MICROWAVE ASSISTED PROCESSING OF PLANT CELLS FOR OPTICAL AND ELECTRON MICROSCOPY

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The technological development of microscope hardware has led to a significant breakthrough by pushing the resolution from the micro to the nano-scale range. During the past century high end electron, optical and atomic force microscopes have been developed for better ultra-structural viewing of the specimen at the atomic level, including 3D visualization. Compared to these impressive hardware developments, progress in specimen processing techniques has been far less dramatic. Despite the increased use of living material in microscopy, numerous applications still require specimens to be fixed and bench time is a major constraint in any kind of sample preparation. The first and one of the most important steps in biological tissue processing for microscopy is fixation. Depending on the specimen, times for chemical fixation may range from half an hour to as much as 24 hours. The entire procedure required for processing biological samples for transmission electron microscopy (TEM) can therefore take between a few days and a week. While this time is necessary to completely fix, dehydrate and embed the specimen, it also allows for unintended processes to occur such as the dissolving of membranes leading to the release of organelle content. This can result in the dilution of the applied solutions and to alterations in their pH value (Russin and Trivett, 2001) which in turn may generate structural artifacts. The fixation time is even more critical in the case of immunohistochemistry where antigen preservation is crucial for successful labeling. Therefore, efforts to minimize fixation and processing times do not only have the aim to reduce bench time but also to prevent structural impact on the tissue.

In the 1970s, the use of microwave (MW) ovens was first introduced to accelerate sample processing (Mayers, 1970; Demaree and Giberson, 2001). The first report on MW-assisted aldehyde tissue fixation for the purpose of light microscopy and TEM was made in the 1980s (reviewed by Giberson, 2001). The early MW ovens had no control of power or temperature and the only variable was the exposure time. Currently, MW ovens are available with variable power (wattage), temperature control and MW transparent vacuum system.

Because of the presence of cell walls, vacuoles, plastids and intracellular air space (Russin and Trivett, 2001), plant cells generally require rather prolonged incubation times with fixation and dehydration solutions and thus would profit greatly from an accelerated protocol. In the present study we aimed at the optimization of MW-assisted sample processing for single plant cells for both optical and electron microscopy. We used leaf trichomes and pollen tubes as test specimens. The former are cells differentiated from the leaf epidermis. They stick out from the leaf surface into the air space and are thus not mechanically stabilized by any surrounding tissue. The latter are cellular protuberances formed by germinating pollen grains upon contact with a receptive stigma. Their function is the generation

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of the two sperm cells and their delivery to the ovary to ensure double fertilization. Pollen tubes are commonly used as a model for the study of anisotropic cell growth and also to understand the structural dynamics and material properties associated with polarized cellular expansion (Geitmann, 2006; Geitmann and Steer, 2006). Due to their extremely rapid growth and active intracellular transport processes, high quality fixation of pollen tubes is critical for the preservation of cellular ultrastructure and polarity. We optimized two important variables critical for MW-assisted sample preparation: wattage, correct adjustment of which is responsible for tissue stabilization, and exposure time, which is sample and treatment dependent (Russin and Trivett, 2001). During experiments, sample temperature was monitored to control the effect of heating (Demaree and Giberson, 2001), and vacuum was used for better infiltration of the cells with the fixation solution (Russin and Trivett, 2001). All experiments were carried out in a PELCO cold spot®.

OPTICAL MICROSCOPY

Due to the highly polarized mode of growth, the pollen tube cell wall has a characteristic spatial profile of non-uniformly distributed cell wall polysaccharides. The tip is composed of methyl-esterified pectins that permit, due to their plastic characteristics, pollen tube elongation at this location (Geitmann and Parre, 2004; Parre and Geitmann, 2005a). On the other hand, the basal part of the tube is composed of non-esterified, stiffer pectins and it is further characterized by the deposition of cellulose and callose (Chebli and Geitmann, 2007). The latter component plays a role in the mechanical resistance of the cell wall against tension and compression stress in the cylindrical part of the cell (Parre and Geitmann, 2005b). In longer pollen tubes, callosic plugs compartmentalize the cell allowing the older parts of the cell to degenerate. To sustain the active growth of the pollen tube, cell wall material is constantly added to the growing tip through the fusion of secretory vesicles which are transported to the growing zone via the highly dynamic actin cytoskeleton.

Visualization of both cell wall components and cytoplasmic structures such as the cytoskeleton can be performed by combining a specific label with fluorescence and confocal microscopy. Due to the extremely fast growth behavior and the polar distribution of cytoplasmic components by selective and directed cytoplasmic streaming, fixation of pollen tubes needs to be rapid for the ultrastructure reality. We tested several staining and immunohistochemical procedures to assess the efficiency and quality of MW-assisted fixation protocols on pollen tubes. Given that we have extensive experience with this cell type and its characteristic labeling profiles we were able to judge the quality of the samples obtained with the MW-assisted protocols comparing them to conventional chemical fixation and rapid freeze fixation.

METHODOLOGY

In the optimized protocol all steps were carried out in the microwave operating at 150 W under 21 inches of Hg vacuum at a controlled temperature of 26 °C ± 2 °C.

PECTIN LABELING

Pollen tubes were fixed for 40 seconds in 3% formaldehyde in phosphate buffer saline (PBS) solution. After 3 washes in PBS with 2% bovine
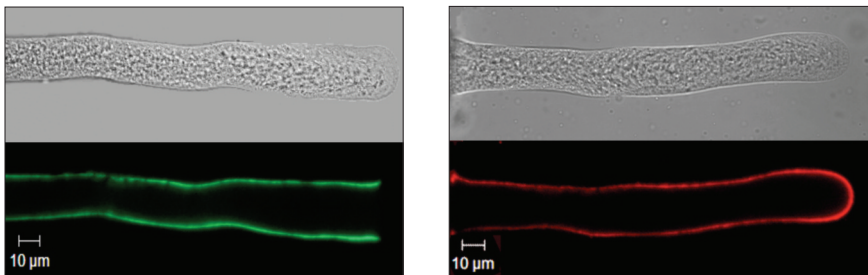
Figure 1: Immunofluorescence label of lily pollen tubes. (A) JIM 5 label of non-esterified pectins reveals the presence of the polymer in the distal region. (B) JIM7 label of esterified pectins is predominantly present at the apex. Pictures represent median pollen tube sections taken with a Zeiss LSM-510 META confocal microsocope.

serum albumine (BSA), they were incubated for 10 minutes in JIM5 (monoclonal antibody specific for pectins with low degree of methyl-esterification; Figure 1A) or JIM7 (monoclonal antibody specific for pectins with high degree of methyl-esterification; Figure 1B) followed by 3 washes of 40 seconds each in a 2% BSA solution. Tubes were then incubated for 10 minutes in Alexa 594 anti-rat secondary antibody (Molecular Probes), washed 3 times and mounted on glass slides for observations.

CALLOSE LABELING
Pollen tubes were fixed in 3 % formaldehyde in PIPES buffer for 40 seconds and washed 3 times in the same buffer. A 10 minute incubation with 0.2 % aniline blue solution was used to label callose. Samples were then washed 4 times in PIPES buffer before observation (Figure 2).

Figure 2: Callose rings observed with aniline blue staining in *Camellia* pollen tubes. These callose rings will develop into callose plugs. Picture represents a Z-stack projection taken with a Zeiss Imager-Z1 microscope equipped with a Zeiss AxioCam MRm Rev 2 camera.
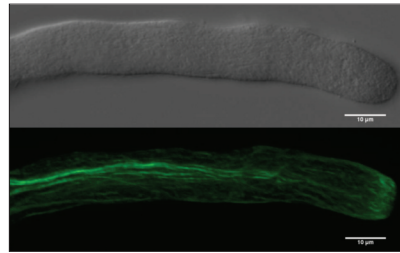
ACTIN LABELING
Pollen tubes were fixed for 40 seconds in a pH 9 PIPES buffer containing 3 % formaldehyde, 0.5 % glutaraldehyde and 0.05 % Triton. After 3 washes with the same buffer, the pollen tubes were incubated with rhodamine phalloidin for 10 minutes in a pH 7 PIPES buffer, washed 5 times and then observed immediately, since actin tends to be unstable even when fixed (Figure 3).

ELECTRON MICROSCOPY
TRANSMISSION ELECTRON MICROSCOPY
Sample preparation for TEM is very critical due to the necessity of preserving the cellular ultrastructure. Any inappropriate handling


Figure 3: *Camellia* pollen tube actin cytoskeleton as visualized by rhodamin-phalloidin label. Picture represents a Z-stack projection taken with a Zeiss Imager-Z1 microscope equipped with a Zeiss AxioCam MRm Rev 2 camera.

during fixation, dehydration or embedding will be flagrantly expressed in the observed samples as artifacts. MW technology has the potential to strongly reduce sample preparation time while preserving tissue subcellular integrity and antigenicity.

We optimized different conditions for microwave sample processing for pollen tubes. Application of a fixative consisting of 2 % formaldehyde and 2.5 % glutaraldehyde in a phosphate buffer (PB) to pollen tubes growing in a 0.5% agar medium for 40 seconds was sufficient for very good fixation. Post-fixation was performed using a 2 % osmium tetroxide solution following 3 washes in PB and 3 additional washes in deionized water. Samples were then washed twice with PB followed by two more washes in water. Dehydration was done using an increasing acetone gradient ranging from 25 to 100 % with the last step repeated thrice. All fixation, washing and dehydration steps were conducted using 150 W and 21 in of Hg vacuum for 40 seconds each.

For resin infiltration we used SPURR resin in 4 steps with increasing resin concentration up to 100 %. These steps were conducted at 300 W for 3 minutes each and under 21 in Hg vacuum. Resin polymerization was done in a regular oven at 64 ºC overnight. This polymerization can also be carried out in a water bath at 60 ºC, 70 ºC and 80 ºC for 10 minutes each then at 100 ºC for 45 minutes (Demaree and Giberson, 2001). Ultrathin sections were cut with a Leica Ultracut and samples were observed with a JEOL JEM 1005 transmission electron microscope operating at 80 kV (Figure 4).
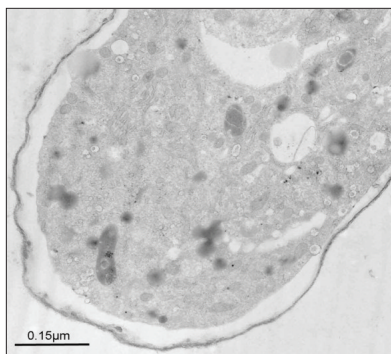
Figure 4: Transmission electron micrograph of a cross-section of a *Camellia* pollen tube. Picture was taken with a JEOL JEM 1005 transmission electron microscope operated at 80 kV.

SCANNING ELECTRON MICROSCOPY

Pollen tubes and trichomes were fixed and dehydrated using the same protocol as that for TEM sample preparation. Samples were then dehydrated, critical point dried, gold-palladium coated and observed with a JEOL JSM 35 (Figure 5).

RESULTS AND DISCUSSION

Plant cell fixation and processing for microscopical observations are a challenge due to the presence of cell wall, vacuoles as well as high internal turgor pressure. Osmotic changes upon the addition of a chemical fixation solution easily causes cellular collapse or bursting, or, less dramatically but nevertheless critical, spatial rearrangement of cytoplasmic contents (e.g. loss of polar distribution of organelles). The cell wall surrounding the plasma membrane hampers effective penetration of the fixative. Using MW-assisted protocols we succeeded in improving and accelerating sample processing while preserving the cellular integrity. Cytochemical labeling of callose in the pollen tube cell wall reproduced the same characteristic profiles as those described previously using conventional chemical fixation. While antigenicity was shown to be enhanced in some cases (Giberson, 2001), in our case, we noticed that it was preserved as revealed by immunolabelling for two different types of pectin. The actin cytoskeleton, a very dynamic and unstable structure, was preserved using MW-assisted fixation and resulted in a spatial configuration similar to that observed after freeze fixation, the gold standard for the quality of fixed plant cell cytoskeleton (Lovy-Wheeler et al., 2005). In electron microscopy, cell structure and subcellular compartments were well preserved when comparing with bench top processed plant cells. This was observed when comparing structural integrity of the subcellular components from pictures taken using both methods.

In addition to this, experimentation time was dramatically reduced using the MW-assisted methods (Figures 6 and 7). For optical microscopy, sample preparation time reduction varied from 5 fold for cytochemical labeling to 8 fold for actin labeling while time reduction for TEM sample preparation was 26 fold.

CONCLUSION

The use of MW-assisted protocols resulted in a dramatic reduction of experimentation time. More importantly, structural integrity and antigenicity were not compromised when comparing to conventional bench-top processing methods
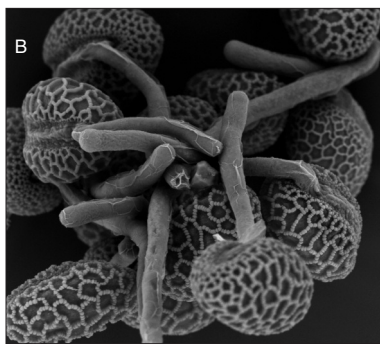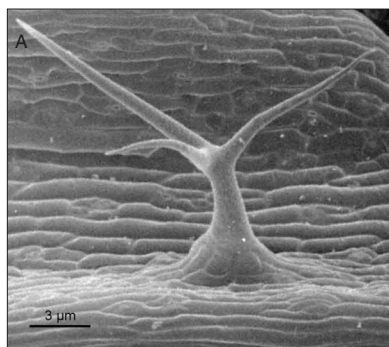
Figure 5: Scanning electron micrograph of an *Arabidopsis* leaf trichome (A) and germinated lily pollen grains (B). Pictures were taken with a JEOL JSM 35 (A) and a Hitachi TEM 1000 (B)
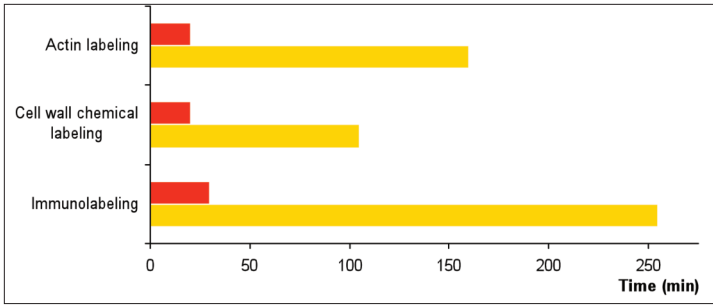
Figure 6: Comparison of experimentation time between bench-top (orange) and microwave assisted (red) methods of sample preparation for optical microscopy.
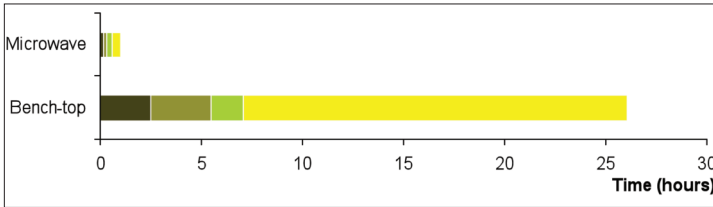
Figure 7: Comparison of experimentation time between conventional bench-top and microwave assisted methods of sample preparation for transmission electron microscopy. Fixation (black), post-fixation (brown), dehydration (green) and resin infiltration (yellow).

for chemically fixed samples. Vacuum MW processing for electron microscopy of pollen tubes gave the same results as compared to the traditional method despite much shorter fixation and incubation times.

On a more practical level, MW technology is affordable, user friendly, it does not require specific installations and older models can be upgraded with a temperature control device. Furthermore, it is very flexible allowing an easy switch mid-protocol to the conventional bench-top method if necessitated by time constraints or logistics.

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