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Optical Tweezers and Multiphoton Microscopies integrated photonic tool for mechanical and biochemical cell processes studies.

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ABSTRACT

The research in biomedical photonics is clearly evolving in the direction of the understanding of biological processes at the cell level. The spatial resolution to accomplish this task practically requires photonics tools. However, an integration of different photonic tools and a multimodal and functional approach will be necessary to access the mechanical and biochemical cell processes. This way we can observe mechanicaly triggered biochemical events or biochemicaly triggered mechanical events, or even observe simultaneously mechanical and biochemical events triggered by other means, e.g. electricaly. One great advantage of the photonic tools is its easiness for integration. Therefore, we developed such integrated tool by incorporating single and double Optical Tweezers with Confocal Single and Multiphoton Microscopies. This system can perform 2-photon excited fluorescence and Second Harmonic Generation microscopies together with optical manipulations. It also can acquire Fluorescence and SHG spectra of specific spots. Force, elasticity and viscosity measurements of stretched membranes can be followed by real time confocal microscopies. Also opticaly trapped living protozoas, such as leishmania amazonensis. Integration with CARS microscopy is under way. We will show several examples of the use of such integrated instrument and its potential to observe mechanical and biochemical processes at cell level.

Keywords: Multiphoton Microscopy, SHG Microscopy, Confocal Microscopy, Optical Tweezers

1. INTRODUCTION

The present research agenda in biosciences is to get a deep understanding of life processes, beginning with the cell, the basic unit of life. That agenda actually came late in the scientific evolution, because the irony of the history was that we learned to destroy the world we live in well before we could understand very simple life processes. Even the DNA structure was discovered after the atomic bomb. Although life is a complex phenomenon it does not seem that the effort to understand cell processes would be harder than to understand nuclear processes. As any material scientists know processes are very hard to copy because its necessary to follow the whole sequence of events in time. The fact that one knows all the components of a sample does not mean he will be able to reproduce it. Companies use spies, or hire others company employees, to learn the competion processes. However, there is no one to ask about life processes other than nature itself. The only way, therefore, to understand life processes is to observe its course in time.

That imposes restrictions about the tools necessary to perform this task. First, we need tools to continuously observe the process in a non destructive way, that is, functional tools. Fixed cells studies are, therefore, ruled out. The observation should be remote, contactless and without any contamination. It is also necessary to be able to trigger the process or to change its course to trace the right cause-effect relationship. The relevant information for life processes are biochemical

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and biomechanical with, at least, sub cellular spatial resolution. The ideal spatial resolution would be the one capable to discriminate colocalization of proteins, that means on the order of a few nm. We believe that photonics tools will be almost mandatory to accomplish this task, although multimodal systems may be necessary at some point. The nm resolution is achievable today only with electron and atomic or tunneling microscopies. Although optical spatial resolution is of order of 200 nm, still below the ideal, optics is in the right energy range because molecular electronic and vibration levels falls in the UV-VIS-IR region. The very poor spatial resolution for the vibrational levels at the IR region, can be solved using Raman that brings the vibration information to the visible. Molecular electronic and vibration levels fall in the UV-VIS-IR region. Techniques based on electrons and x-rays, such as Energy Dispersive X-ray Spectroscopy [EDX or EDS], x-Ray Photoelectron Spectroscopy [XPS] used in Scanning or Transmission Electron Microscopy [SEM or TEM] tends to provide information about the chemical elements and usually require vacuum, incompatible with life. The penetration of an electron beam is too shallow to observe a whole 10-20 μm cell, and they are much more destructive than light beams. Although atomic force and tunneling microscopies present atomic spatial resolution and do not require vacuum, they are contact techniques unable to observe an event inside of a 10-20 μm cell without destroying it. Several photonic schemes are being proposed to enhance spatial resolution, such as Near Field microscopy, plasmonic and tip enhanced microscopies, energy transfer, and fluorescence suppression by stimulated emission. We believe that the non-contact optical techniques will provide the best tools to observe cell processes.

Optics also opened up the way to observe biomechanical events after the pioneer work of Ashkin in the second half of the 1980's. Although the fuel of life is essentially biochemical, the biomechanical events play a very important role. The first infection step is the movement towards the right cell and the adhesion to its walls. Forces, torques, elasticities, membrane viscosities, permeabilities and deformations are important variables that have to be measured. Forces on the micrometer scale are of order of, or smaller, than hundreds of picoNewtons. Very sensitive techniques are necessary to measure such forces. Optical forces are in the right range, from 200 pN down to tens of femtoNewtons. The development of optical tweezers opened up the possibility to observe biomechanical events in the microscopy world never before achievable in 400 years of optical microscopy. However, an integration of different photonic tools and a multimodal and functional approach will be necessary to access the mechanical and biochemical cell processes. This way we can observe mechanically triggered biochemical events or biochemically triggered mechanical events, or even observe simultaneously mechanical and biochemical events triggered by other means, e.g. electrically. One great advantage of the photonic tools is its easiness for integration.

The development of femtosecond lasers in the 1990's opened up the way to use non-linear optics for life sciences. First with Two-Photon Excited Fluorescence [TPFE] of the Multiphoton Microscopy, now a commercial system[1,2]. Smaller photobleaching and deeper penetration are advantages of the multiphoton microscopy. Also the techniques based on the fluorescence lifetime such as Fluorescence Lifetime Imaging [FLIM], Fluorescence Resonant Energy Transfer [FRET] and Fluorescence Correlation Spectroscopy [FCS][3,4,5,6,7,8], that existed before the multiphoton microscopy, were improved with multiphoton process. These lifetime techniques provide information about the local pH/oxygen concentration changes, proximity between molecules and the diffusion and molecular mass inside a femtoliter volume. By the end of 1990's the Second Harmonic Generation [SHG][9] microscopy provide a tool to observe non-centre-symmetric molecules, specially collagen structures and interfaces. Raman spectroscopy had two main difficults for imaging of biological samples. First the presence of fluorescence that completely masks Raman signal, forcing researches to move to the IR region with poor Raman signal, spatial resolution and less efficient detectors. The second was the time required to acquire good resolution images, that could be of order of several hours. These two difficults were solved by enhancing Raman signal with Coherent Antistokes Raman Scattering [CARS][10,11] microscopy . The SHG and CARS microscopies do not require exogenous marker that could spoil the observed cell process. Among the fluorescence techniques only autofluorescence do not require exogenous markers.

The integration of all these biophotonic techniques in one system would allow the simultaneous observation of biochemical and biomechanical events necessary to follow cell processes. Despite the great advantage of this integration there are very few contributions in literature showing optical tweezers in parallel with fluorescence multiphoton or confocal microscopy[11]. In this paper we will demonstrate the integration of a double optical tweezers system with confocal microscopies including single/multi-photon microscopy, SHG microscopy and, for the near future, CARS and fluorescence lifetime microscopies. The system can acquire spectra of specific spots. We will show several examples of the use of such integrated instrument and its potential to observe mechanical and biochemical processes at cell level. Integration of Optical Tweezers with Raman spectroscopy is now a common place, have been demonstrated before and used by several groups[13,14], including ours[15,16].

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2. EXPERIMENTAL SYSTEM

Figure 1 shows the experimental setup. We used an Olympus confocal system [IX-81 inverted microscope, the FV300 scan head, the FV-5 COMB2 laser combiner and four Hamamatsu model 3896 PMTs] with a special port and dichroic mirror to couple the Ti:sapphire laser beams [Tsunami, Spectra-Physics pumped with a 10W XS Millennia]. A Prior Scientific, model ProScan translation stage was used to move the specimens in the x-y direction, while the z-translation was performed with the IX-81 built in system. Single photon confocal microscopy was excited with the 488 nm line of an Argon laser [Omnichrome, Model 170B] coupled to the scan head through an optical fiber. The 10 W XS Millenia deliver 5 W for each Millenia after a beam splitter. One of the Tsunami laser can deliver both, 100 fs or 5 ps pulses, while the other only operate in the 5 ps regime. They are synchronized with a lock-to-clock electronics with less than 300 fs timejitter. For TPFE and SHG microscopy we used only the femtosecond laser. CARS system will use the two picosecond lock-to-clock laser. One telescope is used for each laser to match the beam divergence and the focal plane after the objective, using the single photon confocal system with the argon laser as the reference for the focal plane. By changing the distance between the two telescope lenses we make sure the single and two photon images correspond to the same plane. Images were obtained with a 40x,1.3 NA oil immersion (UPlanFL N Olympus) objective. Single photon fluorescence images were collected in the backscattering geometry after passing through a pinhole, while multiphoton could be collected in both directions, backscattering and forward, simultaneously or not, and SHG signal was collected only in the forward direction. A 3rd Millenium 690SP [Omegafilters] short pass filter was used to reject the ti:sapphire laser line for fluorescence. A 570nm dichroic mirror (DM 570nm, Olympus) further divided the backscattered fluorescence signal in two channels, PMT1 and PMT2. For the forward signals the light was collected with the microscope condenser and focused in an external PMT. The fluorescence and laser light were rejected with a 400 nm band pass filter [ORIEL] right after the condenser for SHG image and spectrum. For the image an extra shortpass colour filter was attached to the PMT. The SHG spectrum was obtained with 30 cm long monochromator (Acton Research, model 300) equipped with a back-illuminated refrigerated CCD detector (Princeton Instruments-LNCCD 1340/ 100 EB/1). The Fluoview software was used to reconstruct 512 x 512 pixels images. Usually we acquired the image five times and used a Kalman filter.

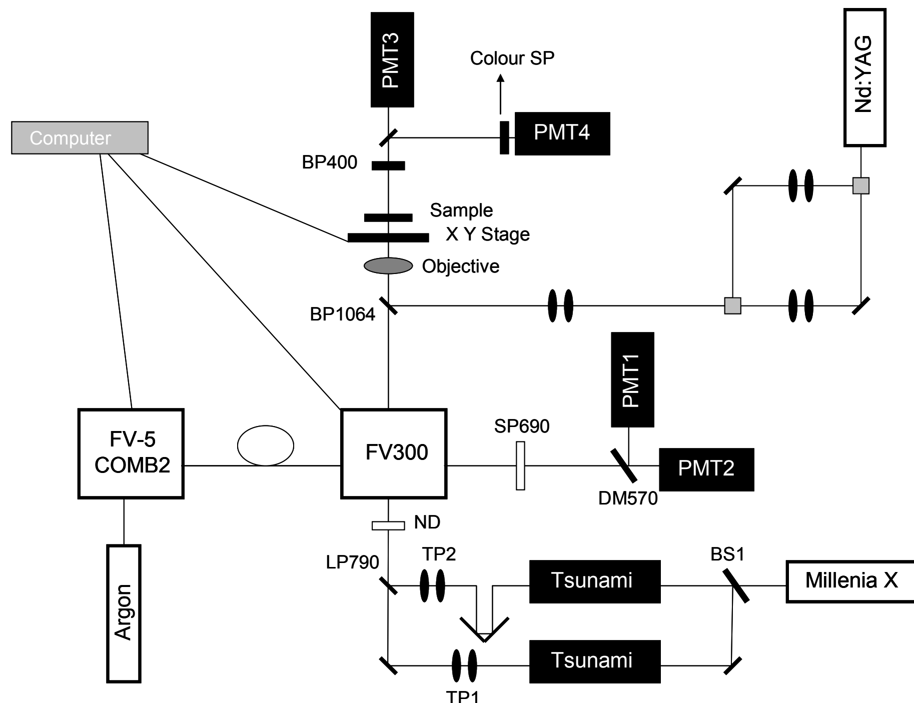

Fig 1. Experimental setup for the integrated TPFE Confocal, SHG, CARS and Optical Tweezers system.

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The double Optical Tweezers system used a Nd:YAG cw laser and it was described before[17]. The Nd:YAG was coupled with a 1064 nm bandpass mirror (Newport) right before the objective using a special turret port of the IX-81 microscope. The two optical tweezers telescopes give us control of the trapping planes with respect to the confocal image planes.

Figure 2 shows the multiphoton confocal image of a triple marked bovine pulmonary artery endothelial cells from Molecular Probes obtained with our system. The nucleus was marked with DAPI [blue], the tubulins were marked with BODIPY FL [green] and the F-actin marked with Texas-red [red]. The two-photon excitation of both, the nucleus and the tubulin fibers, was performed with the fs Ti:sapphire (780nm) laser, while the argon (488nm) excited the actin fibers.

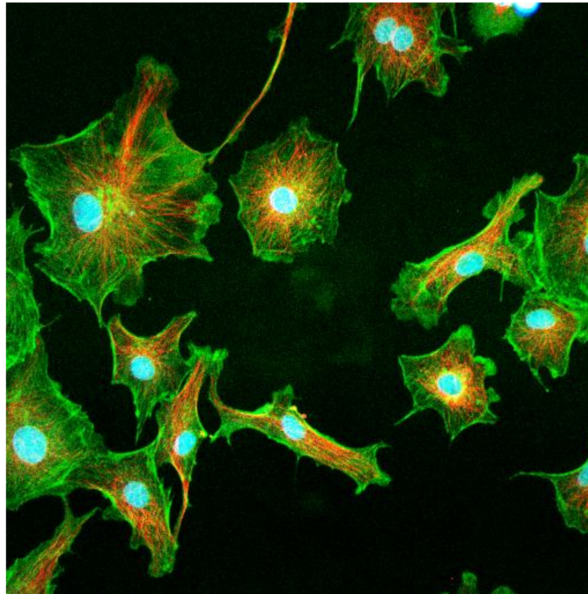

Fig 2. Multiphoton and single photon confocal microscopy of tripled marked bovine pulmonary artery endothelial cells. Blue: nuclei; Green: tubulin fibers; Red: actin fibers.

Figure 3 shows the same cells with multiphoton two colors confocal microscopy adjusting the excitation power to avoid nuclei saturation to be able to distinguish its internal structure.

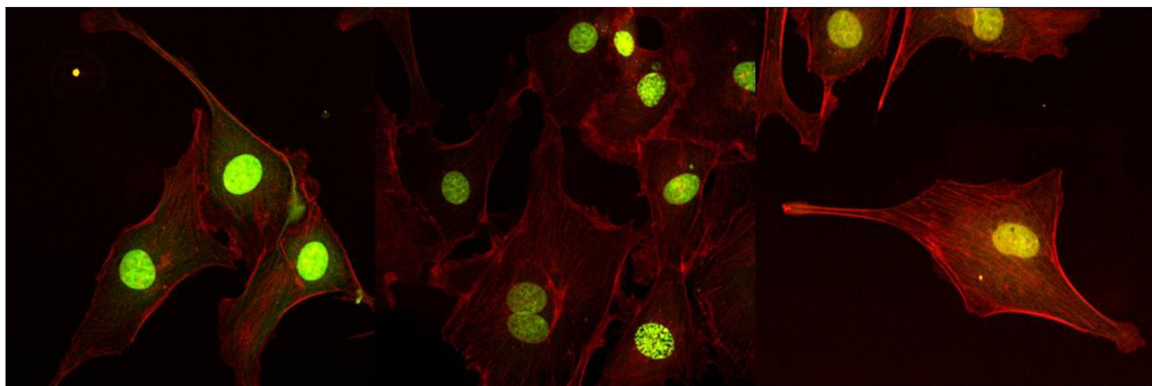

Fig 3. TPFE only two colors image of the previous cell.

The sequence of the following pictures illustrated the contribution that confocal microscopy can bring to Pathology. Figure 4 shows multiphoton and transmission image of elastin fiber of a human aorta stained with eosin. The discrimination of elastin fibers is very in the confocal image compared with the transmission one.

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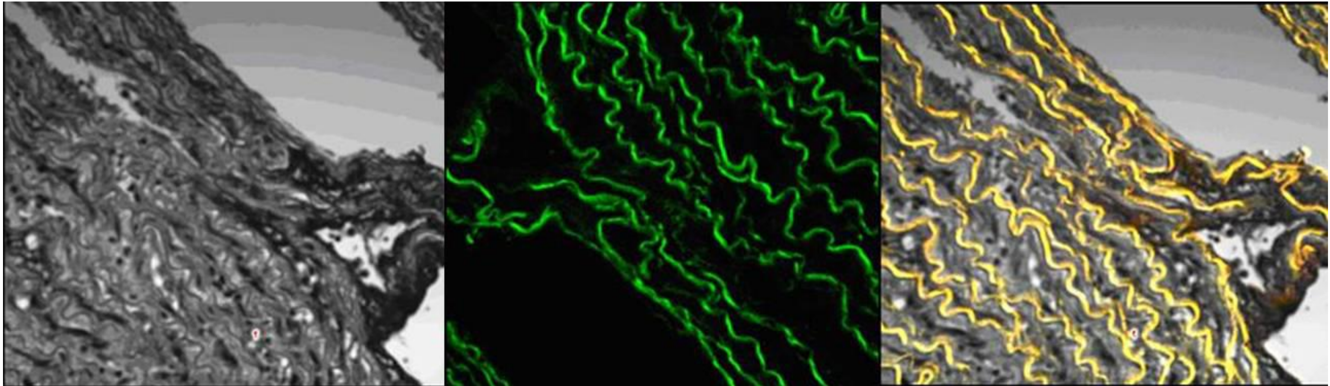

Fig 4. Human Aorta tissue with elastin stained with eosin. Left: transmission image; Mid: fluorescence image; right: overlay of the two images.

Figure 5 shows the nuclei of the aorta cells stained with propidium iodide together with the autofluorescence of the elastin fibers.

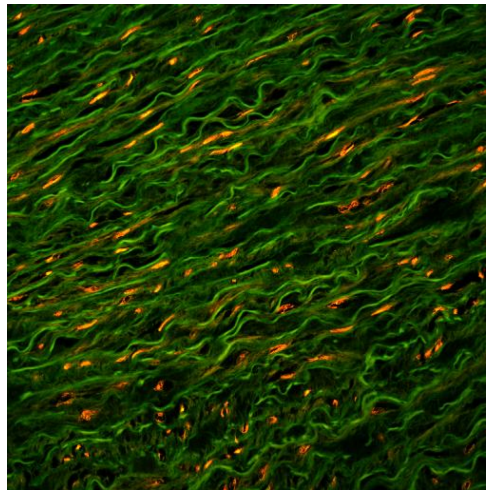

Fig 5. Human aorta. Nuclei: stained with propidium iodide; Elastin: autofluorescence.

Figure 6 shows the clearly marked contours of cancer cells from a frozen specimen of a lung invasive epidermoid carcinoma. Pathologists tend to avoid frozen samples due to the artifacts that can lead to misdiagnostics. However, this process can be performed during the surgery while the conventional one can take days for the diagnostic. The contour of the cancer region is important for the diagnostic showing if the tumor is contained or not.

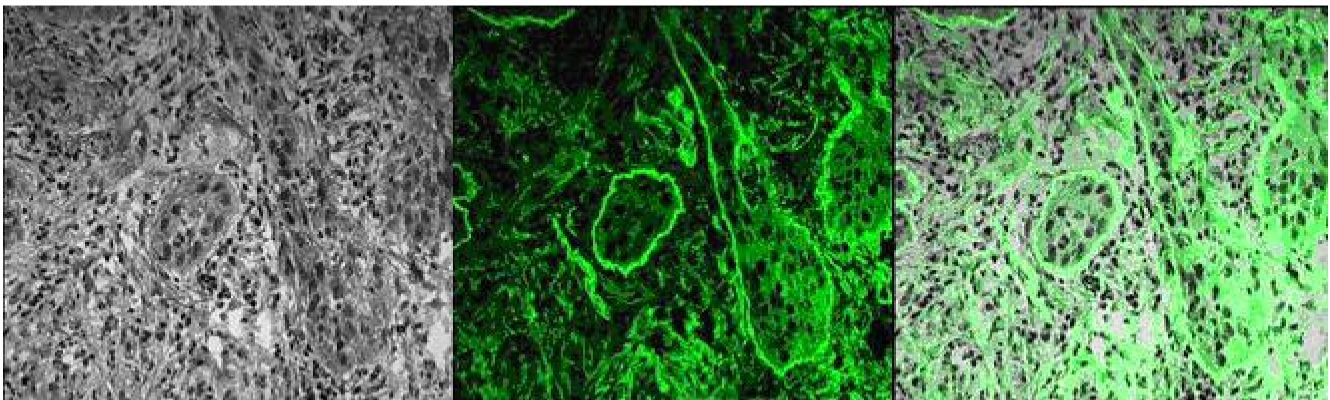

Fig 6. Frozen sample of lung invasive epidermoid carcinoma stained with eosin.

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Figure 7 shows another example of the use of confocal microscopy for pathology. A miscalibration of the staining machining, that happens in about 0.1% of the time, lead to the right image of a human recto colon tissue that could not be used for diagnostics. The only way to correct this by conventional means was to perform another invasive biopsy. However the fluorescence confocal image of the same sample did show clearly all the tissue structure that allowed the clear not cancerous tumor diagnostic.

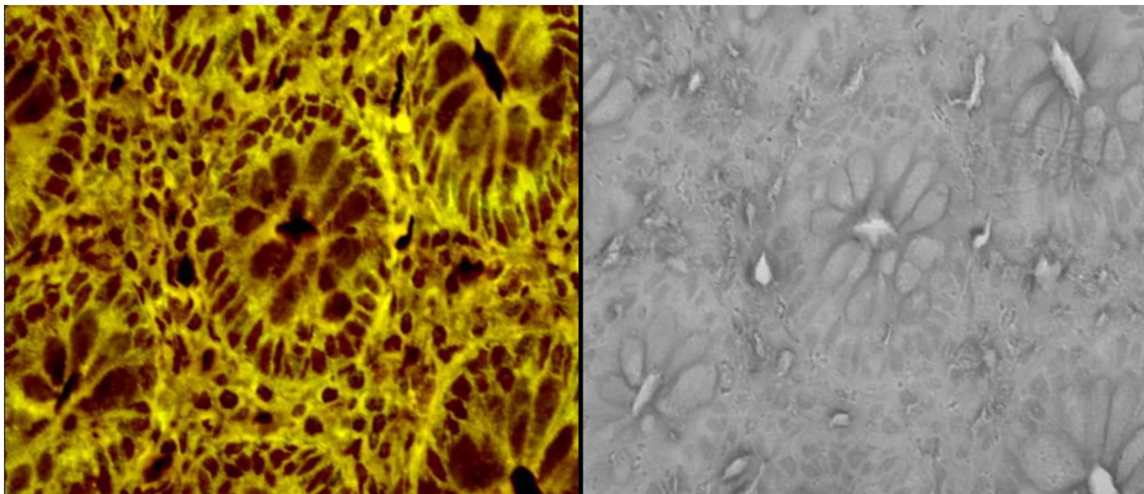
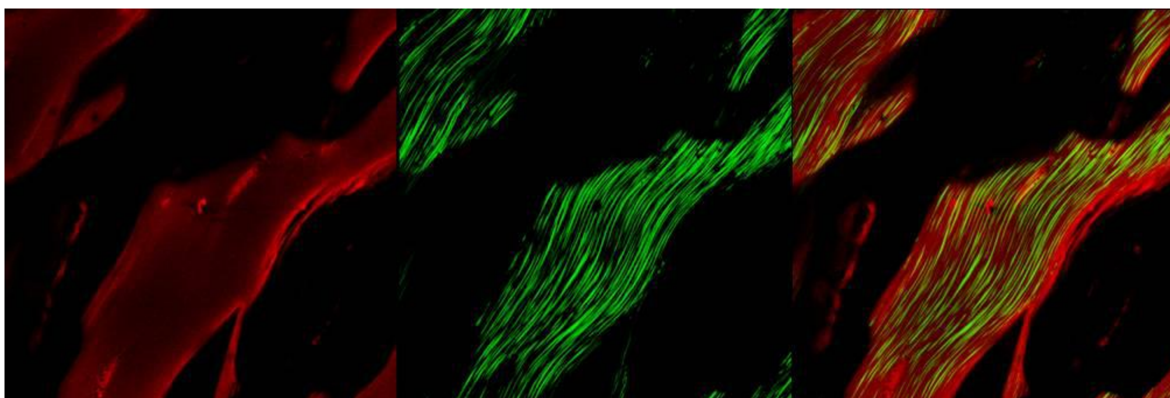


Fig 7. Recto colon mucosa. Left: confocal fluorescence image; Right: transmission image.

Figure 8 shows SHG and TPEF images of rat tendon obtained with the same confocal system. The collagen tendon fiber is clearly shown in the mid green image while the autofluorescence is shown in red at the left. The superposition of the two images is shown at the right. Figure 9 shows the SHG spectrum collected in fixed spot at the microscope.



Fige 8. TPFE autofluorescence image (left), SHG image (mid) and overlay of the two images (right) of a rat tendon tissue sample.

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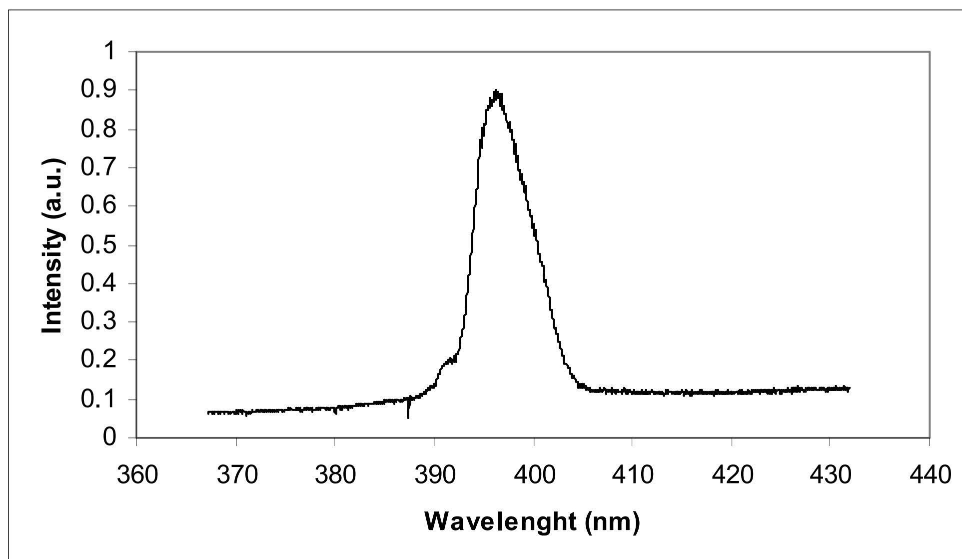

Fig 9. Rat tendon fiber SHG spectrum.

Finally, figure 9 shows the integration of the multiphoton confocal system with the Optical Tweezers system. Red Blood Cells marked with eosin were suspended with the double optical tweezers to the focal plane of the fs laser where they show the TPFE fluorescence. Notice that there are other red blood cells out of focus that do not fluoresce. We then move the tweezer of the right while kept the other fixed.

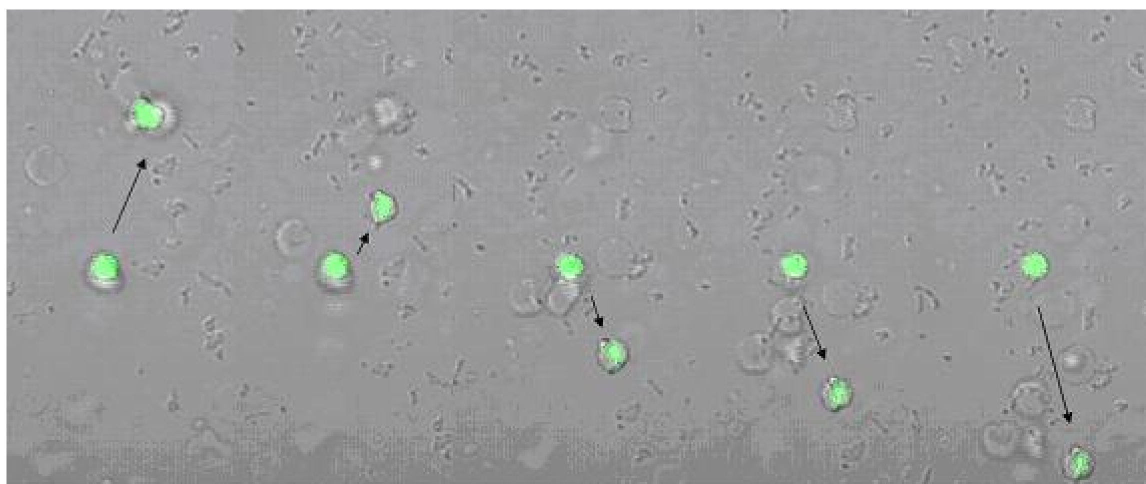

Fig 10. Integration of TPFE confocal microscopy with Double Optical Tweezers system. Red Blood cells stained with eosin moved by optical trap respect to each other.

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