3D CLEM: from Morphology to Membranes

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Keywords: correlative light and electron microscopy (CLEM), 3D imaging, FIB-SEM

Correlative light and electron microscopy (CLEM) has become a powerful tool in life science. The idea is to collect data of different information levels from an identical sample, starting with a general histological description of the specimen down to protein identification via antibody labeling on the electron microscopic level. TEM tomography and recently focused ion beam-SEM (FIB-SEM) have opened up new possibilities to expand morphological context description into to the third dimension at nanometer scale.

Sample preparation for correlative 3D microscopy requires not only optimal structural preservation but a combination of fluorescence and heavy metal staining suitable for each of the microscopy modes: CLSM, TEM, and FIB-SEM. The latter is especially challenging as the sample is investigated en-bloc, and post-staining as usually applied to sections is impossible. This has become available with the establishment of a protocol for freeze-substitution (FS) after high pressure freezing (HPF), including fluorophores, and subsequent embedding in HM20 resin [1]. This not only enables the pre-selection of a region of interest (ROI) but also a 3D reconstruction of the resin-embedded sample by CLSM prior to investigating the identical ROI by EM. For FIB-SEM applications, the addition of osmium tetroxide during FS is indispensable, as uranyl acetate staining [2] alone is not sufficient for imaging membranes with this technique [3].

This method is applicable to a wide variety of biological specimen, e.g. cell culture, tissue, small organisms such as nematodes, as well as plant samples. Figure 1 shows an example of legume root nodules colonized with nitrogen-fixing bacteria (*Bradyrhizobium japonicum*), a symbiosis providing the host plant with nitrogen, which in return provides nutrients for the bacteria. The colonization process of root nodules by wild-type *B. japonicum* and two mutant strains was investigated.

200 µm thick sections of mungbean root nodules (Fig. 1A) were frozen by HPF and stained with acridine orange (Invitrogen) during FS. A clean blockface was cut perpendicular to the section (Fig. 1B), and an ROI was chosen by CLSM (Fig. 1C) including cells filled with bacteria as well as not yet colonized cells. Cells devoid of bacteria show no fluorescence or only few spots, as they usually comprise large vacuoles. The corresponding volume recorded by FIB-SEM proves the direct correlation of the fluorescence signal with the occurrence of bacteria or large vesicles in the cells (Fig. 1G). The fluorescence from the cell walls is rather attributed to an autofluorescence of the plant material. The FIB-SEM images and the 3D reconstruction of this volume (Fig. 1D-F) illustrate the intracellular organization of the bacteria in symbiosomes surrounded by a peribacteroid membrane [4], which is not visible by CLSM. Within the cytoplasm of the bacteria substructures can be distinguished, showing electron dense domains of various contrasts, hence revealing an unknown interior structuring of the bacteria cytoplasm (Fig. 1H).

For further investigation at even higher magnification, ultrathin sections for TEM tomography of the very same sample can be prepared before or even after the FIB-SEM

M.A. Pabst, G. Zellnig (Eds.): MC2009, Vol. 2: Life Sciences,
DOI: 10.3217/978-3-85125-062-6-178, © Verlag der TU Graz 2009

investigation. This would enable a complete 3D characterization of a certain structure from millimeter to nanometer resolution levels.

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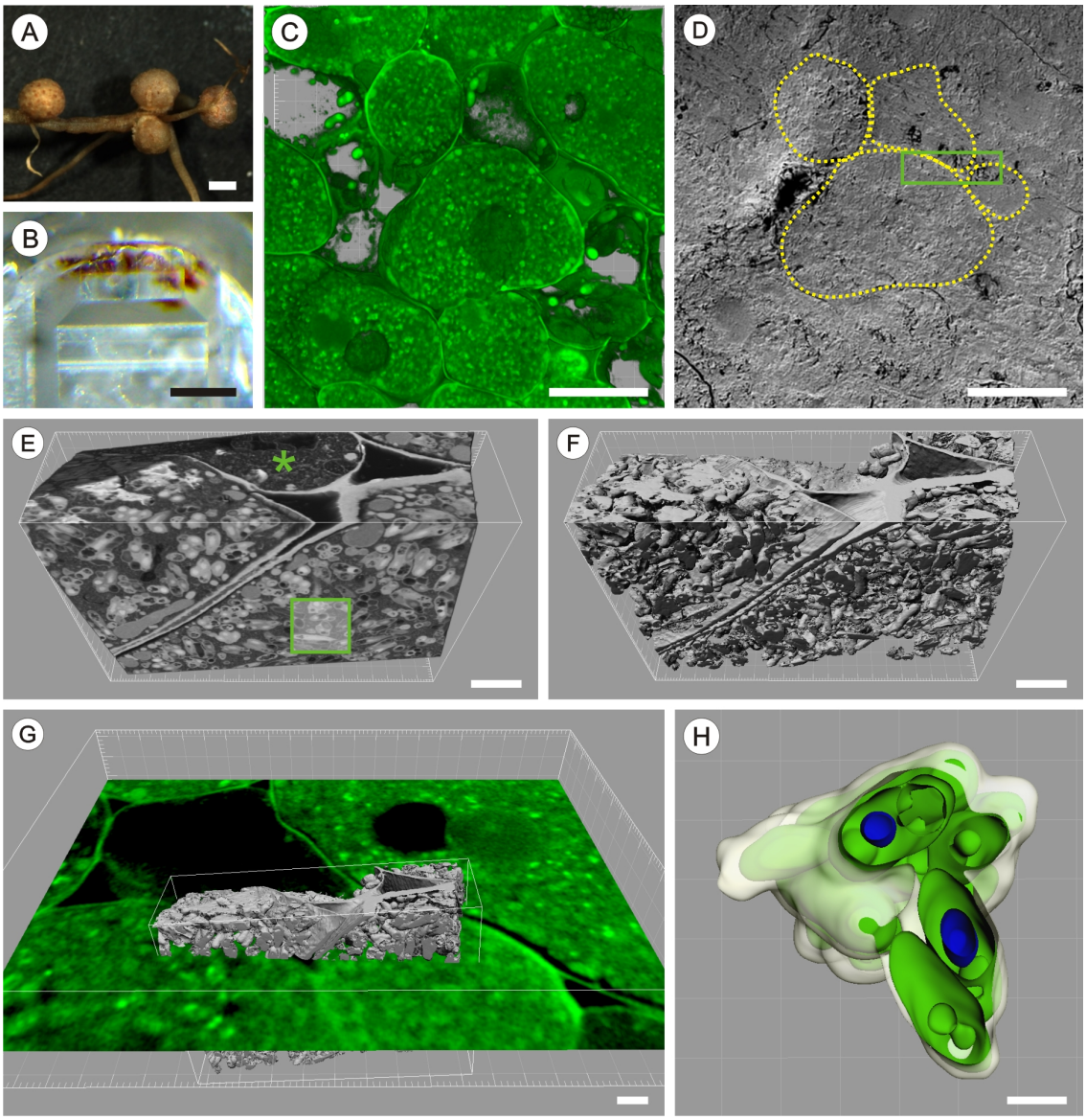

Figure 1. (A) Legume root nodules and (B) blockface of the HM20 embedded sample, stained with acridine orange. (C) 3D projection of a CLSM stack and (D) corresponding SEM image of the sample surface. The area where the FIB-SEM stack was recorded is marked in green. (E) The 3D projection of this stack shows cells densely filled with bacteria, and one cell which is not colonized (marked by asterisk). (F) Isosurface of the cells and included bacteria created by thresholding. (G) Matching the isosurface of the FIB-SEM stack with a single CLSM image of the corresponding stack demonstrates the correlation of the fluorescence signal with the occurrence of bacteria or large vesicles in the cells. (H) 3D reconstruction of a symbiosome, comprising several bacteria (green) within the surrounding peribacteroid membrane (white). Within the bacteria various electron dense areas were observed (blue). *Bars: (A) 2 mm, (B) 1 mm, (C)+(D) 20 µm, (E)-(G) 2 µm, and (H) 500 nm.*

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