivity Detection and Isolation of Circulating Tumor Cells

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In recent years, correlation between the number of circulating tumor cells (CTCs) in peripheral blood and survival in metastatic cancer patients has been reported. These reports demonstrate that the number of CTCs can provide an early and reproducible indication of disease progression and treatment efficacy, and establish CTCs as an emerging and important marker of disease status. The ability to isolate CTCs and subsequently profile the biochemical changes that have occurred in these cells will greatly enhance our understanding both of cancer metastases and in assessing the metastatic risks of cancer patients.

It is, however, difficult to detect and study CTCs. The principal difficulty originates from the rarity of CTCs, especially in peripheral blood where they are the most accessible from a clinical perspective. There is on the order of 1-100 tumor cells per 1 million mononuclear cells. This concentration is equivalent to about 1-100 tumor cells per 0.5 mL of peripheral blood. Practically, this means that if only 1 CTCs is present in 1 million mononuclear cells, a sample with an estimated 100 million cells must be screened in order to detect at least one CTC with 99.995% certainty.
To address this challenge, we have developed a sensitive technique (which we termed eDAR) for the isolation of CTCs from blood. Our approach is based on aliquoting blood into nanoliter volumes, high-sensitivity and high-speed detection of a CTC within the nanoliter volume of blood in a flow-through format, sorting of the volume that contains the CTC, and isolation of the CTCs contained within the sorted volumes. Our approach is particularly well suited for the enrichment of rare cells such as CTCs: By aliquoting blood into nanoliter volumes and by discarding volumes that do not contain any rare cells, we can rapidly and efficiently reduce sample complexity by over a million fold.

To further remove CTCs from unwanted blood cells, our approach also integrates a specially designed filtration system that is hydrodynamically engineered to remove efficiently blood cells from the isolated CTCs. We have determined our integrated system to be extremely sensitive and to have excellent throughput. We can also isolate live CTCs, as well as remove single isolated CTCs for downstream analysis and manipulations. In this presentation, I will describe both the concept behind our technique as well as our recent results.

27. Highly Parallel Edman Sequencing of Individual Peptide Molecules

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Most biological processes are executed by proteins, but no method currently exists to accurately measure protein abundance and post-translational state proteome-wide. To redress this deficiency, we propose Digital Analysis of Proteins by End Sequencing (DAPES), a method that sequences many individual peptide molecules in parallel using Edman degradation. DAPES will be cost-effective, highly sensitive, and quantitative. DPA is based on two innovations: (1) the use of dye-labeled antibodies to inexpensively and robustly detect single peptide molecules and (2) a strategy that uses a universal set of ~20 antibodies to sequence peptide molecules. Our previous work, in which we used fluorescent antibodies to detect and quantify protein levels by single molecule counting, demonstrates that this approach is realistic and powerful.

28. Ice-COLD-PCR Enriches All Low-Prevalence Unknown Mutations Efficiently Using a Wild-Type-Blocking Reference Sequence During COLD-PCR

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Objectives
Molecular profiling of somatic mutations in cancer often requires the identification of low-prevalence DNA mutations in an excess of wild-type (WT) DNA; however, the method selectivity and sensitivity are often limiting factors. COLD-PCR (CO-amplification at Lower Denaturation Temperature) is a new technology funded by IMAT program, that resolves several limitations in low-level mutation detection by using critical denaturation temperatures to enrich mutant-containing amplicons during PCR. However, certain mutation types are not enriched by some COLD-PCR formats. We report a novel enhancement in COLD-PCR that enables Improved and Complete Enrichment (ice-COLD-PCR) for all mutation types in an efficient and robust manner.

Methodology
A Reference Sequence (RS) that preferably forms double-stranded structures with WT sequences, but not with mutant sequences, was added to COLD-PCR. A RS was designed to bind to one WT-sequence strand, avoid primer binding and prevent polymerase extension. To validate the use of a RS in COLD-PCR, we evaluated segments of TP53
exon 8. Serial dilutions of mutant cell-line DNA, or human lung tumor DNA, in WT DNA were analyzed. Mutations that increase, decrease, or retain the amplicon melting temperature were tested. Following conventional-PCR, COLD-PCR (full or fast COLD-PCR formats), and ice-COLD-PCR methods amplicons were sequenced and the degree of mutation enrichment was compared. Several clinical lung adenocarcinoma samples with known low-level mutations were also analyzed with ice-COLD-PCR.

Validation
ice-COLD-PCR yielded ~13-fold enrichment for Tm-increasing and Tm-equivalent mutations, and ~15-fold enrichment for Tm-reducing mutations. In contrast, Full-COLD-PCR demonstrated ~5-fold enrichment for all mutations. Further, fast-COLD-PCR, which can only enrich Tm-reducing mutations, exhibited ~17-fold enrichment for these types of mutations, while the Tm-increasing and Tm-equivalent mutations remained undetectable. Regardless of mutant type and position, after ice-COLD-PCR amplification, all mutation types are strongly enriched and can be reliably sequenced down to a level of 1-3%. Ice-COLD-PCR duration is ~1 hour, compared to several hours for full-COLD-PCR.

Conclusions
The inclusion of an appropriately designed RS within COLD-PCR selectively inhibits WT amplification throughout PCR, while preferentially enriching mutants and reducing time-intensive hybridization times. Ice-COLD-PCR combines high sensitivity, speed, and low-cost, and facilitates direct sequencing for all types of unknown low-prevalence mutations in clinical cancer samples.

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29. Imaging DNA Damage Dynamics for Diagnostics, Screening, and Target Discovery

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Radiation remains an important treatment modality in the therapy of breast cancer. Ionizing radiation induces DNA double strand breaks, which initiates a characteristic response. ATM and related protein kinases recognize each break and phosphorylate adjacent histone H2AX, promoting assembly of ionizing radiation induced foci (IRIF), multiprotein signaling and repair complexes spread over megabases of the surrounding chromatin. The resulting amplification mediates its effects by activating downstream kinases to induce cell cycle arrest, DNA repair, and apoptotic responses.

This project is directed toward developing a novel reporter of DNA damage response that will allow tracking of the formation and resolution of DNA damage foci in normal and breast cancer cells. Toward these ends, we have developed a fluorescent protein fusion based on the checkpoint protein 53BP1 that accumulates at double strand breaks. GFP fused to 53BP1 IRIF binding domain (GFP-53BP1-IBD) forms fluorescent foci at DSBs in living cells, providing a tag for both visualization and biochemistry. In this project, we are using these fusion proteins to follow foci kinetics after ionizing radiation in vitro and in vivo, for high content screening to discover new small molecule modulators of DNA damage repair and as proteomic probes to identify proteins that constitute IRIF.

As an initial application of this technology, we tracked IRIF kinetics in breast cancer cells and tumors. We expressed GFP-53BP1-IBD under tetracycline-inducible control in MCF7, a p53 positive, caspase-3 negative, apoptosis resistant human breast cancer cell line (MCF7Tet-On GFP-53BP1-IBD cells). While IRIF largely resolved within 24 h after 3 or 6 Gy, higher IR doses greatly increased IRIF persistence. Screening candidate shRNAs and small molecules targeting chromatin modifiers revealed that poly(ADP-ribose) polymerase (PARP) inhibitors promote IRIF persistence. When combined with PARP inhibitor ABT-888 (veliparib), currently in clinical trials, the clinically relevant dose of 3 Gy
induced an IRIF response equivalent to > 6 Gy and markedly decreased clonogenic survival. At 7 days, ABT-888 increased residual IRIF numbers, senescence associated beta-galactosidase activity and p21CIP levels. We used the MCF7Tet-On GFP-53BP1-IBD cells to form xenograft tumors in nude mice. Foci kinetics imaged with two-photon microscopy were similar to cells in vitro and ABT-888 induced similar delays in foci kinetics. We treated MCF7 tumors with a single dose of radiation with or without ABT-888 and excised the tumors after 7 d. ABT-888 plus 3 or 6 Gy produced SA-βGal staining in tumors to the level observed for 9 or 12 Gy alone and markedly decreased tumor growth. Our data suggest that ABT-888 and other agents that stabilize IRIF and induce cell senescence may find broad uses as radiosensitizers for cancer therapy.

Based on these studies, we have used high content screening to discover novel small molecule modulators of DNA repair, based on perturbation of GFP-53BP1-IBD foci formation and kinetics after a 6 Gy dose. In a screen of 2000 drugs and natural products, we recovered nine compounds that blocked foci formation, including three previously described radiosensitizers. We are characterizing the novel compounds for effects on radiation response. We have also identified other compounds that increase foci persistence.

We have also performed an initial proteomic screen to determine the protein components of the GFP-53BP1-IBD foci and their dynamics during repair. We adapted standard ChIP protocols using anti-GFP magnetic beads to isolate IRIF-associated chromatin from irradiated MCF7Tet-On GFP-53BP1-IBD cells over time. Mass spectrometry (MS) analysis confirmed that GFP-53BP1-IBD is successfully enriched in our sample, and histones are robustly identified. DNA damage response proteins identified by LC-MS/MS include PARP-1, DNA-PK, FACT complex, Ku70/80, topoisomerases and histone deacetylases (HDACs). Quantitative analysis of identified proteins using 16O/18O labeling is currently underway to identify differences in the IRIF composition at early versus late time points post irradiation, and to identify factors that correlate with radiosensitivity. Also ongoing is the MS analysis on ABT-888 treated MCF7Tet-On GFP-53BP1-IBD cells. From these studies we expect to discover novel IRIF interactors. Functional significance will be examined using shRNA-mediated knockdown in MCF7Tet-On GFP-53BP1-IBD cells to observe changes in IRIF kinetics.

We anticipate that this new technology can be used to determine the radiation responses of tumors and normal tissues, to validate radiosensitizers and other drugs affecting DNA damage response and to discover and validate new biological targets to enhance radiation effects.

30. Improved Designs for a Microfabricated Magnetic Sifter for Biomolecule and Cell Purification

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Magnetic separation of proteins and cells is a method of growing importance in biology and medicine. Previously, we reported preliminary designs for a novel magnetic separation device, the magnetic sifter [1,2]. The magnetic sifter utilizes parallel fluid flow through a dense array of slots in a magnetically soft membrane. Early designs with very narrow slots suffered from low capture efficiencies and irreversible binding of magnetic capture probes, inspiring us to explore modified structures.

We have used finite element modeling to show that the magnetic field and field gradient, and therefore capture force, experienced by magnetic capture probes is sufficiently large when the slot width is increased from 4 μm to 40 μm. The increase in slot width is experimentally found to improves flow uniformity, reduces slot clogging, and allows for efficient rinsing of the sifter following capture. The result is higher capture efficiencies, excellent reproducibility, and complete release of captured magnetic particles.
Our data shows the separation results from three consecutive separations of MACS nanoparticles with a sifter containing 40 μm square-shaped slots. The average capture efficiency is 77 ± 0.2%, and the average elution efficiency of captured magnetic nanoprobes is 99 ± 2.7%. The flow rate for these experiments was 1 ml/hr. Efforts are underway to increase the sifter area, currently 20 mm², to increase volume throughput without an increase in linear velocity of capture probes and the associated reduction in capture efficiency.

The increased slot size of the sifter also enables its use for applications in flow-through magnetic cell separation of magnetic cell. Separations have been carried out with magnetically labeled human umbilical vein endothelial cells (HUVEC). The planarity of the sifter allows for observation of the separation process with a microscope. Optical observations of the capture process indicate capture efficiencies higher than 90% at a flow rate of 1 ml/hr.

References

31. Improved Insertional Mutagenesis for Molecular Analysis of Cancer

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Our goal is to develop a function-based gene discovery platform for forward genetics applications in molecular oncology that is applicable with minor or no changes to any biological system and process, unbiased in regard to the knowledge about candidate genes and properties of their products, affordable, and readily upgradable. We chose insertional mutagenesis as a starting point. We have documented that inclusion of a promoter in an inserted DNA fragment greatly increases the yield of phenotypically detectable mutants. Depending on the site and orientation of an insert, random insertion of a promoter may lead to expression of a complete host gene or generate truncated or anti-sense products. Such events manifest as dominant gain- or loss-of-function mutations. Promoter-dependence, as tested by inactivation of the promoter, may be used to confirm a causative link between insertion and the phenotype of a mutant clone.

We have constructed retroviral vectors for reversible insertional mutagenesis and have used them successfully for studies of NFkB signaling pathway. For the multiple mutants obtained in such experiments we were readily able to establish the relevance of individual targeted loci, even when multiple inserts were harbored by the same cell. Because the function of the targeted locus falls under the control of the inserted regulated promoter, not only do such mutants reveal the identity of a relevant gene, but also represent valuable tools to study the gene’s function. For example, we were able to document the existence of a positive feedback loop, which includes NFkB-dependent secretion of molecules that, in turn, can further activate NFkB by signaling from the outside of a cell.

In addition to the vectors based on gamma-retroviruses and lentiviruses, we have successfully used insertional mutagens based on Sleeping Beauty transposons. Devoid of certain limitations and safety concerns of viral vectors, these constructs proved adequate for tissue culture use and helped us to discover new elements in the regulation of TNFa pathway. Furthermore, these mutagens are especially promising for in vivo applications, where they demonstrate efficient mobilization both in somatic and generative tissues.

Our work on vector design has revealed some pitfalls, which have to be accounted for in the experiments involving transduction and insertional mutagenesis. For example, we have documented that a natural human retrovirus can
restore replicative ability to common retroviral vectors. We have also observed that insertion of a construct harboring a strong enhancer can cause a mutant phenotype by influencing a gene tens or even hundreds of kilobases away from the insertion site. In those cases, the relevance of the immediate target site to the phenotype of the mutant clone cannot be taken for granted. This observation warrants extra caution when vectors are designed for experimental and clinical applications, and questions the validity of claims about the relevance of frequent integration targets that did not undergo functional validation.

In order to increase the throughput of mapping and validating the relevant target sites and to enable the application of reversible insertional mutagenesis to the situations where high background is expected, we have started using vector-derived fusion transcripts as “tags” for individual mutant clones. Tracing the changes in the composition of the pools of the “tags” under various conditions allows us to judge the properties of the respective mutants. We are currently working on establishing the utility of this technique for discovering the genetic determinants of drug response in prostate cancer.

Overall, we have tested and validated many improvements to insertional mutagenesis as a forward genetics tool. The technology enabled numerous findings in ours and other laboratories. The work to further improve its utility continues.

32. Integrated Genomic Approaches to Identify and Validate Cancer Targets

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Recent advances in genomics now make it possible to consider enumerating all of the genetic lesions in specific cancers. While these approaches will yield critical information regarding the identify, number, and types of alterations found in human tumors, a complementary approach to decipher the molecular basis of malignant transformation depends upon the application of genome scale tools to annotate the function of genes involved in cancer initiation and progression. Over the past several years, we have developed genome scale RNAi libraries and open reading frame expression libraries that permit a systematic evaluation of genes involved in cancer initiation and maintenance. Using these libraries, we have now performed screens in a panel of human cancer cell lines to systematically identify cancer vulnerabilities. By combining these functional approaches with information derived from mapping the structural abnormalities present in cancer genomes, we have identified several new oncogenes that contribute to cancer development. In addition, many commonly occurring and well-validated oncogenes and tumor suppressor genes remain refractory to molecularly targeted therapies. For example, the proto-oncogene KRAS is mutated in a wide array of human cancers, most of which are aggressive and respond poorly to standard therapies. An alternative strategy for targeting KRAS is to identify gene products that, when suppressed or inhibited, result in cell death only in the presence of an oncogenic allele. Through the use of systematic RNAi screens, we have identified two kinases, TBK1 and STK33 that act in a synthetic lethal manner to selectively kill cancer cell lines that depend on mutant KRAS. Taken together, these studies suggest that combining forward and reverse genetic approaches with information derived from the cancer genome characterization projects will yield a comprehensive list of cancer vulnerabilities and establish a general approach for the rational identification of oncogenic and co-dependent pathways in cancer.
Mitotic homologous recombination (HR) is a DNA damage repair/tolerance mechanism associated with cancer formation. While HR is usually error free, errors in HR can lead to large-scale sequence rearrangements: greater damage than the pathway originally attempted to process. In addition, defects in the HR pathway are known to promote cancer (e.g., BRCA1 and BRCA2). Due to the importance of this pathway for health, and due to the fact that agents that induce HR are often carcinogenic, we set out to develop methods to monitor HR in animals.

We have developed a mouse model in which cells that have undergone homologous recombination at an integrated transgene can be detected by a fluorescent signal. The fluorescent yellow direct repeat (FYDR) mouse contains a single-copy recombination substrate integrated in the genome. This mouse offers advantages over previous systems: (1) effects of exposures to adult animals can be studied and (2) fluorescence allows easy detection of HR in contrast to laborious processing necessary with earlier methods. HR events are detected by in situ imaging in intact tissue and flow cytometry in disaggregated tissue, as well as in cultured primary cells. Data collection and analysis are facilitated by automated imaging. Furthermore, we have developed a combination one- and two-photon imaging platform that is able to identify and analyze rare recombinant cells within intact tissue.

Using the FYDR mouse, we have shown that HR is induced by several DNA-damaging chemicals in adult mice, and that recombinant cells accumulate with age. Combining DNA-damaging agents and induced cell proliferation revealed that the effect of genotoxic exposure can be modulated by the physiological state of a tissue. This result has implications in assessing the risk from hormone mimetics, widespread environmental contaminants whose carcinogenic potential has been difficult to determine. We are expanding the use of this system to test the role of oncogenes and DNA repair genes in HR induction, and to test the effect of chronic inflammation, an established risk factor for cancer.

Use of the FYDR mouse is limited by the expression pattern of the recombination substrate (pancreas and certain epithelial tissues). We have recently created the next-generation mouse, termed RADR (recombination at a direct repeat), which has been generated by targeting the recombination substrate to the ROSA26 locus, previously shown to permit ubiquitous expression. Recent data show impressive wide-spread expression that opens doors to analysis of many more tissues. In combination with the development of automated foci counting software (currently under development), we have therefore created a rapid method for assessing the impact of genetic and environmental factors on genomic stability in vivo.

This work is supported by CA112151 and the Singapore-MIT Alliance for Research and Technology.
Isolation of Rare Tumor Cells From Blood Using Perfluorocarbon Microbubbles

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During cancer disease, malignant cells are shed into blood (1). These extremely rare cells (few cells/ml blood) could be isolated and analyzed to provide invaluable information for diagnosis and prognosis of cancer patients. Immunomagnetic microbeads are currently used for tumor cell isolation from blood samples of cancer patients. This method is very sensitive (1 cell/ml blood), but produces significant contamination of non-specific cells in the isolated sample (2).

We propose to develop a cell isolation technique based on capture of rare cells in blood by gas-filled microbubbles (MBs). Previously we demonstrated (3) that targeted MBs attach to their target cells in blood, rendering them buoyant and separable from the rest of the sample by flotation. In this project, we propose to test and optimize the efficiency of separation of tumor cells from blood. The method of MB-assisted separation will be benchmarked against other existing methods and combined together with downstream cell analysis such as microfluidic sorting, and molecular analysis using surface cell markers and PCR.

In the first part of this project, microbubble formulation was optimized to achieve efficient binding to beads and cells in both PBS and blood. We used streptavidin-coated microspheres; B-cell lymphoma and lung carcinoma cells (A549) that express EpCAM for the optimization experiments. The efficiency of binding was >90% for beads and cells. Efficient concentration and harvesting of MBs is critical for the technology. The stability of MBs is important for the centrifugal separation from blood. By varying lipid composition and by adding polyethylene glycol, the stability was achieved to withstand gravity forces of up to 500g (up to 95% recovery of MBs after the centrifugation step). A collection device was developed to harvest the separated microbubbles. Finally, the non-specific binding of white blood cells to MBs vs. magnetic beads was tested. MB were incubated with normal mouse or human blood and then separated by centrifugation. Microbubbles containing PEG 5000 Da produced much less non-specific binding than magnetic beads or non-PEGylated MBs (<1000 WBC/ml blood). There was variability in the non-specific binding to MBs as a function of freshness of blood samples and also due to donor-to-donor variability.

In the next stage of the project, the optimized Mb formulation targeted against epithelial marker EpCAM will be used in order to isolate rare cancer cells (A549 lung carcinoma) from various volumes of human blood. The selectivity and specificity, and enrichment factor will be analyzed after separation of cells with MBs. In collaboration with the Department of Electrical Engineering, UCSD, the isolated rare tumor cells will be counted in a microfluidic flow cytometry device and further purified for molecular analysis. This combination has the advantage of high speed and high throughput of microbubble separation and ability to count and separate cells on a small scale by microfluidic cell sorting.

The microbubble method could become a useful strategy for molecular analysis of circulating tumor cells from clinical samples due to: quickstep separation of cancer cells, low level of contamination, concentration of the sample in a small volume for further processing, and the compatibility with downstream single cell analysis.

References
Our prior work has led to several robust, sensitive and specific solid-phase (heterogeneous) assays for tyrosine kinase activity in cellular lysates, using immunodetection or MALDI-TOF MS analysis as a read-out. This project is directed at developing technology to measure oncogenic kinase activity in solid tumor tissue sections with high spatial resolution. Toward addressing this challenge, we have developed methods for tethering peptides onto micron-sized superparamagnetic beads as immobilized substrates in kinase assays. Our concept is to gently permeabilize cells at the surface of tumor tissue sections, apply the beads as local biosensors to allow contact between kinase and substrate, use magnetic surfaces to corral the beads and maintain spatial distribution and then interrogate the beads to determine phosphorylation.

As an attractive label-free approach, MALDI has been demonstrated to be a powerful tool for protein kinase assays because it offers quantitative determination of phosphorylation via comparing intensity of the phosphorylated and unphosphorylated forms of substrate peptides. Further, MALDI-TOF is compatible with multiplexed assays and characterized by high sensitivity, selectivity and speed. However, insofar as MALDI-TOF is confounded by sample complexity, salts and detergents, complex cellular samples are incompatible with this approach. To allow measurement of kinase activity in complex samples such as cell lysates, we have developed label-free kinase assay techniques in which magnetic beads are used as an analytical platform to immobilize peptide substrates via photocleavable linkers for release by UV light and MALDI detection. We chose magnetic beads as solid supports because they facilitate on-bead preparation of peptide conjugates and rapid separation of phosphorylated products. Further, magnetic beads are readily adapted to automated workflows. In addition, the paramagnetic beads may promote the ionization of phosphorylated products during the process of MALDI analysis, and further increase assay sensitivity.

Spacers separating peptide substrates from the support surface affect sensitivity in solid phase kinase assays. Using the magnetic bead support strategy allows straightforward surface modification. We performed a systematic study by introducing different spacers between peptide substrates and magnetic beads to understand their influence on the kinase assays. Both the single polyglycine spacer and PEG spacer were able to enhance the phosphorylation of peptide substrates. Notably, a diblock spacer consisting of polyglycine and PEG greatly improved the degree of peptide phosphorylation as compared with the other spacers due to the synergic effects of the two types of spacers. To validate the assay, we tested the activity of two small-molecule kinase inhibitors, imatinib and dasatinib, which target the oncogenic mutant tyrosine kinase Bcr-Abl to treat chronic myeloid leukemia (CML). Examining inhibition of the purified c-Abl or Bcr-Abl in K562 CML cell extracts, we obtained IC\textsubscript{50} values consistent with the literature. As this new kinase assay technique can rapidly quantify protein kinase activities directly from complex cellular mixtures, it provides a reasonable strategy for the development of cell-based screens for drug discovery. Unlike traditional methods, MALDI can readily differentiate multiple peptides and their modified forms. Thus, the use of the label-free MALDI detection tool provides this new kinase assay with the potential for quantitative evaluation of the relative potencies of a drug to different kinases. Further work will be directed toward achieving highly multiplexed analysis of multiple assays with spatial resolution across a tumor section.
36. Mesoporous Silica Chips for the Selective Enrichment of Low Molecular Weight Proteome to Evaluate Response to Therapy and Disease Progression

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Many studies have investigated how circulating low molecular weight (LMW) proteome can reflect ongoing pathological conditions in patients. The mesoporous silica chip (MSC) technology we developed for this study provides unprecedented performance in: (1) biomolecular analyses, (2) addressing the fundamental bottlenecks that have limited the use of blood proteomics and peptidomics for the early detection of cancer, and (3) for the monitoring of therapeutic efficiency. We introduce a combinatorial approach for the removal of the high molecular weight proteins and for the specific isolation and enrichment of low molecular weight species. This approach is based on mesoporous silica chips able to fractionate, selectively harvest and protect from enzymatic degradation, peptides and proteins present in complex human biological fluids. The MSCs, with fine controlled properties, will provide a powerful platform for proteomics application offering a rapid and efficient methodology for low molecular weight biomarker discovery. We are currently screening the serum peptidome and integrating the use of an optimized set of MSC in combination with mass spectrometry profiling (MALDI-TOF-MS) and sequencing to identify novel biomarkers for malignant transformation in a melanoma model as well as the evaluation of the response to several therapeutic strategies for melanoma tumor growth and metastasis (liposomal IL-8 siRNA in combination with temozolomide, and liposomal IL-8 siRNA delivery from a multistage mesoporous silicon drug delivery particle).

37. Methods for Parallel Crystallization of Steroid Receptor Ligand Binding Domains That Are Important Therapeutic Targets in Cancer

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The estrogen and androgen receptors (ER and AR) represent therapeutic targets for hormone sensitive cancers. Synthetic glucocorticoids targeting the glucocorticoid receptor (GR) are used to induce apoptosis in some blood cancers, and are widely used as part of chemotherapy regimes. In order to speed the development of improved therapies targeting these steroid hormone receptors (SHR), we have developed technology to increase the rate of obtaining x-ray crystal structures of ligand-receptor complexes by several orders of magnitude. The SHR ligand binding domain misfolds when expressed in bacteria, requiring addition of ligand to the fermentation media to facilitate proper folding, and maintenance of the single compound during the purification and crystallization. A second significant barrier to rapid crystallization is conformational heterogeneity, which is compound specific.

Our R33 proposal is based on the development of technology enabled high throughput crystallization of ER in the active conformation via the introduction of a surface mutation, which facilitated proper folding in bacteria, and conformation homogeneity [1].

Our grant is focused on transferring this technology to stabilize ER in the inactive conformation, and to enable high throughput crystallization of GR and AR. Our approach was to analyze published crystal structures and identify surface mutations that would add hydrogen bonds between secondary structural features. These mutations were screened in cell based assays for predicted changes in transcriptional activity of the full length SHRs, and for changes in ligand specific recruitment of transcriptional coregulator proteins. Ligand exchange in the recombinant With GR,
we identified mutations that solved the protein misfolding problem, enabling growth in bacteria with the low affinity ligand, progesterone, and successful ligand exchange with a high affinity synthetic glucocorticoid. Protein folding and ligand exchange were assayed with a thermofluor unfolding assay. With AR, a mutation designed to stabilize the active conformation induced constitutive activity, also an enhancement of activity in response to testosterone. These AR and GR mutants are currently being tested in x-ray crystallography trials.

Other mutations were identified that stabilize the inactive conformation of ER [2]. This enabled us to crystallize the same compound in both the mutant stabilized active and inactive conformations, which has not been done before for any protein-ligand pair. Surprisingly, the partial agonist ligand bound differently to the active and inactive ER conformers. Structural and functional analyses of a series of chemical derivatives demonstrated that altering the ensemble of ligand binding orientations changes signaling output. The coupling of different ligand binding orientations to distinct active and inactive protein conformations defines a novel mechanism for titrating allosteric signaling activity. Further, our technology to sample major conformational substates revealed a fundamental change in how we think about ligand-protein complexes. Current theories of ligand binding suggest that proteins display an ensemble of conformations, and that small molecules select for or bind to a subset of these, as typified by Gleevec binding to c-Abl. In contrast, our findings establish that conformational change can also direct adaptable interactions, where different conformers of a receptor are instructive in selecting for and/or binding to different orientations of a given ligand, giving a mixed population of protein conformation/ligand orientation states that collectively determine biological activity.

References

38. Microfluidic 3D 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 has 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 so far have consisted of cancer cell lines, with potential for input of biopsy specimens, with sources ranging from known libraries of cells to patient samples. Optimized gel parameters have been established to present appropriate chemotactic gradients and physical parameters simulating a tumor microenvironment. The EC layer
mimics the in vivo vascular barrier allowing observation of cancer cell intravasation or extravasation. Optical access permits real time observation of cancer cell migration and intravasation. The optical access combined with image processing techniques has allowed quantified cancer cell morphological and migratory parameters. Initial devices fabricated of polydimethylsiloxane (PDMS) have proven the device conceptually and allowed for data collection. To aid in transitioning the technology to a commercial application and allow material selection other than PDMS, recent work has established a hot-embossing and thermal lamination approach to fabricate the devices of cyclic olefin copolymer (COC). Hot-embossing is a high-throughput and low-cost manufacturing method with the ability to produce devices of numerous thermoplastic materials. The lamination step seals the device using low-cost and durable COC thin films, as opposed to glass cover slips used in the PDMS versions of the device. COC provides excellent optical and chemical resistance qualities, and does not possess some disadvantages of PDMS, namely absorption of small molecules and unstable surface properties.

Development of the hot-embossed COC \( \mu \)MIA has yielded a manufacturing process for device production. Micropatterned photoresist provided a mold to generate a durable embossing mold. The durable mold, formed from an epoxy cast, could be used to emboss many devices without significant degradation. An energetic oxygen plasma provided a means to control surface hydrophobicity of the COC and enhance thermal bonding during the lamination of the thin COC film to the COC base material. The COC material possessed more stable surface hydrophobicity as compared to PDMS, allowing for a more consistent gel- and cell-material interaction. Through control of COC hydrophobicity and collagen gel injection into the device, 3D gel matrices were formed as in the PDMS version. Although oxygen transfer into the COC device is limited by lower oxygen permeability of COC versus PDMS, viability of endothelial cells within the device was similar to that in the PDMS device or a standard cell culture dish. The hot-embossed COC platform will provide a more commercially-viable and stable platform for further studies of cancer cell migration, intravasation, and response to interstitial flow in Specific Aim 2.

39. Microfluidic Perfusion Array for Automated 3D ECM Culture

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We have developed a microfluidic perfusion array for culture of cancer cells in three-dimensional gels of extracellular matrix. Key aspects of this design include the ability to pipette 3 ul samples directly into the open-top culture chambers, a gravity driven continuous perfusion design for long term culture without nutrient limitation, and a 96 well format compatible with existing automation tools. Each 96-well plate contains 32 independent culture units, each with an inlet well, a cell culture well, and an outlet well. The channels are designed for 100 μl/day of medium flow from inlet to outlet via passive gravity flow, eliminating the need for external pumps. The culture chamber is an open-top well of 2 mm diameter and 1 mm height, surrounded by a microfluidic perfusion network. A 4 μm microfluidic mesh prevents cells and gel loaded into the chamber from hindering nutrient flow. The flexible format enables loading cells in 2D, embedded in 3D gel, sandwiched between 2 layers of gel, or with gel overlay. The bottom surface is a #1.5 thickness (170 μm) glass coverslide, enabling high quality imaging with any inverted microscope. Here, we demonstrate 3D culture of MCF-10A cells in Matrigel (BD Biosciences). Culture of these mammary epithelial cells in 3D gel induces acinar morphology similar to in vivo after 4-9 days. This model was used to show a drug toxicity screen compared to the same cells in 2D culture. This robust, easy to use, and flexible 3D perfusion culture format promises to provide an improved tool for studying cancer cell biology in more relevant in vitro environments.
Rationale
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.

We have developed a novel, integrated mass spectrometry strategy for RNA proteomics that enables efficient capture of RNA/protein complexes as they are created in living cells and processes them to discover the complete proteome of those complexes. Several methods have been developed to discover RNAs that associate with proteins in cells (CLIP, RIP-CLIP, PAR-CLIP, etc.). These methods are protein-centric in that they examine a single RNA binding protein and identify the spectrum of RNA molecules associated with it. Our method, MS2-BioTRAP (MS2 in vivo Biotin Tagged RNA Affinity Purification), is RNA-centric, it discovers all the proteins that associate with a single RNA target.

Method
A cluster of RNA stem-loops are engineered into a target RNA to function as a high-affinity tag for co-expressed and in vivo biotinylated MS2 protein (MS2-Bio). Target RNAs are produced by authentic in vivo processes enabling capture of bona fide complexes and in vivo biotinylation of MS2-Bio allows efficient and rapid capture of target RNAs via single-step purification. UV cross-linking prior to cell lysis enables isolation of RNA/protein complexes under denaturing conditions. SILAC-based quantitative mass spectrometry is used to profile proteins that bind specifically to regulatory elements within the RNA target. For proof of principle, we defined the proteome of an Internal Ribosome Entry region (IRES) from Lymphoid Enhancer Factor-1 mRNA, a 1.2 kb RNA sequence in the 5\' untranslated region important for LEF-1 protein production. LEF-1 mRNA and protein is often overexpressed in Wnt- and BcrAbl-driven cancer cells, but the underlying mechanism is not understood. MS2-BioTRAP identified 36 proteins that associate with the LEF-1 IRES in vivo. Some of these proteins are known IRES binding factors, but others are novel. Bioinformatic network analysis showed that the identified proteins have known direct and indirect associations, suggesting that an authentic IRES complex was isolated.

MS2-BioTRAP has broad applicability and versatility. It can be used to study many different types of RNA, and can be modified to focus on complexes in specific subcellular compartments, cell conditions, or cell types. Multiple RNA targets can be studied in parallel from the same MS2-Bio-expressing cell source (MS2-Bio expressed transiently or stably). Currently the method uses a cell line or primary cell source that can be expanded in SILAC media conditions to yield significant RNA/protein starting material (15-25 mg total cell protein). Once the proteome of an RNA complex is defined, it can be studied for response to cell stresses, oncogenic signals, and cancer-relevant drugs to discover how the RNA/protein complex is affected by these treatments.
Cancer diagnostics and prognostics have become increasingly reliant on high-throughput molecular profiling technologies such as RT-PCR, gene chips, and protein microarrays. These technologies can be used to examine the expression levels of many molecular markers in parallel, and hence, allow comprehensive pathway analyses to be harnessed to delineate pathophysiological changes associated with malignant and non-malignant diseases. Such capabilities are critical to the development of preventative and therapeutic strategies that are personalized for individual patients (i.e., optimized for patients possessing different disease status, environmental stresses, and/or ethnic/racial backgrounds). However, in contrast to image-based histological approaches, most molecular profiling methods cannot be used to resolve spatially-dependent distributions of marker levels within cells and tissues sections since markers detection requires their extraction from bulk samples. This type of information has proven invaluable to the diagnosis and clinical management of a wide variety of cancers, which is why image-based histopathological approaches are among the most widely employed cancer assessments strategies. Yet, only a handful of markers can be examined at a time using existing microscopy techniques. Furthermore, biopsies are often small, limited in number and precious; hence, the use of multiple tissue sections from the same biopsy does not necessarily offer opportunities to increase the number of markers that can be studied. Instead, the markers selected for tumor tissue characterization must be carefully prioritized using mostly generalized knowledge and selection criteria that cannot be customized easily for an individual patient. Efforts to personalize cancer diagnosis and therapies would therefore be enhanced significantly if more comprehensive molecular pathway analyses could be performed in situ on individual tissue sections.

Our project addresses two core limitations of current in situ imaging approaches: (1) dye molecules used as reporters of marker levels possess significant spectral overlap, and (2) it is not possible to remove dyes from samples without the use of harsh chemical and/or physical treatments that can perturb cell and tissue morphology, and even disturb marker integrity. The number of markers that can be examined on a single tissue section therefore remains low (typically 3 to 7). To circumvent these limitations, we have developed new reactive DNA complexes that can function as erasable molecular imaging probes. Importantly, these probes facilitate marker labeling and dye removal while using exceptionally mild processing conditions (room temperature reactions in neutral Tris buffers). With this capability, signals generated by each marker can be erased after an image is collected, and the fluorescent channels of a microscope can therefore be used reiteratively to detect different sets of markers. Herein, we provide proof-of-concept for our multiplexed and reiterative markers labeling strategy: we demonstrate that a two color imaging platform can be used to detect 4 different targets. Furthermore, we show that protein markers can be labeled quantitatively yielding linear correlations between markers levels and probe signals. We also demonstrate that the room temperature removal of dyes is efficient and non-perturbative, and therefore, that the same markers on cells can be labeled multiple times. Finally, we will discuss our ongoing efforts to develop synthetic strategies to generate libraries of molecular targeting agents that can be interfaced with our probes and used to profile the status of full marker panels on individual tissue sections.
42. Nanoelectrode Arrays for Study of the Molecular Mechanisms, Triggers, and Inhibitors of Apoptosis

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In this project we aim to develop a nano-electrode technology to interrogate the membrane potential of isolated, vital mitochondria in an on-chip assay. Mitochondria are the central regulator of apoptosis, a process initiated by the activation of the mitochondrial permeability transition pore (mtPTP). When this pore opens, the mitochondrial membrane potential collapses. This process is the “point of no return” for apoptosis. It is generally agreed upon that repression of apoptosis is one of the fundamental steps in tumorigenesis. Cancer cells acquire unresponsiveness to apoptosis facilitating signals, thus enabling uncontrolled proliferation. For this reason, the induction of apoptosis is one of the modes of actions of chemotherapeutic compounds. In order to allow further high throughput studies of the biochemical facilitators and inhibitors of apoptosis, and to determine if changes in individual mitochondrial membrane potential are important to cellular metabolism, we need to develop a system to monitor the membrane potential in individual mitochondria. To accomplish this objective, we propose to extend studies that have monitored the action potentials in neurons using an array of parallel electrodes to which the mitochondria are adhered.

In our preliminary studies [see reference], we have shown the ability to measure the membrane potential of an ensemble of mitochondria using an electrochemical detection method. By using 0.3 ng/mL of isolated mitochondria (Heb7A; a HeLa cell-derived line), we reduced the concentration of mitochondria required (compared to existing assays) by four orders of magnitude, using an on-chip sample chamber that was two orders of magnitude smaller in volume than traditional assays. In the studies currently underway, we are developing technology to probe the membrane potential at the single mitochondrial level. Our current plan of attack is as follow: In order to confine single mitochondria on a nanoelectrode, a nanofluidic channel will be fabricated. The position of mitochondria inside the nanochannel can be precisely controlling by the flow rate inside the channel, thus allowing the positioning of individual mitochondrial over nanoelectrodes which can capacitively sense the membrane potential of individual mitochondria.

A limitation of the technology is that vital mitochondria must be isolated from living cells, which is presently done manually. However, a strength of this technology is that only very small amounts of mitochondria are required. This will enable applications which previously were prohibited because of lack of sufficient amount of sample material.

This ultimate limit of sensitivity will allow assays of mitochondrial function (essential in studies of bio-energetics, as well as studies of the mechanisms, inhibitors, and triggers of apoptosis) to be studied in extremely small sample volumes.

Example applications which require such small sample volumes include, e.g., biopsies for clinical applications assessing patient metabolism, stem-cell research (including cancer stem cell studies), and fundamental studies of apoptosis and cancer biology. Several key features on mitochondrial metabolism are now recognized as important to the alteration of cancer cell mitochondrial function: changes in the Akt signal transduction pathway, induction of hexokinase II, alteration an adenine nucleotide translocator (ANT) isoform expression, down regulation of the SOC2 cytochrome c oxidase (complex IV, COX) assembly factor, mutation in mitochondrial DNA (mtDNA) genes, and modulation of the mitochondrial permeability transition pore (mtPTP) and its interaction with the pro- and anti-apoptotic Bcl2 family proteins. While all of these are important factors in the alteration of cancer cell metabolism, they still fall short of explaining the near universal alterations in mitochondrial function observed in cancer cells. A high throughput technology to monitor the membrane potential in mitochondria will allow further studies of these issues in cancer biology.
A crucial advance enabled by this technology will be to allow studies of how sample pedigree (and sample pedigree variation) affects bioenergetics & metabolism as studied through mitochondrial membrane potential measurements.

Currently, very little is known about the dynamics and variability of the membrane potential from a single mitochondria, and to date no chip-based electronic probe has ever been able to provide high-throughput assays of individual mitochondria. Therefore, this technology will enable studies of the variability of membrane potential within single samples, variations from patient to patient, as well as variations between normal and cancer cells.

Reference

43. Novel Technology for the Preservation of Phosphoproteins in Tissue Specimens: A Necessity for Individualizing Therapy for Molecular Targeted Inhibitors

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An urgent clinical goal is to identify subpopulations of cancer patients that may respond individually to molecular targeted inhibitors. The majority of the current molecular targeted therapeutics are directed at protein targets, and these targets are often protein kinases and/or their phosphorylated substrates. Therefore, measurement of this new class of phosphoprotein signal pathway epitopes in tumor biopsy samples is crucial for individualizing the new generation of molecular therapy aimed at targeting kinase pathways. Phosphoprotein antigen epitopes are not adequately preserved by formalin fixation and paraffin embedding, while freezing of tissue is very expensive and compromises diagnostic accuracy. We are currently in the clinical validation phase of an innovative technology for preserving tissue phosphoproteins and diagnostic histomorphology for clinical cancer molecular profiling. Under NIH 1R21CA125698-01A1, we created a novel tissue preservation chemistry that stabilizes all classes of phosphoproteins through paraffin embedding, while maintaining complete diagnostic histomorphology, and fully preserving critical diagnostic immunohistology (IHC) antigens including Estrogen Receptor, Progesterone Receptor, HER2, and Ki67. These IHC antigens are not preserved by special research fixatives used for tissue RNA preservation. The fixative chemistry can also be used to preserve blood cell morphology and surface antigens for flow or magnetic sorting. Our tissue preservative, termed Biomarker and Histology Preservative (BHP), is designed to be seamlessly introduced into the current community hospital clinical diagnostic workflow with no additional steps or equipment. At the time of procurement, tissue can be immersed directly in the new fixative and processed into a paraffin block for routine diagnosis, obviating the need for costly freezing during shipping or storage. BHP preserves cellular morphology in all major human and murine tissues evaluated. Extraction of protein and phosphoproteins from a BHP paraffin section is up to sixteen times greater yield compared to optimized protein extraction from FFPE. BHP paraffin embedded sections exhibit dramatic enhancement of phosphoprotein antigenicity for IHC, with no increase in background, compared to FFPE. Following objective independent validation by diagnostic pathologists, this preservative chemistry is applicable to biobanking and tissue biomarker research. Adoption of the technology would mean that one diagnostic paraffin block could be used for all classes of molecular profiling. This would increase diagnostic accuracy while substantially reducing costs.
44. Oncogenic Activation of Latent Membrane Protein 1 by Homotrimerization of Transmembrane Domains

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Epstein-Barr virus (EBV), a γ-herpesvirus, establishes lifelong infection by targeting the adaptive immune system of the host through Memory B-cells. The EBV protein, Latent Membrane Protein 1 (LMP-1), is essential for B lymphocyte immortalization by EBV. EBV is associated with both lymphoid malignancies and lymphoproliferative syndromes, and LMP-1 has been identified as a key player in EBV-associated malignancies. The 6 spanning hydrophobic transmembrane region of LMP-1 has been speculated to drive self-aggregation and regulate downstream signaling via a mechanism that has yet to be elucidated at the molecular level. Here we identify the fifth transmembrane helix (TMD5) of LMP-1 that confers upon this helix the ability to self-associate mediated by a buried, charged residue Asp150, to a homotrimer. Mutation of this aspartic acid residue abolishes LMP-1 association in detergent micelles and phospholipid bialyers. The mutants in full-length LMP-1 results in the attenuation of the NF-κB signaling pathway in live cells, suggesting that this transmembrane helix plays a key role in constitutive signal activation of this oncoprotein. Taken together, these results have suggested a novel model of LMP-1-mediated EBV activation and rendered possible new strategy to prevent and treat carcinomas induced by EBV activation.

45. OxMRM: Quantifying the Redox Status of Endogenous Proteins Using Multiple Reaction Monitoring

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Cysteines are often the most easily oxidized amino acid and are prime targets of protein oxidation by reactive oxygen species (ROS). However, while chronic exposure to ROS has traditionally been thought to be deleterious, thiol signaling is essential for normal cellular function suggesting that ROS modification of cysteines may play a complex role in aging and age-related disease biology. Since the key modifications and mechanisms of thiol signaling in these processes are obscure due to limitations in current cysteine oxidation analytical methodologies, we have developed a strategy called OxMRM capable of sensitively quantitating cysteine oxidation status of potentially any protein by integrating differential thiol alkylation, protein purification, and analysis by multiple reaction monitoring. OxMRM has the sensitivity and specificity to perform quantitative analysis of endogenous cysteine oxidation in a targeted manner, such as examining specific proteins of interest or cysteines within key functional domains that are hypothesized to be particularly important for protein regulation and function. OxMRM has allowed quantification of the endogenous oxidation state of 15 cysteines in two different low level transcription factors, human p53 and protein tyrosine phosphatase 1B. OxMRM can detect cysteine peptides in these proteins at low femtomole or attomole levels with <15% coefficient of variation and can measure differential sensitivity of cysteines in these proteins to numerous oxidants in multiple cell lines. We have investigated the intracellular oxidation of p53 under a variety of proxidant treatments in MCF7 cells and find that p53 Cys182 is highly susceptible to diamide oxidation.
46. **PCR for Carbohydrate Cancer Biomarkers**

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The identity and abundance of glycans attached to specific circulating blood proteins change during cancer but the value of using these changes for diagnosis is largely unavailable because state-of-the-art methods to decipher these residues requires expensive equipment and technically challenging protocols. The goal of this R21 project is to develop general-purpose methods to detect and quantify unique glycans attached to individual glycoproteins. Specifically, we propose to label unique glycans with sequence-specific oligonucleotides and count each adduct by real-time polymerase chain reaction (RT-PCR), in effect, converting different glycans into identifying DNA sequences. For glycan labeling and protein counting, we utilize custom-made protein-DNA fusions (with 1:1 stoichiometry) where the protein domain binds specific glycan epitopes and the DNA tail serves as a bar code. By using a simple to implement saturation binding format that controls for differences in binding affinities for all reagents, we quantify bound DNAs by RT-PCR to calculate (1) the total number of glycoproteins present in the sample, and (2) the mean number and standard deviation of specific glycan epitopes per glycoprotein. By analyzing multiple glycans, we generate glycan:protein distributions that constitute a signature and serve as a quantifiable metric of disease risk, possibly earlier than other detectable or overt symptoms. Our envisioned platform is expected to tolerate whole serum, saliva, and urine with little or no preparation because we capture analytes on magnetic beads to remove them from sample fluids for quantification in standardized buffers. Our measurements will provide absolute numbers of glycan epitopes per protein with the quantitative sensitivity of PCR such that sample size and analyte quantities may be extremely low, perhaps as low as nanoliter volumes containing just hundreds of molecules. If successful, we expect this work will enable the discovery of new carbohydrate biomarkers that comprise clinically relevant signatures and enable capturing the value inherent to changes in protein glycosylation for cancer diagnosis.

47. **Probing Cellular DNA/Protein Interactions Using Chromatin DNA Rehybridization for Identification of Oncogene Transcription Factors**

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Identification of transcription factors (TF) assembled at promoter regions of oncogenes is vital to understand how gene expression is altered in cancer and in tumor microenvironments. Most TF identification methods are based on DNA/protein interactions in vitro, which may not reflect DNA/protein interactions in vivo and may overlook protein complex information. Moreover, existing methods for chromatin pull-down target pre-identified proteins rather than DNA, which limits the ability to discover new proteins that may bind to specific genomic sequences. We are pursuing a new approach, chromatin DNA rehybridization (CDR), which preserves and detects DNA/protein and protein/protein interactions that occur in live cells and targets specific DNA sequences rather than associated proteins.

We use ribosomal RNA gene (rDNA), the most actively transcribed gene, and its associated TFs as the model system to establish the CDR technique. Biotinylated oligonucleotides targeting the core and upstream control elements are utilized to pull down rDNA promoter fragments and associated TF complex. TFs are then released from DNA and analyzed using mass spectrometry. Preliminary result suggested that at least 106 live cells were needed to harvest sufficient amount of TFs for identification. This CDR technique will be applied to the detection and discovery of TFs associated with oncogenes (e.g., HER2).
The CDR 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 genes with unknown functions.

48. Real-Time Quantitative Multiplex Analysis of Cell-Cell Interactions

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Approaching cancer from a systems biology perspective has been hindered by the lack of tools for a dynamic multiplexed analysis of surface and secreted proteins from small numbers of tumor cells. Our objective is the development of a platform for the parallel and time dependent analysis of cell-cell communication. This technology can be used to query the cross-talk between cancer cells and cells of their microenvironment on a single cell level. While flow cytometry is most often used to measure surface molecules and detection of intracellular levels of secretants, intracellular levels often do not correlate with secreted amounts and the multiplex capability is limited. Furthermore, the cells need to be fixed and are lost for future studies needing live cells; no time-course can be performed on the same cells. Other flow based methods involve some form of capture of secretants on the cell surface, with subsequent secondary staining. These approaches are limited by the constraints of flow cytometry and are difficult to generalize or multiplex because of either highly specific modalities, or the need for specialized reagents. We have termed our new platform “Single Cell Hyper Analyzer for Secretants” (SiCHAS). Our device will provide measurements of several surface molecules and secretants at the single cell level of heterogeneous cell populations in real time. To develop this technology, we have used microfabrication techniques to create planar arrays of “microwells.” We have fabricated 12x12mm chips with well openings of 50x50μm, 85μm deep, and separated by 10μm wide walls. Smooth walls have been reliably produced with greater than 95% well yield. Several process runs have been performed to fabricate microwells on Si/Pyrex bonded wafers. A 200μm thick Si wafer was bonded to a 500μm thick Pyrex wafer for producing 200μm deep microwells with transparent bottoms. The wafer bound Si/pyrex sample were of good quality. Well depths of 180μm have been fabricated using cryo-etching with further optimizations underway. To attach capture antibody to the SiO2 surface, we first attached protein G to the surface and measured a dose dependent attachment of a fluorophor conjugated capture antibody. Using this approach we made a sandwich adding a secretant capture antibody, the secretant IFN-gamma, and a secretant detection antibody and were able to detect 100ng/ml of the secretant. The coating conditions are currently being optimized to detect lower amounts of 4 different secretants. Subsequently the technology will be evaluated on a cell line measuring cell surface expression and secretants from the same cell over time.

49. Serum Autoantibody Biomarkers for Colorectal Cancer Detection

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Cancer survival rates dramatically increase when diagnosis occurs at an early stage of disease and therefore the development of diagnostic screening tests that can detect the disease at the asymptotic stage is crucial. Efforts toward deciphering the complexity of the tumor specific proteome by profiling immune responses generated against tumor associated antigens (TAAs) holds great promise for predicting the presence of cancer long before the development of clinical symptoms. Our Goal is to develop accurate panels of diagnostic markers using novel discovery technologies and tailored computational methods to allow for the determination of their discriminatory
performance in large scale biomarker validation projects. Our scientific approach is to evaluate the cancer induced humoral immune response by deciphering a patient’s epitomic profile (i.e., autoantibodies). In this project, colorectal human serum samples were interrogated against antigen microarrays to distinguish profiles of immuno-reactivity using a two fluorescent dye system. From our initial antigen set of 3800 phage cDNA clones, we have found 487 tumor-associated antigen clones that exhibited immuno-reactivity to cancer sera from our discovery cohort. The top immuno-reactive antigen clones have been gene identified by DNA sequencing. Through this approach, we have identified a number of novel TAAs as well as re-isolated and confirmed the identity of previously described TAAs from the literature. This group of selected antigen clones has also undergone a second round of interrogation with the original discovery cohort as well as newly acquired sera. Supervised learning algorithms classifier models have been employed on the resulting data set. Using a bundling tool of the two highest performing classifier models utilized, the performance characteristics of this set of markers were 79% sensitivity and 49% specificity. In an adaption of the existing xMAP technology, this group of selected antigen clones are being covalently bound to the microsphere bead arrays and assayed. The peptide sequences encoded in these clones are being expressed in vitro and then covalently bound to microspheres. The detector antibodies are the serum derived autoantibodies (IgGs) with a phycoerythrin labeled goat anti-human IgG secondary monoclonal antibody used for detection. In a pilot test of this system, we used a small set of CRC and related sera to determine the feasibility to conduct immunoassay on microspheres at detectable limits. Our initial results presented here demonstrate that the observed immuno-reactivity to be both reproducible and exhibits a robust signal to noise ratio. The overall objective of this research program is to develop a non-invasive screening test exhibiting high sensitivity and specificity to detect early stage cancer in a heterogeneous population of individuals (screening population) for CRC. The adoption of this technology has the potential of making significant advancements in early detection of CRC due to it ability to produce robust, reproducible epitomic profiles from patients’ sera that are a readily translatable platform for clinical implementation.

50. Single-Molecule Epigenetic Analysis in Nanochannels

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We report on our progress in developing a technology for the epigenetic analysis of whole, genome-size chromatin molecules. The analysis technique will be based on linearizing and stretching molecules, followed by label-free optical readout of the epigenetic properties. The stretching is accomplished by pulling single chromatin molecules into nanochannels with a cross-section of about 100x100 nm², and a length of hundreds of micron. This stretching proceeds without tethering, so that the molecule can be scanned past an optical readout. The optical readout will be provided by label-free Raman scattering, which enables the detection of multiple marks in a single experiment. Due to the small analyte quantity in high-resolution work, we will increase the signal by using uv-illumination resonant with the optical transitions of the biological molecules, and near-field enhancement using metal nanostructures.

In the past funding period we have demonstrated an intermediate step towards the proposed method, in which we observed fluorescent markers for histone modification and 5-cytosine methylation on reconstituted chromatin and DNA that had been stretched inside nanochannels. In particular, we have used a fluorescently labeled methyl binding domain (MBD) protein for the visualization of methylation, and fluorescently labelled antibodies for the visualization of a common histone modification mark (H3K4me3).

Ultimately we aim to monitor such epigenetic changes on a genome-wide level from single molecules, with a sub-gene resolution, without the need for antibodies or labels. Due to the fact that single molecules are measured, the technique lends itself towards the interrogation of heterogeneous cell populations, where it can deliver high-quality data even for rare subpopulations, such as cancer stem cells. The ability to measure very long molecules, potentially whole chromosomes, will also enable the detection of large-scale reorganization of genomic material and haploid data (including epigenetic haplotype).
The current, exploratory grant will only provide a proof of principle for the basic technology without any method for sample preparation, which doubtlessly will be challenging. In its current form we use only well-characterized synthetic samples whose epigenetic state has been deliberately set. In the long-term, we envision high-throughput screening of cell populations for epigenetic variations, resulting in maps with gene-relevant resolution.

51. Specific Detection of Cervical Cancers Using Cytometry-Based Molecular Diagnostics

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Cervical cancer is known to be caused by infection with high risk types of human papilloma virus [1, 2]. Protein products of viral oncogenes E6 and E7 respectively reduce active cellular pools of p53 and pRb, encouraging progression of cell cycle past checkpoints and ultimately preventing complete differentiation of cervical epithelial cells [3-6]. The most severe dysregulation of cell cycle occurs when viral gene E2 is disrupted, causing permanent upregulation of oncogenes E6 and E7 in a cell lineage for which linearization and integration of the viral genome has occurred [7]. HPV types vary in their propensity to linearize by disrupting gene E2; therefore, progression of cervical disease is thought to be dependent in part on genetic type of HPV. Using cervical cancer cell lines as models, we have demonstrated that immunological staining for p16/mcm5 dual over-expression can be used as gating criteria for flow cytometry, enabling sorting and capturing transformed cells from mixed populations [8]. Subsequently, we can detect HPV specifically by type, within sorted cells, using multiplexed PCR primers. Detection is consistently sensitive to 20 cells per reaction, and has detected HPV from sorted clinical samples. We are converting the PCR portion of the workflow to use fluorescently labeled universal primer chemistry from Beckman Coulter, allowing amplicon size analysis by capillary electrophoresis via the Genome Lab GeXP platform. This will allow automated analysis of clinical samples within a 96 well format. Primer sites within the genomes of different HPV types are chosen so that all types can be differentiated by amplicon size, enabling automated analysis by peak detection during GeXP runs. We propose to implement this workflow as a means of providing both added prognostic value to individual cases and reduction of false negative diagnosis. Support for this work has been provided by funds from NIH 1R21CA125370-01 and R33CA140084-01.

References
52. Standardized Nano-Array qPCR of a Lung Cancer Prognostic Panel

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Background
Gene expression for personalized medicine is showing tremendous promise. However, the lack of qPCR standardization, sample limitations and simple workflows currently restrict gene expression based diagnostics to a few highly specialized clinical laboratories.

Methods
Standardized Nano Array PCR (SNAP) combines intrinsic quality control of standardized reverse transcriptase PCR (StaRT-PCR), Pleiades melt probe specificity, and OpenArray scalable throughput to generate a multiplex gene expression platform compatible with molecular diagnostic kits. cDNA was extracted from tissue samples, amplified by multiplex PCR in the presence of a known number of internal standard molecules for each respective gene measured, transferred to the OpenArray to measure native template : internal standard template ratios for each gene by melting curve analysis, and transcript abundance calculated from a fraction NT vs. log [IS] curve. This paper describes SNAP analytic performance for a 21 gene lung cancer prognostic gene expression panel measuring limited cDNA input typical of clinical samples.

Results
SNAP demonstrated a median 15% CV over a dynamic range limited only by the internal standard mixtures employed and sampling error at very low starting copies. Under limiting cDNA input, typical of formalin fixed paraffin embedded clinical samples, SNAP outperformed singleplex quantitative real-time PCR (qPCR) in laboratory reproducibility and analytic sensitivity. Combining results from two laboratories had little impact (2% increase) on the measurement variance of SNAP, whereas qPCR increased by 17%.

Conclusions
These proof-of-principle experiments demonstrated the conversion of an existing singleplex qPCR panel to a scalable qPCR platform possessing the necessary low sample and reagent consumption, dynamic range, reproducibility, and QA/QC for the next generation of complex molecular diagnostics.

53. Suspended Single Cell Morphology With Nanoparticle-Induced Magneto-Rotation

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A change from two dimensional to three dimensional growth conditions radically affects in vitro cell patterns. However, it is still difficult to study the morphology of single cells that are freely suspended, as they would be in cases such as circulating tumor cells (CTCs) and stem cells, where morphological changes are of significance. Here we report a new method for monitoring, on single live suspended cancer cells, the morphology, size and their temporal changes, with a precision in three dimensions comparable to that of optical microscopy, but without the associated confinement to the imaging plane. The method is based on nanoparticle induced magneto-rotation of cells. By using a rotating magnetic field, the magnetically labeled cell is actively rotated, and the rotational period is
measured in real-time. The change in morphology induces a change in the rotational period of the cell (e.g., when the cell gets bigger it rotates slower). The ability to monitor in real time cell swelling and death, at the single cell level, is demonstrated. This method could be used in single cell morphology analysis and thus have implications for studying the effectivity of molecular diagnostics and drugs, with respect to circulating tumor cells in their suspended state. Along with single cell analysis, three dimensional assays permit a better comprehension of cellular dynamics, by narrowing the gap between in vitro and in vivo behaviors. However, single cell analysis techniques previously used are generally restricted to two dimensional models. To overcome this limitation, we use individual cells magnetically rotated in suspension. Our method of Asynchronous Magnetic Bead Rotation (AMBR) for suspended tumor cells uses magnetic nanoparticles, followed by rotation under an external magnetic field of 1 mT, much weaker than the fields of around 1T used for MRI. The cell is rotated in suspension, and the frequency is highly sensitive to morphology change. Magneto-rotation preserves the cell’s viability, and allows for real time analysis to be performed, where changes in the morphology are indicated by a single cell’s rotation period. A cell in suspension is rotated, and the trends in the rotation rate allow one to discriminate between a healthy cell, a dying cell and a swelling cell. In addition, this new technique is very adaptable to any microscope set-up, label-free, and compatible with fluorescence and other optical detection methods.

Reference

54. Switchable Affinity Antibodies for Highly Multiplexed IHC

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For current drug development efforts, it is becoming increasingly clear that the identification of individual biomarkers is insufficient to provide accurate prognosis or to accurately predict drug response [1]. Additionally, several studies have indicated that the prediction of disease outcome may be significantly improved by identifying the specific sub-cellular locations of biomarkers [2]. While traditional immunohistochemistry (IHC) or gene expression arrays have provided valuable biochemical and phenotypic information, neither technique satisfies the necessary demands for an extensive biomarker evaluation strategy that preserves the original tissue context of the sample. In the case of oncology, such a technology would more accurately capture the inherent heterogeneity present within cancer tissue and hence provide more of the detail necessary to achieve truly personalized medicine.

To this end, we at GE have developed a new method that can perform a high-level multiplex analysis of 30-40 biomarkers on a single cell basis in single tissue sections. The method involves repeated cycles of staining, imaging and stain removal to acquire a large number of images with different protein expression profiles that are co-registered and segmented to provide sub-cellular expression results. Despite the power of this technology, however, current methods utilize harsh chemical treatment to remove signal and have proven to be detrimental to some target epitopes. The objective of this IMAT funded project is to design an alternate approach that allows signal removal under very mild conditions. Specifically, biomarker-specific antibodies appended with environmentally sensitive molecular switches are being investigated. These antibodies will ideally retain high affinity and specificity for their targets under one set of conditions, but would be released upon application of a modest environmental stimulus.

Three different molecular switches are being pursued: thermal, UV, and pH. For each of these switches, multiple switch-to-antibody ratios are being assessed in order to identify the effect of modification level on performance. The coupling of the switches to the antibody is achieved using NHS ester/amine coupling chemistry. Despite the non site-specific nature of this labeling strategy, this approach represents well-established chemistry that can be easily tuned.
from one antibody system to another under simple stoichiometric control. The antibody-switch conjugates prepared are characterized by an array of quantitative bioanalytical techniques including gel electrophoresis, UV/VIS spectroscopy and MALDI-TOF mass spectrometry. Two different aspects of performance are being evaluated for each of the conjugates: (1) the effect of switch conjugation on initial binding, and (2) the effect of the switch upon exposure to the environmental trigger of interest. Relative antibody binding and kinetics are being determined by Surface Plasmon Resonance (SPR) analysis using a Biacore system. Although SPR cannot address the degree of nonspecific binding that can potentially occur on tissue samples, the use of this tool to quantify and correlate antibody conjugate performance will allow structure/activity relationships that can enable further optimization of the switch systems under investigation. In parallel with SPR, the staining and imaging of well-characterized control cell pellet slides will be performed prior to proof of concept on clinical tissue samples. As an initial proof-of-concept, we are focusing our efforts on the well-established Her2 biomarker using antibodies that have previously been validated in-house for their specific high affinity binding on both the Biacore platform and on tissue sections. Successful demonstration of the technology will open the possibility of developing a new class of probes for not only multiplex analysis of tissue samples and potentially live cells but also in areas outside of healthcare such as renewable sensors.

References

55. Targeted Bisulfite Sequencing of CpG Island DNA by Solution Hybrid Selection and Massively Parallel Sequencing


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Recent studies using next-generation sequencing have generated genome-wide, single-base resolution DNA methylation maps. However, it is still very costly to conduct whole genome shotgun bisulfite sequencing. Currently, few methods enable targeted bisulfite sequencing of CpG islands (CGIs) or other specific regions of interest in a highly flexible and efficient manner. Here we present an approach that combines solution-phase hybrid selection and massively parallel bisulfite sequencing to profile DNA methylation in targeted CGI and promoter regions. We designed 51,466 single strand DNA oligonucleotides (160-mer) which target 23,441 CGIs and the transcription start sites of 19,369 known genes in the human genome. The synthetic long DNA oligonucleotides were converted into biotinylated RNA probes for solution-phase hybridization capture of target DNA. The captured genomic DNA was treated with sodium bisulfite, amplified by PCR and sequenced using Illumina GA II sequencer. Using this approach, we conducted bisulfite sequencing on captured DNA from three breast cancer cell lines, MCF10A, MCF7, and MDA-MB-231. 23-30 million single-end 75bp sequencing reads were obtained for each cell line. The raw sequencing reads were mapped to bisulfite converted genome and methylation levels for CpG sites covered with at least 10 sequencing reads were extracted, providing accurate quantification on 900,000-1,000,000 CpGs. 77-84% of CpGs analyzed in the these samples fell on or near capture probe sequences; 69-75% lay properly on CGIs. More than 85% of capture probes successfully yielded quantitative DNA methylation information of targeted regions. One lane of Illumina sequence was sufficient to determine the methylation status of over 22,000 CGIs (75% of all annotated CGIs) in the
human genome. For the first time, we generated genome-wide, single-base resolution DNA methylation maps in three most commonly used breast cancer cell lines. Interesting differential methylation patterns were observed among three cell lines; particularly on X chromosome. We demonstrated the targeted bisulfite sequencing approach to be a powerful method to uncover novel aberrant methylation in the cancer genome. Since all targets were captured and sequenced as a pool through a series of single-tube reactions, this method can be easily scaled up to deal with a large number of samples.

56. Technology for Detection and Quantitation of Telomeric DNA Aberrations in Cancer

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Telomeric DNA abnormalities are a critical and universal aspect of carcinogenesis. The gradual replicative loss of telomeres ultimately results in telomere dysfunction and plays a role in many age-related diseases, including cancer. Sporadic telomere deletion events are a universal cell-intrinsic mutational mechanism that can lead to dysfunctional telomeres and chromosome instability; the frequency of these events is believed to depend upon factors such as DNA replication fork stalling and other DNA replication stress, oxidative damage, and homologous recombination events such as unequal sister chromatid exchange (SCE) and T-loop recombination. Sporadic telomere deletion events occur at a very low frequency in normal cells; an increased frequency of these events (and hence dysfunctional telomeres) may be among the very first mutational events in carcinogenesis. Repair of dysfunctional telomeres can result in telomere-telomere fusions, telomere-chromosome arm translocations at sites of internal double-strand DNA breaks, and additional DNA rearrangements as a consequence of repeated fusion-breakage-fusion cycles that result in genome instability and help drive cancer progression. Eventually, activation of telomere maintenance mechanisms (either telomerase-based or ALT-based) are believed to help stabilize dysfunctional telomeres and permit rapid growth of tumor cells.

It is impossible to measure accurately the global frequency of sporadic telomere deletion events and telomere fusions with current technology. As a consequence, telomere mutational data are totally absent from the high-throughput datasets being acquired from tumor samples as part of The Cancer Genome Atlas, and telomere mutational data gleaned from a few labor-intensive studies of telomere function in cellular and organismal cancer models are incomplete and biased. Our lab has focused upon detailed analyses of human telomeric DNA structure and variation; here, we present our initial efforts towards developing a universal, high throughput assay for detection and quantitation of telomeric DNA mutational events in humans. The method couples the physical enrichment and purification of telomeric DNA with quantitative analysis of the telomeric genome fraction by high-throughput paired-end sequencing. It is designed to detect and quantitate single-allele-resolution ultrashort (TTAGGG)n tract profiles, telomere fusions, and subterminal DNA breakage-rejoining events. In addition, it is amenable to future refinement to permit miniaturization and multiplexing of the assays. The quantitative, single-allele-resolution measurements of telomere length and instability will permit unprecedented insights into the role(s) telomere loss and telomere fusion play in carcinogenesis, including mechanistic insights into molecular events mediating these processes and translational insights for the potential prognostic and tumor stratification applicability of the method.
57. Time-Resolved Fluorometric Method for Assay Design Using Plasmonic Nanostructures

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There is a need in the medical field to investigate real-time expression of biological markers to better understand their roles in disease progression. It was found that a large number of diseases are associated with quantitative patterns of cytokine production. Most of the cytokine assays developed over the past decade are based on specific anti-cytokine antibodies using a variety of reporter systems including enzymes and fluorescent tags. Currently there are two main technologies for measurement of multiple of cytokines: multiplex sandwich ELISA and bead based assays. The limited methods for detection of cytokines is because of requirement sensitivity of 1-10 pg/ml. A number of innovative approaches for immunoassay designs have now emerged that exploit the interaction of light with plasmonic nanostructures.

Methods

We are developing an immunoassay platform for detection of cancer biomarkers which is based on amplified fluorescence by localized surface plasmons resonance and implementing time-resolved detection modality. Highly amplified fluorescence signal from the bound probes allows for designs of fluoroimmunoassays with a number of advantages over the current standard approaches.

Results

We developed planar substrates consisting of glass slide and multi-layer of metallic and dielectric layers that provide amplification of fluorescence signal up to 200-fold compared to that on standard glass substrate. The fabrication of substrates involves simple vacuum deposition process of silver and dielectric films. The increase in signal intensity accompany with decrease of fluorescence lifetime which provides significant spectroscopic contrast between bound and unbound probes. We demonstrate the experimental results using panel of cytokines such as TNF-α, IFN, IL-5, IL-8, VEGF and RANTES with sensitivities of about 1-10 pg/ml and dynamic range of 4-5 orders of magnitude.

Strengths and Limitations

The strengths of the developed immunoassay platform are (1) can be extended to other cytokines and cancer biomarkers, (2) assay can be performed in one-step, (3) assays use commercially available components, (4) ability for real-time binding monitoring, (5) assay performance can be easily compared between laboratories.

58. Transformative HTS Cell Migration Assay for Rapid Screening of Cancer Therapeutics

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The goal of this project is to develop a 384-well cell migration assay suitable for high throughput screening (HTS) of chemical libraries for cancer therapeutics. Such an assay, intended for HTS settings, must be robust, reproducible and cost-effective to perform. One of the two standard cell migration assays is a 96-well transmembrane format that is not automation compatible, has high variability, and does not permit real-time visualization of migrating cells. The second is a scratch wound assay which damages the cells and extracellular matrix and also has high variability. The 96-well Oris™ Cell Migration Assay, recently developed by Platypus Technologies, greatly improves on the transmembrane and scratch assays in that it yields reproducible results and robust z-factors. However, the Oris™ assay remains limiting for HTS applications based on its use of a silicone stopper to create a temporary cell exclusion
zone that requires manual removal in order to reveal the migration zone. We propose to redesign the current Oris™
96-well cell assay to make it fully compatible with automated liquid handling systems and high content screening
platforms while also formatting the assay for a 384-well plate. This proposed Oris™ HTS assay is based on the
innovative use of a temporary cell exclusion barrier comprised of a non-toxic, biocompatible gel (BCG) that will be
deposited in a defined central area at the bottom of a tissue culture well. Seeded cells will attach at the perimeter of
the well, and the BCG barrier will dissolve to reveal a zone that becomes permissible for cell migration. This
enhancement over the stopper-based Oris™ 96-well assay will allow assay miniaturization that dramatically improves
testing throughput (allowing increased numbers of compounds to be tested) and efficiency (reducing hands-on time)
while markedly reducing the cost per assay.

To date, we have employed robotics to deposit a BCG formulation in the center of wells in 96-well tissue culture
plates. Preliminary data suggest that the BCG is non-toxic, does not adversely affect cell viability or action of
benchmarked inhibitors. The BCG-based 96-well assay has been compared to the current stopper-based 96-well
Oris™ assay for which we have predicate data. We have now begun to transition the BCG-based assay from 96-wells
to a 384-well HTS format and are in the process of defining the optimal dispensing parameters. The optimized 384-
well assay plates will be tested in-house using 3 cell lines and 2 inhibitors of cell motility. In conjunction with
collaborators at the University of Pittsburgh Drug Discovery Institute (UPDDI), a HTS facility, we will develop a high
content screening protocol for results collection and validate it according to UPDDI acceptance guidelines for assay
performance.

The availability of a 384-well HTS cell migration assay that requires minimal numbers of cells, minute volumes of test
compounds and reduced operator time will transform drug development research for cancer therapeutics. It will be
amenable to primary screens for cell migration inhibitors. This format will also permit subsequent secondary screens
in the same assay wells for multiplexed probing of inhibitor effects on target molecules, viability and morphology
using high content screening instruments. The strength of our proposed assay is that it will fill an unmet need for a
robust and reliable 384-well cell migration assay.

59. Ultrahigh-Sensitive, Personalized Detection of Lymphoma DNA Markers in Blood

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Our goal is to identify the recurrence of non-Hodgkin’s lymphoma at a very early stage through detection of
personalized, lymphoma-specific markers in plasma DNA. Specifically, we used Pyrophosphorolysis Activated
Polymerization (PAP), an ultrahigh sensitive nucleic acid amplification technology, to detect as few as one copy of the
markers in blood. Our procedure includes three steps: (1) identify personalized lymphoma-specific markers, such as
the somatic mutations in the PIM1, cMYC, RhoH/TTF (ARHH), PAX5, and P53 genes, using tumor tissue samples, (2)
collect multi-time point plasma DNA samples from patients, and (3) develop personalized PAP assays to detect as low
as a copy of the markers in the collected plasma. This approach integrates the ultra-high sensitivity of PAP
technology and the biological nature of non-Hodgkin’s lymphoma, providing following advantages: (1) ability to detect
a single copy of cancer-specific mutations in a large volume of plasma, (2) over 1000-times more sensitive than any
current PCR-based methods, (3) not affected by metastatic status because DNA mutations are intrinsic signatures of
lymphoma cells, and (4) non-invasive blood sampling.
Simple static-fluid culture micro chambers are well suited for studies of direct reactions of cells to exogenous ligand introduction and paracrine signaling. Static systems reduce reagent waste and material cost by abrogating the need for flow (and thereby pumps and tubes and they can be easily manipulated by both manual pipetting and fully automated, multi-channel dispensers. Static microfluidic culture carries risks, however. Culture media are not replenished or recirculated, which renders such systems susceptible to evaporative loss. A potential metabolic complication posed by static microfluidic chambers is the relative abundance of oxygen. The commonly employed polymer polydimethylsiloxane (PDMS) is highly gas permeable and may allow oxygen concentrations much higher than that normally seen in vitro. Up-regulation of oxidative stress response pathways may complicate interpretation of data collected from these channels. Dense polymers such as polystyrene are gas impermeable; they represent the opposite problem that cells located sufficiently far from ports will become hypoxic in time. In addition, the reduced volumes, while beneficial for decreased reagent use and increased sensitivity to soluble factor signaling, result in reduced nutrients and growth factors available to each cell. Thus, a goal of our present work is to better understand the way in which micro scale culture modifies cell behavior in response to factors including nutrients, growth factors, hypoxia and osmotic changes. Importantly and as expected, the differences between macro and micro culture baseline cell behavior is often cell type dependent. Examples of results to date include a higher glucose consumption rate in microchannels, upregulation of BiP expression at low serum concentrations and a greater sensitivity to low serum concentration in attachment and spreading properties.

Interestingly, in 3D culture, we typically observe no differences in cell behavior between macro and micro scale culture environments. This may be because cell-matrix and soluble factor-matrix interactions become dominant. We have continued to exploit the capabilities of micro scale culture to precisely manipulate the microenvironment. For example, we have developed a compartmentalized in vitro co-culture model of stromal and cancer cells that supports the transition to invasion (ductal carcinoma in situ to invasive ductal carcinoma). Further, our ability to example distance dependent effects has allowed us to observe a two step process which is initiated by soluble factors alone, but which does not proceed without cell-cell contact. This new observations changes they way one things about the transition to invasion and opens the door to many new avenues of inquiry to better understand the molecular mechanisms that regulate the transition and also enable the discover of inhibitors.

Finally, we have designed a microfluidic platform to simultaneously culture up to five different cell types in individual chambers connected via ports which allow the diffusion of secreted signaling molecules between adjacent chambers. Following culture and treatment, cells can be fixed and stained in the microdevice or removed chamber-by-chamber, enabling independent analysis of each cell type. Initially, microfluidically-cultured breast cancer cells and stromal cells from common metastatic sites (e.g., bone marrow, lung, liver) were cultured individually to establish a baseline for cellular behavior in microfluidic conditions. Cellular proliferation was monitored and expression of several steroid receptors (estrogen, androgen, progesterone, and glucocorticoid receptors) was quantified via RT-PCR and immunofluorescence (IF). In this manner, we were able to confirm that the breast cancer cell line (MCF-7) maintained its expected behavior (e.g., estrogen-dependent growth, estrogen-dependent regulation of ERα and PR) in the microfluidic environment). However, when this cell line was co-cultured with bone marrow stromal cells (HS-5), we found that ERα was down-regulated while estrogen-independent growth was increased. We are currently in the process of comparing these findings with results obtained under macroscale culture conditions (e.g., transwell plates) to determine if microfluidic culture increases the sensitivity of cells to paracrine signaling events (i.e., Does the lack of convective transport in microfluidic systems lead to higher local concentrations of secreted molecules, thus enhancing the response by other cells within the microenvironment?)
61. Using RNAi to Probe Cancer Cells

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Over the past 5 years our lab has, in collaboration with Greg Hannon’s lab, made two major technological advances in RNAi. We have generated a retroviral/lentiviral based, barcoded short-hairpin RNA (shRNA) library targeting the entire human genome that is capable of stable and efficient gene knockdown in most cell lines. Furthermore, we have developed technologies of using barcode microarray to carry out high-throughput screens using this shRNA library in large, complex pools of >10,000 shRNAs/pool. We are now investigating how to increase through-put such that we could screen libraries of greater complexity, a current limitation of the technology. We have begun screening lung cancer cell lines for shRNAs that increase or decrease proliferation. We have also employed an epithelial cell transformation screen which has identified candidate tumor suppressor genes that are deleted across many cancer types including the lung. Secondly, we are trying to develop methods to identify more optimal shRNAs for a given gene.