A Comparison of Optical Microscopy Modalities for Non-Invasive In Vivo Biopsy of Skin

By Will Goth

The prevalence of skin cancers in modern society necessitates the development of a less invasive form of biopsy for diagnosis. Optical microscopy modalities can provide an entirely non-invasive form of virtual biopsy through which suspected cancerous lesions may be diagnosed. Confocal laser scanning microscopy, two-photon fluorescence microscopy, and optical coherence tomography have been developed to a point where they have appropriate contrast, resolution, imaging depth, and scanning rates to replace traditional biopsy and histology in the near future. These characteristics, along with barriers to implementing a new biopsy method, are discussed as a prelude to future conversations in clinical testing.

Keywords: Confocal microscopy, two-photon fluorescence, optical coherence tomography, biopsy, in vivo, skin cancer

I. Introduction

Skin cancer is the most common form of cancer in the United States, with one in five Americans having a probability of developing skin cancer at some point in their lifetime [1, 2]. Despite its prevalence it has one of the lowest mortality rates among cancers, and early diagnosis and treatment results in very few fatalities [3]. However, to determine if further action must be taken on a suspected malignant lesion, current medical protocols call for an invasive biopsy to excise a sample of the lesion and examine it pathologically [4]. This can cause considerable pain, scarring, and disfiguration in the area, especially on sensitive areas such as the face and neck that are particularly susceptible to tissue damage from sun exposure [5].

An alternative to the current method involves using biomedical imaging to diagnose skin lesions. Many current subsurface sensing and imaging techniques, such as ultrasound, magnetic resonance imaging (MRI), and x-ray, are unsuitable for skin imaging due to either low resolution or inability to differentiate any contrast between epidermal constituents. Alternatively, optical microscopy can produce high resolution, high contrast images of skin. Furthermore, due to skin's translucent properties, three-dimensional imaging is achievable to a limited depth. More recent advances in image acquisition using laser light sources, precise lenses, software processing, and new scanning techniques have helped allow several different methods of optically based microscopy with high resolution and coherent signal depth capable of resolving malignant tumors in the epidermis. Confocal laser scanning microscopy (CLSM), optical coherence tomography (OCT), and two-photon fluorescence microscopy (TPFM) are leading methods of diagnosing skin lesions *in vivo* without removing any tissue. This paper will examine these techniques and discuss their respective advantages and limitations in clinical settings.

II. Types of Optical Microscopy

Confocal Laser-Scanning Microscopy

Using a confocal (conjugate-focal) technique for microscopy was first described by Minsky in 1957 [6]. In its rudimentary form, confocal microscopy consists of focusing a light source onto a very small point on a specimen using mirrors and lenses (Figure 1).

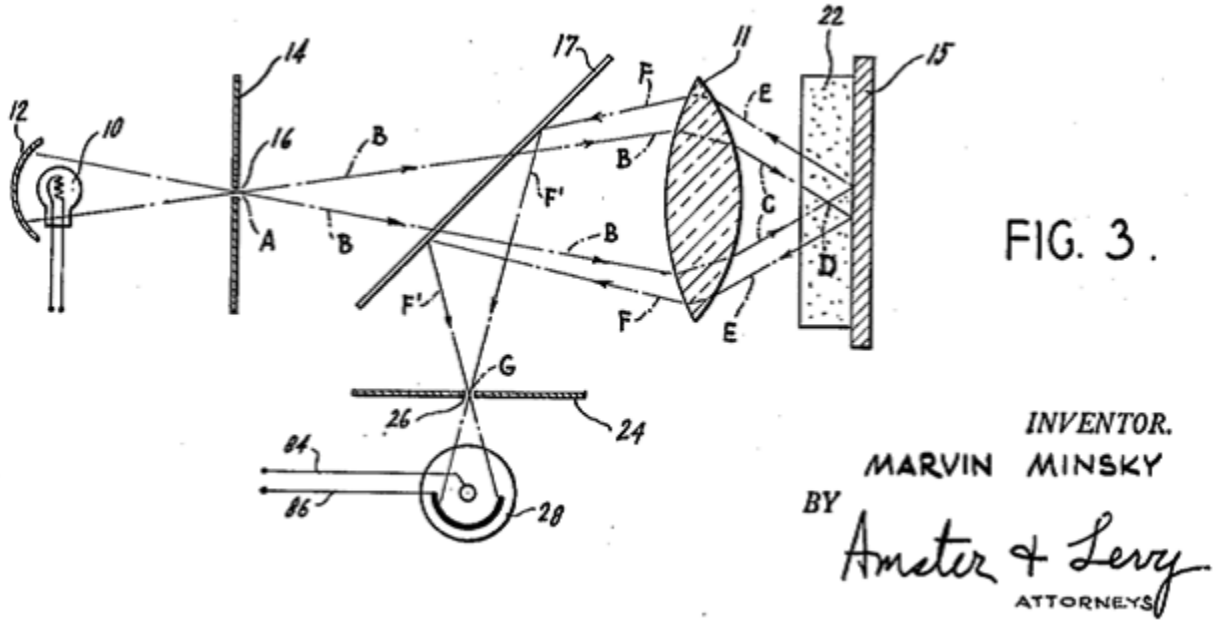

Figure 1 – Original patent drawing of Minsky's confocal microscope design [6]

A point light source propagates first through a polarizing beam splitter, causing the light to be "P" polarized. The beam splitter also contains a special mirror that transmits certain polarities of light while reflecting others at a 90-degree angle. This is necessary to differentiate between the backscattered light from the sample and any other incident light that propagates to a receiver. The light continues through a convex objective lens placed just before the sample, which causes the light to be focused to a single point at a fixed focal distance, which is determined by the lens' geometry. The plane within the sample that is conjugate—the same distance—as this focal point will reflect back extremely focused light, while planes further from this focal distance reflect back more scattered light [7].

Reflected light then returns through the objective lens and back through the beam splitter, which now causes "S" polarization and reflects the light through a pinhole. The pinhole filters any more out of focus light while only allowing the light reflected from the focal plane to be passed through. The remaining light carries discreet, very focused information about the point that was scanned, creating a pixel. However, the resulting information is returned only for this one pixel on the specimen, so a large amount of pixels must be obtained through scanning methods [8]. The size of the individual pixels determines the overall lateral resolution of the image. Multiple focal planes may then be scanned axially by moving either the objective lens or the sample,

resulting in optical sectioning of the object and constituting the information needed to form a 3D image of the object. The axial resolution of the image is controlled by how far apart planes are scanned axially through the depth of the sample.

With the advent of lasers acting as extremely focused light sources, computer aided data analysis, and new and improved techniques of rapid scanning [9], confocal microscopy has become one of the most widely used methods for biological study due to its high levels of resolution and its ability to section a sample in 3D. It also has the capability of capturing the fluorescence in skin samples by simply altering the light source and receiving device, allowing further contrast resolution between particularly fluorescent tissue, like melanin and collagen [10]

Two-photon Fluorescence Microscopy

Though two-photon fluorescence was originally described by Goppert-Mayer in 1931, it was impossible to be observed or confirmed until the appearance of lasers in the 1960s [11]. Typical fluorescence involves a material's tendency to excite to a higher electronic state when it absorbs a single photon of high energy from a light source. As the molecule returns to its original state, it emits a less energetic photon which propagates with a longer wavelength than the light with which it was excited due to vibrational losses. This can be observed when ultraviolet light, invisible to the human eye due to its short wavelength, causes certain materials to fluoresce and glow with visible, longer wavelength light. However, the light source is indiscriminate in the molecules which it fluoresces, exciting all subjected molecules in a process known as photobleaching. In order to achieve any meaningful information about an object's structure, the fluorescence mechanism must affect only a very small area at a single time. [11-13]

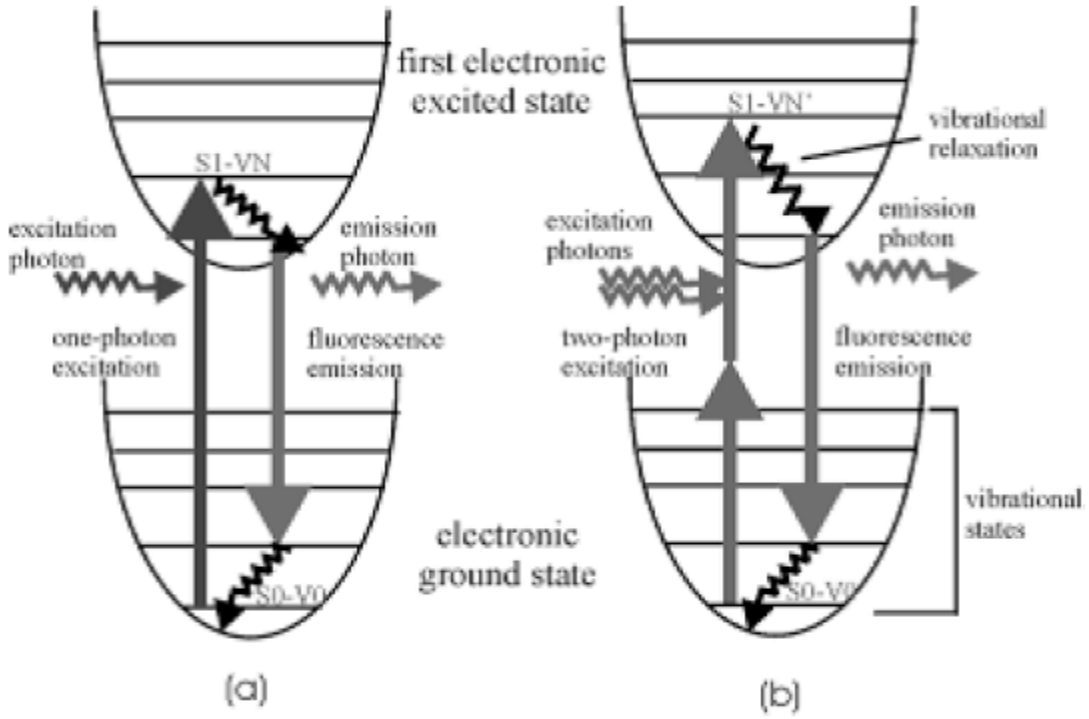

Figure 2 – (a) Single photon fluorescence of a molecule and (b) two-photon fluorescence. Note that the arrow length denotes photon energy, not wavelength. Fluorescence emission has less than the total photon excitation, so that when two less energetic photons are used to excite the molecule, a higher energy photon is released. This allows a high-response signal differentiation from any reflected photons from the original light source, and fluorescence of a finite area in which two photons may be absorbed. [11]

Two-photon excitation solves this problem by fluorescing a molecule using two photons instead of one. Since excitation can only be achieved where two photons are present, the resulting fluorescence can be focused to an extremely small area using an objective lens. Furthermore, photobleaching is minimized due to the fact that the incidence of two-photon fluorescence falls off with the fourth root of the distance from the focal point of the objective, meaning only molecules very close to the focal point are fluoresced [12]. The resulting energy of the emitted photon is less than the total energy of the two absorbed photons combined but greater than the photons' individual energies. This results in a shorter wavelength than the initial wavelength of the pulsed light, allowing differentiation that single-photon fluorescence does not while also boosting the signal strength. Optical sectioning can then be achieved using scanning methods similar to those used in CLSM [14-15].

Optical Coherence Tomography

Optical coherence tomography is an imaging technique that uses the principles of interferometry to achieve tomographic imaging similar to that in ultrasound and CT [16]. In contrast to CLSM and TPFM, tomographic imaging obtains instantaneous axial data and then is sectioned laterally.

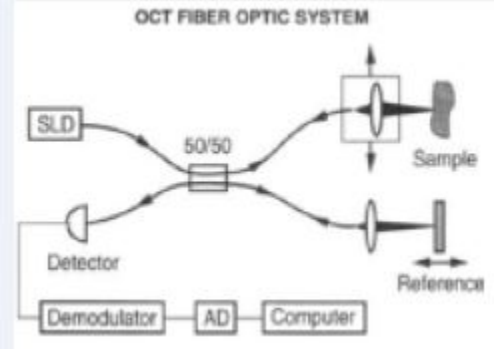

Figure 3 – Basic setup of an OCT System using a fiber-optic light path [17]

In OCT, a laser source is pulsed through a 50/50 beam splitter that transmits half of the light and reflects the other half. One of the resulting beams is directed at a tissue sample, while the other is directed at a reference mirror. The light which is directed at the mirror simply propagates directly back to the beam splitter, with no time delay. The light directed towards the sample, however, has a time delay due to the optical properties of the sample. Passing through a translucent medium such as skin causes a lag in the light propagation, and this information is included in reflected wave. The two light waves are then recombined at the beam splitter, and a resultant light wave is a superposition of the two incident beams. [17]

Interference between the two waves will be apparent where the superimposed signal is amplified or nullified and is indicative of the waves being in phase (amplification) or out of phase (nullification). The phase difference therefore provides information about how long the light took to travel into the sample and to be reflected, and from this, information about the microstructure of the sample is obtained through computational analysis. [16-19]

III. Considerations for Use

Different physical and practical limitations are inherent to each modality. Table (1) compiles a list of comparable characteristics along with further discussion of each in this section.

Contrast Mechanism

Tissue contrast is a hugely important factor when considering possible biopsy replacements, and techniques must at least show equivalency to current staining methods of excised samples to produce contrast. In CLSM, contrast in the tissue is obtained through the refractive differences within the sample. This contrast is comparable if not better than traditional biopsy contrast [20]. TPFM produces the most remarkable contrast of all of the discussed methods through its specific fluorescence of materials by use of carefully selected wavelengths [21, 22]. OCT has lower contrast than both CLSM and TPFM, but is still comparable to traditional biopsy [16].

Resolution

There are two aspects of resolution which are accounted for in optical microscopy: lateral resolution within the x-y field of view and axial resolution along the z-axis of propagation. Both CLSM and TPFM have axial resolution which simply relies on the precision with which their objective lenses are moved towards and away from the sample during optical sectioning. Nanomovers coupled with objective lenses allow very fine axial resolution of a micron or less, much better than conventional biopsy allows [9, 18]. OCT has a lower axial resolution due to the fact that it scans all z-axis points instantaneously, meaning the only limiting factor is the pulse rate of the interferometer [17].

Lateral resolution is again very high in CLSM and TPFM, capable of resolving all micron level tissue structure. OCT lags again in this setting due to the difficulty of its lateral sectioning.

	CLSM	TPFM	OCT
Contrast Mechanism	*Refractive-index variations, especially melanin*	*Individual molecular fluorescence, high-order n-photon excitations*	*Molecular polarization, numerical time shifting, spectral absorption*
Resolution:			
Lateral	*0.3 µm*	*0.2 µm*	*2-10 µm*
Axial	*1.0 µm*	*0.5 µm*	*2-4 µm (pulsed source)* *5-10 µm (typical diode source)*
Field of View	*200 x 200 µm*	*<200 x 200 µm*	*500 x 500 µm*
Maximum imaging depth	*250 µm (tongue)* *350 µm (skin)* *450 µm (lip)*	*>500 µm*	*2 cm (eye)* *1-2mm (dermal tissue)*
Scanning rate	*30 Hz*	*~20 Hz*	*>1 kHz*
Photodamage	*None at visible and near IR*	*EDIT*	*None*

Table 1 – Comparable modality characteristics [9-11, 13-14, 17-18, 20-24]

Field of View

Field of view, while relatively unimportant alone, is key in improving capture rate of images. If a large field of view can be obtained at high resolution, then less scanning samples are needed to be taken. However, FoVs necessitate either longer scanning time at high resolutions or lower resolution at high scanning times, so they are generally kept within three orders of magnitude of the lateral resolution. OCT has a notably larger FoV due to its lower resolution and higher scanning rate.

Maximum Imaging Depth

Maximum imaging depth is another important factor in virtual biopsy. While some sources say the 350 microns which CLSM can penetrate to is acceptable for skin cancer detection, the epidermis can reach thickness of up to 1.5mm. However, this is usually on the ‘padded’ areas of the body such as the soles of feet. OCT shows a remarkable ability to image very deep into

scattering translucent materials such as skin, and can even penetrate layers on the scale of centimeters in highly uniform tissue, such as in the cornea.

Scanning Rate

Scanning rates of each device are important not only to speed image acquisition, but also to provide means of video rate imaging of at least 30 Hz. TPFM is just behind CLSM in achieving video rate imaging [9, 14, 22]. OCT, however, has much higher scanning rates due to the fact that it does not need to section the sample axially. The 1 kHz is exaggerated somewhat as it does not take into account the lateral sweeps that OCT scanning must complete to produce tomography in three dimensions, but even so, OCT produces much higher image acquisition rates.

Photodamage

While CLSM and OCT have no reported photodamage under normal use due to low-energy photon flux, TPFM requires a very high energy, highly concentrated flux of photons focused on a small area of skin. Because of this, photodamage of the imaged cells can occur if proper care is not taken [11, 24]. This would negate the point of a non-invasive biopsy and could also lead to complications in causing, rather than detecting, cancerous lesions.

IV. Discussion

A problem with using any method of optical microscopy to replace biopsy and histology is twofold. While excellent in imaging skin, these new scanning methods have not yet been widely used in clinical settings due to a lack of protocol for interpreting the results. Pathologists have studied tissue using traditional techniques of excision, preparation, and examination (often with traditional ‘wide-field’ microscopes) of excised tissue for centuries, and there is an established guideline for assessing the excised samples [4,20]. An established methodology simply does not yet exist in the new methods of optical microscopy, and pathologists performing the biopsies would need to be retrained fundamentally in interpreting the captured images, which often lack the level of contrast found in traditionally prepared samples. This also leads to a second problem: that both patients and doctors are hesitant to trust the results of new technology. While optical microscopy provides means of avoiding the pain and scarring involved in traditional skin biopsy, it does not yet have a solid history of successful prognosis and treatment. Traditional biopsy has a much larger history of use from which to draw statistical conclusion about its effectiveness, while the new technologies by definition do not have past performance benchmarks. Therefore, sufficient clinical trials must be run using these methods of microscopy as an adjunct screening tool to invasive biopsy and show comparable results.

Acknowledgements

I would like to thank Yair J Mega and Professor Charles W DiMarzio for consultation on imaging modalities, as well as Albert Gelbaum and Amy Doyle for draft review comments and suggestions.

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