by algorithms to remove out-of-focus signals in wide-field fluorescence microscopy (McNally *et al.*, 1999). Compared to wide-field fluorescence microscopy, which works well with relatively thin specimen (<30 μm), both confocal and multi-photon mode microscopy yield crisp images with much thicker samples (Hutzler *et al.*, 1998; Feijo and Moreno, 2004). In addition to localisation of substances, fluorescence microscopy of leaves can yield information about the concentrations of substances as demonstrated in confocal fluorescence microscopic studies on photosystem distribution in leaves from C_4 photosynthesis plants (Pfündel and Neubohn, 1999).

Microscopic analysis of leaf optical properties can be performed by non-destructive observation of the adaxial and abaxial surfaces, as well as by imaging leaf sections: confocal laser scanning microscopy was employed to investigate, non-destructively, the blue–green fluorescence of UV-absorbing phenolics in barley leaves to a depth of 35 μm (Hideg *et al.*, 2002); UV-induced fluorescence microscopic imaging of surfaces of intact wheat leaves was used to evaluate the contribution of epidermis cells and sclerenchyma bands to blue–green emission (Meyer *et al.*, 2003); and fluorescence microimage analysis of leaf surfaces was employed for *in situ* counting of stomata (Karabourniotis *et al.*, 2001).

Photobleaching and photodamage, however, are important limitations in the use of fluorescence microscopy to study living structures since the radiation, used to excite fluorescence, often forms free radicals that react with cellular constituents. Moreover, fluorophores can undergo molecular degeneration or rearrangement to form non-fluorescent species or may produce singlet oxygen, which will efficiently react with nearby bio-molecules. Multiphoton microscopes overcome most of these disadvantages because of their limitation of fluorophore excitation and photodamage to a relatively small focal volume (Diaspro and Sheppard, 2002).

When determining the intracellular location of compounds, non-ruptured cells are required for microscopy and, therefore, cross-sections of at least 50–100 μm thickness are essential. Longitudinal sectioning is required to get intact epidermal cells of monocotyledonous species that can be 200–2000 μm long (Wenzel *et al.*, 1997). Paradermal sections are particularly useful since they show intact cells from the epidermal and mesophyll compartments on the same image (Hutzler *et al.*, 1998).

Micro-spectrofluorimetry, which provides spectrally resolved information on microscopic areas, can identify individual compounds in the presence of several different fluorophores (Schnabl *et al.*, 1986; Opitz *et al.*, 2003) and provides the basis for selection of appropriate acquisition bands for multi-spectral imaging. Multi-spectral imaging, in which spectral information is added to each pixel, is a new and promising technology to identify auto-fluorescent plant molecules and to study their function (Berg, 2004). The introduction of highly sensitive CCD (charge coupled device) detectors permits significant reduction of acquisition times (Agati *et al.*, 2002; see Figure 6.2) and, therefore, ensures wider use of multispectral imaging in the future. Recently, multispectral imaging was successfully used in a study of the intracellular location of polyphenols in species of the Mediterranean basin