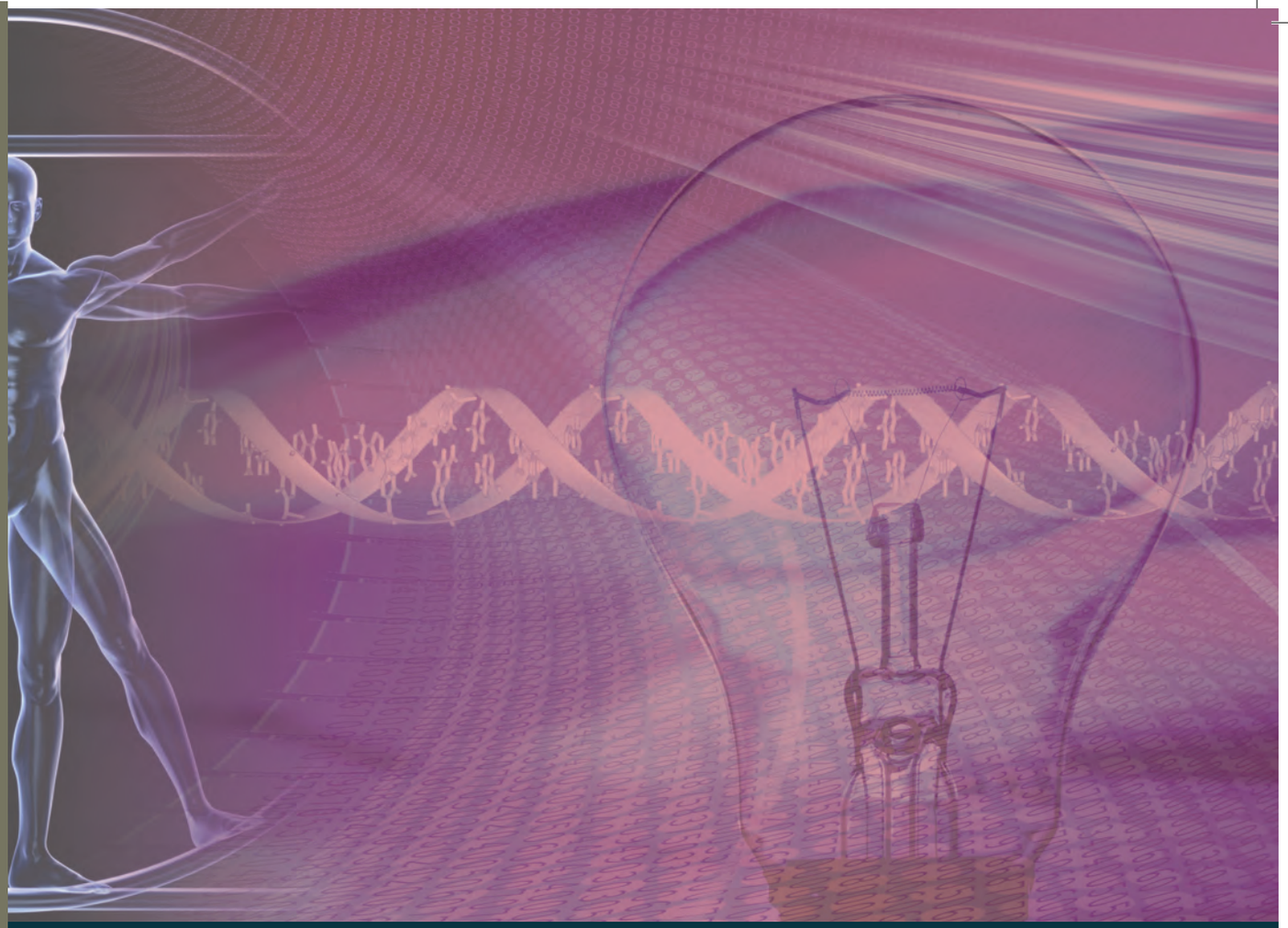
National Cancer Institute



13th Annual Innovative Molecular Analysis Technologies (IMAT) Program Principal Investigators (PI) Meeting

November 27-28, 2012

The Methodist Hospital Research Institute
Houston, Texas

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

Program Overview

Welcome to the 13th Annual Innovative Molecular Analysis Technologies (IMAT) Program Principal Investigators (PI) Meeting. As many of you already know, this annual meeting is organized to address two important aims of the IMAT program: (1) to provide NCI program staff a chance to interact directly with PIs and receive an update on progress to date with supported research and (2) to provide an opportunity for interactions and exchange of ideas among meeting participants. This latter aim is a critical opportunity for sparking potentially transformative project collaborations, receiving critical feedback and guidance from the community, as well as for fostering dissemination of the exciting technologies emerging from IMAT-supported researchers.

The overarching theme for this year's meeting is to *emphasize the importance of engaging technology end-users*, and so the meeting includes various mechanisms for facilitating that communication. To that end, each of the speaker sessions will be chaired by a prominent researcher or clinician engaged in a field relevant to the session topic. They have been asked to provide a brief overview of the various basic and/or clinical research challenges associated with the topic that represent unmet opportunities for innovative technology solutions. Further, our IMAT success story overviews a dissemination path that did not require commercialization of the platform to have a significant impact on cancer research.

As there are more exciting active research projects in the IMAT portfolio than we could possibly allow sufficient speaking time for, we are repeating the "Poster Highlights" session that was piloted last year, in which a number of investigators will give short overview talks on their research posters. An additional change to this year's meeting spurred by our theme is to open the meeting on the afternoon of the first day to all interested local area researchers and clinicians, which will overlap with the Poster Highlights session, and the subsequent poster session in the atrium.

In addition to the agenda and presentation abstracts, a list of resources and funding opportunities that we thought might be of interest to participants are included toward the back of the book. On behalf of the NCI program staff and everyone involved in the planning for this meeting, I thank you for your participation, your interest, and the important work you all do to assist in our collective mission against cancer. I look forward to an exciting and productive meeting.

Sincerely,

Tony Dickherber, Ph.D.
Program Director
Center for Strategic Scientific Initiatives
Office of the Director
National Cancer Institute

Agenda

Day 1: Tuesday, November 27

8:00 a.m. - 8:45 a.m. Registration and Continental Breakfast

Poster Session Setup

8:45 a.m. - 9:00 a.m. Welcome *Auditorium*
Tony Dickherber, Ph.D., Program Director
Innovative Molecular Analysis Technologies Program
National Cancer Institute, NIH

9:00 a.m. - 10:10 a.m. Panel Session: Novel Biosensors I
Chair: Steven Shen, M.D., Ph.D.
The Methodist Hospital Research Institute

9:10 a.m. - 9:30 a.m. ***Measuring Kinase Activity in Intact Cells Using Surface-Enhanced Raman Spectroscopy Nanosensors***
Alyssa Garrelts, Ph.D.
Purdue University

9:30 a.m. - 9:50 a.m. ***Nanoelectrode and Nanofluidic-Based Assay of Mitochondria Membrane Potential and Apoptosis***
Peter J. Burke, Ph.D.
University of California, Irvine

9:50 a.m. - 10:10 a.m. ***Multiplex Cancer Cell Purification With Magnetic Sifters***
Shan X. Wang, Ph.D.
Stanford University

10:10 a.m. - 10:30 a.m. Break

10:30 a.m. - 12 noon Panel Session: Novel Biosensors II
Chair: Rebecca R. Richards-Kortum, Ph.D.
Rice University

10:40 a.m. - 11:00 a.m. ***Quantifying RNA-Protein Interactions In Situ Using Modified-MTRIPs and Proximity Ligation***
Philip J. Santangelo, Ph.D.
Georgia Institute of Technology and Emory University

11:00 a.m. - 11:20 a.m. ***Highly Multiplexed, Spatially Delineated Molecular Imaging in Cancer***
Michael R. Diehl, Ph.D.
Rice University

11:20 a.m. - 11:40 a.m. ***An Integrated Platform for Quantifying Gene Expression in Co-Cultured Cells***
David J. Beebe, Ph.D.
University of Wisconsin

11:40 a.m. - 12 noon

Rapid and Sensitive Multiplex Sequencing of Actionable Cancer Genes in Clinical Samples
Stephen Salipante, M.D., Ph.D.
University of Washington

12 noon - 1:00 p.m. Lunch (on your own)

1:00 p.m. - 2:00 p.m. Success Story: Collaboration Versus Commercialization to Disseminate Your Technology
William C. Hahn, M.D., Ph.D.
Dana-Farber Cancer Institute

David E. Hill, Ph.D.
Dana-Farber Cancer Institute

2:00 p.m. - 3:50 p.m. Poster Highlights Session

Activatable BRET Probes for MMP Enzymatic Activity Detection
Jianghong Rao, Ph.D.
Stanford University

Development of a Methylation-Based Diagnostic Assay for Malignant Melanoma: Defining the Factors Affecting Marker Selection and Assay Performance
Sharon N. Edmiston
The University of North Carolina at Chapel Hill

FRET-Based Biosensors to Monitor Redox in Cell Cycle Regulation
Vladimir Kolossov, Ph.D.
University of Illinois, Urbana-Champaign

Microfluidic Sorting of Blood Cells for SPR and Fluorescence Analysis
Nathaniel C. Cady, Ph.D.
University at Albany

Application of an Innovative Technology to Develop Low-Toxicity Kinase Inhibitors
Xiang Li, Ph.D.
University of Maryland, Baltimore County

Multiple Reaction Monitoring to Profile Biosensor Phosphorylation in Leukemia
Laurie L. Parker, Ph.D
Purdue University

Probing Cancer Cell Chemoinvasion Strategies Using 3D Microfluidic Models
Mingming Wu, Ph.D.
Cornell University

Specific and Reversible Binding of DNA Nanoparticles to Cancer Cells
Bradley T. Messmer, Ph.D.
University of California, San Diego

2D-PCR for Spatially Mapping Gene Changes in Tumor Sections
Daniel Gowetski, Ph.D.
University of Maryland

Discovery Platform for Cancer Antigens
Kevin Claffey, Ph.D.
University of Connecticut Health Center

Digital Analysis of Proteins Through End Sequencing (DAPES)
Tom Cohen, Ph.D.
Washington University in St. Louis

Molecular Diagnostic Tests to Augment Cytomorphologic Diagnosis of Lung Cancer
James C. Willey
University of Toledo

Method for Detection of Secreted Proteins in Single-Cell Assays
Henryk Szmacinski, Ph.D.
University of Maryland

3:50 p.m. - 6:00 p.m.	Poster Session	*First Floor Lobby*

Day 2: Wednesday, November 28

8:00 a.m. - 8:15 a.m. — Recap of Day One and Overview of Day Two — *Auditorium*
Tony Dickherber, Ph.D.
Innovative Molecular Analysis Technologies Program
National Cancer Institute, NIH

8:15 a.m. - 8:45 a.m. — Host Welcome
Mauro Ferrari, Ph.D.
The Methodist Hospital Research Institute

8:45 a.m. - 9:30 a.m. — Keynote Address
Joshua LaBaer, M.D., Ph.D.
The Biodesign Institute
Arizona State University

9:30 a.m. - 10:40 a.m. — Panel Session: Pathway Tools - 1
Chair: TBD

9:40 a.m. - 10:00 a.m. — ***VEC³-Valve Enabled Cell Co-Culture Platforms for Cancer Biology Study***
Deyu Li, Ph.D.
Vanderbilt University

10:00 a.m. - 10:20 a.m. — ***Translational Control Analysis by Translationally Active RNA Capture/Microarray Analysis (TrIP-Chip)***
Jingfang Ju, Ph.D.
Stony Brook University

10:20 a.m. - 10:40 a.m. — ***Scanning Correlation Microscopy Methods for Quantifying DNA Repair Kinetics***
Georgios Alexandrakis, Ph.D.
The University of Texas at Arlington

10:40 a.m. - 11:00 a.m. — Break

11:00 a.m. - 12:30 p.m. — Panel Session: Biomarker Preservation and Discovery
Chair: Ignacio Wistuba, M.D.
The University of Texas MD Anderson Cancer Center

11:10 a.m. - 11:30 a.m. — ***Genome-Scale DNA Methylation Profiling in the Developing Colon and the Impact of Diet***
Lanlan Shen, M.D., Ph.D.
Baylor College of Medicine

11:30 a.m. - 11:50 a.m. — ***Detection of Low-Prevalence Mutations in Solid Tumors via Ultra-Deep Targeted Sequencing***
Olivier Harismendy, Ph.D.
University of California, San Diego

11:50 a.m. - 12:10 p.m. ***Tissue Is Alive: Preserving Biomolecules and Tissue Morphology in Clinical Trial Samples***
Lance A. Liotta, M.D., Ph.D.
George Mason University

12:10 p.m. - 12:30 p.m. ***Sentinel RNAs as a Measure of mRNA Integrity in Clinical Biospecimens***
Curt H. Hagedorn, M.D.
University of Utah

12:30 p.m. - 1:30 p.m. Lunch (on your own)

1:30 p.m. - 2:40 p.m. Panel Session: Pathway Tools - 2
Chair: Stephen Wong, Ph.D.
The Methodist Hospital Research Institute

1:40 p.m. - 2:00 p.m. ***Development and Application of Novel Glycan-Specific Reagents to Facilitate Early Detection of Epithelial Ovarian Cancer***
David C. Muddiman, Ph.D.
North Carolina State University

2:00 p.m. - 2:20 p.m. ***Targeted Selection, Sequencing, and Analysis of Human Telomere and Subtelomere DNA in Cancer***
Harold C. Riethman, Ph.D.
The Wistar Institute

2:20 p.m. - 2:40 p.m. ***Application of Next-Generation Sequencing to Cancer Epigenomics***
Huidong Shi, Ph.D.
Georgia Health Sciences University

2:40 p.m. - 3:00 p.m. Break

3:00 p.m. - 4:50 p.m. Panel Session: Technologies to Assist With Drug Screening and Delivery
Chair: Melissa D. Landis, Ph.D.
The Methodist Hospital Research Institute

3:10 p.m. - 3:30 p.m. ***Magnetorotation: A Rapid Assay for Single Cell Drug Sensitivity of Cancer Cells***
Raoul Kopelman, Ph.D.
University of Michigan

3:30 p.m. - 3:50 p.m. ***Scaffolds for Delivering Deoxycytidine Kinase to HER2 Positive Cancer Cells***
Brian K. Kay, Ph.D.
University of Illinois at Chicago

3:50 p.m. - 4:10 p.m. ***384-Well Cell Migration Assay Suitable for High-Throughput Screening (HTS) of Chemical Libraries for Cancer Therapeutics***
Andreas Vogt, Ph.D.
University of Pittsburgh Drug Discovery Institute

4:10 p.m. - 4:30 p.m. ***Hyperspectral and Structural Microscopy Platform for Therapy of Resistant Cancer***
Conor L. Evans, Ph.D.
Harvard University

4:30 p.m. - 4:50 p.m. ***Ultra-Throughput Multiple Reaction Monitoring Mass Spectrometry for Large-Scale Cancer Biomarker Validation***
Xudong Yao, Ph.D.
University of Connecticut

4:50 p.m. - 5:00 p.m. Closing Remarks and Adjournment
Tony Dickherber, Ph.D.
Innovative Molecular Analysis Technologies Program
National Cancer Institute, NIH

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Measuring Kinase Activity in Intact Cells Using Surface-Enhanced Raman Spectroscopy Nanosensors

Joseph Irudayaraj, Alyssa Garrelts, Laurie Parker

Purdue University, West Lafayette, Indiana

Our long-term goal is to develop a sensitive, multiplexed detection platform for real-time single-cell monitoring of prognostic kinase activity in tumor samples. The objective of this proposal is to develop the first steps toward a multiplex quantification of kinase activity in a breast cancer model system using surface-enhanced Raman spectroscopy (SERS) and peptide-functionalized nanoparticle (NP)-based biosensors. Recent unpublished work in our laboratory suggests that exogenously-added peptide substrates and SERS will allow for sensitive, direct monitoring of kinase activity in biological environment such as cells (without requiring labels such as phospho-specific antibodies). In this multidisciplinary proposal, we combine the kinase biosensor expertise of the Parker laboratory and the extensive SERS experience of the Irudayaraj laboratory to develop a quantitative SERS platform to monitor the activity of Akt, Src, and c-Abl, kinases associated with drug resistance and clinical outcome of breast cancer patients. Our milestone targets are to:

1. Detect two peptide substrates simultaneously in vitro using deuterated peptides and SERS microscopy.
2. Calibrate effect of %phosphopeptide on SERS signal from NPs in vitro.
3. Achieve std. error/variance for the analysis of less than 15%.
4. Achieve detection of two peptide simultaneously from NPs in cells.
5. Calibrate effect of %phosphopeptide on SERS signal from NPs in cells, achieving accuracy and reproducibility such that 0%, 50% and 100% phosphorylation can be detected with statistical significance.
6. Establish conditions for nanoparticle treatment that result in <10% toxicity to cells.

To date, we have established several key elements toward addressing the milestones for this project. These efforts have focused on the substrate and nanoparticle development, and understanding the behavior of these materials for SERS in vitro (not in cells). Outlined below are the main outcomes of the first year of this project:

A. We have further characterized the detection system in vitro using silver nanoparticles (AgNPs) and SERS, obtaining a better understanding of the limitations of the system on reproducibility and quantification under conditions in which dynamic particle aggregation and Brownian motion in solution are factors. These results suggest to us that it may not be possible to achieve Milestones 2 and 3.
B. We have synthesized and tested several variations on the Abl substrate peptide in attempts to optimize the distance of the tyrosine moiety from the metal NP surface and other structural elements contributing to confounding signal.
C. We have reproduced the detection and analysis of dried peptide material using Raman, comparing unphosphorylated to phosphorylated peptide spectra.
D. We have begun the synthesis and characterization of several additional nanomaterials for evaluation as SERS substrates.
E. We have designed and performed initial characterization on a peptide substrate for Src kinase, which will be tested alongside Abl kinase in a multiplexed experiment in the upcoming year by incorporating a deuterated tyrosine (via collaboration with Bart Dahl at University of Wisconsin-Eau Claire, subcontracted for the upcoming budget period).

Future Directions: We continue to examine the conditions for alternative nanomaterial synthesis and functionalization to work towards optimized preparation of AuNRs and AuBPMs for SERS. We are also exploring other nanomaterials, including hollow gold nanospheres and gold nanostars. In the meantime, we are pursuing in-cell experiments using the AgNPs (to address Milestones 4-6), since in preliminary experiments the intracellular environment seemed to stabilize the SERS variability (likely due to confined and controlled organization of the particles in intracellular compartments), and will extend these experiments to other nanomaterials as they are optimized. We also plan to apply the substrate development process to Akt, and will initiate synthesis of deuterium-

labeled Fmoc-tyrosine this summer in collaboration with Bart Dahl at University of Wisconsin-Eau Claire. Once in hand, this monomer will be incorporated into either the Abl or Src substrate and the ability to distinguish between their respective tyrosines via the isotope labeling will be examined.

Nanoelectrode and Nanofluidic-Based Assay of Mitochondria Membrane Potential and Apoptosis

Peter Burke[1], Douglas Wallace[2], Katie Zand[1], Ted Pham[1], Will Wang[1], Antonio Davila[2]

[1]University of California, Irvine, Irvine, California; [2]Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

For the past 70 years, mammalian mitochondrial physiology of has been assayed by following the time course of oxygen consumption for mitochondria suspended in volumes of 0.5 to 2 ml. These studies require several hundred mg of mitochondrial protein representing billions of individual mitochondria. However, because of very limited sample availability, technology advances that require reduced sample size (preferably, many orders of magnitude) will dramatically facilitate and accelerate (and in some, cases, enable for the first time) the evaluation of mitochondrial function in human embryonic stem cells and clinical biopsy samples. Furthermore, it has been assumed that individual mitochondria show the same behaviors as seen in large volumes of billions of mitochondria. However, it is a possibility and a potentially very important finding that an individual mitochondrion might function very differently from the average behavior of billions of mitochondria. For example, it is likely that the membrane potential of an individual mitochondrion will oscillate as the electron transport chain burns reducing equivalents to pump protons out across the mitochondrial inner membrane while the ATP synthase utilizes the membrane potential to convert ADP + Pi to ATP only when ADP is abundant and available. However, the frequency and wavelength, amplitude, and consistency of such oscillation have never been investigated.

To better evaluate the biophysics of the mammalian mitochondrion, we set out to develop microfluidic chambers that would permit us to evaluate mitochondrial metabolic and biophysical parameters on just a few mitochondria with the ultimate goal of assaying a single mitochondrion. During the prior grant period, we reported on two generations of microchambers that were used to interrogate the mitochondrial membrane potential fluctuations in response to various mitochondrial oxidative phosphorylation (OXPHOS) substrates and inhibitors. These chambers were two orders of magnitude less volume than standard respiration chambers and required mitochondrial concentrations that were four orders of magnitude less than conventional assays. Using these chambers, we reported interrogation of the mitochondrial function of human embryonic stem cells (hESCs). This has revealed that human stem cells have a mitochondrial membrane potential that is about 50% less than that of human somatic (adult) or cancer cells (160 mV in somatic cells vs. 120 mV in hESCs). This reduced mitochondrial function is consistent with our demonstration of a 50% reduction in the respiration rate of hESC versus that of control somatic cells using a commercially available high-resolution respirometry system (Oroboros). Furthermore, this reduced mitochondrial function is a feature of the stem cell mitochondria themselves, since transfer of human stem cell mitochondria into somatic cells results in the recipient cell acquiring several stem cell features including their morphology, altered nuclear gene expression, and reduced mitochondrial respiration.

Since our last report, we have developed nanofluidic channels in PDMS of cross-section 500 nm x 2 mm that are capable of trapping an individual mitochondria, delivering a variety of chemical analytes to it and interrogating mitochondrial physiology via a variety of fluorescence probes.Using fluorescence labeling with MitoTracker green we have demonstrated the immobilization of mitochondria at discrete locations along the channel. Interrogation of mitochondrial membrane potential with two different potential-sensitive dyes (JC-1 and TMRE) indicates the trapped mitochondria are vital in respiration buffer. Fluctuations of the membrane potential can be observed at the single mitochondrial level. A variety of chemical challenges can be delivered to each individual mitochondrion in the nanofluidic system. Increases in the membrane potential are seen upon introduction of OXPHOS substrates puruvate/malate into the nanofluidic channel. Introduction of Ca^{2+} into the nanochannels induces mitochondrial membrane permeabilization (MMP), leading to depolarization, observed at the single mitochondrial level. It is believed that once MMP has passed a critical threshold, the phenomenon self-amplifies in an all-or-nothing fashion, resulting in an irreversible cascade causing apoptosis. Thus, this nanofluidic system is the first such on-chip assay of isolated mitochondria for the study of apoptosis at the single mitochondrial level.

At present a variety of signals are known to induce or inhibit MMP, and thus the mitochondria act as a decision-making “gate” and the point of no return for cell death. Cancer cells are often characterized as resistant to MMP induction. Therefore, potential therapeutic actions include enhancers of MMP through proteins such as BCL-2.

However, the interaction mechanism is still under investigation. Therefore, the technology demonstrated in this paper could allow for high-throughput, combinatorial screening for chemical inducers and inhibitors of MMP and, thus, apoptosis, requiring small sample quantities for both the mitochondrial mass and also the candidate drugs. In addition, because of the high throughput, interference among various inhibitors and inducers can be assayed in an economical fashion.

In order to enhance the interrogation capabilities of our chips with improved spatial and temporal resolution, we have integrated electronic sensors into the nanochannels composed of two different nanotechnology based materials: Carbon nanotube mats and graphene sheets. In prior experiments (reported last year on much larger chamber sizes), we reported the ability to sense the opening and closing of single ion channels in artificial lipid bilayers with membrane pore proteins gramicidin A (gA) and α-Hemolysin (α-HL). The nanotubes can electrically detect the opening and closing of individual ion channels in the membrane through changes in the ionic current, the membrane capacitance, and the nanotube conductivity. In our new generation of devices, we have incorporated these electronic sensors into the nanochannels used for single mitochondria trapping and fluorescence interrogation, allowing for simultaneous fluorescence and electronic interrogation in a high throughput system. These new devices have demonstrated ability to sense changes in the electrical potential of a global gate using the channel solution as an ionic conductor, and tests to demonstrate mitochondrial membrane potential sensing are under way. Once perfected, this new generation of chips will be able to deliver vital, isolated, individual mitochondria to nanotube electrodes and interrogate their membrane potential fluctuations with unprecedented spatial and temporal resolution.

Multiplex Cancer Cell Purification With Magnetic Sifters

Chin Ooi, Christopher Earhart, Robert Wilson, Heather Wakelee, Shan Wang

Stanford University, Stanford, California

Magnetic separation of biomolecules and cells has become increasingly common as a means of preparing biological samples as researchers utilize magnetic particles conjugated with specific antibodies to selectively isolate cells and proteins. Purification and isolation of these cells and biomolecules can facilitate further biological analysis such as flow cytometry or protein assays. In applications such as rare cell separation, however, magnetic separation devices must demonstrate high elution efficiencies, and ideally multi-biomarker capabilities, in addition to high capture efficiencies for viable downstream analysis.

Recently we have demonstrated that our magnetic sifter, with a size of only 7 mm by 7 mm, can readily outperform Miltenyi's MACS column. Monoclonal antibodies to mouse H2Kb/d were used to identify and separate mouse cells using our standard sifter, or Miltenyi's protocols for MS columns. Following purification, flow cytometry was used to evaluate the purity of the separated populations. In a comparison of the flow cytometry results, the sifter demonstrated lower levels of cell loss and increased harvest efficiency. This is especially promising as Miltenyi's magnetic separation tools are among the most established commercially available devices.

We also demonstrated 2-plex magnetic sorting with the sifter. Cells labeled with the smaller nanoparticles (~50 nm) are captured at 2ml/hr but not 15ml/hr, whereas cells labeled with 150nm nanoparticles are captured at both 2ml/hr and 15ml/hr. This capture performance difference can be leveraged to enable separation with two distinct cell markers.

Additionally, we also showed that magnetic field configuration and field gradient have a profound effect on the effectiveness of magnetic sorting. In our initial experiments with H1650 lung cancer cell lines labeled with magnetic nanoparticles via the Epithelial Cell Adhesion Molecule (EpCAM) antigen, we can obtain capture efficiencies above 90% even at a sample flow rate of 5ml/hr, but elution efficiencies hover between 50% and 60%. A significant cause of the low elution efficiency is the lateral drift of labeled cells in the device due to magnetic field gradients from the permanent magnets. We obtain improved elution efficiencies close to 90% via optimization of the permanent magnet size and position, and explain the effect via the use of finite element software (Ansoft Maxwell 3D) for magnetic field and field gradient distributions, and a particle tracing algorithm for analyzing the final positions of the particles. This improvement in elution efficiency, allied to previous optimization of sifter geometry to improve capture efficiency, is critical in enabling the sifter to be used for magnetic separation of biologically relevant moieties such as circulating tumor cells and cancer stem cells, which require elution for subsequent analysis.

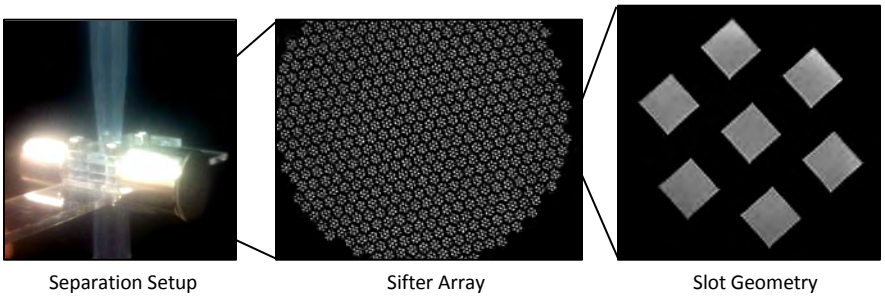

Figure 1. Magnetic sifter assembly.

Quantifying RNA-Protein Interactions In Situ Using Modified-MTRIPs and Proximity Ligation

Jeenah Jung, Aaron W. Lifland, Chiara Zurla, Eric J. Alonas, Philip J. Santangelo

Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia

The regulation of RNA is controlled by its interactions with *trans*-acting factors, such as microRNA and RNA-binding proteins (RBP). In order to investigate the relationships between these events and their significance during cancer pathogenesis [1,2], a method that detects the localization of these interactions within a single cell, as well as their variability across a cell population was needed. To visualize and quantify RNAprotein interactions in situ, we developed a new method using peptide-modified, multiply-labeled tetravalent RNA imaging probes (MTRIPs) (Figure 1). Using this method, we detected and quantified, the localization and frequency of interactions of the human respiratory syncytial virus (hRSV) nucleoprotein (N) with viral genomic RNA (Figure 2), and described the effects of actinomycin D (actD) on the interactions of HuR with β-actin mRNA and with poly(A)+ mRNA.

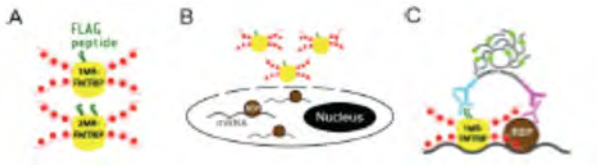

Figure 1. Summary of new methodology. (A) Flag peptides bind to a neutravidin (yellow) containing fluor-labeled antisense oligos to form a FMTRIP. (B) FMTRIPs enter cells via streptolysin O. (C) Antibodies and proximity probes attach to FMTRIP and RNA-binding (brown) on the mRNA (gray); probes join to synthesize a Cy5 equivalent-hybridized product via RCA.

Introduction: Numerous RBPs and their target sites have been identified and studied using immunoprecipitation and microarray analysis; however these techniques cannot provide the localization or variability of these interactions on a per cell or molecule basis. To image and quantify native RNA and RNA-protein interactions simultaneously in situ, we developed a proximity ligation assay (PLA) that combines peptide-modified MTRIPs, which enabled sequence specific imaging of native RNA [3], with proximity ligation and rolling circle amplification (RCA), which allowed the localization of the interactions with single molecule sensitivity [3,4].

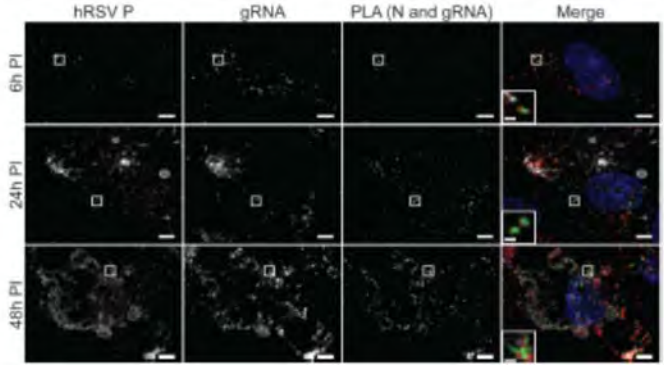

Figure 2. Imaging and quantification of PLA between hRSV N and gRNA at 6, 24, and 48 h PI. hRSV P protein IF (white), gRNA (red), and PLA (green) between N and gRNA in Vero cells 6, 24, and 48 h PI. Scale bar, 5 μm (1 μm in insets). The mean PLA frequency in Vero cells increased from 6 (n=14), mean = 0.28, s.d. = 0.09), 24 (n=14, mean = 0.37, s.d. = 0.06) and 48 h PI (n = 14, mean = 0.28, s.d. = 0.07), 24 (n = 14, mean = 0.41, s.d. = 0.11) and 48 h PI (n = 14, mean = 0.44, s.d. = 0.06). *** $p<0.001$. Error bars, s.d.

Results: Using this assay, we demonstrated that interactions between the hRSV N protein and viral genomic RNA (gRNA) of hRSV increased as the duration of the infection increased (Figure 2). We also imaged and quantified interactions between an important RBP, HuR, and poly(A)+ mRNA and β-actin mRNA (data not shown). Although predominately within the nucleus, HuR shuttles between the nucleus and the cytoplasm when cells are exposed to actinomycin D (actD), and acts to stabilize mRNA. We used PLA to examine changes in the HuR-mRNA interactions with native or overexpressed HuR during transcription inhibition using actD. Under normal growth conditions, cytoplasmic mRNA, as detected using poly(A)-targeted FMTRIPs, were found to be more abundant in wild-type HeLa cells than in the transfected ones. The observed standard deviation was likely due to the heterogeneity in the population. While HuR overexpression did not increase the overall mRNA amount, we found that it slowed the effect of actD in reducing mRNA number, after 30 minutes of exposure. ActD treatment on HuR

transfected cells had more dramatic effects on β-actin mRNA copy number, both as detected by FMTRIPs and imaging, and as analyzed by qRT-PCR. HuR overexpression stabilized the β-actin mRNA and increased its quantity in the cytoplasm. With 60 minute exposure to actD, the β-actin mRNA levels decreased to similar levels in transfected and wild-type cells. Next, given that ActD exposure has been shown to increase HuR binding to mRNA in the cytoplasm, we quantified the HuR-mRNA interactions via PLA. In transfected cells without actD, the frequency of HuR interactions increased 99% with poly(A)+ mRNA and 255% with β-actin mRNA than in wild-type cells. In transfected cells and with actD, the frequency of HuR interactions increased 184% with poly(A)+ mRNA and 250% with β-actin mRNA. No PLA was observed in transfected, MTRIP-delivered (no flag tag) cells. One clear advantage of this methodology is that it allowed us to quantify the cell-to-cell variability in RNA expression and interactions simultaneously, which is very challenging with other methods.

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Highly Multiplexed, Spatially Delineated Molecular Imaging in Cancer

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Molecular imaging methods are critical to numerous fundamental and clinical investigations in various human diseases including cancer. These approaches offer significant advantages over many bulk proteomic techniques since they provide important abilities to characterize spatial distributions of molecular markers in situ within cells and tissues. Such capabilities have been further enhanced by the development of optical microscopy techniques that allow biological samples to be examined with extremely high spatial and spectral resolutions. Nevertheless, in contrast to bulk analytical methods, the majority of molecular imaging approaches still have limited utility for proteomic analyses of diseases since they only allow a handful of molecular pathway components to be visualized simultaneously in the same biological sample. This deficiency largely stems from intrinsic limitations of existing molecular probes technologies. For one, the fluorophores that are coupled to protein recognition agents such as antibodies exhibit significant spectral overlap, which limits the number of proteins that can be labeled and detected simultaneously within a cell. The number of dyes that are coupled to antibodies is also difficult to control and is highly variable, especially when enzymatic signal amplification strategies are employed. These issues can confound multiplexed imaging approaches since distinguishing different spectral signals ultimately requires an appropriate balance of marker intensities within a sample. Finally, selective protein targeting often necessitates the use of high-affinity antibodies, making them difficult to remove without using harsh illumination and/or chemical treatments that can perturb sample morphology and marker integrity. Consequently, most cell samples and tissue sections can only be labeled and examined one time.

With funding support from IMAT, our group has developed a novel class of programmable immunofluorescent imaging probes that offer new levels of control over the coupling of fluorophores to protein recognition reagents. A unique feature of this technology is that it employs dynamic DNA complexes to control the in situ assembly and disassembly of structured fluorescent reporting complexes that can be coupled selectively to DNA-conjugated antibodies. Importantly, these reactions occur through a process called DNA strand displacement which involves the isothermal exchange of oligonucleotides between different thermodynamically stable DNA complexes and allows reporting complexes to be created and removed from a sample at ambient temperatures while using mild reaction buffers (e.g., Tris or PBS). Herein, we demonstrate how strand-displacement can be harnessed to create DNA complexes that can function as erasable molecular imaging probes in order to increase the number of proteins that can be examined within a single cell. We will also describe how these modular reagents can be adapted in a plug-and-play fashion to controllably tune antibody reporting intensities. Finally, methods to change the reporting functionalities of marker-bound probes to allow samples to be examined using conventional and then super-resolution imaging approaches will also be demonstrated. In each case, we will highlight how this technology can be used to increase the multiplexed detection capabilities of molecular imaging techniques and facilitate much more quantitative, proteomic-level in situ analyses.

An Integrated Platform for Quantifying Gene Expression in Co-Cultured Cells

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The quantification of mRNA is a ubiquitous and critical tool for understanding cellular mechanisms in cancer. While RT-PCR is often the endpoint, the success of the analysis depends not only on the PCR reaction, but also on an entire workflow linking cells to the PCR endpoint. For cultured cells, this process flow includes the culture itself, cell lysis, mRNA extraction and purification, and RT-PCR. While much research has been targeted to streamlining and increasing throughput of the PCR process, the remainder of the process flow has remained largely unchanged. In this project, we link a new mechanism for purifying mRNA, Immiscible Filtration Assisted by Surface Tension (IFAST), with co-culture on a single platform. IFAST uses an immiscible liquid barrier (e.g., oil) to separate the cell culture/lysis region from an elution buffer. Using paramagnetic particles (PMPs) that selectively bind mRNA, we extracted mRNA from the lysate by using a magnet to draw the PMP-captured mRNA through the immiscible phase. This process, which takes only seconds, replaces multiple washing steps required by current mRNA isolation protocols. The integrated platform incorporates co-culture of two cell types, breast cancer cells and stromal cells, in separate compartments connected via diffusion ports to facilitate cytokine exchange. Each compartment has an IFAST device, such that mRNA can be collected independently from each cell type without cross-contamination. Breast cancer cells (MCF-7) co-cultured with bone marrow stromal cells (HS-5) showed increased proliferation and morphological changes relative to breast cancer cells cultured alone. Additionally, mRNA extracted from these cells using the integrated device showed transcriptional changes consistent with estrogen response, even in hormone-independent conditions. These responses suggest that this technology could be used to recapitulate pro-growth responses seen in the metastatic microenvironment. Furthermore, the technology streamlines and accelerates two processes ubiquitous in cancer biology (culture and mRNA extraction), enabling the collection of additional endpoints with finite resources. We anticipate that our approach will provide an ~10X reduction in the labor required to perform gene expression experiments enabling studies that are currently cost prohibitive such as transcriptional profiles of entire classes of receptors (e.g., nuclear receptors) over a significant parameter space (e.g., mono/co-culture, dose responses with multiple drugs).

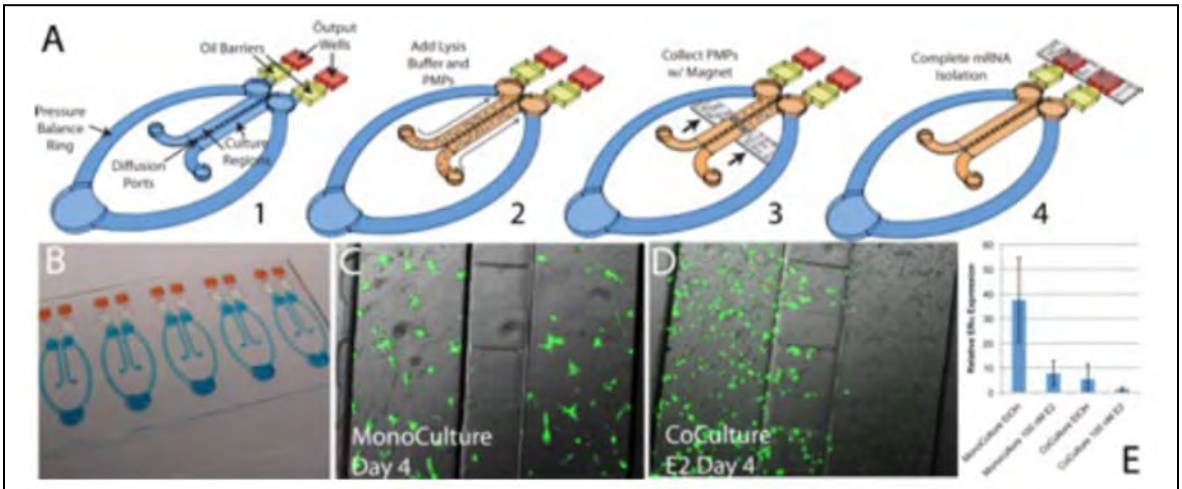

Figure 1. (A) Schematic of device operation. Cells are cultured in cultured regions and cytokines are exchanged through diffusion ports (1). After culture, lysis buffer and PMPs are added to culture regions (2). Magnet is used to collect PMPs (3) and draw them through the IFAST to purify mRNA (4). (B) Array of five devices. (C) Proliferation is low when breast cancer cells (green) are cultured in both culture regions. (D) However, breast cancer proliferation increases when bone marrow stromal cells (uncolored) are added to one culture region. (E) Co-culture with stromal cells down-regulates estrogen receptor in breast cancer cells, even in absence of estrogen (E2).

Rapid and Sensitive Multiplex Sequencing of Actionable Cancer Genes in Clinical Samples

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Despite recent advances in DNA sequencing technology, we continue to lack practical methods for comprehensively detecting actionable cancer mutations in a clinical setting. We have therefore developed smMIP, a massively parallel assay for multiplex targeted sequencing with concurrent single molecule tagging to enable rapid, cost-effective, accurate, and sensitive resequencing of clinically relevant cancer genes. We validated the method by resequencing 33 genes in each of 53 samples (including 45 obtained during routine patient care, 40 of which were FFPE). Single molecule tagging facilitated extremely accurate consensus calling, with an estimated per-base error rate of 8.4x10-6 in cell lines and 2.6x10-5 in clinical samples. Altogether, we detected 134 putative somatic non-synonymous variants. In addition to replicating 25 of 27 (93%) positive results of single mutation tests from a clinical laboratory, we identified 7 low-frequency mutations (0.2% to 4.7%) including BRAF V600E (melanoma, 0.2% alternate allele frequency), KRAS G12V (lung, 0.6%), JAK2 V617F (melanoma, colon, two lung, 0.3% to 1.4%), and NRAS Q61R (colon, 4.7%). We also demonstrate compatibility with a workflow that goes from sample to analyzed result in less than 72 hours. We anticipate that smMIP will be broadly adoptable as a rapid, cost-effective method for accurately detecting actionable cancer mutations in a clinical setting.

Activatable BRET Probes for MMP Enzymatic Activity Detection

Jianghong Rao

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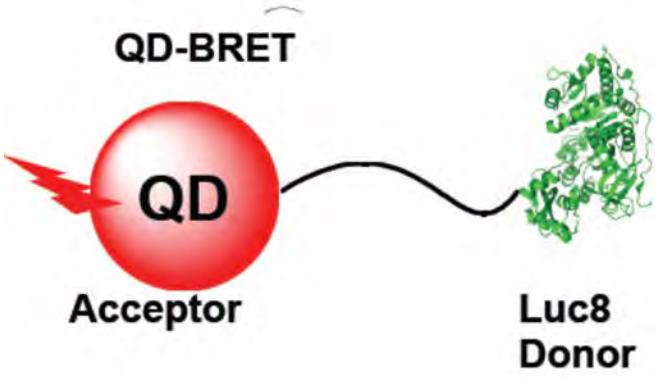

Figure 1. QD-BRET

Matrix metalloproteases (MMPs) are a family of zinc-dependent secreted endopeptidases that play a crucial role in defining the cellular environment through regulated degradation and processing of extracellular matrices (ECM). They have been extensively investigated in the past several decades due to their important roles played in cancer cell invasion and metastasis. MMPs are upregulated in almost every type of human cancer, and their overexpression correlates with advanced tumor stage, increased invasion and metastasis, and shortened survival. Commonly used assay for MMP detection is ELISA, which uses antibody to measure the total MMP concentration in the samples. MMPs are produced in a latent pro-enzyme form and require post-translational activation by the cleavage of an N-terminal pro-domain sequence to generate the active form. It is the active forms of these enzymes responsible for their physiological functions. More and more studies are reporting the correlation of the tumor status with the activity of MMPs. We have developed a new nanoplatform based on fluorescent semiconductor nanoparticles quantum dots (QD) — QD-BRET with QDs as the energy acceptor for a light emitting protein (for example, a bioluminescent protein *Renilla* luciferase) [1]. When QDs are in close proximity to luciferase, the energy released in the oxidation of the substrate by luciferase is transferred to QDs through BRET and enable QD emission (Figure 1). In this work, we applied this QD-BRET nanoplatform to develop sensors for sensitive detection of MMP activity in complex biological samples including serum [2]. This presentation will report our progress on the following grounds: (1) Development of new fluorescent nanoparticles for the BRET assay. The new nanoparticles show great BRET efficiency (the BRET ratio increased from previous 1.29 with QDs to 2.5), high serum stability and do not contain toxic metals, making them attractive replacement of quantum dots in this BRET assay platform. (2) Development of competitive BRET assay for detecting MMP activity. Our previous sensors produced decreased BRET signal after the protease cleavage. In contrast, the new sensor produces positive signal by the use of a non-emitting quenching molecule to the conjugate.

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Development of a Methylation-Based Diagnostic Assay for Malignant Melanoma: Defining the Factors Affecting Marker Selection and Assay Performance

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Melanoma has a predilection to metastasize when only a few millimeters in depth; however, early detection and diagnosis are difficult due to the overlap in clinical and histologic appearances of melanomas with highly prevalent benign moles. High-throughput DNA-methylation array technology holds the promise of discovery of candidate DNA-methylation markers useful for improving melanoma diagnosis but must be valid on small formalin-fixed paraffin-embedded (FFPE) tissues embedded in paraffin blocks, which is typically the only diagnostic tissue available for primary melanomas and moles. Moreover, a diagnostic assay should be sufficiently sensitive and specific to detect cancer even in the presence of some contaminating non-malignant cells, and robust enough to yield accurate results despite variability in patient and tumor characteristics.

Our initial work, under a R21, evaluated DNA methylation in primary melanomas and non-dysplastic benign nevi using the Illumina Cancer Panel I array, which measured promoter methylation at 1402 CpG sites in cancer-related genes after filtering. We identified 26 CpG sites in 22 genes that were significantly differentially methylated between non-dysplastic nevi and melanomas after controlling for age, sex, and multiple comparisons. Independent validation in a dataset that included dysplastic nevi confirmed 14 of the 22 genes to be significantly differentially methylated (1). In an effort to identify factors that could affect the accuracy of our diagnostic marker panel, we expanded our methylation profiling to 40 normal skin samples, in addition to the 47 primary malignant melanomas, 34 non-dysplastic nevi, and 22 dysplastic nevi previously tested. Factors such as patient characteristics, tissue source, tumor subtype or staging criteria, degree of nevus dysplasia, and specimen contamination by non-malignant cells were tested for potential impact on our methylation panel or markers comprising this assay. Our results to date are summarized as follows: Patient/host factors such as age, sex, anatomic site, solar elastosis, pigmentation, and lymphocytic presence were not associated with differential methylation of the 26 CpG loci in our marker panel. In melanomas, distinct sets of CpG loci differed in methylation according to Breslow thickness, mitotic rate, ulceration, and BRAF mutation; however, none of these overlapped with the 22 genes in our diagnostic marker panel, except for BRAF mutation. In melanomas, K-means clustering based on the 235 most variant CpG loci defined 3 clusters, including a subset exhibiting the putative CpG island methylator phenotype (CIMP) in 13% of melanomas. Comparison of melanomas with a larger series of nevi showed that methylation continued to distinguish melanomas from both common nevi and dysplastic nevi. Interestingly, dysplastic nevi did not show significant methylation differences from common nevi. Overlap of CpGs identified through supervised analyses (PAM, logistic regression, ROC) and filtering for the largest differences in β values allowed us to further refine as well as select the most robust loci for our diagnostic panel. These results support the usefulness of methylation for the specific identification of melanocytic malignancies. Comparison of melanocytic lesions (melanomas, dysplastic nevi, non-dysplastic nevi, individually or combined) and normal skin identified CpG site differences that may be used as internal standards to estimate the degree of skin contamination (or % tumor) in melanocytic biopsies. Nevi or melanomas differing in % melanocytic lesion ($\geq$70% vs. <70%) or study site (UNC vs. UNM) did not cluster separately on hierarchical clustering. For the R33 phase of this study, we are in the process of conducting validation and advanced development of methodology to overcome the challenges inherent in analyzing DNA methylation in small formalin-fixed paraffin-embedded specimens. Specifically, we are (1) Identifying candidate DNA-methylation differences that distinguish melanocytic (nevus or melanoma) vs. non-melanocytic (surrounding skin or lymphocytic infiltrate) cells for use as internal quality control standards to quantify sample percent tumor; (2) Identifying valid conditions for high-throughput DNA methylation array profiling of small-sized FFPE melanocytic tissues in order to select the least biased candidate DNA methylation sites for melanoma diagnosis; and (3) Confirming candidate DNA-methylation differences from high-throughput DNA methylation profiling using more quantitative assays.

Reference

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FRET-Based Biosensors to Monitor Redox in Cell Cycle Regulation

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Thiol redox homeostasis is central to the control of cell fate and is associated with various abnormal biochemical processes. Because of its abundance, glutathione is considered to be the major thiol-disulfide redox buffer of the cell and is often used as a proxy of the intracellular redox environment. We recently reported the development of Förster resonance energy transfer (FRET)-based redox-sensitive sensor adjusted for more oxidative environments that enables noninvasive redox measurements of the ER of living cells [1]. This research project aims to validate protocols and evaluate the performance of the FRET-based redox sensors when applied to cancer [2]. A second-generation FRET biosensor (CY-RL7) permitted visual monitoring of glutathione potentials in the relatively high oxidative environment of the endoplasmic reticulum (ER) [2]. We demonstrated that the sensor targeted to the ER of non-tumorigenic (CHO) and tumorigenic (HCT116 p53+/+, HCT p53-/-) cells is equally oxidized at ≈88%, while it is fully reduced in the cytosol. We also observed that the reductive level of the FRET probe was increased twofold to about 28% in cells incubated with N-acetylcysteine (NAC), a substrate for GSH synthesis. Additionally, to expand the basis of the FRET-based sensors used in the present study to monitor redox processes in the cytosol and mitochondria of live cells, we employed recently reported roGFP sensors that have the capability of monitoring redox in the more reduced mitochondrial compartment. Green fluorescent protein probes attached to the glutaredoxin enzyme (Grx1-roGFP2) enable real-time monitoring of the redox events in the cytosol and in mitochondria. We confirmed that probes targeted to these compartments are highly reduced at steady state. Our data demonstrate that mitochondrial as well as cytosolic redox homeostasis of mammalian cells is capable of restoring a reduced steady state redox environment within minutes after an acute oxidative insult (1 mM diamide for 2 min) is removed. Next, the efficacy of the Grx1-roGFP2 probe was ascertained by the probe response to perturbations in glutathione homeostasis. Surprisingly, both NAC and GSH ethyl ester (GSHee) triggered oxidative stress in mitochondria, but not in the cytosol. Most noticeably, the mitochondrial redox environment in cells incubated with NAC and GSHee gained resistance to exogenous oxidants in spite of elevated oxidative stress. Finally, we observed distinct oxidative responses of mitochondrial sensors expressed in isogenic porcine fibroblast 161-p53 and 161+p53 cancer cells after depletion of the glutathione pool with buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis. This observation implies that the sensitivity of the sensor expressed in the mitochondria of cancer cells exposed to the inhibition of glutathione biosynthesis by BSO depends on alterations in p53 expression in cancer cells. In conclusion, our studies demonstrate that individual organelles have different redox requirements principally driven by the reduced (GSH) and oxidized glutathione (GSSG) redox couple. Our data also demonstrate metabolic differences in GSH homeostasis between cancerous and normal cells and may offer suggestions for novel therapeutic approaches to cancer.

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Microfluidic Sorting of Blood Cells for SPR and Fluorescence Analysis

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A microfluidic, size-based sorting array (3 cm x 1 cm) has been adapted to a slide holder that accommodates a 1 cm^2 grating-coupled surface plasmon resonance (GCSPR) chip that can be spotted with hundreds of regions of interests (ROIs) to capture soluble analytes or cells. Based on the size of the cells entering on the left slide in a narrow fluid stream, the cells are bumped to the right. Since monocytes and myeloid derived cells are larger than lymphocytes they move toward the right side. Circulating tumor cells (CTCs) are usually the largest cells so they exit onto the detection chip on the far right. The sorting and detection portions of the device have been fabricated using soft-lithographic patterning and nanoscale lithography/etching, respectively. This has resulted in a monolithic separation/detection chip which has been tested with beads of various sizes to confirm the sorting efficiency. Antibodies used at ROIs to assess capture of CTCs include anti-CD326, CD44, CD49f and CD24. Once captured, the cells can be further characterized for released analytes and then lysed for analysis of intracellular biomarkers with AlexFuor647 conjugated antibodies. The grating coupled surface plasmon coupled fluorescence (GCSPCF) enhances the signal about 100-fold from that of GCSPR.

Application of an Innovative Technology to Develop Low-Toxicity Kinase Inhibitors

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Regulation of all cellular processes requires dynamic regulation of protein phosphorylation. We have developed an unbiased system to globally quantify the phosphorylation index for substrates of a specific kinase by independently quantifying phosphorylated and total substrate molecules in a reverse in-gel kinase assay. Non-phosphorylated substrate molecules are first quantified in the presence and absence of a specific stimulus. Total substrate molecules are then measured after complete chemical de-phosphorylation, and a ratio of phosphorylated to total substrate is derived. To demonstrate the utility of this approach, we profiled and quantified changes in phosphorylation index for Protein Kinase CK2 substrates that respond to a small-molecule inhibitor. A broad range of inhibitor-induced changes in phosphorylation was observed in cultured cells. Differences among substrates in the kinetics of phosphorylation change were also revealed. Comparison of CK2 inhibitor-induced changes in phosphorylation in cultured cells and in mouse peripheral blood lymphocytes in vivo revealed distinct kinetic and depth-of-response profiles. This technology provides a new approach to facilitate functional analyses of kinase-specific phosphorylation events, to facilitate kinase inhibitor target validation studies, and to inform in vivo analyses of kinase inhibitor drug efficacy. We have used this approach to identify high-efficiency substrates of CK2, and have derived an inhibitory peptide based on the major CK2 phosphoacceptor site on Elongation Factor 1ß. The characteristics of this new peptide kinase inhibitor will be discussed.

Multiple Reaction Monitoring to Profile Biosensor Phosphorylation in Leukemia

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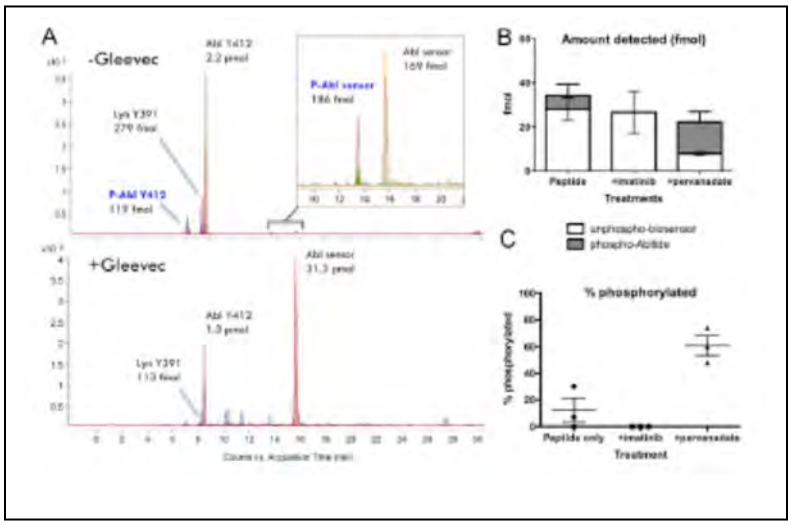
Drugs that target signaling mechanisms have shifted the paradigm for cancer chemotherapy. The Bcr-Abl inhibitor imatinib (Gleevec) is the most prominent example of this. Treatment of chronic myelogenous leukemia (CML) with imatinib inhibits the Bcr-Abl kinase and lowers the levels of phosphorylated substrates (such as CrkL) in patient cells. However, while treatment is frequently successful, a patient's clinical response is not always predictable. Some evidence suggests that decreased Bcr-Abl substrate phosphorylation in mononuclear cells is a reliable indicator of initial response, could inform dosage adjustment, and predicts longer term outcomes. Such "real-time" monitoring of drug response during treatment is not widely performed, because technical hurdles present a challenge to efficient analysis of patient material. Therefore, there is a need for technologies that can detect multiplexed, specific kinase activities from cells derived from patient material. The objective of this proposal is to apply two emerging technologies to this challenge: our recently published intracellular kinase "biosensor" strategy along with multiple reaction monitoring (MRM) mass spectrometry, to develop a sensitive, multiplexed kinase assay for leukemia-related signaling in patient material. This technology should be adaptable for basic research, drug development, and even potentially diagnostic use. To date we have focused on optimizing the technical parameters of the assay and have made substantial progress on the technical optimization; however, we have not yet moved into testing of patient samples. Our milestone targets are:

1. To detect ≤250 nM peptide detected, using ≤2-5 x 104 cells per experiment
2. To achieve statistically significant, reproducible quantification of %phosphopeptide for intervals of 20%.
3. To achieve detection of Bcr-Abl activity and inhibition with statistical confidence that is appropriate for the effect size.



We have been able to detect endogenous Abl kinase, Lyn kinase and the biosensor peptide in an MRM workflow (Figure A) from amounts of injected material that are equivalent to ~15K cells (below the 20K-50K cell range we proposed in Milestone 1). In replicate experiments (Figure B), we have achieved sensitivity as low as the 10-30 fmol (per µl trypsin digested lysate) range for detecting the biosensor peptide from cultured K562 human CML cells (which express Bcr-Abl) treated with biosensor in the presence or absence of imatinib (Gleevec) or pervanadate (a phosphatase inhibitor). This corresponds to ~10-30 nM concentration per lysate digest. Accordingly, we consider Milestone 1 to be achieved. We have also been able to achieve quantification of % phosphorylated peptide (Figure C) with sufficient accuracy to determine the difference between ~10-20% phosphorylation (peptide only) and 50-70% phosphorylation (+pervanadate) with statistical significance; however, the reproducibility of % phosphorylation is still unacceptable under the current conditions; while coefficient of variation is as low as 22% in the best cases (which would be acceptable), it ranges as high as 127% (which is unacceptable). This seems to be related to signal to noise in the SRM measurement, as these low-level fmol-scale signals are accompanied by a large number of noise peaks. Therefore although we can distinguish ~20-30% intervals with the assay, we do not consider Milestone 2 to be achieved as of yet.

In the next year of the project, we will improve our sample handling conditions and introduce enrichment steps to improve signal to noise. Data shown above were from whole cell lysate digests, however, it is becoming clear in the MRM field that sample enrichment prior to MS is important to achieving reproducible and reliable results, especially for low abundance proteins. In addition to examining CD34-based hematopoietic stem cell enrichment, we will use avidin- and antibody-based enrichment of our endogenous proteins of interest and our biosensor peptide from lysates, followed by column-based inline protease digestion (which has also been shown to dramatically improve reproducibility for MRM experiments). Once this reproducibility is achieved, we will begin analysis of patient material in collaboration with Hamid Sayar, M.D. (Indiana University Simon Cancer Center) to address Milestone 3.

Reference

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Probing Cancer Cell Chemoinvasion Strategies Using 3D Microfluidic Models

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Chemokine-mediated directed tumor cell migration, or chemoinvasion, is an important early step in cancer metastasis. Despite its clinical importance, the role of cancer cell microenvironment in regulating chemoinvasion is largely unknown. This is primarily hindered by the lack of tools for probing cancer cell dissemination strategies in a physiologically realistic, 3D, and well defined microenvironment. Current state of art for migration assay, the Boyden chamber assay, is population and end-point based. It is not suitable for cancer cell migration studies, in which cancer cell heterogeneity (variation in space) and plasticity (variation in time) are the two hallmarks of cancer. To overcome these limitations, we have developed a set of microfluidic models to re-create the key biophysical (interstitial fluid flows and biomatrix stiffness) and biochemical (chemokine and growth factor gradients) factors within the cancer cell environment, and 3D imaging techniques for probing cancer cell dynamics in three dimensional space and time. Lymph nodes are known to be the first metastasis sites of many cancer types, for example, breast, melanoma and prostate cancers. Using a malignant breast tumor cell line, MDA-MB-231, as a model system, we studied the roles of lymph node microenvironment in cancer cell chemoinvasion. Our experimental results have revealed (1) the synergistic roles of chemokines and growth factors in regulating cancer cell migration. Specifically, chemokine SDF-1α was found to be a chemo-attractant to breast tumor cell lines (MDA-MB-231), and a background of epidermal growth factors (EGF) abrogated this chemotaxis effect. (2) Slow interstitial fluid flows within a 3D biomatrix promote the heterogeneity and plasticity of tumor cell morphology and motility. We found that MDA-MB-231 cells evolved from mostly mesenchymal types to amoeboid types when subjected to slow interstitial flows. This heterogeneity and plasticity of cancer cells may be implicated in their ability to invade and survive in a foreign environment. (3) Malignant tumor cells (MDA-MD-231) were found to use rare event statistics to disseminate, a more efficient strategy for random walkers to spread in space, in contrast to the diffusive Gaussian statistics that is common for passive microscale particles. Using the dynamic data collected from the microfluidic models, we plan to formulate a set of rules that cancer cells use to invade to a distant site including lymph nodes and vascular vessels, both through migration within a 3D biomatrix, and intra/extravasation through the blood or lymphatic endothelium layers. The eventual goal of the research is to translate this knowledge into improvements of cancer treatments through the modulation of the cancer microenvironment.

Specific and Reversible Binding of DNA Nanoparticles to Cancer Cells

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DNA nanoparticles (DNA-NP) are produced by enzymatic DNA synthesis using a strand displacing DNA polymerase (phi29) and a circular oligonucleotide template. The long single stranded product collapses into a particle containing many iterations of the complement of the template sequence. Template oligonucleotides containing random sequences generate highly diverse libraries of unique DNA nanoparticles. Since DNA can adopt secondary structures that exhibit molecular specificity, DNA-NPs with specific cell or other target binding properties can be selected by the biopanning process diagrammed in figure 1. DNA-NP that bound to the mouse pancreatic cancer cell line panc02 and the human breast cancer line MDA-MB-231 were selected over 6 rounds of biopanning and several binding particles recovered in each case. All of the panc02 binding particles contained an 8 nucleotide motif (AAnGGGGCG) and did not bind to other cell lines tested. Molecular modeling suggested that at least three of the four consecutive G were unpaired. Saturating that motif with a complementary locked nucleic acid could both inhibit the particle binding as well as release particles already bound to cells. Preliminary in vivo analysis in mice bearing subcutaneous panc02 tumors has shown that the panc02 binding particles are enriched in the tumor compared to control particles following both intravenous and intra-tumoral administration. Experiments to determine if the panc02 binding particles can inhibit primary or metastatic tumor development are ongoing. In addition, selection is being performed on panc02 tumors in vivo and those particles will be compared to those obtained by ex vivo selection. The MDA-MB-231 binding particles were found to be hybrid dimers and trimers.. This was surprising since the PCR amplification typically deconstructs hybrids and multimers back to their monomer components in the absence of an intentional primer design to preserve hybrids from different libraries. Cloning revealed that the dimers and trimers were all hybrids of different monomer units. One such clone was studied further as both monomer components and the dimer sequence. Modeling the predicted structure suggested the hybrid was able to preserve itself through the amplification because the primer binding sites are included in a large stem loop structure that out-competed the PCR primers for binding. When made from synthetic templates, particles based on each of the monomer template sequences as well as the hybrid dimer bound to MDA-MB-231 and not to THP-1, an unrelated cell line. Binding of the dimer appeared stronger. Competition with oligonucleotides that bind to the internal 60bp sequences of the particles showed that each monomer was completely inhibited by its complementary oligo, whereas the dimer was reduced in binding when either of the monomer complements was used, but required both competitors to completely abrogate cell binding. The ability to form hybrid particles is a key benefit of this technology since the modular nature of the DNA-NPs allows for multiple discrete sequences to be displayed on a given particle. Combinatorial selection methods can then be used to "breed" particles with optimized activity by re-assorting the individual modules in each round. In this case, it happened spontaneously, further emphasizing the potential benefit of multimodule particles.

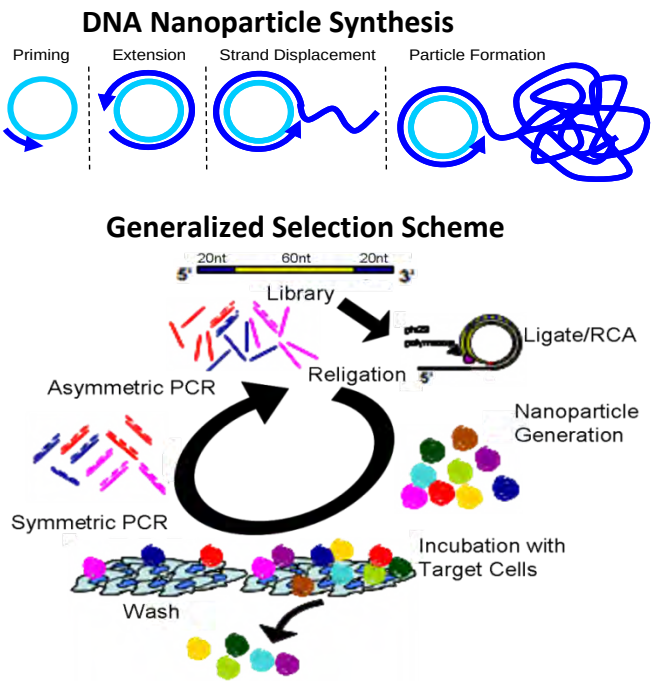

Figure 1. DNA nanoparticle synthesis and selection.

2D-PCR for Spatially Mapping Gene Changes in Tumor Sections

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Our research is focused on developing a novel technology to spatially map genetic changes in human tissue sections with high spatial resolution. Our previously published results have mapped DNA to a 1 mm resolution. Our technology aims to combine the benefits of PCR (amplification), laser capture microdissection (high spatial resolution), and FISH (multiplexing). This technology aims to elucidate the genetic changes occurring in tumor microenvironments: tumor cells and the cells surrounding the tumor. We have developed techniques to study modifications in the genome as well as epigenome of histological sections obtained from cancerous tissue and biopsies. Our method of evaluating the methylation status of cancer relevant genes utilizes the methylation restrictive enzymes digestion of DNA. Our innovative protocol is optimized for performing restriction analysis and PCR in the same reaction mix on a tissue lysate while maintaining spatial resolution. Our results show a significant difference in methylation between tumor and non-tumor cells. In addition, we have made significant strides toward miniaturizing our previously published 2D-PCR procedure. Our innovative process involves direct lysis of tumor section followed by DNA capture onto a hydrogel. The DNA is amplified within the hydrogel and the image is resolved to analyze the variation in DNA across the tissue section. We are currently working to adapt the procedure for investigating changes in gene expression.

Discovery Platform for Cancer Antigens

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Abstract and Specific Aims: The main goal of this proposal is to implement a platform-based method to isolate and identify patient-derived tumor-specific antibodies and their cognate cancer antigens. It is the intention of these investigators to use these reagents to expand our understanding of breast cancer biology, detection of cancer specific biomarkers and as novel therapeutic treatments. The single domain antibodies isolated are infinitely renewable and hold great promise as tools for population based biomarker screening and individualized patient-specific targeted therapy for advanced and metastatic disease. The specific aims of this proposal will focus on four specific goals:

Specific Aim 1: Perform throughput platform screening of at least 40 breast cancer cases representing up to 65 total libraries and identification of an estimated 1000-1500 novel VH antibodies.

Specific Aim 2: Identify the cognate antigens for 200-400 antigen driven VH single domain antibodies identified in Aim 1 based upon clonal expansion and somatic hypermutation properties.

Specific Aim 3: Validate identified antigens and their presentation in human breast cancer using multiplex large scale arrays.

Specific Aim 4: Assemble multiplex protein arrays of recombinant soluble domain antibodies and matching arrays of their cognate antigens for use in cancer diagnostic, screening and clinical targeting applications.

Results and Observations: Synthesis and banking of 22 total patient-derived sentinel lymph node libraries of VH single domain antibodies has been performed. Fourteen of these were effective at producing large volume clones during transformation. Approximately 12,000 clones have been screened for orientation and 6,000 individual clones directly sequenced using library 96-well sequencing format. 5,800 open reading frame clones were compared to genomic databases for somatic hypermutation and V-D-J gene usage clonal expansion. Current accumulation of data from these large scale screens indicate that each library has 3-4% of clones that reach criteria for antigen-driven selection and further analysis (175-220 clones). To date we have developed screening mechanisms for selection of these VH single domain antibodies according to the following criteria: (a) cell surface immunofluorescence of ER+ and ER- human breast cancer cell lines under optimal and low glucose conditions, (b) positive threshold signal in direct ELISA of breast cancer cell and primary tumor lysates, (c) immunohistochemistry signal on tumor tissue array (80 sample), and (d) immune-selection of target antigen from cell or tumor lysates. A total of 46 VH clones have been synthesized and screened through methods a-c above and 6 have been processed through d. Immunoselection and identification of VH antigen targets by mass spectrometry methods are in progress. Direct screening for antigens using our collaboration with Arizona State University Biodesign Institute is ongoing and on-slide transcription/translation and antigen detection are being performed to control background and specificity thresholds.

Goals and Future Directions: The goal for the coming year is to screen and identify cognate antigens from approximately 200-250 VH clones with high scores of antigen-driven somatic hypermutation and/or clonal expansion. Secondly, in an effort to streamline library VH synthesis and antigen identification we are developing SV40 large T Ag expressing MCF-7 and MDA-MB-231 surrogate cells. Direct transfection of library plasmid containing individual VH clones will effectively express the VH antibody in the transfected cell provide for immunoprecipitation from a whole cell lysate as a rapid and medium throughput method to identify cognate antigens. Recombinant VH antibodies and their cognate antigens will be used to determine their distribution in human breast and ovarian cancer as novel diagnostic reagents and therapeutic targets.

Digital Analysis of Proteins Through End Sequencing (DAPES)

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We are developing DAPES (Digital Analysis of Proteins Through End Sequencing), a single peptide sequencing technology, which, if successfully completed, would identify and quantify all proteins in biological fluids. The potential applications of DAPES are widespread, it should allow researchers to find signatures in a variety of human tissue samples that diagnosis disease, determine prognosis, or predict responses to a particular kind of therapy. The DAPES procedure begins by cleaving cellular protein content into short peptides and covalently attaching them to a glass surface through a PEG based nanogel. The terminal amino acid in these attached peptides is then identified through the use of fluorescently labeled N-terminal Amino Acid Binding molecules (NAABs) and single molecule detection (SMD). A cleavage reaction is next used to remove the N-terminal amino acid and the cycle is repeated to sequence 10 amino acids from the N-terminus of each peptide on the slide. The peptide sequences are then mapped back to the human proteome and the number of different peptide sequences observed which map to a particular protein is then divided by the total number of possible peptides that could be produced by that protein to yield a quantitative measure of the protein's abundance. To serve as the NAAB probes integral to the DAPES procedure we have been cloning *E. coli* tRNA Synthetase (RS) enzymes and now have 8 probes capable of binding specific N-terminal amino acids. We have begun to assay the specificity of these probes and found that they bind specific subsets of amino acids, an observation in agreement with previous work studying the mis-acetylation patterns of the RS enzymes. We hope to utilize the cross-reactivity of the RS probes to help simplify the DAPES procedure by distinguishing between all 20 amino acids through as few rounds of binding as possible. As part of our effort to develop DAPES our laboratory has also produced probe stripping and N-terminal amino acid cleaving protocols and developed an optimal imaging technology. The stripping of NAAB probe bound to N-terminal amino acids is a necessary step between successive rounds of probe binding. We have developed an efficient stripping protocol using a detergent wash that removes probe but does not affect the PEG surface or the binding of peptide by additional probe. To remove the N-terminal residue of bound peptides after its identity has been elucidated we have established a cleavage protocol based on a modified Edman reaction. Lastly, significant effort has been made to optimize our SMD peptide imaging platform; we have reduced background fluorescence and nonspecific interactions of probe with the surface while maximizing the accessibility of bound peptide to probing by NAAB molecules. We are now working to fully characterize our existing RS probes while continuing to expand our library with new N-terminal binders. Efforts are also ongoing to develop software which calls N-terminal residue identity based on probe binding signature and maps the 10-mer sequences generated by DAPES to the human proteome. We will soon combine these different efforts and begin performing single molecule sequencing experiments.

Molecular Diagnostic Tests to Augment Cytomorphologic Diagnosis of Lung Cancer

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Morphologic analysis of cytology samples obtained by fine needle aspiration (FNA) is a primary method for diagnosing bronchogenic carcinoma. However, across multiple studies the false negative rate for cytomorphological analysis ranges from 0.2-0.3, and among those diagnosed with lung cancer, the false positive rate for diagnosing the sub-class of small cell carcinoma averages 0.09. These false results lead to additional invasive diagnostic studies and delay treatment. In previous studies the MYCXE2F1/p21 index correctly classified 72/78 non-malignant lung tissue and 50/57 lung cancer samples for a sensitivity of 88%, and specificity of 92%. A CDKN2C/FOSL1 test for distinguishing non-small cell lung cancer (NSCLC) from small cell lung cancer (SCLC) had a PPV of 100% with 63% sensitivity. The goal of this study was to optimize these tests for assessment of RNA extracted from FFPE samples In order to optimize the robustness and utility of these tests for assessment of RNA extracted from FFPE samples including cell block FFPE samples from FNA, we developed qPCR methods that enable simultaneous measurement of each target gene and reference gene transcript relative to a known number of internal standard competitive template (CT) molecules within a standardized mixture of internal standards (SMIS) using two-color fluorometric analysis on real-time platform. For each gene, the native template was quantified with a sequence-specific FAM-labeled probe and the CT was quantified with a sequence-specific Quasar670 labeled probe. Use of multiple base changes in CT ensured specificity of each internal standard. External standard corrected for inter-experimental and inter-probe difference in cycle threshold. Pre-amplification with 20cycles increased signal to background. Results for each gene thus far demonstrate excellent linearity (R2 > 0.99, slope 1.0 +/- 0.05). Percent error as relative accuracy showed 12.1% in constant CT with diluting NT until 1/20 fold difference and 11.9% in vice versa. We obtained excellent signal-to-analyte response (1.0 +/- 0.05) and precision (CV < 30%) over six orders of magnitude, and reliable detection of as few as 10 molecules. We then conducted a validation study of this two-color fluorometric assay in 10 malignant and 10 benign surgically removed FFPE samples. Consistent with previous results in fresh samples, the MYCXE2F1/p21 index optimal cut-off value had 90% specificity and 90% sensitivity. The Receiver Operator Characteristic (ROC) area under the curve (AUC) was 0.94 with 95% confidence interval of 0.8331 to 1.047 and p-value of t-test for stratification of malignant from non-malignant was 0.00017. Based on these data, we expect that this quality-controlled two color fluorometric qPCR approach will enable reliable analysis of the promising MYCXE2F1/p21 and CDKN2C/FOSL1 assays in small degraded RNA extracted from FFPE and FNA cell block FFPE samples.

Disclosures: JCW has equity interest in and serves as a consultant for Accugenomics, which develops and licenses the use of standardized mixtures of internal standards used in this study. There are no other potential conflicts of interest.

Method for Detection of Secreted Proteins in Single-Cell Assays

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A major goal of biological research is to provide a greater understanding of human physiology as it relates to pathological process involved in disease. Because cytokines play an important role during inflammation and disease, they are the best tool to measure the activation of immune cells. Cytokines are small regulatory proteins and peptides (8-30 kDa) that exhibit a wide range of biological activities. Cytokines are released in unique profiles in response to inflammation, infection, systemic infections such as sepsis, chronic wound healing, and even as predictors of mortality. Measurements of cytokines are not without problems. The detection of cytokines is hampered by their biological properties, e.g., local secretion, rapid uptake and utilization, and short half-life. These properties have led to the development of limited techniques commonly used to measure cytokines based on single cell level, such as flow cytometry, intracellular cytokine staining (ICCS), and enzyme-linked immunospot (ELISPOT) assays. The current methods for single-cell assays as described above require complex procedures, cell treatment, and inconvenient enzymatic or chemical signal amplification. Moreover, none of the above methods has real-time detection capability and potential quantitation of secretion of cytokines per single-cell. Our goal is to develop the method for single-cell assays that relies on nanofabrication of plasmonic substrates allowing optical amplification of fluorescence from surface bound dye-labeled detection antibodies. Our technology, called MEFspot, includes collection of intensity and lifetime images for each fluorophore. If the spot is really generated by the protein secreting cell, it displays characteristic values for two measured parameters: an intensity value greater than the background and lifetime shorter than background. Fluorescence lifetime imaging software allows fast processing and visualization of cells that secrete proteins. The background signal corresponds to unbound fluorophore labeled detection antibody. The strengths of our technology is simplified procedure (no washing out cells), ability for kinetics of secretion, ability for multiplexing (dual secretion and cell viability control), and potential for quantitative analysis. Previously we have reported the development of planar plasmonic substrates that allow fluorescence amplification of about 200-fold for fluorophores assembled in the immunoassay geometry. Here we provide additional numerical calculations that provide physical explanation of amplification factors and guidance for rational design of substrates with regard to geometrical composition of metallic/dielectric nanolayers and spectral properties (excitation/emission) of fluorescent materials. Also, optimization was performed for surface chemistry for surface stabilization and immobilization of capture antibodies to maximize the optical signal. We also demonstrated detection of cytokines (TNF-α and IFN-γ) from microphages stimulated with lipopolysaccharide (LPS). Here we focused on detection of cytokines (IFN-γ and IL-2) secreted from T cells isolated from human peripheral blood monocyte cells (PBMC) stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Single and dual cytokine assays were designed and tested using fluorophores in two spectral ranges; blue-green (AlexaFluor 532) and red (AlexaFluor 649). We envision that MEFspot method can be an excellent alternative to other methods for single cell assays providing additional valuable features as described above.

VEC^3-Valve Enabled Cell Co-Culture Platforms for Cancer Biology Study

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Tumor microenvironment is being recognized to play critical roles in tumor progression. While some approaches have been developed to study the tumor microenvironment, a major gap still exists as the lack of a reliable method to study the interactions between tumor cells and adjacent cell populations in real-time where physiologic and signaling events can be measured quantitatively. We are developing Valve Enabled Cell Co-Culture (VEC^3) platforms, a new class of microfluidic devices for analyzing interactions between adjacent cell populations seen in the tumor microenvironment. The technology allows for separate culture and treatment of distinct cell populations in close proximity and cell-cell interactions through either soluble factors or physical cell contact while maintaining fluidic control over the microenvironment. Using two-dimensional (2D) VEC^3 platforms, we have demonstrated the function of VEC^3 in studying the effects of hypoxia in tumor cell-endothelial cell cross-migration through creating hypoxic microenvironment for tumor cells. We have also verified the effects of EphA2 receptor on tumor cell-endothelial cell cross-migration. The platform will also be used to dissect signaling pathways in vascular endothelial cells by observing the cross-migration between tumor cells and wild-type or rictor-deficient vascular endothelial cells. We have found that rictor-deficient endothelial cells are defective in VEGF-induced proliferation, migration, and vascular assembly in vitro and angiogenesis in vivo. We are currently testing if tumor-endothelial cross-migration is also affected in rictor-deficient endothelial cells. Additionally, we discovered that rictor-deficient cells secrete elevated levels of certain inhibitory chemokines. Through introduction of nanoporous collagen gel with embedded biotin-coated polystyrene microparticles (0.7-0.9 μm diameter) between two cell chambers, we have successfully demonstrated the selective blockage of avidin with concurrent perfusion of ovalbumin between two cell populations. Design rules have been developed for this novel concept of "ligand traps," which will not perturb the optimal culture conditions, to work for weeks. In addition to 2D platforms, three-dimensional (3D) co-culture has been achieved in the VEC^3 platform by loading cells within biogels. We have demonstrated this with co-culture of cancer cells with stromal fibroblasts. Several types of head and neck cancer cells, including SCC61 and JHU, have been co-cultured with primary fibroblasts from human cancers (CAFs) or from normal tissue (NAFs) in 3D type I collagen matrices. Time-lapse imaging shows that SCC61 cells migrate directionally along CAFs, while they migrate randomly when co-cultured with NAFs. To quantify this, we calculated an association index between SCC61 cells and fibroblasts by measuring the intersecting angles between the cells and taking the cosine of this angle. The association index for SCC61 cells and CAFs was 0.76±0.07, whereas SCC61 cells and NAFs had an index of 0.43±0.03. These results suggest that SCC61 cells more closely associate with CAFs than with NAFs, and this association regulates the directional movement of the SCC61 cells. Interestingly, CAFs from various tissue, including prostate and breast, similarly influence the migration of SCC61 cells, suggesting a common mechanism by which stromal fibroblasts regulate cancer cell migration. In addition, through immunostaining fibronectin, it was found that CAFs organized the fibronectin into a parallel fiber structure, which is distinct from the meshwork-like arrangement of fibronectin found with NAFs.

Translational Control Analysis by Translationally Active RNA Capture/Microarray Analysis (TrIP-Chip)

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We have developed a new approach to systematically study post-transcriptional regulation in a small number of cells. Actively translating mRNAs are associated with polysomes and the newly synthesized peptide chains are closely associated with molecular chaperones such as hsp70s, which assist in the proper folding of nascent polypeptides into higher ordered structures. These chaperones provide an anchor with which to separate actively translating mRNAs associated with polysomes from free mRNAs. Affinity capture beads were developed to capture hsp70 chaperones associated with the polysome complexes. The isolated actively translating mRNAs were used for high throughput expression profiling analysis. Feasibility was demonstrated using an in vitro translation system with known translationally regulated mRNA transcript thymidylate synthase (TS). We further developed the approach using HCT-116 colon cancer cells with both TS and p53 as positive controls. The steady state levels of TS and p53 mRNAs were unaltered after 5-fluorouracil treatment as assessed by real time qRT-PCR analysis. In contrast, the protein expression and polysome-associated mRNA levels of both genes were increased. These differences in translational rate were revealed with our new approach from 500 cells. This technology has the potential to make investigation of translational control mediated by RNA binding proteins or non-coding miRNAs feasible with limited quantities of cells or clinical specimens.

Scanning Correlation Microscopy Methods for Quantifying DNA Repair Kinetics

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DNA double strand breaks (DSBs) formed by ionizing radiation or by radiomimetic agents are considered as the most cytotoxic of DNA lesions and if misrepaired may lead to chromosomal translocations and genomic instability. Deletion or translocation of chromosome fragments could lead to inactivation of tumor suppressor genes or activation of oncogenes. Both events can trigger the onset of carcinogenesis in surviving cells. Research work that will explain in detail the function of wild type and mutant proteins involved in DSB damage sensing, signaling and repair will be an invaluable contribution to the understanding of repair deficiency mechanisms, which can direct the design of optimal cancer treatment strategies. Live cell fluorescence microscopy methods have great potential for helping decipher the detailed spatio-temporal relationships among the different factors participating in DNA repair. The major hurdle currently faced by these methods is that they are not able to quantify the kinetics of DNA repair proteins after creation of sparse DNA damage by γ-rays or radiomimetic agents, which is the basis of many cancer treatments today. In this work we develop quantitative fluorescence microscopy methods that can overcome this major hurdle in the DNA repair field and enable quantification of repair kinetics due to sparse DNA damage in living cells. More specifically we apply and further develop methods for the quantification of sparse DNA damage based on the raster image correlation spectroscopy (RICS) principle. Simply put, RICS analysis enables quantification of protein kinetics in images that appear uniformly green as no visible fluorescent foci are being formed after DNA damage. In this work we apply and develop RICS-based methods to the specific challenge of quantifying the repair kinetics of DNA-PKcs, a key DSB repair protein, after exposure to γ-rays and to the radiomimetic chemical bleomycin. Importantly, this technique is generalizable to any other repair and signaling protein. In the 1st year of the funding period, we have been working in parallel on all three Specific Aims of the study:

Aim 1. Studying DNA Repair After γ-ray and Radiomimetic Agent Exposure With Confocal RICS RICS was applied to the quantification of DSB repair kinetics of DNA-PKcs, in its wild type and repair-deficient 7A forms. We showed that it is indeed possible to quantify DNA repair kinetics after sparse DNA repair damage has occurred, which is what happens after radiation therapy or chemotherapy. Extensive studies for repair kinetics were also done for another key DNA repair protein, Ku70/80, which is the first to recognize and bind to a DSB and on which DNA-PKcs then binds onto. The kinetics of the two repair proteins after irradiation and chemical damage were compared and contrasted. A manuscript is under preparation for this work.

Aim 2. Improving Protein Kinetics Quantification Using Photoactivatable GFP (PA-GFP) With RICS Quantification of fluorescently tagged protein dynamics by RICS is most accurate at low concentrations, but control of GFP expression levels inside cells is very difficult with standard transfection methods. We have used photoactivatable (PA)-GFP to reliably control fluorescently tagged protein concentration independent of protein expression levels. We have explored the limits of photoactivation versus photobleaching in cells with different expression levels to arrive at the optimal laser energy density for photoactivation at 405 nm. We have subsequently established a step-wise photoactivation procedure that produces very similar fluorescent protein concentration in every cell studied, which we have found to reduce significantly the variance in the RICS deduced protein diffusion coefficient in cells.

Aim 3. Improving Protein Kinetics Quantification by Use of Two-Photon Coherent Control With RICS Quantification accuracy of fluorescently tagged protein dynamics by RICS can be maximized if one has very high photon count rate per molecule at low concentrations. To that end, we have used pulse compression and phase control as a means to maximize the two-photon cross-section of GFP and PA-GFP. More specifically we have purchased non-linear fibers generating supercontinuum pulses, which we then compress with a multiphoton intra-pulse interference phase scan (MIIPS) device that was purchased with funds from this grant. By using coherent control of pulses generated by supercontinuum fiber we have increased fluorescent intensity for GFP by a factor of 3 at constant excitation power. We are now using a different non-linear fiber that we have found to generate higher coherence pulses and are finding that we can increase fluorescent intensity for GFP by a factor

of 10 at constant excitation power. The significance of the RICS techniques developed in this work is that they will enable researchers to study the kinetics of a wide range of DNA damage sensing, signaling, and repair proteins in the nucleus under conditions mimicking radiation therapy and/or chemotherapy. This is not possible to achieve using existing methods.

Genome-Scale DNA Methylation Profiling in the Developing Colon and the Impact of Diet

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Background: Methylated CpG island Amplification (MCA) is a sensitive and robust method for generating a reduced representation of DNA methylome, and provides good coverage of genes and gene promoters [1]. Coupling MCA with next-generation sequencing (MCA-Seq) provides a high-resolution tool for genome-wide DNA methylation profiling. In a mouse model, we performed MCA-Seq to test whether during critical ontogenic periods, nutrition influences developmental epigenetics in the colon, causing persistent alterations in epigenetic regulation that affect susceptibility to colon cancer. We focus on two likely critical periods for colon epigenetic development: embryonic day 15.5 (E15.5)-postnatal day 0 (P0) and P0-P21. To test for nutritional influences on developmental epigenetics, we used an established model of maternal pro-methylation dietary supplementation [2].

Method: We used the Illumina Hi-Seq 2000 sequencer, and incorporated sample-specific barcodes into sequencing libraries to assay up to 12 samples in a single lane. To identify developmental changes in DNA methylation in mouse colon, two independent sex-matched MCA-Seq comparisons were performed at E15.5 and P0 C57BL/6J mice. To determine if gene regions undergoing developmental changes in DNA methylation during fetal or postnatal development are affected by maternal supplementation, virgin female mice, age 8 weeks, were provided either control (NIH-31) or pro-methylation diet (NIH-31 supplemented with folic acid, vitamin B12, betaine, and choline) for 2 weeks before mating and during pregnancy and lactation. In the offspring, two independent sex-matched supplemented vs. control MCA-Seq comparisons were performed at weaning (P21) and adulthood (P100). We used quantitative bisulfite pyrosequencing to measure DNA methylation at a subset of identified genomic regions in the entire set of samples collected (N=10 per sex and age). In addition, we compared developmental methylation changes separately for colonic epithelial stem cells, differentiated epithelial and stromal cells.

Results: We identified 7568 genomic regions undergoing significant developmental changes in DNA methylation (FDR adjusted *P*-value <0.05 by Benjamini-Hochberg). We validated methylation differences at a subset of intervals including several Wnt target genes. Further results regarding the consequences of maternal supplementation will be presented.

Conclusion: Our data confirm that MCA-Seq provides a powerful and sensitive approach to detect methylation changes on a genome-wide scale. Our preliminary data indicate that important epigenetic changes are under way during late fetal colon development, and support a potential link between developmental epigenetics and later risk of colon cancer.

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Detection of Low-Prevalence Mutations in Solid Tumors via Ultra-Deep Targeted Sequencing

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Cells within a tumor sample are known to be heterogeneous, due to the contamination from nonmalignant tissue or the presence of multiple subclones, each carrying different somatic mutations. Currently available diagnostic assays that are able to detect these rare somatic mutations are not comprehensive, limited to a few mutations only. We propose to leverage two innovative technologies, microdroplet PCR and high-throughput sequencing, and an original and robust analysis method, to implement a clinically compatible assay (UDT-Seq) able to screen ≥100kb of DNA, to detect mutations present in 1% of a tumor sample. UDT-Seq can therefore work on poor cellularity samples commonly encountered in the clinic and help address clonal heterogeneity, which is thought to drive cancer progression and drug resistance. We designed a library of 676 PCR primer pairs targeting 42 cancer genes representing 71.1kb of cancer mutational hotspots, as defined by the most commonly reported mutations in the COSMIC database. In addition, these primers target 29 kb of polymorphic amplicons, carrying known SNPs in a blend of 4 Coriell DNA samples used for calibration. We developed a streamlined protocol easy to implement in a clinical setting that seamlessly combines highly uniform microdroplet PCR amplification with deep sequencing. We first verified the technical validity of the approach. We used the calibration samples to experimentally measure the error rate at each position and rank the candidate mutations by statistical significance. A machine learning approach trained on ~200 calibration SNPs at known prevalence complements empirical filters and reliably identifies 89% of the expected variants at prevalence ≥1% with a specificity of 99.99% and less than 2.4% of false positives. The observed prevalence is highly correlated with the expected one (r=0.97) and this performance is maintained even at coverage of 3000x on the Illumina GAII. We observed that whole genome amplification introduces an allelic bias for the mutations present at 5% or less, which resulted in reduced sensitivity at lower coverage. Finally, we were able to perform early validation of the approach on fast sequencer Illumina MiSeq that shows a lower error rate and as a consequence reduces by six fold systematic noise [see reference]. With the intent of a clinical implementation, we analyzed 6 frozen cancer samples from 4 different cancer patients. All tumor samples as well as matched blood DNA were used as a template for microdroplet PCR. We were able to detect only one mutation shared by the breast cancer primary and matched xenograft samples (HRAS-G12V), one mutation in the ovarian xenograft (TP53-R248Q) and 13 mutations in the colon primary and xenograft samples, with the lowest present at 10% of the primary tumor DNA. Interestingly the change of mutation prevalence indicates a purifying selection in the xenograft. We validated 100% of the mutations investigated using an independent assay. We have instigated the proper human subject and logistical framework to carry out such clinical sequencing at our institution (IRB approved protocol). Our current work expands the number of clinical sample to 40 breast cancer patients. We are developing cross-validation of the low prevalence mutations using digital PCR and improving the usability of our analysis pipeline as a stand-alone tool and to make it broadly accessible. We believe UDT-Seq provides significant improvements over previous reports of similar approaches either by increased breadth or more meticulous evaluation. Featuring several innovative ideas, the assay and analysis we present is now very well suited for clinical applications including screening patients for molecularly targeted clinical trials, identifying and targeting tumor subclones that are resistant to standard treatment and enabling improved clinical care for uncommon molecular findings targeted by therapies approved for alternative indications.

Reference

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Tissue Is Alive: Preserving Biomolecules and Tissue Morphology in Clinical Trial Samples

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To facilitate clinical trial molecular profiling, where immediate snap-freezing of tumor biopsies is not feasible, we have created a novel, one-step, room temperature preservative that stabilizes proteins/phosphoproteins/nucleic acids equivalent to snap-freezing and tissue/cell morphology equivalent to neutral buffered formalin fixation. Sources of pre-analytical variability in clinical specimens include tissue (cellular) heterogeneity and biomolecule stability. Laser capture microdissection has solved the issue of cellular heterogeneity. Our molecular fixatives Biomarker and Histology Preservative (BHP-Cell and BHP-Tissue) solve the problem of biomolecular instability in blood and tissue in one step. Technologies now exist that can measure protein phosphorylations and map cell signaling pathways in a single core-needle biopsy, thus relying on the inhibition of any kinase/phosphatase activity within the sample immediately following excision. Unfortunately applying these technologies to clinical samples has been hindered because phosphoprotein epitopes are not adequately preserved by formalin fixation and paraffin embedding, while freezing tissue samples may not be feasible in multi-center clinical trial sites and cannot adequately preserve morphology. Our preservative solution simultaneously fixes and decalcifies bony tissue, thus permitting molecular profiling of bony tissues that was never before possible. We are expanding our international clinical validation of this innovative technology for preserving tissue biomarkers (proteins/nucleic acids) in bone and brain tissue, as well as immuno-LCM applications for cell-type specific nucleic acids. We have collected twenty-two types of human tissues, representing 54 specimens to validate biomolecule and histomorphology preservation. Thirty-one different murine tissue types have also been evaluated, as well as whole mouse embryos at 13.5, 14.5 and 18 days gestation. Six international pathology practices have favorably and independently evaluated the fixative in 126 samples: Bone Cancer (50), Lung Cancer (19), Ovarian Cancer (11), Breast Cancer (10), Renal Cancer (10), Colon Cancer (4), and Other (22). Preservation of tissue morphology has been demonstrated with 56 antigens using standard IHC protocols. Samples of BHP have been distributed to 20 different institutions through either research collaborations (MTA) or formal requests for evaluation-size samples of our Biomarker and Histology Preservative. RNA preservation in laser microdissected brain blood vessels has been verified using an innovative protocol for directly amplifying RNA without prior RNA extraction. We have utilized BHP-Cell in a breast cancer multi-site clinical trial (US Oncology 05-074/GSK LPT109096) and a clinical research multiple myeloma trial. BHP has been validated to (a) function as a transport medium while preserving histomorphology, (b) maintain full antigenicity for clinical immunohistochemistry (such as Ki-67, ER, PR, Her2, p63, and phosphorylated epitopes), (c) preserve phosphoprotein epitopes for cell signaling pathway profiling by reverse phase protein microarray (RPMA), (d) be compatible with frozen sections or paraffin embedding, and (e) obviate the need for additional decalcification. RPMA data from LPT10906 shows differentially deranged signaling networks in the pre-treatment biopsies for patients that did not have a pathologic complete response compared to responders. We are currently exploring the capabilities of BHP Cell and BHP Tissue to preserve the integrity of nucleic acids, in parallel with proteins, thereby providing a universal one step biospecimen tissue fixative for clinical trials.

Sentinel RNAs as a Measure of mRNA Integrity in Clinical Biospecimens

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Diagnostic or predictive panels of gene expression biomarkers are increasingly being measured in cancer, inflammatory or premalignant disorders to guide therapy of individual patients. The integrity of mRNA is a critical factor in the accuracy of such clinical gene expression biomarker tests. The primary measure of mRNA integrity in biospecimens currently used is the integrity of rRNA, a surrogate marker for mRNA integrity. The purpose of our study was to develop an approach for identifying *sentinel RNAs* that improves measures of the intactness of mRNA in biospecimens to determine if reliable gene expression biomarker testing can be performed on a biospecimen. mRNA decay generally proceeds from the 3' end and is likely to be underestimated by analyzing polyA+ selected mRNA. To overcome this obstacle we used an efficient 5' cap dependent purification of Pol II RNA (PNAS PMID 12777618). 5' capped RNA isolated from human liver specimens thawed at room temperature for 0, 5, 10, and 15 minutes was analyzed by RNA-sequencing (RNA-seq, see Viruses PMID 22590687). Total RNA from these samples had RIN (bioanalyzer) values of 9.5, 8.9, 7.9, and 6.7, respectively. Surprisingly, RNA-seq analysis showed that only half of protein coding mRNAs at 10 and 80% at 5 minutes were ≥70% intact. Mining the RNA-seq datasets for 3'/5' end sequence ratios (number of sequence reads in the last and first 200 nt) of each mRNA at each time point identified a total of 304 mRNAs with rapid decay of their 3’ ends relative to their 5’ ends. These mRNAs represent candidate sentinel RNAs. qPCR analysis of the 3' and 5' ends of candidate sentinel RNAs was done to develop a rapid assay for their integrity. We identified several sentinel RNAs, for the conditions cited, that reflect general mRNA decay that could be measured by 3'/5' qPCR ratios. We are conducting similar studies of normal colon and colon cancer biopsies to identify sentinel RNAs for this tumor and tissue type and to determine there may be common sentinel RNAs between liver and colon. We conclude that RNA-seq analysis of differentially selected RNA in clinical biospecimens can identify *sentinel RNAs* that provide improved measures of general mRNA integrity prior to clinical gene expression testing.

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Development and Application of Novel Glycan-Specific Reagents to Facilitate Early Detection of Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is currently the most lethal of gynecologic cancers.[1] Today, nearly 80% of women are diagnosed at advanced stages of EOC, and of these 80%, there is only a 20% survival rate (after 5 years).[1] In stark contrast, of the ~20% of women diagnosed with early stages of EOC, 90% of women survive longer than 5 years.[1] Thus, finding a biomarker (or panel of biomarkers) that can detect early-stage EOC cheaply and non-invasively has the potential to significantly increase EOC survival rates. The central hypothesis of this study is that N-linked glycosylation is significantly altered in diseased patients in comparison to their healthy counterparts, and this is supported by numerous studies showing the correlation between aberrant glycosylation and diseased tissue. However, the chemical tools currently available in the glycomics arena are insufficient in quantitatively profiling such biomolecules. This study aims to (1) address a key and critical step to ultimately become the mainstream strategy for the analysis and relative quantification of glycans in plasma and (2) apply these methods to the assay of N-linked glycans in EOC biomarker discovery. We have currently developed a novel method for the relative quantification of plasma N-linked glycans via stable-isotope labeled hydrazide reagents. A pair of reagents has been synthesized such that two glycan samples can be labeled with either the 'light' or 'heavy' reagent, mixed together, and relatively quantified in the same mass spectrum. Furthermore, these reagents have two additional advantages: a large amount of hydrophobic surface area has been synthesized on the terminal end of the reagents allowing us to take advantage of 1) the hydrophobic electrospray bias and detect larger ion abundances for derivatized vs. native N-linked glycans in ESI,[2-4] and 2) the increased interaction with a reverse phase stationary phase allow N-linked glycans to be characterized by reverse phase LC. We have shown that these reagents are capable of increasing the ion abundance of N-linked glycans in ESI MS by > 10–fold and increase the amount of glycans indentified by ~10%.[3] Additionally, the separation of N-linked glycans by reverse phase chromatography has allowed for improved separation efficiency over HILIC and a further increase in the number of glycans detected (by nearly 50%). This is an important advance because of the ubiquity of reverse phase LC-MS used in the bioanalytical mass spectrometry community. In other words, this labeling strategy fits into existing LC-MS workflows that dominate biological mass spectrometry. A final point is that although we have not yet demonstrated this tagging approach to biological samples other than plasma, there is no foreseeable technical barriers that will prohibit its use for tissues, cell lines, and biologics (biological therapeutics) further broadening the impact of this technology. Studies are currently underway to determine the bioinformatic strategies necessary to extract the glycan data and relatively quantify glycans using the SIL reagents. Additionally, we are utilizing one of the newest cutting edge technologies, a Q-Exactive Orbitrap mass spectrometer, to further enhance the glycan profiling and extend the detection limits of the glycan analytes (beneficial to EOC biomarker discovery efforts). By coupling the N-linked glycan tagging technology with advanced mass spectrometry instrumentation, we have been able to detect numerous undocumented N-linked glycans. Finally, the utility and innovation of the relative quantification strategy has been recognized by industry, and we are currently in active discussions with Pierce (part of Thermo Fisher Scientific) to manufacture and market this strategy. This is an exciting, new collaboration that is capable of realizing one of the studies main goals: developing a mainstream strategy for relatively quantifying glycans that can be implemented in nearly any laboratory on a wide range of biological substrates. The future of this study will involve the N-linked glycan relative quantification strategy being applied to a novel model system for spontaneous EOC, the domestic chicken. The chicken model of spontaneous EOC affords several advantages: (1) chickens develop EOC spontaneously between 2-4 years of age at a prevalence that can exceed 35%,[5] (2) chickens are raised in a controlled environment, (3) sample procurement is controlled throughout the study, (4) the chickens are from a single strain reducing biological variability of the model, (5) the ability to procure longitudinal plasma samples and tissue-matched samples after necropsy, and (6) the similar histopathogenesis to humans. The glycan relative quantification strategy combined with the longitudinal sampling of spontaneous EOC will allow us to quantitatively detect the up- and down-regulation of N-linked glycans as a function of the onset of EOC. These changes will allow us to identify possible glycan biomarkers (or a panel of biomarkers) that will be indicative of the onset of EOC.

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Targeted Selection, Sequencing, and Analysis of Human Telomere and Subtelomere DNA in Cancer

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Telomeric DNA abnormalities are a critical and universal aspect of carcinogenesis. It is impossible to measure accurately the global frequency of sporadic telomere deletion events and telomere fusions with current technology. We are developing a method that 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 ultrashort (TTAGGG)n tract profiles, telomere fusions, and subterminal DNA breakage-rejoining events. Quantitative, single-allele resolution measurements of telomere length and instability would permit important 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. We report on the results of experiments applying this targeted selection and sequencing strategy to DNA from one lymphoblastoid and three cancer cell lines (HT1080, UMUC3, and COLO-829). DNA from each sample was first telomere-tagged with an oligo linker, and fragmented to 600 bp using a Covaris DNA fragmentation device. Following ligation of sequence-tagged Illumina linkers to the fragmented DNA, the telomeric and subterminal fragments from these genomes were selected using a full panel of subterminal as well as the telomeric biotinylated long RNAs (custom-designed by us and synthesized by Agilent), then subjected to paired-end sequencing. Analysis is ongoing: the preliminary results indicate that 58-64 % of all reads are subtelomere-derived, and that a small but measurable fraction of telomeres from the cancer cell lines are very short (less than 600 bp). Sequencing coverage of subtelomere regions corresponding to the oligonucleotide “baits” used for targeted sequence selection ranges from 250x to 750x for the cell lines, with 94-97% of these subtelomere regions covered by at least two independent reads. We developed and are incorporating several novel read-mapping algorithms into our paired-end analysis of these data, with the goals of characterizing the telomere tracts and identifying and quantitating critically short telomeres as well as telomeric and subtelomeric fusions, deletions and translocations in these datasets.

Application of Next-Generation Sequencing to Cancer Epigenomics

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Summary of Progress to Date

Aim 1: We have developed a solution hybrid selection approach to the enrichment of CpG islands (CGIs) and promoter sequences from the human genome for targeted high-throughput bisulfite sequencing [1]. This novel approach allows accurate and quantitative analysis of approximately 1 million CpGs in more than 21,408 CGIs and 15,946 transcriptional regulatory regions. 77-84% of CpGs analyzed fell on or near capture probe sequences; 69-75% fell within CGIs. More than 85% of capture probes successfully yielded quantitative DNA methylation information of targeted regions. Differentially methylated regions (DMRs) were identified in the 5'-end regulatory regions, as well as the intra- and intergenic regions, particularly in the X chromosome among the three breast cancer cell lines analyzed. We chose 46 candidate loci (762 CpGs) for confirmation with PCR-based bisulfite sequencing and demonstrated excellent correlation between two data sets. Targeted bisulfite sequencing of three DNA methyltransferase (DNMT) knockout cell lines and the parental HCT116 colon cancer cell line revealed a significant decrease in CpG methylation for the DNMT1 knockout and DNMT1, 3B double knockout cell lines, but not in DNMT3B knockout cell line. We demonstrated the targeted bisulfite sequencing approach to be a powerful method to uncover novel aberrant methylation in the cancer epigenome. 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.

Aim 2-3: We have applied the next-generation sequencing to generate genome-wide DNA methylation map in chronic lymphocytic leukemia (CLL) [2]. CLL is the most common adult leukemia in the United States. The clinical course of CLL patients is highly variable. Patients with unmutated immunoglobulin heavy-chain variable (IGHV) gene show a shorter progression-free and overall survival than patients with mutated IGHV gene. We analyzed genome-wide DNA methylation patterns in CD19+ B-cells isolated from more than 40 CLL patients. While global CpG methylation was similar between CLL samples with mutated and unmutated IGHV gene, 849 differentially methylated regions (DMRs) were identified between the IGHV mutated and unmutated CLL subgroups (FDR<0.05, average methylation difference >0.25). Of those, ~65 percent of the DMRs were hypermethylated in the unmutated IGHV subset. The majority of the DMRs were located in the intron or intergenic regions of the genome, and over 60% of them overlapped with enhancer and DNase I hypersensitive sites identified in the ENCODE B-cell line GM12878. RNA-seq analysis performed using the same CLL samples demonstrated an inverse correlation between gene expression and DNA methylation for a subset of genes. 47 genes were identified as being differentially methylated and at the same time being differentially expressed between IGHV mutated and IGHV unmutated CLL patients, including ZAP70, CYR1, LDOC1, TCF7, and SEPT10 which were previously reported to be differentially expressed in CLL. These results further support the role of DNA methylation in defining the molecular subgroups of CLL. This comprehensive DNA methylation analysis will further our understanding of the epigenetic contribution to cellular dysfunction and pathogenesis in CLL.

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384-Well Cell Migration Assay Suitable for High-Throughput Screening (HTS) of Chemical Libraries for 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. None of the current products offered in industry, e.g., 96-well transmembrane, scratch wound assays, and Oris™ stopper-based assays meet these requirements. Platypus Technologies developed the Oris™ Pro 384 Cell Migration Assay that is completely compatible with automated liquid handling systems and high content analysis (HCA) instrumentation, to enable HTS of anti-cancer compounds and wound healing agents on adherent tumor and endothelial cell lines. This assay format utilizes a centrally located, self-dissolving, non-toxic biocompatible gel (BCG) to form a uniformly sized, cell-free detection zone on tissue culture treated or collagen I coated cell culture surfaces. Cells are seeded into 384-well plates and patterned in an annular monolayer surrounding the BCG. Once the BCG dissolves, cells can migrate into the detection zone previously occupied by the BCG. Following the successful development work completed in year 1, Platypus has launched (2) 96-well and (2) 384-well Oris™ Pro Cell Migration Assay products. In year 2 we developed image and data acquisition schemes that fully exploit the unique feature of the Oris platform to permit key microscopy observations on cell morphology and toxicity of the cells as they migrate. Using a panel of six agents with known mechanisms of action and predicted cellular outcomes, we documented the assay's ability to distinguish antimigratory activity from necrotic and programmed cell death. We also initiated HTS development and validation studies according to HTS guidelines in place at the University of Pittsburgh Drug Discovery Institute (UPDDI). The assay was optimized for cell seeding density, plating volume, and length of migration time. The assay was implemented on automated liquid handling equipment, establishing a workflow suited for HTS. Using a full 384 well microplate of cells fixed pre-migration and at the end of the migration period, the assay gave Z-factors > 0.5, Signal to Background ratio (S/B) > 14, and coefficients of variance (CV) of maximum control <10%. The assay performed equally well under manual and automation conditions, and was not sensitive to cell passage effects in culture. These results indicate that the Oris™ Pro 384 Cell Migration Assay can achieve HTS performance with laboratory automation. Based on these data, the assay is ready to progress into HTS validation studies encompassing multi-day variability studies and screening of the LOPAC 1280 test cassette of compounds with known mechanisms of action.

Magnetorotation: A Rapid Assay for Single-Cell Drug Sensitivity of Cancer Cells

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Heterogeneity inside a supposedly homogeneous cell population is one of the reasons behind drug resistance associated with cancer. This called for the emergence of single cell assays to study specific subpopulations of cancer cells. Circulating cancer cells (CTCs), responsible for metastasis, do not escape the rule. However, in order to develop a single cell assay for CTCs, they need to be kept in suspension, otherwise phenotypic changes might occur, and this alters the relevance of the assay. To answer this problem, we developed the magnetorotation method for quantitative real time monitoring of cell size and morphology, on single live suspended cancer cells, unconfined in three dimensions. The *Cell Magnetorotation* (CM) method is made possible by *nanoparticle induced cell magnetization*. By using a rotating magnetic field, the magnetically labeled cell is actively rotated, and the rotational period is measured in real-time. Any morphology change induces a change in the rotational pattern of the suspended cell (e.g., when the cell gets bigger it rotates slower). So far, we have demonstrated the proof of concept of the method on cancer cells, which led to a publication (Elbez et al., PlosONE Dec. 2011). We showed that the presence of magnetic nanoparticles inside the cells (at the order of a few picograms per cells) and the rotation under a magnetic field were not detrimental to cell viability, neither to cell division. We have demonstrated the ability to use cells as rotating magnetic microplatforms, through the uptake of functionalized magnetic nanoparticles, and the ability to control and measure their rotation under near real-time conditions. Also, and most importantly, we proved on single cells that we could discriminate between cell death, cell swelling and a live cell, by monitoring in real time the trend of evolution of their rotation period. We also compared the effect of a toxic environment under different conditions (5% ethanol and 100µg/ml of Cisplatin). The efficiency of different drugs can thus be qualitatively compared in terms of death time. Following these results, we went on multiplexing the assay. To do so, we use a microfluidic chip in order to trap the cell, which is achieved thanks to the laminar flow of the cells above the traps. The efficiency of the trapping allows us to follow between 100 and 150 cells at the same time while they are rotating. This has led us to the ability to realize statistical studies on single cell death, such as death time distribution curves. Some cell adherence issues need however to be solved. Considering potential applications, it often takes weeks or months, and multiple bone marrow biopsies, to determine whether a patient is having a meaningful response to a kinase inhibitor. Thus, a key unmet medical need remains the ability to make an early assessment as to whether the patient is receiving the right drug and dose to achieve an optimal response. Magnetorotation is likely to meet such needs. We note that Magnetorotation provides more information on cell morphology than buoyancy, and can measure changes below the resolution limit of confocal microscopy, with a much simpler and less expensive set-up.

Hyperspectral and Structural Microscopy Platform for Therapy of Resistant Cancer

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The central goal of our research program is to create a platform for the rapid screening and identification of optimal therapeutics for cancer patients. Cancers are not only heterogeneous between tumor types and individuals, but also even within a single patient, significantly complicating therapy. Personalized therapeutic regimens are needed to optimally treat patients, but finding the right combination of therapies remains difficult, with trial-and-error approaches predominantly used in the clinic. Current ex vivo screening techniques show promise, but most rely on simple adherent monolayer culture systems that do not adequately replicate the heterogeneity and biological complexity of tumors in vivo. We are developing an approach whereby patient-derived samples are cultured in 3D in vitro models designed for compatibility with an optical imaging platform enabling high-content, high-throughput therapeutic screening to determine optimal personalized therapeutic regimens. In the first year of this project, we have made considerable progress in creating the imaging systems and screening methodology required for robust, rapid, high-content and high-throughput therapeutic optimization in vitro. In our initial studies, we used a 3D culture model of metastatic ovarian cancer that rapidly grows into a heterogeneous mixture of biologically relevant tumor nodules, complete with acidic and hypoxic tumor cores. We developed an optical imaging-based, tiered screening method, and tested this new approach for the identification of optimized hypoxia-selective photodynamic therapy agents. Our results demonstrate that tiered screening platforms based on 3D in vitro models can not only be used to select optimal therapeutics, but are also advantageous for creating treatment regimens targeting select cellular subpopulations. While this first study helped build our screening methodology, more rapid imaging systems are needed to screen the many thousands of nodules targeted in our grant milestones. To achieve this screening rate, we created a modular, custom-built video-rate confocal imaging system optimized for deep, near-IR imaging in our in vitro 3D tumor cultures. Based on a resonant galvanometric mirror, this microscope platform is highly customizable and can be readily combined with other imaging modalities such as two-photon microscopy and optical coherence tomography (OCT). One key limitation in visualizing numerous molecular factors in complex biological systems is the spectral overlap between the myriad emission sources in a given sample. While careful selection of fluorophores can partially alleviate this issue, autofluorescence emission can present a significant barrier to the interpretation and quantitative analysis of samples. To overcome these difficulties, we have additionally developed a video-rate capable hyperspectral detector. A programmable spectrometer disperses the emission light onto a custom-built 16 channel, multianode PMT for rapid, parallel spectral acquisition. A major remaining problem in understanding therapeutic response in these large nodules (300 μm to 1 mm in diameter) is that many optical technologies suffer from depth-dependent penetration problems. As the goal of our work is to overcome the impact of microenvironmental heterogeneity on therapeutic response, we have worked to develop new OCT-based approaches for continuous longitudinal tracking of treatment response over the course of days and weeks. We have found that, with OCT, therapeutic structural decomposition appears as two major changes: (1) the structural decomposition of nodular archetecture into recognizable sub-structures and (2) increased scattering at cellular length scales due to apoptosis in the in vitro system. To take advantage of these structural alterations, we have developed a morphometic analysis routine that reports cellular(-level) therapeutic response throughout the entirety of tumor nodules. Our results from this first year are most promising and indicate that our work may have a significant impact in current therapeutic screening methodologies, especially those investigating the use of agents targeting otherwise treatment-resistant cellular populations. The platforms we are developing can be readily adapted to accommodate a wide variety of patient tumor types for the development and optimization of personalized therapeutic compounds and combinatorial treatment regimens.

Ultra-Throughput Multiple Reaction Monitoring Mass Spectrometry for Large-Scale Cancer Biomarker Validation

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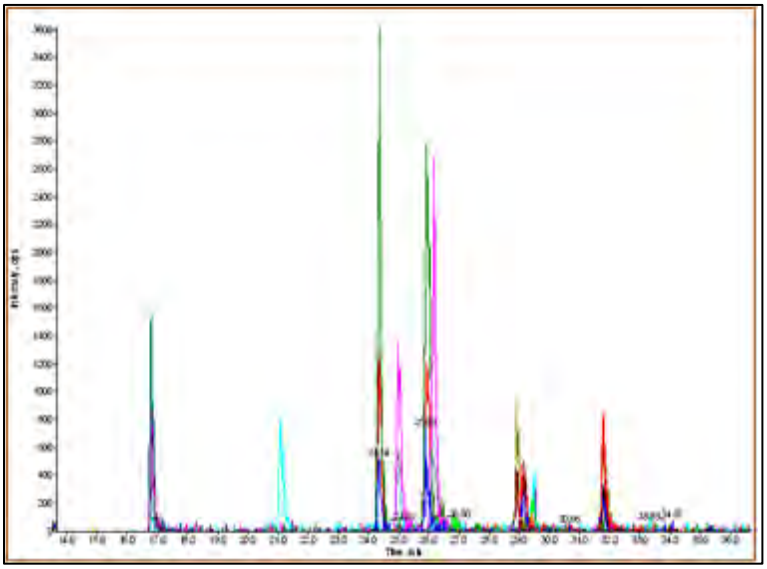
This project develops a technology to significantly improve the sample throughput for mass spectrometry-based quantitation of cancer protein biomarkers, i.e., the number of patient samples that can be quantified by mass spectrometry in a unit time. In the first year of the project we worked on Specific Aim 1 to identify suitable peptide derivatizations for LC-MRM MS measurements. We have explored a variety of strategies for synthesizing chemicals to derivatize signature peptides of protein markers. There are two major classes of approaches that are investigated. One is to synthesize magnetic nanoparticle reagents to facilitate the peptide derivatization and associated sample cleanup. Although this study produced desired derivatized peptides with free N-terminal amines, the product yields were low at this stage of the study. Our current efforts focus on the originally proposed approach, which develops a large collection of peptidyl reagents for derivatizing peptides via conventional solution reactions. We have, via solid-phase synthesis, generated a pool of short peptides, which are 2-4 amino acids in length and have hydrophobic amino acid residues in composition. This pool of peptidyl reagents have been further diversified via the addition of more than 10 different capping groups at the N-termini of the peptidyl reagents. We currently have made more than 200 different reagents which are being screened for the suitability assignature peptide derivatizing reagents. More importantly, we have established standard synthesis routes, which allow us to generate much larger numbers of reagents fast. These reagents will be used, together with the existing ones, for screening and selecting the needed derivatization reagents to implement the Specific Aim 2 in the second year of the project. The selection of peptidyl reagents is done by LC-TOF-MS or LC-MRM MS. In the figure are chromatograms for underivatized and derivatized peptides with the same amino acid sequence and at the equal molar ratio. The chromatograms are plotted for the peptide signal intensities against the elution times. The first group of peaks on the left is for an underivatized peptide and the others are for the derivatized ones. When a mass spectrometric peak is stronger than the peak for the underivatized counterpart peptide on the left, the corresponding derivatizing reagent becomes a candidate. In the set of reagents used for derivatizing the peptides in this figure, several have the increased signals, thus qualified, and several have the decreased signals, thus failed. Multiple reagents can be screened in a single analysis, and we have performed a one experiment screening of up to 24 peptidyl reagents. We have made key progress towards the successful implementation of the project: (1) finalized the selection of signature peptides for the model marker protein; (2) established a fast, general route for synthesizing derivatizing reagents; (3) optimized the procedure and conditions for the peptide derivatization; and (4) developed methods for quantitative comparison of the derivatization efficiency and the mass spectrometry signal yield.



Scaffolds for Delivering Deoxycytidine Kinase to HER2 Positive Cancer Cells

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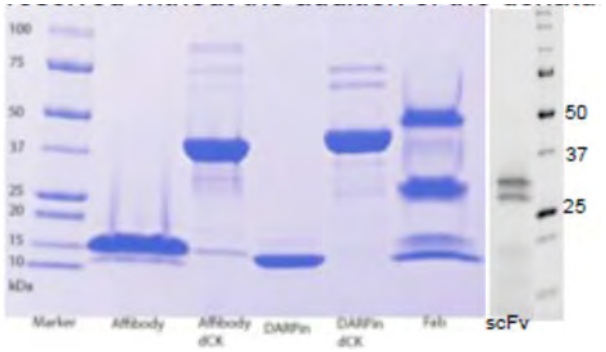

Figure. Scaffold proteins expressed and purified from bacteria and yeast.

A major challenge in cancer therapy is the targeting of the neoplastic cells while sparing healthy tissue. Antibodies are one strategy used to select for cancer cells, and in fact, the FDA approved monoclonal trastuzumab (Herceptin) does just that, by binding to HER2 (epidermal growth factor receptor 2), a marker of some breast and ovarian cancers. However, such naked antibodies are not efficient at cancer cell killing. To increase the cytotoxicity of this approach, one needs to conjugate the antibody (the targeting moiety) with a cell-killing moiety. In fact, very recently a monoclonal-toxin conjugate (brentuximab vedotin) has been approved [1]. Our approach is to conjugate the prodrug-activating enzyme deoxycytidine kinase (dCK) to allow the cancer-cell specific activation of nucleoside analog prodrugs [2]. To develop a model system for testing our novel approach, we are targeting breast and ovarian cancer cells that express HER2 on their cell surface. We propose to test four different scaffolds that bind HER2, with and without fusion to the enzyme, dCK, for their ability to deliver a 'knock-out punch' to the cancer cells. As a first step towards this goal, we cloned the coding regions for human singlechain Fragments of variable regions (scFv), fragment of antigen binding (Fab), designed ankyrin repeat proteins (DARPins), and affibodies that bind to the human HER2 cell surface protein.The anti-HER2 scFv was cloned into the pPICZαC' expression vector for expression of soluble protein in the yeast, *Pichia pastoris*. The DARPin, affibody and their dCK fusion variants were cloned into the pET14b vector for expression as a SUMO fusion protein in C41 strain of *Escherichia coli*. Finally, the Fab scaffold was cloned into the phagemid pAPIII6ΔpIII vector [3] for expression in BL21Codon+ strain of *E. coli*. Expressed, purified protein was resolved on a SDS-PAGE gel. As the Fab protein contains two disulphide bonds it was resolved without the addition of the denaturant, β-mercaptoethanol; the intact Fab runs at 55 KDa, while the heavy and light chains resolve as ~28 KDa species. Epitopes of the ectodomain of HER2 protein [4] will be binned for the various recombinant affinity reagents using the BIND® Reader [5]. Binding of scaffold proteins to HER2+ human breast cancer cell line, SKBR-3, will be assessed by immunofluorescence and flow cytometry. Once confirmed, we will evaluate the efficiency of the reagents to direct receptor-scaffold-enzyme internalization and cell death.

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1. 2D-PCR for Spatially Mapping Gene Changes in Tumor Sections

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Our research is focused on developing a novel technology to spatially map genetic changes in human tissue sections with high spatial resolution. Our previously published results have mapped DNA to a 1 mm resolution. Our technology aims to combine the benefits of PCR (amplification), laser capture micro dissection (high spatial resolution), and FISH (multiplexing). This technology aims to elucidate the genetic changes occurring in tumor microenvironments: tumor cells and the cells surrounding the tumor. We have developed techniques to study modifications in the genome as well as epigenome of histological sections obtained from cancerous tissue and biopsies. Our method of evaluating the methylation status of cancer relevant genes utilizes the methylation restrictive enzymes digestion of DNA. Our innovative protocol is optimized for performing restriction analysis and PCR in the same reaction mix on a tissue lysate while maintaining spatial resolution. Our results show a significant difference in methylation between tumor and non-tumor cells. In addition, we have made significant strides toward miniaturizing our previously published 2D-PCR procedure. Our innovative process involves direct lysis of tumor section followed by DNA capture onto a hydrogel. The DNA is amplified within the hydrogel and the image is resolved to analyze the variation in DNA across the tissue section. We are currently working to adapt the procedure for investigating changes in gene expression.

2. A Flow-Based Selection Procedure for Analysis of Cytopathology for Cervical Cancer

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Cervical cancer is known to be caused by infection with high risk types of human papilloma virus. Protein products of viral oncogenes E6 and E7 respectively reduce active cellular pools of p53 and pRb, promoting progression of cell cycle past checkpoints and, ultimately, preventing complete differentiation of cervical epithelial cells. Using cervical cancer cell lines combined with cytopathology-negative clinical samples as model cell populations, we have demonstrated that immunological staining for p16/MCM5 dual over-expression can be used as gating criteria for cell sorting, enabling capture of diseased cells from mixed populations. Subsequently, we can detect HPV specifically by type within sorted cells, using multiplexed PCR primers. A critical objective, demanded by the integrated concept of this two-part assay, is demonstration that sorting cells according to protein biomarker expression affects the subsequent detection sensitivity of specific HPV genotypes. We demonstrate this dependency with an experimental system in which residual fixed cells, from cytopathology-negative clinical cervical lavage samples, are spiked with decreasing ratios of fixed cervical cancer cells known to contain three different HPV types (16, 18 and 45). Cell sorting is performed by either gating upon two biomarker immunofluorescence signals or by using “ungated” sorting in which the gate is simply the widest possible setting on forward scatter/side scatter. As fixed cervical cancer cells diminish in frequency, “ungated” sorting produces samples in which subsequent HPV genotype detection fails. For the same cell mixtures, gating upon biomarkers results in consistent recovery of a wide range of HPV genotypes, enabling HPV detection even at the very lowest frequency of spiked cancer cells. We further observe high variability for distribution of $p16^{INK4a}$/MCM5 immunofluorescence signal among negative clinical samples, indicating that computational gating may benefit from a more information rich, spectral analysis signal interpretation method.

3. A Strategy for Phage Display Selection of Functional Domain-Exchanged Immunoglobulin Scaffolds With High Affinity for Glycan Targets

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Monoclonal antibodies are essential reagents for deciphering gene or protein function and have been a fruitful source of therapeutic and diagnostic agents. However, the use of anticarbohydrate antibodies to target glycans for these purposes has been less successful. Glycans contain less hydrophobic functionality than do proteins or nucleic acids, thus individual glycan-antibody interactions are relatively weak. Information encoded by glycans often involves subtle variations of branched oligosaccharides that cannot be detected with conventional antibodies. Here we describe a new phage display selection strategy for identification of high affinity and specific glycan antibodies. We designed and characterized a phage clone that functionally displays the unique architectural scaffold of 2G12, an antibody that targets oligomannoses on the HIV-1 glycoprotein gp120. The two heavy chain variable domains of 2G12 exchange positions to create an extended recognition surface containing four oligomannose binding sites per IgG molecule. We designed and characterized a phage clone in which this domain exchange architecture was recapitulated as a antigen binding fragment dimer [$(Fab)_2$] on the phage surface by protein engineering. The functional display of the 2G12 $(Fab)_2$ fragment was validated by Western blot and phage enzyme-linked immunosorbent assay. Furthermore, we demonstrate that this 2G12 $(Fab)_2$ display system is amenable to selection of functional clones using a mock selection. These results provide proof of concept that the privileged 2G12 domain-exchanged scaffold can be used for design of novel antibody libraries that are biased toward glycan recognition. Current efforts are focused on screening libraries based on the 2G12 scaffold to identify which side chains have particular bias for glycan recognition, and how this information can be used to create de novo 2G12-based libraries to target tumor-associated glycans.

4. Activatable BRET Probes for MMP Enzymatic Activity Detection

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Stanford University, Stanford, California

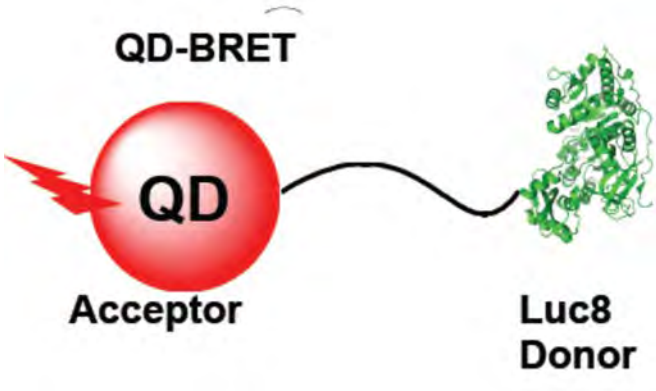

Figure 1. QD-BRET.

Matrix metalloproteases (MMPs) are a family of zinc-dependent secreted endopeptidases that play a crucial role in defining the cellular environment through regulated degradation and processing of extracellular matrices (ECM). They have been extensively investigated in the past several decades due to their important roles played in the cancer cell invasion and metastasis. MMPs are upregulated in almost every type of human cancer, and their over-expression correlates with advanced tumor stage, increased invasion and metastasis,and shortened survival. Commonly used assay for MMP detection is ELISA, which uses antibody to measure the total MMP concentration in the samples. MMPs are produced in a latent pro-enzyme form and require post-translational activation by the cleavage of an N-terminal pro-domain sequence to generate the active form. It is the active forms of these enzymes responsible for their physiological functions. More and more studies are reporting the correlation of the tumor status with the activity of MMPs. We have developed a new nanoplatform based on fluorescent semiconductor nanoparticles quantum dots (QD) — QD-BRET with QDs as the energy acceptor for a lightemitting protein (for example, a bioluminescent protein *Renilla* luciferase) [1]. When QDs are in close proximity to luciferase, the energy released in the oxidation of the substrate by luciferase is transferred to QDs through BRET and enable QD emission (Figure 1). In this work, we applied this QD-BRET nanoplatform to develop sensors for sensitive detection of MMP activity in complex biological samples including serum [2]. This presentation will report our progress on the following grounds: (1) Development of new fluorescent nanoparticles for the BRET assay. The new nanoparticles show great BRET efficiency (the BRET ratio increased from previous 1.29 with QDs to 2.5), high serum stability and do not contain toxic metals, making them attractive replacement of quantum dots in this BRET assay platform.
(2) Development of competitive BRET assay for detecting MMP activity. Our previous sensors produce decreased BRET signal after the protease cleavage. In contrast, the new sensor produce positive signal by the use of a non-emitting quenching molecule to the conjugate.

We acknowledge the support of the IMAT award (1R21CA138353-01A2) for the work presented here.

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5. Advanced Development of an Integrated CTC Enrichment Technology

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The long-term objective of this research proposal is to perform advanced development and analytical validation of a technology for highly efficient enrichment of circulating tumor cells (CTCs). This new CTC-based diagnostic platform will pave the way not only for CTC enumeration to serve as a biomarker for prostate cancer to better predict clinical outcomes, but also as a source of clinical material (i.e., CTCs as a "liquid biopsy") for sequential molecular analyses that can be used to direct appropriate therapies for individual patients. Over the first 10-month grant period, our joint team has been able to make good progress on the following aspects:

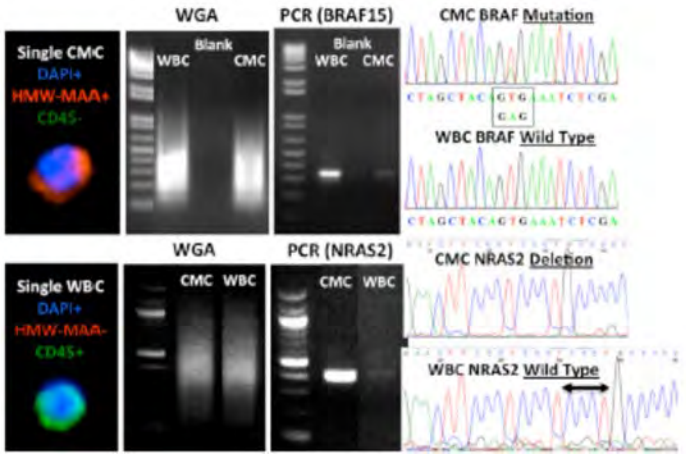

Figure 1. SINGLE CMCs and WBCs were captured and isolated from a melanoma patient's blood sample. After PCR amplification, BRAFV600E mutation and NRAS deletion in intron (37bp 3' of NRAS exon 2; nucleotides CCCT deleted) were detected. Here WBCs were employed as control samples (Wild Type).

A. Our CTC Enrichment Technology. Our team at UCLA has demonstrated a highly efficient, inexpensive CTC assay capable of enriching, identifying and isolating CTCs in whole-blood samples collected from patients with different solid tumors (e.g., prostate, breast, gastric and colon cancer). Initially, our joint team at UCLA pioneered a unique concept of "NanoVelcro" cell-affinity substrates1-3, by which anti-EpCAM-coated SiNWs surfaces were utilized to immobilize CTCs in a stationary device setting. By integrating the NanoVelcro substrate with an overlaid microfluidic component that can generate vertical flows, further improved CTC capture efficiency4,5 (>85%) has been achieved as a result of the enhanced collisions between CTCs and the substrate. A 3-color ICC protocol for parallel staining of DAPI, FITC-labeled anti-CD45 and PE-labeled anti-CK for identifying substrate immobilized CTCs. Single-cell image cytometry data covering CK/CD45 expressions and CTC footprint sizes can be used to identify CTCs (DAPI+/CK+/CD45-, sizes>10 μm) from nonspecifically captured WBCs (DAPI+/CK-/CD45+, sizes<10 μm) and cellular debris. Side-by-side analytical validation studies using both artificial and patient CTC samples suggested that the sensitivity of NanoVelcro CTC Assay outperformed that of CellSearchTM. Our continuous endeavors have been devoted to explore NanoVelcro CTC Chip as a highly sensitive CTC enumeration tool for monitoring therapeutic responses and recurrence in metastatic castration resistance PC (mCRPC) patients who were treated with Abiraterone acetate (i.e., a testosterone synthesis inhibitor). So far, the team has conducted more than 200 CTC tests, detecting CTCs at concentrations ranging from 0 to 112 CTCs/mL blood. Our results suggested that variations in CTC counts (determined by NanoVelcro Assay) and PSA values were employed to monitor therapeutic outcomes of a mCRPC patient, who received two different treatment interventions.

B. Single-CTC Isolation. A new generation of NanoVelcro CTC Chip has been created by replacing the *nontransparent* NanoVelcro substrate (with embedded vertically oriented silicon nanowire array) with a *transparent NanoVelcro substrate* (based on electrospun Poly(Lactic-co-Glycolic Acid) (PLGA) polymer nanofibers on a glass slide). Again, anti-EpCAM can be introduced onto the transparent NanoVelcro substrate for capturing CTCs. The electrospun PLGA nanofibers can be deposited onto a commercial Laser MicroDissection (LMD) slide (a regular microscope slide coated with a 1.2-μm-thick Poly(Phenylene Sulfide), PPS membrane), thus enabling isolation of single CMCs using LMD technique. Using a fluorescent microscope equipped with an LMD module (Leica LMD7000), currently available in CNSI Advanced Microscopy Core, single CTCs (DAPI+/CK+/CD45-, sizes>10 μm) were first identified, and a 355-nm laser beam was then employed to cut the PLGA/polymer substrate underneath the CTCs.

The individual CTCs (along with their LMD-cut polymer substrates) were directly collected in 500-μL Eppendorf tubes for long-term storage (in liquid N2) or subsequent molecular analyses.

Molecular analyses of single CMCs. By using the prototype single-CTC isolation approach, we were able to isolate 12 SINGLE circulating melanoma cells (CMCs without contamination of WBCs) from a metastatic melanoma patient (anti-CD146 is employed as capture agent here), and each CMC was transferred into a 500-μL Eppendorf tube for molecular analyses in sequence. We were able to carry out PCR and exome sequencing on these CMCs to confirm that BRAFv600E mutation and NRAS deletion are present in these SINGLE CMCs (Figure 1). More exciting these mutations have been observed in the biopsy tissue of the same patient. We have been applying this single-CTC sequencing approach to study the drug resistance mechanisms in mCRPC patients who receive AR-targeted therapy.

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6. Application of Next-Generation Sequencing to Cancer Epigenomics

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Summary of Progress to Date

Aim 1: We have developed a solution hybrid selection approach to the enrichment of CpG islands (CGIs) and promoter sequences from the human genome for targeted high-throughput bisulfite sequencing [1]. This novel approach allows accurate and quantitative analysis of approximately 1 million CpGs in more than 21,408 CGIs and 15,946 transcriptional regulatory regions. 77-84% of CpGs analyzed fell on or near capture probe sequences; 69-75% fell within CGIs. More than 85% of capture probes successfully yielded quantitative DNA methylation information of targeted regions. Differentially methylated regions (DMRs) were identified in the 5'-end regulatory regions, as well as the intra- and intergenic regions, particularly in the X chromosome among the three breast cancer cell lines analyzed. We chose 46 candidate loci (762 CpGs) for confirmation with PCR-based bisulfite sequencing and demonstrated excellent correlation between two data sets. Targeted bisulfite sequencing of three DNA methyltransferase (DNMT) knockout cell lines and the parental HCT116 colon cancer cell line revealed a significant decrease in CpG methylation for the DNMT1 knockout and DNMT1, 3B double knockout cell lines, but not in DNMT3B knockout cell line. We demonstrated the targeted bisulfite sequencing approach to be a powerful method to uncover novel aberrant methylation in the cancer epigenome. 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. Aim 2-3: We have applied the next-generation sequencing to generate genome-wide DNA methylation map in Chronic lymphocytic leukemia (CLL) [2]. CLL is the most common adult leukemia in the United States. The clinical course of CLL patients is highly variable. Patients with unmutated immunoglobulin heavy-chain variable (IGHV) gene show a shorter progression-free and overall survival than patients with mutated IGHV gene. We analyzed genome-wide DNA methylation patterns in CD19+ B-cells isolated from more than 40 CLL patients. While global CpG methylation was similar between CLL samples with mutated and unmutated IGHV gene, 849 differentially methylated regions (DMRs) were identified between the IGHV mutated and unmutated CLL subgroups (FDR<0.05, average methylation difference >0.25). Of those, ~65 percent of the DMRs were hypermethylated in the unmutated IGHV subset. The majority of the DMRs were located in the intron or intergenic regions of the genome, and over 60% of them overlapped with enhancer and DNase I hypersensitive sites identified in the ENCODE B-cell line GM12878. RNA-seq analysis performed using the same CLL samples demonstrated an inverse correlation between gene expression and DNA methylation for a subset of genes. 47 genes were identified as being differentially methylated and at the same time being differentially expressed between IGHV mutated and IGHV unmutated CLL patients, including ZAP70, CYR1, LDOC1, TCF7, and SEPT10 which were previously reported to be differentially expressed in CLL. These results further support the role of DNA methylation in defining the molecular subgroups of CLL. This comprehensive DNA methylation analysis will further our understanding of the epigenetic contribution to cellular dysfunction and pathogenesis in CLL.

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7. Application of an Innovative Technology to Develop Low-Toxicity Kinase Inhibitors

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Regulation of all cellular processes requires dynamic regulation of protein phosphorylation. We have developed an unbiased system to globally quantify the phosphorylation index for substrates of a specific kinase by independently quantifying phosphorylated and total substrate molecules in a reverse in-gel kinase assay. Non-phosphorylated substrate molecules are first quantified in the presence and absence of a specific stimulus. Total substrate molecules are then measured after complete chemical de-phosphorylation, and a ratio of phosphorylated to total substrate is derived. To demonstrate the utility of this approach, we profiled and quantified changes in phosphorylation index for Protein Kinase CK2 substrates that respond to a small-molecule inhibitor. A broad range of inhibitor-induced changes in phosphorylation was observed in cultured cells. Differences among substrates in the kinetics of phosphorylation change were also revealed. Comparison of CK2 inhibitor-induced changes in phosphorylation in cultured cells and in mouse peripheral blood lymphocytes in vivo revealed distinct kinetic and depth-of-response profiles. This technology provides a new approach to facilitate functional analyses of kinase-specific phosphorylation events, to facilitate kinase inhibitor target validation studies, and to inform in vivo analyses of kinase inhibitor drug efficacy. We have used this approach to identify high-efficiency substrates of CK2, and have derived an inhibitory peptide based on the major CK2 phosphoacceptor site on Elongation Factor 1ß. The characteristics of this new peptide kinase inhibitor will be discussed.

8. Applying Molecular Phylogeny to Predict Patient Survival

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We propose that tumor diversity, not order of acquired mutations, is a better clinical predictor of cancer patient outcome. Previously we have used DNA methylation patterns to characterize tumor diversity, and applied cancer molecular phylogeny to estimate tumor age. Recently, we have analyzed colon tumor DNA using the Illumina HumanOmniExpress BeadChip. DNA samples are obtained from opposite halves of a single tumor, and tumor copy number alterations compared between halves. Using a new computer simulation we model the heterogeneity in tumor copy number as a function of tumor growth, allowing us to address questions related to cancer cell survival and the relative importance of neutral changes versus selection. Our novel measures of tumor diversity will be assessed as clinical predictors of patient outcome.

9. CITP-Based Selective Tissue Proteome Enrichment

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University of Maryland, College Park, Maryland

The proposed research aims to develop a capillary isotachophoresis (CITP)-based multidimensional separation platform, capable of providing selective analyte enrichment and extremely high resolving power, to achieve comprehensive and comparative tissue proteome analysis including the characterization of low abundance proteins. Furthermore, complicating proteomic analysis of whole tissues is the obvious problem of cell heterogeneity in tissues, which often results in misleading or confusing molecular findings. Thus, the proposed coupling of tissue microdissection for targeting tumor cells (in an effort to reduce proteome complexity and protein concentration dynamics) with CITP selective enrichment of trace proteins not only represents a synergistic strategy toward the detection and characterization of low abundance proteins, but also presents a novel biomarker discovery paradigm in support of cancer research, diagnosis, and treatment. In order to investigate the proposed coupling of the CITP proteomic platform with tissue microdissection technology, a set of fresh frozen brain biopsies were selectively microdissected to provide an enriched, high quality, and reproducible sample of tumor cells. Despite sharing many common proteins, there are significant differences in the protein expression level among different grades of astrocytomas. A large number of proteins, such as plasma membrane proteins EGFR and Erbb2, are up-regulated in glioblastoma. Besides facilitating the prioritization of follow-on biomarker selection and validation, comparative proteomics involving measurements in changes of pathways are expected to reveal the molecular relationships among different pathological grades of gliomas and potential molecular mechanisms that drive gliomagenesis.

10. Deep Clonal Profiling of Formalin Fixed Paraffin Embedded Clinical Samples

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We have applied DNA content based flow cytometry assays to identify and sort nuclei of diploid and aneuploid populations from a variety of archived formalin fixed paraffin embedded (FFPE) tissues. We optimized sample preparation protocols and used the output from a step gram algorithm (ADM2) to measure the reproducibility of our aCGH data in matching FFPE and fresh frozen (FF) samples of pancreatic ductal adenocarcinomas (PDAs). The overlap of two aCGH intervals is defined as the genomic length of their intersection divided by the genomic length of their union. We selected the top 20 ranked amplicons in the FFPE sample. In 19 of these 20 amplicons the overlap was >0.9 with the same ADM2 defined interval in the sorted FF sample. We subsequently interrogated FFPE samples from a variety of tissues, including triple negative breast carcinomas, glioblastomas, melanomas, sarcomas, bladder carcinoma, and small cell carcinoma of the ovary. We validated the use of sorted FFPE samples for whole exome mutational analysis by next generation sequencing (NGS) with matching FF and FFPE samples from a rapid autopsy PDA sample, and a matching cell line with a previously published exome sequence. A comparison of the paired end read alignments against the reference genome showed that almost 80% of the target areas had ≥20x coverage in all three samples. The 34 previously reported non-synonymous mutations in the cell line were compared across the 3 samples. In 12 cases the regions of interest were not targeted by the capture oligonucleotides while the remaining 22 mutations were all detected in each sample preparation. We also developed flow assays combining DAPI with cytokeratin 19, vimentin, or ZEB1 to assess and sort the epithelial and mesenchymal content of each tumor sample. These assays are done while collecting up to 6 simultaneous sorting streams thus optimizing the use of each clinical sample. The total diploid sorted fractions from the matching FF and FFPE PDA tissues were non-aberrant by aCGH analysis. However a low (<5-10%) number of reads for some mutations present in the aneuploid fraction (e.g., *KRAS*) were observed in the NGS data for the total diploid fractions. To determine whether these low frequency mutation reads represent subpopulations of neoplastic cells we used a DAPI/cytokeratin 19 and a DAPI/vimentin flow assay to resort the biopsy. The cytokeratin 19^+ and the $vimentin^+$ diploid populations each had the heterozygous *KRAS* mutation detected of the aneuploid population and cell line. However, only the small (5-10%) cytokeratin 19^+ diploid population had the clonal homozygous *TP53* mutation. The aCGH profile of the diploid cytokeratin 19^+ population was identical to the aneuploid and cell line. Thus the 2N $cytokeratin^+$ population represents a co-existing tumor population with a DNA content that is diploid by flow cytometry. In contrast the diploid $KRAS^{mut}$, $TP53^{wt}$ population was normal by aCGH and represents a third clonal population in this biopsy that is either from an earlier stage of disease or is a non-progressing neoplastic population. Our ability to resort this tissue provides a unique approach to validate our NGS results and confirms the presence of distinct clonal populations. We propose that this iterative approach can exploit the detection of low frequency reads in NGS data to provide deep clonal analysis.

11. Detection of Low-Prevalence Mutations in Solid Tumors via Ultra-Deep Targeted Sequencing

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Cells within a tumor sample are known to be heterogeneous, due to the contamination from non-malignant tissue or the presence of multiple sub-clones, each carrying different somatic mutations. Currently available diagnostic assays that are able to detect these rare somatic mutations are not comprehensive, limited to a few mutations only. We propose to leverage two innovative technologies, microdroplet PCR and high-throughput sequencing, and an original and robust analysis method, to implement a clinically compatible assay (UDT-Seq) able to screen ≥100kb of DNA, to detect mutations present in 1% of a tumor sample. UDT-Seq can therefore work on poor cellularity samples commonly encountered in the clinic and help address clonal heterogeneity, which is thought to drive cancer progression and drug resistance. We designed a library of 676 PCR primer pairs targeting 42 cancer genes representing 71.1kb of cancer mutational hotspots, as defined by the most commonly reported mutations in the COSMIC database. In addition, these primers target 29 kb of polymorphic amplicons, carrying known SNPs in a blend of 4 Coriell DNA samples used for calibration. We developed a streamlined protocol easy to implement in a clinical setting that seamlessly combines highly uniform microdroplet PCR amplification with deep sequencing. We first verified the technical validity of the approach. We used the calibration samples to experimentally measure the error rate at each position and rank the candidate mutations by statistical significance. A machine learning approach trained on ~200 calibration SNPs at known prevalence complements empirical filters and reliably identifies 89 % of the expected variants at prevalence ≥1% with a specificity of 99.99% and less than 2.4% of false positives. The observed prevalence is highly correlated with the expected one (r=0.97) and this performance is maintained even at coverage of 3000x on the Illumina GAII. We observed that whole genome amplification introduces an allelic bias for the mutations present at 5% or less, which resulted in reduced sensitivity at lower coverage. Finally, we were able to perform early validation of the approach on fast sequencer, Illumina MiSeq, that shows a lower error rate and as a consequence reduces by 6-fold systematic noise [see reference]. With the intent of a clinical implementation, we analyzed 6 frozen cancer samples from 4 different cancer patients. All tumor samples as well as matched blood DNA were used as a template for microdroplet PCR. We were able to detect only one mutation shared by the breast cancer primary and matched xenograft samples (HRAS-G12V), one mutation in the ovarian xenograft (TP53-R248Q) and 13 mutations in the colon primary and xenograft samples, with the lowest present at 10% of the primary tumor DNA. Interestingly the change of mutation prevalence indicates a purifying selection in the xenograft. We validated 100% of the mutations investigated using an independent assay. We have instigated the proper human subject and logistical framework to carry out such clinical sequencing at our institution (IRB approved protocol). Our current work expands the number of clinical sample to 40 breast cancer patients. We are developing cross-validation of the low prevalence mutations using digital PCR and improving the usability of our analysis pipeline as a standalone tool and to make it broadly accessible. We believe UDT-Seq provides significant improvements over previous reports of similar approaches either by increased breadth or more meticulous evaluation. Featuring several innovative ideas, the assay and analysis we present is now very well suited for clinical applications including screening patients for molecularly targeted clinical trials, identifying and targeting tumor sub-clones that are resistant to standard treatment and enabling improved clinical care for uncommon molecular findings targeted by therapies approved for alternative indications.

Reference

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12. Development and Application of Novel Glycan-Specific Reagents to Facilitate Early Detection of Epithelial Ovarian Cancer

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Epithelial ovarian cancer (EOC) is currently the most lethal of gynecologic cancers [1]. Today, nearly 80% of women are diagnosed at advanced stages of EOC, and of these 80%, there is only a 20% survival rate (after 5 years) [1]. In stark contrast, of the ~20% of women diagnosed with early stages of EOC, 90% of women survive longer than 5 years [1]. Thus, finding a biomarker (or panel of biomarkers) that can detect early-stage EOC cheaply and non-invasively has the potential to significantly increase EOC survival rates. The central hypothesis of this study is that N-linked glycosylation is significantly altered in diseased patients in comparison to their healthy counterparts, and this is supported by numerous studies showing the correlation between aberrant glycosylation and diseased tissue. However, the chemical tools currently available in the glycomics arena are insufficient in quantitatively profiling such biomolecules. This study aims to (1) address a key and critical step to ultimately become the mainstream strategy for the analysis and relative quantification of glycans in plasma and (2) apply these methods to the assay of N-linked glycans in EOC biomarker discovery. We have currently developed a novel method for the relative quantification of plasma N-linked glycans via stable-isotope labeled hydrazide reagents. A pair of reagents has been synthesized such that two glycan samples can be labeled with either the 'light' or 'heavy' reagent, mixed together, and relatively quantified in the same mass spectrum. Furthermore, these reagents have two additional advantages: a large amount of hydrophobic surface area has been synthesized on the terminal end of the reagents allowing us to take advantage of (1) the hydrophobic electrospray bias and detect larger ion abundances for derivatized vs. native N-linked glycans in ESI [2-4], and (2) the increased interaction with a reverse phase stationary phase allow N-linked glycans to be characterized by reverse phase LC. We have shown that these reagents are capable of increasing the ion abundance of N-linked glycans in ESI MS by >10-fold and increase the amount of glycans indentified by ~10%[3]. Additionally, the separation of N-linked glycans by reverse phase chromatography has allowed for improved separation efficiency over HILIC and a further increase in the number of glycans detected (by nearly 50%). This is an important advance because of the ubiquity of reverse phase LC-MS used in the bioanalytical mass spectrometry community. In other words, this labeling strategy fits into existing LC-MS workflows that dominate biological mass spectrometry. A final point is that although we have not yet demonstrated this tagging approach to biological samples other than plasma, there are no foreseeable technical barriers that will prohibit its use for tissues, cell lines, and biologics (biological therapeutics) further broadening the impact of this technology. Studies are currently underway to determine the bioinformatic strategies necessary to extract the glycan data and relatively quantify glycans using the SIL reagents. Additionally, we are utilizing one of the newest cutting edge technologies, a Q-Exactive Orbitrap mass spectrometer, to further enhance the glycan profiling and extend the detection limits of the glycan analytes (beneficial to EOC biomarker discovery efforts). By coupling the N-linked glycan tagging technology with advanced mass spectrometry instrumentation, we have been able to detect numerous undocumented N-linked glycans. Finally, the utility and innovation of the relative quantification strategy has been recognized by industry, and we are currently in active discussions with Pierce (part of Thermo Fisher Scientific) to manufacture and market this strategy. This is an exciting, new collaboration that is capable of realizing one of the studies main goals: developing a mainstream strategy for relatively quantifying glycans that can be implemented in nearly any laboratory on a wide range of biological substrates. The future of this study will involve the N-linked glycan relative quantification strategy being applied to a novel model system for spontaneous EOC, the domestic chicken. The chicken model of spontaneous EOC affords several advantages: (1) chickens develop EOC spontaneously between 2-4 years of age at a prevalence that can exceed 35% [5], (2) chickens are raised in a controlled environment, (3) sample procurement is controlled throughout the study, (4) the chickens are from a single strain reducing biological variability of the model, (5) the ability to procure longitudinal plasma samples and tissue-matched samples after necropsy, and (6) the similar histopathogenesis to humans. The glycan relative quantification strategy combined with the longitudinal sampling of spontaneous EOC will allow us to quantitatively detect the up- and down-regulation of N-linked glycans as a function of the onset of EOC. These changes will allow us to identify possible glycan biomarkers (or a panel of biomarkers) that will be indicative of the onset of EOC.

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13. Development of a Methylation-Based Diagnostic Assay for Malignant Melanoma: Defining the Factors Affecting Marker Selection and Assay Performance

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Melanoma has a predilection to metastasize when only a few millimeters in depth; however, early detection and diagnosis are difficult due to the overlap in clinical and histologic appearances of melanomas with highly prevalent benign moles. High-throughput DNA-methylation array technology holds the promise of discovery of candidate DNA-methylation markers useful for improving melanoma diagnosis but must be valid on small formalin-fixed paraffin-embedded (FFPE) tissues embedded in paraffin blocks, which is typically the only diagnostic tissue available for primary melanomas and moles. Moreover, a diagnostic assay should be sufficiently sensitive and specific to detect cancer even in the presence of some contaminating non-malignant cells, and robust enough to yield accurate results despite variability in patient and tumor characteristics. Our initial work, under a R21, evaluated DNA methylation in primary melanomas and non-dysplastic benign nevi using the Illumina Cancer Panel I array, which measured promoter methylation at 1402 CpG sites in cancer-related genes after filtering. We identified 26 CpG sites in 22 genes that were significantly differentially methylated between non-dysplastic nevi and melanomas after controlling for age, sex, and multiple comparisons. Independent validation in a dataset that included dysplastic nevi confirmed 14 of the 22 genes to be significantly differentially methylated [see reference]. In an effort to identify factors that could affect the accuracy of our diagnostic marker panel, we expanded our methylation profiling to 40 normal skin samples, in addition to the 47 primary malignant melanomas, 34 non-dysplastic nevi, and 22 dysplastic nevi previously tested. Factors such as patient characteristics, tissue source, tumor subtype or staging criteria, degree of nevus dysplasia, and specimen contamination by non-malignant cells were tested for potential impact on our methylation panel or markers comprising this assay. Our results to date are summarized as follows: Patient/host factors such as age, sex, anatomic site, solar elastosis, pigmentation, and lymphocytic presence were not associated with differential methylation of the 26 CpG loci in our marker panel. In melanomas, distinct sets of CpG loci differed in methylation according to Breslow thickness, mitotic rate, ulceration, and BRAF mutation; however, none of these overlapped with the 22 genes in our diagnostic marker panel, except for BRAF mutation. In melanomas, K-means clustering based on the 235 most variant CpG loci defined 3 clusters, including a subset exhibiting the putative CpG island methylator phenotype (CIMP) in 13% of melanomas. Comparison of melanomas with a larger series of nevi showed that methylation continued to distinguish melanomas from both common nevi and dysplastic nevi. Interestingly, dysplastic nevi did not show significant methylation differences from common nevi. Overlap of CpGs identified through supervised analyses (PAM, logistic regression, ROC) and filtering for the largest differences in β values allowed us to further refine as well select the most robust loci for our diagnostic panel. These results support the usefulness of methylation for the specific identification of melanocytic malignancies. Comparison of melanocytic lesions (melanomas, dysplastic nevi, non-dysplastic nevi, individually or combined) and normal skin identified CpG site differences that may be used as internal standards to estimate the degree of skin contamination (or % tumor) in melanocytic biopsies. Nevi or melanomas differing in % melanocytic lesion ($\geq$70% vs <70%) or study site (UNC vs. UNM) did not cluster separately on hierarchical clustering. For the R33 phase of this study, we are in the process of conducting validation and advanced development of methodology to overcome the challenges inherent in analyzing DNA methylation in small formalin-fixed paraffin-embedded specimens. Specifically, we are (1) Identifying candidate DNA-methylation differences that distinguish melanocytic (nevus or melanoma) vs. non-melanocytic (surrounding skin or lymphocytic infiltrate) cells for use as internal quality control standards to quantify sample percent tumor;
(2) Identifying valid conditions for high-throughput DNA methylation array profiling of small-sized FFPE melanocytic tissues in order to select the least biased candidate DNA methylation sites for melanoma diagnosis; and
(3) Confirming candidate DNA-methylation differences from high-throughput DNA methylation profiling using more quantitative assays.

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14. Development of an In Vivo Screening Technology for Cancer Vaccine Immunogens

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The Specific Aim of the project is to create a microbial genomics-based high throughput in vivo mucosal immune screening system to identify immunogens for use as potential cancer vaccines. We propose to develop a new technology that will employ DNA barcoded plasmids with Gram(-) autotransporter (AT) surface expression cassettes expressing tumor vaccine antigen candidates, a method to quantitate the relative abundance of plasmids (qPCR, high throughput sequencing), an intact mucosal immune system, and Darwinian principles to identify and compare potentially immunogenic peptides and proteins. We hypothesize that bacteria transformed with plasmids directing the synthesis of more immunogenic proteins will be selected against within the GI tract by the GI immune system and will therefore over time show a relative decrease in abundance in feces. This technology will enable investigators to identify candidate tumor vaccine antigens with enhanced immunogenicity and likely anti-tumor activity. The project can be divided conceptually into four parts: (1) Design and production of surface expression screening plasmids. (2) Pilot level demonstration that the in vivo mucosal immunogen screening approach can identify immunogenic vs. non-immunogenic proteins. (3) Use of the screen on an exhaustive collection of survivin subpeptide-expressing plasmids to find maximally immunogenic peptides. (4) Testing the identified subpeptides in an in vivo tumor model to determine if peptides identified in the screen serve as a better tumor vaccine than existing survivin-based tumor vaccines. In the first year, we have essentially completed part 1, are actively working on part 2, and have designed and commissioned the production of the exhaustive collection of the survivin peptides for use in part 3. We commissioned the production of 50 plasmids. They have pUC high copy oris, AmpR, TetR, and ThyA genes, a DNA barcode based on the V1V2 16S variable region of extremophiles, and an AIDA-I autotransporter surface expression cassette. We also commissioned synthesis of survivin-derived inserts. These include full length survivin, 3 previously studied MHC class I-binding survivin peptides, and 26 15-mer overlapping peptides spanning the survivin sequence. We verified surface expression of full length survivin with immunoblots. We also produced versions of the plasmids that express selected immunogenic and non-immunogenic proteins and a negative control plasmid with stop codons in the surface expression cassette. We obtained strain *E. coli* MG1655 from ATCC, and other strains thought to be good GI colonizers. We also isolated 5 additional different *E. coli* strains (strains JC1-6) from mice living in our vivarium, reasoning that *E. colis* that are best at colonizing our mice may be those already living in the GI tracts. We also obtained a derivative strain of MG1655 that is StrepR/NalR. We have produced *ThyA*- versions of all these strains, as well as DH5α for use as a negative control strain unlikely to colonize the mouse GI tract, using the Sigma-Aldrich Targetron technology. We showed that our pVISIA plasmids, supplying ThyA in *trans,* can rescue the bacteria back to prototrophy. We partnered with Roy Curtiss, Arizona State University, to use the Asd+ plasmid pYA3342, maintained as a balanced lethal in attenuated *Salmonella* Typhimurium vaccine vector strains, which display excellent immunogenicity, for use in subsequent immunization experiments. We have begun our initial inoculations of the mice with different *E. coli* strains and with MG1655 transformed with plasmids expressing immunogenic and non-immunogenic proteins, and are monitoring the colonization kinetics of the different *E. coli* strains and the *E. coli* expression different immunogenic and non-immunogenic proteins. We have found that ThyA provided in *trans* stabilizes the plasmids in the bacteria for many weeks. We are analyzing the colonization kinetics of the bacteria expressing different immunogenic and non-immunogenic proteins to determine whether we can observe in vivo immune-dependent selection.

15. Digital Analysis of Proteins Through End Sequencing (DAPES)

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We are developing DAPES (Digital Analysis of Proteins Through End Sequencing), a single peptide sequencing technology, which, if successfully completed, would identify and quantify all proteins in biological fluids. The potential applications of DAPES are widespread, it should allow researchers to find signatures in a variety of human tissue samples that diagnosis disease, determine prognosis, or predict responses to a particular kind of therapy. The DAPES procedure begins by cleaving cellular protein content into short peptides and covalently attaching them to a glass surface through a PEG based nanogel. The terminal amino acid in these attached peptides is then identified through the use of fluorescently labeled N-terminal Amino Acid Binding molecules (NAABs) and single molecule detection (SMD). A cleavage reaction is next used to remove the N-terminal amino acid and the cycle is repeated to sequence 10 amino acids from the N-terminus of each peptide on the slide. The peptide sequences are then mapped back to the human proteome and the number of different peptide sequences observed which map to a particular protein is then divided by the total number of possible peptides that could be produced by that protein to yield a quantitative measure of the protein's abundance. To serve as the NAAB probes integral to the DAPES procedure we have been cloning *E. Coli* tRNA Synthetase (RS) enzymes and now have 8 probes capable of binding specific N-terminal amino acids. We have begun to assay the specificity of these probes and found that they bind specific subsets of amino acids, an observation in agreement with previous work studying the mis-acetylation patterns of the RS enzymes. We hope to utilize the cross-reactivity of the RS probes to help simplify the DAPES procedure by distinguishing between all 20 amino acids through as few rounds of binding as possible. As part of our effort to develop DAPES, our laboratory has also produced probe stripping and N-terminal amino acid cleaving protocols and developed an optimal imaging technology. The stripping of NAAB probe bound to N-terminal amino acids is a necessary step in between successive rounds of probe binding. We have developed an efficient stripping protocol using a detergent wash that removes probe but does not affect the PEG surface or the binding of peptide by additional probe. To remove the N-terminal residue of bound peptides after its identity has been elucidated we have established a cleavage protocol based on a modified Edman reaction. Lastly, significant effort has been made to optimize our SMD peptide imaging platform, we have reduced background fluorescence and non-specific interactions of probe with the surface while maximizing the accessibility of bound peptide to probing by NAAB molecules. We are now working to fully characterize our existing RS probes while continuing to expand our library with new N-terminal binders. Efforts are also ongoing to develop software which calls N-terminal residue identity based on probe binding signature and maps the 10-mer sequences generated by DAPES to the human proteome. We will soon combine these different efforts and begin performing single molecule sequencing experiments.

16. Discovery Platform for Cancer Antigens

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Abstract and Specific Aims: The main goal of this proposal is to implement a platform-based method to isolate and identify patient-derived tumor-specific antibodies and their cognate cancer antigens. It is the intention of these investigators to use these reagents to expand our understanding of breast cancer biology, detection of cancer specific biomarkers and as novel therapeutic treatments. The single domain antibodies isolated are infinitely renewable and hold great promise as tools for population based biomarker screening and individualized patient-specific targeted therapy for advanced and metastatic disease. The specific aims of this proposal will focus on four specific goals:

Specific Aim 1: Perform throughput platform screening of at least 40 breast cancer cases representing up to 65 total libraries and identification of an estimated 1000-1500 novel VH antibodies.

Specific Aim 2: Identify the cognate antigens for 200-400 antigen driven VH single domain antibodies identified in Aim 1 based upon clonal expansion and somatic hypermutation properties.

Specific Aim 3: Validate identified antigens and their presentation in human breast cancer using multiplex large scale arrays.

Specific Aim 4: Assemble multiplex protein arrays of recombinant soluble domain antibodies and matching arrays of their cognate antigens for use in cancer diagnostic, screening and clinical targeting applications.

Results and Observations: Synthesis and banking of 22 total patient-derived sentinel lymph node libraries of VH single domain antibodies has been performed. Fourteen of these were effective at producing large volume clones during transformation. Approximately 12,000 clones have been screened for orientation and 6,000 individual clones directly sequenced using library 96-well sequencing format. 5,800 open reading frame clones were compared to genomic databases for somatic hypermutation and V-D-J gene usage clonal expansion. Current accumulation of data from these large scale screens indicate that each library has 3-4% of clones that reach criteria for antigen-driven selection and further analysis (175-220 clones). To date we have developed screening mechanisms for selection of these VH single domain antibodies according to the following criteria: (a) cell surface immunofluorescence of ER+ and ER- human breast cancer cell lines under optimal and low glucose conditions, (b) positive threshold signal in direct ELISA of breast cancer cell and primary tumor lysates, (c) immunohistochemistry signal on tumor tissue array (80 sample), and (d) immune-selection of target antigen from cell or tumor lysates. A total of 46 VH clones have been synthesized and screened through methods a-c above and 6 have been processed through d. Immunoselection and identification of VH antigen targets by mass spectrometry methods are in progress. Direct screening for antigens using our collaboration with Arizona State University Biodesign Institute is ongoing and on-slide transcription/translation and antigen detection are being performed to control background and specificity thresholds.

Goals and Future Directions: The goal for the coming year is to screen and identify cognate antigens from approximately 200-250 VH clones with high scores of antigen-driven somatic hypermutation and/or clonal expansion. Secondly, in an effort to streamline library VH synthesis and antigen identification we are developing SV40 large T Ag expressing MCF-7 and MDA-MB-231 surrogate cells. Direct transfection of library plasmid containing individual VH clones will effectively express the VH antibody in the transfected cell provide for immunoprecipitation from a whole cell lysate as a rapid and medium throughput method to identify cognate antigens. Recombinant VH antibodies and their cognate antigens will be used to determine their distribution in human breast and ovarian cancer as novel diagnostic reagents and therapeutic targets.

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

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Thiol redox homeostasis is central to the control of cell fate and is associated with various abnormal biochemical processes. Because of its abundance, glutathione is considered to be the major thiol-disulfide redox buffer of the cell and is often used as a proxy of the intracellular redox environment. We recently reported the development of Förster resonance energy transfer (FRET)-based redox-sensitive sensor adjusted for more oxidative environments that enables noninvasive redox measurements of the ER of living cells [1]. This research project aims to validate protocols and evaluate the performance of the FRET-based redox sensors when applied to cancer [2]. A second-generation FRET biosensor (CY-RL7) permitted visual monitoring of glutathione potentials in the relatively high oxidative environment of the endoplasmic reticulum (ER) [2]. We demonstrated that the sensor targeted to the ER of non-tumorigenic (CHO) and tumorigenic (HCT116 p53+/+, HCT p53-/-) cells is equally oxidized at ≈88%, while it is fully reduced in the cytosol. We also observed that the reductive level of the FRET probe was increased two-fold to about 28% in cells incubated with N-acetylcysteine (NAC), a substrate for GSH synthesis. Additionally, to expand the basis of the FRET-based sensors used in the present study to monitor redox processes in the cytosol and mitochondria of live cells, we employed recently reported roGFP sensors that have the capability of monitoring redox in the more reduced mitochondrial compartment. Green fluorescent protein probes attached to the glutaredoxin enzyme (Grx1-roGFP2) enable real time monitoring of the redox events in the cytosol and in mitochondria. We confirmed that probes targeted to these compartments are highly reduced at steady state. Our data demonstrate that mitochondrial as well as cytosolic redox homeostasis of mammalian cells is capable of restoring a reduced steady state redox environment within minutes after an acute oxidative insult (1 mM diamide for 2 min) is removed. Next, the efficacy of the Grx1-roGFP2 probe was ascertained by the probe response to perturbations in glutathione homeostasis. Surprisingly, both NAC and GSH ethyl ester (GSHee) triggered oxidative stress in mitochondria, but not in the cytosol. Most noticeably, the mitochondrial redox environment in cells incubated with NAC and GSHee gained resistance to exogenous oxidants in spite of elevated oxidative stress. Finally, we observed distinct oxidative responses of mitochondrial sensors expressed in isogenic porcine fibroblast 161-p53 and 161+p53 cancer cells after depletion of the glutathione pool with buthionine sulfoximine (BSO), an inhibitor of glutathione biosynthesis. This observation implies that the sensitivity of the sensor expressed in the mitochondria of cancer cells exposed to the inhibition of glutathione biosynthesis by BSO depends on alterations in p53 expression in cancer cells. In conclusion, our studies demonstrate that individual organelles have different redox requirements principally driven by the reduced (GSH) and oxidized glutathione (GSSG) redox couple. Our data also demonstrate metabolic differences in GSH homeostasis between cancerous and normal cells and may offer suggestions for novel therapeutic approaches to cancer.

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18. Genome-Scale DNA Methylation Profiling in the Developing Colon and the Impact of Diet

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Background: Methylated CpG island Amplification (MCA) is a sensitive and robust method for generating a reduced representation of DNA methylome, and provides good coverage of genes and gene promoters [1]. Coupling MCA with next-generation sequencing (MCA-Seq) provides a high-resolution tool for genome-wide DNA methylation profiling. In a mouse model, we performed MCA-Seq to test whether during critical ontogenic periods, nutrition influences developmental epigenetics in the colon, causing persistent alterations in epigenetic regulation that affect susceptibility to colon cancer. We focus on two likely critical periods for colon epigenetic development: embryonic day 15.5 (E15.5)-postnatal day 0 (P0) and P0-P21. To test for nutritional influences on developmental epigenetics, we used an established model of maternal pro-methylation dietary supplementation [2].

Method: We used the Illumina Hi-Seq 2000 sequencer, and incorporated sample-specific barcodes into sequencing libraries to assay up to 12 samples in a single lane. To identify developmental changes in DNA methylation in mouse colon, two independent sex-matched MCA-Seq comparisons were performed at E15.5 and P0 C57BL/6J mice. To determine if gene regions undergoing developmental changes in DNA methylation during fetal or postnatal development are affected by maternal supplementation, virgin female mice, age 8 wk, were provided either control (NIH-31) or pro-methylation diet (NIH-31 supplemented with folic acid, vitamin B12, betaine, and choline) for 2 wk before mating and during pregnancy and lactation. In the offspring, two independent sex-matched supplemented vs. control MCA-Seq comparisons were performed at weaning (P21) and adulthood (P100). We used quantitative bisulfite pyrosequencing to measure DNA methylation at a subset of identified genomic regions in the entire set of samples collected (N=10 per sex and age). In addition, we compared developmental methylation changes separately for colonic epithelial stem cells, differentiated epithelial and stromal cells.

Results: We identified 7568 genomic regions undergoing significant developmental changes in DNA methylation (FDR adjusted *P*-value <0.05 by Benjamini-Hochberg). We validated methylation differences at a subset of intervals including several Wnt target genes. Further results regarding the consequences of maternal supplementation will be presented.

Conclusion: Our data confirm that MCA-Seq provides a powerful and sensitive approach to detect methylation changes on a genome-wide scale. Our preliminary data indicate that important epigenetic changes are underway during late fetal colon development, and support a potential link between developmental epigenetics and later risk of colon cancer.

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19. Highly Multiplexed, Spatially Delineated Molecular Imaging in Cancer

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Molecular imaging methods are critical to numerous fundamental and clinical investigations in various human diseases including cancer. These approaches offer significant advantages over many bulk proteomic techniques since they provide important abilities to characterize spatial distributions of molecular markers in situ within cells and tissues. Such capabilities have been further enhanced by the development of optical microscopy techniques that allow biological samples to be examined with extremely high spatial and spectral resolutions. Nevertheless, in contrast to bulk analytical methods, the majority of molecular imaging approaches still have limited utility for proteomic analyses of diseases since they only allow a handful of molecular pathway components to be visualized simultaneously in the same biological sample. This deficiency largely stems from intrinsic limitations of existing molecular probes technologies. For one, the fluorophores that are coupled to protein recognition agents such as antibodies exhibit significant spectral overlap, which limits the number of proteins that can be labeled and detected simultaneously within a cell. The number of dyes that are coupled to antibodies is also difficult to control and is highly variable, especially when enzymatic signal amplification strategies are employed. These issues can confound multiplexed imaging approaches since distinguishing different spectral signals ultimately requires an appropriate balance of marker intensities within a sample. Finally, selective protein targeting often necessitates the use of high-affinity antibodies, making them difficult to remove without using harsh illumination and/or chemical treatments that can perturb sample morphology and marker integrity. Consequently, most cell samples and tissue sections can only be labeled and examined one time. With funding support from IMAT, our group has developed a novel class of programmable immunofluorescent imaging probes that offer new levels of control over the coupling of fluorophores to protein recognition reagents. A unique feature of this technology is that it employs dynamic DNA complexes to control the in situ assembly and disassembly of structured fluorescent reporting complexes that can be coupled selectively to DNA-conjugated antibodies. Importantly, these reactions occur through a process called DNA strand displacement which involves the isothermal exchange of oligonucleotides between different thermodynamically-stable DNA complexes and allows reporting complexes to be created and removed from a sample at ambient temperatures while using mild reaction buffers (e.g., Tris or PBS). Herein, we demonstrate how strand-displacement can be harnessed to create DNA complexes that can function as erasable molecular imaging probes in order to increase the number of proteins that can be examined within a single cell. We will also describe how these modular reagents can be adapted in a plug-and-play fashion to controllably tune antibody reporting intensities. Finally, methods to change the reporting functionalities of marker-bound probes to allow samples to be examined using conventional and then super-resolution imaging approaches will also be demonstrated. In each case, we will highlight how this technology can be used to increase the multiplexed detection capabilities of molecular imaging techniques and facilitate much more quantitative, proteomic-level in situ analyses.

20. Hollow Perfluoropentane Filled Silica Nano and Microparticles for Breast Conservation Therapy

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Introduction: Excising non-palpable breast cancers in breast conservation therapy is difficult because surgeons must rely on mammographic localization. Wire localization has been the standard method to localize tumors, but the reported positive margin rate from wire-localized excisions of breast cancers is approximately 20-50%. There is a need for a better localization procedure to completely remove the tumor with one operation. Microbubble based contrast agents are clinically used to enhance the ultrasound echo signals. These are commercially available but have a short half life. Our group has developed hollow perfluoropentane filled silica nano and microparticles of a highly uniform size which can be used as a contrast agent under color Doppler and CPS imaging for extended periods of time. The shells of these particles are rigid thereby allowing for extended storage and in vivo survival. We hypothesize that these microparticles will be more suitable for this intraoperative application.

Methods: For testing of locally injected microshells, nano shells and microbubbles, a rabbit model was employed. Four New Zealand White Rabbits where used to study the particles in vivo over five days. 50 µl of either 2 mg/ml of 2 µm and/or 2 mg/ml of 500 nm particles were injected into both thighs. 50 µl of Definity microbubbles, a commercially available US contrast agent, were injected at a concentration of 10^8 microbubbles/ml or 10^{10} microbubbles/ml as controls to duplicate the particle concentration of 100 µg of the 2 µm and 500 nm particles, respectively. Controls and both shell sizes were imaged using the Siemens Sequoia at a MI of 1.9 and a frequency of 7 MHz for optimal particle detection.

Results: Commercially available microbubbles could only be imaged within a few of minutes of the injection on Day 0. Conversely, both the gas filled 2 µm and 500 nm shells could be detected four days in vivo after the initial injection. Moreover, these in vivo data are highly correlated to the previous results shown in an in vitro model, consistent with the hypothesis that the perfluoropentane gas does not readily diffuse out of the particles both in vitro and in vivo. Furthermore, while the signal diminishes over several days, signal decay is substantially similar to the in vitro data suggesting that blood flow and muscle movement have a limited effect on particle and/or gas loss. The results also indicate that the particles do in fact stay stationary once injected into tissue and do not excavate from the injection site despite being in a high strain zone such as thigh muscle tissue.

Conclusion: Based on our preliminary data the commercially available microbubbles would not be a viable option for intra-operative localization of breast tumors since they can only be reliable imaged for a few minutes. The micro particles developed at UCSD are imageable in vivo for at least five days and are a viable alternative for intra-operative tumor localization. Future studies will examine the optimal dosing of the microparticles and toxicity studies in animal models prior to human testing.

21. 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 or excision of the promoter, may be used to confirm a causative link between insertion and the phenotype of a mutant clone. Previously, we have successfully used a variety of vector systems (gamma retroviruses, lentiviruses and transposons) as backbones for insertional mutagens, and have identified new components, as well as new functions for already known components of several mammalian signaling pathways. Our work has also revealed some pitfalls, which have to be accounted for in the experiments involving transduction and insertional mutagenesis. For example, we have 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. Most recently, 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. In this manner, we have used the improved technology to discover drug resistance genes in prostate cancer cells, including the determinants of Taxol response.

22. Isolation and Analysis of Circulating Tumor Cells

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We have developed a method for isolating circulating tumor cells (CTCs) in blood based on positive selection, where cell-surface markers are labeled with fluorescent antibodies, ranked by aliquots, and sorted. We call this process ensemble-decision aliquot ranking (eDAR), because we perform the ranking by looking at an ensemble of cells within each aliquot. In our method, we break down a blood sample into nanoliter aliquots that get rapidly ranked for the presence or absence of CTCs in a flow-through format; the ranking helps us to decide which aliquots of cells are worth closer investigation. Our current microfluidic platform can routinely analyze 1 mL of whole blood in 20 minutes with a recovery efficiency of greater than 93% and a false positive rate of zero. Importantly, this platform concentrates CTCs into a small field-of-view (< 1mm2) within a filtration chamber for microscopic imaging and allows easy isolation of and access to individual live CTCs. The small size of the filtration chamber allowed for complete imaging in a single frame using a 10× objective and a camera with a large CCD sensor. The chamber was open and easily accessible from the top. Additional reagents, such as antibodies against various cellular targets, could be pipetted onto the filter and perfused over the targeted cells. Micropipettes could also be positioned above the filter to remove individual CTCs for analysis. To further improve throughput and sensitivity of the proposed technique, in the past year we have developed new generations of microfluidic chips for eDAR, new types of fluorescent probes for labeling the CTCs, and new sorting schemes for sorting the aliquots for downstream manipulation and analysis. Using a model system where cultured cancer cells were spiked into blood, we have also demonstrated the ability to isolate and culture individual cancer cells that were isolated by eDAR.

23. Magnetorotation: A Rapid Assay for Single-Cell Drug Sensitivity of Cancer Cells

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Heterogeneity inside a supposedly homogeneous cell population is one of the reasons behind drug resistance associated with cancer. This called for the emergence of single cell assays to study specific subpopulations of cancer cells. Circulating cancer cells (CTCs), responsible for metastasis, do not escape the rule. However, in order to develop a single cell assay for CTCs, they need to be kept in suspension, otherwise phenotypic changes might occur, and this alters the relevance of the assay. To answer this problem, we developed the magnetorotation method for quantitative real time monitoring of cell size and morphology, on single live suspended cancer cells, unconfined in three dimensions. The *Cell Magnetorotation* (CM) method is made possible by *nanoparticle induced cell magnetization*. By using a rotating magnetic field, the magnetically labeled cell is actively rotated, and the rotational period is measured in real-time. Any morphology change induces a change in the rotational pattern of the suspended cell (e.g., when the cell gets bigger it rotates slower). So far, we have demonstrated the proof of concept of the method on cancer cells, which led to a publication (Elbez et al., PlosONE Dec. 2011). We showed that the presence of magnetic nanoparticles inside the cells (at the order of a few picograms per cells) and the rotation under a magnetic field were not detrimental to cell viability, neither to cell division. We have demonstrated the ability to use cells as rotating magnetic microplatforms, through the uptake of functionalized magnetic nanoparticles, and the ability to control and measure their rotation under near real-time conditions. Also, and most importantly, we proved on single cells that we could discriminate between cell death, cell swelling and a live cell, by monitoring in real time the trend of evolution of their rotation period. We also compared the effect of a toxic environment under different conditions (5% ethanol and 100µg/ml of Cisplatin). The efficiency of different drugs can thus be qualitatively compared in terms of death time. Following these results, we went on multiplexing the assay. To do so, we use a microfluidic chip in order to trap the cell, which is achieved thanks to the laminar flow of the cells above the traps. The efficiency of the trapping allows us to follow between 100 and 150 cells at the same time while they are rotating. This has led us to the ability to realize statistical studies on single cell death, such as death time distribution curves. Some cell adherence issues need however to be solved. Considering potential applications, it often takes weeks or months, and multiple bone marrow biopsies, to determine whether a patient is having a meaningful response to a kinase inhibitor. Thus, a key unmet medical need remains the ability to make an early assessment as to whether the patient is receiving the right drug and dose to achieve an optimal response. Magnetorotation is likely to meet such needs. We note that Magnetorotation provides more information on cell morphology than buoyancy, and can measure changes below the resolution limit of confocal microscopy, with a much simpler and less expensive set-up.

24. Method for Detection of Secreted Proteins in Single-Cell Assays

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A major goal of biological research is to provide a greater understanding of human physiology as it relates to pathological process involved in disease. Because cytokines play an important role during inflammation and disease, they are the best tool to measure the activation of immune cells. Cytokines are small regulatory proteins and peptides (8-30 kDa) that exhibit a wide range of biological activities. Cytokines are released in unique profiles in response to inflammation, infection, systemic infections such as sepsis, chronic wound healing, and even as predictors of mortality. Measurements of cytokines are not without problems. The detection of cytokines is hampered by their biological properties, e.g., local secretion, rapid uptake and utilization, and short half-life. These properties have led to the development of limited techniques commonly used to measure cytokines based on single cell level, such as flow cytometry, intracellular cytokine staining (ICCS), and enzyme-linked immunospot (ELISPOT) assays. The current methods for single-cell assays as described above require complex procedures, cell treatment, and inconvenient enzymatic or chemical signal amplification. Moreover, none of the above methods has real-time detection capability and potential quantitation of secretion of cytokines per single cell. Our goal is to develop the method for single-cell assays that relies on nanofabrication of plasmonic substrates allowing optical amplification of fluorescence from surface bound dye-labeled detection antibodies. Our technology, called MEFspot, includes collection of intensity and lifetime images for each fluorophore. If the spot is really generated by the protein secreting cell, it displays characteristic values for two measured parameters: an intensity value greater than the background and lifetime shorter than background. Fluorescence lifetime imaging software allows fast processing and visualization of cells that secrete proteins. The background signal corresponds to unbound fluorophore labeled detection antibody. The strengths of our technology is simplified procedure (no washing out cells), ability for kinetics of secretion, ability for multiplexing (dual secretion and cell viability control), and potential for quantitative analysis. Previously we have reported the development of planar plasmonic substrates that allow fluorescence amplification of about 200-fold for fluorophores assembled in the immunoassay geometry. Here we provide additional numerical calculations that provide physical explanation of amplification factors and guidance for rational design of substrates with regard to geometrical composition of metallic/dielectric nanolayers and spectral properties (excitation/emission) of fluorescent materials. Also, optimization was performed for surface chemistry for surface stabilization and immobilization of capture antibodies to maximize the optical signal. We also demonstrated detection of cytokines (TNF-α and IFN-γ) from microphages stimulated with lipopolysaccharide (LPS). Here we focused on detection of cytokines (IFN-γ and IL-2) secreted from T cells isolated from human peripheral blood monocyte cells (PBMC) stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Single and dual cytokine assays were designed and tested using fluorophores in two spectral ranges; blue-green (AlexaFluor 532) and red (AlexaFluor 649). We envision that MEFspot method can be an excellent alternative to other methods for single cell assays providing additional valuable features as described above.

25. Methods of Systematic MicroRNA Target Validation and Identification

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Overwhelming evidence has indicates that microRNAs can play a fundamental role in regulation of diverse cellular functions and dysgulation of microRNA expression could lead to a variety of disorders including human cancer. Although significant progress has been made in the past years in discovery of microRNAs and their biogenesis, and their role in many cellular phenotypes, it is not fully understood how microRNAs exert their cellular functions because a single microRNA can have hundreds of targets. Hence, identification of microRNA targets is a critical step toward understanding of molecular mechanisms of microRNA-mediated gene expression in normal and disease processes. In this application, we propose to develop a selection method for microRNA target validation and identification through two complementary approaches. In the past year, our major effort was on construction of microRNA library so that these microRNAs can be used for selection purpose and so far we have cloned over 900 individual microRNA precursors in a lentiviral vector. By transient transfection of 293T cells combined with qRT-PCR, we verified that most of these clones expressed corresponding microRNAs. These microRNAs will be used in our selection experiments. As proposed, we decided to make the use of Sleeping Beauty (SB) system to generate selection vectors. SB is molecularly reconstructed from evolutionarily defunct sequences in salmonid fish to generate the first cut-and-paste DNA transposon system known to exhibit significant activity in vertebrate cells. The first generation of SB-based vector is named pSSMT-5/6 which carries tetO-EF1a promoter driving GFP/or mCherry-T2A-Pu and tetR-Krab under CMV promoter. We examined the feasibility of these new constructs in 293T and HeLa cells. Using GFP or m-Cherry along with Pu (puromycin resistance) as a reporter, we found that the construct carrying either a given UTR or microRNA sponge provides a significant selection advantage over the vector alone. For example, we cloned 6 different UTRs into the construct and identified 3 of them, CDH1, p53 and p27, revealed stronger mCherry signals and are more resistant to puromycin. Thus, we will test our microRNA library to identify microRNAs capable of targeting these genes (i.e., one known UTR construct against the microRNA library). At the same time, we will start to generate a UTR library so that we will be able to test an individual microRNA against the UTR library to identify its biologically relevant targets.

26. Microfluidic Sorting of Blood Cells for SPR and Fluorescence Analysis

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A microfluidic, size-based sorting array (3 cm x 1 cm) has been adapted to a slide holder that accommodates a 1 cm^2 grating-coupled surface plasmon resonance (GCSPR) chip that can be spotted with hundreds of regions of interests (ROIs) to capture soluble analytes or cells. Based on the size of the cells entering on the left slide in a narrow fluid stream, the cells are bumped to the right. Since monocytes and myeloid derived cells are larger than lymphocytes they move toward the right side. Circulating tumor cells (CTCs) are usually the largest cells so they exit onto the detection chip on the far right. The sorting and detection portions of the device have been fabricated using soft-lithographic patterning and nanoscale lithography/etching, respectively. This has resulted in a monolithic separation/detection chip which has been tested with beads of various sizes to confirm the sorting efficiency. Antibodies used at ROIs to assess capture of CTCs include anti-CD326, CD44, CD49f and CD24. Once captured, the cells can be further characterized for released analytes and then lysed for analysis of intracellular biomarkers with AlexFuor647 conjugated antibodies. The grating coupled surface plasmon coupled fluorescence (GCSPCF) enhances the signal about 100-fold from that of GCSPR.

27. Molecular Diagnostic Tests to Augment Cytomorphologic Diagnosis of Lung Cancer

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Morphologic analysis of cytology samples obtained by fine needle aspiration (FNA) is a primary method for diagnosing bronchogenic carcinoma. However, across multiple studies the false negative rate for cytomorphological analysis ranges from 0.2-0.3, and among those diagnosed with lung cancer, the false positive rate for diagnosing the sub-class of small cell carcinoma averages 0.09. These false results lead to additional invasive diagnostic studies and delay treatment. In previous studies the MYCXE2F1/p21 index correctly classified 72/78 non-malignant lung tissue and 50/57 lung cancer samples for a sensitivity of 88%, and specificity of 92%. A CDKN2C/FOSL1 test for distinguishing non-small cell lung cancer (NSCLC) from small cell lung cancer (SCLC) had a PPV of 100% with 63% sensitivity. The goal of this study was to optimize these tests for assessment of RNA extracted from FFPE samples In order to optimize the robustness and utility of these tests for assessment of RNA extracted from FFPE samples including cell block FFPE samples from FNA, we developed qPCR methods that enable simultaneous measurement of each target gene and reference gene transcript relative to a known number of internal standard competitive template (CT) molecules within a standardized mixture of internal standards (SMIS) using two-color fluorometric analysis on real-time platform. For each gene, the native template was quantified with a sequence-specific FAM-labeled probe and the CT was quantified with a sequence-specific Quasar670 labeled probe. Use of multiple base changes in CT ensured specificity of each internal standard. External standard corrected for inter-experimental and inter-probe difference in cycle threshold. Pre-amplification with 20cycles increased signal to background. Results for each gene thus far demonstrate excellent linearity ($R2 > 0.99$, slope 1.0 +/- 0.05). Percent error as relative accuracy showed 12.1% in constant CT with diluting NT until 1/20 fold difference and 11.9% in vice versa. We obtained excellent signal-to-analyte response (1.0 +/- 0.05) and precision ($CV < 30\%$) over six orders of magnitude, and reliable detection of as few as 10 molecules. We then conducted a validation study of this two-color fluorometric assay in 10 malignant and 10 benign surgically removed FFPE samples. Consistent with previous results in fresh samples, the MYCXE2F1/p21 index optimal cut-off value had 90% specificity and 90% sensitivity. The Receiver Operator Characteristic (ROC) area under the curve (AUC) was 0.94 with 95% confidence interval of 0.8331 to 1.047 and p-value of t-test for stratification of malignant from non-malignant was 0.00017. Based on these data, we expect that this quality-controlled two color fluorometric qPCR approach will enable reliable analysis of the promising MYCXE2F1/p21 and CDKN2C/FOSL1 assays in small degraded RNA extracted from FFPE and FNA cell block FFPE samples.

Disclosures: JCW has equity interest in and serves as a consultant for Accugenomics, which develops and licenses the use of standardized mixtures of internal standards used in this study. There are no other potential conflicts of interest.

28. Multiplex Cancer Cell Purification With Magnetic Sifters

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Magnetic separation of biomolecules and cells has become increasingly common as a means of preparing biological samples as researchers utilize magnetic particles conjugated with specific antibodies to selectively isolate cells and proteins. Purification and isolation of these cells and biomolecules can facilitate further biological analysis such as flow cytometry or protein assays. In applications such as rare cell separation, however, magnetic separation devices must demonstrate high elution efficiencies, and ideally multi-biomarker capabilities, in addition to high capture efficiencies for viable downstream analysis. Recently we have demonstrated that our magnetic sifter, with a size of only 7 mm by 7 mm, can readily outperform Miltenyi's MACS column. Monoclonal antibodies to mouse H2Kb/d were used to identify and separate mouse cells using our standard sifter, or Miltenyi's protocols for MS columns. Following purification, flow cytometry was used to evaluate the purity of the separated populations. In a comparison of the flow cytometry results, the sifter demonstrated lower levels of cell loss and increased harvest efficiency. This is especially promising as Miltenyi's magnetic separation tools are among the most established commercially available devices. We also demonstrated 2-plex magnetic sorting with the sifter. Cells labeled with the smaller nanoparticles (~50 nm) are captured at 2ml/hr but not 15ml/hr, whereas cells labeled with 150nm nanoparticles are captured at both 2ml/hr and 15ml/hr. This capture performance difference can be leveraged to enable separation with two distinct cell markers. Additionally, we also showed that magnetic field configuration and field gradient have a profound effect on the effectiveness of magnetic sorting. In our initial experiments with H1650 lung cancer cell lines labeled with magnetic nanoparticles via the Epithelial Cell Adhesion Molecule (EpCAM) antigen, we can obtain capture efficiencies above 90% even at a sample flow rate of 5ml/hr, but elution efficiencies hover between 50% and 60%. A significant cause of the low elution efficiency is the lateral drift of labeled cells in the device due to magnetic field gradients from the permanent magnets. We obtain improved elution efficiencies close to 90% via optimization of the permanent magnet size and position, and explain the effect via the use of finite element software (Ansoft Maxwell 3D) for magnetic field and field gradient distributions, and a particle tracing algorithm for analyzing the final positions of the particles. This improvement in elution efficiency, allied to previous optimization of sifter geometry to improve capture efficiency, is critical in enabling the sifter to be used for magnetic separation of biologically relevant moieties such as circulating tumor cells and cancer stem cells, which require elution for subsequent analysis.

29. Multiple Reaction Monitoring to Profile Biosensor Phosphorylation in Leukemia

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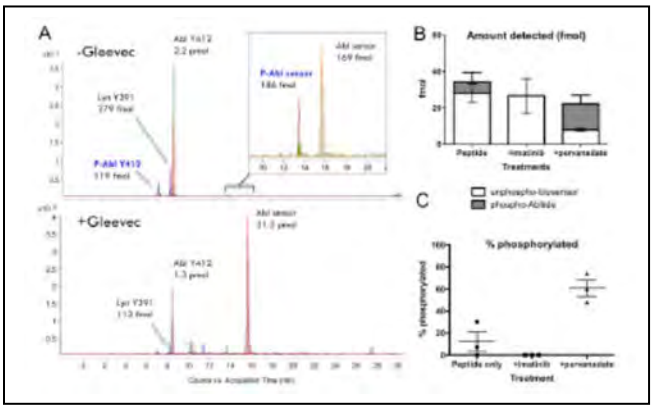
Drugs that target signaling mechanisms have shifted the paradigm for cancer chemotherapy. The Bcr-Abl inhibitor imatinib (Gleevec) is the most prominent example of this. Treatment of chronic myelogenous leukemia (CML) with imatinib inhibits the Bcr-Abl kinase and lowers the levels of phosphorylated substrates (such as CrkL) in patient cells. However, while treatment is frequently successful, a patient's clinical response is not always predictable. Some evidence suggests that decreased Bcr-Abl substrate phosphorylation in mononuclear cells is a reliable indicator of initial response, could inform dosage adjustment, and predicts longer-term outcomes. Such "real-time" monitoring of drug response during treatment is not widely performed, because technical hurdles present a challenge to efficient analysis of patient material. Therefore, there is a need for technologies that can detect multiplexed, specific kinase activities from cells derived from patient material. The *objective of this proposal* is to apply two emerging technologies to this challenge: our recently published intracellular kinase 'biosensor' strategy along with multiple reaction monitoring (MRM) mass spectrometry, to develop a sensitive, multiplexed kinase assay for leukemia-related signaling in patient material. This technology should be adaptable for basic research, drug development, and even potentially diagnostic use. To date we have focused on optimizing the technical parameters of the assay and have made substantial progress on the technical optimization; however, we have not yet moved into testing of patient samples. Our milestone targets are:

1. To detect ≤250 nM peptide detected, using ≤2-5 x 104 cells per experiment.
2. To achieve statistically significant, reproducible quantification of %phosphopeptide for intervals of 20%.
3. To achieve detection of Bcr-Abl activity and inhibition with statistical confidence that is appropriate for the effect size.



We have been able to detect endogenous Abl kinase, Lyn kinase and the biosensor peptide in an MRM workflow (Figure A) from amounts of injected material that are equivalent to ~15K cells (below the 20K-50K cell range we proposed in Milestone 1). In replicate experiments (Figure B), we have achieved sensitivity as low as the 10-30 fmol (per µl trypsin digested lysate) range for detecting the biosensor peptide from cultured K562 human CML cells (which express Bcr-Abl) treated with biosensor in the presence or absence of imatinib (Gleevec) or pervanadate (a phosphatase inhibitor). This corresponds to ~10-30 nM concentration per lysate digest. *Accordingly, we consider Milestone 1 to be achieved.* We have also been able to achieve quantification of % phosphorylated peptide (Figure C) with sufficient accuracy to determine the difference between ~10-20% phosphorylation (peptide only) and 50-70% phosphorylation (+pervanadate) with statistical significance; however, the reproducibility of % phosphorylation is still unacceptable under the current conditions; while coefficient of variation is as low as 22% in the best cases (which would be acceptable), it ranges as high as 127% (which is unacceptable). This seems to be related to signal to noise in the SRM measurement, as these low-level fmol-scale signals are accompanied by a large number of noise peaks. Therefore although we can distinguish ~20-30% intervals with the assay, we do not consider Milestone 2 to be achieved as of yet. In the next year of the project, we will improve our sample handling conditions and introduce enrichment steps to improve signal to noise. Data shown above were from whole cell lysate digests, however, it is becoming clear in the MRM field that sample enrichment prior to MS is important to achieving reproducible and reliable results, especially for low abundance proteins. In addition to examining CD34-based hematopoietic stem cell enrichment, we will use avidin- and antibody-based enrichment of our endogenous proteins of interest and our biosensor peptide from lysates, followed by column-based inline protease digestion (which has also been shown to dramatically improve reproducibility for MRM

experiments). Once this reproducibility is achieved, we will begin analysis of patient material in collaboration with Hamid Sayar, M.D. (Indiana University Simon Cancer Center) to address Milestone 3.

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30. OxMRM: Quantifying Oxidation of Endogenous Redox-Sensitive Cysteines In Targeted Proteins Using Multiple Reaction Monitoring

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Introduction: Thiol signaling by reactive oxygen species (ROS) regulates many aspects of carcinogenesis including tumor growth, migration, invasion, survival, angiogenesis, and metastasis. However the key modifications and mechanisms of thiol signaling in cancer remain obscure. Although chronic exposure to RN/OS has traditionally been thought to be deleterious, thiol signaling is essential for normal cellular function suggesting that ROS modification of cysteines plays a complex role in cancer biology. Insights into cysteine oxidation will further our understanding of cancer progression as well as aid development of new anti-cancer therapeutics.

OxMRM Technology: Multiple reaction monitoring (MRM) is a targeted quantitative technique with the highest sensitivity and reproducibility among mass spectrometry-based approaches [1]. Our laboratory has developed an approach, termed OxMRM, which combines: (1) differential alkylation of samples with unlabeled d_0 and a generic d_5 stable isotope labeled NEM, (2) purification of a protein or proteins of interest, and (3) analysis by MRM to quantify the percent oxidation of virtually any targeted cysteine or protein from a cellular source. In addition, since MRM quantifies at the MS/MS level, it can distinguish between the oxidation of two cysteines within a peptide if a fragment ion between the two cysteines is quantified. The sensitivity of OxMRM analysis allows a unique workflow to quantify the redox status of low-abundance target proteins that are undetectable by unbiased mass spectrometry approaches. We have begun expanding the OxMRM assay to quantify larger numbers of oxidized cysteines per sample, including Complex I of the electron transport chain isolated from brain in a mouse model of Parkinson's disease [2].

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31. Platform for High-Throughput Analysis of Protein Adducts for Carcinogen Exposure Assessment

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The long-term goal of this project is to develop a high-throughput, multiplexed technology to globally characterize an individual's protein covalent modification profile (i.e., the "adductome") with high sensitivity and specificity. Adducts are important as potential cancer mediators and as short-term, long-term, and/or cumulative markers of exposure to carcinogens. Current methods of adduct quantitation are expensive, structure-specific, and labor-intensive. A technology for rapid and comprehensive profiling of macromolecular adduction would be an important tool for carcinogen exposure assessment and epidemiological investigation and may ultimately also be useful in clinical diagnostics, biomarker discovery, and assessment of cancer treatment efficacies. This project ultimately combines three technologies; combinatorial chemical synthesis, antibody (scFv) phage display, and antibody microarray, into a multiplexed system to rapidly profile protein adducts, both on a global and site-specific basis. Efforts in Year 1 have focused on aspects of Specific Aim 1 (adducted peptide synthesis; Milestones 1 and 2) and Specific Aim 3 (synthesis of standard adducted proteins; Milestone 5). For Aim 1, priority has been given to synthesis of a generic model Cys peptide, to be followed by synthesis of peptides specific to the reactive Cys-93 of hemoglobin (Hb) and Cys-34 of serum albumin (SA), with a focus on adduction by three model aromatic amines. As an initial step, we have chosen to synthesize carbon isosteres of the sulfinamide peptide adducts. This approach allows us to make the adducted peptides using simple amide bond coupling methods as compared to the more complex chemistry required for sulfinamide synthesis. We anticipate, based on similar structural parameters, significant crossreactivity of the resulting isolated scFv probes with sulfinamide linked adducts, a hypothesis to be confirmed in Specific Aim 2 of the project. In addition, instead of synthesizing each of the peptides on a synthetic resin, removing and purifying them by HPLC, and then recoupling them to the Tentagel screening resin, we have chosen to perform the peptide synthesis directly on the screening resin. To date, we have successfully synthesized two of the carbon isosteres of the adducted peptides, i.e., the aniline and 4-aminobiphenyl adducts of the generic Cys peptide isostere (i.e., AAG-Asp[Adduct]-GAA) on a Rink Tentagel resin. In addition, for peptides composed of amino acids that require protecting groups, the moiety linking the peptide to the resin cannot be sensitive to TFA treatment. For this reason, we have chosen to incorporate a commercially available photo-labile linker between the resin and the peptide. This linker enables us to treat the peptides with TFA after synthesis, in order to remove the protecting groups, without removing the peptides from the resin. A sample of the deprotected peptide can then be removed from the resin-bound product by UV irradiation. For Aim 3, our initial focus has been to obtain Hb and SA adducted in vitro with aniline, 4-aminobiphenyl, and 2-aminobiphenyl for use as future standards in the microarray platform. The metabolic activation of aromatic amines in vivo takes place by N-hydroxylation of the exocyclic group, followed by oxidation to the nitroso form. The nitroso form then reacts with the free thiol moiety and produces the adducted protein via formation of the sulfinamide. Aniline is a very weak base and cannot directly form protein adducts in vitro. Therefore, aniline requires oxidation to N-phenylhydroxylamine and nitrosobenzene. Additionally, the free thiol moiety of SA and Hb can be oxidized in vitro to sulfenic, sulfinic, or sulfonic acid via exposure of the protein to H_2O_2. Oxidized sulfur products are obtained at varying concentrations depending on the incubation time and concentration of peroxide used. Consequently, our initial approach has been to explore the relevant protein adduction chemistry using aniline, N-phenylhydroxylamine and/or nitrosobenzene. To obtain human SA adducts under different chemical conditions, reactions were carried out in presence of aniline or N-phenylhydroxylamine added at a protein:amine molar ratio of 1:2 under a controlled oxidative environment. Additionally, we performed reactions in the absence of H_2O_2 and in presence of β-mercaptoethanol to avoid disulfide protein dimerization. Aliquots of each sample were used for tryptic digestion and peptide mapping, SDS-PAGE, and direct mass spectrometric analysis of intact proteins. MS analyses were carried out by LC coupled to accurate mass Q-TOF instrumentation. As anticipated, SDS-PAGE of modified SA did not indicate significant change in MW due to adduct formation, since the expected modification masses are not large enough to detect by this approach. Preliminary MS/MS peptide analysis data showed a high percentage (56-80%) of protein sequence coverage corresponding to SA. In reactions between SA/H_2O_2 and the amines, the tryptic peptide corresponding to Cys-34 was not detected, in contrast to the control SA where it was readily observed. This result, although consistent with specific modification of Cys-34 in SA, will require further MS/MS analysis for

confirmation. Work planned for Year 2 of the project includes completion of adducted peptide target molecule synthesis, initial screenings by phage display to obtain adduct specific scFv probes, and continued synthesis of adducted protein standards for the immunoassay platform validation to be performed in Year 3.

32. Probing Cancer Cell Chemoinvasion Strategies Using 3D Microfluidic Models

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Chemokine-mediated directed tumor cell migration, or chemoinvasion, is an important early step in cancer metastasis. Despite its clinical importance, the role of cancer cell microenvironment in regulating chemoinvasion is largely unknown. This is primarily hindered by the lack of tools for probing cancer cell dissemination strategies in a physiologically realistic, 3D, and well defined microenvironment. Current state of art for migration assay, the Boyden chamber assay, is population and end-point based. It is not suitable for cancer cell migration studies, in which cancer cell heterogeneity (variation in space) and plasticity (variation in time) are the two hallmarks of cancer. To overcome these limitations, we have developed a set of microfluidic models to re-create the key biophysical (interstitial fluid flows and biomatrix stiffness) and biochemical (chemokine and growth factor gradients) factors within the cancer cell environment, and 3D imaging techniques for probing cancer cell dynamics in three dimensional space and time. Lymph nodes are known to be the first metastasis sites of many cancer types, for example, breast, melanoma and prostate cancers. Using a malignant breast tumor cell line, MDA-MB-231, as a model system, we studied the roles of lymph node microenvironment in cancer cell chemoinvasion. Our experimental results have revealed (1) the synergistic roles of chemokines and growth factors in regulating cancer cell migration. Specifically, chemokine SDF-1 [illegible] attractant to breast tumor cell lines (MDA-MB-231), and a background of epidermal growth factors (EGF) abrogated this chemotaxis effect. (2) Slow interstitial fluid flows within a 3D biomatrix promote the heterogeneity and plasticity of tumor cell morphology and motility. We found that MDA-MB-231 cells evolved from mostly mesenchymal types to amoeboid types when subjected to slow interstitial flows. This heterogeneity and plasticity of cancer cells may be implicated in their ability to invade and survive in a foreign environment. (3) Malignant tumor cells (MDA-MD-231) were found to use rare event statistics to disseminate, a more efficient strategy for random walkers to spread in space, in contrast to the diffusive Gaussian statistics that is common for passive micro-scale particles. Using the dynamic data collected from the microfluidic models, we plan to formulate a set of rules that cancer cell use to invade to a distant site including lymph nodes and vascular vessels, both through migration within a 3D biomatrix, and intra/extra-vasation through the blood or lymphatic endothelium layers. The eventual goal of the research is to translate this knowledge into improvements of cancer treatments through the modulation of cancer microenvironment.

33. ProCure System

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The specific aim of this project remains to create a reusable actively-cooled system to conveniently and reproducibly refrigerate multiple tissue samples to <-70°C, 4°C and 15°C and precisely maintain temperatures during shipment. The practical benefits of this "ProCure System" to the field of personalized medicine are to: (1) substantially increase the reliability and reproducibility of biospecimen preservation and shipping; (2) create broader access to the collection of a wide range of tissue types; (3) improve the convenience and flexibility of tissue acquisition; and, (4) reduce the cost and environmental impact over current single-use solutions. In the long-term, these benefits support the mission of NCI OBBR: "to guide, coordinate, and develop the Institute's biospecimen resources and capabilities and ensure that human biospecimens available for cancer research are of the highest quality." [1] Successful development of the ProCure System requires several key innovations, including the modification of commercially available lightweight liquid pressure vessels, such that liquid CO_2 can be withdrawn from the cylinder while it is in any spatial orientation as could be experienced during express package shipment. A second key innovation involves refrigeration of the cryogenic biospecimen payload unit through evaporation of liquid carbon dioxide in the most efficient way possible to meet system performance targets. Research efforts on these two innovation priorities are underway at Shipman Technologies in Research Triangle Park, North Carolina (CO_2 vessel) and Design Concepts in Madison, Wisconsin (liquid CO_2 expansion). Liquid carbon dioxide is a very well understood cryogen, long used in refrigeration applications. Detailed theoretical thermodynamic modeling and predictive analysis using computational fluid dynamic modeling have been employed in this study, but actual bench experiment results are often different than predicted. Adapting a well-understood technology to this specific application remains a very challenging combination of "art" as well as science. Unlike the development of a potential life-saving disease therapy, the expected production and use cost of the ProCure System must be an integral part of these investigations, in order to make biospecimen preservation and shipment broadly available to research and clinical enterprises. This adds a frustrating challenge, such as dismissing the "ideal" component available because it will not support the achievement of cost targets. However, with diligent Internet searches, innovative materials and solutions have been identified. A key and recurring topic at the Office of Biorepositories and Biospecimen Research (OBBR) Biospecimen Research Network (BRN) symposia has been pre-analytical variability in biospecimen handling. Except for groups investigating room-temperature fixation of biospecimens, ProCure System development represents the only effort addressing pre-analytical variability associated with biospecimen fixation and shipment. Recent BRN symposium participants welcomed the ProCure system poster presentation with requests to be beta-testers of the ProCure system when it becomes available.

Reference

National Cancer Institute (NCI), Office of Biorepositories and Biospecimen Research (OBBR) Web site.

34. Prussian Blue Nanoparticles as Cellular T1 MRI Contrast Agents

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Prussian blue is a mixed-valence iron hexacyanoferrate compound (abbreviated as PB) with a face-centered cubic structure. Despite the venerable history and numerous applications of PB in materials science since its discovery in 1703, the use of PB nanoparticles (PBNPs) as a superparamagnetic platform to deliver diagnostic and/or therapeutic agents has remained completely unexplored. We have discovered that PB nanoparticles can act as an effective T_1 MRI contrast agent for cellular imaging (Figure 1, U. S. Patent 61/154,457). Specifically, the lattice vacancies and water coordination to the paramagnetic Fe^{3+} centers in $Fe_4^{III}[Fe^{II}(CN)_6]_3 \cdot nH_2O$ renders the inner-sphere relaxation mechanism active and tunable for optimizing the r_2/r_1 relaxivity ratio (Figure 2). We have also shown that PB nanoparticles are extremely stable in aqueous and even strong acidic solutions (e.g., 12 M HCl). These nanoparticles release a very low level of free CN^- ions in serum after 24-h incubation (i.e., ≤0.1 μg/mL vs. the maximum allowed cyanide concentration of ~0.2 μg/mL in drinking water established by EPA), cannot trigger the production of reactive oxygen species (ROS), and exhibit no detectable cytotoxicity, but have the ability to penetrate cells as demonstrated by us in various studies at the cellular level. We have found that doping the PB lattice with Gd^{3+} ions can dramatically increase the relaxivity of the nanoparticles to as high as ten times of the commercial T_1 contrast agents for imaging applications in high-field scanners (e.g., B_0=7 or 9 T). By modifying the particle surface with a targeting agent (e.g., folic acid) and/or an anticancer drug (e.g., doxorubicin), we are investigating their use in the image-guided drug delivery. In addition, we have moved to incorporate Ga-67, Ga-68 and bismuth into the lattice to prepare PB analogue nanoparticles for multi-modal imaging studies involving positron emission tomography (PET) and x-ray computed tomography (CT) modalities.

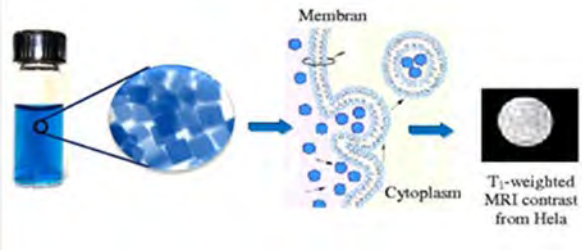

Figure 1. PB nanoparticles as the cellular T_1 MRI contrast agent.

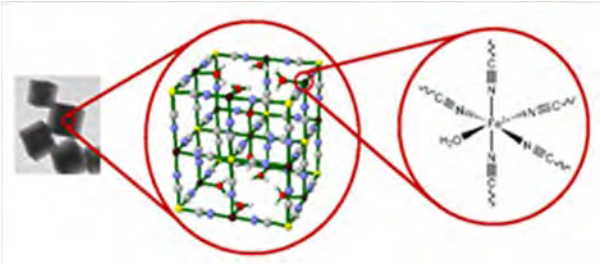

Figure 2. Water coordination to the Fe^{3+} center in PBNPs.

35. Pushing the Limit of Sensitivity for LC-MRM MS Quantification of Low-Abundance Protein Biomarkers

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Mass spectrometry (MS)-based proteomics has emerged as a powerful tool for high throughput quantitative analyses of protein biomarkers in biofluids. Most disease-related protein biomarkers tend to exist at extremely low concentrations and are frequently "masked" by non-significant species present in orders of magnitude higher concentrations. A promising platform for quantitative biomarker analysis involves the combined use of liquid chromatography (LC) separation, electrospray ionization (ESI), and triple quadrupole MS operating in multiple reaction monitoring (MRM) mode. At present, the effectiveness of the technique is still limited by the achievable sensitivity and measurement dynamic range. The research and development effort in the first year of the project was mainly focused on developing new instrumentation and method to significantly improve the measurement sensitivity and dynamic range. A novel workflow, named as high Pressure high Resolution Separation with Intelligent Selection and Multiplexing (PRISM)-MRM MS strategy, has been developed for effective detection and quantification of low abundance protein biomarkers (<1 ng/mL) in human blood plasma/serum. Advanced ESI source and MS interface technologies developed at Pacific Northwest National Laboratory (PNNL) over the years were incorporated into the PRISM-MRM MS workflow to improve the assay limit of quantitation (LOQ). In PRISM-MRM MS, human blood plasma/serum samples are first depleted using immunoaffinity depletion IgY14 column to remove 14 most abundance proteins followed by tryptic digestion to increase the dynamic range of MRM MS measurements. After sample clean up, the peptide mixture spiked with heavy peptide internal standards (for absolute sample quantification) is then fractionated by a high resolution high pH reversed phase LC, a key component of PRISM workflow for effective enrichment of targeted peptides, that has been demonstrated to provide significant advantage over the conventional strong cation exchange chromatography (SCX) due to its much better resolution. Only the fractions that contain specific targeted peptides are intelligently selected (iSelection), based on the elution times of the internal standards simultaneously monitored by an on-line MRM MS during the fractionation, for subsequent high resolution low pH reversed phase LC-MRM MS measurements. Detailed evaluation of the PRISM-MRM MS based assay, incorporating PNNL's multi-capillary/tandem ion funnel interface technology, showed a significant improvement in sensitivity as compared to using the commercial LC-MRM MS instrument platforms. Experimental data acquired from analyses of three standard proteins spiked in a depleted human female serum with concentrations ranging from 10 pg/mL to 100 ng/mL indicated that the LOQ of ~50 to 100 pg/mL could be consistently achieved for all three proteins by the new PRISM-MRM MS platform. Specifically, two peptides with the best ionization efficiencies related to each standard protein were selected and three MRM transitions for each selected peptide were used in the MRM MS. Isotope labeled heavy peptides were used as the internal standards for each targeted peptides to quantify their concentrations in the samples. The intelligent selection of the sample containing fractions during the fractionation stage also increased the sample analysis throughput. To further test the capability of the platform in quantifying protein biomarker in clinical samples, the PRISM-MRM MS was applied to quantify PSA levels in serum samples from prostate cancer patients with the PSA concentrations premeasured by ELISA, an antibody based immunoassay. The PRISM-MRM MS was shown to be able to quantify PSA concentrations in all randomly selected patient samples with good measurement CVs (<20%). A good correlation ($R^2 > 0.90$) was also observed between PRISM-MRM MS assay and ELISA, indicating the reliability of the PRISM-MRM MS measurements.

36. Screening of Novel Small Molecule Candidates to Augment Formalin Fixation

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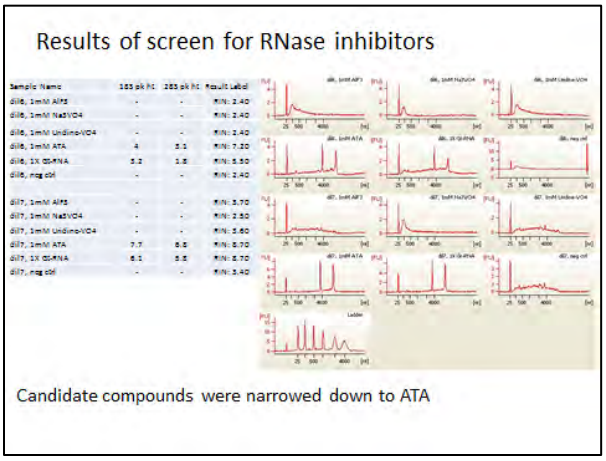

Studies and Results: Screening of Novel Small Molecule Candidates to augment formalin fixation was aimed at preserving RNA in vitro. A promising chemical stabilizer was identified, aurin tricarboxylic acid (ATA), and it is now being tested in two formulas (with or without added Malonate at 1M as an antioxidant and 10mM EDTA as a metal chelator. A Rat Liver Model was used to test impact on RNA quality when added to Formalin. Fixation was performed at two temperatures (4°C and 25°C) for two durations (6hr and 24hr) with or without each chemical additive. RNA was isolated from these tissues by standard Qiagen column extraction, and is now being reattempted using Trizol to improve RNA yield. Pilot data suggests that cold temperature fixation alone improved RNA quality, as tested by two methods: Agilent Bioanlyzer data (with RIN score) and reverse transcription PCR (rtPCR) of the 18S RNA transcripts. The impact of chemical stabilizers is still being examined.

Significance: The original hypothesis which has driven this application is that formalin fixation of tissue does not itself damage nucleic acid. We propose instead that the damage incurred during formalin processing of tissue is an independent result of endogenous tissue nuclease action upon DNA and RNA. More specifically, as would have been expected based on many studies over the years with RNA in solution, the chemical reaction between formaldehyde and RNA is fully reversible and does not produce in itself either strand breaks or other permanent chemical lesions. This hypothesis is consistent with our pilot results showing no measureable RNA damage after 24hrs of formalin fixation at 4°C. There is already data in the literature suggesting that tissue fixed at 4°C is amenable for standard histopathology. If confirmed to promote RNA quality in our study, without adversely affecting histologic and histochemical stain interpretation, then 4°C fixation may by itself serve as a practical solution to the problem of preserving RNA in human tissue during FFPE production. The observation also defines the nature of the chemistry problem remaining to be solved-- namely, whether small molecule inhibitors such as ATA or AME can substitute for or add value beyond what is achieved by chilling the fixative. Testing small molecule inhibitor cocktails remains important for two reasons: (1) 4°C fixation may prove to be unacceptable for some human tissues, e.g. producing inadequate morphology or immunostain quality. (2) Some tissues may possess high RNAse activity: a drop in temperature may not provide enough protection.

Plans: Complete Small Molecule Screening in the Rat Model. This work is in progress and is predicated on improving nucleic acid extraction yields. We do not expect to screen new candidate molecules other than those related to the ATA and AME leads until the rat data are made more consistent. Initiate Human FFPE Studies Comparing 4°C vs 25°C Fixation: Use RNA and DNA Endpoints as well as Histochemical Endpoints. This work is now beginning at UNC. Downstream assays to be applied include Bioanalyzer tracings with RIN score, rtPCR of 18srRNA, quantitative real time PCR of a 2.4kb segment of the human mitochondrial genome (qmtDNA PCR), and pathologist-rated histopathology (H&E and immunohistochemical) stain quality. Carry out Human Tissue Studies Comparing 25°C to 4°C Fixation plus or minus the Best Candidate Small Molecule (ATA) Combination. This work will be done as described in the original application, once a protocol is optimized in rat tissue.

Summary: The study is progressing well, and we anticipate being able to achieve our aims and meet all milestones.

37. Sentinel RNAs as a Measure of mRNA Integrity in Clinical Biospecimens

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Diagnostic or predictive panels of gene expression biomarkers are increasingly being measured in cancer, inflammatory or premalignant disorders to guide therapy of individual patients. The integrity of mRNA is a critical factor in the accuracy of such clinical gene expression biomarker tests. The primary measure of mRNA integrity in biospecimens currently used is the integrity of rRNA, a surrogate marker for mRNA integrity. The purpose of our study was to develop an approach for identifying *sentinel RNAs* that improves measures of the intactness of mRNA in a biospecimens to determine if reliable gene expression biomarker testing can be performed on a biospecimen. mRNA decay generally proceeds from the 3' end and is likely to be underestimated by analyzing polyA+ selected mRNA. To overcome this obstacle we used an efficient 5' cap dependent purification of Pol II RNA (PNAS PMID 12777618). 5' capped RNA isolated from human liver specimens thawed at room temperature for 0, 5, 10, and 15 minutes was analyzed by RNA-sequencing (RNA-seq, see Viruses PMID 22590687). Total RNA from these samples had RIN (bioanalyzer) values of 9.5, 8.9, 7.9, and 6.7, respectively. Surprisingly, RNA-seq analysis showed that only half of protein coding mRNAs at 10 and 80% at 5 minutes were ≥70% intact. Mining the RNA-seq datasets for 3'/5' end sequence ratios (number of sequence reads in the last and first 200 nt) of each mRNA at each time point identified total of 304 mRNAs with rapid decay of their 3' ends relative the their 5' ends. These mRNAs represent candidate sentinel RNAs. qPCR analysis of the 3' and 5' ends of candidate sentinel RNAs was measured to develop a rapid assay for their integrity. We identified several sentinel RNAs, for the conditions cited, that reflect general mRNA decay that could be measured by 3'/5' qPCR ratios. We are conducting similar studies of normal colon and colon cancer biopsies to identify sentinel RNAs for this tumor and tissue type and to determine there may be common sentinel RNAs between liver and colon. We conclude that RNA-seq analysis of differentially selected RNA in clinical biospecimens can identify *sentinel RNAs* that provide improved measures of general mRNA integrity prior to clinical gene expression testing.

This work is supported by CA148068.

38. Specific and Reversible Binding of DNA Nanoparticles to Cancer Cells

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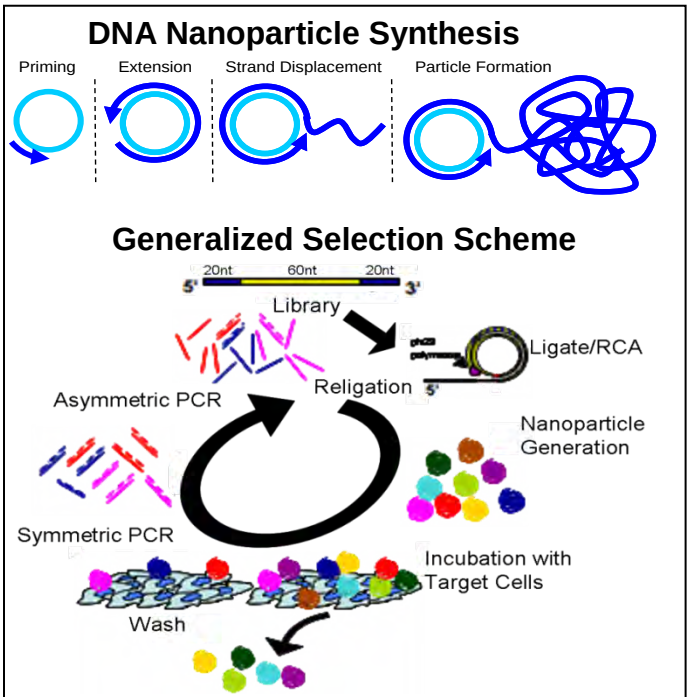

Figure 1. DNA nanoparticle synthesis and selection.

DNA nanoparticles (DNA-NP) are produced by enzymatic DNA synthesis using a strand displacing DNA polymerase (phi29) and a circular oligonucleotide template. The long single stranded product collapses into a particle containing many iterations of the complement of the template sequence. Template oligonucleotides containing random sequences generate highly diverse libraries of unique DNA nanoparticles. Since DNA can adopt secondary structures that exhibit molecular specificity, DNA-NPs with specific cell or other target binding properties can be selected by the biopanning process diagrammed in figure 1. DNA-NP that bound to the mouse pancreatic cancer cell line panc02 and the human breast cancer line MDA-MB-231 were selected over 6 rounds of biopanning and several binding particles recovered in each case. All of the panc02 binding particles contained an 8 nucleotide motif (AAnGGGGCG) and did not bind to other cell lines tested. Molecular modeling suggested that at least three of the four consecutive G were unpaired. Saturating that motif with a complementary locked nucleic acid could both inhibit the particle binding as well as release particles already bound to cells. Preliminary in vivo analysis in mice bearing subcutaneous panc02 tumors has shown that the panc02 binding particles are enriched in the tumor compared to control particles following both intravenous and intra-tumoral administration. Experiments to determine if the panc02 binding particles can inhibit primary or metastatic tumor development are ongoing. In addition, selection are being performed on panc02 tumors in vivo and those particles will be compared to those obtained by ex vivo selection. The MDA-MB-231 binding particles were found to by hybrid dimmers and trimers.. This was surprising since the PCR amplification typically deconstructs hybrids and multimers back to their monomer components in the absence of an intentional primer design to preserve hybrids from different libraries. Cloning revealed that the dimmers and trimers were all hybrids of different monomer units. One such clone was studied further as both monomer components and the dimer sequence. Modeling the predicted structure suggested the hybrid was able to preserve itself through the amplification because the primer binding sites are included in a large stem loop structure that out competed the PCR primers for binding. When made from synthetic templates, particles based on each of the monomer template sequences as well as the hybrid dimer bound to MDA-MB-231 and not to THP-1, an unrelated cell line. Binding of the dimer appeared stronger. Competition with oligonucleotides that bind to the internal 60bp sequences of the particles showed that each monomer was completely inhibited by its complementary oligo, whereas the dimer was reduced in binding when either of the monomer complements were used, but required both competitors to completely abrogate cell binding. The ability to form hybrid particles is a key benefit of this technology since the modular nature of the DNA-NPs allow for multiple discrete sequences to be displayed on a given particle. Combinatorial selection methods can then be used to “breed” particles with optimized activity by re-assorting the individual modules in each round. In this case, it happened spontaneously, further emphasizing the potential benefit of multi-module particles.

39. Standardized Mixtures of Internal Standards in Quantitative Sequencing Enables Inter-laboratory and Inter-platform Concordance and Reduces Costs by Log-Order Magnitudes

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Background: Next Generation Sequencing promises to enable cost-effective qualitative assessment of clinically important genetic variation as well as quantitative nucleic acid measurement of transcripts, breakpoint lesions and relative makeup of polymicrobial communities. However, there is still an unmet need to reduce quantitative sequencing costs and ensure inter-laboratory and inter-platform concordance. Discordance in quantitative sequencing data results, in part, from bias introduced during library preparation and platform-specific variation in sequence analysis (up to >1,000-fold variance). Cost of quantitative sequencing is primarily determined by method of library preparation and depth of sequencing required.

Approach: We addressed the challenges in quantitative sequencing library preparation through a novel combination of well-established methods, including (1) massively multiplexed PCR with low primer concentration and touchdown thermal cycling conditions, and (2) inclusion of a standardized mixture of internal standards at known concentration. The massively multiplexed PCR under low primer concentration and touchdown thermal cycling conditions enables specific amplification of both high and low-abundance target amplicons to equimolar endpoint concentration. Because each nucleic acid target is at approximately equal abundance at endpoint, this promises to markedly reduce the required read depth and thereby reduce quantitative sequencing costs by log-order magnitudes. However, without appropriate quality control the original quantitative relationships between nucleic acid templates will be lost during multiplex PCR-based library preparation. We propose to solve this challenge through introduction of a standardized mixture of internal standards into each sample prior to PCR. Based on extensive published experience, by measuring each native target relative to a known number of internal standard molecules, the values measured after multiplex PCR will accurately represent the relationship among nucleic acid templates in the original sample.

Results/Discussion: In preliminary studies, using multiplex PCR we bar-coded 333 unique target libraries from six equal aliquots of normal gDNA, each combined with a known concentration of serially titrated synthetic internal standard mixture (10^7-10^2 copies). A 15x read coverage at each internal standard titration point provided a 10^8x equivalent quantitative sequencing depth even for genes in balance with the most dilute concentration of internal standard and preserved the original inter-gene copy number relationship. Based on these results we conclude that, compared to other commonly used quantitative sequencing library preparation methods, the novel approach reported here will reduce required read depth for quantitative sequencing by log-order magnitude. Further, when the same standardized mixture of internal standards is used, results will be concordant across different laboratories and platforms.

Disclosures: JCW has equity interest in and serves as a consultant for Accugenomics, which develops and licenses the use of standardized mixtures of internal standards used in this study. There are no other potential conflicts of interest.

40. Successful Construction and Validation of Fluorescent Sensors for Monitoring GTP Levels in Living Cells

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GTP is the most common allosteric modulator of protein function and is therefore involved in numerous cellular processes important in both healthy function and disease. Methods to monitor GTP levels inside living cells, with high temporal and spatial resolution, would reveal how local and temporal variations in GTP concentrations are involved in regulating cell function, but such methods have not previously been available. We report the construction of a set of fluorescent GTP sensors in which a circularly permuted GFP variant is fused to a protein that undergoes a rapid conformational change upon GTP binding. By varying linker length and fusion position we were able to identify constructs in which the GTP induced conformational change in one protein was transmitted to the fused GFP to cause a 2-fold decrease in fluorescence from the latter. Mutations have been introduced into the GTP binding protein to alter its affinity for GTP so as to make the sensors responsive to GTP concentrations over a 10-1000 μM range. A second GFP variant, with distinct spectral characteristics, has been fused via a flexible linker to the termini of these sensors (so that it is insulated from the GTP induced conformational change) to provide a normalization signal so that GTP concentrations can be measured as the ratio of fluorescence between the two GFPs. These constructs therefore have all the required biochemical properties to act as sensors of GTP levels. We have now introduced these constructs into cells and shown that they show the expected changes in fluorescence when intracellular GTP levels are manipulated by adding either inhibitors of GTP synthesis (mycophenolic acid) or providing guanosine in solution. This approach to constructing a sensor that can report on GTP levels in living cells is therefore validated.

41. Targeting Cancer Cells With Functionalized Nanoparticle Libraries

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The surface of nanoparticles represents a unique environment for the presentation of ligands and allows for multiple low affinity interactions from multiply displayed ligands to develop high affinity through avidity effects. However, approaches to adapt, functionalize and optimize nanoparticles with targeting ligands rely on empirical testing to determine optimal ligand surface concentrations. Moreover, biological systems which often rely on avidity effects for interactions typically do not utilize high affinity ligands, but make use of multiple coordinated low affinity ligands to achieve high affinity and specificity. The goal of our work is to develop a novel system for the direct selection of functionalized nanoparticles capable of targeting human cancer cells. To do this, we will combine two extant technologies: nucleic acid based affinity agents known as aptamers and a methodology called in vitro compartmentalization (IVC). By combining these technologies, we will be able to generate large libraries ($10^{10\text{-}11}$) of nanoparticles which display multiple copies ($10\text{-}10^3$) of a single (clonal) aptamer sequence. The resulting functionalized nanoparticle libraries can then be screened directly for a desired function – in our case, binding and internalization by cancer cells.

42. Temperature-Tolerant COLD-PCR Enables Mutation Enrichment Using a Single Cycling Protocol For Diverse DNA Sequences

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Objectives: Identification of low-level mutations in cancer is of crucial importance for the diagnosis, prognosis and treatment of the disease. Often, these low-abundance mutated alleles fall below the limit of detection of current molecular methods, thus depriving the opportunity for optimal clinical management. We developed COLD-PCR, an IMAT-funded technology that magnifies unknown mutations during PCR prior to downstream mutation identification by sequencing or other methods (Li et al., *Nature Medicine,* May 2008). Since its original development, COLD-PCR has been used by several groups for diverse applications in DNA diagnostics such as somatic mutation detection, pre-natal diagnosis, infectious diseases and plant floral genetics. We recently demonstrated that COLD-PCR enables detection of 0.04% mutant alleles when combined with next generation sequencing technology (Milbury et al., *Clin Chem* 2012;58:580-9). However, a practical drawback of COLD-PCR is that it requires a stringent denaturation temperature control for a given amplicon. This hinders amplification and enrichment of diverse amplicons in different wells of a single thermocycler and complicates multiplex application of COLD-PCR prior to downstream sequencing. Here we describe a temperature-tolerant (TT-COLD-PCR) approach that circumvents this issue (*Clin Chem*, May 2012, Advance Online Publication).

Methodology: We developed a PCR cycling protocol that applies a modified, gradual increase in the denaturation temperature during TT-COLD-PCR. This enables preferential amplification of mutant DNA during PCR cycling. When the correct denaturation temperature is attained for a given amplicon, amplification *and* mutation enrichment are both initiated for this amplicon. TT-COLD-PCR is validated for *KRAS* mutations and *TP53* exon 6-9 mutations using dilutions of mutated DNA, clinical cancer samples and plasma-circulating DNA.

Results: Different DNA targets with various T_m values were enriched simultaneously using a single thermocycling program that spanned a denaturation-temperature window of 2.5-3.0ºC. Mutation enrichments observed were between 6-9-fold using a TT-*full*-COLD-PCR approach, while higher values were attained with other forms of COLD-PCR (*fast*-COLD-PCR and *ice*-COLD-PCR). We also present early results from the application of TT-COLD-PCR in emulsion, where multiple DNA targets are simultaneously enriched for mutations in a single tube format using TT-COLD-PCR.

Conclusions: TT-COLD-PCR enriches low-level mutations in amplicons with diverse T_m. This development enables simultaneous enrichment of mutations in different amplicons or in single-tube multiplex formats, and increases greatly the versatility of COLD-PCR. Application of COLD-PCR with microfluidic PCR technology (e.g., Fluidigm) or with emulsion-PCR (e.g., RainDance) prior to amplicon-based next generation sequencing becomes possible using TT-COLD-PCR.

43. Tissue Is Alive: Preserving Biomolecules and Tissue Morphology in Clinical Trial Samples

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To facilitate clinical trial molecular profiling, where immediate snap-freezing of tumor biopsies is not feasible, we have created a novel, one-step, room temperature preservative that stabilizes proteins/phosphoproteins/nucleic acids equivalent to snap-freezing and tissue/cell morphology equivalent to neutral buffered formalin fixation. Sources of pre-analytical variability in clinical specimens include tissue (cellular) heterogeneity and biomolecule stability. Laser capture microdissection has solved the issue of cellular heterogeneity. Our molecular fixatives Biomarker and Histology Preservative (BHP-Cell and BHP-Tissue) solve the problem of biomolecular instability in blood and tissue in one step. Technologies now exist that can measure protein phosphorylations and map cell signaling pathways in a single core-needle biopsy, thus relying on the inhibition of any kinase/phosphatase activity within the sample immediately following excision. Unfortunately applying these technologies to clinical samples has been hindered because phosphoprotein epitopes are not adequately preserved by formalin fixation and paraffin embedding, while freezing tissue samples may not be feasible in multi-center clinical trial sites and cannot adequately preserve morphology. Our preservative solution simultaneously fixes and decalcifies bony tissue, thus permitting molecular profiling of bony tissues that was never before possible. We are expanding our international clinical validation of this innovative technology for preserving tissue biomarkers (proteins/nucleic acids) in bone and brain tissue, as well as immuno-LCM applications for cell-type specific nucleic acids. We have collected twenty-two types of human tissues, representing 54 specimens to validate biomolecule and histomorphology preservation. Thirty-one different murine tissue types have also been evaluated, as well as whole mouse embryos at 13.5, 14.5 and 18 days gestation. Six international pathology practices have favorably and independently evaluated the fixative in 126 samples: Bone Cancer (50), Lung Cancer (19), Ovarian Cancer (11), Breast Cancer (10), Renal Cancer (10), Colon Cancer (4), and Other (22). Preservation of tissue morphology has been demonstrated with 56 antigens using standard IHC protocols. Samples of BHP have been distributed to 20 different institutions through either research collaborations (MTA) or formal requests for evaluation-size samples of our Biomarker and Histology Preservative. RNA preservation in laser microdissected brain blood vessels has been verified using an innovative protocol for directly amplifying RNA without prior RNA extraction. We have utilized BHP-Cell in a breast cancer multi-site clinical trial (US Oncology 05-074/GSK LPT109096) and a clinical research multiple myeloma trial. BHP has been validated to (a) function as a transport medium while preserving histomorphology, (b) maintain full antigenicity for clinical immunohistochemistry (such as Ki-67, ER, PR, Her2, p63, and phosphorylated epitopes), (c) preserve phosphoprotein epitopes for cell signaling pathway profiling by reverse phase protein microarray (RPMA), (d) be compatible with frozen sections or paraffin embedding, and (e) obviate the need for additional decalcification. RPMA data from LPT10906 shows differentially deranged signaling networks in the pre-treatment biopsies for patients that did not have a pathologic complete response compared to responders. We are currently exploring the capabilities of BHP Cell and BHP Tissue to preserve the integrity of nucleic acids, in parallel with proteins, thereby providing a universal one step biospecimen tissue fixative for clinical trials.

44. Ultra-Throughput Multiple Reaction Monitoring Mass Spectrometry for Large-Scale Cancer Biomarker Validation

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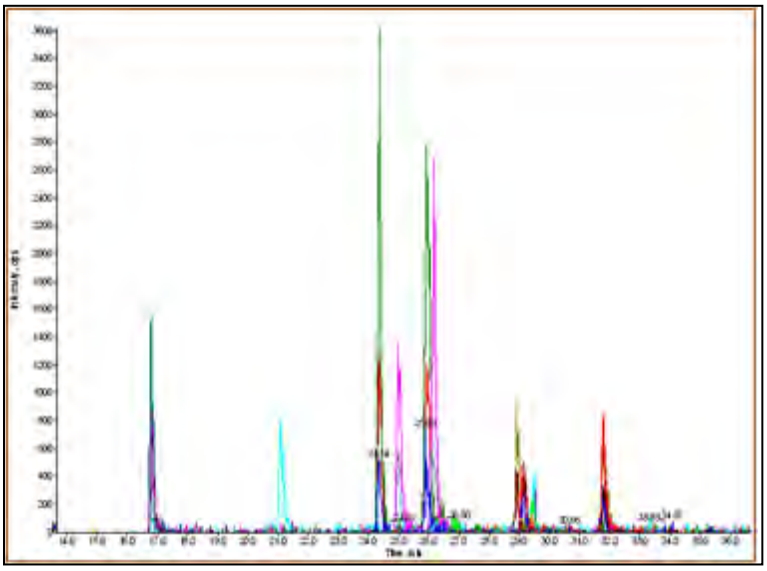

This project develops a technology to significantly improve the sample throughput for mass spectrometry-based quantitation of cancer protein biomarkers, i.e., the number of patient samples that can be quantified by mass spectrometry in a unit time. In the first year of the project we worked on the Specific Aim 1 to identify suitable peptide derivatizations for LC-MRM MS measurements. We have explored a variety of strategies for synthesizing chemicals to derivatize signature peptides of protein markers. There are two major classes of approaches that are investigated. One is to synthesize magnetic nanoparticle reagents to facilitate the peptide derivatization and associated sample cleanup. Although this study produced desired derivatized peptides with free N-terminal amines, the product yields were low at this stage of the study. Our current efforts focus on the originally proposed approach, which develops a large collection of peptidyl reagents for derivatizing peptides via conventional solution reactions. We have, via solid-phase synthesis, generated a pool of short peptides, which are 2-4 amino acids in length and have hydrophobic amino acid residues in composition. This pool of peptidyl reagents have been further diversified via the addition of more than 10 different capping groups at the N-termini of the peptidyl reagents. We currently have made more than 200 different reagents which are being screened for the suitability assignature peptide derivatizing reagents. More importantly, we have established standard synthesisroutes, which allow us to generate much larger numbers of reagents fast. These reagents will be used, together with the existing ones, for screening and selecting the needed derivatization reagents to implement the Specific Aim 2 in the second year of the project. The selection of peptidyl reagents is done by LC-TOF-MS or LC-MRM MS. In the figure are chromatograms for underivatized and derivatized peptides with the same amino acid sequence and at the equal molar ratio. The chromatograms are plotted for the peptide signal intensities against the elution times. The first group of peaks on the left is for an underivatized peptide and the others are for the derivatized ones. When a mass spectrometric peak is stronger than the peak for the underivatized counterpart peptide on the left, the corresponding derivatizing reagent becomes a candidate. In the set of reagents used for derivatizing the peptides in this figure, several have the increased signals, thus qualified, and several have the decreased signals, thus failed. Multiple reagents can be screened in a single analysis, and we have performed a one experiment screening of up to 24 peptidyl reagents. We have made key progress towards the successful implementation of the project: (1) finalized the selection of signature peptides for the model marker protein; (2) established a fast, general route for synthesizing derivatizing reagents; (3) optimized the procedure and conditions for the peptide derivatization; and (4) developed methods for quantitative comparison of the derivatization efficiency and the mass spectrometry signal yield.

45. Understanding Stromal-Cancer Cell Interactions via a Microscale 3D Model of DCIS

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The progression of early breast cancer advances through atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). The progression from DCIS to IDC is considered a lifethreatening step because it is directly associated with poor prognosis. Mounting evidence shows that the DCIS progression is actively driven by complex interactions with the surrounding microenvironment (both the matrix and various stromal cells). There is a need for an in vitro model of DCIS progression that captures key features of the in vivo microenvironment. Functional in vitro systems can lead to new insights that have been overlooked due to the limitations of traditional in vitro systems - in particular understanding the role of the stroma is understudied yet the stroma holds great potential for new therapeutic approaches.

We developed an efficient 3D microfluidic system that supports the transition from DCIS to IDC (Fig. 1). The in vitro system employs arrays of microchannels each with two inputs and one output enabling MCF10-DCIS.com cells (MCF-DCIS) and human mammary fibroblasts (HMFs) to be loaded into two adjacent (side-by-side) compartments. Importantly, the compartmentalized platform enables monitoring of both MCF-DCIS cells and HMF independently, including quantitative measures of the collagen architecture associated with each cell type. The arrayed microchannel platform is compatible with existing HTS infrastructure such as pipetting robots and plate scanners. Because of the multiple endpoints obtainable from a single assay, this platform enables a high content and high throughput screening assay that can be used to discover how the interplay between cancer cells and stromal cells regulate progression.

Of particular importance is the ability to dissect the relative role of fibroblasts which is understudied, but could lead to novel targets for therapy. Very few studies have explored how cancer cells alter fibroblasts, which may in turn foster DCIS progression. This is largely due to the lack of an in vitro system that facilitates monitoring of the changes in both cancer cells and fibroblasts. Using the compartmentalized system, we observed that the HMFs near MCF-DCIS became more protrusive compared with HMFs that were relatively far from MCF-DCIS. We have also begun to identify how the HMFs become activated and protrusive when co-cultured with MCF-DCIS and to understand the biological function and impact of protrusive HMFs during DCIS progression to IDC. We show that migration of HMFs is strongly reduced after exposure to MCF-DCIS conditioned media, and that it inversely correlates with the switch to a more protrusive activity. We developed novel imaging analysis algorithms to quantify the morphology of HMFs, the protein localization within them, and the alterations in the surrounding ECM. We verified that the signaling based on Cathepsin D (CTSD) produced from MCF-DCIS and low-density lipoprotein receptor-related protein-1 (LRP1) in

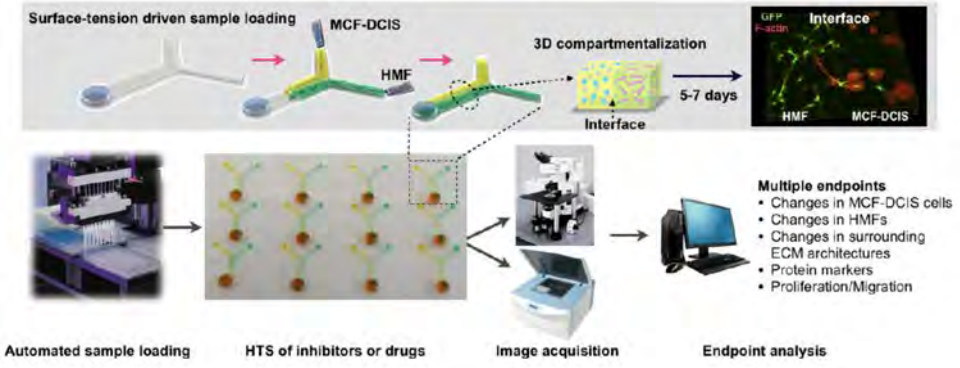

Figure 1. 3D compartmentalized system to investigate the progression of DCIS to IDC. After 5-7 days of co-culture, MCF-DCIS cells (Red) at the interface show invasive transition. Moreover, GFPHMFs (Green) at the interface show more protrusions. The system is compatible with existing HTS infrastructures and provides multiple endpoints.

HMFs was necessary for the protrusive activity of HMFs. Our model demonstrates one possible route through which MCF-DCIS activates pre-existing fibroblasts and subsequently, leads to the modification of the ECM and the progression to IDC. Improved understanding of the mechanisms involved in DCIS progression has the potential to guide the development of new anti-cancer strategies that target the stroma. The simplicity of the platform makes it accessible even to researchers without prior experience with microfluidics enhancing its potential impact.

46. Use of Nanogels to Target Delivery of siRNA to Cancer Cells in Mice

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The potential utility of small regulatory RNAs (siRNAs, miRNAs) as cancer therapeutic agents is dependent upon effective mechanisms to target their delivery to cancer cells. We have previously established that functionalized hydro nanoparticles can efficiently transport therapeutic RNAs to ovarian cancer (OC) cells in vitro. To facilitate extension of these studies to *a xenograft (intraperitoneal) mouse model of OC*, we have (1) modified the surface of our hydro nanoparticles with polyethylene glycol chains (PEG) to minimize uptake by macrophages and increase uptake by targeted OC cells; (2) established an accurate range of non-toxic levels of hydro nanoparticles to be used in the in vivo studies; (3) established a highly metastatic OC cell line (Hey A8-F8) stably transfected with the pGL4.51[luc2/CMV/Neo] luciferase reporter vector; (4) demonstrated that these stably transfected cells grow aggressively in nude (nu/nu) mice after intraperitoneal implantation.

47. Validation of MALDI-MS-Based Inhibitor Screening Technologies for Cancer Targets

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Mass spectrometry (MS) has a long history as a transformative technology. Two specific examples include quantitative MS to measure the fate of drug compounds in biological systems and the development of proteomics techniques for protein identification and characterization. Recently MS-based applications have been demonstrated to be highly effective technologies for high throughput screening (HTS). The major advantages reported over the commonly used fluorescence and chemi-luminescence readout is the paucity of false positive or false negative readout, reduced reagent costs and the ability to multiplex assays such that multiple therapeutic targets can be screened for inhibitor hits with one pass through the compound repository, thus achieving substantial savings in reagent and personnel costs. We have shown previously that MALDI-MS readout is amenable to enzyme assays and inhibitor screening on a small scale. If however, the MALDI-MS technology can be scaled to true HTS levels while maintaining all the advantages seen in the small-scale studies, then this technology would likely transform the way we approach compound screening is drug discovery applications. Thus the primary goal of this proposal is to scale up the MS-based readout technology to include a library of >30,000 compounds to systematically validate the reliability of the MALDI-MS readout head-to-head versus traditional methods of HTS. Validation measurements include the primary screening hit rate, validation rate, false positive rate, signal to background, coefficient of variance (CVs) and Z' values (Aim 1). The target enzymes for these comparative assays include PKC-zeta and PKC-iota, since PKC-zeta has been reported to act as a tumor suppressor while PKC-iota has been shown to have oncogenic potential in some cell types. A secondary outcome (Aim 2) is to assess the effectiveness of the MALDI-MS technology to distinguish inhibitors that have selectivity for PKC-iota over PKC-zeta. The primary goal of Aim 1 was to scale up the MS-based screening technology (using PKC-iota) to compare traditional screening readout with the newly developed MS-based technology. As a first step in the process, the PKC-iota assays was re-optimized for both a fluorescence- and MS-based readout using three known kinase inhibitors (RO-318220, GF109203X, 981261). Next, the assays were adapted and validated in a 384-well format using the fully-automated PerkinElmer Plate:Explorer HTS system. For the fluorescence assay, the DiscoveRx ADP HitHunter fluorescence assay was used. The primary screen with the fluorescence assay included 30,400 compounds from a diversity set library selected from our 300K compound repository. Compounds were tested in the single-point primary screen at 21.8μM for their effect on PKC-iota activity. From the 30,400 compounds tested in the fluorescence assay, 276 compounds inhibited PKCiota by more than 25% (a 0.9% hit rate). These compounds were tested in triplicate for confirmation at 21.8 μM. 180 compounds were confirmed as hits causing more than 30% inhibition of enzyme activity (a 65% hit confirmation rate). To determine the false positive rate of the ADP HitHunter assay, a triplicate assay of the 180 confirmed hits was also conducted in the absence of PKC-iota. From the 180 compounds tested, 105 compounds inhibited detection of ADP using HitHunter Assay in the absence of PKC to > 30% (a 58% false positive rate). The overall quality control measurement included a mean Z' value of 0.73 for the primary screen, 0.77 for triplicate confirmation and 0.89 for the false positive evaluation (Z' value > 0.5 is considered to be an excellent HTS assay method). For the MS-based assay, adapting the readout to a fully automated analysis included transforming the enzyme reaction and sample preparation onto the automated PerkinElmer Plate:Explorer HTS platform. The MALDI-MS method required proper spotting and drying of sample on MALDI steel sample plates. We have now successfully customized and integrated the MALDI plates with an effective drying mechanism into the HTS system in a 384-well format and optimized all the assay parameters (e.g. enzyme and substrate concentrations, incubation time) and we are on track for the full screen of the 30,400 compounds (slated for July 2012). The overall significance of this research will not be fully realized until the completion of the comparative studies with the MS-based technology and the generation of any compounds that may show selectivity between PKC-iota and PKC-zeta. However from our pervious smaller scale studies (1000 compounds) of the MS-based technology that maintained a hit confirmation rate of 100% with no detected false positives, it is expected that the full scale screening data from the MS-based technology will show far superior results to the 65% hit confirmation rate and the 58% false-positive rate displayed so far for the HTS standard fluorescence-based assay of the 30,400 compounds. To conclude, significant progress has been made on Aim 1 of the project with the completion of the fluorescence-based screen and the staging the comparative MS-based screen. Our next steps will include completing the primary and secondary screens using the

MALDI-based technology to generate all the quality control data (hit rate, confirmation rate, false positive rate and Z'-values) needed to assess the comparative advantages of each. In addition, we will have a pool of candidate compounds that will then be available to evaluate selectivity for PKC-iota versus PKC-zeta as described in Aim 2.

48. VEC^3-Valve Enabled Cell Co-Culture Platforms for Cancer Biology Study

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Tumor microenvironment is being recognized to play critical roles in tumor progression. While some approaches have been developed to study the tumor microenvironment, a major gap still exists as the lack of a reliable method to study the interactions between tumor cells and adjacent cell populations in real-time where physiologic and signaling events can be measured quantitatively. We are developing Valve Enabled Cell Co-Culture (VEC^3) platforms, a new class of microfluidic devices for analyzing interactions between adjacent cell populations seen in the tumor microenvironment. The technology allows for separate culture and treatment of distinct cell populations in close proximity and cell-cell interactions through either soluble factors or physical cell contact while maintaining fluidic control over the microenvironment. Using two-dimensional (2D) VEC^3 platforms, we have demonstrated the function of VEC^3 in studying the effects of hypoxia in tumor cell-endothelial cell cross-migration through creating hypoxic microenvironment for tumor cells. We have also verified the effects of EphA2 receptor on tumor cell-endothelial cell cross-migration. The platform will also be used to dissect signaling pathways in vascular endothelial cells by observing the cross-migration between tumor cells and wild-type or rictor-deficient vascular endothelial cells. We have found that rictor-deficient endothelial cells are defective in VEGF-induced proliferation, migration, and vascular assembly in vitro and angiogenesis in vivo. We are currently testing if tumor-endothelial cross migration is also affected in rictor-deficient endothelial cells. Additionally, we discovered that rictor-deficient cells secret elevated levels of certain inhibitory chemokines. Through introduction of nanoporous collagen gel with embedded biotin-coated polystyrene microparticles (0.7-0.9 μm diameter) between two cell chambers, we have successfully demonstrated the selective blockage of avidin with concurrent perfusion of ovalbumin between two cell populations. Design rules have been developed for this novel concept of 'ligand traps', which will not perturb the optimal culture conditions, to work for weeks. In addition to 2D platforms, three-dimensional (3D) co-culture has been achieved in the VEC^3 platform by loading cells within biogels. We have demonstrated this with co-culture of cancer cells with stromal fibroblasts. Several types of head and neck cancer cells, including SCC61 and JHU, have been co-cultured with primary fibroblasts from human cancers (CAFs) or from normal tissue (NAFs) in 3D type I collagen matrices. Time-lapse imaging shows that SCC61 cells migrate directionally along CAFs, while they migrate randomly when co-cultured with NAFs. To quantify this, we calculated an association index between SCC61 cells and fibroblasts by measuring the intersecting angles between the cells and taking the cosine of this angle. The association index for SCC61 cells and CAFs was 0.76±0.07, whereas SCC61 cells and NAFs had an index of 0.43±0.03. These results suggest that SCC61 cells more closely associate with CAFs than with NAFs, and this association regulates the directional movement of the SCC61 cells. Interestingly, CAFs from various tissue, including prostate and breast, similarly influence the migration of SCC61 cells, suggesting a common mechanism by which stromal fibroblasts regulate cancer cell migration. In addition, through immunostaining fibronectin, it was found that CAFs organized the fibronectin into a parallel fiber structure, which is distinct from the meshwork-like arrangement of fibronectin found with NAFs.

49. Development of a Nanoscale Calorimeter

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The phenomenon of extraordinary optical transmission (EOT) coupled to a microfluidics system serves as the basis for the creation of a new approach to calorimetry used in pharmaceutical development processes. EOT enables extremely small, sensitive, noncontact temperature sensors that can be used to determine thermodynamic properties of reacting compounds. When these sensors are placed in a grid, the resulting system has the potential to reduce compound usage 500 to 1000-fold and deliver significant improvements in sample processing throughput. A microfluidic delivery design for performing a co-flow reaction in a microchannel will be discussed. The reported data and developed algorithms confirmed that enthalpy of reactions can be measured in the picojoule range and will enable the researcher to gain deep insight into the nature of a binding reaction. Calorimetry technology is extremely valuable in the context of drug development, but conventional ITC finds very limited usage because the amount of sample required is prohibitive and it cannot be multiplexed. Improvements to the microfluidic system used in the EOT-based calorimeter have proved to be more accurate than the volumetric sample injection method. The nanoscale calorimeter technology has the potential for multiplexing and obtaining more informative high-throughput screening data.

Resources and Funding Opportunities

Resources

- TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp) provides a platform for researchers to search, download, and analyze datasets generated by TCGA. It contains clinical information, genomic characterization data, and high-throughput sequencing analysis of the tumor genomes.
- NCI Proteomics Data Portal (https://cptac-data-portal.georgetown.edu/cptacPublic/) provides proteomics datasets of breast, ovarian, and tumor tissue that have also been genomically characterized by TCGA datasets.
- The Antibody Characterization Laboratory (http://Antibodies.cancer.gov) provides access to a large number of reagents and accompanying characterization data. Antigens and antibodies are expressed, purified, and characterized using standard operating procedures, with all accompanying protocols and data.
- NCI Best Practices for Biospecimen Resources guiding principles define state-of-the-science biospecimen resource practices, promote biospecimen and data quality, and support adherence to ethical and legal requirements (http://biospecimens.cancer.gov/practices/default.asp).

Funding Opportunities

- Innovative Molecular Analysis Technologies (IMAT) reissuance
  - Early-stage innovative molecular analysis technologies for cancer research (R21, up to 3 years and $500k in direct costs) – RFA-CA-13-001
  - Advanced development and validation of emerging molecular analysis technologies for cancer research (R33, up to 3 years and $900k in direct costs) – RFA-CA-13-002
  - Early-stage innovative technologies for biospecimen science (R21, up to 3 years and $500k in direct costs) – RFA-CA-13-003
  - Advanced development and validation of emerging technologies for biospecimen science(R33, up to 3 years and $900k in direct costs) – RFA-CA-13-004

- NCI Provocative Questions (PQ) initiative (http://provocativequestions.nci.nih.gov/), to support research projects designed to use sound and innovative research strategies to solve specific problems and paradoxes in cancer research
  - Group A covered by RFA-CA-12-015 (R01) and RFA-CA-12-016 (R21) that generally relates to cancer prevention and risk
  - Group B covered by RFA-CA-12-017 (R01) and RFA-CA-12-018 (R21) that generally relates to mechanisms of tumor development or recurrence
  - Group C covered by RFA-CA-12-019 (R01) and RFA-CA-12-020 (R21) that generally relates to cancer detection, diagnosis, and prognosis
  - Group D covered by RFA-CA-12-021 (R01) and RFA-CA-12-022 (R21) that generally relates to cancer therapy and outcomes

- Informatics Technology for Cancer Research (ITCR) program (http://itcr.nci.nih.gov)
  - Early-stage development including initial development (prototyping) and modification of existing methods for new applications: collaborate with NCI grantees and target naïve users for up to $150k DC/yr for 2 years - PAR-12-286[R01 supplement], PAR-12-290[P01 supplement], PAR-12-289[U01 supplement]
  - Early-stage development (U01) at the prototyping and hardening stages, for up to $250k DC/year for 3 years - PAR-12-288
  - Advanced development (U24) at the enhancement, dissemination, and maintenance stages: target both naïve users and power users, for up to $500k DC/year for 5 years - PAR-12-287

- NCI Small Business Innovation Research Development Center offers an array of grant and contract awards and other resources for the cancer research community. Find out more at http://sbir.cancer.gov

- Bioengineering Research Grants program (http://www.nibib.nih.gov/Funding/Bioengineering)
  - Multidisciplinary research that applies an integrative, systems approach to develop knowledge and/or methods to prevent, detect, diagnose, or treat disease or to understand health and behavior. Exploratory BRG (R21, up to $275k DC/2 years), BRG (R01), and BRP (large partnerships R01).

- Research Supplements to Promote Diversity in Health Related Research
  - Research supplements to support and recruit students, postdoctorates, and eligible investigators from groups that have been shown to be underrepresented in cancer and cancer health disparities research (PA-12-149)

- Other Current NCI FOAs
  - PA-11-158 & -159 Biomarkers of Infection-Associated Cancers (R01 and R21, respectively)
  - PA-12-213 & -214 Identifying Non-coding RNA Targets for Early Detection of Cancer (R01 and R21, respectively)
  - PA-12-221 & -220 Biomarkers for Early Detection of Hematopoietic Malignancies (R01 and R21, respectively)
  - PAR-12-039 Small Grants Program for Cancer Epidemiology (R03)
  - PA-11-297 & -298 Pilot studies in Pancreatic Cancer (R21 and R03, respectively)
  - PA-10-290 & -291 Research on Malignancies in the Context of HIV/AIDS (R01 and R21, respectively)
  - PA-11-073 & -074 Mitochondria in Cancer Epidemiology, Detection, Diagnosis and Prognosis (R01 and R21, respectively)
  - PA-11-151 Strategic Partnering to Evaluate Cancer Signatures (SPECS II) (U01)
  - PA-12-013 & -014 Validation of Molecular Diagnostics to Predict Patient Outcomes Using Specimens from Multi-Site Cancer Trials (R01 and R21, respectively)

- Coming soon (approved but no FOA assigned by time of printing)
  - Bridging the gap between cancer mechanism and population research: brings together systems biology + epidemiology to connect mechanism to population outcomes
  - Collaborations with the Integrative Cancer Biology Program (ICBP): multi-PI U01s, one from within ICBP one from outside ICBP to collaborate on anything related to cancer systems biology
  - Pending challenge/prize topics on BIG DATA from the NIH Common Fund in the next year or so

- And please always check the following for opportunities
  - NCI Center for Strategic Scientific Initiatives @ http://cssi.cancer.gov/resources-current_funding.asp
  - NCI Research Funding Opportunities @ http://www.cancer.gov/researchandfunding/funding/announcements
  - NIH Common Fund Initiatives @ http://commonfund.nih.gov, especially the "High-Risk Research" programs @ http://commonfund.nih.gov/highrisk/index.aspx

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