# Laser scanning third-harmonic-generation microscopy in biology

**D. Yelin and Y. Silberberg**

*Department of Physics of Complex Systems, Weizmann Institute of Science, Rehovot 76100, Israel.*

*yelin@wis.weizmann.ac.il*

http://www.weizmann.ac.il/~feyaron

**Abstract:** A laser scanning microscope using third-harmonic generation as a probe is shown to produce high-resolution images of transparent biological specimens. Third harmonic light is generated by a tightly focused short-pulse laser beam and collected point-by-point to form a digital image. Demonstrations with two biological samples are presented. Live neurons in a cell culture are imaged with clear and detailed images, including organelles at the threshold of optical resolution. Internal organelles of yeast cells are also imaged, demonstrating the ability of the technique for cellular and intracellular imaging.

**OCIS codes:** (170.3880) Medical and biological imaging; (180.6900) Three-dimensional microscopy; (180.5810) Scanning microscopy; (170.7160) Ultrafast Technology.

## References and links

1. T. Wilson, *Confocal microscopy*, (Academic, London, 1990).
2. W. Denk, J. H. Stricker and W. W. Webb, "Two-photon laser scanning fluorescence microscopy," Science **248**, 73-76 (1990).
3. S. Maiti, J. B. Shear, R. M. Williams, W. R. Zipfel and W. W. Webb, "Measuring serotonin distribution in live cells with three-photon excitation," Science **275**, 530-532 (1997).
4. M. Schrader, K. Bahlmann and S. W. Hell, "Three-photon-excitation microscopy: theory, experiment and applications," Optik **104**, 116-124 (1997).
5. D. L. Wokosin, V. E. Centonze, S. Crittenden and J. White, "Three-photon excitation fluorescence imaging of biological specimens using an all-solid-state laser," Bioimaging **4**, 208-214 (1996).
6. R. Hellwarth and P. Christensen, "Nonlinear optical microscopic examination of structure in polycrystalline ZnSe," Opt. Comm. **12**, 318-322 (1974).
7. J. N. Gannaway and C. J. R. Sheppard, "Second-harmonic imaging in the scanning optical microscope," Opt. and Quant. Elect. **10**, 435-439 (1978).
8. R. Gauderon, P. B. Lukins and C. J. R. Sheppard, "Three-dimensional second-harmonic generation imaging with femtosecond laser pulses," Opt. Lett. **23**, 1209-1211 (1998).
9. G. Peleg, A. Lewis, O. Bouevitch, L. Loew, D. Parnas and M. Linial, "Gigantic optical non-linearities from nanopartical-enhanced molecular probes with potential for selectively imaging the structure and physiology of nanometric regions in cellular systems," Bioimaging **4**, 215-224 (1996).
10. Y. Barad, H. Eizenberg, M. Horowitz and Y. Silberberg, "Nonlinear scanning laser microscopy by third-harmonic generation," Appl. Phys. Lett. **70**, 922-924 (1997).
11. R. Boyd, *Nonlinear Optics*, (Academic, New York, 1992).
12. M. Müller, J. Sqier, K. R. Wilson and G. J. Brakenhoff, "3D-microscopy of transparent objects using third-harmonic generation," J. Microsc **191**, 266-274 (1998).
13. J. A. Squier, M. Muller, G. J. Brakenhoff and K. R. Wilson, "Third harmonic generation microscopy," Opt. Express **3**, 315-324 (1998). http://www.opticsexpress.org/oearchive/source/5872.htm
14. D. Yelin, Y. Silberberg, Y. Barad, and J. S. Patel, "Depth resolved imaging of nematic liquid crystals by third-harmonic microscopy," Appl. Phys. Lett. **74**, 3107-3109 (1999).
15. Y. R. Shen, *The principles of nonlinear optics*, (Wiley, New York, 1984), Chap. 27.

#13710 - $15.00 US Received September 27, 1999; Revised October 04, 1999

## 1. Introduction

In laser scanning microscopy [1], an image is formed by scanning the sample point-by-point with a tightly focused laser beam. The scattered laser light, or, more commonly, fluorescence induced by the laser light is detected and collected to form a digital image. Utilizing nonlinear optical effects, such as two-photon [2] and three-photon [3-5] fluorescence, significantly improved the depth resolution and reduced the background noise. Second-harmonic generation was also used for microscopy of certain crystal samples [6-8] and for specialized biological imaging [9]. We have recently proposed and demonstrated a novel nonlinear scanning laser microscope that uses Third-Harmonic Generation (THG) to characterize transparent specimen [10].

In THG microscopy, third harmonic light is generated at the focal point of a tightly focused short-pulse laser beam. When the medium at the focal point is homogenous, the third harmonic waves generated before and after the focal point interfere destructively, resulting in zero net THG [11]. However, when there are inhomogeneities near the focal point, such as an interface between two media, the symmetry along the optical axis breaks and measurable amount of third harmonic is generated. Due to its nonlinear nature, the third harmonic light is generated only in a close proximity to the focal point. Therefore, high lateral resolution can be obtained, allowing THG microscopy to perform sectioning and to construct three-dimensional images of transparent samples. Since all materials have non-vanishing third-order susceptibilities, THG microscopy can be utilized as a general-purpose microscopy technique.

Since our initial demonstration of THG microscopy [10], significant progress was reported by Squier and coworkers [12, 13]. They, too, illuminate the sample with a focused short-pulse laser beam; however, they integrate the resulting THG image on a CCD camera. Using this direct imaging method, depth-resolved images of live biological samples were recently reported [13]. That work demonstrated the applicability of THG microscopy to biology, and in particular, that useful images can be obtained at power levels that do not harm live specimens. Their technique has the advantage that lateral resolution is determined by the shorter third-harmonic wavelength, but there are several disadvantages. For example, since the microscope has to operate in a transmission mode, good quality, high NA objective lenses should be used on both sides of the sample, requiring thin samples and special mounting. Also, scattering of the short-wavelength THG light will be deleterious to the image.

In *laser scanning* THG microscopy the image is collected point-by-point. This permits optimized collection and detection schemes which can be implemented for high sensitivity and short collection times. The digitized image thus obtained can be then processed and enhanced digitally, as in other types of scanning laser microscopes. In addition, scattering of the THG light does not affect the image quality; hence it is possible to image through thick samples. The lateral resolution is slightly lower as compared with direct imaging, since it is determined by both the fundamental and the third harmonic light.

We have recently demonstrated [14] depth-resolved imaging of liquid crystal cells by laser scanning THG, where various internal structures could be clearly observed. We report here a study of laser scanning THG microscopy for biological imaging. Our results show that THG microscopy is applicable to high-resolution intracellular biological imaging. We show that the inhomogeneity inherent to most biological specimen, and, in particular, the internal structure of various cells, lead to generation of good quality THG images without any preconditioning such as labeling or staining that might induce undesirable effects in the live cell. We have performed these experiments with a high repetition-rate short-pulse laser system, which enables image acquisition time of much less than a minute.

#13710 - $15.00 US Received September 27, 1999; Revised October 04, 1999

## 2. Experiment

As an imaging platform, we have used a Zeiss Axiovert-135 microscope, which was modified into a THG microscope. The experimental setup is shown schematically in Fig. 1. The laser source is a synchronously pumped OPO (Spectra-Physics Tsunami-Opal system) which provides 130 fs pulses at a wavelength of 1.5 μm at a repetition rate of 80 MHz. The laser beam is coupled through one of the microscope ports and is focused into the sample by the microscope objective. The focal point is scanned in the x-y plane using two optical scanners, and along the z-axis using the motorized stage of the microscope. The third harmonic light at the wavelength of 0.5 μm is collected by the original condenser (NA=0.63) and measured by a photomultiplier tube (PMT, Hammamatsu R4220) after filtering out the fundamental wavelength using a band-pass interference filter (Center wavelength 500 nm, FWHM = 25 nm). We used a condenser with 5 mm working distance, which allow access to the sample, a feature that is most desirable when dealing with monitoring complex biological experiments. The current generated by the photomultiplier was amplified, digitized and fed into a computer, which synchronizes the scanning process and the data collection.

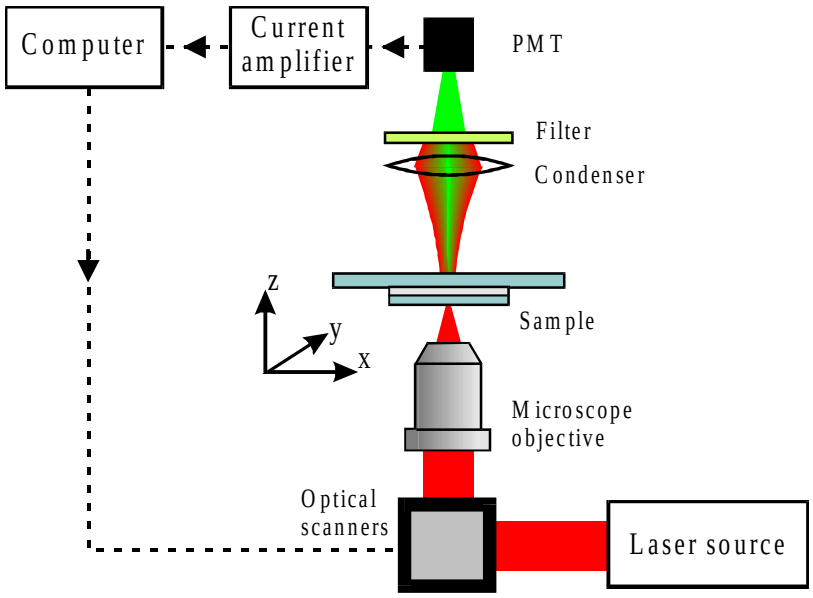


Fig. 1. Optical setup for laser scanning THG microscopy.

One of the main issues in biological imaging is possible damage to the specimen from the high intensity pulses. In the experiments reported below, the average laser power was 50 mW. Focusing the beam into an area of 1 $\mu m^2$, using the strongest microscope objective (NA=1.4oil) results in a peak intensity of 0.5 $TW/cm^2$, which is still below the threshold for ionization of atoms or molecules [15]. When the focused beam is illuminating a single point for a few seconds, local heating often damages the sample. However, we have verified that scanning the sample at high rates eliminates this problem. Images composed of 300x300 pixels were taken in 30 seconds, i.e. only 0.3 ms per pixel. During consecutive scans we could not observe any changes in the sample. These results agree with the observations of Squire *et al*. [13]. The upper power limits due to heating or ionization damage and the lower power limit, in which the THG signal becomes too weak for useful imaging, leave a fairly narrow interval of power that can be used for practical THG microscopy.

#13710 - $15.00 US
Received September 27, 1999; Revised October 04, 1999

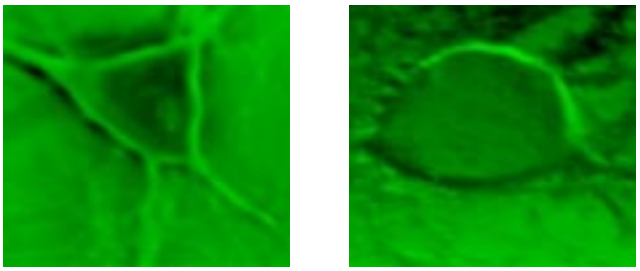

Fig. 2. THG images of neurons in a cell culture. The size of the cell's soma is about 15 μm.

We first present THG images of single neurons. The study of single neurons and small networks of neurons in the central nervous system is one of the main tools of neurobiology. High-resolution, non-destructive imaging of the live neurons and their structures without any staining or markers can be very helpful.

Shown in Fig. 2 are THG images of two neurons, taken with NA=0.6 (Left) and NA=1.4oil (Right) microscope objectives. These neurons were grown in a culture of cells from a mice brain, and were fixated and prepared on a microscope slide. The neuron's soma (cell body), the dendrites and the axon can be seen clearly. The plane in which these images were taken was chosen to be very close to the bottom of the cell. The shadowing effect seen in both images is caused probably by the varying separation between the lower membrane of the neuron and the glass substrate. Since the amount of THG is very sensitive to the presence of more than one interface in the focal depth, very small variations, of about 0.5 μm, in the distance between the glass and the bottom of the cell, can induce constructive or destructive interference, and create this "shadowing" effect. This effect looks quite similar to the shadowing effect which is characteristic to DIC microscopy, and it could help to enhance the image in certain cases.

The ability of the microscope to obtain three-dimensional images of *live* neuron soma is demonstrated in Fig. 3. Twelve consecutive horizontal sections along the axial direction (z-axis), separated 0.5-μm apart, are shown. The top-left section was taken from the bottom of the soma, while the bottom right image is from its very top. The neurons were grown on a layer of Glia cells on a thin cover glass. A strong THG signal from the Glia cells can be seen at the lower sections. Note that very strong THG signals from certain organelles within the cell saturate the detector. These organelles give rise to THG that is 3 orders of magnitude stronger than the background signals from the cytoplasm, which is less than 10 detected photons per pixel. A large variety of organelles in the soma can be observed. The nucleus is the dark round region inside the soma. The nucleolus is easily observed inside the nucleus. We could not observe any kind of change or damage to the cell even after tens of scans, although the effect of the laser beam on a living cell should be studied more carefully.

By exploiting the axial resolution of THG microscopy, it is possible to obtain 3-D structures of thick specimens. Shown in Fig. 4 is a computer generated vertical section from the middle of the neuron in Fig. 3. This section shows the location of the different organelles in the depth of the cell, as well as the shape of the cell membrane.

#13710 - $15.00 US Received September 27, 1999; Revised October 04, 1999

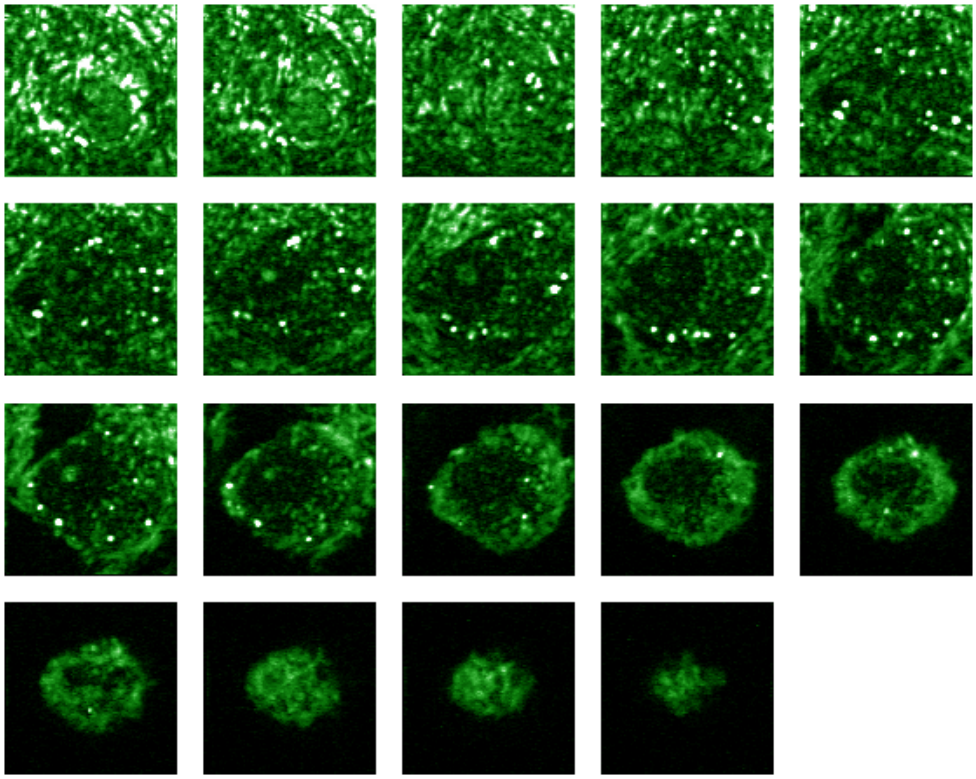

Fig. 3. Sectioning of live neurons in a cell culture. Each image is a horizontal section of the neuron's soma. The sections are separated by 0.5 µm, where the top-left section is closer to the glass substrate and the bottom-right section is the top of the cell. The dimensions of each image are 20 x 20 µm.

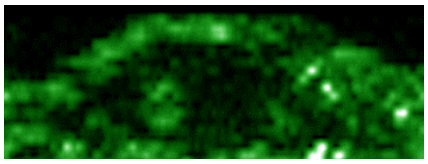

Fig. 4. Vertical sectioning of the neurons in Fig. 3. The bright nucleolus, the dark nucleus and organelles outside the nucleus can be seen.

#13710 - $15.00 US
Received September 27, 1999; Revised October 04, 1999

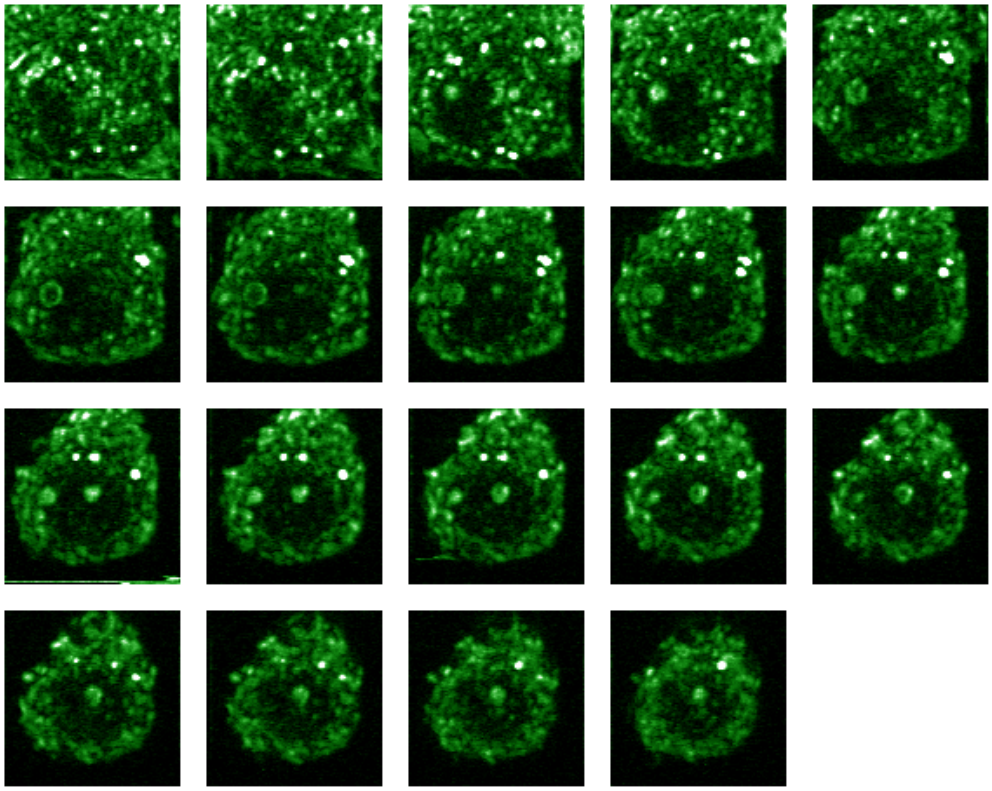

Fig. 5. Sectioning of live neurons. This cell has two nucleoli. The dimensions of each image are 20 x 20 μm.

Shown in Fig. 5 is another series of z-sections of a different neuron. These sections are separated by 0.5-μm. In this neuron the nucleus appears to be in the upper region of the soma. Two nucleoli are observed in different planes.

The dendritic spines are the primary loci of synaptic interaction between neurons. These are very small organelles, at the threshold of optical resolution, extending about 0.5-1 μm from the dendrite. THG microscopy allows the imaging of dendritic spines without any fluorescence labeling. Shown in Fig. 6 are three dendritic spines on a single dendrite. Although these spines are extremely small and are practically invisible by standard phase techniques, here their size and shape is clearly resolved. In this case third harmonic is generated not as a combination of interfaces, as in the case of large objects, but by being the

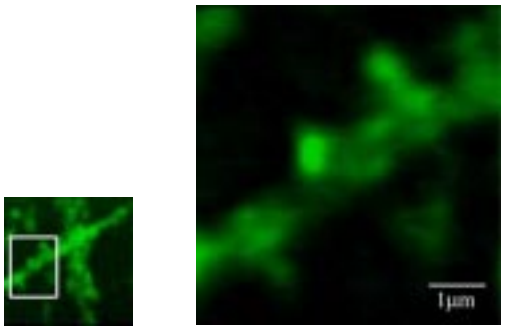


Fig. 6. THG images of three dendritic spines on a single dendrite.

#13710 - $15.00 US Received September 27, 1999; Revised October 04, 1999

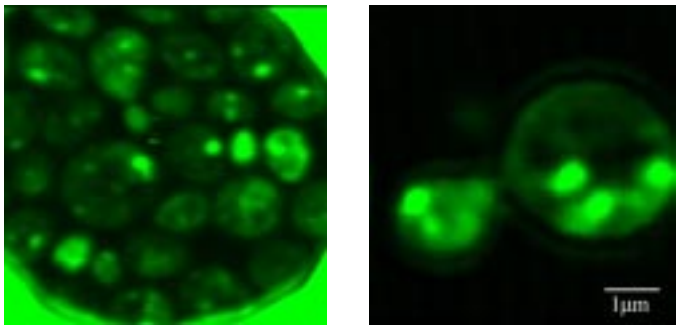

Fig. 7. THG images of yeast cell.

spine itself a small inhomogeneity at the focal volume.

In Fig. 7 we present THG images of yeast cells. Yeast cells are small (3-4 μm in diameter). The images clearly show the cells, as well as organelles inside the cells. Yeast cells in the middle of the budding process are magnified in Fig 7 (Right). Internal organelles and the cell's membrane are clearly noticed. We note that some organelles in these yeast cells appear much brighter than the rest of the structure.

## 3. Conclusions

In conclusion, laser scanning THG microscopy is shown to yield clear three-dimensional images of cellular and sub-cellular transparent biological structures. We demonstrated that this method could be used to identify even minute biological organelles, at the threshold of optical resolution. When an organelle is very small, and if its molecular structure lead to high $\chi^{(3)}$, it may be detected even when its size is below optical resolution. This means that THG microscopy is potentially capable of locating submicron-size organelles, similar to the techniques of fluorescence labeling.

High repetition rate short-pulse laser is used with peak intensities that are slightly below the ionization damage threshold of organic molecules. No observable damage to the inspected samples was observed. The live neurons appear to be unaffected, although the effect of the high intensity illumination on the function of the cells should be examined more carefully. We believe that the results presented here further demonstrate that laser scanning THG microscopy is a promising general-purpose imaging technique with many possible application in biological imaging.

## Acknowledgments

We thank M. Segal and E. Korkotian for supplying the neuron samples, S. Silberberg for the yeast samples, and for their help in image interpretation. We also thank D. Leibovitz and J. Shopen for their valuable technical assistance. This research was supported by the Israeli Ministry of Science and Technology and by the Minerva Foundation.

#13710 - $15.00 US Received September 27, 1999; Revised October 04, 1999