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<p>Specific Aim 1: Develop and test plaque characterization criteria based on birefringence. Coronary arteries were obtained at autopsy and imaged with the existing OCT system in our laboratory. The specimens were opened and imaged at normal body temperature (37 -C) in saline. To ensure proper registration of OCT images with histology the image location was marked with ink viewable on the OCT image and in the histology section. Routine histologic processing was performed using H&amp;E, Elastic and Movat's pentachrome stains. Trichrome was used for fibrous tissue identification. Individual images were oriented and scaled to match histology. In the OCT images, the axial periodicity of the birefringence banding was measured using image processing software. A set of 20 correlated OCT and histology pairs were used to identify characterization criteria based on birefringence. Our results indicated that fibrous plaque provides a strong birefringence as measured by OCT while lipid rich and calcific plaques can be identified by a complete loss of birefringence.</p>					
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## ANNUAL PROGRESS REPORT

PRINCIPAL INVESTIGATOR: John A. Parrish, M.D.

INSTITUTION: Massachusetts General Hospital

GRANT TITLE: Research To Develop Biomedical Applications of Free Electron Laser Technology

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**Investigators:** Brett E. Bouma, Ph.D., G.J. Tearney, M.D. Ph.D.

**Title:** Contrast enhancement through birefringence in OCT

**Objective:**

The overall goal of this work is to develop methods and apparatus for exploiting intrinsic tissue birefringence as a contrast mechanism in optical coherence tomography (OCT). Although these methods and devices have multiple applications, our focus has been to investigate their use for the characterization of atherosclerotic plaque. In particular, our goal has been to use birefringence as a measure of collagen content and integrity in coronary plaque and through this to gain insight into the mechanical aspects of plaque rupture and acute myocardial infarction.

**Approach:**

Polarization is one of the defining characteristics of light and influences its propagation and interaction with matter. Polarization is described by a vector oriented in a plane that is orthogonal to the direction of propagation. Homogeneous media with no structure interact with all possible polarization orientations equivalently. Structured media, however, can have a property known as birefringence that will give rise to a rotation of the polarization vector about the propagation direction. Since the polarization of the light returning to typical OCT systems influences detection, the interpretation of images of structured media is complicated. Returned light having a polarization direction aligned with the incident light will be detected while returned light having an orthogonal orientation will be rejected. An OCT image of a homogeneous birefringent medium will show artifactual banding. At the surface of the medium, the polarization state that is returned to the system will be unaltered and detected accurately. Light returning from deeper within the sample will have a rotated polarization vector and will give rise to an artifactually altered signal. At still greater depths the polarization state of the returned light will continue to rotate until it is once again aligned with the incident polarization.

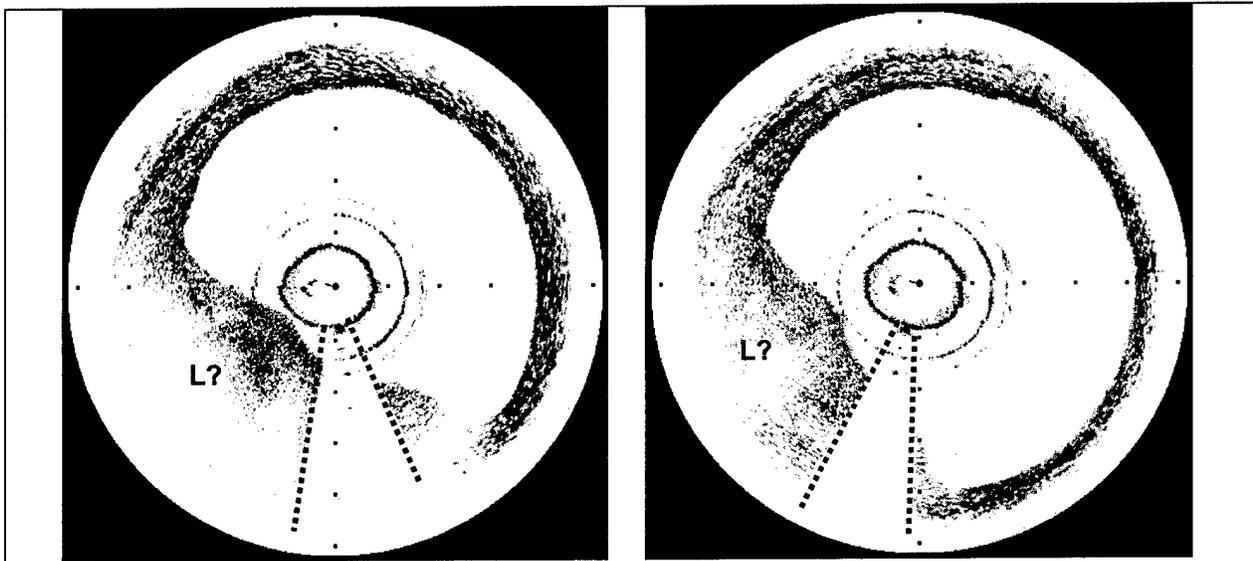
Our approach has been to develop and characterize a polarization sensitive OCT system and intravascular catheter suitable for use in patients undergoing percutaneous coronary intervention. The system was tested and image diagnostic criteria were developed *in vitro*. This system has recently been used in clinical studies funded by industry.

**Accomplishments:**

Specific Aim 1: Develop and test plaque characterization criteria based on birefringence. Coronary arteries were obtained at autopsy and imaged with the existing OCT system in our laboratory. The specimens were opened and imaged at normal body temperature (37 -C) in saline. To ensure proper registration of OCT images with histology the image location was marked with ink viewable on the OCT image and in the histology section. Routine histologic processing was performed using H&E, Elastic and Movat's pentachrome stains. Trichrome was used for fibrous tissue identification. Individual images were oriented and scaled to match histology. In the OCT images, the axial periodicity of the birefringence banding was measured using image processing software. A set of 20 correlated OCT and histology pairs were used to identify characterization criteria based on birefringence. Our results indicated that fibrous plaque provides a strong birefringence as measured by OCT while lipid rich and calcific plaques can be identified by a complete loss of birefringence.

Specific Aim 2: Develop a catheter suitable for intracoronary polarization sensitive imaging. We have developed an intravascular catheter for polarization sensitive OCT imaging. The catheter is based on the design of an FDA approved intravascular ultrasound catheter and is suitable for use with a guide wire. The diameter of the catheter is 1.0 mm (3.0 F) and its mechanical characteristics are identical to IVUS catheters and to the previous OCT catheters used in patients at the MGH. We have tested the catheters for imaging and birefringence detection in phantoms and in porcine coronary arteries *in vitro*.

A recent pilot study, funded by industry, has deployed the system and catheter developed by the MFEL sponsored research. The figures below depict preliminary results demonstrating the power of polarization sensitive imaging for improving plaque characterization. Two vessel cross-sections are shown in Fig. 1. Both exhibit features that by previously established criteria would suggest the presence of a lipid-rich plaque. In the polarization maps, however, one of these plaques is clearly seen to be a fibrous plaque.



**Figure 1** Images of atherosclerotic plaque acquired in a human subject. Two distinct locations are shown that exhibit plaques with signal-poor regions (lower left quadrant). Does this signal drop-out indicate a lipid rich plaque? The sectors delineated by dotted lines are obscured by the guide wire.

**Significance:**

Our previous work in orthopedics demonstrated that contrast between cartilage and bone can be enhanced by analyzing the intrinsic birefringence in cartilage. We also demonstrated that the integrity of cartilage is associated with the strength of birefringence and can therefore be evaluated with polarization sensitive imaging. Our work in cardiology has suggested that these methods may have a significant impact when applied to the characterization of coronary atherosclerotic plaque. The current objective of our project has been to apply these methods for the investigation of plaque rupture and acute myocardial infarction.

Cardiovascular disease is the leading cause of death in the industrialized world.<sup>1</sup> It is currently believed that acute myocardial infarction is a result of a specific type of coronary plaque characterized by the presence of a large necrotic core underlying a thin, structurally compromised fibrous cap.<sup>2,3</sup> Through vasospasm or elevated pressure, this cap can be disrupted exposing blood to the necrotic debris. The rapid clot that subsequently forms leads to catastrophic closure of the coronary artery. There is, however, no method available to cardiologists to identify these 'vulnerable' lesions in living patients. We have demonstrated that intracoronary imaging with OCT provides the ability to characterize plaque types and can potentially identify vulnerable plaque but suffers from limited contrast between fibrous tissue and lipid-rich tissue.<sup>4-6</sup> Our preliminary data suggests that polarization sensitive imaging can significantly enhance this contrast without the use of exogenous contrast agents.

**Publications:**

Tearney G, Jang I and Bouma B. Evidence of Cholesterol Crystals in Atherosclerotic Plaques by Optical Coherence Tomography. *European Heart Journal* 2003;In Press.

Tearney GJ, Yabushita H, Houser SL, Aretz HT, Jang IK, Schlendorf KH, Kauffman CR, Shishkov M, Halpern EF and Bouma BE. Quantification of macrophage content in atherosclerotic plaques by optical coherence tomography. *Circulation* 2003;107:113-9.

MacNeill BD, Jang IK, Bouma BE, Iftimia N, Takano M, Yabushita H, Shishkov M, Kauffman CR, Houser SL, Aretz HT, DeJoseph D, Halpern EF, Tearney GJ. Coronary plaque macrophage distributions in living patients. Accepted for publication in the *Journal of the American College of Cardiology*.

**Patents:**

No technology disclosures have resulted from this work.

**Investigators:** Brett E. Bouma, Ph.D., G.J. Tearney, M.D. Ph.D.

**Title:** Development of a miniature flexible endoscope

**Objective:**

To develop an ultra small diameter(300  $\mu\text{m}$ ), flexible probe for video imaging. The image quality of current narrow diameter probes is limited by the relationship between the number of picture elements and the diameter ( $N \sim D^2$ ). As the diameter of these probes is reduced below 1 mm, the image quality of these probes becomes severely compromised. Our approach applies spectral encoding and can be applied through a single mode optical fiber. We refer to this technology as spectrally encoded endoscopy (SEE).

**Approach:**

Our research has been divided into three distinct technological efforts. The first is the development and characterization of a 300  $\mu\text{m}$  diameter catheter probe. The second focuses on the combination of light source and optical receiver. The third effort is system integration to develop a portable imaging console suitable for clinical deployment.

**Accomplishments:**

Computer modeling studies were conducted to investigate the use of in-fiber gratings, holographic Bragg gratings, blazed gratings, commercially available gradient index, aspherical, and drum lenses to determine which combination of elements provides the lowest insertion loss and the highest transverse resolution in a confined space. These studies indicated that the combination of a holographic Bragg diffraction grating and a gradient index lens provided sufficient resolution and field of view. A prototype catheter configuration was developed based on these results and validation of the resolution of the device was accomplished by slit scanning techniques and imaging standard resolution charts.

The prototype SEE system utilized a broadband light source and spectroscopic detection based on a diffraction grating. One aim of this work was to optimize the sensitivity of this source/receiver configuration to allow real-time imaging. In the first quarter of work, however, our research led to the conclusion that affordable multi-channel detector arrays had insufficient sensitivity. Although this limitation may be rectified in future generation linescan cameras currently under development, we redirected our immediate efforts to the development of a new paradigm for the source/receiver configuration.

An alternative source/receiver configuration utilizes a wavelength-swept, monochromatic laser and a single InGaAs photodiode receiver. In essence, this approach moves from a simple light source and complicated receiver to a more sophisticated light source and a simplified receiver. In order to achieve sufficient imaging speed and sensitivity, the light source must provide an instantaneous linewidth of  $< 0.1$  nm, be tunable over a spectral range of  $> 50$  nm, have a wavelength-sweep speed of 0.1 ms, and an average power of  $> 1$  mW. We have developed a novel laser configuration that exceeds all of these specifications.

The laser resonator comprises a unidirectional fiber-optic ring, a semiconductor optical amplifier as the gain medium, and a novel scanning filter based on a polygon scanner. Variable tuning rates up to 1,150 nm/ms (15.7 kHz repetition frequency) are demonstrated over a 70 nm

wavelength span centered at 1.32  $\mu\text{m}$ . This tuning rate is more than an order of magnitude faster than previously demonstrated and is facilitated in part by self-frequency shifting in the semiconductor optical amplifier. The instantaneous linewidth of the source was  $<0.1$  nm for 9 mW cw output power and a low spontaneous-emission background of  $-80$  dB.

Endoscopes employing a single optical fiber may have advantages over conventional fiber-bundle or CCD array imaging techniques, including the potential for greater flexibility and miniaturization. Although single-mode fibers can provide superior resolution compared with multimode fibers, they are prone to increased speckle noise and suffer from limited optical throughput and reduced depth-of-field. We have developed a novel approach in which a double-clad fiber is utilized for single-mode illumination and multi-mode detection to achieve high-resolution, reduced-speckle imaging with high optical throughput and a large depth of field.

We have extended our previous miniature endoscope work to include the capability of three-dimensional imaging. Our approach relies on phase-sensitive spectral encoding to relay both transverse and depth information through a single fiber channel. This capability makes three-dimensional imaging within the confines of a miniature flexible probe possible for the first time. Our prototype instrument was capable of measuring surface profiles within a volume of 50 mm x 50 mm x 30 mm with 250  $\mu\text{m}$  transverse and 100  $\mu\text{m}$  axial resolution.

**Significance:** Laparoscopy and endoscopy are invaluable tools for tissue diagnosis and surgical guidance. Use of these techniques has decreased patient morbidity and significantly reduced the cost of treatment of a variety of common diseases. Unfortunately, the diameter of currently available laparoscopes and endoscopes limits their use. Traditional laparoscopy is an operative procedure that requires general anesthesia due to the large diameter of these scopes. Small diameter imaging probes open up the possibility of performing many procedures such as assessment of pelvic adhesions, diagnosis of appendicitis, tubal sterilization, and fertilization, under local anesthesia. Many diagnostic imaging methods including fetoscopy, fetoscopic surgery, and pediatric surgery, require small diameter endoscopic probes for diagnosis or guidance of surgery. However, in many cases, the complication rate of these techniques is unacceptably high due to the size of available imaging devices. Moreover, the resolution provided by these probes has severely limited the clinical utility of these techniques. In addition to procedures currently in practice, recently introduced minimally invasive diagnostic imaging techniques, such as mammary ductoscopy, neuroendoscopy, pancreatoscopy, and coronary angioscopy, need imaging devices with smaller diameters before they can be effectively utilized to improve patient care. We expect that second generation SEE probes could be as small as 125  $\mu\text{m}$ .

endoscopic confocal microscopy in large diameter ( $\sim 1$  cm diameter) probes.

#### **Publications:**

1. Yun S, Boudoux C, Pierce M, de Boer J, Tearney G and Bouma B. Extended-cavity semiconductor wavelength-swept laser for biomedical imaging. *IEEE Photonics Technology Letters* 2003;16:293-5.
2. Yun S, Boudoux C, Tearney G and Bouma B. High-speed wavelength-swept semiconductor laser with polygon-scanner-based wavelength filter. *Optics Letters* 2003;28:1981-3.

3. Yelin D, Bouma BE, Ifimia N, Tearney GJ. Three-dimensional spectrally encoded imaging. Optics Letters 2003;28:2321-3.
4. Yelin D, Bouma BE, Tearney GJ. Double-clad fiber for endoscopy. Accepted for publication, Optics Letters.

Patents: None

**Investigators:** Michael R Hamblin Ph.D. Tatiana N Demidova, B.S.

**Title:** Photodynamic inactivation of anthrax surrogate spores

### **Objective**

Investigate use of targeted photodynamic therapy for inactivation of *Bacillus* spores that could have applications against biowarfare and bioterrorism.

### **Approach**

Anthrax spores can be prepared in a dry form, and milled into a powder of a sufficiently small particle size to produce an aerosol that can be widely dispersed into the air. Inhalation will then cause a serious and frequently fatal disease, while entry of the spores into cuts and abrasions on the skin produces a less fatal but still serious disease, cutaneous anthrax. For these reasons anthrax spores they have been proposed as a major component of biological weapons and potential bioterrorism. Spores are highly resistant to damage by heat, radiation, and many of the commonly employed anti-bacterial agents, and can only be destroyed by some severe chemical procedures including oxidizing vapors such as peracetic acid, chlorine dioxide and ozone, and DNA cross-linking vapors such as ethylene oxide and glutaraldehyde. These reagents are too toxic to be used to decontaminate wounds or broken skin and additional non-toxic sporicides are needed. Previously it has been reported that *Bacillus* spores were resistant to inactivation using a highly active photosensitizer (PS), Rose Bengal. We used various *Bacillus* spores as *B. anthracis* surrogates, as availability of *B. anthracis* is now strictly controlled. *B. cereus* is very closely related to *B. anthracis* and a recent report suggests that from a genetic viewpoint they are the same species. A similar argument is made regarding *B. thuringiensis* which is widely used as a biological insecticide. In fact there is mention of the *B. anthracis* "cluster" that includes all *B. anthracis* strains (both pathogenic and non-pathogenic) together with numerous *B. cereus* and *B. thuringiensis* strains.

### **Accomplishments:**

We have extended our discovery that many spores of *Bacillus* species are efficiently photoinactivated by PDT mediated by a wide range of phenothiazinium dyes. We have found that dimethylmethylene blue is most active followed by Toluidine blue O and new methylene blue. Azures A, B, and C and methylene blue are less active. Alternative PS such as poly-L-lysine chlorin(e6) conjugate, benzoporphyrin derivative and Rose Bengal that are highly active in mediating photoinactivation against bacteria in the vegetative state are completely inactive against spores. *B. cereus* spores are exceptionally sensitive to photoinactivation, followed by *B. thuringiensis*, *B. subtilis* and *B. atrophaeus*. *B. megaterium* spores are least sensitive. PDI was still effective after spore suspensions were centrifuged and resuspended, implying the dye bound to and penetrated into the spore. Binding and penetration of dyes into spores appear to be largely diffusion controlled since the degree of PDI increases with dye concentration, incubation time and incubation temperature.

### **Significance:**

This relatively mild procedure to kill spores may be applicable when tissue has been contaminated with spores (e.g. in battlefield wounds). Phenothiazinium dyes have long been

used to stain tissue in living humans and are generally considered as safe. It may also be possible to use this technology to decontaminate external surfaces of inanimate objects such as vehicles and buildings and vehicles by spraying them with a phenothiazinium dye and exposing them to sunlight.

### **Publications And Abstracts**

Hamblin MR, Demidova T, Photodynamic destruction of anthrax-surrogate spores, 10th Congress of the European Society for Photobiology, Vienna, Austria, 2003.

Demidova TN, Tegos GP, and Hamblin MR. Anthrax surrogate spores can be destroyed with photodynamic therapy. 44th ICAAC Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington, DC, 2004.

**Patents:** None

**Investigators :** Michael R Hamblin Ph.D., Tayyaba Hasan Ph.D., Faten Gad, M.D.  
**Project Title:** Photodynamic inactivation of pathogenic microorganisms in wounds

### **Objective**

Investigate use of targeted photodynamic therapy for wound and other localized infections.

### **Approach**

We have developed a system of targeting photosensitizers to bacteria using poly-L-lysine chlorin e6 conjugates. The polycationic nature of these conjugates ensures that the photosensitizer can penetrate the outer membrane permeability barrier to reach sensitive intracellular sites, and subsequent illumination with red light kills >99.99% of the bacteria. Multi-antibiotic resistant strains of bacteria are as sensitive as naïve strains. In collaboration with Stanford University we have investigated the use of luminescent bacteria as targets to give a means of following bacterial numbers in living animals in real-time. A sensitive “photon-counting” camera gives an image derived from an optical signal that is proportional to bacterial contamination in wounds. In previous reporting periods of this grant we have investigated the structure-function relationship between the length of the poly-L-lysine chain, the substitution ratio and the efficacy for killing various species of pathogenic bacteria. We established a mouse model of infected excisional wounds on the mouse back suitable for imaging and for testing locally delivered PDT. Targeted PDT using pl-ce6 conjugates was able to eradicate Gram (-) bacterial infections from wounds after delivery of 100 µl of a 200 µM solution of conjugate and illumination with 300 Jcm<sup>-2</sup> 660 nm light Both infections with non invasive *Escherichia coli* and also with invasive *Pseudomonas aeruginosa* could be effectively cured. In the latter case PDT treated mice were saved from death due to sepsis experienced by mice whose infected wounds received either no treatment or had conjugate alone or light alone.

### **Accomplishments:**

We have developed a new type of polycationic antimicrobial PS conjugates. In contrast to the previously used poly-L-lysine conjugates, the novel group of compounds is based on polyethyleneimine (PEI). PEI is available in linear or branched forms and in a wide range of molecular weights. We have developed a synthetic route to prepare, purify and characterize the conjugates covalently joined to the PS chlorin(e6). A conjugate between a small linear PEI (average molecular weight = 400) and ce6 has exceptional activity in mediating PDI of Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, while a PEI-ce6 conjugate prepared from a branched PEI (average molecular weight = 25,000) has exceptional activity against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. We are currently evaluating the activity of these PEI-ce6 conjgates and others for their activity against pathogenic fungi such as *Candida albicans* and *Aspergillus fumigatus*

### **Significance:**

PDT for localized infections and contaminated wounds and burns has a potentially wide area of application. In order to maximize the utility of this therapeutic approach it is necessary to have specific and powerful antimicrobial PS. The PEI conjugates described above are probably the world's most powerful antibacterial PS and with potential activity against pathogenic fungi. In preliminary experiments we have shown that they are also active in mouse models of infection using bioluminescent bacteria and low-light imaging. We propose that topical PDT could be used

by fast-progressing soft tissue infections such as necrotizing fasciitis. Typically surgery (frequently amputation) is the only realistic option as there is insufficient time for antibiotics to take effect. In burn infections and infections in crushed and lacerated tissue the presence of non-viable tissue and the consequent lack of perfusion due to compromised capillaries reduces the effectiveness of systemically delivered antibiotics, thus necessitating the application of topical antimicrobial therapy such as PDT.

### **Publications And Abstracts**

Hamblin MR, Zahra T, Contag CH, McManus AT, and Hasan T. Optical monitoring and treatment of potentially lethal wound infections *in vivo*. *J Infect Dis* 2003;187:1717-25.

Gad F, Zahra T, Francis KP, Hasan T, and Hamblin MR. Targeted photodynamic therapy of established soft-tissue infections in mice. *Photochem Photobiol Sci*, 2004; 3: 451 – 458

Hamblin MR and Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochem Photobiol Sci*, 2004; 3: 436 – 450

Gad F, Zahra T, Hasan T, and Hamblin MR. Photodynamic inactivation of Gram-positive pathogenic bacteria: effect of growth phase and extracellular slime. *Antimicrob Agents Chemother*, 2004; 48:2173-2178.

Gad F, Zahra T, Hasan T, and Hamblin MR. Targeted photodynamic therapy of established soft-tissue infections in mice. In: Kessel D, Editor, *Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy*, Jan 24-29 2004, Bellingham, WA, The International Society for Optical Engineering, Proceedings of SPIE, 2004 in press.

Hamblin MR, Gad F, Anderson RR, Hasan T. Photons for therapy: Targeted photodynamic therapy for infected and contaminated wounds. NATO Research and Technology Organisation Symposium: Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures. HFM-109 /RSY organised by the Human Factors and Medicine Panel in cooperation with the US Department of Defense Advanced Technology Applications for Combat Casualty Care Conference. *In press*

Demidova TN and Hamblin MR. Strong microbe-photosensitizer binding leads to efficient photoinactivation. 32nd Annual Meeting of American Society for Photobiology, Seattle, WA, 2004.

**Patents:** None

**Principal Investigators:** Tayyaba Hasan, PhD, Gerard Nau MD. PhD, Jerome Gross MD  
**Title:** Photochemical Destruction of *Mycobacterium tuberculosis* (MTB)

**Objective:**

The broad goal of this project is to develop photochemistry-based techniques for the destruction of MTB in localized granulomatous lesions. In the pilot project, there were two Specific aims:

Aim 1: To establish the most efficient photosensitizing agent for the destruction of the mycolic acid rich MTB by studying existing and synthesizing new photosensitizers (PS) constructs for pathogen targeting in cultures of *Mycobacterium bovis* (BCG).

Aim 2: To study the effect of photodynamic agents and therapy on the intracellular bacteria. Here the uptake/phototoxicity properties of the PS on MTB/BCG in live macrophages would be established in vitro.

**Approach:**

Photodynamic therapy (PDT) is reasonably well developed for a number of neoplastic and non-neoplastic diseases and interest in its application to infections is increasing. The overall approach here was to rationally test a variety of photosensitizing agents based on the interaction of the mycolic acid rich coat of the mycobacterium. BCG would be used as models for MTB for the initial testing. In the first step the the PS would be evaluated on naked bacteria. As clinically the MTB are intracellular pathogens, the next step will be to test macrophages infected with BCG. Estimate of PDT dosimetry would require not only the measurement of delivered fluence but also of the PS content in bacteria both extra- and intra- cellularly.

**Accomplishments:**

We have tested a number of photosensitizing agents that vary in size, charge and hydrophobicity and confirmed the preliminary results that benzoporphyrin derivative (BPD) was the most effective PDT agent. The previous report presented data from these first studies. In addition a series of new phenothiazine compounds that are positively charged were tested. Surprisingly these were 10-100-fold less effective than the BPD. It also appeared that BPD packaged as a liposomal preparation was the most effective; free BPD was slightly less efficient at killing of bacteria. Having established BPD to be the most effective molecule in this pre-screen we went on to determine the dose response of the photosensitizer for at a fixed light dose and these data were also presented in the previous report. In the current period, we have embarked on the second step of the proposed experiments. We have demonstrated that we can incorporate BCG into macrophages and have also shown, in initial experiments that we can kill the bacteria containing macrophages in a light and drug dose dependent fashion. We now plan to confirm these findings and establish if the killing of the macrophages releases live BCG into the medium.

**Significance:**

Our findings so far are encouraging in that they demonstrate that a clinically approved photosensitizing agent is effective in killing of BCG strain of mycobacterium effectively not only directly but also when the bacteria are incorporated into macrophages.

**Publications:** None

**Patent:** Issued. Photodynamic Destruction of Intracellular Pathogens.

**Investigator:** Charles P. Lin, PhD

**Title:** New developments for in vivo microscopy

### **Objective**

Our goal is to develop methods to extend imaging depth and improve image contrast for in vivo optical microscopy.

### **Approach**

We have developed a video-rate scanning laser microscope with multiple excitation sources and detection channels to enable optical sectioning/imaging of tissue microstructures using both linear and nonlinear contrast mechanisms: confocal (backscattering and fluorescence), two-photon, and second harmonic generation (SHG, also in the backscattering geometry for live tissue imaging). In the last year we have introduced a new nonlinear contrast mechanism for in vivo imaging based on Coherent Anti-Stokes Raman Scattering (CARS). This work is done in collaboration with Professor Sunney's Xie's group at Harvard.

### **Accomplishments**

Video-rate CARS microscopy was successfully performed in a live animal for the first time. Figure 1 shows some of the first CARS images obtained when the lasers were tuned to excite the lipid transition ( $\text{CH}_2$  vibration at  $2845\text{ cm}^{-1}$ ). The panel on the left shows cells in the sebaceous gland 70-100  $\mu\text{m}$  from the skin surface. Dark nuclei are visible within the lipid rich cell bodies. The panel on the right shows dermal adipocytes (fat cells) about 150  $\mu\text{m}$  from the surface. Scale bar = 100  $\mu\text{m}$  in each frame.

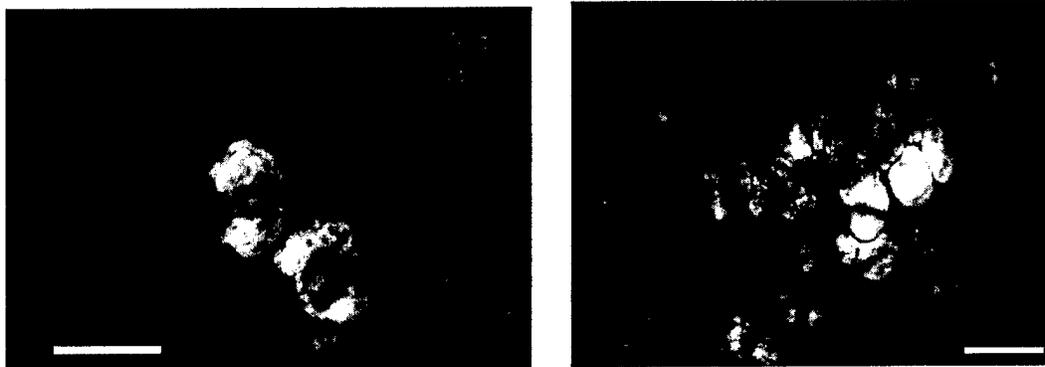


Fig. 1

The CARS images were obtained using a optical parametric oscillator (OPO) pumped by a modelocked Nd:vanadate laser emitting 76 MHz, 7 psec pulse trains at 1064 nm. The OPO output was tunable from 780-920 nm with 5 psec pulse width and 76 MHz pulse repetition frequency synchronized to the pump. Picosecond laser pulses were used instead of femtosecond pulses because they provided better spectral overlap with the vibrational linewidths. An optical delay line was used to adjust the temporal overlap of the pump and the Stokes pulses. The wavelength of the OPO was tuned so that the difference frequency between the two lasers matched the vibrational frequency of interest, e.g.  $2845\text{ cm}^{-1}$  for lipids and  $1650\text{ cm}^{-1}$  for proteins (Amide I band). The spatially and temporally overlapped laser beams were sent into the video rate scanner and focused into tissue using a 60X 1.2 NA water immersion objective lens. The CARS signal was collected in the epi direction by the same microscope objective lens and

detected by a photomultiplier tube without descanning.

### **Significance**

CARS microscopy provides high resolution imaging in tissue (optical sectioning) with molecular specificity, without the need to introduce exogenous dyes. This is a powerful method for imaging molecular transport in the skin as well as for imaging cellular uptake and cellular response to transdermally delivered drugs.

### **Publications and Abstracts**

Potma E, Evans C, Nan X, Lin C, and Xie S. Manuscript in preparation.

### **Patents**

None.

**Investigator:** Johannes F. de Boer

**Title:** Burn depth determination using polarization sensitive optical coherence tomography

**Objective:**

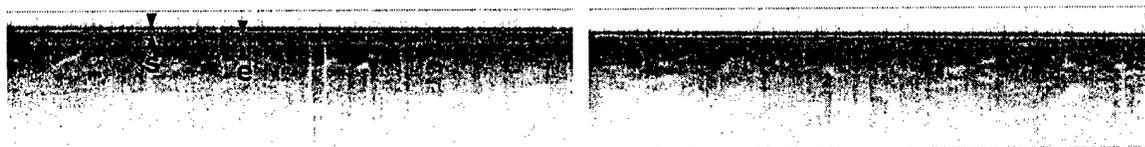
When burn depth can be established at an early stage, any necessary excision and closure can be performed before the onset of infection, minimizing inflammation, shortening hospital stay and improving the long-term functional outcome. However, identifying which burns require surgery often presents problems, with burns covering a complete range of depth and severity proving similar in appearance. The objective of this study is to establish burn depth in skin using polarization sensitive optical coherence tomography (PS-OCT), a recently developed non-invasive imaging technique.

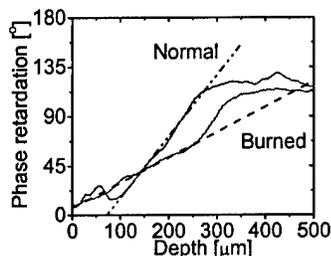
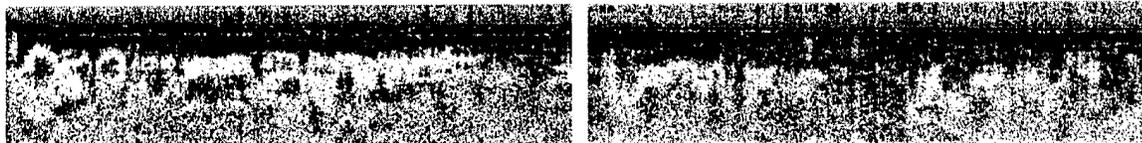
**Approach:**

PS-OCT can detect and measure changes in tissue birefringence, which we aim to correlate with the extent of thermal injury. It is known that on heating to elevated temperatures above around 55 °C, the regular arrangement of collagen macromolecules is lost, resulting in a partial or total loss of birefringence. We previously reported that this loss of birefringence can be quantified in *ex-vivo* burned human skin [1], obtained from burn patients undergoing excisional surgery as part of their routine medical care. The next stage of this project aims to determine whether our *ex-vivo* findings can be extended to *in-vivo* burn imaging. A pilot human study protocol for *in vivo* imaging of burn injuries was approved by the hospital IRB. This protocol allowed for PS-OCT imaging of 25 burn patients in the operating room and at the bedside, both on initial admission, and subsequent days during their hospital stay. Up to 5 burned skin sites are imaged, with corresponding images obtained at neighboring or contra-lateral unburned sites for baseline measurements.

**Accomplishments:**

We have begun imaging patients at Shriners Burns Hospital, Boston, specialized in pediatric burn care. Examples of conventional (top) and polarization-sensitive (bottom) OCT images are shown below, obtained from unburned (left) and burned skin (right) on the legs of a 14 year old patient, within 48 hours of suffering a flame injury. A sterile, disposable plastic sheath is used with our scanning OCT handpiece to reduce the risk of infection, and is indicated (s) in the upper-left image. The epidermal skin layer (e) is apparent, approximately 100  $\mu\text{m}$  thick at this location. Beneath the epidermal layer, the regularly-arranged collagen fibers in the dermis produce a strong backscattering signal (darker coloring in the OCT image) and can change the polarization state of light, resulting in a transition from black to white in the lower (PS-OCT) image. In the OCT image of burned skin (upper-right), the plastic sheath is again visible, but the epidermal layer is almost completely absent. In the PS-OCT image (lower-right), the transition from black to white appears to take place deeper than in the corresponding normal skin.





Information contained within the PS-OCT images is quantified, as shown (left) for the examples above. The mean phase retardation is calculated for each image ( $0^\circ = \text{black}$ ,  $180^\circ = \text{white}$ ). Within  $250 \mu\text{m}$  of the surface, the burned skin shows a reduced slope compared to the normal skin, indicating reduced birefringence, before displaying a similar slope to the normal skin. At greater depths, multiple scattering results in slopes tending towards zero.

### Significance:

Imaging burn patients at various time points in the clinic enables the relationships between non-invasive birefringence measurements and clinical outcome to be established for a wide range of variables, including patient age, burn location, and burn mechanism. These relationships may then enable decisions concerning clinical burn care to be made at earlier stage than is currently possible.

### Publications And Abstracts:

[1] M. C. Pierce, R. L. Sheridan, B. H. Park, B. Cense, J. F. de Boer, "Collagen denaturation can be quantified in burned human skin using polarization-sensitive optical coherence tomography," *Burns* (2004), *in press*.

**Patents:** None

**Investigator:** Robert W. Redmond, Ph.D.

**Project Title:** Bystander Effects

**Objective:**

The aim of this project is to determine the mechanism behind the "bystander effect", whereby cells that are not directly exposed to a given stress, e.g., oxidative stress, undergo cell death or other biological changes in response to neighboring cells that were targeted by the stress. This has been observed in ionizing radiation treatment and also in photosensitization experiments in cell culture. Our hypothesis is that reactive oxygen species (ROS), initially produced in a targeted cell may act as mediators to trigger responses in the bystander cells and that the species responsible for the mediation are derived from lipid peroxidation.

**Approach:**

We have shown that secondary ROS produced by photosensitizers localized in particular intracellular domains can drastically extend the range of biochemical effects in cells. Specifically, we have shown that a plasma membrane localized photosensitizer (Deuteroporphyrin, DP) produced DNA lesions, even though the effects of the primary ROS produced (singlet oxygen) are confined to the membrane itself. The logical extension of this finding is that the bystander effect may also be due to secondary ROS diffusing between cells, rather than intracellularly and the project addresses this potential mechanism for the bystander effect.

Photosensitization provides the perfect approach for studying bystander effects as mixed populations of cells with or without photosensitizer are easily prepared and treated with light. Following treatment, the differentiation of directly stressed cells and bystanders is easily achieved on the basis of cells exhibiting photosensitizer fluorescence. This has not been possible in ionizing radiation studies where a direct differentiation is not possible except in studies using microbeam irradiation where cell numbers are necessarily small and analysis is difficult. The experimental approach used in this project was to utilize illumination of mixed populations of WTK1 human lymphoblastoid cells, some of which were doped with photosensitizer (DP) and some with no photosensitizer. Two end-points were studied for potential correlation. In the first, the amount of ROS produced was measured using a reduced fluorescein probe (dichlorodihydrofluorescein, DCF) that becomes fluorescent on oxidation by reactive oxygen species (ROS). This allowed us to evidence ROS production in bystander cells. The second end-point was DNA damage, monitored using the single cell gel electrophoresis (comet) assay. Inhibitors and quenchers have also been added to the system to obtain mechanistic information.

**Accomplishments:**

We have adopted a new and powerful approach to the study of the bystander effect by taking advantage of photosensitizer not only to selectively stress a fraction of cells but by using the photosensitizer fluorescence as a discriminator in flow cytometry to separate directly affected and bystander studies for further study. We have shown that DCF levels increase in bystander as well as directly photosensitized cells. To gain some mechanistic information samples were pretreated with vitamin E, a membrane antioxidant, which significantly reduced DCF fluorescence. This antioxidant is co-localized in the membrane with the photosensitizer and inhibits lipid peroxidation. The water soluble free radical scavenger, trolox, did not cause a

similar reduction when present in the extracellular medium, evidence that direct inter-cellular diffusion of free radicals is not responsible. Extracellular catalase was also shown to be ineffective in reducing the bystander effect; thus, hydrogen peroxide can also be ruled out as a mediator. Our current hypothesis is that lipid hydroperoxide products resulting from photosensitized lipid peroxidation are the mediating species and give rise to free radicals and/or initiate arachidonic acid release and metabolism in bystander cells. We are currently testing the effects of extracellular glutathione peroxidase (Gpx) and superoxide dismutase (SOD) in modulating the bystander effect. If lipid hydroperoxides are involved we would expect Gpx to reduce the bystander effect but SOD to have no effect.

This data has been instrumental in successfully obtaining a NIH PO1 Program Project Grant entitled "Cellular responses to localized oxidative stress" with funding from the National Cancer Institute due to be initiated 8/1/04 for a four year period.

**Significance:** This project has confirmed the existence of a bystander effect in photosensitization and provided valuable mechanistic information regarding its nature. We hypothesize that diffusing lipid hydroperoxides may be responsible and these are subsequently activated to ROS by metal catalyzed breakdown to oxy radicals to elicit biochemical changes in the bystander cells. The use of cell sorting by flow cytometry under sterile conditions allows us to generate these distinct cell populations and their progeny for future investigation. This has important ramifications not only for the bystander effect but also for studies of induced genomic instability where oxidative stress is also thought to play a role and leads to increased mutagenesis and carcinogenesis. These studies will allow us to test the hypothesis of a common oxidative pathway in the bystander effect and genomic instability. This mechanistic information may be useful in understanding mutagenic and carcinogenic consequences of increased oxidative stress and in the application of cancer therapies that involve oxidative stress and potential optimization through bystander cell killing.

**Publications And Abstracts:** None

**Patents:** None

**Investigators:** Irene E. Kochevar, Ph.D. and Robert R. Redmond, Ph.D.

**Title:** Photochemical Tissue Bonding

**Objective:**

The overall goal is to develop a rapid and efficient method for bonding or reattachment of tissues that does not rely on the use of mechanical means such as sutures, staples or clips. The use of mechanical methods is complex in certain surgical scenarios, with potential complications including inflammation, foreign body response and infection. A non-mechanical method for attachment of tissue could overcome these adverse effects and have application on many different tissues in different medical specialties.

**Approach:**

Photochemical tissue bonding (PTB) involves application of a photochemically-active dye to the tissue to be joined, approximation of these surfaces in close contact, followed by activation with visible light. Activation causes the formation of reactive intermediates that cause chemical crosslinks to be formed between the tissue surfaces at the molecular level. These "nanosutures" are responsible for the strength of the intimate bond or seal formed. The specific aims in the last period were:

1. To explore PTB as a means to seal incisional wounds in skin.
2. To explore PTB for peripheral nerve repair

**Accomplishments:**

1. Demonstrated that closure of skin incisions and excisions in a pig model with PTB occurs with little scarring. Experiments were carried out using male Haniford mini-pigs. In the incisional model Full thickness linear incisions of 2 cm length and 7 mm depth were created with a calibrated scalpel instrument. For excision studies an elliptical excision of 2.5 cm x 1 cm x 0.7cm deep was removed with a surgical scalpel. PTB treatments were carried out by administration of Rose Bengal (0.1%, w/v) to the wound surfaces and irradiating with 532 nm light from a CW Nd/YAG laser. PTB was compared to repair alternatives such as sutures and Dermabond adhesive. All wounds were treated with epinephrine to leave a bloodless field and air cooling was employed for PTB treatments to allow a relatively high irradiance to be used without concomitant thermal damage. Typically 8 sites were used for each treatment type.

PTB was shown to efficiently close incisional and excisional wounds with a few minutes of light irradiation. Visual examination was carried out at 2, 4 and 6 weeks by three blinded dermatologists to determine wound healing parameters such as contour, visible appearance, and inflammation. Histology was also carried out to determine scar thickness as a function of treatment mode. Results showed that PTB treatment did not result in enhanced inflammation or an adverse scarring response compared to other modalities, although there was no statistically significant improvement. Similarly, PTB did not produce a thicker scar than the other modalities but an improvement was noticeable over sutures where a number of the sutured wounds failed, resulting in wound opening and a thicker scar by histology. No failures occurred in the PTB groups, even in the excisional model, which is under considerable tension. Thus, PTB is a promising candidate for repair of surgical incisions and excisions with minimal scarring without

inflammation of foreign body response. A human clinical trial is now in preparation for repair of excisions to remove skin cancer.

2. PTB was previously shown in ex vivo pilot experiments to seal severed peripheral nerves following administration of RB and green (532 nm) light and a methodology was developed using an epineurial cuff approach to provide greater surface area for bonding. The epineurial cuff method was applied in the rat sciatic nerve model where nerve transection was followed by four types of treatment (no repair, sutured repair, epineurial cuff alone or epineurial cuff with PTB). The gold standard for nerve repair at present is microscopic suture repair; hence this group has been used as our positive control while the no repair group was used as our negative control. Rats were followed for 90 days and end points included gait analysis, gastrocnemius muscle mass (innervated by the sciatic nerve) and histological measurement of regrowth of myelinated (functional) axons through the repair site. In all end points the PTB + cuff treatment approached the efficacy of the microsurgical repair and no evidence for adverse inflammation or scarring was seen at the repair site. Histology showed extensive regrowth of myelinated axons with densities proximal and distal to the repair site being similar.

#### **Significance:**

PTB has been expanded to multiple tissues and has shown potential application in each tissue in animal models. The data above support the use of PTB to seal tissues without need for sutures that can cause adverse effects due to foreign body reaction and scarring. To date, PTB has proven not to stimulate inflammation and scarring in any tissue studied. This opens the door for a better cosmetic outcome in skin repair and also a better outcome for recovery of function in peripheral nerve repair where sutures can induce scarring that is a known inhibitor of recovery of function. In addition, the process can be rapid and effective and produce a complete seal, rather than the interrupted seal produced by sutures. This is important in nerves and blood vessels to prevent inflammation and neroma formation in the nerve. Similar arguments apply to reanastomosis of blood vessels, a target of ongoing studies.

#### **Publications And Abstracts**

Photochemical keratodesmos for bonding corneal incisions. Proano, CE, Mulroy L, Jones E, Azar DT, Redmond RW and Kochevar IE. I.O.V.S. 2004, 45 2177-2181.

Photochemical tissue bonding: Photons for healing. Redmond RW, Kochevar, IE, Amann C, Chan BP, Farinelli WA, Anderson RR, Azar DT, Johnson TS, Winograd J and Randolph, MA. NATO HFM-109/RSY Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures"

**Patents** None

**Investigator:** Hensin Tsao, MD PhD

**Title:** Ultraviolet Radiation and Nucleotide Excision Repair in the Pathogenesis of Cutaneous Melanoma

**Objective:**

The broad goal of this project is to understand the genetic determinants of cutaneous melanoma tumorigenesis in response to excessive ultraviolet radiation (UVR). Since nucleotide excision repair (NER) has evolved to rectify UVR-induced genetic lesions, we will specifically explore the role of the NER apparatus in the pathogenesis of cutaneous melanoma. At the current time, no adequate model of melanoma photocarcinogenesis exists. We will generate mice that are potentially susceptible to UVR-induced melanoma formation by genetically ablating both the melanoma tumor suppressor gene, *INK4a*, and nucleotide excision repair gene, xeroderma pigmentosum C (*XPC*).

**Approach:**

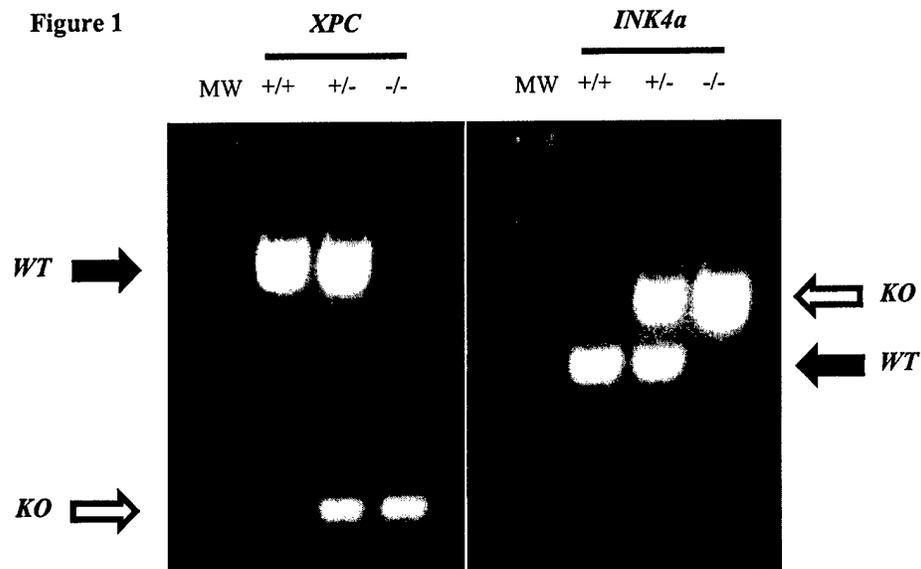
1. To create mice deficient for both *XPC* and *INK4a* genes.
2. To develop, *in vivo*, a model of stepwise melanoma photocarcinogenesis.
3. To compare, genetically, murine melanomas caused by excessive UVR with those spontaneously induced by an oncogenic *RAS* transgene.

**Accomplishments:**

1. To create mice deficient for both *XPC* and *INK4a* genes.

Previously, we only had sequence information within the mouse *XPC* gene but not the targeting vector; this makes it tremendously difficult to score for heterozygosity. In other words, we knew whether the gene was there or not but could not tell how many copies were present. As shown in Fig 1 on the left, with our new

sequence information, the targeted allele (labeled, knockout or KO) is a much smaller fragment than the wild type *XPC*. Thus, the 3 lanes on the left gel show genotypes for mice that have two normal copies (+/+), one normal copy (+/-) or and no normal copies, respectively. A similar genotype



approach is shown on the right hand gel for the other gene, *INK4a*.

In the last 12 months, we have been able to complete our breeding strategy for deriving *XPC*<sup>-/-</sup>; *INK4a*<sup>-/-</sup> mice. Viability studies are very encouraging that these mice can continue to reproduce without ostensible compromises in health.

2. To develop, *in vivo*, a model of stepwise melanoma photocarcinogenesis. In order to study melanoma photocarcinogenesis, we propose to define parameters that will produce melanomas in *XPC*<sup>-/-</sup>; *INK4a*<sup>-/-</sup> mice. Since the mice have different levels of pigmentation that may confer different levels of UV sensitivity, we have spent a great deal of time breeding the targeted alleles into appropriate backgrounds.

The original *XPC* *-/-*; *INK4a* *+/+* mice are in a darkly pigmented B6/129J background while the FVB *XPC* *+/+*; *INK4a* *-/-* mice are in an albino background. Consequently, we have to breed the albino *XPC* *+/+*; *INK4a* *-/-* mice into a wild-type B6/129J control mouse (from Taconic Farms, NY) and interbreed the F1 heterozygote generation in order to generate F2 *XPC* *+/+*; *INK4a* *-/-* mice with a dark coat. Since it is significantly easier to diagnose murine melanomas if melanin is detectable histologically, studies are under way to examine the photocarcinogenesis of these dark coat mice.

We have also adjunctively isolated murine embryonic fibroblasts (MEFs) in order to better characterize the in vitro mechanisms that dictate UV effects in these mice. In terms of growth, the loss of *INK4a* appears to enhance the proliferation of the primary MEFs (Fig 2). This increase rate of growth is to be expected since loss of *INK4a* abrogates both the RB and p53 pathways through p16 and ARF, respectively. Although preliminary, the additional loss of *XPC* appears to partially limit the enhanced growth effects seen with loss of *INK4a*. This may be due to increased mutational events from loss of *XPC* or minor strain variation since our *INK4a*-null mice are on a pure FVB background. We are currently correcting for the strain differences by breeding our *INK4a* targeted allele into a B6/129 background. Loss of *XPC* does not appear to increase or decrease the rate of proliferation as compared to normal controls.

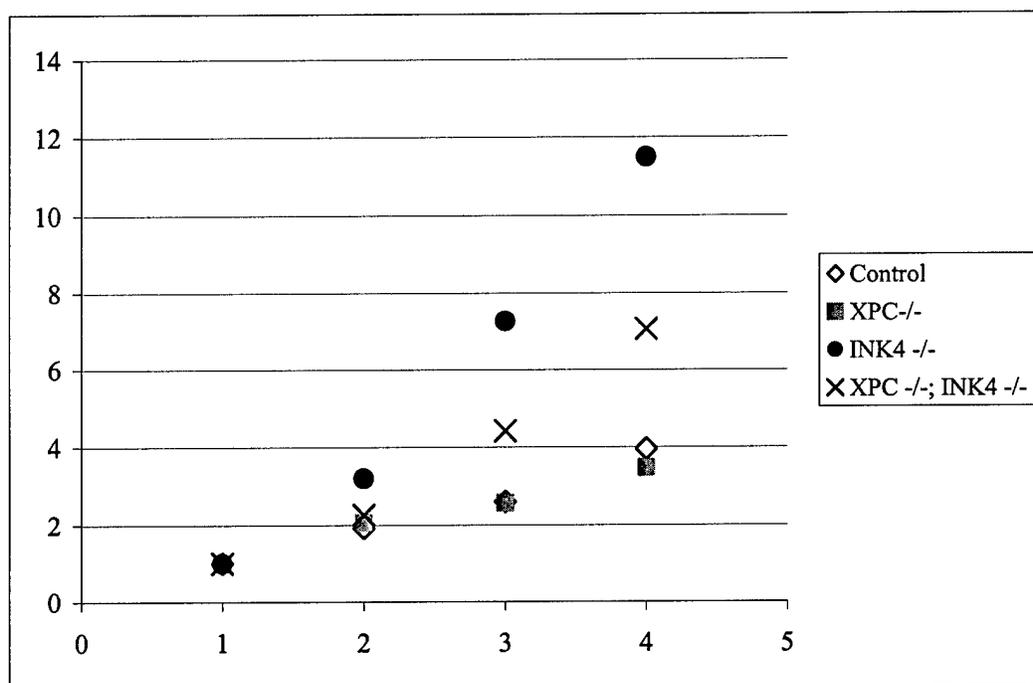


Figure 2. 70,000 MEFs were plated on Day 0 in triplicate and cells were counted in triplicate relative to attachment on Day 1.

We have also begun to examine ultraviolet sensitivity of the MEFs. We use UVA/UVB lamps which have filtered out UVC. We initially hypothesized that loss of ARF would increase MDM2 levels in the cell and interfere with p53 function thereby potentially enhancing the survival of *XPC* *-/-* mice. However, this hypothesis will also allow us to test the possibility that UV-stimulated cells phosphorylate p53 thereby possibly rendering the protein resistant to MDM2. In this second scenario, MEFs from *XPC*-null mice will exhibit the same UV sensitivity as MEFs from *XPC*, *INK4a*-doubly null mice. We were thus very intrigued to see that MEFs which lacked both *XPC* and *INK4a* did not exhibit a significant difference in UV sensitivity when compared to MEFs that lacked only *XPC* (Fig 3).

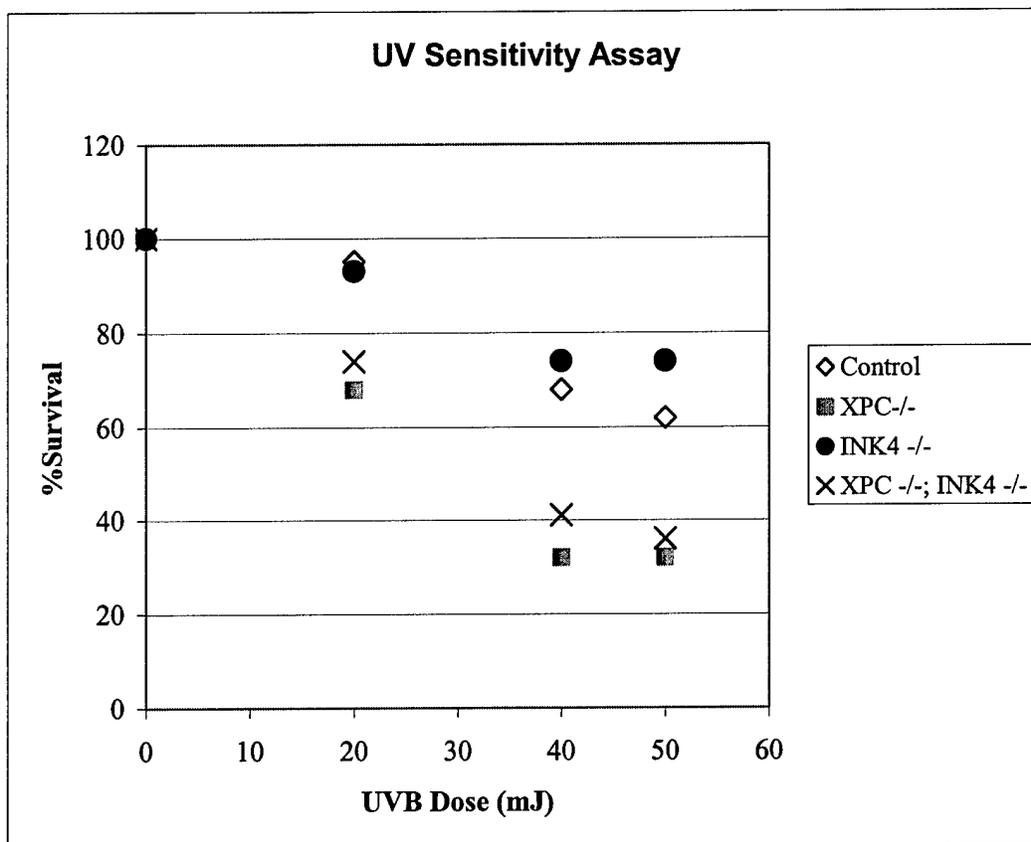


Figure 3. 70,000 MEFs were plated in triplicate on Day 0 and exposed to the designated amount of UVB on Day 1. Cells were allowed to grow until Day 4 and the total number of cells were counted. Survival is shown as % Survival relative to un-irradiated cells.

3. To compare, genetically, melanomas caused by excessive UVR with those spontaneously induced by an oncogenic RAS transgene. As part of a larger genetic analysis of melanomas from these mice, we have banked murine melanomas derived from 3 strains- tyr-RAS; XPC +/+; INK4a -/- (common); tyr-RAS; XPC -/-; INK4a -/- (common); XPC -/-; INK4a -/- (rare). Given the evolution of more powerful genomic approaches, we have withheld the tumors from simple mutational analyses of loci, but rather, reserved these specimens for expression profiling. Furthermore, we have also derived at least 10 melanoma cell lines from these mice for future tumorigenesis and in vitro analysis.

**Significance.**

This first pilot year has given the project the necessary momentum to move forwards in terms of technical and resource development. We devoted a significant amount of effort to generating the MEF lines in order to dissect the UV mechanism at *in vitro* level. We are also at a point where the necessary controls are available to more precisely examine the *in vivo* effects. An understanding of UV sensitivity in the context of deficient cell cycle regulators represents a critical step towards the development of a more effective, highly targeted, solar protectant.

### **Publications and Abstracts**

\*Rheinwald JG, Hahn WC, Ramsey MR, Wu JY, Guo Z, Tsao H, De Luca M, Catricala C, O'Toole KM. A two-stage, p16(INK4A)- and p53-dependent keratinocyte senescence mechanism that limits replicative potential independent of telomere status. *Mol Cell Biol* 2002; 22:5157-72

\*Goggins WB, Tsao H. A population-based analysis of risk factors for a second primary cutaneous melanoma among melanoma survivors. *Cancer* 2003;97:639-43

\*Tsao H, Bevona C, Goggins W, Quinn T. The transformation rate of moles (melanocytic nevi) into cutaneous melanoma. *Arch Dermatol* 2003;139:282-8.

\*Tsao H, Feldman M, Fullerton JE, Sober AJ, Rosenthal D, Goggins W. Early detection of asymptomatic pulmonary melanoma metastases by routine chest radiographs is not associated with improved survival. *Arch Dermatol*; In Press

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\*Bevona C, Goggins W, Quinn T, Tsao H. A Multivariate analysis of cutaneous melanomas associated histologically with melanocytic nevi. *Arch Dermatol*; In Press

\*Tsao H, Sober AJ. Chapter 6. Precursor lesions and markers of increased risk. In: Balch C, Houghton AN, Sober AJ, Soong SJ, eds. Cutaneous Melanoma 4<sup>th</sup> Edition. St Louis: Quality Medical Publishing, Inc, 2003: 121-135

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\*Tsao H, Sober AJ. Chapter 91. Atypical Melanocytic Nevi. In: Fitzpatrick TB, et al, eds. Fitzpatrick's Dermatology in General Medicine, 6<sup>th</sup> Edition. New York, NY: McGraw Hill, Inc., 2003: 906-916

\*Tsao H. Chapter 61. Neurofibromatosis and Tuberous Sclerosis. In: Bologna JL, et al, eds. Dermatology, 1<sup>st</sup> Edition . London, UK; Harcourt Health Sciences, 2003: 853-868.

### **Patents**

None.

**Investigators:** R. Rox Anderson, MD, Rieko Tachihara, MD PhD, Anna Yaroslavsky, MD, Agustina Echague, MD PhD

**Title:** Light-induced Vasodilation and Wound Healing

**Objectives:**

1. Demonstrate photovasodilation *in vivo*, as a potential mechanism for light-stimulated wound healing.
2. Determine optimal wavelength and irradiance.
3. Design and construct photovasodilation light source.
4. Determine effect of photovasodilation light source on wound healing.

**Approach:**

As reported previously, the rabbit ear model was used to demonstrate and study photovasodilation *in vivo*, for aims 1 and 2. Arterioles showed the most reproducible vasodilation response, occurring within seconds of exposure to low-level visible light, as measured by NIR laser Doppler blood flowmetry. A filtered Xe arc lamp was used to compare response at different wavebands from 350 – 500 nm. Maximum efficacy was noted with wavelengths from 380~420 nm. Response occurred at 488 nm, occasionally occurred at 514 nm, and did not occur at 532 nm or longer wavelengths. In the waveband near 400 nm, irradiances as low as 10-30 mW/cm<sup>2</sup> were effective. This waveband and irradiance can be achieved with small LED sources, which may provide a practical, fieldable source. For Aim 3, a commercial prototype 420 nm LED array emitting up to 100 mW/cm<sup>2</sup> was chosen, which has been modified for use in a forthcoming clinical trial of wound healing at MGH. For aim 4, as previously reported, the diabetic mouse model of ischemic wound healing showed too much variability. Therefore, a clinical study of ischemic and diabetic chronic leg ulcer wound healing will be performed. Going forward, this work will continue under other funding (dermatology department, industry, NIH). Non-healing ischemic leg ulcers are a common and significant clinical problem among civilians and veterans, but do not *per se* pose a threat to combat readiness or casualty care. We will approach aim 4 in patients who are readily accessible in the dermatology service here. Briefly, the rate of ulcer healing under exposure to the 420 nm LED array will be compared with that of a placebo source, in a prospective double-blinded pilot trial. L-arginine, the only natural substrate for NO synthetase, will also be tested as a topical enhancer for photostimulation and wound healing. MFEL funds will not be used for this first human trial. If successful, we hope it may motivate future DoD support for wound photostimulation in military burn and trauma patients.

**Accomplishments:**

Aims 1-3 have been accomplished or nearly accomplished. We have decided to test aim 4 in chronic diabetic and ischemic non-healing leg ulcer patients. This project will be pursued with other support. If indicated, DoD support may be sought for future studies in military medical care settings.

**Significance:**

Rapid wound healing is an essential challenge for combat casualty care, and combat readiness of war fighters who are able to re-enter action after a period of healing. The studies in this project

have shown that photovasodilation does occur *in vivo*, that a waveband from about 380-420 nm is the most efficient stimulus, and that irradiances achievable with a small LED array or even filtered sunlight might be used for ischemic wound stimulation. Potentially, a deployable wound stimulator will result from this work, which is expected to enter a clinical trial in early 2004.

#### **Publications And Abstracts**

Tachihara R, Farinelli WA, Anderson RR. Low Intensity Light-Induced Vasodilation In Vivo. *Lasers Surg Med Suppl* 14:39, 2002.

#### **Patents Filed:**

None.