NON-LINEAR MICROSCOPY AND ITS APPLICATIONS FOR THE STUDY OF THE STRUCTURE AND DYNAMICS OF BIOLOGICAL SYSTEMS.

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This paper is aimed at introducing non-linear microscopy and some of the applications that this technique has been applied to in investigating biological phenomenon. The article gives a broad overview of how some non-linear processes are generated and how this can be used to extract information from biological systems and processes.

There has been a recent increase in the use of light microscopy in the study of biological systems. This is due to the fact that optical microscopy is one of the very few techniques by which live cells can be studied giving high spatial resolution. These techniques also enable scientists to study sub-surface and intra-cellular processes. Confocal microscopy over recent years has become a powerful tool for investigating live cells and providing high resolution images. A limitation of confocal microscopy is that the laser energy required for optimal resolutions the can produce cell damage (Eisenstein 2006).

In recent years new optical microscopy techniques have been developed which are referred to as non-linear microscopy. This group of techniques include multiphoton-excitation fluorescence (MPF) (Denk et al.1990), second harmonic generation (SHG) (Sheppard 1997) and third harmonic generation (THG) (Barad et al 1997). Non-linear microscopy really became an established technique with the development of mode-locked femtosecond lasers. Such lasers are able to produce an excitation source with sufficient stability and high photon intensity to enable the efficient generation of non-linear effects (Barzda 2008). Even so these effects are only generated by the use of a tightly focused femtosecond

laser source (Prent et al 2005b) and thus these signals are propagated to the volume of the focal point (Cheng et al. 2001).

There has also been a shift to using infrared wavelengths. Due to low absorption and reduced scattering of the light through biological materials using infrared light enables deeper penetration of the biological sample. There is also a concomitant reduction in photo-damage of the sample (Cheng et al. 2001) Thus using non-linear microscopy information can be obtained up to a depth of up to 600 microns in some tissues (Campagnola et al. 2003) with 500 microns being standard. This is about a three fold increase compared to confocal microscopy (Theer and Denk 2004). The wavelength region that is usually used is between 800 – 1500nm. The longer wavelengths are strongly absorbed by water while the shorter wavelengths are absorbed by biological molecules. Furthermore, if THG is being used the resulting signals are of ultraviolet wavelengths which adds another limiting factor (for a review see Barzda et al 2008). Figure 1 shows the mechanism as to how these non-linear events can be propagated.

As well as principally being a tool for imaging a microscope can also be adapted to measure spectral data.

Multi-photon excitation fluorescence (MPF).

The most commonly used form of non-linear microscopy is MPF such as two and three photon fluorescence. To a large extent this is due to the fact that the widely used confocal fluorescence microscope systems are readily up-gradable to multi-photon excitation (Barzda 2008). MPF has a number of advantages over confocal microscopy. The wavelength used in non-linear microscopy is outside the absorption band of the sample molecule (Barzda et al. 2005). For multiphoton absorption to occur the combined energies of two or even three photons needs to equal the ground state to excited state transition energy. The conditions for this to occur exist only at the laser focus. Thus the fluorescence is propagated in the very confined region of the laser focus volume which is in the order of femtoliters (Barzda 2008). The excitation that occurs in confocal microscopy along the whole pathlength of the beam as it passes through the sample thus does not occur with MPF. As a result the photobleaching that commonly accompanies this phenomenon also does not occur in non-linear MPF (Denk et al. 1990).

Multiphoton microscopy has been used to image dynamic events in a number of biological systems. Examples are, imaging of Ca^{2+} accumulation in neurons (Kloppenburg et al. 2000), dynamic changes have been observed in dendritic spines of rat hippocampus pyramidal cells and Purkinje cells (Denk et al. 1996) and calcium waves in Zebrafish embryo (Gilland et al. 2003). Saggau has used mulitphoton microscopy to investigate neurons from rat brains and collected 3D images of neurons firing in real time (Reddy et al. 2008). Myocyte contraction dynamics with sub-video rate resolution has been investigated with SHG microscopy (Prent et al. 2008).

Second Harmonic Generation (SHG).

SHG can be observed when the symmetry at an interface is broken. The two lipid layers of a biological membrane often has this arrangement. The lipid and protein composition of the two layers of a lipid bilayer is often different. Under these conditions there is a non-centrosymmetry of the membrane organisation. Thus there can be sufficient asymmetry to enable SHG to be efficiently propagated. This makes SHG very suitable to study biological systems and structures which exhibit macrochirality (Prent et al 2005b). Mertz and Moreaux (2001) used SHG to investigate membranes and measure inter-membrane separation. This technique has also been used by Campagnola et al. (1999) and Pons et al. (2003) to characterize membrane potentials. Other examples of where SHG has been used to investigate biological samples are tubulin (Campagnola et al. 2002), collagen (Freund et al 1986 and Campagnola and Loew 2003), actin and mysin complexes (Campagnola et al 2006, Barzda et al 2005) chloroplasts (Prent et al 2005) light harvesting chlorophyll a/b pigment-protein complexes of Photosystem II (Prent et al. 2005) and the formation of polysaccharides such as starch (Mizutani et al. 2000, Cox et al. 20005).

Third Harmonic Generation (THG).

THG may be propagated at an interface of materials which have different refractive indices. The THG signal can be enhanced by the presence of a multilayered structure as found in the grana of thylakoid membranes in chloroplasts (Prent et al. 2005) and crista within motochondria (Barzda et al. 2005).

Here again THG has been used to investigate a variety of biological samples which includes yeast cells (Yelin and Silberberg 1999), muscle cells (Barzda et al. 2005), glial human cells (Barille et al. 2001), erythrocytes (Millard et al. 1999) mitochondria (Barzda et al. 2005) and neurons (Yelin et al. 2002).

Multi-modal microscopy.

It is possible to generate these non-linear (MPF, SHG and THG) signals from the same focal volume simultaneously by using ultra-fast pulses (Barzda 2008). Thus microscope systems have been developed quite recently that can record these signals simultaneously. This is being referred to as multimodal microscopy (Chu et al. 2001, Barzda et al 2005, Prent et al. 2005).

Previously MPF and SHG have been used in conjunction to take images from muscle and tubulin (Campagnola et al. 2002) and a variety of labelled samples such as neuroblastoma cells (Campagnola et al. 1999), lipid vesicles (Moreaux et al. 2000) and neurons (Moreaux et al. 2001). Similarly SHG and THG have been used to investigate mitosis in zebrafish embryo (Chu et al. 2003).

The microscope system.

The laser

Picosecond and femtosecond lasers are used for non-linear microscopy as these ultra-short pulses have a high photon density. As these pulses are so short the average power that the sample is exposed to is low and thus not damaging to biological materials. Even so there is a limit to the peak power that a biological sample can be exposed to before damage occurs (Müller et al. 1998). The repetition rate of the laser is also of importance as the time between pulses needs to be sufficiently long to allow the excited state to completely relax back to the ground state (Barzda et al. 2001).

The detectors

The non-linear signals are often very low from many biological samples. This could be as little as a single photon per 1 to 10 excitation pulses delivered to the sample. In these cases photon counting is the favoured method. The types of detectors that have been used are photomultiplier tubes, avalanche photodiodes and charge-coupled device cameras.

Figure 2 shows the general schematic diagram of a multi-modal microscope system.

Clinical applications.

In recent years a number of groups have been investigating the potential for non-linear microscopy for clinical applications. Skala et al. (2005) experimented on squamous epithelial cells using multiphoton microscopy. They were able to distinguish between normal, pre-cancerous and cancerous tissue from their endogenous fluorescence. Multiphoton microscopy allows structural and functional changes to be tracked in the same sample over time scales ranging from seconds to weeks. Molitoris and Sadoval (2005) used this property to study glomerular permeability, proximal tubule endocytosis and microvascular renal function. Multiphoton microscopy has been used *in vivo* to monitor intrasomal corneal laser surgery. Here both MPF and SHG were used. This technique enabled 3D submicron tomographic data to be obtained without requiring mechanical sectioning or staining of the tissue (Wang and Halbhuber 2006). The combination of MPF and SHG has also been applied by Gueta et al. (2007) to produce a 3D structure of the tectorial membrane in the cochlea. This is a structure that covers the sensory cells and is a non-cellular matrix composed of 97% water and 3% protein. The majority of this protein is collagen and they were able to detect differences collagen assembly within the membrane. MPF and SHG have been used to investigate blood vessel wall microstructure (Zoumi et al. 2004). The MPF from elastin and smooth muscle can be spectrally separated from the SHG signal propagated from collagen. This potentially will enable a greater understanding of the bio-mechanics of vascular tissue under healthy and pathological conditions. THG has been used to investigate haemoglobin and has been shown that in physiological solutions the various ligand binding states can be differentiated (Clay et al. 2006).

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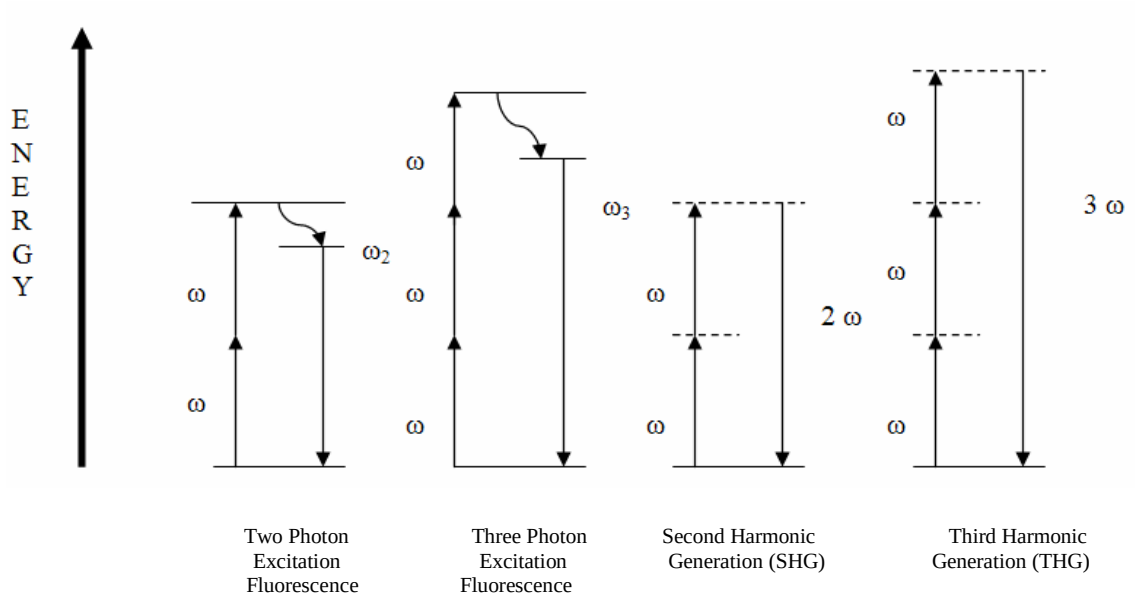
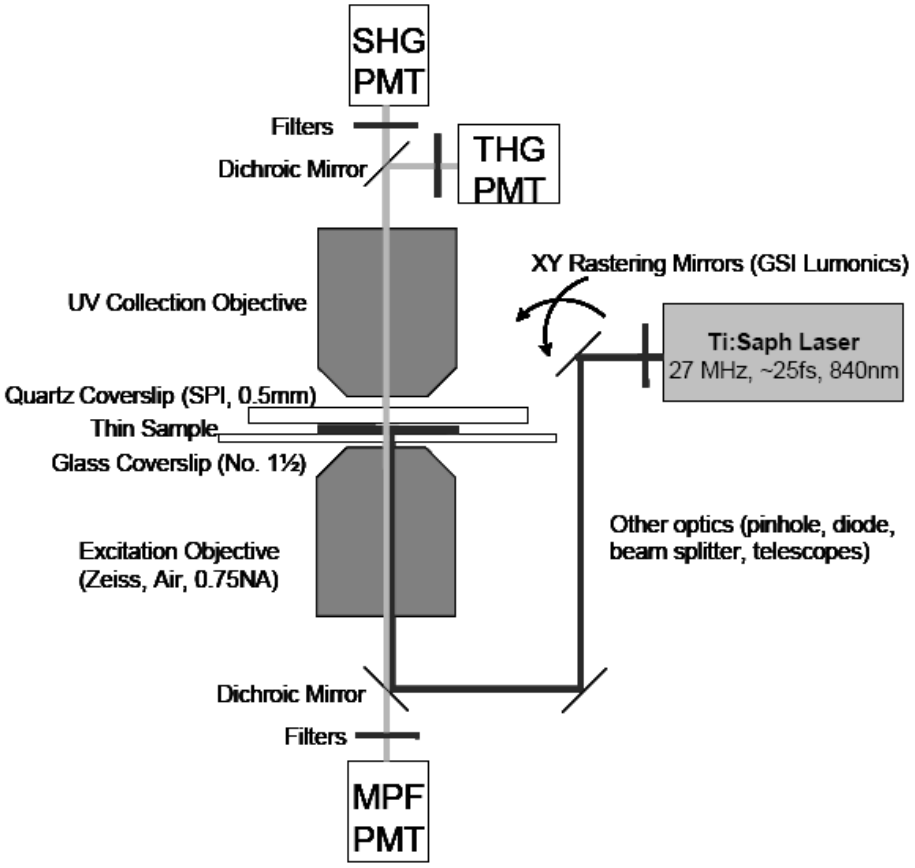


Figure 1. Energy level diagrams for two-photon and three photon absorption and the resulting multi-photon fluorescence (MPF) as well as second harmonic generation (SHG) and third harmonic generation (THG). The solid horizontal lines represent real energy levels. The dashed horizontal lines represent virtual energy levels. ω shows the frequency of absorbed photons. 2ω, 3ω, ω_2 and ω_3 show the frequency of fluoresced photons.



Prent et al. 2005

Figure 2 shows a typical optical set-up for a multi-modal non-linear microscope system. Here simultaneous MPF, SHG and THG data can be collected.

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