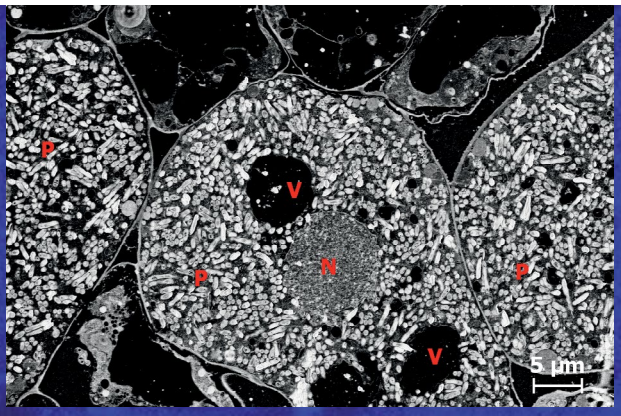
White Paper



Multiscale Analysis of Bacteria Population in Legume Root Nodules with "Shuttle & Find"

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Multiscale Analysis of Bacteria Population in Legume Root Nodules with "Shuttle & Find"

Authors: Dr. Christian Thomas
Carl Zeiss AG, Corporate Research and Technology, Germany
Dr. Miriam Lucas, Dr. Roger Wepf
EMEZ, ETH Zurich, Switzerland

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Introduction

Legumes are an important protein supplier, especially in developing countries. Therefore, the enhancement of efficiency in legume cultivation would help to improve the food supply in those areas. Very important for the growth of legumes are symbiotic bacteria living inside of so-called root nodules (Figure 1). The rhizobia bacteria reside there and live in an endosymbiotic relationship within the root cells. Rhizobia have the ability to convert atmospheric nitrogen into ammonia, a nitrogen compound which can be utilized by the plant. In return, the host plant provides the rhizobia with nutritive compounds. In root nodules, high levels of the plant-derived oxygen-binding protein leghaemoglobin are present which ensures a microaerobic environment that is critical for the physiology of rhizobial nitrogen fixation [1].

Figure 1: *Legume plants (a) often show so-called nodules at their roots (b) where rhizobia bacteria reside and live in an endosymbiotic relationship with the plant.*

For a better understanding of infection and colonization of the legume hosts by their rhizobial symbionts Correlative Light and Electron Microscopy (CLEM) is very useful as it allows combining the information of an overview in Fluorescence Light Microscopy (FLM) with structural details in the Scanning Electron Microscope (SEM). While FLM gives an overview which and how many cells are infected with bacteria, SEM images show the intracellular distribution and relationship of those bacteria in subclusters. The higher resolution allows to see membranes and hence the organization of these bacteria in membrane-enclosed subclusters (symbiosomes). Therefore CLEM enables statistic analysis over several cells in the large context of the root nodule tissue. For that reason, CLEM becomes an essential tool for the collection of relevant data in systems biology approaches.

Sample Preparation

The sample preparation was performed according to the protocol described in [2]. Root nodules of a mung bean plant *(Vigna radiata)*, inoculated with the rhizobia *Bradyrhizobium japonicum*, were fixed by high-pressure freezing and afterwards stored in liquid nitrogen until further processing.

Then freeze-substitution was performed in acetone with 2% uranyl acetate and 1% osmium tetroxide (28 h at -90° C, 8 h at -60° C and 4 h at -35° C). Uranyl acetate and osmium tetroxide are used as fixatives which preserve ultrastructure and lipid composition in the sample and simultaneously work as heavy metal stains to enhance SEM contrast.

After freeze-substitution, the sample was washed twice with acetone (1 h at -35° C each) and then once with ethanol (1 h at -35° C). For embedding, the samples were infiltrated with HM20 (Polysciences) in ethanol (30 % for 2 h at -35° C, 70 % for 2 h at -35° C, and twice in 100 %, overnight and for 2 h at -35° C), followed by UV-polymerization in fresh pure HM20 for at least 24 h at -35° C.

After curing of the resin, 70 nm ultrathin sections were cut from the sample using an ultramicrotome equipped with a diamond knife (Diatome). The sections were transferred onto cover slips coated with ITO (Indium Tin Oxide, 8 Ω/sq). This coating reduces charging in SEM, while a high optical transparency is still assured. Now the sections were stained with DAPI for 15 min to enable FLM imaging, followed by post-staining with uranyl acetate and lead citrate for SEM imaging.

Imaging

The cover slip was placed into the sample holder especially designed for CLEM by ZEISS. This holder can be used in LM as well as SEM so that the sample is stably fixed in the holder during the whole imaging process. The holder has three fiducial markers which define a coordinate system that can be calibrated very fast and semi-automatically in the Shuttle & Find module of the AxioVision Software.

FLM of the sections was performed with Axio Imager.M1 (Carl Zeiss Microscopy GmbH) using a 40 x objective (EC Plan-Neofluar 40 x/0.75 M27) and a filter set with 365 nm excitation and 445/50 nm emission (Filter set 49). The microscope was equipped with an AxioCam MRm and an exposure time of 1000 ms was set for imaging. Regions of interest (ROIs) were defined and selected in the fluorescence image.

Then the sample was transferred to a GEMINI 1530 FE-SEM (Carl Zeiss Microscopy GmbH). After the semi-automatic calibration of the sample holder and subsequent fine calibration the ROI imaged in the FLM was relocated within a few seconds at a precision below 5 µm. SEM imaging was done at an acceleration voltage of 2 kV with the in-lens secondary electron detector.

Results

A widefield FLM image of an ultrathin section of a root nodule area is shown in Figure 2. The bacteria show DAPI fluorescence, the fluorescence signal of the cell walls is rather attributed to autofluorescence. Thus, particular root cells infected with bacteria can clearly be identified and the cellular assembly can be analysed. A ROI is chosen containing a cell filled with bacteria adjacent to uninfected cells.

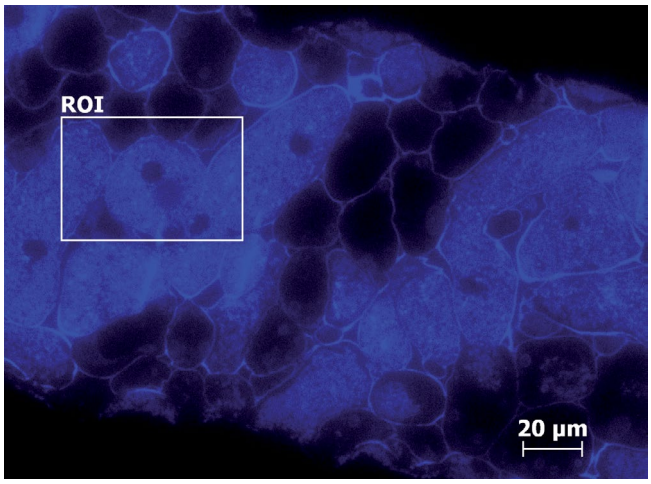

Figure 2: *FLM image of a 70 nm section of a mung bean root nodule showing cells with and without bacteria infection; ROI is indicated by frame.*

The selected ROI is displayed in more detail in Figure 3: Figure 3a shows the enlarged ROI of the fluorescence image, while the corresponding SEM image is presented in Figure 3b. Structure, orientation and intracellular organization of the bacteria in symbiosomes can clearly be observed. Also other cell compounds can be precisely allocated in comparison of FLM and SEM images, e.g. the nucleus showing a weak fluorescence or vacuoles. Such nuances cannot be worked out from the FLM image.

Furthermore the high signal-to-noise ratio of the SEM images allows segmentation of specific structures enabling semiautomated collection of statistical data e.g. to compare the bacteria density in different cells.

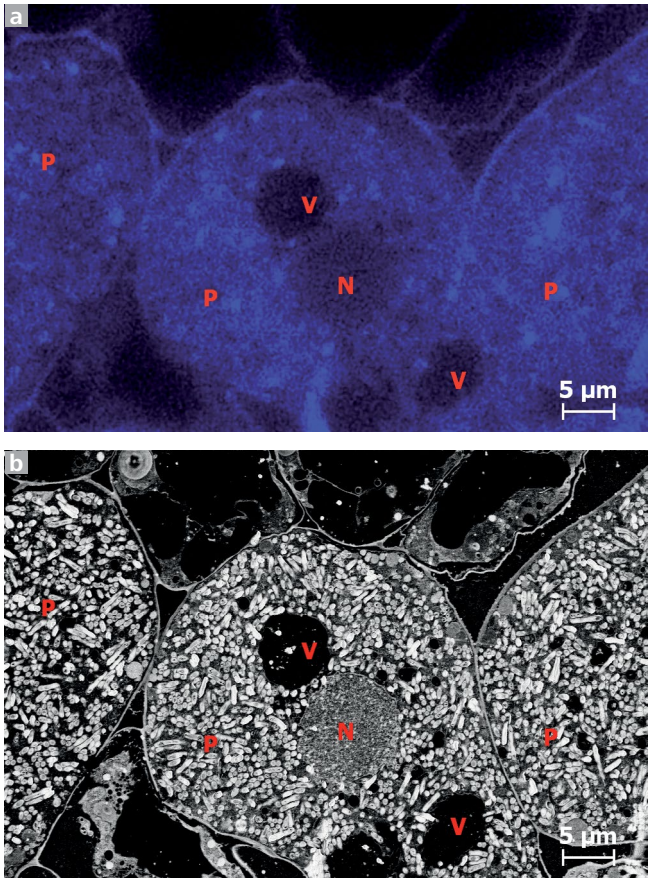

Figure 3: *FLM (a; enlarged) and SEM (b) images of ROI selected in Fig. 2. Bacteria population (P), cell nucleus (N) and vacuoles (V) can clearly be identified.*

Conclusion and Outlook

The Shuttle & Find interface for CLEM enables fast and reliable analysis of the bacterial infection of legume root nodule cells. Ultrathin sections of resin embedded root nodule samples are stained with fluorescent dyes and imaged in FLM as well as in SEM. FLM images provide an overview of the plant tissue showing the grade of infection of the cells. At the same time SEM imaging of the same ROI gives ultrastructural information, e.g. number and intracellular context of the bacteria.

With Shuttle & Find the workflow is sped up significantly as the process of searching the same ROI in both microscopy systems is now automated. Therefore also serial section imaging can be performed within a moderate time allowing reconstruction of the 3D organization of biological systems. Further, this solution enables new possibilities especially for statistical data analysis in microscopic images which can now be carried out systematically.

Acknowledgements

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References

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Carl Zeiss Microscopy GmbH
07745 Jena, Germany
BioSciences
microscopy@zeiss.com
www.zeiss.com/microscopy

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