

**INNOVATIVE MOLECULAR ANALYSIS TECHNOLOGIES (IMAT)  
PROGRAM  
13<sup>TH</sup> PRINCIPAL INVESTIGATORS (PI) MEETING**

November 27-28, 2012  
Houston, TX

Center for Strategic Scientific Initiatives  
National Cancer Institute (NCI)  
National Institutes of Health (NIH)  
U.S. Department of Health and Human Services

**DAY 1 - NOVEMBER 27, 2012**

**WELCOME**

*Tony Dickherber, Ph.D., Program Director, IMAT Program, National Cancer Institute, National Institutes of Health*

Dr. Dickherber opened the meeting by welcoming all participants on behalf of the trans-divisional team that collectively manages the IMAT program. He provided several program updates, including the news that the new IMAT Requests for Applications (RFAs) are now available. He also noted that IMAT is up for renewal in 2013 and the program is undergoing evaluation. He explained that the NIH is shifting to a new technical progress reporting mechanism (the Research Performance Progress Report [RPPR]). Given that this new format is generally more helpful to NIH program officers, Dr. Dickherber encouraged everyone to complete these reports rather than the old technical progress report format. The IMAT is seeking approval from the Office of Management and Budget to issue post-project surveys. The questionnaire, if approved, will be used only for information gathering, and will not have any impact on current or future grant funding.

The structure of this year's meeting is to inject the perspective of the end-user into the IMAT conversation. The goal is to provide a better sense of end-users' needs and how IMAT investigators can address these needs. Dr. Dickherber noted that the IMAT team has come to appreciate that this annual PI meeting is an important mechanism for improving current projects and to developing new projects. He stressed the importance to broaden those conversations to include the end-user perspective. He added as a final note that this meeting will mark the first time that an afternoon's worth of sessions will be open to local investigators, again with the goal of further catalyzing discussions that could lead to new ideas and new collaborations.

**PANEL SESSION: NOVEL BIOSENSORS I**

*Chair: Steven Shen, M.D., Ph.D., The Methodist Hospital Research Institute*

As an introduction to this session, Dr. Shen presented statistics revealing the magnitude of the cancer problem. Cancer, he said, is now approaching heart disease as the leading cause of death in the United States, and cancer is already the leading cause of death in many Asian countries. Out of all cancers, lung cancer is the biggest killer now of both men and women, accounting for more deaths annually than breast, prostate, and colorectal cancers combined. The reason for cancer's lethality, he said, is that too many cancers are diagnosed too late, something that as a pathologist he sees on a daily basis. As a result, developing more effective methods for detecting cancer early would have the single biggest impact on cancer survival. However, today there are very few good markers for early-stage cancer, particularly for pancreatic, kidney, ovarian and liver cancers. He presented a long list of "potential" biomarkers, but none of these have the specificity or sensitivity to make them truly useful.

There are several key challenges to biomarker development, said Dr. Shen. First, there are over 200 cancer types, each with substantial heterogeneity and complex genetics both across the disease and within a given tumor. Also, cancer is a

chronic disease that starts slowly with small morphological changes, something he illustrated with an image of a colon tissue sample showing all four stages of development within a single biopsy sample. Overcoming these challenges and enabling early detection is the biggest opportunity for technology, and the key to making meaningful progress against the disease.

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

*Alyssa Garrelts, Ph.D., Purdue University*

Dr. Garrelts, a post doctoral fellow working with Laurie Parker, Ph.D., began the meeting's first scientific presentation by describing the multiplexed assay system that she and her colleagues are developing to map protein kinase activity in breast cancer patient biospecimens. Kinases are of interest, she explained, because of their important roles in cancer development, as well as in drug effectiveness, resistance, and side-effects. The assays she and her colleagues are developing use Surface Enhanced Raman Spectroscopy (SERS) in conjunction with a panel of peptide-functionalized metal nanoparticles, each member of which serves as a substrate for a specific to probe kinase activity. This project is being conducted in collaboration with Joseph Irudayaraj, Ph.D., and his group.

*In vitro* data show that cells internalize these peptide-functionalized nanoparticles, and that each peptide-nanoparticle combination produces a unique SERS signal. She is now working to establish limits of detection and develop calibration curves that will enable quantification of kinase activity in cells. She and her colleagues have synthesized and tested several variations of the Abl kinase and Src kinase substrate peptides in order to optimize the SERS signal from silver nanoparticles. She and her colleagues are using the Abl kinase-silver nanoparticle to map Abl function in tissues and are exploring the use of other SERS-responsive nanoparticles, such as hollow gold nanospheres and gold nanostars, in order to improve the performance of the assay. In response to a question from the audience, Dr. Garrelts noted that one of the strengths of SERS is the availability of techniques to deconvolute the signal peaks from multiple substrates. She added that Dr. Parker's laboratory is working to develop specific substrates for tyrosine kinases.

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

*Peter J. Burke, Ph.D., University of California, Irvine*

Dr. Burke's group has developed a microfluidic device for evaluating metabolic and biophysical parameters of small numbers of functional mitochondria. In the initial stage of this IMAT project, his team demonstrated that they could measure the membrane potential of mitochondria from human embryonic stem cells. Using this device, he and his collaborators showed that the membrane potential of human embryonic stem cells is about 50 percent less than that of human somatic or cancer cells. Since then, his team has developed nanofluidic channels that are narrow enough to trap individual mitochondria. Once trapped in the channel, the

mitochondria can be exposed to a variety of chemical analytes and interrogated using fluorescent probes.

Before discussing the experiments his group has conducted using this device, Dr. Burke reminded the audience that the bioenergetics of a cell is based on metabolizing glucose. Once imported into the cytoplasm, glucose is converted to pyruvate through a series of enzymatic reactions, yielding 2 ATPs, in a process termed glycolysis. Pyruvate is then directed down one of two pathways: In one case, the pyruvate is reduced by lactate dehydrogenase using NADH to generate lactate and the lactate is then excreted from the cell, which has the added effect of acidifying the extracellular matrix. In this case, no oxygen is consumed, and the mitochondria are not involved. In the second case, the pyruvate is imported into the mitochondria, and the chemical energy stored in the pyruvate is used to drive proton pumps in the mitochondrial membrane by oxidizing the pyruvate (through the Krebs cycle, and using oxygen) to H<sub>2</sub>O and CO<sub>2</sub>. The mitochondrial membrane potential is then used to generate ATP. This process (termed OXPHOS for oxidative phosphorylation) generates much more ATP than glycolysis (typically 36 ATPs molecules per glucose imported into the cell). Thus, proton pumps are key players in converting stored energy into ATP, and proton pumps generate a membrane potential, which explains his interest in measuring that physical parameter.

A prevailing view in cancer metabolism for the last several decades has been that tumor cells predominantly use glycolysis as their energy source, whereas somatic cells use OXPHOS. This is thought even to occur in the presence of normoxia (termed the Warburg effect). With the advent of interest in stem cells, recent work has indicated that pluripotent stem cells predominantly use glycolysis prior to differentiation, and OXPHOS after differentiation. This raises the intriguing question of the metabolic state of cancer stem cells, which to date has not been measured. The cause/effect relationship between the metabolic shift from OXPHOS to glycolysis in tumorigenesis is not clear. Similarly, the cause/effect relationship between the metabolic shift from glycolysis to OXPHOS upon differentiation of stem cells is not clear. Finally, the generality and universality of these metabolic shifts in tumorigenesis (in the context of cancer) and differentiation (in the context of stem cells) have recently been questioned, and dependence on cell type, tissue type, and other experimental conditions makes such sweeping generalizations difficult to comprehensively defend. It is in the context of this controversy that the need for a high-throughput metabolic assay technology becomes very clear, and justifies the IMAT technology development work, which Dr. Burke then went on to present.

What is known is that mitochondrial membrane potential changes rapidly during apoptosis, though the molecular mechanism involved in this rapid depolarization is still not known. Dr. Burke's group is developing spatially resolved methods to use in conjunction with its mitochondria trap to study this phenomenon. The goal is to determine if this is a site-specific event or a general event.

He then described the individual components used to measure membrane potential and the work his group is doing to miniaturize these components. So far, he and his collaborators have developed on-chip electrochemical methods that work on

approximately 1000 cells' worth of mitochondria. In relation to the stem cell mitochondrial membrane potential, with the IMAT chips, his co-PI D. Wallace has shown that stem cell mitochondria lose their membrane potential when they are studied in the absence of oxidizable substrates such as pyruvate, glutamate, or malate. When they have an oxidizable substrate, their membrane potential is almost as high as that of other cell types. Because the chips require such a small amount of mitochondria compared to prior technologies (which required 10 million cells worth of mitochondria), this can be viewed as an enabling technology for studying metabolism and stem cells in ways that were not possible prior to the IMAT funded project. In related experiments on the same cell line, his co-PI Wallace discovered that telomerase expression in stem cells was upregulated in a manner indicating that mitochondrial epigenetics must be involved. Results from both of these experiments in which mitochondria from stem cells were implanted in somatic cells lacking mitochondria indicate that the mitochondria play an intimate role in controlling cellular respiration. Dr. Burke added that two laboratories have since generated findings consistent with his team's results. Additional measures show that stem cell mitochondria, while fully functional, are on what he called respiratory standby when cells are in a pluripotent state.

In the last part of his talk, Dr. Burke described the work his team is doing with nanochannels that trap individual mitochondria. Using two different fluorescent dyes, his team has been able to make qualitative assessments of membrane potential and watch that potential change when various substrates are added to the nanochannels. He and his collaborators are now developing nanoprobe that are comparable in size to the proteins in the electron transport chain in membranes to produce high-resolution images. Using these nanoprobe, which consist of graphene coated with a lipid bilayer, they have been able to observe the transition between on and off states of an ion channel. They are now working to incorporate these nanoprobe into the nanochannel device. He said in closing that it should be possible to develop probes that measure electrons as they move down the electron transport chain in the membrane.

### **Multiplex Cancer Cell Purification with Magnetic Sifters**

*Shan X. Wang, Stanford University*

To begin the last talk of this session, Dr. Wang reviewed the working hypothesis that circulating tumor cells (CTCs) are related to cancer metastasis. While potentially valuable as a means of detecting cancer early and monitoring therapeutic response in a minimally invasive manner, the challenge in working with CTCs is that there are very few of them in circulation, particularly in early-stage advanced cancer. He explained that researchers have developed several novel approaches to isolating CTCs, and the most powerful of these techniques capture CTCs based on cell-surface markers, particularly the protein EpCAM. Dr. Wang's approach to collecting CTCs is to use a magnetic sifter that captures magnetically labeled cells using an anti-EpCAM antibody labeled with magnetic nanoparticles, though he noted that these magnetic nanoparticles can be used with any capture molecule.

The magnetic sifter, he explained, works by generating a strong magnetic field gradient across a metal grid structure with 40 micron pores. The magnetic field gradient runs in the direction perpendicular to the flow of blood through the pores, trapping only labeled cells. Once the unlabeled cells have flowed through the device, the magnetic field is turned off and the CTCs are released for collection and further study. While the grid can efficiently capture all labeled CTCs at high flow rates, Dr. Wang said that flow rates are restricted to 5-15 mL/hr to protect the integrity of the cells. He showed a video of real-time capture of cancer cells flowing through and then being captured by the sifter with the magnetic field off and on. Capture efficiency is not affected by the background of whole blood, he said, and the pores do not clog when the device is used with whole blood.

Dr. Wang then showed the results of standard staining experiments his team conducted to prove that the capture cells are indeed CTCs and not white blood cells. His team has also developed an automated image analysis system to identify CTCs based on size, shape, and color criteria.

His team is now using the sifter to enhance the purity and capture efficiency of cancer stem cells (CSCs) from epithelial ovarian cancer ascites and tissue samples while ensuring their viability. The design challenge is to use just two antibodies, rather than the usual five or more used with standard magnetic-activated cell sorting (MACS) to enrich putative ovarian CSCs. Dr. Wang and his colleagues have successfully demonstrated that they have achieved this milestone using antibodies against CD44 and CD49f and on mouse xenograft and human clinical samples. Comparing the results obtained with a MACS column and the magnetic sifter, Dr. Wang's team showed that the sifter's recovery ratio was 66.8 percent compared to 45 percent for the MACS column, and the harvest ratio was 42.3 percent for the sifter compared to 24.2 percent for the MACS column. His team is now developing a two-plex sorting system that works with different flow speeds and magnetic nanoparticle labels.

#### **PANEL SESSION: NOVEL BIOSENSORS II**

*Chair: Rebecca R. Richards-Kortum, Ph.D., Rice University*

Before introducing the first speaker of this second session on biosensors, Dr. Richards-Kortum remarked that it is important to consider the context of these analyses – early detection, selecting therapy, monitoring therapeutic response, guiding resection, and the like - when designing technology. The choice of setting, she explained, is as important to consider as is a gold standard endpoint. Histology is a typical gold standard endpoint for the clinic because it's easy to obtain. The question, though, is whether this is the right endpoint because histology is not often conclusive, nor does it provide much evidence for progression. A second consideration is analytical techniques must have high-specificity when it comes to low-prevalence cancers in order to not produce too many false positive, as this is often proves problematic for regulatory validation of new platforms. She concluded her remarks by asking the attendees to consider when creating new technologies that cancer is growing in less-developed nations. Early detection and prevention will be even more

important in those countries, so cost and ease of operation should be an important consideration when developing technology.

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

*Philip J. Santangelo, Ph.D., Georgia Institute of Technology and Emory University*

In the first presentation of this second session on novel biosensors, Dr. Santangelo gave a brief overview of mRNA regulation during cancer pathogenesis, highlighting the fact that mRNA regulation occurs at both the transcriptional and post-transcriptional level. In general, he said, methods for studying mRNA regulation do not assess both mRNA copy number and mRNA-RNA binding protein (RBP) interactions in single cells and at the single molecule levels, both of which are critical to characterizing their translational potential. As an example of the influence of RBP in cancer, Dr. Santangelo cited the case of HuR, an RBP that binds to adenine-uracil (AU) rich sequences. HuR moves from the nucleus to the cytoplasm in cancer, and its appearance in the cytoplasm appears to be associated with the severity of cancer and its response to therapy. This particular RBP has also been shown to bind to different gene clusters in different types of the same cancer.

The goal of Dr. Santangelo's IMAT project is to use imaging to assess transcript copy number and regulation at the single cell and single molecule sensitivity. Given that most RNAs are present in copy numbers of less than 100, any imaging approach will require probes with exquisite sensitivity. His team's approach is to develop what he calls multiply-labeled tetravalent RNA imaging probes (MTRIPs), which is coupled with proximity ligation assay (PLA) technique to detect RBPs. Each RNA of interest is targeted with three to six probes, and approximately 30-50 oligo probes in total bind to the RNA-PLA complex, providing sufficient sensitivity for detection at the single molecule level. He listed the wide range of experiments his group has conducted to confirm MTRIP specificity and to demonstrate their non-intrusive nature, and he showed examples of images obtained using these agents, including images from which mRNA knockdown can be quantified.

Dr. Santangelo's team used a modified probe labeled with the FLAG peptide to quantify RNA-protein interactions using proximity ligation. He noted that it takes approximately 10 minutes to deliver the probes into live cells using streptolysin O membrane permeabilization. The FLAG-labeled probes can detect RNA-protein interactions in a distance dependent fashion with single interaction sensitivity. He noted that both mRNA levels and mRNA-protein interactions can be quantified using automated counting routines, and that mRNA-protein interactions can be identified even when RBP localization is diffuse. To illustrate these points, he ran through the results of a variety of experiments examining RNA-protein interactions and the effect of various treatments on these interactions. He closed his talk by noting that his group is now developing a multiplexed format to examine multiple RNA-protein interactions simultaneously. They are also about to begin a study using human biopsy samples.

### **Highly Multiplexed, Spatially Delineated Molecular Imaging in Cancer**

*Michael R. Diehl, Ph.D., Rice University*

Dr. Diehl, who noted that much of this work was done in collaboration with Walter Hittelman's group at MD Anderson Cancer Center, said that his IMAT project aims to develop a highly multiplexed, spatially delineated molecular imaging system to enable the study of networks rather than just single molecules. The problem with most approaches to *in situ* studies of this type is that they are limited by dye spectral overlap, the available species for monoclonal antibody, the inability to remove probes from samples in order to carry out multiple sequential analyses, and sample scarcity.

His approach is to develop a multiplexed and reiterative molecular imaging system to repeatedly assay for all biomarkers. This is enzyme-free, isothermal, programmable, and regenerative system uses no harsh chemicals, but it does use a new class of DNA technology called Dynamic DNA complexes as programmable probes. He described how this technology can be used to build logic gates from displacement reactions involving programmable immunofluorescent imaging probes, and he explained how he and his collaborators discovered how to conduct these displacement reactions *in situ* in live cells. The probes consist of designed DNA sequences that engage in DNA strand displacement involving the isothermal exchange of oligonucleotides between different thermodynamically stable DNA complexes. This allows reporting complexes to be created and removed from a sample at ambient temperatures using mild reactions buffers. Using these optimized structures and DNA probes targeted with monoclonal antibodies, his group can multiplex and reiteratively image using immunofluorescence imaging with a cycle time of 10 minutes to label and 10 minutes to erase the signal and start a new cycle.

Dr. Diehl noted that hyperspectral imaging requires balancing tunable amplification methods, otherwise crosstalk between markers will produce small errors from bright markers and larger errors from dim markers. Enzymatic methods aren't suitable for this type of assay, he said, so he and his group adapted a convergent approach using dendrimers to create a tunable, convergent, and erasable amplification system. He briefly described this system and showed how it produced more useful hyperspectral data. His team is using these reagents to combine dynamic DNA labeling with super-resolution microscopy to provide information on hundreds of molecules in a couple of rounds of high-resolution stochastic optical reconstruction microscopy (STORM) imaging.

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

*David J. Beebe, Ph.D., University of Wisconsin*

The focus of Dr. Beebe's work is to develop a screening platform to probe the role that paracrine signaling plays in regulating cancer progression. He noted that fibroblasts control breast cancer cell growth via paracrine signaling that regulates the estrogen receptor. He explained that a paracrine signaling screening platform would be useful for developing therapies targeted at the stroma and for creating -targeted treatment based on paracrine signaling. He noted that while RT-PCR transcriptional



analysis is good for assaying many genes from a single sample, it is too cumbersome for use on the multiple samples that would flow into a clinical setting.

After reviewing the role of the tumor microenvironment in cancer and how critical it is in the context of cancer to understand how one cell type influences another is essential to understanding cancer, Dr. Beebe discussed his team's approach for using microfluidics to co-culture two types of cells and study their interactions. In an earlier IMAT project, his group developed a tubeless microfluidics system that uses a passive pumping mechanism and automated pipetting. This system is patented and commercially available now.

His group is now using this same simplified approach to construct a micro-co-culture system. Dr. Beebe described the system and demonstrated that this group can reproduce *in vitro* some intercellular signaling seen *in vivo* in both humans and animal systems. He and his collaborators have also developed a sample preparation technique that greatly reduces the complexity of nucleic acid isolation on the microscale. This method, which he called Immiscible Filtration Assisted by Surface Tension (IFAST), uses oil and an immiscible liquid barrier to the cell culture/lysis region of the microfluidic device from the elution buffer. He explained how this system uses paramagnetic particles that selectively bind mRNA to drag labeled materials through the oil-water interface using a simple magnet. This mRNA purification process takes seconds, replaces the multiple washing steps used in current mRNA isolation protocols, and is so simple that it should be useful in a global health setting.

Dr. Beebe then described the integrated co-culture/mRNA purification device his team built. Breast cancer and stromal cells are loaded into independent culture regions that are connected via diffusion ports. Each culture well has its own connected IFAST device so enable mRNA to be collected from each cell type without cross-contamination. To demonstrate the utility of this device, Dr. Beebe described an experiment in which bone marrow-derived stromal cells were co-cultured with breast cancer cells. The results showed that breast cancer increased their proliferation rate in presence of stromal cells, compared to when they were cultured alone. In addition, mRNA extracted from these cells revealed that the estrogen receptor was down-regulated by the stromal cells even in the absence of estrogen, and that estrogen receptor down-regulation correlated with the growth promoting activity of stroma. He noted that estrogen receptor mRNA levels can be quantified at the single cell level using this system.

His group is now studying the transition to invasion and early studies have shown that there is a distance-dependent invasive transition. These experiments revealed that there is an initial step that only involves soluble factors, but completing the transition requires soluble factors and cell-cell contacts. This suggests that there is a two-step transition. Dr. Beebe finished his talk with the comment that his team is now building lumen on the device and using it to study angiogenesis, both in two and three dimensions, and eventually CTC extravasation from blood vessels. Preliminary studies have shown that putting cells into a round structure rather than a rectangular one produces different results in terms of growth patterns.

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

*Stephen Salipante, M.D., Ph.D., University of Washington*

To start the final presentation on novel biosensor development, Dr. Salipante noted that today's clinical diagnostic assays for genetic changes associated with cancer are not ideal because they are incapable of comprehensively detecting all actionable mutations in a clinical sample. The goal of his IMAT project, in collaboration with Dr. Joseph Hiatt and Dr. Jay Shendure, is to use massively parallel DNA sequencing to develop such an assay. He described the general properties of today's high-throughput DNA sequencing technologies and discussed the challenges of using massively parallel DNA sequencing as a cancer diagnostic. One major issue is that many samples are formalin-fixed, paraffin-embedded (FFPE) samples that are generally unsuited for analysis using today's sequencing technologies. Another issue is that only a small subset of the genome is actionable, so there is a need to confine sequencing to those specific regions. And finally, the turnaround time for today's technologies is too long, about four to eight weeks. There is also the problem of dealing with tumor heterogeneity and the fact that clinical samples are mixtures of tumor and non-tumor cells, which are not issues specific to massively parallel sequencing. The inherent error rate of sequencing may also mask rare mutations in cancer, making it impossible to distinguish between rare variants and sequencing error.

The strategy that Dr. Salipante is taking to address these challenges is to integrate two sequencing modalities using molecular inversion probes (MIPs) and molecular tagging to yield single-molecule MIP (smMIP) capture. This approach allows for highly accurate targeted sequence capture and the quantification of rare mutations. He explained how smMIP technology works and showed how it is useful with FFPE samples because it works with small DNA fragments common with FFPE.

Dr. Salipante's group has evaluated this approach in pilot experiments with two HapMap cell lines and six mixtures of those cell lines, as well as in 47 clinically characterized samples. The fixed cost of building a panel is about \$15,000, he noted, but that supply lasts a lifetime, he said, and produces results for much less than \$1/gene per sample. The HapMap controls showed excellent agreement with the expected allele frequencies at all dilution. The dynamic range of the system was very high, he said, and its sensitivity exceeded that of any available technology. The use of smMIPs reduced errors to below one per 100,000, and nearly all of the mutations detected in the clinical sample were detected using this assay. In addition, this combined system recovered a number of additional mutations that had been noted in other databases. In total the assay detected seven sub-clonal variants at actionable sites.

His group has since streamlined the library to use Illumina MiSeq to produce releasable results in less than 72 hours. When his team applied their technology to eight samples in one MiSeq run, the results were good, but they did not detect the low-frequency actionable genes. Dr. Salipante noted, though, that, Illumina recently released a new version of MiSeq that greatly reduces the time for analysis and

therefore will allow for the use of the larger probe library. He expects, then, that this change will solve the issue of not detecting low-frequency actionable genes.

## **IMAT SUCCESS STORY**

### **Collaboration Versus Commercialization to Disseminate Your Technology Functional Genomics, Interactomes, and Cancer Targets**

*William C. Hahn, M.D., Ph.D., and David E. Hill, Ph.D., Dana-Farber Cancer  
Institute*

Speaking first, Dr. Hahn said that the idea driving his work is that cancer is in the midst of a revolution enabled by next-generation sequencing. This revolution is impacting the research world, but to become an important clinical tool the field needs to realize that there is a need to go beyond simply having a list of genomic changes in cancer. Given that cancer is a complex disease genomically, it is necessary to know the functions of all of the mutations being identified, and to achieve that level of knowledge requires an equally high-throughput method to determine function. There are two approaches to studying function - gain of function and loss of function experiments – and the key will be developing methods to systematize these experiments.

The approach that Dr. Hahn and his colleagues took was to make extensive use of two sets of tools - an RNA interference (RNAi) library of shRNA's that is amenable to be arrayed in pooled format screens, and an expression library of human open reading frame (ORF) clones. The RNAi library arose from a consortium of groups that decided to make all of these reagents available to everyone who wanted to use them, and so all of the shRNA clones are available from Sigma-Aldrich, Open Biosystems, and Addgene, and all the methods and protocols are available on the RNAi Consortium Web site (<http://www.broadinstitute.org/rnai/public/>). The ORFeome expression library was developed as a collaboration between the DFCI Center for Cancer Systems Biology (CCSB), which contributed their Human ORFeome collection, and the Broad Institute, which carried out a next generation sequencing approach to identify full length wild type ORFs that were then individually transferred into a viral expression vector. As with the shRNA library, the ORFeome collection is also made publicly available.

Dr. Hahn noted that when his team started using these tools they quickly realized that the tools and technologies were powerful but not perfect. One question that came up was how to address false negatives. He gave an example where his team performed both loss of function and gain of function screens and identified three genes in the two subsets. They then ran another set of experiments in which they performed two different loss of function studies and integrated those data with data from The Cancer Genome Atlas (TCGA) to identify a single gene in colorectal cancer. He said that he and his collaborators are finding that using multiple approaches is enabling them to identify these high-probability genes.

Dr. Hahn then discussed Project Achilles, which is conducting genome-wide RNAi screens in a large number of cell lines to generate a database with all of the genomic alterations in cancer. To accomplish this task, he and his colleagues

developed a pooled short hairpin RNA (shRNA) barcode screening technique that looks for changes in abundance of shRNA, not phenotype. He noted that these studies showed that screening just a few cell lines yields the right answer most of the time, but screening eight to nine cell lines produces the right answer all of the time.

He then presented two quick vignettes. The first involved identifying amplified oncogenes. This study identified the gene ID4 as being essential for ovarian cancer cell proliferation and survival. This was confirmed by experiments showing that this gene transformed normal cells. Then, using a targeted tumor-penetrating siRNA nanocomplex developed by Sangeeta Bhatia, his team showed that an anti-ID4 siRNA stopped and reversed tumor growth in an animal model of human ovarian cancer.

The second vignette illustrated how the large datasets generated using these functional assays can identify new unexpected targets. His team was studying the Wnt pathway, which is mutated in almost all colorectal cancers and appears to be an undruggable target. Closer examination of their data showed that there was an enrichment in genes related to the transcriptional factor YAP1. This gene, it turns out, interacts with beta-catenin in colon cancer cell lines, and that both are needed to transform cells. Through a complex series of experiments, Dr. Hahn and his colleagues demonstrated that loss of the gene TCF4 forces beta-catenin into an alternative complex that drives cancer.

Next, Dr. Hill spoke about CCSB's journey towards understanding genotype and phenotype relationships with the goal of comprehensively mapping interactomes. The interactome, he explained, is the full spectrum of all possible macromolecular interactions that can take place within the context of the cell. Mapping the interactome will help decipher how these networks of macromolecules are organized and how this organization affects cell function. He said that the key to understanding how the interactome will move the field forward is by identifying the full spectrum of interactions, termed "edges" from graph theory parlance, among each macromolecule ("node") of the interactome.

Building the interactome requires identifying all of the parts, and ORFs are the tool that can generate this parts list. The Center for Cancer Systems Biology's (CCSB) ORFeome project, which is organized around the principle of minimizing intellectual property restrictions and open collaboration, has already found that there is likely to be, on average, an order of magnitude more splice isoforms than there are individual protein-coding genes. The CCSB is now characterizing binary protein-protein interactions for all possible interactions between all the proteins. He noted that there are tools now for doing large-scale interaction mapping and that different techniques yield non-overlapping data sets that his team is now working to integrate; however the only platform today capable of performing the >200,000,000 combinations is the yeast two-hybrid system. Many of the cloning and Y2H techniques in use at CCSB, he added, were developed with IMAT support. His team is working to reduce the cost of running these screens by several orders of magnitude and is also developing quality control methods, several of which he described. These studies have shown that no single assay is sufficient, and that false negative results for

any binary interaction assay exceed 50 percent. He noted that a new human interactome dataset is available for anyone to use on the Human Interactome Project Website ([http://interactome.dfci.harvard.edu/H\\_sapiens/host.php](http://interactome.dfci.harvard.edu/H_sapiens/host.php)).

Dr. Hill then discussed how the analysis of the human interactome has shown that the protein hubs identified in the literature also show up in this type of unbiased screening, and the CCSB unbiased Y2H screening has identified new hubs. He noted that this analysis shows that all of the networks that interactome mapping research has identified over the years seem to be similar in terms of the global organization properties that appear to be conserved. Among the lessons learned from these studies are that graph theory properties relate to biology, that interactomes and transcriptomes appear globally co-regulated, and that hub proteins are highly pleiotropic and are likely to be essential.

He then briefly discussed studies to characterize the gains and losses in interactome edges as they relate to biological outcomes, which he calls edgotyping, and listed the relationships between different types of genotypes and edgotypes. He described one example in which an edgotype analysis found that different sets of interactions observed with different disease alleles of one gene known to be involved in two distinctly different diseases correlated with the respective phenotypes in the two unrelated diseases. He finished his remarks by saying that there is a need for additional tools that can deal with domain-based fragments and splice isoforms, as well as tools that extend the capabilities of the yeast-two hybrid system.

**POSTER HIGHLIGHTS SESSION**

In a series of eight-minute presentations, twelve investigators presented summaries of the posters they were presenting at this IMAT meeting.

**Activatable BRET Probes for MMP Enzymatic Activity Detection (Poster 4)**

*Adam Shuhendler, Ph.D., Stanford University*

Dr. Shuhendler noted that matrix metalloproteases (MMPs) are overexpressed in most cancer, and that they play a crucial role in defining the cellular environment in a way that enables increased tumor invasion and metastasis. Their overexpression has also been correlated to shortened survival time. The goal of this project, he said, was to develop a method that could be used to develop a better understanding of the spatial and temporal role of MMPs in cancer.

He then reviewed the advantages and disadvantages of bioluminescent imaging as tool for measuring protein levels in complex biological samples. His work focuses on using bioluminescence resonance energy transfer (BRET) in conjunction with gold nanoparticles, quantum dots, and polymer dots to generate near-infrared light signals that could pass through biological tissues. He described the fundamentals of the BRET experiment and then presented an overview of the platform that he and his colleagues in Jianghong Rao's laboratory have developed using gold nanoparticles and an MMP-cleavable linker. He showed how a de-quenching construct was designed to incorporate a collagen binding protein to keep the signal fixed at the location of MMP activity. In this assay, the BRET signal decreases as a result of MMP activity. He discussed the use of quantum dots as a means of developing a multiplexed assay, and then described the work he and his collaborators are doing to replace quantum dots with conjugated polymer dots. The latter would not only have fewer safety issues related to their use in humans, but they also produce a more intense signal with greater depth of tissue penetration.

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

*Sharon N. Edmiston, The University of North Carolina at Chapel Hill*

This IMAT project, said Ms. Edmiston, was developed in response to a need identified by pathologists for a means of discriminating between malignant melanoma and nevi in FFPE tissues. The approach that she and her collaborators in the laboratories of Kathleen Conway-Dorsey and Nancy Thomas took was to develop a high-throughput methylation assay for use with the Illumina BeadChip system. She summarized the results of the initial R21 phase of this project that included the identification of 12 CpG methylation sites in nine genes that provided accurate discrimination between melanoma and nevi with no misclassification. In this phase of the study, she and her colleagues identified a set of candidate DNA methylation markers for use as an internal quality control to distinguishing melanocyte tissue from skin. They also developed a high-throughput methylation assay that they validated in FFPE tissues.

Using this assay, she and her collaborators showed that patient/host factors such as age, sex, anatomical site, or pigmentation were not associated with differential methylation at the CpG markers in their panel. They also found that while five CpG sites distinguished melanoma from skin, methylation similarities between skin and nevi will require the identification of additional markers for use as internal standards.

**FRET-Based Biosensors to Monitor Redox in Cell Cycle Regulation (Poster 17)**

*Vladimir Kolossov, Ph.D., University of Illinois, Urbana-Champaign*

Dr. Kolossov's presentation described the development of a genetically encoded, green fluorescent protein (GFP)-based redox-sensitive sensor to measure glutathione levels in cells. In contrast to the use of redox-sensitive dyes, this approach enables spatiotemporal measurements in live cells. After describing the construction of the GFP-glutaredoxin enzyme construct used as the sensor, Dr. Kolossov showed how the sensor could be used to monitor oxidation of glutathione that occurred only in mitochondria upon application of a specific inhibitor of glutathione synthesis. He also showed how this sensor enabled the identification of complex III in the mitochondrial electron transport chain as the site of oxidation induced by reductive stress.

**Microfluidic Sorting of Blood Cells for SPR and Fluorescence Analysis (Poster 26)**

*Nathaniel C. Cady, Ph.D., University of Albany*

The focus of this IMAT project is to develop a microfluidics platform for capturing and analyzing CTCs and to use that platform as a diagnostic and prognostic instrument. This project, said Dr. Cady, is being conducted in partnership with Ciencia, a company that manufactures a grating-coupled surface plasmon resonance (GCSPR) imaging system. He explained that a typical gold-coated grating chip can be spotted with hundreds of capture agents using a robotic spotter and inserted into a microfluidic device designed to fractionate blood. When used in conjunction with antibody-linked fluorescent dyes, the sensitivity and dynamic range of detection of this system easily surpasses that of ELISA, he noted.

Data from a first-generation device that simply ran whole blood over the GCSPR grid spotted with CTC capture antibodies found inconsistencies with FACS data, largely because of interference from soluble markers in blood. To eliminate these false positives, Dr. Cady's group has created a cell sorting device comprising an array of micron-sized posts. This device operates according to a deterministic hydrodynamic design credited to Robert Austin at Princeton University. As blood flows through the grid, soluble markers move through unimpeded. Meanwhile, cells bounce from post to post, with larger cells having a greater displacement perpendicular to the flow. As a result, cells exit this "bump array" separated laterally by size. Dr. Cady demonstrated this separation and said that his group is now integrating into the microfluidics containing the GCSPR grid.

**Application of an Innovative Technology to Develop Low-Toxicity Kinase Inhibitors (Poster 7)**

*Xiang Li, Ph.D., University of Maryland, Baltimore County*

In this project, Dr. Li and his collaborators have developed an unbiased gel-based system for identifying low-toxicity kinase inhibitors. This system globally quantifies the phosphorylation index for substrates of specific kinases 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 molecule numbers are then measured after complete chemical de-phosphorylation, which yields the ratio of phosphorylated to total substrate. Dr. Li and his collaborators have used this system to identify high-efficiency substrates for the protein kinase CK2. These experiments identified an inhibitory peptide based on the major CK2 phosphorylation site on elongation factor 1 $\beta$ .

**Multiple Reaction Monitoring to Profile Biosensor Phosphorylation in Leukemia (Poster 29)**

*Laurie L. Parker, Ph.D., Purdue University*

Kinase inhibitors have revolutionized chemotherapy for leukemia, said Dr. Parker, but they still cannot cure cancer, largely because resistance develops to these agents in most patients. Evidence suggests that decreased Bcr-Abl substrate phosphorylation in mononuclear cells is a reliable indicator of initial response and predicts longer-term outcomes. Data also suggest that Bcr-Abl substrate phosphorylation in mononuclear cells could be used to make dosage adjustments during therapy. Such “real-time” monitoring of drug response during treatment is not currently performed, however, because technical hurdles present a challenge to efficiently analyze large numbers of patient samples.

To address this issue, Dr. Parker’s group is attempting to merge its intracellular kinase biosensor assay (described in an earlier presentation) with multiple reaction monitoring (MRM) mass spectrometry to create a sensitive, multiplexed assay for leukemia-related signaling in patient samples. She described the biosensor peptide that her group has developed and the MRM experiments they conducted demonstrating that they could quantify Abl kinase activity on about 10,000 cells. The sensitivity of this assay was as low as 10 to 30 femtomoles when working with cultured chronic myelogenous leukemia cells. She described the workflow for this assay and noted that she and her team are working on improved sample-handling and enrichment procedures to improve the assay’s signal to noise performance. She and her team are also working on a multiplexed assay that will measure the activity of more than one kinase in the same assay.



**Probing Cancer Cell Chemoinvasion Strategies Using 3D Microfluidic Models (Poster 32)**

*Mingming Wu, Ph.D., Cornell University*

Tumor cells migrate in response to chemokine or growth factor gradients, said Dr. Wu, and this migration plays an important role in cancer metastasis. To better understand the role that chemokine-mediated tumor cell migration plays in metastasis, Dr. Wu and the members of her laboratory are using a three-dimensional microfluidic device to model key cancer cell migration under changing interstitial fluid flows and biomatrix stiffness, two key biophysical parameters thought to influence metastasis. The goal is to develop a microfluidic model with predictive power and use it to create a microenvironment that suppresses chemoinvasion-mediated metastasis.

Using this device, Dr. Wu and her collaborators have shown tumor cell morphology is heterogeneous and that it displays plasticity in the presence or absence of fluid flow. They also found that tumor cell motility was also regulated by fluid flow. She and her team also developed an assay that tracks tumor cells migrating in a fluorescent bead embedded in three-dimensional collagen bed. The data from this assay can be used with a forward computation algorithm to map a single cell traction field in real time.

**Specific and Reversible Binding of DNA Nanoparticles to Cancer Cells (Poster 38)**

*Bradley T. Messmer, Ph.D., University of California, San Diego*

Dr. Messmer and his colleagues have developed a process that creates highly diverse libraries of selectable, self-assembling DNA nanoparticles (DeNA<sup>no</sup>) with unique secondary structures capable of binding to specific types of cells. He described how these nanoparticles are created using rolling circle amplification and how he and his colleagues use a SELEX-like approach to identify nanoparticles that bind to distinct cells types with high specificity. The first successful selection identified a DNA nanoparticle that binds to dendritic cells, said Dr. Messmer, and he showed other examples of successful selections, including a DNA nanoparticle that binds specifically to pancreatic cancer cell lines and breast cancer cell lines. He and his colleagues have also created an antagonist that can block particle binding to the pancreatic cell line or trigger bound particle release. They are now exploring various *in vivo* and *in vitro* applications for these DNA nanoparticles.

**2D-PCR for Spatially Mapping Gene Changes in Tumor Sections (Poster 1)**

*Daniel Gowetski, Ph.D., University of Maryland*

Dr. Gowetski described a system that he and his collaborators have developed for creating two-dimensional PCR maps of genomic DNA in laser capture microdissection tumor specimens. This team has also developed an enzymatic method using methylation-sensitive restriction endonuclease digestions that can epigenetically map a tumor section. He briefly described the newest methods they have developed to streamline this process in order to minimize sample loss. This procedure involves affixing the sample to a multi-well plate containing lysis buffer, performing an

overnight digestion, and then conducting qPCR to determine methylation. The goal, explained Dr. Gowetski, is to create a methylation map of a target gene across an entire tissue sample in a single run. He is now working on developing the mapping technique and is working to miniaturize the qPCR system to obtain a resolution of 100 microns or less. He and his colleagues are also developing a multiplexed version of this system to assess methylation of several genes in the same tissue sample.

**Discovery Platform for Cancer Antigens (Poster 16)**

*Kevin Claffey, Ph.D., University of Connecticut Health Center*

The technology being developed in this IMAT project, explained Dr. Claffey, takes advantage of the biology that humans have to detect cancer antigens. The idea is to isolate antibodies produced by the patient and use them to identify the matching patient-specific antigens. The process started with small slices of sentinel lymph nodes taken from patients. Dr. Claffey and his team used three molecular screens on B cells to identify those that may be making antibodies against tumors. They then isolated 14 libraries of variable region single-domain antibodies that were effective at producing large volumes of clones during transformation. They have screened approximately 12,000 clones and sequenced 6,000 of them, identifying around 200 clones that may be useful for identifying the cognate antigens. In total, Dr. Claffey's group has synthesized 46 variable region single-domain antibodies and used six of these to select target antigens from cell or tumor lysates. Plans call for using these antibodies to determine antigen distribution in human breast and ovarian cancers with the eventual aim of creating novel diagnostic reagents and identifying new therapeutic targets.

**Digital Analysis of Proteins Through End Sequencing (DAPES) (Poster 15)**

*Tom Cohen, Ph.D., Washington University in St. Louis*

Dr. Cohen described the work that he and his colleagues in Rob Mitra's laboratory are doing to develop the Digital Analysis of Proteins Through End Sequencing (DAPES) method for single peptide sequencing. He explained that the goal is to use DAPES to produce proteome-wide sequence information from complex mixtures such as blood. This technique, which is modeled on the approach used in next-generation DNA sequencing, starts by using a set of enzymes to cleave protein samples into peptide fragments that are then immobilized on a nanogel-coated glass coverslip. The nanogel is a polyethylene glycol-based material that has been optimized to bind peptide fragments while dramatically reducing nonspecific interactions of fluorescent probes with the glass surface.

Once immobilized, the terminal amino acid in these attached peptides is identified using a fluorescently labeled N-terminal amino acid binding molecule (NAAB) - engineered proteins developed in collaboration Jim Havranek's laboratory at Washington University – and single molecule fluorescence imaging. That amino acid is then cleaved and the imaging process repeated for a total of 10 amino acids from the N-terminal of each peptide on the nanogel. The peptide sequences are then mapped back to the human proteome. Experiments have shown that there is some

cross-reactivity of the probes, which enables the use of fewer than 20 probes to identify the 20 amino acids through the use of probe binding signatures.

Dr. Cohen noted that it will also be possible to take the number of peptide sequences that map back to a particular protein and divide that by the total number of possible peptides that could be produced by that protein to yield a quantitative measure of the protein's abundance. He also described experiments confirming that it is possible to remove probes from the immobilized protein, cleave single residues using the Edman reaction, and introduce the next probe. Though further optimization of each step is still needed, data show that this protocol produces an 80% cleavage rate.

### **Molecular Diagnostic Tests to Augment Cytomorphologic Diagnosis of Lung Cancer (Posters 27 and 39)**

*James C. Willey, M.D., and Thomas Blomquist, M.D., University of Toledo Health Sciences Campus*

This presentation described the highlights of two posters from Dr. Willey's laboratory, both featuring work on developing robust quality controlled molecular diagnostic tests with intrinsic quality control metrics for qPCR and next-generation sequencing diagnostic methods as applied to human biospecimens. Dr. Willey began by noting that there are large archives of FFPE samples from subjects with known outcomes and response to specific drugs, but the RNA quality in these samples is variable and typically bad. Therefore, there is a need for quality-controlled RT-PCR methods to make use of these samples. Toward this goal, Dr. Willey described a two-color fluorometric real-time PCR method that generates small (60-80 bp) products suitable for analysis of degraded RNA, and measures each gene relative to a known number of synthetic internal standard molecules to control for interfering substances. For each gene, an internal standard was synthesized with 4-6 nucleotides different from the native template, then a QUASAR-labeled probe homologous to the internal standard and a FAM-labeled probe homologous internal standard probe was prepared. He described the validation tests that his group ran on a qPCR test prepared with this method showing promise for diagnosing lung cancer, the [MYC X E2F1]/p21 assay to augment cytomorphologic diagnosis of lung cancer, In analysis of 38 FFPE biopsy samples (20 surgical tissues and 18 fine needle aspirate samples) with the [MYC X E2F1]/p21 assay the receiver operator characteristic area under the curve was 0.87 with 95% confidence interval of 0.74 to 0.99 and the statistically optimal LCDT cut-off value had 92.9% specificity and 75.0% sensitivity.

Next, Dr. Blomquist spoke about the project he has been working on to develop methods for identifying and controlling bias associated with RNA sequencing technology. He described the Standardize RNA SEQuencing (STARSEQ) technology that uses libraries prepared through massively multiplexed PCR of each target in the presence of a known number of synthetic internal standard copies to control for inter-library variation in transcript abundance measurement. PCR is done under low primer concentrations and touchdown thermal cycling conditions to enable which results in convergence of high- and low-abundance target amplicons toward equimolar

concentration at PCR endpoint. He explained that because the PCR product for each nucleic acid target is at approximately equal abundance, this approach promises to markedly reduce the required read depth and thereby reduce sequencing costs by log-order magnitude. He then described one experiment showing that the measured abundance of nucleic acids in a sample prepared by mixing two FDA SEQC standards of known nucleic acid abundance agreed with the expected abundance.

**Method for Detection of Secreted Proteins in Single-Cell Assays (Poster 24)**

*Henryk Szymacinski, Ph.D., University of Maryland*

In the last presentation of the poster previews, Dr. Szymacinski described the development of an assay system for secreted proteins that uses plasmonic fluorescence enhancement to achieve single-digit picogram/mL sensitivity. He briefly commented on his group's work creating a plasmonic substrate for amplifying optical signals and then described what he calls the MEFspot technology as a direct, multi-color technique that uses a fluorescence lifetime imaging microscope to collect intensity and lifetime images for each fluorophore present on an array spot. If the spot is really generated by a protein-secreting cell, it displays characteristic values for these measured parameters – an intensity value greater than background and a lifetime shorter than background.

He then showed data from multiplexed experiments quantifying real-time TNF- $\alpha$  and interferon- $\gamma$  secretion by macrophages. He also described the Phasor Plot analytical method based on fluorescence lifetime measurements that accurately identifies secreting cells. In a final note, he said that this MEFspot technology is simpler than the ELISPOT method for single cell assays.

**DAY 2 - NOVEMBER 28, 2012**

**HOST WELCOME**

*Mauro Ferrari, Ph.D., The Methodist Hospital Research Institute*

To start the second day of the Principle Investigators Meeting, Dr. Ferrari welcomed everyone to The Methodist Hospital Research Institute and noted that IMAT has contributed in a largely unrecognized but absolutely essential way to the development of many of the most important technologies that are at the heart of today's revolution in genomics and diagnostics. The development of platform technologies is not appreciated enough, he said, but this type of bricks and mortar research is absolutely essential to enable the research that will solve the problems of cancer and other major human illnesses.

Dr. Ferrari then reviewed the history of the development of the Texas Medical Center, which started with an enormous donation of cash and land by M.D. Anderson and is now part of the largest medical center in the world, employing over 200,000 people and handling over 6.5 million patient visits a year. He then discussed The Methodist Hospital Research Institute and described some of the infrastructure that is available to collaborators, including a Good Manufacturing Practices (GMP) manufacturing facility, a GMP cyclotron and cell therapeutics facility, imaging suites, and robotic surgery facilities. The Institute also has its own Clinical Research Organization (CRO) to run clinical trials.

The Methodist Hospital Research Institute, explained Dr. Ferrari, is set up as a collaborative institution structured around interdisciplinary fields, and he encouraged IMAT investigators to work with Institute faculty and to make use of its facilities. He closed his remarks by emphasizing the strong emphasis that the Institute places on translational studies. The entire infrastructure of the Institute is set up to move products into the clinic and dovetails with the clinical trials infrastructure at the Methodist and throughout the Texas Medical Center.

**KEYNOTE ADDRESS**

*Joshua LaBaer, M.D., Ph.D., The Biodesign Institute, Arizona State University*

In his introductory remarks, Dr. LaBaer commented that IMAT is a rare program in that it focuses on developing the enabling platforms that drive research throughout the biomedical enterprise. He also provided some background on the Biodesign Institute at Arizona State University, noting that it is problem-oriented with a strong emphasis on translation.

Turning to the main subject of his talk – how to develop and apply new protein microarray technologies – Dr. LaBaer said that is essential when designing a new platform technology to always remember the problem that this new technology is meant to solve. In his case, the problem that he wanted to address was the early detection of cancer, the key to reducing the mortality of cancer, and Dr. LaBaer's focus is on biomarkers. Biomarkers, he explained, provide biometric measurements that convey information about the subject's biological or clinical state, and they are different from biological differences. He showed an example of how a test value can

display a huge uncertain zone between normal and disease, and explained that the size of that uncertain zone is related to the specificity and sensitivity of a test value.

Biomarkers can be classified by their clinical use, the source and type of test material, and the level of validation. He listed the different types of biomarkers by clinical use, the variety of source materials, and the different molecular and cellular types of biomarkers, and then spoke about the different validation levels that a biomarker must go through to become clinically useful. The first step in biomarker development is to define the clinical need and the necessary performance characteristics to meet that need. Next comes finding an observable difference between disease and normal that can lead to the identification of a candidate biomarker by comparing properly matched cases and controls. The marker is verified by repeating the comparison with a fully independent and blinded set of cases and controls and then validated in a large, blinded, prospective study conducted at more than one location. The utility of the marker is then validated in additional large, randomized, blinded, prospective studies encompassing more than one research site, and then submitted for approval, typically with a commercial partner.

He then described the extensive range of skill sets that are needed to successfully develop a biomarker and listed the common pitfalls that scuttle biomarker development efforts. The latter includes discovery without defining clinical need, conducting underpowered studies using inappropriate statistics, the use of inappropriate samples or poorly selected controls, a failure to account for over-fitting or false discovery in “omics” studies, and a failure to ensure that the biomarker assay is robust and reproducible in settings other than the developer’s laboratory.

As a final note before discussing protein biomarkers, Dr. LaBaer said that there are thousands of papers reporting promising biomarker results, but that FDA approves about one new biomarker-based diagnostic a year. To remedy this situation, academia needs to take the lead role in developing biomarkers. Industry, he said, does not have the funds to invest in discovery, but it will take on biomarker development after a biomarker has been validated. He added that the field needs to set a higher bar for biomarker discovery publications, perhaps requiring preliminary validation data. He also said that there is a need to publish negative results – killing a bad biomarker is important and researchers need recognition for this negative validation work.

Dr. LaBaer then spoke in praise of proteins as cancer biomarkers. Nucleic acids, he said, are terrific but the initial biopsy is usually the only source of nucleic acids for analysis. Proteins, however, can reflect an ongoing clinical state. From a practical perspective, nucleic acid microarrays do not have a great track record with the FDA, while protein-based diagnostics are well-established with a well-defined path toward regulatory approval. In addition, our best biomarkers today would not have been predicted by genetics, he said.

He then described the libraries of expressed genes, including full-length expression ready (FLEX) clones, that his group has developed and that are available

to anyone who wants to use them with no restrictions. These libraries, which are created using robotic techniques, can be ordered via the Web at <http://dnasu.asu.edu>. He noted that DNASU has delivered 300,000 samples to 38 countries and 46 states.

Turning to the discussion of the protein microarrays his group is developing, he recounted how his laboratory gets around the challenge of printing purified proteins when many proteins do not purify well. Their approach is to print the cDNAs that codes for the proteins of interest, and he noted that these arrays can be stored dry for at least six months. Using the array entails adding a cell free expression reagent to the array, expressing the proteins *in situ*, and capturing them using the co-printed protein capture reagent. This technology, called nucleic acid-programmable protein array (NAPPA), has many advantages over assays that use printed proteins. For example, proteins are expressed in the mammalian milieu, so they fold naturally. Shelf-life is not an issue since the proteins are produced on demand, and protein expression levels, at about 700 picograms per spot, are about 1000-fold higher than in the typical protein-spotting arrays.

His group has developed NAPPA 2.0, which is more robust and scalable than the original platform. His team has developed high-density printing, improved capture chemistries, and better DNA preparation methods, and they have improved detection on the array. He noted that the result, a high-density array, produces very even levels of protein with no protein class or size biases and with a very tight range of protein levels. He added that it is not necessary to make full-length proteins if the goal, for example, is to map antibody epitopes.

Dr. LaBaer then presented a few brief examples of some of the applications of this technology, including the use of protein microarrays for immune profiling and for measuring protein response to therapy. The first application of the former was to identify patterns of proteins that cystic fibrosis patients produce in response to infection. The idea was to identify good antigens as either biomarkers of disease or candidates for a vaccine, but it turned out that no one antigen was observed in all patients.

NAPPA technology has also been used to identify the antibodies that cancer patients produce against tumor antigens, which are proteins that are not usually encountered by the immune system. The advantages of looking for antibodies, versus peptides, as biomarkers is that antibodies persist long after the triggering antigen is gone and their levels are stable in blood. Individual autoantibodies of this type are prevalent in low levels in blood, but they are observable nonetheless. What is required, though, is a panel of these antibodies, since each appears in about 15-20 percent of women with breast cancer. In a blinded study of women with stage 1 breast cancer, Dr. LaBaer's group, over three stages of screening, found a 28-antigen panel that was diagnostic of stage 1 breast cancer. He noted that a new study is underway looking just at basal-like breast cancer.

His group is currently working on developing NAPPA 3.0, which will feature better and faster DNA preparation, improved protein production, and reduced background; include functional assays; and be printed at higher density. Toward this end, he noted that his team is developing a DNA factory that will perform 4600

preparations in 70 hours, and they have discovered that adding a lysate of *E. coli* to the array reduces the background signal of the array. They have also increased protein yield on the array by using a human protein expression system. He then described an example of a functional array that measures auto-phosphorylation on the NAPPA platform that can be used to screen for kinase inhibitors for specific kinases. He also mentioned a new covalent protein capture technology that not only improves assay performance, but also allows proteins to be denatured on the array in order to search for additional epitopes.

Development work showed that the density of the arrays was limited by diffusion between spots, so Dr. LaBaer and his collaborators are using lithographic techniques to create nanowells on silicon that can be sealed with silicone to stop diffusion and spillover between wells. Using this approach, his group can now print 8,000 features per array and he believes that the density can be increased to as high as 20,000 features per array using high-density piezo printing to fill the nanowells.

During the brief discussion session following his talk, an audience member asked about the sensitivity of detection of these NAPPA arrays. Dr. LaBaer said that sensitivity is not a major limitation because the arrays can be used with a wide range of detection technologies. As an example, he said he was confident that he could use the magnetic nanotags that Dr. Wang described in the first session to approach single molecule detection. The major concern, he said, is identifying markers that are present in many patients, not just one.

#### **PANEL SESSION: PATHWAY TOOLS I**

*Chair: Joshua LaBaer, M.D., Ph.D. The Biodesign Institute, Arizona State University*

In his introduction to the first of two sessions on pathway tools, Dr. LaBaer noted that one of the findings from TCGA and similar efforts is that there not just 15 to 20 important proteins in cancer, but hundreds whose presence or absence varies tremendously from patient to patient. However, what these analyses are also showing is that these proteins occur in common pathways, making it clear that cancer is a disease of pathways, which raises the question of how to identify which pathways are affected in a particular cancer, and how to treat that pathway.

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

*David Muddiman, Ph.D., North Carolina State University*

The focus of Dr. Muddiman's research is to identify biomarkers in stage 1 and stage 2 ovarian cancer patients with the goal of developing a diagnostic assay for the early detection of ovarian cancer. He began his talk by reviewing ovulation biology and the similarity between human and chicken ovarian physiology. Chickens, he said, are a good model for studying ovarian cancer because they develop spontaneous ovarian cancer – about 35 percent of chickens develop ovarian cancer after age two – and they ovulate daily, providing a good research opportunity for conducting longitudinal studies for biomarker discovery.



He described the study he and his team conducted in which they collected five biospecimens between the age of two and three, and a new study that started in April 2012 that will collect 12 time points over 2.5 years starting at age six months. His group is looking at post-translational modification in blood proteins, focusing in particular on glycosylation. Aberrant glycosylation has long been linked to cancer, but the technology for assaying native N-linked glycans is plagued by too much variability in the analytical measurements. To address this limitation, his group is developing a <sup>13</sup>C-tagging method for use with mass spectrometry. He described the multifunctional, tunable hydrazide-based reagent his group developed to efficiently derivatize glycans and maximize ionization efficiency in the mass spectrometer. This hydrophobic reagent also enabled the use of reverse phase liquid chromatography to separate glycans, resulting in a much cleaner separation of individual glycans and an improved mass spectrometry signal.

By using an internal standard, Dr. Muddiman's group showed that it could correct for analytical variability. The dynamic range of this technique is approximately four orders of magnitude, and his laboratory is working to improve the lower limit of detection. Horseradish peroxidase, which has a xylose that is group not present in animal samples, serves as an effective internal standard to control for digestion efficiency. In closing, Dr. Muddiman noted that his laboratory makes these reagents available and is transitioning them to a commercial synthesis company.

### **High-Throughput Translational Control Analysis by TrIP-chip**

*Jingfang Ju, Ph.D., Stony Brook University*

At the start of his talk, Dr. Ju noted that his laboratory has a long history of interest in post-transcriptional and translational control. He reviewed the pathways of gene regulation and how they are studied by simultaneously monitoring the translation state of mRNAs. The problem with the old approach is that it requires a large number of cells. He and his group have now developed an approach that works on a small number of cells so that they could better understand the mechanism of resistance to 5-fluorouracil and antifolate therapy. The key target for this therapy is the enzyme thymidylate synthase, and regulation of expression of this enzyme plays a central role in the development of resistance.

The technique his team developed, called TrIP-Chip/Seq, uses magnetically tagged antibodies to pull out the translational complex by targeting the translational chaperone protein hsp70, that can then be analyzed using any suitable technology. He described the method and discussed how his team used a colon cancer model when developing the technique. He presented data showing that the TrIP approach and ultracentrifugation produced about 85 percent overlap. An *in vitro* proof of principle experiment was successful at pulling down a known translational complex, as was an *in vivo* experiment involving expression of a known regulator in colon cancer cells. He closed his talk by showing data from a study of global translational control by 5-fluorouracil treatment in which the method was able to identify post-transcriptionally regulated mRNAs.

## **Scanning Correlation Microscopy Methods for Quantifying DNA Repair Kinetics**

*Georgios Alexandrakis, Ph.D., The University of Texas at Arlington*

Before discussing his team's work on developing technology to quantify DNA repair kinetics, Dr. Alexandrakis reviewed the mechanism of radiation injury to DNA. During S-phase, DNA injury involves template-driven homologous end-joining, while at all other times the mechanism involves non-homologous end-joining. Most studies of DNA repair, he explained, use focused high-power lasers to create visible protein accumulation at DNA damage sites. As soon as cells are irradiated, time-lapse video imaging reveals an increase in concentration of repair protein at the site of damage followed by a gradual decrease. The problem with this approach is that it requires triggering massive DNA damage, not the sparse DNA damage that more closely resembles what occurs during radiation therapy.

The approach that Dr. Alexandrakis employs uses raster image correlation microscopy and a statistical method known as number and brightness analysis. The former is a scanning microscopy technique that detects fluorescence fluctuations resulting from molecular interactions. This technique measures fluctuation intensities as molecules go through the focal length of a confocal microscope, and from that data it is possible to calculate a correlation function and use biophysical modeling to determine reactant concentrations, diffusion coefficients, and binding rate kinetics. Number and brightness analysis is a statistical method that provides information about the relative change in the mobile fraction of the reactants. Using both these techniques, Dr. Alexandrakis's team monitored and quantified the kinetics of DNA damage repair after gamma irradiation and for DNA repair kinetics after bleomycin treatment.

In his final remarks, he discussed the use of two-photon microscopy to increase image brightness. He described the commercial pulse shaper his group used and showed how this approach increased the brightness of a fluorophore by 10-fold, which enables rapid data acquisition. He also discussed the use of two-color cross-correlation technique to study both partners of a binding pair. He noted that this technique can be used with FRET to reduce cross-talk among cells.

## **PANEL SESSION: BIOMARKER PRESERVATION AND DISCOVERY**

*Chair: Ignacio Wistuba, M.D., The University of Texas MD Anderson Cancer Center*

As an introduction to this session, Dr. Wistuba discussed some of the challenges in preserving biomarkers in clinical samples. The new paradigms in the molecular pathology of cancer, he said, are based on the detection of multiple clinically relevant molecular abnormalities in tumors and that these "driver alterations" can be used to direct targeted therapy and improve patients' outcomes. As an example, he listed 11 molecular alterations observed in non-small cell lung cancer biospecimens, all but two with a prevalence of five percent or less, and their association with specific therapies. The number of such alterations is likely to grow as a result of efforts such as TCGA.

For the pathologist, tissue is king, said Dr. Wistuba, but for the researcher looking for biomarkers, tissue leaves much to be desired as a source material. Most tissue available for research comes from late stage cases and is a combination of tumor and non-tumor. In addition to the heterogeneity of the sources of tissue and in terms of its gross composition of tumor and non-tumor, tissues for biomarker discovery are often processed differently than those used for pathological diagnosis. While significant progress has been made on mutation analysis of tumor tissue specimens, not much progress has occurred for gene expression or proteomic analyses, and little is known about the impact on biomarkers of intra-patient and intra-tumor heterogeneity.

Another problem confronting biomarker discovery efforts is that such studies require access to tissue samples across all stages of disease, but in most cases, biopsies are not taken repeatedly. He noted in closing that the MD Anderson BATTLE program is now collecting samples from patients at all disease stages, as well as before treatment and during treatment.

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

*Lanlan Shen, M.D., Ph.D., Baylor College of Medicine*

Cancer is a complex interaction among genetics, environment, and epigenetics, said Dr. Shen. DNA methylation is a major epigenetic mechanism that requires dietary methyl donors and cofactors absorbed through the colon, raising the question of when and what epigenetic changes occur as a part of normal colonic differentiation and functional maturation, and what role do those changes play in cancer susceptibility. She outlined the experiments her laboratory is conducting to identify developmental changes in DNA methylation, determine the impact of diet on developmental epigenetics, and to compare developmental methylation changes separately for colonic epithelial stem cells. Her team combined methylated CpG-island amplification (MCA) with next-gen sequencing technology to create the MCA-Seq platform. This novel technology is highly sensitive and specific for DNA methylation, to conduct genome-wide screening and validates the results using quantitative bisulfite-pyrosequencing. MCA-Seq, she noted, can detect changes in methylation as small as 10 percent, and costs a relatively modest \$400 per sample.

Her group's studies showed there was a jump in methylation with age that starts during post-natal development, and that the methylation changes occurred largely in repeated regions of the genome as opposed to unique regions. This age-associated methylation increase affects all repeat classes similarly, she said. The majority of the changes in the unique genomic regions involved developmental loss of methylation post-natally, and many of the changes were involved in the Wnt signaling pathway.

The *Apc2* gene locus proved to be of particular interest. When diet was supplemented during the fetal period with methyl donors, the loss of methylation was blocked post-natally, an effect that persisted at least three months. Given that the role of *Apc2* is well documented in colon cancer, Dr. Shen and her team repeated these

experiments in a larger population of mice. Again, methyl supplementation prevented the loss of Apc2 methylation. She noted that methylation was not occurring in the promoter region, but at the gene's 3' end, which was a puzzling finding. Additional experiments showed that this decrease in methylation was taking place in colon stem cells. One possible conclusion from these findings, Dr. Shen said in summary, is that changes in methylation could provide a link between developmental epigenetics and later risk of colon cancer.

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

*Olivier Harismendy, Ph.D., University of California, San Diego*

The most important paradigm shift occurring in the cancer world today, said Dr. Harismendy, is associated with the idea that molecular profile more than tissue of origin matters more for therapeutic success, even though most clinical care is still organized by tissue. He discussed PIK3CA, BRAF, and KRAS as examples of genes that are mutated across cancers, and noted that intra-tumoral heterogeneity is an important confounding issue in cancer research and therapy. He reviewed the past and current methods being used to detection mutations and discussed the tradeoffs between number of samples, coverage per sample, and region size. His group's approach, which he said is best suited to heterogeneous samples, is to use a fixed gene panel that provides ultra-deep coverage of a few genes.

After describing the desired specifications for this assay, the most important of which are that it detects mutations with five percent prevalence at a sensitivity of 95 percent and a false positive rate under five percent, that the turn-around time for results is under two weeks, and that the cost per patient is under \$2,000, Dr. Harismendy described the microdroplet PCR technique and UDT-Seq assay that his group is using. He noted that this is a multiplexed assay that provides direct and directional sequencing with uniform coverage, an important consideration for keeping costs low. He presented details of the assay designed to study 47 actionable genes by examining 894 exons and 1736 amplicons. He also briefly described the Mutascope analytical package that his group developed to analyze UDT-Seq data and identify germline and somatic variants.

His group has now sequenced 38 samples and found that 20 out of 38 have less than 50 percent invasive tumor cells. He showed the somatic coding landscape for the 38 patients and noted that 14 of 38 had P53 coding mutations and 10 of 38 had PIK3CA hotspot mutations. In total, there were 173 germline variants observed in the 38 patients. His group has started to use this information to match patients with therapies.

### **Tissue is Alive: Preserving Biomolecules and Tissue Morphology in Clinical Trial Samples**

*Lance A. Liotta, M.D., Ph.D., George Mason University*

Molecular profiling of samples in the clinic is hampered by formalin fixation for many reasons, said Dr. Liotta, but he claimed that the field can break free of the

chains of formalin thanks to work that IMAT has funded in his laboratory. His group is now commercializing a fixative that preserves tissues, including phosphoproteins, better than formalin, and that achieves the exquisite preservation of tissue morphology demanded by pathologists.

Before discussing the new fixative, he reviewed the work that his group had done looking at the stability of phosphoproteins under the oxidative stress conditions that tissues experience immediately after removal from the body. During the first hour alone, a large number of phosphoproteins increased in abundance, while others decreased. To protect tissue against these reactive changes, Dr. Liotta's laboratory developed what he calls biomarker and histology preservative (or BHP), a one-step snap preservative that can be used with both snap-freezing and paraffin embedding. The fixative works rapidly to preserve biomarkers – within one minute of tissue exposure to BHP, both phosphatase and kinase activities are greatly inhibited, and protein synthesis is halted completely. The yield of extractable protein from BHP fixed tissue is 10- to 16-fold greater than with formalin fixation, and extraction can be accomplished in an eight minute standard protocol.

Equally important, diagnostic histology with tissues preserved using BHP was equal or superior to that obtained with formalin fixation. BHP preserves nuclear volume, color, and chromatin for every major tissue in the human body, as judged by outside pathologists who conducted an independent review. In addition, the new fixative can be used with bone without the need for prior decalcification, opening an opportunity to profile bone and bone metastases for biomarkers. Finally, Dr. Liotta's group has demonstrated that the fixative does not impact immunohistochemistry results, and in fact, produces better results in some instances. As an example, he noted that Ki-67 antigenicity is maintained with this fixative, addressing a significant issue in pathology.

Dr. Liotta then presented data demonstrating that BHP can preserve RNA in a manner similar or equivalent to snap freezing and about equivalent to RNALater or Qiagen methodology. BHP also affords a high level of preservation of DNA for high-fidelity single-nucleotide polymorphism and copy number variation analysis. He concluded his talk by describing the validation workflow and listing the wide range of collaborators who have provided independent validations. In summary, he said that with the development of BHP it is now possible to use one paraffin block for both histology and biomarker discovery and validation.

### **Sentinel RNAs as a Measure of mRNA Integrity in Clinical Biospecimens**

*Curt H. Hagedorn, M.D., University of Utah*

In the final talk of this session, Dr. Hagedorn addressed the stability of RNA in biospecimens, an issue that he called vexing. Next generation technologies to measure gene expression, such as RNA sequencing, represent promising new ways of analyzing biospecimens but the quality of RNA in specimens is a real issue. In general, he said, the RNA species of interest are RNA polymerase II (Pol II) transcripts, and he described the general pathway for eukaryotic Pol II RNA decay. What his group has done over the years is study a critical protein, the mRNA 5' cap

binding protein (eIF-4E), that regulates mRNA translation and identified a mutation that increases 5' cap binding dramatically. This high affinity variant was used to purify 5'-capped RNA. The idea is to use this technology to develop an analytical method for identifying sentinel transcripts that decay at the 3' end as a means of better assessing biospecimen suitability for RNA analysis.

Dr. Hagedorn recounted the results of a study in which his laboratory took snap-frozen liver biopsy samples and measured RNA Integrity Numbers (RIN) over time at room temperature. He noted that any RIN value greater than seven is considered to be of high enough quality to be useful for diagnostic tests. However, when he and his team used more powerful RNA sequencing analysis to measure RNA quality in samples with RIN values exceeding this threshold, they found that after 10 minutes at room temperature, about 15 percent of the RNA in a biospecimen was degraded, but by 15 minutes over 70 percent of the RNA was degraded. If the cutoff for diagnostic use is 50 percent intact RNA, about 30 percent of transcripts are lost within 10 minutes.

He then described the bioinformatics criteria for identifying sentinel RNAs. The key point, he said, is that his group focused on abundantly expressed transcripts and transcripts that had significant loss of 3' integrity compared to 5' integrity. He and his collaborators found a small number of slow phenotypes, such as cytochrome c oxidase subunit 1, that serve as controls and 304 candidate sentinel RNAs, such as beta-2-microglobulin, enolase 1, and  $\alpha_1$ -antichymotrypsin, that display fast 3' degradation. As an aside, he discussed a study on adenomatous and serrated polyps that identified Mucin 17 and other RNAs as a potential biomarkers capable of distinguishing between these two types of polyps, which are often misidentified.

Dr. Hagedorn noted in his concluding remarks that his group has switched from liquid nitrogen flash freezing to immediate RNALater preservation in their studies. He added that the fixative developed by Dr. Liotta's group looks very promising.

## **PANEL SESSION: PATHWAY TOOLS II**

*Chair: Stephen Wong, Ph.D., The Methodist Hospital Research Institute*

In his introduction to the second session on pathway tools, Dr. Wong noted that there is no shortage of databases for the disease-specific analysis. However, there are a number of challenges to turning these databases into useful insights about cancer, particularly with regard to constructing disease- and patient-specific pathways from general ones. He suggested that there may be a new type of signaling component, which he called cellular signaling bridges, that if elucidated would uncover downstream signaling molecules or mechanisms for known signaling pathways.

Another challenge, said Dr. Wong, is to integrate genetic and epigenetic information into pathway analysis. Computational modeling strategies to elucidate disease- and patient-specific signaling mechanisms could prove useful in addressing this challenge. Multidimensional data analysis and integration could yield patient-specific signaling pathways that could then be used to derive personalized treatment

plans using drugs repositioned to meet the genetic or epigenetic characteristics of that patient's cancer. He cited modeling work being done in his laboratory that has identified a new specific pathway, a set of 31 prognostic genes, and repositioned a drug for phase II clinical trials for the treatment of metastatic breast cancer. One limitation to this approach today is the cost of performing such a modeling exercise. If the cost of multi-dimensional data generation for modeling can come down significantly, this will become a very powerful approach, Dr. Wong predicted.

### **VEC3-Valve Enabled Cell Co-Culture Platforms for Cancer Biology Study**

*Deyu Li, Ph.D., Vanderbilt University*

The motivation for this study, explained Dr. Li, was to use microfluidics for co-culturing cells in a way that provides exquisite control of conditions that each cell receives and allows for cell-cell interactions. Toward this end, he and his colleagues have developed a device that can completely cut off cell-cell interactions yet be manipulated to allow for direct cell-to-cell contact or contact via the diffusion of soluble factors between the two cell types, all while maintaining fluidic control over the microenvironment of both cell types. The valve-enabled cell co-culture (VEC<sup>3</sup>) platform consists of a coverslip coated with layers of PDMS that can be flexed through a change in pressure to lift a PDMS barrier between two growth chambers.

After describing the construction of the VEC<sup>3</sup> platform, Dr. Li discussed an experiment in which he co-cultured tumor and endothelial cells on a VEC<sup>3</sup> device under hypoxic conditions. He explained that under normoxic conditions, the two cells each migrate into the other chamber, but under hypoxic conditions, only endothelial cells migrated into the tumor cell chamber. The device was also used to demonstrate knocking out EphA2 production stopped tumor-endothelial cell cross migration, that Slit2 has a chemorepulsive effect on migrating cells, and that the Rictor gene suppresses tumor cell migration into endothelium but has a positive effect on endothelial cell migration into tumor cell populations.

Dr. Li also designed a device that keeps two cell populations separate but allows soluble factor exchange when the valve is activated. In addition to having a PDMS barrier to stop all direct and indirect contact between cell populations, this device adds an agarose-based barrier to separate cells but allow soluble factor diffusion. The agarose barrier can be modified further through the addition of nanoparticles coated with molecules that bind specific soluble factors. The latter device configuration can be used to study the effect of specific diffusible molecules on cell-cell interactions. Dr. Li also noted that he and his colleagues have created a system for studying three-dimensional migration in collagen gels within the device microchambers.

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

*Harold C. Riethman, Ph.D., The Wistar Institute*

To better enable his group to measure accurately the global frequency of sporadic telomere deletion events and telomere fusions, Dr. Riethman and his team

have been developing a universal, high-throughput assay to detect and quantify single-allele resolution ultrashort (TTAGGG)<sub>n</sub> tract profiles, telomere fusions, and sub-terminal DNA breakage-rejoining events. Quantitative, single-allele resolution measurements of telomere length and instability would enable research that could provide important insights into the roles that telomere loss and telomere fusion play in cancer initiation, progression, and metastasis.

The assay that Dr. Riethman's team is developing is based on targeted DNA sequence capture using an array of biotinylated RNA baits from sub-telomeric regions and high-throughput paired-end sequencing. Between 57 and 64 percent of the targeted reads map to subterminal or telomeric regions, and between 94 and 97 percent of the sequences, are covered by at least two independent reads. The biggest challenge that this effort has faced is in tagging the physical ends of the chromosome.

From the data collected so far, it appears that a small but measurable fraction of telomeres from cancer cell lines are smaller than 600 base pairs in length. His group has identified a number of telomere fusion events in cancer cells, and for sub-telomeric region they've identified redundant mate pairs that were either structural variants that exist naturally or mutations; so far, they have been unable to distinguish between these two possibilities. His group plans to analyze data sets from normal and tumor of the same individual to see if they can resolve that issue. In closing, he noted that it is unclear whether telomere end-tagging and 600 base pair fragment mate-pair sequencing is going to work sufficiently well for sequence-based single-telomere length analysis. His group is now exploring alternative methods for accomplishing this challenging task.

### **Application of Next-Generation Sequencing to Cancer Epigenomics**

*Huidong Shi, Ph.D., Georgia Health Sciences University*

The specific aims of Dr. Shi's project are to develop a multiplexed method for high-throughput, ultra-deep bisulfite sequencing and develop a genome-scale approach for bisulfite sequencing of methylation-enriched genomic DNA libraries. Before describing the method his laboratory has developed, he discussed briefly the role that aberrant epigenetic changes play in cancer and the basics of bisulfite sequencing for detecting methylation. This method has been widely used for single gene analysis, he noted, but it is laborious and throughput is low. He noted, too, that methods exist for whole genome sequencing of the methylome, but again, these methods are slow and expensive, about \$5,000 to \$6,000 per genome.

To reduce these costs and accelerate sequencing, his group developed targeted bisulfite sequencing for use with the Illumina sequencing platform. This approach uses two sets of probes attached to magnetic beads to capture both the forward and reverse strands of the DNA. His laboratory worked with a group at the University of Missouri to develop software to design 35,746 probes for capturing every 500 base pairs within approximately 83 percent of the 28226 CpG islands in the human genome and another 15,000 probes to capture the regions surrounding transcription start sites of some 19,000 known genes in the human genome. Data from experiments with four cancer cell lines showed that this technique can measure about one million CpGs and



the probe success rate was about 85 percent. Reproducibility, noted Dr. Shi, was high, using only 2-3 micrograms of DNA for this analysis. He concluded from this data that this solution-based hybrid capture methods can be a powerful technique to uncover aberrant methylation alterations in the cancer genome.

To demonstrate the utility of their method, Dr. Shi and his group conducted a methylation analysis of DNA isolated from blood samples taken from patients with chronic lymphocytic leukemia (CLL). The DNA methylation landscape in CLL patients indicates that CLL B cells possess an active B-cell phenotype, but that they are faithfully committed to their lineage resembling either naïve or memory B-cells. Dr. Shi's group also found that DNA methylation plays an important role in defining the gene expression patterns of these prognostic genes such as ZAP70, CRY1, and LDOC1.

**PANEL SESSION: TECHNOLOGIES TO ASSIST WITH DRUG SCREENING AND DELIVERY**

*Chair: Melissa D. Landis, Ph.D., The Methodist Hospital Research Institute*

Before introducing the first speaker of last panel session, Dr. Landis noted that the increasing knowledge about the multiple mechanisms involved in cancer pathogenesis creates opportunities for drug discovery but also challenges for screening drugs that can target many aberrant pathways involved in cancer. In particular, new drug screens must be able to account for the heterogeneity of cancer, both at the stem cell and somatic cell level, as well as in the microenvironment surrounding a tumor. Above all, targeting the hallmarks of cancer requires personalized screening technologies that can help physicians choose the right cocktails of targeted therapies in order to ensure that patients that the biggest therapeutic effect with the fewest side effects and at the lowest cost to the nation's healthcare system.

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

*Raoul Kopelman, Ph.D., University of Michigan*

To begin his presentation, Dr. Kopelman said that a major challenge in cancer research was develop methods for tackling metastasis, which is responsible for 90 percent of all cancer related deaths. His group's approach is to control metastasis by stopping CTCs from adhering to blood vessel walls, a key step needed for CTCs to extravasate from circulation and colonize new tissues. A key to understanding how to interfere in this process, said Dr. Kopelman, is to understand the evolution of a CTC's morphology between the time it is in circulation and when it adhere to the endothelial wall. CTCs, he noted, are often isolated using a specific biomarker, such as EpCAM, and so any assay that measures therapeutic effectiveness by counting the number of EpCAM-positive cells, for example, may not account for the heterogeneity of the CTC population. In particular, he said, cells undergoing the epithelial to mesenchymal transition (EMT) may be missed using current biomarker-based approaches to isolating CTCs, and data from numerous experiments suggest that these are the most aggressively metastatic of the CTCs.

Dr. Kopelman's approach is to trap cells in single well traps and asynchronously rotate them to mimic the shear stress and attachment cues they experience in circulation. He and his team do this by transfecting the captured cells with poly-L-lysine coated iron oxide nanoparticles and then subjecting the cells to a low field, low frequency magnetic field that generates low torque and low drag conditions. They then measure changes in the rotation rate of the cells, which reflects even the slightest change in cell morphology. Changes in rotation rate also reflect the drug sensitivity for a given cell, an effect his laboratory demonstrated using cisplatin. Dr. Kopelman noted that magnetorotation does not affect cell growth rate or stop cell division for cell rotation periods longer than one second at frequencies below 30 Hz.

After describing the design of a microfluidic device with single cell traps that produces turbulent flow once the cell is captured in the chip, he discussed how his group assays cell shape in a multiplexed diagnostic assay system using an automated multiplexed shape recognition algorithm. He noted that amoeboid-like and protrusive cells are thought to have higher metastatic potential, an idea that experiments using this device with epithelial and mesenchymal prostate PC-3 cells confirmed. He added that experiments using Lantruculin A, a cytoskeletal inhibitor that prevents polymerization of actin monomers, showed that cells can adapt to their environment and switch between different morphological pathways, in this case between protrusive to amoeboid. These experiments also showed that a drug that inhibits myosin light chain kinase effectively reduced both amoeboid and protrusive behaviors. In closing, Dr. Kopelman said that his group is now going to conduct molecular and genomic analyses on cells with different morphological phenotypes with the goal of identifying biomarkers of metastatic potential. His group also plans to use their automated magnetorotation assay to test the potency of different drugs for preventing metastatic CTC attachment to vascular endothelium.

### **Scaffolds for Delivering Deoxycytidine Kinase to HER2 Positive Cancer Cells**

*Arnon Lavie, Ph.D., and Brian K. Kay, Ph.D., University of Illinois at Chicago*

To start their presentation, Dr. Lavie described the problem that he and his colleague Dr. Kay are attempting to solve. Nucleoside analogs such as AraC (cytarabine) and Gemcitabine have been used successfully to treat hematological malignancies, but have not had much success in treating solid tumors. The reason for this failure is that many solid tumors lack the enzyme needed to convert these analogs into active drug molecules. The approach that these two researchers and their laboratories are taking to overcome this problem is to create a fusion molecule between a HER2-binding proteins and deoxycytidine kinase (dCK), the enzyme that activates AraC and other nucleoside analogs. The researchers developed constructs using designed ankyrin repeat proteins (DARPin) and affibodies that bind to the human HER2 cell surface protein. The goal of this work is to develop a new therapeutic agent that might augment breast cancer therapy.

Dr. Kay then described how the two research groups created this scaffold and presented data showing that the fusion constructs bind to and are taken up by HER2-positive cells. Confocal microscopy showed that the DARPin-dCK construct is taken

up by cells and that the fusion did not interfere with enzymatic activity. Images of the affibody-dCK fusion were slightly different, which Dr. Kay said suggests that this construct is internalized, too, but that it does not escape the endosomal compartment. Experiments in which HER2-positive breast cancer cells were incubated with the DARPIn construct and a nucleoside analog demonstrated that this approach does have potential as this combination produced cell death that was not seen with either enzyme or drug alone. These experiments also showed that the combination of affibody construct and drug did not trigger cell death, which again suggests that the affibody construct was unable to escape the endosomal compartment.

### **384-Well Cell Migration Assay Suitable for High-Throughput Screening (HTS) of Chemical Libraries for Cancer Therapeutics**

*Andreas Vogt, Ph.D., The University of Pittsburgh Discovery Institute*

The emphasis of Dr. Vogt's research is on phenotypic drug development and phenotypic assays, and for this project, cell motility. He explained that the assays that Platypus Technologies (Madison, WI) and his group have developed – the Oris and Oris Pro Cell Migration Assays – are now commercially available from Platypus Technologies. The Oris Pro Cell Migration assay, which was developed with IMAT funding, uses no mechanical manipulation. The heart of the assay system is a biocompatible gel to which cells seed and adhere. Cells are seeded into 384-well plates and patterned in an annular monolayer surrounded by the gel. As the gel biodegrades, cell migration can be visualized and quantified. The gel, he noted, is non-toxic to mammalian cells and is compatible with a wide range of stains and can be used in a wide range of drug experiments. This system can also be used to make morphometric measurements and develop multi-parametric, high-content data including variables such as cell migration, necrosis, cell density, and nuclear area.

Dr. Vogt described the experiments performed to document the high-throughput screening performance of the Oris Pro 384-well cell migration assay using a panel of six agents with known mechanisms of action and predicted cellular outcomes. He discussed the reproducibility of these assays over a three-day period and noted that the cell migration assay generated reliable and repeatable dose-response curves on two different instruments on multiple days, a key performance metric for a commercial assay. He then discussed the results obtained when the Oris Pro was used to evaluate the Sigma-Aldrich Library of Pharmacologically Active Compounds, a panel of 1280 well-characterized chemical compounds with various modes of action. The data formed several distinct clusters, including selective migration inhibitors, inactive compounds, those that triggered apoptosis, and cytotoxic compounds. Dr. Vogt noted that the assay delivery Z-factors of greater than 0.5, signal-to-background ratios exceeding 10, and low intra-plate and inter-plate variability in multi-day studies.

### **Hyperspectral and Structural Microscopy Platform for Therapy of Resistant Cancer**

*Conor L. Evans, Ph.D., Harvard Medical School/Massachusetts General Hospital*

In his presentation, Dr. Evans reviewed the multiplexed assay system that his group is developing to screen potential therapeutics and measure how those agents differentially impact live cells via a novel molecular and structural optical microscopy platform. The assay has the capability to use cells taken directly from ovarian cancer patients. The seeded cells form nodules that model the avascular tumor nodules found *in vivo* - complete with acidic and hypoxic tumor cores. Various microscale factors such as hypoxia, pH, secreted matrix, and therapeutic uptake and treatment response can be imaged using near-infrared confocal microscopy and optical coherence tomography (OCT). The advantage of using OCT, he explained, is that it detects contrast changes that arise from minute changes in tissue scattering and refractive index over an entire three-dimensional volume with substantially better depth of penetration than fluorescence imaging.

Dr. Evans and his team have shown that OCT can detect structural decomposition that occurs with therapeutically active molecules. This structural decomposition shows up as two major changes: the structural decomposition of the nodular architecture into recognizable sub-structures, and increased scattering at cellular length scales arising from apoptosis. On the same platform, specific molecular targets can also be tracked using high-resolution fluorescence microscopy correlated with the spatio-temporal OCT images to further elucidate the mechanisms for therapeutic efficacy. Using this assay, he and his colleagues have identified a family of potent, light-activatable molecules that trigger widespread apoptotic damage in nodules greater than 250 microns from the inside out. These compounds were effective even under conditions of complete anoxia, which is atypical of photodynamic therapies.

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

*Xudong Yao, Ph.D., University of Connecticut*

In the last presentation of this year's annual meeting, Dr. Yao described his group's work developing technology to significantly improve sample throughput for mass spectrometry so that this powerful analytical tool can be used for large-scale validation of cancer biomarkers. While mass spectrometry has always had potential as a tool for multiplexed analysis, sample throughput has been a major limitation.

Dr. Yao's solution has been to develop a library of 158 non-isotopic peptidyl reagents, which are two to four amino acids in length modified with 10 different capping groups at their N-termini. These reagents are used to derivatize target biomarker proteins in human clinical samples and enable the simultaneous analysis of over 100 samples in a single mass spectrometry run. He described the experiments his group performed to optimize derivatization conditions and to select those peptidyl reagents that produced a high-yield signal.