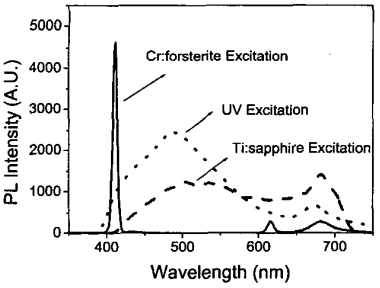
**CTuR2** **3:30 pm**

### Multi-modality non-linear microscopy

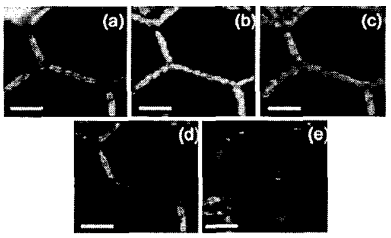
Chi-Kuang Sun, Shi-Wei Chu, I-Shiu Chen, Tze-Min Liu, Ping-Chin Cheng,* Bai-Ling Lin,**
*Graduate Institute of Electro-Optical Engineering, National Taiwan University, Taipei, 10617 Taiwan, R.O.C.; Email: sun@cc.ee.ntu.edu.tw.;*
**Department of Electrical Engineering, State University of New York, Buffalo, NY 14260–2050, USA; **Institute of Molecular Biology, Academia Sinica, Taipei 11529, TAIWAN, R.O.C.*

Two-photon fluorescence microscopy (TPFM) meets wide applications in biology with high axial resolution without using a confocal aperture.[1] In TPFM, strong fluorescence emission is restricted to the vicinity of the focal point with the advantage of minimum out-of-focus absorption, photo-bleaching and damage. The use of near infrared (NIR, generally around 800 nm) leads to deeper penetration in most biological specimens than conventional light sources, providing an opportunity for tissue imaging. Fluorescent stains are generally required for imaging of biological structures except when cellular organelles or contents are auto-fluorescing. However, this widely accepted microscopic technique requires in-focus non-linear absorption in order to stimulate fluorescence emission, which induces in-focus photo-damage to both fluorescing and non-fluorescing absorbers. Different from most animal tissues, plant material has significantly higher linear absorbance in the 700–800 nm. Such high absorption makes the use of NIR in TPFM not much advantageous from single-photon-fluorescence microscopy in terms of depth penetration. Moreover, the photo-damage in multi-photon mode is significantly worse than single-photon mode. The photo-damage caused by NIR points to the need of finding alternative light source and imaging modality. Studies on human skin[1] and plant tissues[2] indicated that the light attenuation (absorption and scattering) in *live* biological specimen reaches a minimum around 1200–1250 nm. It is thus advantageous to develop alternative multi-photon microscopy based on a femtosecond 1200–1250 nm laser system for which Cr:forsterite is an ideal choice. Due to the low attenuation of biological sample in the 1200–1250 nm infrared range, Cr:forsterite laser not only achieves deep penetration in living samples, but also opens imaging modalities in second-harmonic-generation (SHG), in the red wavelength, and in third-harmonic-generation (THG), in the blue wavelength. The marked advantage of virtual state transition in SHG/THG makes no energy deposition during the process, thus no photo-damage and bleaching are resulted. In addition, both visible and NIR regions are open to efficient detection while the pump wavelength is far away from the sensitivity range of the silicon-based detectors. Furthermore, the use of long illumination wavelength dramatically reduces the background auto-fluorescence and photo-damage.

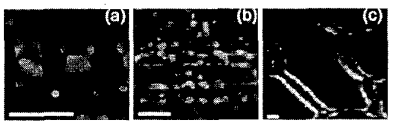
Figure 1 is an emission spectrum of parenchyma cells in the stem of maize (*Zea mays* L., var. Ohio43) excited by 365 nm UV light (dotted line), 810 nm femtosecond light from a Ti:sapphire laser (dashed line), and 1230 nm femtosecond light from a Cr:forsterite laser (solid line). Board-band auto-fluorescence is evident for both UV and NIR excitation. In contrast, when 1230 nm femtosecond light was used, a weak residual red auto-fluorescence and a prominent SHG signal centered at 615 nm and THG centered at 410 nm were observed. Figure 2 shows (a) THG, (b) SHG and (c) TPF images obtained from parenchyma cells in a transverse sectional view of maize stem taken at 63 μm from the surface of the specimen using a 110 MHz femtosecond Cr:forsterite laser operating at 1230 nm. Three images obtained from different modalities were combined and shown in false color(d). The multi-modality longitudinal sectional image of the same region was also shown (e). Figure 3 shows multi-modality images (THG: blue, SHG: green and TPF: red) from adaxial surface of rice (*Oryza sativa* L.) leaf and from abaxial epidermal peel of onion (*Allium cepa* L.) bulb. Since THG signal is generated in the region with optical inhomogeneity,[4] THG picks up structures such as thin transverse walls and cuticular papillae. On the other hand, SHG process picks up optical interfaces and optically active structures, where centro-symmetry is broken.[5] Cell walls, both primary and secondary, consist of highly organized cellulose micro-fibrils and are generally birefringent. Such high-order organization behaves similarly to photonic crystals and may accounts for the SHG (Fig.3b,c). The silica dioxide deposition commonly found in the silica cells in many grasses is also birefringent and is responsible for the strong SHG signals (Fig. 3a).




**CTuR2 Fig. 1.** Emission spectrum of parenchyma cells in maize stem. The specimen was excited by 365 nm UV light (dotted blue line), 810 nm femtosecond light from a Ti:sapphire laser (dashed green line), and 1230 nm femtosecond light from a Cr:forsterite laser (solid red line).




**CTuR2 Fig. 2.** (a) THG, (b) SHG, and (c) TPF images of parenchyma cells in transverse optical section of maize stem taken with a Cr:forsterite laser operating at 1230 nm. Multi-modality image in RGB false color (blue: THG, green: SHG, red: TPF) is shown in (d). (e) shows longitudinal sectional view taken in the same sample. Scale bar, 50 μm.




**CTuR2 Fig. 3.** Multi-modality images in RGB false color (blue: THG, green: SHG, red: TPF) taken from rice leaf epidermis, showing (a) silica cells (dumbbell-shaped cells, left), and (b) epidermal cells in adaxial furrow and the adjacent mesophyll cells, and from (c) abaxial epidermal peel of onion bulb. Scale bar, 50 μm.

### References

1. W. Denk, J.H. Strickler and W.W. Webb, "Two-photon laser scanning fluorescence microscopy," Science **248,** 73–76 (1990).
2. R.R. Anderson and J.A. Parish, "The optics of human skin," J. Invest. Dermatol. **77,** 13–19 (1981).
3. P.C. Cheng *et al.*, "Highly efficient upconverter for multiphoton fluorescence microscopy," J. Microscopy **189,** 199–212 (1998).
4. Y. Barad, *et al.*, "Nonlinear scanning laser microscopy by third harmonic generation," Appl. Phys. Lett. **70,** 922–924 (1997).
5. J.N. Gannaway and C.J.R. Sheppard, "Second-harmonic imaging in the scanning optical microscope," Opt. Quantum Electronics **10,** 435 (1978).

**CTuR3** **3:45 pm**

### Imaging intracellular $Ca^{2+}$ dynamic with Third Harmonic Generation microscope

L. Canioni, S. Rivet, L. Sarger, and R. Barille, *Centre de Physique Moléculaire et ondes Hertziennes, 351, Crs de la libération, 33405 Talence Cedex, France*

Pierre Vacher, *Laboratoire de Physiologie et Physiopathologie de la Signalisation, 146, rue Léo Saignat, 33076 Bordeaux Cedex*

Pierre Voisin, *Laboratoire de résonance magnétique des systèmes biologiques, 146, rue Léo Saignat, 33076 Bordeaux Cedex*

The validity of the Third Harmonic Generation (THG) microscopy for imaging has been shown and its potential use in material science and more precisely in biology.[1–2] All these studies have demonstrated that THG microscopy is a method which has the advantage that no labeling is necessary.

In order to develop further imaging instruments, biological dynamic events has to be measured and especially $Ca^{2+}$ dynamics which is a special importance in cellular biology. For the biological experiments, we have used a human astrocytome cell line: U-87 MG.

Intracellular calcium increase was obtained using thapsigargin, a cell permeable inhibitor of the endoplasmic reticulum calcium-ATPases. The calcium-ATPases enable the endoplasmic reticulum filling. As they are inhibited, the endoplasmic reticulum releases its calcium ions so that the calcium concentration increases in the cytosol.[3] The effect of 10 μM thapsigargin has been analyzed as a function of time. The thapsi-