HARMONIC IMAGING ADDS NEW DIMENSIONS TO MULTIPHOTON MICROSCOPY (MPM)

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Second Harmonic Geneation (SHG) and Third Harmonic Generation (THG) are non-linear optical phenomena, known for decades for their potential utility in imaging (1). For a long time, due to the lack of suitable light source, harmonic imaging had not had any practical use in biology. However, the emergence of multiphoton technology during the past decade has brought about renewed enthusiasm in this area. Biological applications continue to emerge as well. Such imaging modality compliment MPM as being a powerful tool for probing living cells and tissues.

Background

Molecules lacking any center of symmetry are able to convert light to its second harmonic, at twice the frequency and half the wavelength (third harmonic at three times the frequency and a third the wavelength, and so on, although the high order harmonics are rare occurrence). However, such happenings relies on extremely high incident light sources such as that by pulsed lasers (SHG depends on the square of incident beam power and THG has a cube relationship). In fact, the "frequency doubling" of certain anisotropic crystals in generating high power lasers is a SHG process (2). In a microscopy system, harmonic generation can only be detected at the focused volume, where photon flux is at its highest. Therefore, it has intrinsic optical sectioning capability, much like MPM. Instrumentation used for MPM is well suited for harmonic imaging. Among the applications, imaging collagen with SHG is among the most studied and documented.

Something similar, something different

Although there are many similarities between harmonic imaging and MPM, there are also significant differences between the two. The following comparison of SHG and 2-photon fluorescence (2PF) highlights their major similarities and differences

	SHG	2PF
Similarities	◦ Conversion of two identical photons of weaker energies to a single photon of twice the energy	
	◦ Requires short laser pulses	
	◦ output intensities proportional to the square of incident laser power	
	◦ Intrinsic optical sectioning capability	
	◦ uses similar instrumentation	
	◦ comparable image resolution	
Differences	◦ Exactly double the frequency and half the wavelength of the incident beam	◦ Range of frequencies less than twice, and longer wavelengths than half, the incident beam
	◦ Non-resonance scattering only; no energy loss; no fluorescence; no photo-chemistry involved; no photo-bleaching/damage	◦ Resonance absorption with energy loss, resulting in fluorescence emission and photo-bleaching/damage
	◦ Narrow bandwidth of emission	◦ Broad emission curve
	◦ Does not require sample staining	◦ Sample staining required (except in auto-fluorescence)
	◦ Frequency independent	◦ Fluorescence absorption is frequency dependent
	◦ Coherent as the incident beam, propagates forward	◦ Emission is incoherent, propagates in all directions

Utilities of harmonic imaging in biology

THG is very sensitive to reflective index differences and therefore useful in imaging unstained cells and tissue much like phase contrast microscopy does. However, with current equipment capability, THG is more difficult to observe and resource in this area of research has been scarce. On the other hand, there is abundance of recent research in SHG imaging in the literature. Due to the non-centrosymmetric requirement, molecules with repeated patterns and orderly arranged structures (eg. membranes, starch granules, cell wall fibrils, etc.) are good candidates for SHG. However, these repeated patterns have to be arranged in a certain way and separated within a certain distance from each other. Type I collagen, in particular, is known for its large second-order harmonic response (3). This structural protein is an essential component of extracellular matrix in higher vertebrates, is involved in cell differentiation, proliferation, growth and migration (Fig 1 and 2). It has also been linked to certain pathological conditions (Fig 3).

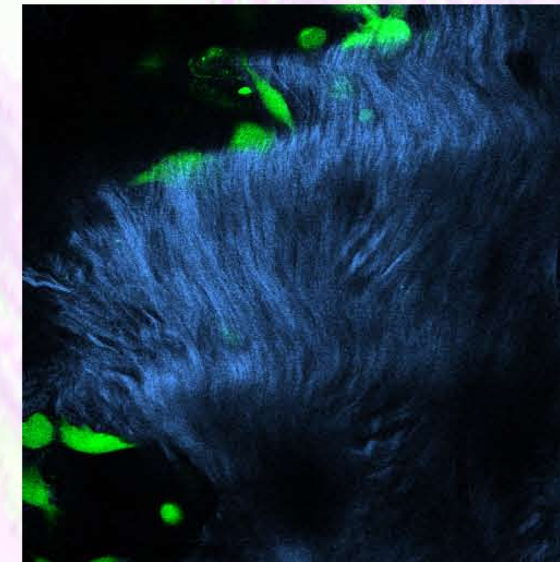

Fig 1. HT-1080/MT1 tumor cells (green) migrating through a dense matrix of collagen fibers (blue) in the dermis of a live mouse. (data from the von Andrian Lab)

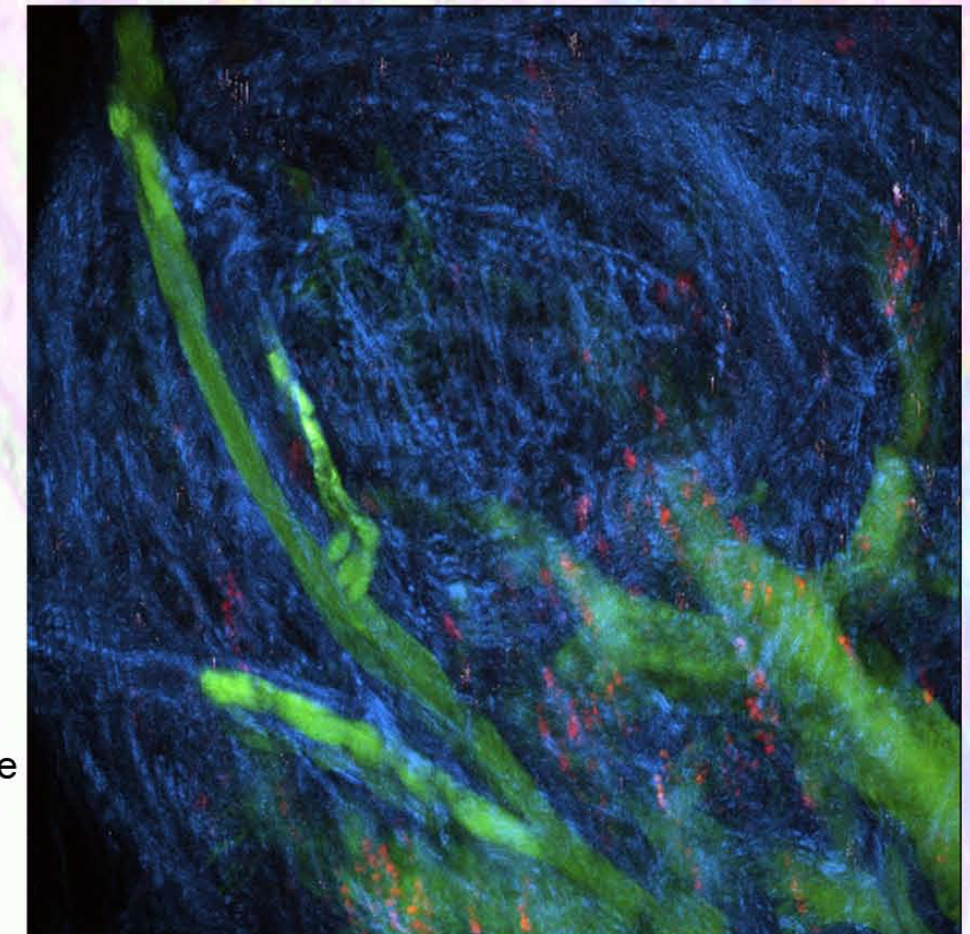

Fig 2. T-lymphocytes (red) traveling in the abdominal lymph node of a live mouse amidst a network of collagen fibers (blue). (data from the von Andrian Lab)

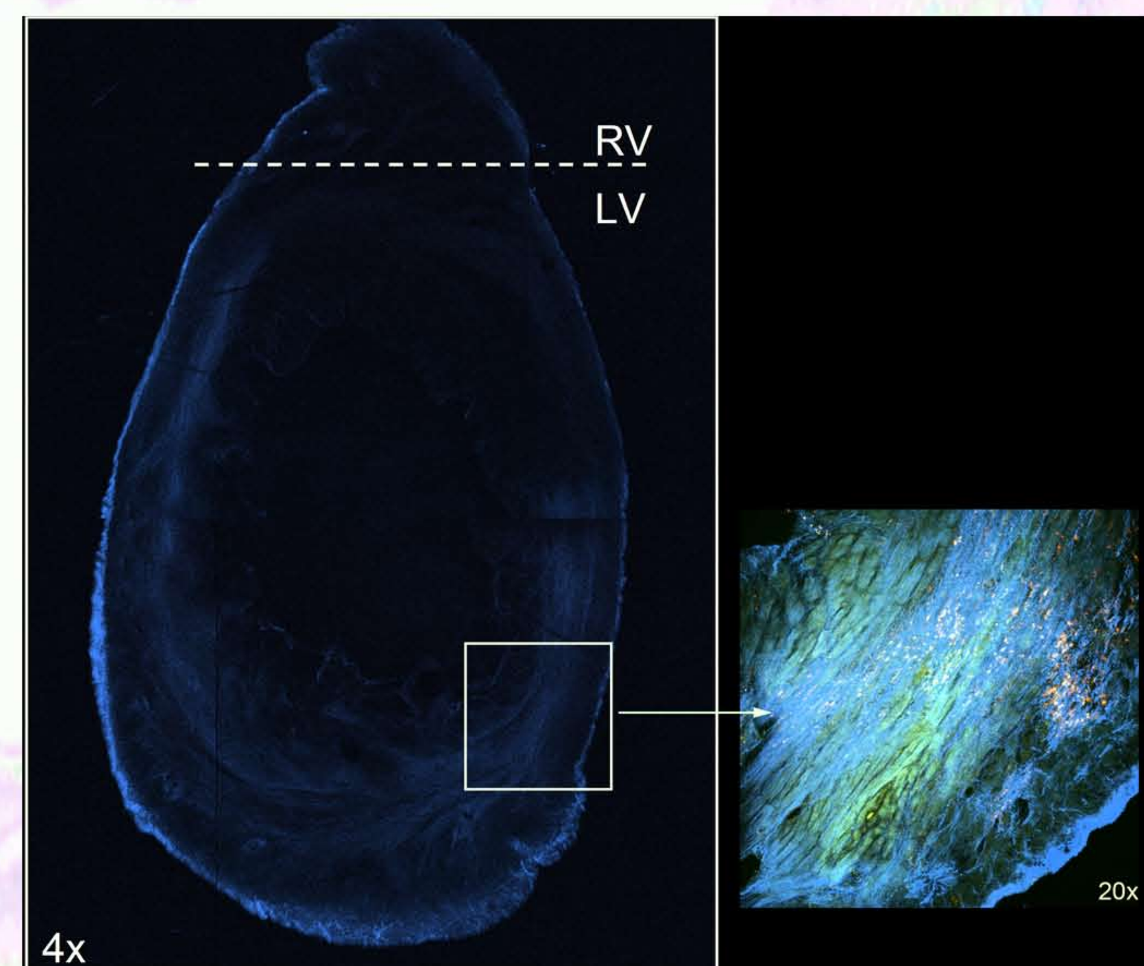

Fig 3. Excised murine heart showing deposition of collagen (blue) after myocardial ischemia and reperfusion. LV= left ventricle; RV= right ventricle. Inset shows collagen fibers (blue) among underlying muscle fibers (green autofluorescence). (data from the Carroll Lab)

More applications ....

◦ **Clinical diagnosis -** To help diagnose osteoarthritis: in a diseased stage, there is increased fibrillation with the deposition of collagen (increased SHG signal) and lost of chondrocytes (decreased THG signal) (4).

◦ **Modeling the synthesis of artificial skin -** Found how collagen formed around fibroblasts (use SGH for collagen and 2PF for fibroblasts) to form a stable scaffold under optimal culture conditions (5).

◦ **Assessment of cutaneous photo-aging under continual UVB exposure -** Findings: in mice, such photo-aging could be reversed after UVB withdrawal (6)

Conclusions

Since harmonic imaging is non photo-toxic, and without the need to use stains, it is extremely useful for intravital imaging. For that matter, collagen has been made valuable as an intrinsic marker for tissues and organs. Refinements to MPM during the last decade have brought about 'cheaper' and readily available instrumentation for further development of these techniques. The future is promising.

References

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