Two-photon fluorescence microscopy (TPFM) meets wide applications in biology with high axial resolution without using a confocal aperture. 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; 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 and plant tissues 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.

Figure 1 shows 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 Tisapphire 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 65 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 in-homogeneity, 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. 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. 3c). 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).

References