# Speaker Abstracts

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## Abscription-Based Bisulfite-Free CpG Island Detection

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We have developed a rapid and sensitive method for detection of methylated CpG islands that does not involve bisulfite treatment. Genomic DNA is fractionated to separate methylated from unmethylated DNA. Targeted islands are then amplified from the methylated and unmethylated fractions. The amplification step is coupled to a linear signal amplification process called Abscription. By coupling DNA target amplification with a linear signal amplification process that produces up to 10,000 signals per target per minute, very sensitive and rapid detection assays are possible. Abscription stands for abortive transcription in which an RNA polymerase binds to and becomes trapped at an artificial promoter and reiteratively makes short oligonucleotides rather than full length transcripts. The products of abscription are abscripts (abortive transcripts) which can range in size from 3 to 12 nucleotides. Uniform populations of abscripts can be made by synthesizing specific Abortive Promoter Cassettes (APCs) designed via their sequence to encode specific abscripts.

Because different APCs encode different abscripts, multiplexed biomarker detection is possible by attaching different APCs to different molecular targets in a sample (DNA, RNA, protein, SNPs or Me-CpG islands). Abscripts can be detected by a variety of methods including thin-layer-chromatography (TLC), mass spectrometry (MS), capillary electrophoresis (CE) and fluorescent molecular-beacon opening. We have attached APCs to CpG Islands that have been fractionated to separate methylated from unmethylated DNA and have detected and quantified the abscripts produced to determine the level of methylation of different islands in samples. Analysis by TLC allows for very rapid and affordable qualitative and semi-quantitative analysis of methylation levels. Analysis by mass spec provides a higher level of sensitivity and allows for accurate quantitation of abscripts.

The detection process begins with the isolation of methylated DNA fragments using a glutathione-S-transferase fusion protein that is linked to the mouse MBD2 methyl-CpG binding domain. This has been incorporated into a commercially available MethylMagnet mCpG DNA Isolation Kit, with which methylated DNA is captured to glutathione magnetic beads followed by elution under conditions that allow amplification of CpG islands by PCR without further purification. The performance of this system was validated with synthetic DNAs containing defined patterns of CpG methylation. Binding of unmethylated DNA was undetectable even at the lowest stringency tested. Our standard stringency requires 6 or more methylated CpG sites per island for efficient capture, although a requirement for more or fewer methylated CpGs can be imposed by altering the NaCl concentration of the binding and wash buffers.

Abscription-based detection was incorporated by amplifying CpG islands with primers with attached APCs. The APCs are inactive in the single-stranded form and became activated when copied during PCR amplification of the islands. PCR reactions were subjected to abscription for up to 1 hr following amplification. The yields of abscripts from the methylated and methylated fractions were quantified by MS which agreed with the direct detection of amplicons by gel electrophoresis and detection of abscripts by TLC. At low DNA inputs it was possible to detect signals by MS that were not detectable by electrophoresis. The added sensitivity provided by abscription-based signal amplification allows the use of 1 ng or less of genomic DNA in the initial MethylMagnet isolation step and as little as 40 pg, or 12 copies, to be detected rapidly by abscription and TLC and fewer than 3 copies by abscription and mass spec. Analysis of DNA from tumors and paired normal cells revealed aberrant methylation patterns of some islands in tumor samples, as well as a few islands that were also methylated in the matched normal samples. These similarities presumably reflect low levels of early methylation events that occurred before formation of the tumors.

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

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

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

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

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

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

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

## Architectural Proteomics for Early Risk Assessment in Breast Cancer

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

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

## Bead Array-Mass Spectrometer: Equipment and Method Development

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Technologies that drive bio-discovery are progressively shifting toward highly parallel, high-throughput, and highly multiplexed approaches that are able to extract larger amounts of data from smaller samples at increasing cost effectiveness. We are developing a new technology that takes advantage of the high resolution and sensitivity of Inductively Coupled Plasma Mass Spectrometry (ICP-MS), combined with the many available stable isotopes of elements of the periodic table, to simultaneously determine many proteins and gene transcripts in samples through the quantification of stable isotope tags bound to a wide variety of bioaffinity molecules. The instrumental heart of this integrated massively multiplexed technology is the Bead Array-Mass Spectrometer (BA-MS).

The BA-MS derives, in part, from our earlier prototype of an ICP-MS analog of flow cytometry, called a Mass Cytometer. In the single cell cytometer configuration, elements of the periodic table are used as tags in a manner similar to flow cytometry, and the tagged cells are vaporized, atomized, ionized and quantified in the ICP-MS. The BA-MS addresses the challenge of massively multiplexed analyses of cell lysates. It "reads" metal-encoded functionalized polymer microbeads that incorporate various concentrations and ratios of metal ions: ten elements at 4 distinct concentrations provide for more than a million distinguishable beads. The beads are functionalized to expose affinity reagents (antibodies, oligonucleotides, etc.) in a manner that the bead encoding identifies the target analyte. After exposure to the sample, bound analytes are sandwiched with a reporter tag element, and the ensemble is analyzed one bead at a time (at up to 1000 beads per second) correlating the intensity of the reporter tag element (concentration of the analyte) with the encoding that identifies the analyte.

The BA-MS concept provides opportunities for enhancement of sensitivity, throughput and simultaneous reduction of cost relative to the Mass Cytometer. This is largely because a limited number of pre-determined elements are to be analyzed (e.g., 10 encoding elements and one reporter element). This allows optimization of the ion optics, vacuum and data acquisition systems. We have performed early investigative experiments to determine the cost and performance opportunities that this provides.

We will also report proof of principle of the technology. A variety of metal-encoding strategies have been explored, and the resultant beads have been analyzed on the Mass Cytometer platform. It is demonstrably clear that metal-encoding of beads is feasible and quantifiable. A significant challenge is the functionalization of the beads, for which we report first successful results.

The technology represents a significant challenge for software development. For the BA-MS application of many-dimensional measurements, the principal need is for recognition of specific multivariate bead signatures. We will discuss our approach to the development of a suitable mathematical approach to data analysis with limited reliance on visualization.

Metal encoded beads coupled with BA-MS have the potential to revolutionize immunoassays, gene expression studies, cancer research and clinical diagnostics. The technology overcomes some limitations of fluorescent-based suspension assays. The number of unique beads is theoretically limitless. The beads are insensitive to light, and do not leach or bleach. They are stable and do not require special storage conditions. There is no background interference from sample containers and biological molecules. However, issues remain to be addressed, such as the complexity and expense of BA-MS systems; the uniformity of microbead size, which influences the analysis; and the development of sophisticated software for multiplex assays.

## Capillary Electrophoresis for Analysis of Barrett's Esophagus Biopsies

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The study of the protein expression has shown promise to predict disease progression. In order to exploit the potential of protein expression as a prognostic tool, the proteins must be quickly separated and detected at very low concentrations. We performed Capillary Isoelectric Focusing (cIEF) on a post-column sheath flow cuvette system with ultrasensitive laser-induced fluorescence detection. Proteins were labeled with Chromeo P503, and we obtained detection limits in the low attomole (10-18 mol) range. A standard solution of four proteins was separated with high resolution; we achieved a correlation coefficient of 0.9 for the relationship of pI and molecular weight. A protein homogenate from a Barrett's esophagus biopsy was also analyzed; over 150 components were resolved within 35 minutes. However, due to background fluorescence

produced by trace impurities within the ampholytes used in the separation, detection limits for cIEF were four orders of magnitude poorer thenCE methods. In order to improve detection limits, we explored different method to decrease the fluorescent background such as photobleaching, oxidation reaction, and carbon treatment. Nearly two orders of magnitude improvement in detection limits resulted from shifting the excitation and emission wavelengths to 532/580 nm and by photobleaching the ampholytes before use. Detection limits are 150 zeptomole (1 zmol = 10-21 mole) for standard proteins.

## Development of Dynamic Single Molecule Analysis Platform Using Nanochannel Array

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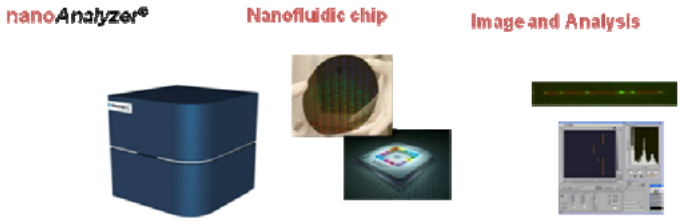
BioNanomatrix is developing the world's first commercial system (NanoAnalyzer®) for high throughput dynamic single molecule analysis using a high density nanochannel array platform. The core technology consists of a penny sized nanofluidic chip with thousands of massive parallel channels of less than 100 nm fabricated on the surface of semiconductor or other solid state material. Long native state genomic DNA molecules of 100's kbs to multi-megabases even chromosomal length can be streamed continuously into these long channels and individually confined and linearized for analysis. With sequence specific single molecule labeling technique developed at BioNanomatrix, this system can allow direct imaging of genome structural information with a unique barcode pattern along long linear DNA molecules, the barcode patterns and the change of them could be correlated to reference databases and biomarkers such as disease and cancer associated genomic arrangements and copy number variations. Due to the massive parallel nature of long genomic sample anaysis, unprecedented throughput can be achieved such as imaging of 1-10X human genome coverage within 1 hour while patient's valuable genomic structure information preserved. Because of single molecule level sensitivity, rare sample derived from needle biopsy or stem cell research containing as few as a single cell could be analyzed on this platform.




BioNanomatrix technology can potentially deliver direct comprehensive image analyses of genome and epigenome on single-cell and single-molecule level, while avoiding the fragmentation and complex data re-assembly required by other approaches. The affordability, speed, and simplicity of the technology are expected to make the routine use of genetic information feasible in broad-ranging applications including genetic diagnostics, personalized medicine, and daily biomedical research.

## Fluorescent Tracking of DNA Damage and Repair in Living Cells and Tumors: A Novel Tool for Cancer Biology and Drug Discovery

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

directed toward developing a novel reporter of DNA damage response that will allow tracking of the formation and resolution of DNA damage foci in normal and breast cancer cells. Toward these ends, we are developing fluorescent protein fusions based on 53BP1 that accumulate at double strand breaks. GFP fused to the checkpoint protein 53BP1 IRIF binding domain (GFP-53BP1-IBD) forms fluorescent foci at DSBs in living cells, providing a tag for both visualization and biochemistry. In this project, we are using these fusion proteins to follow foci kinetics after ionizing radiation in vitro and in vivo, for high content screening to discover new small molecule modulators of DNA damage repair and as proteomic probes to identify proteins that constitute IRIF. As an initial application of this technology, we tracked IRIF kinetics in breast cancer cells and tumors. We expressed GFP-53BP1-IBD under tetracycline-inducible control in MCF7, a p53 positive, caspase-3 negative, apoptosis resistant human breast cancer cell line. While IRIF largely resolved within 24 h after 3 or 6 Gy, higher IR doses greatly increased IRIF persistence. Screening candidate shRNAs and small molecules targeting chromatin modifiers revealed that poly(ADP-ribose) polymerase (PARP) inhibitors promote IRIF persistence. When combined with a PARP inhibitor (PARPi) currently in clinical trials, the clinically relevant dose of 3 Gy induced an IRIF response equivalent to >6 Gy and markedly decreased clonogenic survival. At 7 days, PARPi increased residual IRIF numbers, senescence associated beta-galactosidase activity and p21CIP levels. We used the GFP-53BP1-IBD expressing MCF7 cell line to form xenograft tumors in nude mice. Foci kinetics imaged with two photon microscopy were similar to cells in vitro and PARPi induced similar delays in foci kinetics. We treated MCF7 tumors with a single dose of radiation with or without PARPi and excised the tumors after 7 d. PARPi plus 3 or 6 Gy produced SA-βGal staining in tumors to the level observed for 9 or 12 Gy alone and markedly decreased tumor growth. Our data suggest that PARPi and other agents that stabilize IRIF and induce cell senescence may find broad uses as radiosensitizers for cancer therapy. Based on these studies, we have used high content screening to discover novel small molecule modulators of DNA damage response, based on perturbation of GFP-53BP1-IBD foci formation and kinetics after a 6 Gy dose. In a screen of 2000 drugs and natural products, we recovered nine compounds that blocked foci formation, including three previously described radiosensitizers. We are characterizing the novel compounds for effects on radiation response. We have also identified other compounds that increase foci persistence. We have also performed an initial proteomic screen to determine the protein components of the GFP-53BP1-IBD foci and their dynamics during repair. We used formaldehyde cross-linking, chromatin preparation, pull-down with anti-GFP magnetic beads, gel electrophoresis and Western analysis and mass spectrometry to identify IRIF proteins. Histones were readily identified. DNA damage response proteins identified by LC-MS/MS include PARP-1, DNA-PK, FACT complex, Ku70/80, topoisomerases and histone deacetylases (HDACs). We anticipate that this new technology can be used to determine the radiation responses of tumors and normal tissues, to evaluate radiosensitizers and other drugs affecting DNA damage response and to discover and validate new biological targets to enhance radiation effects.

## Genome Sequencing Technology for Studying Melanoma

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We have made significant progress on further developing polony sequencing technology during the past year. Namely, we have currently sequenced ~24 invasive Group A Streptococcus (GAS) clinical isolates. The GAS strains we have sequenced served as a test system for optimizing the sequencing library construction, sequencing biochemistry, basecalling algorithms, and data analysis pipeline. Our preliminary efforts have proved very successful and our major accomplishments are summarized below. (1) We have developed an optimal genome sequencing library construction protocol. Our original protocols were barely adequate for sequencing bacterial genomes due to stochastic bias in the construction, which resulted in only ~70% genome coverage. We have identified the inefficient steps and optimized the entire protocol, now we are

able to generate libraries that are sufficient for human genome sequencing. (2) We have optimized the sequencing biochemistries for high quality sequencing. The optimal biochemistry allows for 30 base reads. We are now moving toward 50 base reads. (3) Our basecalling algorithms have been optimized to maximize the amount of sequence obtained from a sequencing run. Currently we are able to identify and call bases from ~70 million beads per lane in a sequencing run. We have the potential to run 16 lanes simultaneously.(4) We have now established a data analysis pipeline that takes the raw data from the polonator and maps the raw reads to a similar known genome and makes final assembled base calls. The final assembly calls every base with a probability, or P-value. Therefore, in the end we can call SNPs or somatic mutations with a defined P-value. Specifically, at this point in time, we have obtained patient blood samples, and generated mate-paired genome sequencing libraries. We have initiated sequencing of the libraries and are on schedule to complete the sequencing within the next 2-3 months.

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

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

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

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Reversible protein phosphorylation is a ubiquitous post-translational modification playing a vital role in the control of many biological processes. Aberrant phosphorylation is believed to be one of the underlying mechanisms for cancer. While mass spectrometry (MS) techniques have been successfully applied to determine the phosphorylation state of single proteins/peptides, proteome-wide MS analysis of phosphorylation still poses substantial challenges due to the low abundance of phosphoproteins and substoichiometric phosphorylation. Therefore, isolation and enrichment of the phosphoproteins/peptides are essential for MS based phosphoproteomics. Immobilized metal ion affinity chromatography (IMAC) and microparticles of titanium dioxide ($TiO_2$) and zirconium dioxide ($ZrO_2$) have demonstrated specificity for trapping phosphate. Here we describe the synthesis of large surface area mesoporous $ZrO_2$ and hafnium dioxide ($HfO_2$) nanomaterials for highly selective enrichment of phosphoproteins/peptides for MS-based phosphoproteomics.

The synthesized mesoporous nanostructured materials have small pores on the order of 6 nm with a periodicity of about 10 nm and display small ordered domains of 2D hexagonal structure and a large surface area of 72 $m^2/g$. Materials were first tested for their ability to enrich peptides from a tryptic digest of a

standard phosphoprotein, α-casein. The enrichment using mesoporous metal oxides are extremely effective as shown by the high resolution ESI/FT mass spectra of the protein digest acquired before and after the enrichment. Only 6 phosphopeptides were detected without enrichment, all of which are low abundance peaks owing to ion suppression from abundant non phosphopeptides. In contrast, 18 and 20 phosphopeptides, respectively, corresponding to 20 unique phosphorylation sites were detected in a single mass spectrum with mesoporous $ZrO_2$ and $HfO_2$ enrichment with much higher signal-to-noise ratios. Furthermore, we evaluated the use of these materials with a more complex tryptic digest of a 6-protein mixture (bovine serum albumin, α-casein, troponin C, ubiquitin, ribonuclease B, and β-lactoglobulin). Without enrichment many non phosphopeptides in this mixture dominate the MS spectrum so that phosphopeptides are hardly observable. A single enrichment with mesoporous $ZrO_2$ or $HfO_2$ identified 18 or 20 phosphopeptides, respectively. While both mesoporous $HfO_2$ and $ZrO_2$ materials show highly effective and specific enrichment of phosphopeptides (almost like purification).

## Microdialysis Combined With Proteomics for Protein Identification in Breast Tumor Microenvironment In Vivo

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Tumor microenvironment constitutes a reservoir for proteins released from tumor cells and the host, which can contribute significantly to tumor growth, invasion, and metastasis. A better understanding of the proteins presented in tumor microenvironment will advance our understanding of tumor-host interactions. This study aims to explore the in vivo microdialysis technique combined with proteomics for identification of proteins in mammary tumor microenvironment. In vivo microdialysis was performed in polyomavirus middle T antigen (PyVmT) transgenic mouse mammary tumors and wild-type control mammary glands to collect the soluble proteins in tissue interstitial fluids. Over three hundred proteins were identified from the microdialysis perfusates using the Multidimensional Protein Identification Technology. Bioinformatic analysis revealed extracellular proteins as a major portion of the proteins identified. Three proteins that were overexpressed in the tumor perfusates, including osteopontin (OPN), were further confirmed using ELISA. OPN was further found to be present in tumor-associated stroma in both PyVmT and human breast tumors using immunohistochemistry. In addition, OPN was positively expressed, at both mRNA and protein levels, in primary mouse mammary cancer associated fibroblast cultures. OPN was also shown to stimulate PyVmT breast carcinoma cell proliferation and migration. Finally, the expression of OPN was significantly higher in the peripheral blood of mice bearing breast tumors compared to wild-type mice, indicating it may serve as a serum tumor protein biomarker candidate. Overall, OPN was identified from mammary tumor microenvironment using microdialysis and proteomics, and its tumor promoting effects may present a new opportunity for inhibiting mammary tumor progression.

## Microfluidic Devices for Analysis of Circulating Tumor Cells

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Performing biochemical analysis on circulating tumor cells (CTC) can provide critical information for medical decision. Single-cell analysis allows multiple biochemical analysis to be performed with a limited number of the rare CTC. We have developed microfluidic devices to perform nanoliter scale biochemical analysis to minimize material loss on single-cells analysis. Our microfluidic devices convert single-cell mRNA-to-cDNA in 10-nanoliter reactors, and can simultaneously process 32 single cells with 5-fold higher efficiency compared to bulk assay. However, manipulating single cells via controlling fluid flow inside microfluidic

devices is complicate and inefficient, especially when manipulating a large number of cells. To address these issues for enabling large scale single-cell analysis of CTC, we recently constructed devices with individual addressable reactors and integrated Optoelectronic Tweezers (OET) technology. These new features enable precise and non-contact manipulation of a large number of live single cells inside microfluidic devices. It not only reduces the complexity of our microfluidic devices, but also significantly improves the simultaneous processing capacity. With these new devices, a population of circulating tumor cells (CTC), isolated from patient blood, can be processed simultaneously for multiple assays inside a single integrated device.

## 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. To address this we proposed a strategy called OxMRM capable of sensitively quantitating cysteine oxidation status of potentially any protein by integrating differential thiol alkylation, protein purification, and analysis by multiple reaction monitoring.

**Method:** To "freeze" the thiol oxidation state, cells are precipitated in situ using TCA to denature proteins and protonate the thiolate anions. The protein pellet is washed and resuspended in urea and sodium dodecyl sulfate. Free cysteines are initially alkylated using D0 NEM. The cell lysate is re-precipitated with TCA, washed to remove the D0 NEM, and again resuspended and denatured with urea. This is followed by reduction with TCEP and alkylation with D5 NEM. The sample is desalted using a 6kd gel filtration column and proteins of interest can be immunopurified and proteolytically digested. Sets of MRM transitions are used to quantify the relative oxidation level of cysteine containing peptides in different samples as well as irreversible oxidation.

**Data:** OxMRM has the sensitivity and specificity to perform quantitative analysis of endogenous cysteine oxidation in a targeted manner, such as examining specific proteins of interest or cysteines within key functional domains that are hypothesized to be particularly important for protein regulation and function. MRM is a highly sensitive and rigorous quantitative method, allowing us to quantify the oxidation state of 12 cysteines in two different low level transcription factors, human p53 and protein tyrosine phosphatase 1B. OxMRM can detect cysteine peptides in these proteins at low femtomole or attomole levels with <15% coefficient of variation and can measure differential sensitivity of cysteines in these proteins to different oxidants in different cell lines. We will specifically report on how various oxidants such as diamide, $H_2O_2$, rotenone, and BSO in the epithelial cultured cells lines MCF-7 and HCA-2. Moreover, to examine the interface between regulated and irreversible oxidation, we can measure the sulfinic and sulfonic acid forms of these same cysteines. Measuring these different oxidation states will allow insight into which therapies, antioxidant systems, and environmental exposures lead to, and prevent, oxidation of specific proteins. In addition to the unique sensitivity and specificity of this method, our approach is also able to individually quantitate the oxidation of two separate cysteines that are very closely spaced and on a single peptide. However, a weakness of this method is that you must know beforehand which protein you are interested in. It is not a "discovery" based approach.

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

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

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

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

pancreatitis, pancreatic cysts, etc.). We are planning to investigate the role of new optical parameters in combination with previously reported parameters can develop better prediction rules for different neoplastic processes. The technique will be cross-validated on an independent patient population in a double-blinded manner. If successful, a prospective multi-center trial is warranted.

### The Prevalence and Nature of Glycan Alterations on Specific Proteins in Pancreatic Cancer Patients Revealed Using Antibody-Lectin Sandwich Arrays

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Changes to the glycan structures of proteins secreted by cancer cells are known to be functionally important and to have potential diagnostic value. However, an exploration of the population variation and prevalence of glycan alterations on specific proteins has been lacking due to limitations in conventional glycobiology methods. Here we report the use of a previously-developed antibody-lectin sandwich array method to characterize both the protein and glycan levels of specific mucins and CEA-related proteins captured from the sera of pancreatic cancer patients (n = 23) and control subjects (n = 23). The MUC16 protein was frequently elevated in the cancer patients (65% of the patients) but showed no glycan alterations, while the MUC1 and MUC5AC proteins were less frequently elevated (30% and 35%, respectively) and showed highly-prevalent (up to 65%) and distinct glycan alterations. The most frequent glycan elevations involved the TF antigen, fucose, and Lewis antigens. An unexpected increase in the exposure of alpha-linked mannose also was observed on MUC1 and MUC5ac, indicating possible N-glycan modifications. Because glycan alterations occurred independently from the protein levels, improved identification of the cancer samples was achieved using glycan measurements on specific proteins, relative to using the core protein measurements. The most significant elevation was the CA 19-9 antigen on MUC1, occurring in 19/23 (87%) of the cancer patients and 1/23 (4%) of the control subjects. Follow-up studies using blinded samples from patients with benign and malignant pancreatic disease also have been performed and will be discussed. This work gives insight into the prevalence and protein carriers of glycan alterations in pancreatic cancer and points to the potential of using glycan measurements on specific proteins for highly effective biomarkers.

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

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

We recently described Co-amplification at Lower Denaturation temperature (COLD-PCR), a novel form of PCR that amplifies minority alleles selectively from mixtures of wild-type and mutation-containing sequences irrespective of the mutation type or position on the sequence (1). In COLD-PCR, an intermediate annealing temperature is used during PCR-cycling to allow cross-hybridization of mutant and wild type alleles; hetero-duplexes, which melt at lower temperatures than homo-duplexes, are then selectively denatured and

amplified at Critical Denaturation Temperature, while homo-duplexes remain double-stranded and do not amplify efficiently.

By replacing regular PCR with COLD-PCR prior to application of downstream mutation detection assays (Sanger sequencing; Pyrosequencing; MALDI-TOF; dHPLC; RFLP; Taqman; HRM) we improved mutation detection sensitivity up to 100-fold. Thereby we identified several additional p53/Kras/EGFR mutations in heterogeneous cancer samples that were not detected when preceded by regular-PCR, but that were detectable following COLD-PCR. When followed by High Resolution Melting, COLD-PCR enables identification of low-level (~0.1-1%) variants that can be directly sequenced. COLD-PCR in real time 'Taqman' format increases detection sensitivity for the resistance-causing EGFR mutation T790M in lung cancer by 250-fold (2). By COLD-PCR-based deep-sequencing of 48 snap-frozen lung cancer specimens for p53 alterations, we identified a novel spectrum of low-level of unknown missense somatic mutations some of which were not detectable by any previous technologies (3). DNA from FFPE specimens leads to reduction of COLD-PCR detection sensitivity depending on specimen 'age', however, in all cases the sensitivity is highly improved compared to conventional PCR, irrespective of the downstream method used. COLD-PCR performed from whole genome-amplified DNA retains the ability to identify low-level mutations. Finally, combination of COLD-PCR with second generation sequencing enables both 'deep-sequencing' and 'high-throughput performance', thereby allowing the application of new sequencing to 'real' patient samples that invariably involves stromal cells or tumor heterogeneity.

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

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## Smart Nanoparticles Overcome Basic Roadblocks to Biomarker Discovery and Validation

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The focus is novel nanotechnology that directly overcomes the fundamental physiologic roadblocks to cancer biomarker discovery, measurement and validation. Cancer-associated blood biomarkers exist in exceedingly low concentrations within complex mixtures of high-abundance proteins such as albumin and immunoglobulins. For this reason, existing methods for biomarker discovery and measurement may not have the sensitivity to detect important biomarkers of early stage cancer. A further roadblock to biomarker development is biomarker instability. Biomarkers in the blood may be highly labile and subjected to degradation during blood collection, transportation, and storage. Biomarker instability is a significant source of bias for validation, and can prohibit practical routine clinical use. The nanotechnology developed under this proposal overcomes these serious roadblocks that prevent promising biomarkers from being discovered, or hinder biomarker utility for patient benefit. We have created N-isopropylacrylamide (NIPAm) hydrogel porous sieving nanoparticles containing an internal affinity bait. The nanoparticles perform three

independent functions within minutes, in one step, in solution (serum, plasma, or urine): a) molecular size sieving, b) affinity capture of all solution phase low abundance target analyte molecules, and c) complete protection of harvested proteins from enzymatic degradation. The captured analytes can be readily eluted for analysis. We have developed a variety of novel high affinity chemical bait chemistries that appear to have the capability to sequester six major classes of biomarkers. Six bait categories with fourteen different chemistries, will extend analyte targets to the following major categories of body fluid cancer biomarkers: 1) basic proteins and peptides, 2) acidic proteins and peptides, 3) glycoproteins, 4) hormone metabolites, 5) phosphoproteins, and 6) micro RNA. These novel baits now offer the opportunity to create a family of nanoparticle "flavors" that can be mixed together in one sample collection vessel. The nanoparticle family will capture, in one step, in the same fluid volume, a wide variety of proteins, peptides, metabolites and nucleic acids that constitute potential cancer biomarkers for both proteomic and genomic applications. The envisioned technology is a panel of dry lyophilized, sub-micron sized harvesting particles that carry specific affinity baits for known classes of biomarkers. Following introduction of the blood or body fluid, the respective particle populations will remove all of their target molecules, in one step, in solution, from the entire volume of the sample and concentrate the sequestered analytes inside the particles. Analytes can then be eluted from the particles in a small volume to yield a much higher concentration and purification compared to the starting sample. Depending on the starting volume, this technology can concentrate a biomarker many hundred fold, and fully prevent biomarker degradation, within minutes. We propose the development of two classes of novel technology to be used at the bedside or in the field: a) blood collection tubes that fully stabilize biomarkers and concentrate them 100 fold, at the time of venipuncture collection, and b) a diagnostic skin patch which harvests, concentrates, and stabilizes a panel of biomarkers derived from skin transudate or sweat. Our published studies supported under this funded study document a) Sequestration of greater than 99% of the target low abundance biomarker in less than 5 minutes in serum, with complete exclusion of albumin; b) Full protection (>95%) against degradation of labile biomarkers at 37 °C for at least 24 hrs; and c) Amplification of the effective concentration of low abundance endogenous or spiked biomarkers such that biomarkers at femptomole concentrations well below the lower detection limits of clinical grade 3rd generation immunoassays and mass spectrometry, could now be measured in the linear range of detection with high precision.

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

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Folate receptors (FR) are over-expressed in some cancers, such as ovarian, lung, kidney, and brain cancer. Therefore folic acid (FA) and folate conjugates have been frequently used to deliver diagnostic and therapeutic agents to cancer cells through FR-assisted endocytosis. Towards the goal of developing efficient and cancer specific siRNA delivery reagents, we have developed a series of multivalent, biocompatible FA-conjugated polymers that complex with small interfering (siRNA) and protect it from enzymatic degradation. Herein, we demonstrate the cell-specific delivery of FA-polymer/siRNA complexes and subsequent gene suppression. Firstly, using a fluorophore-labeled siRNA (against human survivin) and fluorescence microscopy, we show the internalization of the FA-polymer/siRNA by Z-axis scanning and time-lapse analysis in KB cells (human nasopharyngeal cancer cells). Two other cancer cell lines have also been tested, including SKOV3 cells (Human ovarian cancer cells), and Hela cells (Human cervical cancer cells). Lyso-tracker, a specific endosome/lysosome fluorescence probe, is used to study colocalization of the fluorophore-labeled siRNA and endosome in KB cells, which indicates the FR-mediated siRNA delivery via endocytosis. Secondly, siRNA accumulates in cytoplasm at 36 hr post incubation, further indicating internalization of polymer/siRNA complexes. Additionally, KB cells are transfected with FA-polymer/siRNA complexes to examine the induction of gene silencing. We have observed significant gene suppression

starting at 24 hr to 72 hr post transfection. A reduction of 60% in surviving mRNA has been achieved at 48 hr post incubation with 200nM siRNA and no cytotoxicity has been observed under the conditions. Furthermore, cell-specific targeting has been established by the following experiments: i) polymers without FA conjugation can complex siRNA, but do not deliver siRNA; ii) free FA competitively blocks the uptake of complexes and gene knockdown; and iii) FR-negative cancer cell line A549 (Human lung cancer cell) does not show uptake of FA-polymer/siRNA complexes. Taking together, we have illustrated a cell-specific siRNA delivery system that can achieve significant gene down regulation with low cytotoxicity. The system does not display some common drawbacks of conventional transfection reagents, and may lead to the development of therapeutic siRNA delivery systems for cancer therapy.

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

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Cancer and other disease biomarkers and targets may provide key diagnostic, prognostic and therapeutic opportunities including clinical trial surrogates and screens for patient treatment assignment. Drugs, gene vectors, and nanoparticles may benefit greatly from improved in vivo delivery through homing to specific disease biomarkers. Yet in vivo barriers limit access to most disease targets including cancer. We have developed novel systems biology approaches that integrate nanotechnology-based subcellular fractionation, quantitative organellar and subtractive proteomics, bioinformatic interrogation, antibody generation, expression profiling, and various in vivo imaging modalities to quickly identify and validate target candidates for pre-clinical and clinical testing. Analysis of rodent and human tumor samples have been compared to focus on clinical meaningful targets and to understand model relevance to human disease. Tissue and tumor microenvironmental influences on endothelial cell expression are extensive. We have developed quantitative proteomic analysis using a new spectral intensity index to identify proteins specific to tumor vs. normal endothelium as well as concentrated in caveolae; many of which are confirmed by immuno-electron microscopy. Novel targets in caveolae enable antibodies to penetrate deep into solid tumors and single organs and were utilized to improve tissue-specific imaging and treatment. Our recent findings reveal that caveolae not only express tissue-specific proteins but also function to rapidly and actively pump specifically targeted antibodies and nanoparticles across the endothelial cell barrier and into the tissue interstitium. This targeted penetration of the antibody into the tissue (transcytosis) occurs within seconds to minutes in normal tissues and with in minutes to a few hours in various tumor models tested. Such pervasive access inside the tumor improves the efficacy of radioimmunotherapy in destroying both stromal and tumor cells and in treating a wide variety of solid tumors. Various rodent tumors are imaged rapidly and specifically after intravenous injection of specific monoclonal antibodies. Because the antibody not only targets the tumor endothelium but also crosses it to penetrate deep and throughout the tumor tissue, this radioimmunotherapy effectively destroys tumors in rodent models to increase survival and even apparently cure the disease. Blood flow stops selectively within the tumor within 24 hrs and after most of the injected antibody has been pumped inside the tumor where it is now trapped to kill not only tumor cells but also stromal cells surrounding the tumor and constituting the critical tumor microenvironment. This tumor penetration and treatment efficacy requires the presence of caveolae on the tumor endothelium and only occurs when the antibodies deliver their attached radionuclides directly and rapidly into the tumors. So far, we have tested breast, lung, ovarian, prostate, and liver tumors with similar success. We have antibodies that recognize this target in humans. A wide variety of human tumors express this novel accessible endothelial cell surface target in a pattern quite similar to the rodent models. We are testing different radionuclides to evaluate which one is most effective. Toxicology studies are ongoing. Our antibody appears useful in tumor-specific imaging as well as in treating a wide variety of solid tumors. This work represents a novel discovery, validation and delivery strategy that so far provides promising and unprecedented results.

Testing in humans is now necessary to understand limitations and possibilities for clinical translation to imaging and treating human disease.

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

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

### Using Synthetic Dosage Lethality to Find Novel Anti-Cancer Targets

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A significant challenge in cancer therapy is to selectively kill cancer cells without harming normal cells. We are taking a novel approach to identify genes that are essential only in cancer cells. Our scheme is based on synthetic dosage lethality (SDL), which defines a genetic interaction where a non-essential gene becomes essential only when a second gene is over-expressed. The normally non-essential gene becomes a target for drugs to inactivate it. Such drugs should selectively kill cancer cells and not cells from normal tissue. Since it is commonly found that cancers often increase expression of specific genes due to translocations or gene amplifications, uncovering SDL interactions may identify specific targets for selective drug therapy. Many essential functions that are deregulated in cancer are conserved between yeast and humans. Therefore yeast can be used as a surrogate to identify SDL interactions. We have developed an innovative new method called SPA, for "selective ploidy ablation," to screen for SDL interactions. By creating yeast strains that over-express the yeast orthologs of several genes that are over-expressed in tumors, we have used SPA to facilitate our search for SDL interactions amongst the 4827 non-essential yeast genes. Two specific examples are described below.

The human Rad9 gene is over-expressed in 33% of non-small cell lung carcinomas (NSCLC) and plays a role in cell cycle checkpoint control, DNA repair and genome stability. We performed an SDL screen with the yeast ortholog of Rad9 (*DDC1*) by transferring a copper-inducible *DDC1* expression plasmid into every strain

of the yeast gene disruption library. *DDC1* shows a strong SDL interaction with *cik1*. Cik1, a binding partner of the kinesin motor protein Kar3, functions in the assembly and/or maintenance of the mitotic spindle. NuMA is the functional human ortholog of Cik1. Like Cik1, NuMA is important for proper mitotic spindle assembly and regulation of microtubule dynamics and cross-linking. We showed that the *DDC1-cik1* SDL interaction is conserved in human cells by exogenously over-expressing human Rad9 in Hela cells in which NuMA was down-regulated by shRNA. We find that cells with reduced levels of NuMA are highly sensitive to increased Rad9 levels. In contrast, control cells are unaffected by increased Rad9. We next tested the effect of NuMA down-regulation in lung cancer cells by transducing two different NSCLC cell lines (A549 and SKLU) with a lentivirus containing a GFP reporter, or a GFP reporter + NuMA shRNA. The A549 cells are sensitive to NuMA down-regulation, while the SKLU cells are not. In addition, we tested 4 breast cancer cell lines and 5 prostate cancer cell lines that exhibit increased *RAD9* expression and found that they do not show decreased viability upon NuMA knockdown.

We performed a second SDL screen using a constitutively active oncogenic mutation of *ras* (G12V) constructed at the conserved position in yeast *RAS1* (G19V). Overexpression of $ras1_{V19}$ in every strain of the yeast gene disruption library identified 230 deletion strains showing significantly repressed growth. The list of mutations includes many genes previously identified as having genetic interactions with *ras1* or *ras2*. In addition, the list is enriched for GTPases and kinases suggesting that components of signaling cascades can partially compensate for expression of the oncogenic allele. Finally, the list contains components of the vesicle trafficking pathways, including genes involved in autophagy, which is down-regulated in cells expressing activated ras. We are currently testing these interactions in mammalian cells.

Recently we performed 10 additional SDL screens with genes that are over-expressed PTEN- breast tumors. Several have yielded interesting targets for verification in mammalian cells. Some of the genes that we have screened are involved in DNA replication - MCM5 (*MCM5*), BOB1 (*BOB1*), MCM6 (*MCM6*), CDC7 (*CDC7*), checkpoints - TOPBP1 (*DPB11*), ATAD2 (*YTA7*), splicing - SFRS1 (*NPL3*), centrosomes - SGOL2 (*SPC110*), sumolyation - SUMO3 (*SMT3*) and cohesion - SGOL2 (*USO1*), where the gene in parentheses is the yeast homologue. We are in the process of carrying out additional screens to identify those genetic interactions that are conserved in human cells.