**LASER SCANNING CONFOCAL MICROSCOPY (LSCM): AN APPLICATION FOR THE
DETECTION OF MORPHOLOGICAL ALTERATIONS IN SKIN STRUCTURE**

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By

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in Skin Structure

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Abstract

LASER SCANNING CONFOCAL MICROSCOPY (LSCM): AN APPLICATION FOR THE DETECTION OF MORPHOLOGICAL ALTERATIONS IN SKIN STRUCTURE

BY

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Laser scanning confocal microscopy (LSCM) is an optical imaging technique that provides improved resolution and sensitivity over conventional methods of optical microscopy. However, the cost of most commercial LSCM systems exceeds the financial limitations of many smaller laboratories. The design of a custom LSCM created at a fraction of the cost of a commercial model is discussed in this paper.

The increase in the incidence rate of skin cancer in the world today is alarming, as such, it is essential to provide an early, rapid and effective method for in vivo diagnostics of human skin tissue. LSCM is capable of detecting alterations in skin morphology and configuration, as well as providing chemical composition information which may be indicative of the development of skin cancer. If developed successfully, LSCM could replace the current invasive biopsy procedures performed today with a quick, non-invasive optical scanning method that would prove beneficial for both patients and physicians alike.

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Chapter 1: Introduction

The incidence of skin cancer is increasing at an alarming rate with one in six Americans developing skin cancer at some point in their lives.¹ The capacity to effectively screen for invasive cancers in its early stages of development provides doctors and physicians with the ability to immediately begin administering treatment in an effort to decrease the chance of the carcinoma becoming malignant and significantly improve the patient's chance of survival. Although numerous means of detection have been developed in an attempt to create an ideal early recognition technique, it is evident that an optimal method is still yet to be established.

The need for improved sensitivity and specificity in cancer diagnoses has led to an interest in an optical biopsy where qualitative and quantitative differences in cellular fluorophores would distinguish between diseased and normal tissue. Also, the optical biopsy could be performed *in vivo* and would provide real-time feedback as to the status of the tissue in question.

The purpose of this paper is to explore the ability to use laser scanning confocal microscopy technology to detect morphological and configurational alterations in the cellular structure of skin tissue which may be used as an indication of the onset of malignant melanoma or other skin cancer invasion. This paper will discuss the background information needed to better understand the optical biopsy including, a description of the skin, an overview of the most common types of skin cancer, the present

techniques used in a skin cancer diagnosis, light and tissue interactions, light microscopy techniques, fluorescence microscopy techniques and the components and applications of a confocal fluorescence microscope. Following the background information, this thesis will discuss my design for a custom *fluorescence* microscope upgrade to a standard optical laboratory microscope intended to elicit fluorescence from naturally occurring fluorophores in the skin tissue. Finally, this paper will detail our experiment involving the irradiation of engineered skin samples and the results observed utilizing confocal *reflectance* microscopy.

Chapter 2: Background

Section 2.1 Human Skin

Fig. 1 A cross-sectional diagram of the skin³

The skin is the largest organ of the human body accounting for 12% to 16% of our body weight and covering approximately 12 to 20 square feet. The chemical composition of the skin is about 70.0% water, 25.5% protein, 2.5% lipids and 2.5% trace minerals and other compounds.² It covers the internal organs

and protects them from injury; serves as a

barrier between germs, such as bacteria, and internal organs; and prevents the loss of too much water and other fluids. The skin regulates body temperature and helps the body get rid of excess water and salts. It also has numerous nerve endings that make the skin a sensory organ which is able to detect warmth, cold, light, taste and touch.¹ The skin is comprised of three primary layers, the epidermis, dermis and subcutis from outer to inner, respectively.

The epidermis is composed of four different cell types: keratinocytes, melanocytes, Langerhans cells and Merckels cells with keratinocytes constituting 95% of

the epidermis. The epidermis is the thinnest layer of the skin at a maximum of about one millimeter and can be subdivided into 4-5 layers depending on the area of interest. The layers in descending order are the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale.¹ The layers of the epidermis are typically differentiated by the size and shape of the skin cells of which they are comprised. In addition, the state of the components within the individual skin cells, for example live versus dead keratinocytes, can also be used as an indicator of the layer type. It should also be noted that thicker areas of skin may contain all five sub layers of the epidermis while thinner areas may only be comprised of 3 or 4.

The innermost layer of the epidermis, which is the closest to the dermis and makes up a part of the stratum germinativum, is also called the basal layer. The basal layer contains stem cells which can renew themselves and continually divide pushing older cells up toward the surface of the skin, where they are eventually shed. The basal cell layer also contains cells called melanocytes. Melanocytes produce the skin coloring or pigment known as melanin, which gives skin its tan or brown color and helps protect the deeper layers of the skin from the harmful effects of the sun.

The stratum spinosum is also called the squamous cell layer and is located above the basal layer. Within this layer are the basal cells that have been pushed upward, however these maturing cells are now called squamous cells, or keratinocytes. Keratinocytes produce the tough and protective protein keratin that makes up the structure of the skin, hair and nails giving them strength and flexibility. The squamous cell layer is the thickest layer of the epidermis, and is involved in the transfer of certain

substances in and out of the body. The keratinocytes from the squamous layer are then pushed up through two thin epidermal layers called the stratum granulosum and the stratum lucidum. As these cells move further towards the surface of the skin, they become flatter, more scale-like and adhere together, and then eventually become dehydrated and die. The stratum corneum is made up entirely of these dead and dehydrated cells which shed about every two weeks.

The epidermis is separated from, and adhered to, the dermis by the basement membrane. The membrane acts as a physical barrier to prevent invading molecules from reaching the deeper tissues. The basement membrane is an important structure in skin cancer because when a carcinoma becomes more advanced and malignant, it generally grows through this barrier.⁴

The dermis is located beneath the epidermis and is the thickest of the three layers of the skin. The dermis contains the structural components of the skin including collagen, elastic fibers and extracellular matrix. The dermis is held together by collagen which provides the matrix that holds individual skin cells in place and forms the structural network of the skin. Collagen is the most abundant protein in the body and is primarily composed of glycine, proline and hydroxyproline.⁵ It is one of the strongest proteins in nature and gives the skin its strength and durability. Elastin is similar to collagen but is a more stretchable protein that maintains the skin's elasticity. Elastin also contains two unique amino acids, desmosine and isodesmosine that together permit the skin to stretch and then regain its original shape.²

The subcutis is the innermost layer of the skin, and consists of a network of fat and collagen cells. The subcutis is also known as the hypodermis or subcutaneous layer, and functions as both an insulator, conserving the body's heat, and as a shock-absorber, protecting the inner organs. It also stores fat as an energy reserve for the body. The blood vessels, nerves, lymph vessels, and hair follicles also cross through this layer. The thickness of the subcutis layer varies throughout the body and from person to person.⁵

Section 2.2 Skin Carcinoma

One in six Americans develop skin cancer at some point in their lives, most of which are classified as non-melanoma carcinomas, usually occurring in either basal or squamous cells. There are more than 1 million cases of non-melanoma skin cancer diagnosed yearly in the United States that are considered to be sun-related. Melanoma, the most deadly type of skin cancer, accounts for more than 75% of deaths due to skin cancer each year. It is estimated that 116,500 new cases of melanoma were diagnosed in the United States in 2008; however, for melanoma and non-melanoma type skin carcinomas, a cure is highly likely with an average five-year survival rate of 99%, if the cancer is detected and treated when the cancer is still localized and has not spread to the inner layers of the skin.² However, the survival rate drops to 36% after the cancer has spread to the lymph nodes and falls as low as 5% for those with distant metastasis.

Section 2.2.1 Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common form of skin cancer worldwide. BCC originates in the basal keratinocytes, and is thought to be caused by overexposure to the ultraviolet rays of the sun. The exact origin and primary cause of BCC is unknown, however many believe that BCC arises from stem cells in the basal layer and occasionally the outer root sheath of hair follicles, specifically in hair follicle stem cells residing just below the sebaceous gland duct.⁷ Basal cell cancer is considered a less life-threatening form because it is slow growing and rarely will metastasize, or travel in the bloodstream, but it will affect the surrounding area by destroying tissue. Basal cell tumors have many different appearances, usually dome-shaped with a pearly white color, and generally occur in sun exposed areas such as the face, scalp, chest, back and legs.⁸ BCC lesions can take on a variety of shapes and sizes, some with indistinct borders making them difficult to identify and remove.

Basal cell carcinoma

Fig. 2 Image of Basal Cell Carcinoma⁸

After treatment, a basal cell carcinoma patient can have recurrence in the same place on the skin and is also more likely to get new ones elsewhere on their body. As many as half of the people who are diagnosed with basal cell cancer will develop a new skin cancer within 5 years.⁸

Section 2.2.2 Squamous Cell Carcinoma

Squamous cell carcinoma (SCC) is the second most common form of skin cancer accounting for about 250,000 of the new cases each year. SCC is a faster moving malignant tumor of the epidermal keratinocytes and is also associated with overexposure to ultraviolet light. SCC is more aggressive than BCC in its invasion of fatty tissues beneath the skin layer and the lymph nodes. The metastasis rate for SCC is considered to be relatively low; however, if the disease goes untreated SCC can reach the lymph nodes or lungs and the morbidity rate increases significantly.⁹ The skin change caused by squamous cell carcinoma most often looks like a scab. There may be a thick, adherent scale on a red, inflamed base.⁸

Squamous cell carcinoma

Fig. 3 Image of Squamous Cell Carcinoma⁸

Section 2.2.3 Other Types of Non-Melanoma Skin Cancers

There are some other types of non-melanoma-type skin cancers that are much less common including Merkel cell carcinoma, Kaposi sarcoma, cutaneous lymphoma, Adnexal tumors and other sarcomas.⁸ These less common types of skin cancer will not be discussed in detail in this paper.

Section 2.2.4 Melanoma

Although melanoma accounts for only a small percentage of skin cancer, it is far more dangerous than other skin cancers and causes the most skin cancer deaths.⁷ The cells that become cancerous in malignant melanoma are called

Fig. 4 The Warning Signs of Melanoma¹⁰

melanocytes which synthesize melanin, the pigment or coloring for the skin, and can be found in the basal layer of the epidermis.¹¹ Melanoma is capable of metastasizing and tends to show up in lymph nodes, but can also spread to the liver, lungs and brain.

However, melanoma is also almost always curable when it is detected before it has metastasized. Physicians using the current detection techniques are able to diagnose about 80% of melanomas at the localized stage.⁷

Section 2.2.5 Diagnosis

The diagnosis of a potential cancer area is nearly always based on a physician examining the patient's skin for unusual moles and other suspect areas. If the physician determines that an area should be tested, a section of the suspect area must be removed and the tissue sample more closely examined under a microscope. The procedure of taking a sample for testing is called a biopsy. In an excisional biopsy, a surgeon cuts through the skin to remove the entire tumor or an incisional biopsy can be performed where a small part of a larger tumor is removed. This often can be done using local or general anesthesia depending on the location of the necessary biopsy. There are several ways to take a biopsy of the skin and a doctor will choose the method best suited to the type of skin tumor suspected. Shave biopsies remove the outer layers of skin with a scalpel and are fine for some basal cell or squamous cell skin cancers, but they are not recommended for suspected melanomas of the skin. Punch biopsies use a blade to remove small, round core of the deeper layers of the skin, and can reveal the depth of melanoma penetration into the skin which is an important factor in choosing the appropriate type treatment for that type of cancer.¹²

Following a biopsy, a histology, or study of cells and tissues, is performed by the physician or pathologist. The histology requires the sectioning, staining and examination of the skin sample under a microscope. The pathologist then analyzes the sample for any abnormal appearance of the tissues and identifies areas with the potential for skin carcinoma. In basal cell carcinoma, the pathologist will observe cohesive groups of basal

tumor cells, palisading, or aligning of nuclei in tumor cell groups, clefts around cell groups which can appear as projections from the nucleus or as intranuclear tubular structures, as well as variable inflammation and ulceration. In squamous cell carcinoma, there will be signs of abnormal keratin production, thickening of the skin layer, invasion of the dermis into the skin layer and the appearance of abnormal keratinocytes.¹⁰ Finally, in a melanoma diagnosis the pathologist will observe a predominance of singular melanocytes above the basal layer of the epidermis, irregularly sized and distributed nests of melanocytes and, if the melanoma is more advanced, a descent of the melanocytes into the dermis. Determining an accurate pathologic diagnosis can be challenging, even for an experienced pathologist, making this a time-consuming and expensive procedure.¹² After a positive diagnosis is made, a patient's entire body needs to be examined to determine whether the cancer cells have spread to other areas such as nearby lymph glands, and whether there are any additional areas affected with cancer.¹³ The uncertainty one may feel waiting for biopsy and histology test results can be a source of much anxiety. Not knowing when the results will be ready and not understanding why testing sometimes takes longer than expected can cause extra concern.

Currently, the most practical early detection methods include patient self-tests, physical examinations and routine tests by a physician. A doctor can determine a patient's cancer risk depending on age, medical history and other risk factors and may suggest other exams for patients with an increased risk. Images of areas inside the body can help determine if cancer or a tumor is present. Imaging detection methods include, CT or CAT scans, radionuclide scans, ultrasonography and MRI. Also, laboratory tests,

such as blood tests, can show the effects of the disease on the body and indicate that cancer is present.¹³ Despite the numerous methods that have been developed, it is evident that an optimal method for early, efficient and accurate detection is still yet to be established.

As an alternative to invasive biopsies and other early detection techniques used today, an “optical biopsy” could be developed that would employ an optical probe (i.e. laser) scanned over suspect areas of the patient’s tissue and a spectrometer to analyze the collected wavelengths of light from the skin to determine the nature of the tissue (i.e. cancerous or non-cancerous). This technique would provide the physician and patient with a diagnostic tool that is non-invasive while still being able gather information below the skin surface, would not require the removal of any skin tissue and could provide real-time recording and feedback as the optical probe is moved from site to site of successive suspect areas. Also, the high spatial resolution offered by optical methods provides an ability to detect very small tumors comparable to that of histology.¹⁵ This diagnostic technique offers the potential for improved disease management with reduced risks to the patient and the potential for earlier diagnosis and immediate treatment.¹³

Section 2.3 Light and Tissue Interaction

In order to fully explore the potential of an optical biopsy, we must first understand the interaction between light and the tissue of the skin. When a photon of light

encounters tissue there are five ways in which the light can interact with the tissue, it can be transmitted, reflected, refracted, scattered or absorbed.

Transmission and reflection of light waves occurs when the frequency of the light wave does not match the natural

frequency of vibration of the atoms

in the tissue. In both transmission

and reflection, the electrons will

vibrate for a short period of time with small

amplitude of vibration; then reemit the energy

as a light wave. If the specimen is transparent, the vibrations are passed on to neighboring

atoms through the material and are reemitted on the opposite side of the object. If the

specimen is opaque, the vibrations are not passed from atom to atom through the

material, but rather the atoms on the material's surface vibrate for short periods of time

and reemit the energy as a reflected wave. In transmission and reflection, the resulting

light maintains the same wavelength as the incident light since the majority of the energy

remains undiminished in these processes.¹⁷

Absorption by molecules of the skin is a process of borrowing energy from, or lending it to, the electromagnetic field to which it is exposed. In the microwave region of the EM spectrum, molecules usually change the way they rotate in space. Exposure in the infrared region will change the way the molecules vibrate. Ultraviolet and visible light can change the distribution of electrons within the molecules and may change the shape of the molecules. These electronically excited states can undergo chemical reactions that

Fig. 5 Transmission and Reflection of Light¹⁶

would not otherwise occur in the unexcited configurations. Since there are many possible vibrations and rotations in most molecules, these transitions often result in broad composite absorption and emission spectra. Typically, the energy absorbed by a molecule or material will be dissipated as heat.¹⁸

Refraction of light by the skin has to do with the change in the path of the light when it is transferred from one media with a certain refractive index to another. The change in the path of the light occurs as a result of the change in the wave's phase velocity at the boundary between the different media.

Scattering is the tendency of light to deviate from a straight path caused by non-uniformities within the medium through which it is passing. In scattering, the light is deviated in such a way that can be described by a probability distribution and rarely follows a single angular projection as can be predicted by the law of reflection.¹⁶

Section 2.4 Fluorescence

A certain class of molecules exists that when illuminated by light of a specific wavelength will momentarily absorb the light and then emits a different wavelength of light in a process called fluorescence. For fluorescence to occur in a molecule a specific wavelength of light must be incident on the molecule and, if its structural configuration is susceptible to fluorescence, it may absorb the incident light and emit light of a different wavelength. At ordinary temperatures most molecules are in their lowest energy state, the ground state. However, they may absorb a photon of light (for example, ultraviolet laser

light) that increases their energy causing an electron to jump to a higher-energy excited state. Typically, the molecule will quickly dissipate some of the absorbed energy through collisions with surrounding molecules causing the electron to drop back to a lower energy level. However, if the surrounding molecules are not able to accept the energy needed to lower the molecule to its

Fig. 6 The Jablonski Energy Diagram¹⁹

ground state, it may undergo spontaneous emission, thereby losing the remaining energy, by emitting light of a longer wavelength.¹⁸ The generation of light by a molecule stimulated through the absorption of light of a different wavelength is termed photoluminescence, which is formally divided into two categories, fluorescence and phosphorescence, depending on electronic configuration and emission pathway (phosphorescent emission occurs more gradually than fluorescent emission due to differing excited states and the probability of returning to the ground state from an excited state). Fluorescence is a characteristic of various molecules to emit light from electronically excited states created by physical (absorption of light), mechanical (friction), or chemical mechanism (combination of fluorescent compounds). The various energy levels involved in the absorption and emission of light by a fluorescent molecule are shown in the Jablonski Energy Diagram. Fluorescence is a natural feature of many

proteins and enzymes of the human dermis and epidermis including collagen, elastin, NADH, melanin and even DNA, as a result of the structure and configuration of these molecules. An ultraviolet wavelength, 100-400 nm, can be used to excite these molecules to elicit fluorescence emission in the range of 400-425 nm. Because the energy associated with fluorescence emission transitions is typically less than that of absorption, the resulting emitted photons have less energy and are shifted to longer wavelengths, a phenomenon known as the Stokes Shift. The Stokes Shift can be measured as the difference between the maximum wavelengths of the excitation and emission spectra.¹⁹ Stokes Shift is a critical principle of confocal microscopy because it is the fluorescent emission that is filtered from the excitation wavelength by the dichromatic mirror that is used for image formation which will be discussed later.

Section 2.5 Traditional Light Microscopy Techniques

Armed with the knowledge of the interactions between light and matter and the principles of optical magnification, scientists were able to take advantage of these different interactions to generate images to study biological specimens. The first microscopes used transmitted and reflected light from a sample to greatly magnify the image of those samples and provided a novel perspective of our world at a level of detail that had not been previously possible. The earliest techniques used in these traditional microscopes (i.e. brightfield) involved simply passing light through or reflecting it off of a sample and viewing the magnified image through a pair of oculars. As the scientific

community gained more understanding of the behavior of light, more advanced techniques were developed including phase contrast and differential interference microscopy which provided added contrast and clarity to the final images. The next section explains in more detail the different techniques involved in both transmitted and reflected microscopy.

Section 2.5.1 Transmitted Light Microscopy Techniques

Transmitted light microscopy is the general term used for any type of microscopy where light is transmitted from a source on the opposite side of a specimen from the objective. Usually the light is passed through a condenser to focus it on the specimen to obtain a very strong point of illumination. After the light passes through the specimen, the image of the specimen goes through a lens or mirror that gathers and focuses light from the object being observed to produce a real image, also called the objective lens. The image is then enlarged and can be observed through the oculars. The wavelengths of light used to illuminate the sample are the same as those collected for image formation, as they are unaltered by the optics and processes involved in this technique. Transmitted light microscopy techniques were the first developed as the microscope was evolving and, in order to perform correctly, require a relatively translucent or optically thin sample.

The microscopy techniques that are utilized with a transmitted light path include brightfield, darkfield, phase contrast and differential interference contrast or Nomarski

microscopy. Other not as commonly used transmitted light techniques include Hoffman modulation, Varel optics, and polarization optics.²⁰

Section 2.5.1a Brightfield Microscopy

In brightfield microscopy, the sample is illuminated from one side and all scattered and unscattered light is observed from the opposite side. Brightfield microscopes are easy to use and setup and are relatively inexpensive since they require only basic equipment. This technique is used extensively in classroom settings with biology, microbiology and chemistry specimens and works especially well with a sample that contains structures that will

Fig. 7 Diagram of a Brightfield Microscope²⁰

significantly scatter and diffract the illumination light creating good contrast in the final image. A brightfield microscope can be used with either stained or unstained specimens, however, if the sample is transparent an image may not be discernable against the white field created by the illumination light.²¹ Also, light that is out of focus around the focal plane will significantly reduce the clarity of the image.

Section 2.5.1b Darkfield Microscopy

Darkfield microscopy also uses an illumination source on the opposite side of the

specimen from the observer

however, the microscope is

designed so that it will not

collect the illumination light

and only scattered light is

collected to form an image

Fig. 8 Diagram of a Darkfield Microscope²¹

creating a bright image against a dark

background. Darkfield is useful with

unstained and colorless specimens and offers high resolution, but it is limited in the imaging of colored specimens with images displaying primarily shades of black and white. The main limitation of this technique is the low light levels seen in the final image. This means that the sample must be very strongly illuminated to achieve the necessary amount of light in the final image which may be sufficient enough to cause damage to the sample. Also, converting between bright field and darkfield modes requires the switching of the condensers which requires additional hardware, can be expensive and, if converted manually, can be quite time consuming.²¹ Ideal specimens for use with darkfield microscopy include living aquatic organisms, small insects, bone, fibers, hair, bacteria and protozoa. Extra care must be used when preparing a sample for dark field microscopy since any dust or debris on the slide will scatter light and contribute to image degradation.

Section 2.5.1c Phase Contrast Microscopy

In phase contrast microscopy, the sample is illuminated by wavefronts created by a specialized condenser annulus, which is typically an opaque disc with a transparent circular ring that is positioned in the focal plane of the

condenser. The annulus creates parallel, defocused wavefronts which either pass through undeviated or are diffracted in phase by structures in the sample. The undeviated and diffracted light is collected by the condenser, separated and focused to form the final phase contrast image.²² Phase contrast yields image intensity values as a function of specimen optical path length, with dense regions, those having large path lengths, appearing darker than the background. The

specimen features with relatively low

thicknesses or small path lengths, are rendered much lighter when superimposed on the medium gray background.²³ Phase contrast microscopy is preferable to brightfield

microscopy when high magnifications, from 400 to 1000x, are needed and the specimen is colorless and has very fine detail. This characteristic of phase contrast microscopy may also be perceived as a drawback since it is really only suitable for samples that do not cause major changes in absorption which restricts this technique to only very optically thin samples. Cilia and flagella are a good example of a sample that appears as a vague

Fig. 9 Diagram of a Phase Contrast Microscope²²

outline in brightfield microscopy, but appear with sharp contrast using phase contrast microscopy. This technique is used extensively in cytology and histology and in applications where the visualization of internal cellular structures is necessary. Phase contrast microscopy is also found useful in the fields of hematology, virology, bacteriology, parasitology, paleontology, and marine biology.²²

Section 2.5.1d Differential Interference Contrast Microscopy

In differential interference contrast microscopy, polarized light is separated into two beams with perpendicular vibrations by a Wollaston prism. The two beams take slightly different paths through the sample and, as the light recombines and is collected, it is passed through a second Wollaston prism to reorient the beams to their identical vibrational state and original path difference. This design results in an image being formed corresponding to the optical density of the sample. Steep gradients in path length

Fig. 10 Diagram of a Differential Interference Contrast Microscope²³

generate excellent contrast and features in the specimen display the appearance of three-dimensional relief shading due to one side of a feature appearing bright and the other side dark, a unique characteristic of this technique. Regions having very shallow optical path

slopes, such as those observed in extended, flat specimens, produce insignificant contrast and often appear in the image at the same intensity level as the background. This technique is able to make better use of the numerical aperture of the system than with phase contrast and can be used with optical staining to provide color in the image. DIC is not suitable for thick specimens in biology, such as skin tissue, since it requires a nearly transparent sample with a fairly similar refractive index to its surroundings. The equipment used for DIC is expensive and certain specimen containers, such as culture vessels and petri dishes, cannot be used because of their effect on polarized light.²³

Section 2.5.2 Reflected Light Microscopy Techniques

Reflected microscopy techniques are considered the method of choice for samples that remain opaque at a thickness of around 30 microns or more. Since light is unable to pass through these specimens it is instead directed at the surface of the sample and eventually returned to the objective by reflection. Most modes of transmitted light illumination are possible with reflected: brightfield, darkfield, polarized light and differential interference contrast. Brightfield illumination results in absorption and diffraction of the incident light rays by the specimen which often leads to easily discernible variations in the image, from black through various shades of gray, or color if the specimen is colored. Other specimens show such subtle difference in intensity and color that their feature details are extremely difficult to discern and distinguish in brightfield reflected light microscopy. Such specimens behave like the phase specimens

that are familiar from transmitted light microscopy, and are suited for darkfield and reflected light differential interference contrast applications.²⁰ Reflected microscopy use ranges from fairly modern semiconductor applications to classical applications such as metallography, petrography and other materials research.²² Again, the light used to illuminate the sample and those collected for image formation have the same wavelength as they are unaltered by the optics and processes involved in this technique.

The basic techniques involved in transmitted and reflected microscopy require only basic equipment and very little sample preparation; however they offer low contrast in most biological samples and low apparent resolution due to the blur created by out of focus material. More advanced techniques are expensive and inflexible in biological applications since they typically require thin samples and are not suited for *in vivo* applications.²⁰

Section 2.6 Fluorescence Microscopy Techniques

Fluorescence microscopy uses incident light to excite fluorescence in a specimen which is focused at the detector by the same objective that is used for excitation. There are three main techniques employed in fluorescence microscopy; widefield, confocal and multiphoton.

Section 2.6.1 Widefield Fluorescence Microscopy

In conventional widefield microscopy, the entire specimen is bathed in light from a broadband source, and the resulting emitted fluorescence is collected by the objective to form an image. A bright source that produces the correct wavelengths for excitation of the sample is required, usually a mercury arc or xenon lamp. The key feature of fluorescence microscopy is the dichromatic mirror cube which is capable of reflecting the shorter wavelength excitation light and transmits the longer wavelength emission light. This is required because both wavelengths follow the same optical path from the sample to the dichroic mirror where the emission wavelength must be isolated to be used for image formation. After the emission light is separated, the image can be viewed directly by the eye or can be projected onto an image capture device. The nature of widefield illumination used in traditional widefield fluorescence microscopy means that the final image is plagued by secondary fluorescence occurring away from the focal region, which contributes to a high background noise signal, often obscuring important specimen details.

Fig. 11 Illumination above and below the plane of focus¹⁷

Widefield fluorescence is very useful for observation of living cells and is often used in toxicology studies where cell populations are imaged after treatments of various types of chemicals. It is also used extensively for cell culture studies where ongoing biochemical and physiological changes can be observed over time.¹⁹

Section 2.6.3 Multiphoton Fluorescence Microscopy

In multiphoton fluorescence microscopy, the sample is illuminated with a laser at a wavelength of about twice the

absorption peak, and half the frequency,

of the fluorophore being targeted. The

fluorophore will not be excited by a single

photon at this wavelength, however, if a

high-peak power, pulsed laser is used it is

possible for the fluorophore to absorb two

photons simultaneously. The multiphoton absorption is equivalent in the sum of their

energies to a single photon absorption. The laser induces fluorescence only at the point

where coherently interfering photons converge in time and space creating an

electromagnetic field that excites the chromophores within the sample. In this way,

fluorophore excitation will only occur at the point of focus eliminating the need for a

confocal aperture to spatially filter out of focus emission.³⁰ Additionally, the use of

infrared wavelength light sources allows for deeper penetration into samples than with

visible and ultraviolet wavelengths because the Rayleigh scattering of light is

proportional to the inverse fourth power of the wavelength of the excitation light.

Therefore, longer wavelength near infrared light will be scattered much less than the

shorter wavelength ultraviolet or visible light.³⁵ High photon densities are necessary in

multiphoton fluorescence to ensure a sufficient level of fluorophore excitation. In fact,

Fig. 12 The Two-Photon Jablonski Energy Diagram¹⁹

photon concentration must be approximately a million times that required for an equivalent number of single-photon absorptions. This is accomplished with high-power mode-locked pulsed lasers, which generate a significant amount of power during pulse peaks, but have an average power that is low enough not to damage the specimen. The introduction of multiphoton fluorescence microscopy provides a new alternative to confocal microscopy through selective excitation coupled to a broader range of detection choices.³⁰

The main limitations of multiphoton fluorescence microscopy are that it is only capable of fluorescence imaging because reflected light imaging is not possible and it is not suitable for imaging cells or tissues that are highly pigmented or absorb near infrared wavelengths that are typically used for this technique.

Multiphoton fluorescence microscopy encompasses most of the same applications as confocal fluorescence microscopy, but it is a particularly useful technique in applications where phototoxicity is an issue, for example embryonic studies and in vivo imaging of human skin.³²

Section 2.6.2 Laser Scanning Confocal Microscopy

In laser scanning confocal microscopy (LSCM), illumination of the sample is achieved by scanning one or more focused beams of light, usually from a laser, across the specimen. The resulting fluorescence transmission is collected by the objective and sent to the detector in the same manner as the widefield illumination mode. In conventional fluorescence microscopy, the light arising from each image point produces significant intensity within a solid cone that reaches a considerable distance above and below the plane of focus (see Figure 11). The light scattering objects that are out of focus produce undesirable light that is collected by the objective and reduces the contrast of the signal from the region in focus.

Fig. 13 Schematic of Confocal Fluorescence Microscopy System

The key differences between widefield and confocal microscopy are the use of a high-intensity monochromatic illumination source and an aperture placed confocally, at the point of focus of the detector lens, in the path of fluorescence transmission to the detector. The confocal aperture spatially filters out-of-focus light from objects not within the plane of focus.²⁵ In other words, the unwanted light that expands the apparent depth of field is eliminated and only the fluorescent and light scattering objects from within the

desired depth of field are collected for image formation. The confocal aperture (see Fig 13) in combination with a scanning apparatus means that although only a portion of the sample is in focus at any particular point in time, an “extended focus” image can be compiled from consecutive scans with a high degree of spatial resolution of features within the range of the entire scan. The aperture, which could be considered only a minor alteration to the widefield configuration, allows the ability for the system to control the depth of field of the image, the capability to collect serial optical sections and a significant reduction of background signal that typically leads to image degradation.²⁸ Also, with the ability to control the depth of field and collect serial optical sections, three-dimensional views at very high resolutions are attainable by compiling consecutive optical sections along the length of the z-axis. In order to gain additional perspective, brightfield or phase contrast images can be obtained concurrently from the transmitted portion of beam providing additional images in exact register with the fluorescence image.¹⁹

An inherent drawback that exists with the confocal fluorescence microscope system is the need for a laser illumination source which can be damaging to some tissue samples used in physiological measurements. The regions of the specimen above and below the focal plane are also exposed to intense excitation light in point scanning confocal microscopy and can suffer from photodamage and the rapid bleaching of fluorescence. Photodamage caused by high intensity light exposure has been shown to result in changes in pH²⁵, loss of plasma membrane integrity²⁵, DNA denaturation²⁶, apoptosis²⁷, and other losses of specific cell functions.²⁸ It is essential to establish a

balance between excitation illumination intensity and emission signal when attempting to produce a well-defined image while minimizing the amount of photodamage to the sample.

A broad range of applications are available to laser scanning confocal microscopy including neuroanatomy and neurophysiology, stem cell research, bioluminescent protein and photobleaching studies, DNA hybridization and morphological studies of a wide variety of cells and tissues.¹⁹

Confocal fluorescence microscopy provides the resolution and sensitivity required to be a viable screening technique for early skin cancer detection. Also, with the use of an ultraviolet laser source, this technique could be used without the need for labeling of the specimen with specialized fluorophores prior to a scan or examination which significantly increases its potential value as a screening technique. The cost of the materials required to upgrade a standard laboratory optical microscope to a confocal fluorescence microscope are appropriate for the allotted resources of this project. For these reasons, confocal microscopy will be the focus of the subsequent portion of this paper as it is employed in our custom microscope design and is the technique utilized for our experiments.

Section 2.7 The History of Confocal Microscopy

The concept of confocal microscopy was developed by Marvin Minsky in the mid-1950s when he was a postdoctoral student at Harvard University and patented in

1957. Minsky was determined to figure out how our brains were able to feel, learn and think. He believed that if he was able to image neural networks in unstained preparations of brain tissue and biological events as they occur in living systems he might discover how the components of the brain worked and what they do. Here Minsky ran into a significant hurdle, the tissue of the central nervous system is so solidly packed with interwoven parts of cells that if he was

Fig. 14 An Airy Disc pattern created by out of focus light³⁴

successful in staining all of them, opacity and scattering factors would keep him from seeing anything useful with the current microscopy techniques of the day. Unless he was able to confine each view to a thin enough plane, nothing would come out in the image except a meaningless blur. Fortunately, Minsky realized that scattered and reflected light in the specimen formed an Airy disc pattern (see Fig. 14) at the detector and that a pinhole aperture could reduce the amount of this light by orders of magnitude without reducing the focal brightness.

Minsky's research remained unnoticed for some time, due most probably to the lack of intense light sources necessary for imaging and the computer horsepower required to handle large amounts of data. However, following Minsky's work in the late 1960s, a multiple-beam confocal microscope was fabricated that utilized a spinning disk for examining unstained brain sections and ganglion cells and, in 1973, the first mechanically scanned confocal laser microscope imaged the first recognizable images of cells. During the 1990s, advances in optics and electronics allowed for more stable and powerful

lasers, very efficient scanning mirror units, fiber optics, better thin film dielectric coatings, and detectors having reduced noise characteristics. In addition, fluorophores that are designed to fluoresce when illuminated by certain laser excitation wavelengths were beginning to be synthesized. Coupled with the rapidly advancing computer processing speeds, enhanced displays, and large-volume storage technology emerging in the late 1990s, the stage was set for an explosion in the number of applications that could be targeted with laser scanning confocal microscopy.²⁴

Today, most modern commercial confocal microscopes are often considered as “completely integrated electronic systems with an optical microscope as the basis of the configuration”. The system consists of one or more electronic detectors, a computer (for image display, processing, output, and storage), and several laser systems combined with wavelength selection devices and a beam scanning assembly. In most cases, integration between the various components is so thorough that the entire confocal microscope can even be collectively referred to as “a digital or video imaging system capable of producing electronic images”. These microscopes are now being employed for routine investigations on molecules, cells, and living tissues that were not possible just a few years ago.¹⁹

Section 2.8 Components

The design of a laser scanning confocal fluorescence microscope is centered on a conventional research-level optical microscope, but its ability to stimulate and capture a

signal in the form of light and compile and process that signal into an image relies on a different set of principles and techniques than those that are used with conventional light microscopy. First, a focused light source, typically a laser, is scanned across a specimen using a moving stage or scanning optics. The scanned light excites fluorescence emission from a sample either by exciting fluorescent probes added to the sample or, if applicable, by targeting naturally occurring auto-fluorescent molecules in the sample. The fluorescent light is emitted in all directions, part of which is collected and focused into a pinhole aperture in front of the detector. Image information is gathered point-by-point with a specialized detector, a photomultiplier or CCD camera. This information is digitized for processing by the host computer, which also controls the synchronization between the motorized stage and detector. Finally, after a series of data points is acquired the image processing software of the computer combines the data into a single image or three-dimensional model.¹⁹

Section 2.8.1 Laser

Laser light is used as the excitation source for confocal microscopy applications because the light can be focused to a very fine point of illumination (about 0.2 micron) with a high intensity which is necessary to acquire well-defined pixels for a high resolution image. Also, a laser allows the user to select a specific desirable wavelength of monochromatic light that will be most beneficial for the particular application. A laser works by stimulating emission of photons of a certain wavelength that is dependent on

the energy difference between the excited and ground states of the material/s within the laser being excited. The lasing medium is typically excited by a flash lamp or electrical discharge. If the emitted photon encounters another atom with an electron in the same excited state, the original photon can induce atomic emission such that the second photon will vibrate with the same frequency and direction as the first photon. On the ends of the laser cavity is a pair of mirrors that reflect the photons back and forth through the lasing medium, stimulating other atoms to emit photons of the same wavelength and phase. One of the mirrors is half-silvered which means about half the light is reflected and half is transmitted. The half that is transmitted is the laser light that emits from the laser and is used in this application as the excitation source.³¹

Most of the laser types in existence are able to be used in laser scanning microscopy as they operate similarly with a gain medium, excitation source, and resonator and are able to achieve similar output wavelengths. However, consideration must be given to the type of laser appropriate to the application since these lasers can vary significantly in size, cost, output power, beam quality, power consumption and operating life.¹⁹ The laser type depends on the material/s being used to create the laser light which can currently be accomplished in a variety of ways. Solid state lasers use a solid substance as the lasing medium stimulated by a flash lamp or other source of light. These lasers can have an average output power of about one watt to several kilowatts and up to one gigawatt in pulsed mode operation. An example of this type of laser used in laser scanning microscopy is the Titanium-Sapphire laser which uses crystals of sapphire (Al_2O_3) doped with titanium ions as the lasing medium. Ti-sapphire lasers are in general

use in multi-photon excitation systems offering ultra-fast pulsing and wavelength tunability (700 to 1100 nm); however, these lasers are typically more expensive than other types. Gas state lasers use a gas or combination of gases as the lasing medium and are typically excited by an electrical discharge. Argon gas lasers are often used in confocal microscopy with output wavelengths between 450 and 500 nm. Semiconductor or diode lasers create laser light by electrically exciting nearly microscopic semiconductor chips with the output wavelength determined by the semiconductor materials and the device's physical structure. The energy level differences between the valence and conduction band electrons are what provide the mechanism for laser action. Diode lasers are compact, low power, relatively inexpensive, efficient and long lasting (10,000 to 50,000 hours) with proper care and maintenance.⁴⁰

Section 2.8.2 Dichromatic Mirror

The dichromatic mirror is one of the most critical components in a fluorescence microscopy filter combination because the wavelength transition, or Stokes Shift, region between reflection and transmission is often limited to around 20 to 30 nanometers. Dichromatic mirrors resemble longpass interference-type filters with multiple layers of dielectric materials and close fabrication tolerances to make it able to achieve this high level of discrimination. The dichromatic mirror is typically chosen based on the fluorophore or dye being used and the wavelength of its matching excitation source. A strong ability to discriminate will result in a sufficient amount of emission signal

reaching the detector with a limited amount of reflected or scattered excitation and noise signal. The dichromatic mirror cube is comprised of three components: a dichroic beam splitter (partial mirror), an excitation filter and a barrier filter. Specific filters are used to isolate the excitation and emission wavelengths for each fluorophore. The dichromatic mirror reflects shorter wavelengths of light and allows longer wavelengths to pass and is required because the objective acts as both the condenser lens (excitation light) and objective lens (emission light); therefore the beam splitter isolates the emitted light from the excitation wavelength. The major difference between a dichromatic mirror and a standard interference filter is that the mirror is specifically designed for reflection and transmission at defined boundary wavelengths, and must operate at a 45-degree angle with respect to the microscope and illuminator optical axes. Dichromatic mirrors are positioned with the interference coating facing the excitation light source in order to reflect short excitation wavelengths at a 90-degree angle through the microscope objective to the specimen. Since the emission signal follows the same path from the specimen to the objective as the original excitation signal, the same mirror must also act as a transmission filter to pass long wavelength fluorescence emission from the objective to the image plane.¹⁹

Section 2.8.3 Confocal Aperture

Traditional wide-field fluorescence microscope objectives focus a wide area of illumination over a large volume of the specimen and all emitted fluorescence is collected

by the objective and sent to the detector. The resulting signal contains a significant amount of background light and undesirable fluorescence signal which considerably reduces resolution and image contrast.

A confocal microscope is able to discard much of the extraneous signal collected in a wide-field fluorescence

microscopy setup by the use of a pinhole aperture which can be any variety of diameters depending on the necessary optical section thickness and wavelength of light used for the application. The aperture is placed in relation to the image plane to spatially filter undesirable light from the photomultiplier or other detector. Emission light that passes through the dichromatic mirror is focused at the confocal aperture by a lens usually called the detector lens. Out of focus fluorescence signals positioned above and below the focal plane form an Airy disk pattern when focused by the detector lens that have diameters much larger than that of the confocal aperture and, therefore, are excluded from the detected light used to form the image. The desirable signal passes through the aperture and reaches the detector relatively undiminished.¹⁹

The size of the confocal aperture is set for an optimal signal-to-noise ratio when the diameter of the aperture allows only the inner, light circle of the Airy disc diffraction pattern to pass through to the detector. The diameter of the Airy disc is dependent on the

Fig. 15 Spatial Discrimination of Light by the Pinhole Aperture³⁵

excitation wavelength (λ), the numerical aperture (NA) and magnifying factor (M) of the objective and is described by the equation;

$$D_{\text{airy}} = (2.5 * \lambda / \pi) * M/NA$$

The confocal aperture is positioned relative to the detector lens at a distance equal to the focal length of the detector lens.³⁵

Section 2.8.4 Scanner

In laser scanning confocal microscopy, the collection of several points of data must occur to create the final image of a specimen. There are several ways for this data to be collected, all of which involve the raster scanning of the excitation laser over the specimen. One way is to use two high-speed oscillating mirrors driven by galvanometer motors to translate the beam along the x- and y-axis. Most commercial systems that use galvanometer systems will have scan rates up to ~500 Hz. Video-rate image acquisition can be achieved with acousto-optic deflectors which can reach scan rates of >15 kHz. Although the speed of the scanning deflectors in either situation are very slow relative to the speed of light and the captured fluorescence emission has time to follow a linear light path along the optical axis to the detector, faster scanning can still result in a lower transmission frequency and high chromatic aberration. Another way to scan an area of a sample is to use a motorized stage with a small step size relative to the distance between

areas of interest and the different scan lines. A high quality scanning stage can achieve step increments of 0.01 micrometers. The stage is controlled by computer software, such as MATLAB or other similar software package, to control the stage and create a precise scan over the sample. This method reduces the complexity of the system by removing the need for any moving optics and computations needed to de-scan the data or to correct for flatness of field. For both methods of scanning, the scanned area is only a small fraction of the total area of the sample; however, the excitation beam is focused to an area of interest only a fraction of a micrometer in diameter offering enough room for several scan lines over the sample area to be achieved.

As each scan passes over the sample, the emitted fluorescence is collected by the objective, sent back through the dichromatic mirror and focused at the detector pinhole. Unlike the raster scanning pattern of excitation light, the fluorescence emission holds a steady position at the pinhole aperture, but fluctuates with intensity over time as the excitation beam scans over the sample.¹⁹ The detected light originating from an illuminated volume element within the specimen represents one pixel in the resulting image. As the laser scans over the plane of interest, a whole image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. This scanning method usually has low reaction latency and the scan speed can be varied. Slower scans provide a better signal-to-noise ratio, resulting in better contrast and higher resolution. Information can be collected from different focal planes by raising or lowering the microscope stage. The

computer can then generate a three-dimensional picture of the specimen by assembling a stack of these two-dimensional images from successive focal planes.³²

Section 2.8.5 Detector

After the secondary emission passes through the confocal aperture, it is directed to a specialized detector where it is collected, measured and digitized before that information is sent to the computer to be recorded and processed by the image formation software. After being filtered through the dichromatic mirror and passed through the confocal aperture, the amount of light available for image formation is significantly diminished requiring a highly sensitive photon detector to sufficiently amplify the signal enough to attain adequate contrast for a well-defined image. There are several classes of photosensitive detectors that can be used in a fluorescence microscopy setup, including photomultipliers, photodiodes, and charge coupled devices.

Photomultipliers are the most popular choice of detector in commercial microscopes and they work by creating an amplified electric charge from detected incident photons. Because of the point scanning nature of the microscope, the photomultiplier does not need to discriminate spatially between detected photons, but must respond quickly to a continuous flux of varying light intensity.¹⁹ If a photodiode detector is to be used, it will typically be an avalanche photodiode variety because this type provides the gain necessary to achieve sufficient amplification of the signal for a good contrast ratio and a well-defined image. The avalanche photodiode contains a

silicon based semiconductor with a voltage applied to the diode. When a photon is incident on the junction, a current will flow in proportion to the number of photons at the junction. These detectors are capable of gains between 500-1000, require low currents and are difficult to overload making them useful in low light level applications.¹⁹

CCDs operate by capturing light from an image projected on to a capacitor array. The capacitor array is photoactive causing an electric charge to accumulate on the capacitor proportional to the light intensity at each location. CCDs are capable of very high sensitivities making them useful in low-light applications; however, they are susceptible to high frequency interference and care must be taken to ensure that noise does not affect the final image quality.¹⁹

Section 2.9 Fluorophores

A specimen that is to be imaged that does not possess the intrinsic capability of fluorescence can still be labeled with fluorescent probes that will make it suitable for fluorescence microscopy. Fluorophores are constructed to localize in a particular region or bind with specific structures within cells and molecules providing a useful tool for monitoring dynamic progressions, such as, cellular integrity, membrane fluidity, protein transportation and several other processes. More than one fluorophore can be used at a time to label various structures within a specimen each with a different emission wavelength. Combinations of fluorophores are used to investigate neurological pathways, nuclear function, organelle detection and membrane integrity studies.¹⁹

The 2008 Nobel Peace prize in chemistry was awarded to the scientists who have pioneered and advanced the ability to replicate and manufacture the fluorescent compounds they first discovered in jellyfish to create a useful tool for fluorescence microscopy technology. The original protein, called green fluorescent protein because of its green emission when illuminated with ultraviolet light, has been engineered to produce variously colored mutations, fusion proteins and biosensors.³³

Another common fluorophore, Fluorescein, acts this way emitting green light when stimulated with blue excitation light. Fluorescein isothiocyanate (FITC) has an absorption maximum of about 495 nanometers and, when excited by a source at 488 nm, produces an emission efficiency of approximately 87 percent.³⁴

The amount of fluorescence emission in a fluorophore is limited, fluorescent compounds are unable to absorb another incident photon until they emit a lower-energy photon through the fluorescence process.

When the rate of fluorescent excitation exceeds the rate of emission decay, the molecules

become saturated and a majority of the laser energy passes through the specimen as a result. By balancing fluorophore saturation with laser light intensity levels the best signal-to-noise ratio in confocal experiments can be achieved. Also, many fluorophores can repeat the excitation and emission cycle until the molecule becomes photobleached,

Fig. 16 Illustration of Fluorophore Laser Excitation Efficiency¹⁹

resulting in the destruction of the fluorescence capability, but typically this will not occur for hundreds to thousands of cycles.¹⁹

The wavelengths of the excitation light and the color of the emitted light are material dependent; however, despite all the advances made in fluorescent dye synthesis, there is little evidence about the molecular design rules for developing new fluorophores. In combination with a limited number of excitation wavelengths, the number of fluorophores finding widespread use in confocal microscopy is very few compared to the thousands that have been discovered. Although an exact match between a particular laser line and the absorption maximum of a specific probe is not always possible, the excitation efficiency of lines near the maximum is usually sufficient to produce a level of fluorescence emission that can be readily detected (see Fig. 15).³⁴

Section 2.10 Advantages and Disadvantages

Confocal microscopy offers several advantages over conventional optical microscopy. The confocal aperture improves the signal-to-noise ratios by reducing background reflection and other extraneous signals that cause image degradation and works to improve contrast and definition. The aperture also provides the user the ability to control the depth of field which allows the capability to collect optical sections from thick specimens (50nm or more) The ability to collect serial optical sections also enables the creation of a 3D image allowing for a more dynamic sample model. Time lapse experiments may also be performed to observe the dynamics of a structure over certain

periods of time.⁴¹ Confocal microscopy lends itself well as a rapid cancer detection technology because it is non-invasive, which means there is no need for a biopsy and with an ability to control the depth of field to a certain degree, some exploration can be performed below the surface of the skin.

Many commercial and custom-built confocal *fluorescence* microscopes can be converted easily to a confocal *reflection* mode with little reconfiguration necessary. Confocal reflection microscopy requires a beam splitter in place of the dichromatic mirror because the same wavelength of light is detected as is used to excite the sample. The confocal aperture is still used to filter out of focus light and provide the control over the depth of field. A major attraction of confocal reflection microscopy for biomedical imaging is the ability to image unlabeled living tissue. Confocal reflection microscopy is usually employed in addition to fluorescence to add context to fluorescence images, which can be rather abstract when viewed in isolation especially fluorescence images which often times can consist of only a few white pixels on a black background.¹⁹

A limited number of excitation wavelengths are available with common lasers, which occur over very narrow bands and are relatively expensive to produce in the ultraviolet region. There is a harmful nature of high-intensity laser irradiation to living cells and tissues especially in the ultraviolet range, although this concern has been alleviated somewhat with the development of multiphoton microscopy. The high cost of purchasing and operating commercial multi-user confocal microscope systems ranges up to an order of magnitude higher than comparable widefield microscopes, limiting their presence in smaller laboratories. However, the recent demand for personal confocal

systems has competitively driven down the price of low-end confocal microscopes and increased the number of individual users.¹⁹ The depth into a sample that can be achieved with confocal microscopy is more limited than with multiphoton microscopy as a result of the increased Rayleigh scattering effect due to the excitation wavelengths used in confocal microscopy. This technique is also only suitable for scans of small areas ($>1 \text{ mm}^2$) due to limitations in scanning speed and the large amount of data that needs to be collected in order to produce the high resolution images characteristic of this technique.

Section 2.11 Applications

Laser scanning confocal microscopy used in biological applications relies almost entirely on fluorescence for imaging, although reflectance microscopy can also be applicable and beneficial.¹⁹ Confocal microscopy offers a high degree of sensitivity and an ability to target specific structural components and processes in living cells and tissues. Commonly, fluorescent probes are constructed and used to bind with a biological macromolecule (protein or nucleic acid) or localize within a specific structural region (nucleus or mitochondria). Also, probes can be used to monitor and explore dynamic processes, such as concentrations of ions or pH. Fluorescent dyes can also be made useful in monitoring cellular integrity (live versus dead or healthy versus cancerous). In addition, fluorescent probes have been applied to genetic mapping and chromosome analysis.³⁶

Experiments have been performed by exciting naturally occurring autofluorescent molecules within a specimen. Studies have shown that the autofluorescence of nictotinamide adenine dinucleotide (NADH) can be used to monitor the metabolic state of living tissues in various species. NADH acts as the hydrogen transferring molecule in the respiratory chain and has an excitation maximum of 365 nm and an emission maximum of 450 nm.³⁷

Fluorescence microscopy has been established as an effective technology for *in vivo* non-invasive characterization of pathological states in human tissue. It has been found that fluorophores, such as NADH and FAD, the coenzymes associated with tissue metabolism, and structural proteins, collagen and elastin, the dominant fluorophores in the structure under the epithelium, can be used as *in vivo* quantitative fluorescent biomarkers of precancerous changes.³⁸

Confocal fluorescence microscopy is a state-of-the-art technology and when the technique is performed properly the resulting images can be so astonishing they are often even considered to be artistic. Researchers who entered the Olympus BioScapes Digital Imaging Competition in 2007 put images on display

Fig. 17 The winning image from BioScapes 2007 titled "Brainbow"³⁹

to be judged on technical merit and aesthetics that represented the state of the art in light

microscopy for biological research. Images included a cross section of the striated tissue of a rat tongue, muscle fibers from a drosophila larva, the inner ear structures of a rat and chromosomes during the interphase stage of cell division. The winning image which was titled “Brainbow”, displayed each of the various neurons of a mouse brain in a different color, the very concept Marvin Minsky had envisioned when he invented the first confocal microscope. The image is not only stunning visually but also signifies the realization of Minsky’s original dream to map the wiring of the brain. The resulting mesh of color that traces individual axons within the brain offers an incredible new perspective that was impossible to visualize using earlier imaging techniques.³⁹

Chapter 3: Our Custom Microscope

Section 3.1 Custom Components

Laser scanning confocal microscopy has become an invaluable tool in medicine and biology as a result of its several advantages over conventional light microscopy. Many systems are in use today particularly in fluorescence and reflection applications which require little sample preparation and offer the capability for three dimensional representation of the sample after the compilation of serial optical sections. However, accurate and well-engineered commercial laser scanning systems are often expensive, on the order of \$250,000-\$500,000, and sometimes unaccommodating to the changing needs of modern research. Often times the multitude of operations offered by a commercial system are excessive and inflexible to experimental requirements. Alternatively, a custom-built upgrade to an existing optical research microscope can be much more affordable, \$15,000-\$30,000, and appropriate for the needs of the user. The initial purpose for this project was to build a custom laser scanning confocal fluorescence microscope as a tool to study the morphological changes that occur in engineered human skin as a result of exposure to ultraviolet light. The resources required to purchase a commercial LSCM were beyond those available for this project; however, it was within our means to design a custom microscope at a fraction of the cost of a commercial model. Our confocal system is designed to be used primarily for autofluorescence excitation

which does not require any special sample preparation before imaging and could potentially be used for *in vivo* imaging. Also, the system can be made to image in the reflected mode quickly and with very little reconfiguration necessary. As of the onset of this project there were no resources offering the capability for fluorescence microscopy available on campus; however, a commercial system was acquired by the department during the course of the project and due to various time delays, we decided to perform our experiments using the commercial Olympus system and postpone the construction of

Fig. 18 Sketch of our custom confocal microscope design

our custom confocal system.

Our custom confocal system design is centered on an Olympus BX41 research level microscope which sits on an optical mounting board supported by four vibration dampening legs. One laser source is used with this microscopy system, a 375 nm, 16mW ultraviolet diode laser with a

Fig. 19 Olympus BX41 research microscope¹⁹

circular profile (Coherent, \$9,450). A diode laser is different from a traditional laser in that coherent laser light is generated by nearly microscopic semiconductor chips with the output wavelength determined by the semiconductor material and the device's physical structure. The energy level differences between the valence and conduction band electrons are what provide the mechanism for laser action. Diode lasers are compact, low power, efficient and long lasting (10,000 to 50,000 hours) with proper care and maintenance.⁴⁰

This type of laser fit our criteria for selection with a low power, continuous output and ultraviolet wavelength. The Coherent model was chosen from a selection of other models based on the price quotes obtained from three laser manufacturing companies.

Fig. 20 Diagram of custom laser configuration

The laser sits on the optical mounting board and the beam height is raised 9 in. to the dichromatic mirror by a periscope assembly (ThorLabs, \$250). The periscope system consists of a pair of 45° mirror mounts with lockable adjustment knobs to provide a convenient way to redirect and change the elevation of the beam of incoming light. This component was selected rather than a fiber optic cable coupling assembly because we are using only one fixed laser source and the periscope assembly is considerably less expensive than the available fiber optic cable assemblies.

Fig. 21 Diagram of periscope assembly

The beam is then expanded to fill the back aperture of the objective in order to obtain complete illumination at the focal plane. The expansion of the beam is

accomplished with two plano-convex lenses (ThorLabs, \$55) with different focal lengths placed with a distance equal to the sum of their focal lengths between them. The focal lengths of our lenses are 30mm and 300mm and the beam expansion factor is equal to the ratio of these lengths.

Fig. 22 Diagram of beam expansion lenses

The custom dichromatic mirror (Chroma, \$350) is specially designed to reflect the ultraviolet excitation wavelength at our sample stage and transmit the resulting fluorescence emission to the detector. The UV laser used

Fig. 23 Diagram of dichromatic mirror assembly

in our application outputs a wavelength of 375nm and we expect the natural fluorophores of our skin samples, primarily elastin, collagen and NADH, to emit fluorescence in the range of 400-425nm. The particularly small Stokes Shift expected to occur in our application required a custom dichromatic mirror to be produced. The transmission spectrum for the dichromatic mirror is shown in Fig 25.

Fig. 24 Transmission spectrum for our custom dichromatic mirror

The scanning stage we are using for our custom system is manufactured by Prior Scientific. The H101A model motorized stepper stage (\$6,000) is capable of a travel range of 114mm x 75mm and a minimum step size of 0.04um. This component is appropriate for our application because of its ease of use and reduced cost compared to an oscillating mirror system. Although an oscillating mirror system would significantly reduce the time required to scan a sample, this was not considered a financially justifiable benefit for our application.

Fig. 25 Diagram of the scanning sample stage

The emission light is then passed through the detector lens which focuses the light at the confocal aperture creating the Airy disk pattern necessary for the spatial filtration of scattered light.

Fig. 26 Diagram of the detector lens and aperture

Our detector is a Retiga-EXI charge coupled device (Q-Imaging, \$11,205). This

Fig. 27 Diagram of the CCD camera

detector offers a high quantum efficiency and low noise electronics providing high sensitivity and sharp imaging for the weak-signal fluorescence detection demanded by our application.

Section 3.2 Alternatives

The components selected for our custom microscope design were selected based on the characteristics necessary for our application and with our financial limitations in mind. However, with an expanded budget more advanced options become available to increase the ease of use and efficacy of our design.

As an alternative to the periscope assembly, a fiber optic cable could be coupled to the laser and positioned such that the cable directs the excitation light to the dichromatic mirror. The fiber optic coupling system becomes particularly useful when using several different excitation sources because the attachment can be interchanged from source to source with little recalibration necessary. The coupling system is

expensive compared to the periscope and is currently beyond the needs of our custom setup.⁴¹

As mentioned earlier in the text, lateral scanning of the excitation beam may be performed by two oscillating mirrors driven by galvanometer motors. The galvanometer setup allows a real-time image to be displayed of the current focal plane which is helpful when locating an appropriate area to begin collecting optical sections. The galvanometer system can also perform the lateral scanning during image capture, so that the motorized stage is only responsible for z-direction movement for each optical section. This greatly reduces the time required for each scan to complete and would be beneficial to our custom system when the time and cost allows for the upgrade.⁴¹

Another method of decreasing the time to acquire an image is to use a Nipkow disc array instead of scanning a single point across the entire area of interest. The Nipkow disc works by passing light through a mask of thousands of pinholes on a spinning disc. The light travels through the pinholes to the specimen and back through the same pinhole for collection and image formation. The entire specimen can be covered several times in a single rotation allowing for speeds of up to 600 frames per second. The drawback to this technique is that only a small percentage of the excitation light makes it through the pinholes to the specimen which can lead to a weak signal and poor imaging, especially in the fluorescence mode.¹⁹

Chapter 4: Experiments and Data

Section 4.1 Purpose

After the design phase had been finalized and during the purchasing phase of the components of our custom confocal microscopy system from our selected suppliers, a commercial system was acquired by the university that provided a beneficial tool for our study of the change in skin morphology from UV radiation. The commercial system is well-engineered and efficient, equipped with three fiber-optic coupled lasers, an automated scanning stage and oscillating scanning mirror system, however, the custom system was not outfitted with an ultraviolet laser source at the time of the acquisition. Although our initial goal had been to use confocal *fluorescence* microscopy with our custom microscope for this study, in the interest of obtaining our experimental data we decided to perform our experiments in the *reflectance* mode with the commercial system and gain experience in the operation of the commercial model.

Numerous strategies have been developed over the past few decades to screen for premalignant tumors and early invasive cancers including immune and cytological techniques, circulating tumor markers and advances in diagnostic radiology. It is evident from the increasing number of advanced cancer diagnoses however, that an optimal method is still yet to be established. An intriguing possibility is to use the native cellular

reflectance and fluorescence as a means of distinguishing normal from premalignant tissue.

Fluorescence is a natural feature of many proteins and enzymes of the human dermis and epidermis including collagen, elastin, NADH, melanin and even DNA, as a result of the structure and configuration of these molecules. Reflectance occurs as a result of light rebounding from the surface of structures in the skin and can provide information about the configuration and morphology of the skin tissue. If a malignant process interferes with the normal cellular makeup of the skin tissue, the biosynthesis, composition and structure of the matrix can be extensively altered.⁴² The innate capacity of tissue to absorb, transmit and reflect light has thus led to an interest in native cellular fluorescence and reflectance as a means of distinguishing normal from premalignant tissue. The concept that qualitative and quantitative differences in cellular fluorescence and reflectance could discriminate between diseased and normal tissue has been explored since the 1950s, however, an optimal method must still be established to effectively screen for premalignant and early invasive cancers.⁴³

Exposure to solar ultraviolet radiation (UVR) is recognized as a major risk factor in the development of skin cancer. Although the relationship between the two is not well understood, evidence suggests that early childhood exposure and intermittent intense exposure rather than cumulative exposure are important factors in determining risk. The sun emits radiation across the UVR spectrum, however, UVC (100-280nm) and a major portion (70-90%) UVB (280-315nm) are blocked by the atmosphere from reaching the surface of the earth. The toxicity in the UVA and UVB regions have been considered to

result in oxidative DNA damage or in direct energy transfer to the DNA producing irregular and hazardous dimers.⁵⁰

Many studies have been conducted to examine the effects of several compounds believed to possess protective properties against UV-induced photodamage. Recently, studies were conducted to test the protective potential of soybean symbiosome extract⁴⁶, soy derived phytoestrogen compounds⁴⁷, and liposomal endonuclease⁴⁸. The studies showed the compounds were able to provide the skin protective effects against UV photodamage through various pathways, including influencing the production of oxidation dimers resulting from irradiation⁴⁷ and other DNA photo products⁴⁸, and increasing the repairing ability of DNA at the sites of UV-induced photodamage⁵⁰. At California Polytechnic University, Dr. Rafael Jimenez has developed an experimental procedure to generate liposomes from bovine milk phospholipids and has an interest in testing the protective effect of these milk phospholipid liposomes against UV-induced photodamage.

The purpose of our experiment is to effectively disrupt the morphology of a number of skin samples using irradiation by an ultraviolet lamp, simulating an intense overexposure to the UVA and UVB regions of solar ultraviolet radiation, in order to examine the extent of disruption in irradiated samples against non-irradiated skin tissue samples. Concurrently, a study examining the protective effects of milk phospholipids against UV radiation using confocal microscopy technology was performed.

Section 4.2 Methods and Materials

Section 4.2.1 Sample Preparation

Engineered skin tissue equivalents (EpiDermFT-212, Mattek Corporation, Ashland, MA) consisting of normal, human-derived epidermal keratinocytes were used for this study. These samples possess uniform

Fig. 28 Engineered tissue equivalent section image

epidermal and dermal structures and extracellular matrix comparable to normal human skin that are highly reproducible. The samples are metabolically active and have in-vivo like lipid profiles. The epidermis is approximately 100-120 microns thick while the dermis is approximately 1 mm thick. The surface area of the tissue is approximately 1 cm². Engineered samples eliminate the difficulty of obtaining tissue samples from human volunteers and the ethical problems associated with human testing. Also, the results are more applicable than animal samples due to biological differences that exist between the species.⁴⁴

Our study compared the images and data of four different categories of skin specimens at three separate time intervals for a total of 12 samples. Each sample was either; a) unexposed to UV light, b) exposed to UV light, c) fed milk phospholipids or d)

exposed to UV light and fed milk phospholipids. The samples that include the milk phospholipids were fed the phospholipids for 24 hours before being irradiated with ultraviolet light.

Section 4.2.2 Ultraviolet Irradiation

Sunlight is a part of everyday life and plays an important role in our well being and good health specifically by stimulating the synthesis of vitamin D. There are, however, several indications of the harmful effects of sunlight, in particular the high-energy ultraviolet (UVR) component. UV light is harmful to human skin in its ability to penetrate the epidermis and the dermis reaching the dermal papillae of the hair and the vascular network in the deeper layers of the dermis.⁴⁴ The UVR component itself has been subdivided into three separate categories based on wavelength, UVA (315-400nm), UVB (280-315nm), and UVC (100-280nm) with the high-energy UVC range being capable of causing the most damage to mammalian skin.⁴⁵ Fortunately, all UVC and a substantial proportion (70-90%) of UVB are prevented by the ozone layer from reaching the earth's surface. The resulting 10% UVB and 90% UVA light that reaches earth is still serious cause for concern of photodamage and cancer especially in individuals with light skin, red or blonde hair, and blue eyes. Even though the relationship of exposure to solar UVR and the risk of malignant melanoma is not well understood, there is still strong evidence that the UVA and UVB components of sunlight plays a significant part in the wrinkling and aging of skin and, in more serious cases, the shorter wave radiation of

UVB can be absorbed directly by DNA temporarily inhibiting synthesis of new DNA and creating targets for mutagenesis.⁴⁴

Sunburn is an acute inflammatory reaction that follows excessive exposure of the skin to ultraviolet radiation (UVR) and is generally classified as a superficial or first-degree burn. The minimal erythema dose (MED) for a given skin type is the approximate dosage of solar UVR required to obtain noticeable swelling and reddening of the skin in reaction to UV sun damage.⁴⁶

Skin Type	Description	MED	Skin Color
I	Always burns, never tans	200 J/m ²	White
II	Always burns, tans minimally	250 J/m ²	White
III	Burns minimally, tans slowly	350 J/m ²	White
IV	Burns minimally, tans well	450 J/m ²	Olive
V	Rarely burns, tans profusely/darkly		Brown
VI	Rarely burns, always tans		Black

Table 1 Fitzpatrick Skin Type Chart with corresponding MED⁴⁶

Our experiment was designed to irradiate our 12 skin tissue samples at three times the MED for skin type I to ensure a significant erythema response is achieved. The

changes in UV irradiated skin samples were then observed against the control samples without UV exposure.

In this study, the skin equivalent models, which were irradiated with light from a UV lamp (QPanel Industries, UV340), were done so while still in the tissue culture insert in which they were contained. The wattage of the UV lamp was determined using a power meter positioned under the lamps at five various locations and calculated as an average of the five values collected. Beforehand, a dark calibration was applied to the power meter to zero the meter and give a consistent basis for the collected values. Two sets of values were collected, one for UVA and another for UVB, and the average values were combined to find the total average wattage reaching the sample. The equation used to calculate the dose and time of exposure is;

$$\text{UV dose (J/m}^2\text{)} = [\text{lamp wattage (W)/area of lamp (m}^2\text{)}] \times \text{time exposure (s)}$$

A total dose consisting of 60 mJ/cm² of UVB exposure and an average of 1730 mJ/cm² of UVA exposure was directed at the samples. The total dosage corresponds to a desired percentage of the MED (300% MED for our experiment) which was then used to determine the amount of exposure time for each sample. The engineered skin sample containers were provided with a clear plastic protective cap to keep dust and other particles from reaching the samples. The dosage calculations were performed for both capped and uncapped sample configurations.

Section 4.2.3 Imaging

Prior to imaging, each skin sample was removed from the tissue culture insert and placed between a microscope slide and a coverslip. A secure seal gasket (Invitrogen, C-18136) was used as a spacer to prevent an excessive amount of pressure on the sample against the slide. The samples were oriented with the surface to be imaged, the outer layer of the epidermis, against the coverslip.

The images for this experiment were taken using a commercial version Olympus IX81 FluoView 1000 laser scanning confocal microscope 40X, 1.30 N.A. oil objective. This microscope is inverted, motorized and fully automated. It has three fiber-optic coupled lasers, two galvanometer scanning mirrors for x-y-

Fig. 30 Olympus IX81 FluoView 1000

scanning, a motorized stage and a photomultiplier detector. An effective lateral resolution of 0.15 μm and effective axial resolution of 0.58 μm can be expected from this setup. Images were taken using confocal reflection microscopy because the commercial system was not equipped with the necessary laser source in the ultraviolet range. Typically, confocal reflection microscopy can be utilized, in addition to fluorescence, to gather

additional information from a specimen with relatively little extra effort, since the technique requires minimum specimen preparation and instrument re-configuration. Reflection microscopy can add context to fluorescence images, which can be rather abstract when viewed in isolation especially confocal fluorescence images. In addition, information from unstained tissues is readily available with confocal reflection microscopy.

Two different wavelengths of laser light were used in this experiment; Channel 1 (CHS1) and Channel 2 (CHS2) have excitation wavelengths of 405 nm and 488 nm, respectively. The shorter wavelength laser yields stronger reflection intensities while the longer wavelength is able to penetrate deeper into the skin samples. Also, the dual-channel setup helps to obtain a well defined image and compensate for overexposure in the case that one wavelength begins to over saturate the detector at the given sensitivity level. The graphs of the results illustrate the difference in the values of reflected intensity collected between the two channels.

In order to obtain our images, the microscope was directed at a suitable area of the sample and focused to a depth sufficiently below the surface of the skin so that no reflection was yielded due to opacity and scattering. The sample was then scanned at a resolution of 0.621 μ m/pixel in both the x- and y- plane at a sampling speed of 10 μ s/pixel. The resulting image size is approximately 955 μ m in the x-plane by 955 μ m in the y-plane. To create a series of sections, the microscope was refocused after each x,y-scan by the microscope software to a distance of 2 μ m above the previous scan. Each image stack consisted of about 40-45 optical sections and a total of nine image stacks were captured

in a 3x3 grid pattern which were stitched together at the edges in order to obtain a sufficient representative area of each sample.

Section 4.3 Results

Figure 30 displays representative images of each of the four different categories of the samples tested. These images are all of the stratum spinosum layer located approximately 60-70 microns deep into the tissue at the 0hr time interval. Figure 30b, UV 0hr, shows a significant increase in reflected intensity from the 0hr control image, Figure 30a. Figure 30b reveals what appear to be the nuclei of keratinocytes, the cells that make up 95% of the epidermis, which are not as pronounced as in the other categories, Figure 30a, 30c, and 30d. A comparison of the UV exposed and control samples suggest that the cells have been photodamaged and the nuclei, and possibly the DNA within, have been disrupted and altered in such a way that resulted in increased reflection.

30a)

30b)

30c)

30d)

Fig 30 Images of the 0hr sample group; a) Control b) UV c) Milk and UV and d) Milk

The graphs of our results show the average reflected intensity for each category of sample at their respective time interval as a function of depth into the skin sample. The average reflected intensity of

Fig 31 0hr sample group results for Channel 1

each region of interest was obtained by calculating the sum of the reflected intensity from each pixel in that region. We were able to calculate the average intensity of each 3x3 slide by summing the nine areas of interest and dividing by nine. This calculation was applied to every slide for each image stack and

Fig 32 0hr sample group results for Channel 2

then plotted as a function of the depth into the sample. This process was completed for both Channel 1 and Channel 2 results.

The quantitative results show a significant disparity in the maximum reflected intensity between the 0hr and the other sample categories. This result is especially apparent in the graph of Channel 2 which captured the maximum average intensity for all sample categories. Figure 32 shows an increased reflected intensity in the UV irradiated

sample compared to the other three sample categories whose maximums occurred at relatively similar values. This result adds evidence that unprotected UV exposure elicits morphological and configurational changes within the skin cells that lead to an increase in reflected intensity.

The results for the samples exposed to both milk phospholipids and UV irradiation show maximum intensity values similar to that of the non-irradiated sample categories, the control and milk exposed samples, at all three time intervals. This suggests a protective effect of the milk phospholipids against UV irradiation on the skin. It is also interesting to note the return of the UV exposed reflected intensity to normal levels in the 24hr and 48hr time intervals as we would expect the healing process in the cells to occur after sunburn inducing UV overexposure to return the cell back to a normal healthy state.

33a)

33b)

33c)

33d)

Fig 33 Images of the 24hr sample group; a) Control b) UV c) Milk and UV and d) Milk

Figure 33 shows images from the 24hr sample group with 33a) displaying only the CH2 wavelength due to saturation in the Channel 1 results. Figure 33b) shows a return to normal levels of reflection in the epidermal keratinocytes most probably due to a repair mechanism that has occurred within the cells similar to that which would occur in normal human skin following overexposure to solar radiation.

Fig 34 24hr sample group results for Channel 1

Fig 35 24hr sample group results for Channel 2

The spots that were visible Fig. 30b) are less apparent in Fig. 33b) indicating the repair may have been centralized in the nuclei of the keratinocytes and possibly could be attributed to the DNA within the nuclei. The black and red area in the bottom right hand corner of figure 33c) is most likely due to an air bubble between the sample and coverslip and should not be considered as an abnormality within the sample.

The graphs of the 24hr group confirm a return to normal averages of reflected intensity with the maximum peak of the UV light exposed group occurring at relatively the same levels as the other sample groups.

With a longer exposure to UV light more thymine dimers would be expected to form in the DNA and, as a result, the risk of an incorrect or missed repair would increase.⁴⁹ It can be inferred that not all of the damaged DNA has been successfully repaired and in the case of numerous successive exposures there would appear an increased number of dimers increasing the magnitude of reflected intensity.

36a)

36b)

36c)

36d)

Fig 36 Images of the 48hr sample group; a) Control b) UV c) Milk and UV and d) Milk

The graphs of reflected intensity for the 48hr sample group also show a return to consistent levels between the various test categories. Although the maximum values occur off of the graph for the MilkUV, Milk and UV test categories it is reasonable to infer that they reside at relatively similar values based on the proximity and similarity of their respective slopes.

Fig 37 48hr sample group results for Channel 1

Fig 38 48hr sample group results for Channel 2

The images of the 0hr UV irradiated samples display a significant increase in reflected intensity from localized areas within the sample that can be easily identified by eye. It is useful to quantify and compare the number of apparent nuclei with increased reflection by counting the visible nuclei from a well-defined image of each sample group. A comparison of the apparent number of nuclei displaying increased reflection from select images of each sample class and time period is shown in Figure 39. The graph shows a definitive alteration in the reflection of UV irradiated nuclei as compared to the other sample groups. The repair of UV damage is also illustrated by the return of reflection values in the UV irradiated cells to baseline values in the 24hr and 48hr groups.

Number of Apparent Nuclei Showing Increased Reflection

Fig 39 Graph of Apparent Nuclei Showing Increased Reflection

Section 4.4 Conclusions

It is known that solar ultraviolet light can penetrate and damage the skin resulting in swelling and cell death.

Ultraviolet light can cause mutations in skin cells by

altering the DNA within the nuclei of the cells in such a way that the damage goes

Fig 40 Dimer formation between two Thymins in a DNA molecule⁵⁰

unrepaired while the DNA continues transcribe and multiply resulting in new, mutated skin cells. The most common DNA photoproduct is called a thymine dimer formed when UV light induces a reaction between two molecules of thymine, one of the bases that make up DNA.⁵⁰ Thymine-DNA glycosylase acts as a DNA repair enzyme which associates with the tumor suppressor gene family and has been shown to directly alter suppression activity.⁵¹ An alteration in thymine configuration within the cell DNA at the molecular level could result in the increased reflection intensity seen in our UV exposed samples at the cellular level and may represent a very early step in skin carcinogenesis that can be detected using LSCM technology.⁵⁰

In our images captured by confocal reflectance scanning, the engineered skin samples displayed an increase in reflected light intensity in what appear to be the nuclei of keratinocyte skin cells for the UV exposed sample group. The graph of the 0hr test

group confirms an increase of reflected light intensity in the UV irradiated samples.

These results support our ability to induce morphological and configurational changes in engineered skin tissue samples using 90% UVA and 10% UVA irradiation and, also, the ability to detect the occurrence of these changes with the use of confocal reflectance microscopy. In the clinical setting, an increase in reflection of a patient's tissue *in-vivo* could signify areas of significant photodamage and indicate the onset of malignant skin carcinoma.

The engineered skin samples behaved as would be expected in normal mammalian skin in the 24hr and 48hr sample groups with the UV exposed samples making a marked return to normal levels of reflected light intensity suggesting a repair mechanism occurring within those cells. Further detailed studies are necessary to verify these results and determine the origin and mechanisms involved in the increase of reflected intensity arising from what are believed to be the nuclei of keratinocyte skin cells and also to determine if this alteration can be utilized as a marker for photoaging and early skin carcinoma detection.

The study involving the protection of skin cells from UV photodamage by milk phospholipids also provided encouraging results. Although the mechanisms involved in this process are also not very well understood, this study demonstrated the potential of the milk phospholipids as a protective agent against the harmful effects of ultraviolet radiation. In the 0hr test group, the MilkUV sample returned only slightly greater intensity values than the Milk and Control samples in the same group indicating that the keratinocyte nuclei were protected by the milk phospholipids from UV damage. This

result is especially apparent in the graph of the Channel 2 readings. The milk phospholipids appear to provide a protective effect rather than a reparative effect since the difference between the reflected intensities were apparent immediately in the 0hr results rather than an accelerated return to baseline intensity values in the 24hr and 48hr sample groups. Again, additional tests would have to be performed in order to confirm these results.

Section 4.5 Future Work

The experiments performed in this study provide fundamental support for the use of laser scanning confocal microscopy as an early skin cancer detection technology. Additional experiments using larger sample groups need to be performed in order to verify the results of our initial experiments and provide a sufficient amount data to demonstrate the value of LSCM as an early detection technology in the clinical setting. As well as additional experiments, it would be beneficial to complete the assembly and calibration of the custom confocal microscope system and perform experiments using confocal fluorescence microscopy which can be used to elicit chemical composition information from naturally occurring fluorophores in the skin. In a future study, confocal fluorescence microscopy could be used to detect selectively stained thymine dimers in UV irradiated samples. The results could then be used to validate our conclusion that dimers were formed as a result of UV exposure resulting in an increase of reflection in our confocal reflectance microscopy study.

In order to better understand the mechanisms behind the protection of the skin cells from UV damage by milk phospholipids, there is currently work in progress studying histology results of these samples. Also, electrophoresis and MTT assays are being performed to detect DNA damage and other metabolic dysfunctions. These studies will add data to further verify our conclusions as well as provide more insight into the mechanisms that occur resulting in the reflection differences between the protected and unprotected sample groups that we observed in our experiments.

The relationship between UV overexposure and the formation of a malignant carcinoma is recognized however it is not well understood. A malignant tumor can occur if UV-induced mutations occur in genes involved with several different signaling pathways including, cell cycle control, proliferation, apoptosis, and/or DNA repair. The fact that cancer does not occur immediately after UV exposure but within a period of years supports the theory that a combination of multiple alterations and mutations are necessary to initiate malignant carcinogenesis. Also, UV radiation has an immunosuppressive effect which can allow DNA damage and tumor formation to go unrecognized and unrepaired.

Although the mechanisms and process involved in the formation of skin cancer are not well understood, many of the disrupted functions and alterations that occur as a result of UV irradiation, which may be representative of the early stages of skin carcinogenesis, can be readily detected and quantified. Mutations caused by UV exposure can be detected in pathogenically important tumor suppressor genes from basal cell and squamous cell type carcinomas in human skin.⁵² Also, thymine dimers in mutated

keratinocyte clones are often found adjacent to BCCs and SCCs and might be an early indication of skin carcinogenesis.⁵³ It would be interesting to attempt to detect these alterations in carcinoma affected skin tissues using LSCM in both the fluorescence and reflectance mode. The high resolution and specificity of this technique could provide the desired ability to detect and locate these mutations early in the carcinogenesis process allowing for earlier and more accurate diagnosis and treatment.

Appendix

Table 2 Custom Confocal Microscope – Bill of Materials

Laser	1 Cube 375nm 16mW with heat sink	Coherent	1112774	\$9,392.50
Dichroic Mirror	1 UV reflection with longpass transmission	Chroma	ZQ375RDC	\$325.00
Mirror	2 UV range protected aluminum coated mirror	ThorLabs	PF10-03-F01	\$47.90
Periscope	1 Complete Periscope Assembly	ThorLabs	RS99	\$246.00
Riser	1 10 in. Riser Post (1" diameter)	ThorLabs	RS6	\$29.00
Pinhole Aperature	1 30 um diam. Pinhole aperature	ThorLabs	P30S	\$59.30
Lens Tube	2 2" Lens Tube 1" diam.	ThorLabs	SM1L20	\$17.10
	4 3" Lens Tube 1" diam.	ThorLabs	SM1L30	\$26.00
Adjustable Lens Tube	1 1" travel	ThorLabs	SM1V10	\$32.60
Spanner Wrench	1 for 1" SM1 Lens Tube	ThorLabs	SPW602	\$28.40
Retaining Ring	10 SM1 1" diam. Lens tube compatible	ThorLabs	SM1RR	\$5.30
Cage Plate	1 SM1 0.35" thick	ThorLabs	CP02	\$15.63
Cage Cube	1 30mm Cube	ThorLabs	C4W	\$57.00
Cover Plate	2 for C4W	ThorLabs	B1C	\$15.00
Cage Rod	8 6" Cage Rod	ThorLabs	ER6	\$8.27
CCD camera	1 Retiga-EXI	Q-Imaging	Retiga-EXI	\$11,205.00
Beam expand Lens	1 Plano-Convex Lens, d = 25.40mm, f = 30mm	ThorLabs	LA1805-A	\$30.15
Beam expand Lens	1 Plano-Convex Lens, d = 25.40mm, f = 300mm	ThorLabs	LA1484-A	\$26.75
Detector Lens	1 Bi-Convex, d=25.40mm, f=30mm	ThorLabs	LB1757	\$23.30
Scanning Stage	1 Motorized Stepper Stage	Prior Sci	H101A	\$6,000.00
Total :				\$27,853.79

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