

FLUORESCENT PROTEINS

June 23-24, 2005 • Doubletree • Philadelphia, PA

THE USE OF

FLUORESCENT PROTEINS FOR *IN VIVO*, PRE-CLINICAL, & SMALL ANIMAL IMAGING

*Discover Powerful Insights into Cellular Imaging, RNA Interference,
Protein-Protein Interaction, and Multispectral Imaging*

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	<i>University of Maryland Biotechnology Institute</i>
	<i>University of Texas Southwestern Medical Center</i>
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- ◆ Discuss Intravital Imaging of Immunity Using Fluorescent Proteins and Fluorescence Microscopy
- ◆ Analyze the Involvement of siRNA in Directing Histone Modifications to Targeted Promoters Insinuates siRNAs and/or miRNAs as Potential Mediators Implicated in Writing the Histone Code
- ◆ Using Multispectral Imaging to Obtain Increased Contrast for *In Vivo* Fluorescence Imaging of Small Animals and Using Multicolor FISH, and Multiplexed Use of Different Fluorophores with Overlapping Emission Spectra

Featured Sessions

<p>Multi-Colored Fluorescent Protein-Based Imaging in Live Animals: The New Cell Biology <i>Dr. Robert Hoffman, President and Founder, AntiCancer, Inc.</i></p>	<p>Visualizing Bioluminescence Sources in Small Animals in Three-Dimensions by Light Emission Tomography <i>Edmond Richer, PhD, Assistant Professor, Department of Radiology, University of Texas Southwestern Medical Center</i></p>
<p>siRNA Mediated Transcriptional Gene Silencing in Human Cells <i>Kevin V. Morris, PhD, Assistant Research Scientist, Division of Molecular Biology, Beckman Research Institute</i></p>	<p>Increasing Contrast and Multiplexing Using Multispectral Imaging <i>James Mansfield, Senior Spectral Imaging Scientist, Product Manager, MSI Systems, Cambridge Research & Instrumentation, Inc.</i></p>

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Thursday, June 23rd, 2005

8:00 Registration and Coffee

8:45 Chairperson's Opening Remarks
Dr. Robert Hoffman, President and Founder, AntiCancer, Inc.

9:00 **Gene Function Screening with Dynamic GFP Sensors**
Nick Thomas, PhD, Principal Scientist, GE Healthcare
Recent developments in instrumentation, cellular sensors, and image analysis software have produced integrated systems for cellular analysis, which allow complex cellular processes to be investigated with previously unprecedented speed and efficiency. We have used high-content imaging of fluorescent fusion proteins in stable cell lines and transient expression assays to investigate a number of key cellular processes. Procedures used to validate model systems expressing EGFP fusion proteins will be discussed, and data from two cDNA over-expression and siRNA high-throughput functional screens will be presented.

9:45 **Visualizing Bioluminescence Sources in Small Animals in Three-Dimensions by Light Emission Tomography**
Edmond Richer, PhD, Assistant Professor, Department of Radiology, University of Texas Southwestern Medical Center
To date, BLI is widely used as an efficient planar technique, although its effectiveness is partially limited for visualization (localization of cell population) and quantitation (tumor size estimation). The limits are due to the fact that the intensity and distribution of the signal captured by the BLI camera at the animal's skin surface are strongly influenced by the intervening tissues, resulting in an attenuated and diffused signal at the skin surface of the animal through both scattering and absorption. A goal of many research groups has been small animal optical tomography, an essential tool to overcome these shortcomings, but to our knowledge none has succeeded to date. Furthermore, bioluminescence is a dynamic process, and the light intensity varies significantly and rapidly after substrate injection limiting the amount of time available for imaging. To overcome these problems, it is important to observe the animal from as many directions as possible simultaneously. We therefore designed and built an optical imaging system with multiple cameras that surround the subject in the transverse plane permitting the simultaneous acquisition of images from different angular directions. Currently we employ twenty orientations provided by a computerized system, which rotates the cameras around the longitudinal axis. The system is complemented with innovative analysis and reconstruction software based on Maximum Likelihood Expectation Maximization method (MLEM), capable of providing full 3-D reconstruction and tomographic imaging. Validation studies using phantoms verified the utility of the approach and the accuracy of the 3D reconstruction algorithms. Three phantoms of increasing complexity are described: a homogeneous diffusing cylinder and both homogeneous and heterogeneous mouse-shaped phantoms. As an *in vivo* imaging example, A549-LUC human lung tumor cells (106) were injected IV in a nude mouse (BALBc/nu/nu). The lung-colonizing pseudo-metastases were imaged following D-luciferin injection in the anesthetized mouse. Data reconstruction provided a 3-D model of the lung tumors. The algorithm was also applied to images obtained using light reflected from the skin under external illumination, and the two data sets were combined for co-registration. Coronal cross-sections emphasize the structured nature of the volumetric data. Intense local foci are apparent with a more diffuse background distribution outlining the anatomy of the lungs. Pathologic studies after sacrifice confirmed that A549-LUC tumor cells dispersed throughout the lobes of the lungs with multiple metastatic foci. We have demonstrated the feasibility of visualizing in 3D luciferase expressing cells at depth in a living mouse. The method can provide not only volumetric but also tomographic representations of the data. It also provides better quantification of the light emission than simple planar imaging techniques, thus enhancing the ability to assess and monitor spatio-temporal characteristics of tumor growth, identifying metastases, and potentially determining the effectiveness of cancer treatment.

10:30 Mid-Morning Break

siRNA and DNA: Examining the Link

10:45 **siRNA Mediated Transcriptional Gene Silencing in Human Cells**
Kevin V. Morris, PhD, Assistant Research Scientist, Division of Molecular Biology, Beckman Research Institute
In mammalian cells, small interfering RNA (siRNA) is known to produce post-transcriptional gene silencing (PTGS) in the cytoplasm. We recently reported transcriptional gene silencing (TGS) mediated by siRNAs when targeted to the EF1alpha promoter and delivered to the nucleus of human cells [1]. We have expanded our initial observation to address the mechanism of siRNA mediated TGS by screening the binding potential of DNA methyltransferases (DNMT) 1, 3A, 3A2, 3B1, 3B2, and heterochromatin proteins (HP1-alpha, beta, and gamma) to the promoter targeted EF52 siRNAs. Interestingly, DNMT3A, 3A2, and 3B2 bound EF52 with DNMT3A displayed the most robust binding. The observed binding appeared to be strand-specific and correlated with a silent state histone methylation mark. These data implicate a link between siRNA mediated targeting of genomic regions (promoters), DNA methylation (DNMTs),

and chromatin remodeling complexes (Suv39H1 and HDACs) in human cells. As such, a model for siRNA targeted regulation of DNA expression has been proposed and will be discussed. Importantly, the observation that siRNAs can regulate DNA via epigenetic modifications significantly shifts the current paradigm of gene expression and regulation. Moreover, the involvement of siRNA in directing histone modifications to targeted promoters insinuates siRNAs and/or miRNAs as potential mediators implicated in writing the histone code. Taken together, these data strongly suggest that siRNA mediated control of DNA through epigenetic modifications plays a pivotal and under-appreciated role in regulating the cell that could be conceptualized to be used therapeutically in treating virtually any ailment affecting humans.

11:30 Interactive Panel Q&A Session with Morning Speakers

12:00 Luncheon

Special Presentation by Chairman Dr. Robert Hoffman

1:15 **Multi-Colored Fluorescent Protein-Based Imaging in Live Animals: The New Cell Biology**
Dr. Robert Hoffman, President and Founder, AntiCancer, Inc.
The use of green fluorescent protein and other fluorescent proteins has enabled a revolution for both *in vitro* and *in vivo* biology. The main features of the new *in vivo* cell biology enabled by fluorescent proteins will be discussed. These features include: the use of fluorescent proteins for whole-body imaging of tumors and metastasis, as well as gene expression in the living animal; and imaging of critical aspects of metastasis including angiogenesis and host cells that interact with the tumor. Imaging of tumor and host is affected through the use of fluorescent proteins of different colors distinguishing tumor and host. *In vivo* imaging of single cells is carried out using cells that are labeled with green fluorescent protein in the nucleus and red fluorescent protein in the cytoplasm. The dual-colored cells allow the visualization of cellular and nuclear dynamics of tumor cells as they migrate in and out of blood vessels. Real-time movies of these processes will be presented. The applications of fluorescent protein-based *in vivo* imaging for drug discovery and evaluation will be demonstrated. The advantages of fluorescent protein-based imaging over other types of imaging will be discussed.

- Fluorescent proteins are extremely bright thereby allowing imaging on deep organs in small animals
- Fluorescent proteins come in multiple colors, allowing *in vivo* color coding of tumor and host or tumor cells with different properties
- Individual cells can be multiple-colored to visualize the nucleus and cytoplasm and their dynamics during steps of metastasis
- Fluorescent protein-based *in vivo* imaging is highly useful for drug discovery and evaluation

2:00 **Optical Imaging of Biomarkers Using Femtosecond Lasers**
Warren S. Warren, Ralph W. Dornste Professor of Chemistry, Department of Radiology, Princeton University, and Adjunct Professor of Radiology, University of Pennsylvania
Recent advances in laser technology have permitted exquisite control over optical fields (phase and amplitude modulation of laser pulses on a femtosecond timescale). While there are many applications of this technology in fields as diverse as high-speed communications and laser selective chemistry, we have found biomarker detection and deep tissue imaging in particular can be dramatically enhanced. The presentation will focus on the uses of shaped laser pulses to detect particular molecular markers in anthrax (at large distances), and on developments of imaging approaches that can quantify multiphoton absorption, quantum yield, and self phase modulation in addition to molecular fluorescence. The combination of these markers significantly increases the amount of extractable molecular information.

2:45 Mid Afternoon Break

Recent Developments in Biotechnology

3:00 **Metal-Enhanced Fluorescence: An Emerging Tool in Biotechnology**
Dr. Chris D. Geddes, Associate Professor, University of Maryland Biotechnology Institute
Over the last 15 years, fluorescence has become the dominant detection/sensing technology in medical diagnostics and biotechnology. Although fluorescence is a highly sensitive technique, where single molecules can readily be detected, there is still a drive for reduced detection limits, such as for small copy-number DNA detection. Fluorophore detectability is usually limited by its quantum yield, auto-fluorescence of the samples, and/or the photostability of the fluorophores. However, during the last three years, there has been an explosion in the use of metallic nanostructures to favorably modify the spectral properties of fluorophores and alleviate some of the fluorophore photophysical constraints. The use of fluorophore-metal interactions has been termed radiative decay engineering (RDE), metal-enhanced fluorescence (MEF), and also surface-enhanced fluorescence (SEF). In this presentation, we review our progress to date.

3:45 Interactive Panel Q&A Session with Afternoon Speakers

4:15 Closing Remarks and Conclusion of Day One

Friday, June 24th, 2005

8:30 Registration and Coffee

9:15 Chairperson's Opening Remarks
Dr. Robert Hoffman, President and Founder, AntiCancer, Inc.

Multispectral Images: Discuss the Insights and Debate the Advantages

9:30 **Increasing Contrast and Multiplexing Using Multispectral Imaging**
James Mansfield, Senior Spectral Imaging Scientist, Product Manager, MSI Systems, Cambridge Research & Instrumentation, Inc.

Non-invasive *in vivo* imaging of small animals is a rapidly growing part of drug discovery, with new technologies and techniques being constantly developed. *In vivo* fluorescence imaging has not done nearly as well as bioluminescence imaging despite having several potential advantages such as the ability to multiplex fluorophores, the lack of a need for a timed injection, and the potential to use injected labeled antibodies. The ability to image and quantitate fluorescently labeled tumors and other fluorescently labeled markers *in vivo* has generally been limited by the autofluorescence of the tissue, which reduces the sensitivity of detection and accuracy of quantitation of the labeled tumor. This session will address using multispectral imaging to obtain increased contrast for *in vivo* fluorescence imaging of small animals:

- Using multispectral imaging methodology to spectrally characterize and computationally eliminate autofluorescence, revealing otherwise invisible labeled targets, which now appear bright against a near-black background – this technique increases sensitivity by orders of magnitude, allowing much smaller or fainter targets to be detected
- The application of multispectral imaging methods to several small animal tumor models
- Recently developed automated methods for determining the spectral signatures of the fluorophores that greatly simplify the application of this technology

In addition, we will also cover aspects related to multispectral imaging for fluorophore multiplexing and tissue autofluorescence removal for microscopy applications, which allows for the detection of multiple molecular species without requiring the use of multiple filter cubes or filter wheels. We will discuss the advantages of multispectral imaging:

- Precise optical spectra at every pixel
- Nuance™ liquid crystal tunable filter-based multispectral imaging systems that can be used in the analysis of chromogenically stained slides in brightfield mode and of samples stained with a variety of light-emitting dyes (from the visible to the NIR range) in fluorescence mode
- Suitability for quantum dot-based labeling
- Multicolor FISH, and multiplexed use of different fluorophores with overlapping emission spectra
- The identification and elimination of interfering autofluorescence and the ability to accurately determine the spectral qualities of dyes *in situ*

10:15 **Visualization of Protein Interactions *In Vivo* by Bimolecular Fluorescence Complementation**
Claudius Vincenz, PhD, Research Specialist,
Howard Hughes Medical Institute

Bimolecular fluorescence complementation is based on the principle that green fluorescent protein, and its color variants, can be expressed as two non-fluorescent fragments that re-associate and yield a fluorescent protein if they are fused to interacting domains. Your speaker will describe application of this principle to the visualization of two types of post-translational modifications. First, ubiquitination will be monitored by directly labeling the post-translational modification with a fragment of GFP. Secondly, methylation of histones will be visualized by tagging specific methyl-histone binding proteins with a GFP fragment. The novel insights provided by these novel applications will be discussed.

11:00 Mid Morning Break

11:15 **Regulation of Caveolar Membrane Trafficking by Myosin Vc**
George S. Bloom, PhD, Professor of Biology and Cell Biology,
University of Virginia

Previous work from our laboratory implicated actin filaments in regulating caveolar membrane trafficking by a mechanism that resembles myosin Va-dependent control of the intracellular transport of pigment granules and melanosomes (Mundy, et al. 2002. *J. Cell Sci.* 115: 4327-4339). To determine if a similar mechanism applies to actin-mediated regulation of caveolar trafficking, caveolin-1 dynamics were studied in cells over expressing fluorescently-tagged myosin V tails. By light microscopy, over expression of GFP-tagged tails of myosins Va, Vb or Vc, or of myosin Vc tail-DsRed caused accumulation of large, intensely fluorescent, spherical structures in the cytoplasm of several cell lines. Immunofluorescence demonstrated extensive co-localization of caveolin-1 with myosin Vc tails, but not with tails of myosins Va or Vb. Myosin Vc-tail-DsRed also co-localized with

caveolin-1-GFP in CHO cells stably expressing the latter. In addition to caveolin-1, the myosin Vc tail-positive structures accumulated transferrin receptor, which also are known to collect in a myosin Vb tail-positive compartment. The myosin Vc tail-positive structures were not labeled by light microscopic markers for lysosomes, auto-phagosomes, exosomes, or late endosomes. At the EM level, cells expressing myosin Vc tails contained an abundance of unusual, morphologically variable structures, many of which appeared multilamellar. To determine whether endocytosis was affected by over expressing myosin Vc tails, rhodamine-labeled transferrin was used as an uptake marker for clathrin-mediated endocytosis. The highest expressors were unable to endocytose transferrin, consistent with the hypothesis that the transferrin receptors in such cells were entirely sequestered in myosin Vc tail-positive structures. Taken together, these results imply that myosin Vc tails inhibit recycling of a membrane compartment through which caveolin-1 and transferrin receptors normally pass, and that transferrin receptors, but not caveolin-1, also normally pass through a myosin Vb tail-sensitive compartment that may correspond to the recycling endosome. The myosin Vc tail-sensitive compartment appears to represent an intersection of the caveolar membrane and clathrin-mediated endocytotic pathways. Caveolar membranes traffic by a mechanism that resembles myosin Va-regulated transport of pigment granules and melanosomes. To determine if the mechanism actually involves a myosin V, caveolin-1 trafficking was studied in cells over-expressing fluorescent tail domains of myosins Va, Vb, or Vc. All tail domains accumulated cytoplasmically in large, intensely fluorescent structures that appeared multilamellar by EM. Caveolin-1 and transferrin receptor (TfR) co-localized with Vc tail, and TfR also co-localized with Vb tail. Highly motile vesicles containing caveolin-1-GFP and endocytosed TRITC-transferrin were frequently observed, but uptake of TRITC-transferrin was inhibited by Vc tail. The Vc tail compartment did not contain detectable levels of markers for early and late endosomes, lysosomes, autophagosomes, or exosomes. Collectively, these data indicate the presence of a novel compartment containing materials derived separately from the caveolar and clathrin-dependent endocytotic systems, and suggest that exit of material from this compartment requires myosin Vc and is blocked by over-expression of its tail domain.

12:00 Interactive Panel Q&A Session with Morning Speakers

12:30 Luncheon

1:45 **Intravital Imaging of Immunity Using Fluorescent Proteins and Fluorescence Microscopy**

Michael L. Dustin, Irene Diamond Associate Professor of Immunology, Department of Pathology,
New York University School of Medicine

Intravital microscopy allows examination of cellular dynamics in live animals in real-time or time-lapse modes. One lab has generated mice in which the chemokine receptor CXCR6 is disrupted by green fluorescent protein (GFP). Another lab has demonstrated that NKT cells in the liver of these mice express high levels of GFP. CXCR6 deficient mice have reduced numbers of NKT cells in the liver and reduced susceptibility to ConA induced hepatitis. In collaboration with other scientists, our staff has shown that NKT cells actively patrol the liver sinusoids at ~15 $\mu\text{m}/\text{min}$. The NKT cells did not extravasate, but moved with and against blood flow in a manner consistent with amoeboid locomotion. Intravenous injection of ConA or anti-CD3 caused rapid stopping of migration. We propose a model where NKT cells patrol the sinusoids and sense lipid antigens. Yet another lab has generated transgenic mice in which the CD11c promoter is used to express high levels of YFP on myeloid dendritic cells. Imaging of dendritic cells in the lymph nodes by other researchers revealed the presence of sessile networks of dendritic cells in the T cell zones that are contacted by migrating T cells. The mechanism by which steady state dendritic cells induce tolerance of T cells involves early stable interactions as T cell encounter the sessile networks after extravasation, followed by transient interactions with the DC network. Tolerance requires days to establish so it is likely that both modes of interaction are functionally important.

2:30 **New Approaches for *In Vivo* Imaging of Protein-Protein Interaction**
Dr. Brian Herman, Vice President for Research,
Professor, Cellular and Structural Biology,
University of Texas Health Science Center

High throughput technology has now been developed that allows assays of protein-protein interactions in intact cells and tissues in a non-destructive manner. This allows investigators to study cell physiology and pathology in real-time and in numerous intact animal models. Coupling this instrumentation with transgenic expression of multiple regulatable fluorescent fusion proteins allows the use of high throughput technology and the sensitivity of fluorescence to be applied to the real-time observation of protein-protein interactions. Recent advances in this area will be discussed.

3:15 Interactive Panel Q&A Session with Afternoon Speakers

3:45 Closing Remarks and Conclusion of Day Two

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