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Technical Note

Applying CLSM to increment core surfaces for histometric analyses: a novel advance in quantitative wood anatomy

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Abstract

A novel procedure has been developed to conduct cell structure measurements on increment core samples of conifers. The procedure combines readily available hardware and software equipment. The essential part of the procedure is the application of a confocal laser scanning microscope (CLSM) which captures images directly from increment cores surfaced with the advanced WSL core-microtome. Cell wall and lumen are displayed with a strong contrast due to the monochrome black and green nature of the images. Consecutive images are merged into long images representing entire increment cores which are then analysed for cell structures in suitable software.

Keywords: wood anatomy; cell structures; confocal laser scanning microscopy; CLSM; dendrochronology; surface preparation

Introduction

The study of wood anatomical characteristics measured in tree rings has proved to be useful in many ways. The use of time series of vessel features to characterize the past climate conditions has become more popular. Intra-annual features have the potential to provide additional climatic information (Fonti and García-González, 2004, 2008; Fonti et al., 2009a; Campelo et al., 2010). The application of qualitative wood anatomy has also added understanding and provided supplementary information of past environmental events such as mechanical impacts on tree growth due to landslides and debris flows (Heinrich et al., 2007a,b; Heinrich and Gärtner, 2008), fires (Schweingruber, 1996), and frosts (LaMarche and Hirschboeck, 1984; Gärtner, 2007). While most quantitative wood anatomical studies have been conducted on angiosperm wood (Sass and Eckstein, 1995; García-González and Eckstein, 2003; Eckstein, 2004; Fonti and García-González, 2004; Bhattacharyya et al., 2007; Fonti et al., 2007; Fonti and García-González, 2008; Giantomasi et al., 2009; Campelo et al., 2010), comparable studies on conifer wood are less common (Yasue et al., 2000; Wang et al., 2002; Vaganov, 2006; Eilmann et al., 2006, 2009; Seo et al., 2012). These studies have highlighted a close relationship between cell structure variables and seasonal climatic conditions. Hence the main reason for the scarcity of such studies seems not to be the lack of environmental signals in series of cell structure measurements but the difficulty of developing long series of conifer cell structure measurements with standard methods of quantitative wood anatomical research. Measurements are usually made on thin-section slides prepared arduously in order to be used for light microscopy analysis or using x-ray photos of special lathes (e.g., Eilmann et al., 2006; Vaganov, 2006). In contrast, if the cells are large enough, for instance when analysing earlywood vessels of ring-porous species, digital images of relatively small magnifications can be taken directly from the wood surface by means of a flatbed scanner or a digital camera mounted on a macroscope, thereby allowing efficient structural analyses (García-González and Eckstein, 2003; Fonti et al., 2007;

Fonti and García-González, 2008; Fonti et al., 2009b). Hence it would be most desirable, if quantitative wood anatomy could be conducted on increment cores of conifers as well.

Recently, progress has been made in two important parts of wood anatomical research, that is, surface preparation and microscopy. Gärtner and Nievergelt (2010) have presented the new advanced WSL core-microtome for the wood surface preparation of entire 40 cm increment cores. With the core-microtome it is feasible to prepare the surface of increment cores which is preferred to sanding as the cell lumen of the annual rings remain open and cell walls are clearly visible. Confocal Laser Scanning Microscopy (CLSM) has the ability to rapidly and nondestructively generate high quality images of wood with micron-level resolution. CLSM has the advantage that it can acquire images from the surface of relatively thick specimens of wood such as increment cores or small blocks, and it can avoid distortion and cell damages resulting from sectioning thin fragile samples. Recent technical advancements in CLSM have resulted in affordable systems, such as the Olympus system used for this study. The application of CLSM to the visualization of xylem structures was first described by Knebel and Schnepf (1991) and CLSM was subsequently used to analyse thin-sections of wood in detail, e.g. to measure the dimensions of xylem cells (Donaldson and Lausberg, 1998; Moëll and Donaldson, 2001), to examine the cell structure of differentiating xylem (Kitin et al., 2003), to study the morphology and positions of intervessel pits related to the conduction of water (Kitin et al., 2004), to study bordered pit aspiration in *Cryptomeria japonica* (Matsumura et al., 2005), and to assess shading correction methods for digital image analyses (Moëll and Donaldson, 2007). However, to our knowledge, CLSM has not been used for the wood anatomical analysis of tracheid features in order to build long chronologies.

In this technical note we describe the application of CLSM for capturing images directly from conifer wood blocks. The individual procedures comprise sample preparation with the WSL core microtome, image acquisition with CLSM and image analysis with the software WinCELL. The

individual steps are explained in the following parts, and problems and possible solutions are also mentioned.

Sample preparation and cutting with core microtome

After the increment cores are taken with a standard 40 cm increment corer of 4 mm inner diameter they are first prepared and analysed for tree-ring widths following dendrochronological procedures described by Stokes and Smiley (1968), Fritts (1976), Schweingruber (1983) and Cook and Kairiukstis (1990). Then the long cores are subdivided into pieces of 5 cm length because shorter wood samples are easier to handle during the following wood anatomical preparation. Moreover, the complete core would be too long for capturing images on the moving stage of the CLSM. The pieces are glued on wooden sample holders in such a way that the samples are cut rectangular to the growth direction of the wood fibers for the analysis of transversal sections. This is important because only perpendicular surfaces guarantee ideal images with sharp contrasts and without background noise for exact analysis processes using digital analysis software such as WinCELL, ROXAS or ImageJ (Regent Instruments Ins., Canada; Abramoff et al., 2004; von Arx and Dietz, 2005). It is also essential to use waterproof glue because samples might disintegrate during the latter cutting procedure when water is used to soften the wood sample.

A plane surface is the prerequisite for acquiring accurate images of the cores with CLSM. The cores are surfaced with the advanced WSL core-microtome. Since the cores are usually dry, some water is needed which is applied with a brush to the top of the wood surface before cutting to keep it soft and easy for cutting. Preferably, cores are cut when they are still fresh because then the cell walls are soft and less fragile during the cutting procedure resulting in good surfaces. For a high quality cutting the blade needs to be sharp. Otherwise, the cell walls will be deformed or torn into the lumen which will hinder the following automatic image analysis. During the cutting procedure the cutting angles of the WSL core microtome can be adjusted in

several directions. The adjustments need to be applied always in relation to the type of material (e.g., hardwood versus softwood). Subdivided core samples of 5 cm length with plane core surfaces are obtained with the WSL core-microtome and facilitate high quality surface images of the cores by means of CLSM.

During the image acquisition with the CLSM autofluorescence background noise from the cell lumen can be quite strong reducing the contrast between cell wall (green) to lumen (black). In normal light microscopy, this contrast is usually enhanced by e.g. staining the wood surface with dark ink and subsequently filling the cell lumina with a bright substance such as plastics, white chalk powder or wax (Sass and Eckstein, 1995; García-González and Eckstein, 2003; Fonti and García-González, 2004). However, these procedures have only been applied to the wood surfaces of angiosperm wood because the vessels are relatively large and thus are easy to fill. Conifer wood cells are much smaller but nevertheless we tried to solve this problem by filling the cells with various components such as bee wax heated and colored with black ink, PEG2000 and dark painting wax to reduce autofluorescence and increase the contrast between cell wall and cell lumen. However, these methods are time consuming and not efficient enough. Therefore another solution has been found instead. The cut surfaces of the cores are stained slightly by applying a strongly diluted safranin solution (safranin concentration depends on which samples will be used) to the top of the wood surface. This method does not only reduce the autofluorescent background noise but also enhances the contrast between cell wall and cell lumen.

Digital imaging with CLSM

The confocal laser scanning microscope used here is connected to an Olympus FluoView FV300 microscope using transmitted visible light or excitation by incident-light from a helium neon laser with a wavelength of 543 nm, which activates particularly well the autofluorescence of wood (Fig. 1). Autofluorescence is the natural emission of light after the wood has absorbed

the light beam of the laser. In the CLSM system, the helium neon laser beam, operated in the epi-illumination mode (illumination and detection from one side of the sample), passes through a light source aperture and then is focused by an objective lens on the wood surface. Reflected laser light as well as the autofluorescent light from the illuminated spot is then re-collected by the objective lens. The confocal aperture (pinhole) obstructs the light that is not coming from the illuminated spot. The out-of-focus light is suppressed and most of the returning light is blocked by the pinhole, which facilitates sharper images than those from conventional microscopy techniques. A beam splitter separates some portion of the light into the detector, which also has a filter that selectively passes the autofluorescent wavelengths while blocking the original excitation wavelength. After passing a pinhole, the light intensity is detected by a photodetector (Photomultiplier Tube = PMT), transforming the light signal into an electrical signal which is then recorded by a computer. The detected light originating from the illuminated spot represents one pixel in the resulting image. As the laser scans over the wood surface, an image is obtained pixel-by-pixel and line-by-line. Such scans are then composed into distortion-free images.

Fig. 1. Confocal Laser Scanning Microscope (CLSM) used for quantitative wood anatomy at the GFZ DendroLab

The system allows for adjustments of the parameters PMT, Gain and Offset which ensures that all images have the same level of brightness and contrast. The PMT enables individual photons to be detected when the incident flux of light is very low. The functions Gain and Offset brighten and darken the image by the ratio set at the time of image acquisition, respectively. The confocal aperture of the FluoView FV300 can be set to five different positions. A small confocal aperture diameter can obtain high z-resolutions but low signal strength and a large confocal aperture diameter results in low z-resolutions but a brighter signal. Since wood anatomical

research of the wood surface is focusing on the xy dimensions of cell structures, we use larger confocal apertures. Because of its laser scanner the CLSM has a better lateral resolution than conventional optical microscopy. Distortion-free images can easily be merged without the problem of different levels of distortion in the merged images. Sequences of images in relatively small magnification (100×) are taken semi-automatically by means of a motorized XY-sample-stage which is connected electronically to the Olympus Fluo View system software controlling the microscope, so that the images are always stored with internal XY-coordinates ensuring an accurate merging process (Fig. 2).

Fig. 2. CLSM in action while imaging the surface of a pine increment core sample in 100× magnification

After all individual successive images of one core sample have been taken they are precisely merged into one long image using image editing software (e.g., Adobe Photoshop, CorelDRAW Graphics Suite). The image then can either be imported directly into analysis software or the quality of the image may first need some enhancements by digitally improving the brightness and contrast in the Olympus image acquisition software package cellB (Fig. 3). CellB is linked to the Olympus CLSM system however other manufacturers of microscopes (e.g., Leica or Nikon) are likely to offer similar software packages.

Fig. 3. Example of merged images containing several consecutive overlapping images

The digital imaging with CLSM results in green monochrome images with a black background and green cell structures. Through so-called look-up-tables other colors could be selected within the system. Since in light microscopy green would be the normal color of fluorescent light,

black-and-green images seem to be a suitable solution. Monochrome images sharply enhance the contrast between cell wall and cell lumen for the image analysis system to automatically distinguish the single cell structures. However, two major problems, shading and background autofluorescence noise, can occur during the digital imaging. In this regard, the evenness of the sample is crucial since uneven surfaces result in images of mixed brightness with darker and brighter sections indicating regions partly out of focus (Fig. 4). Such brightness variations have been found elsewhere and identified as shading which can significantly affect the image quality and impact the accuracy of the image analysis (Moëll and Donaldson, 2007). Shading decreases the contrast between the target objects and the background which is, however, an essential precondition for image analysis systems to automatically detect single cell structures. Therefore, after merging, a shading correction step is often of importance for the following exact image analysis. In cellB two general shading correction methods are offered, that is, multiplicative implementations and additive implementations. Moëll and Donaldson (2007) found that for confocal images of wood where the shading is only present in the cell wall pixels, a multiplicative shading correction avoided any alteration to the unshaded lumen pixels, which mainly have values of zero. Additive shading corrections removed the shading from the cell wall pixels but added new shading to the lumen pixels and, according to the authors, were less effective. Therefore, in cellB we used the NxN average filter method which is a multiplicative implementation to correct for shadings. The NxN filter is a smoothing filter to eliminate noise. The name of the filter refers to the size of the image area whose pixels' grey values are average. The larger the matrix of the filter is, the bigger the shading fluctuations being equalized. The NxN filter averages the values of all pixels surrounding a central pixel and assigns them to that central pixel. When determining the filter size "N" it should be kept in mind that the smaller the matrix, the finer are the details that can be edited. The NxN average filter method avoids any alterations to the pixels of the unshaded lumen (Moëll and Donaldson, 2007).

Fig. 4. CLSM image with shading (left) and after shading correction (right); magnification 100×

Fig. 5. Example demonstrating pit structures in a thin section (50µm) of *Pinus sylvestris* (200× magnification). Gaps due to the pit structures can be noted (white arrows).

During the digital imaging process one important advantage of CLSM over light microscopy is the fact that core samples rather than thin sections are used. When images are captured from thin sections clear pit structures are visible which have also been shown by Matsumura et al. (2005) (Fig. 5). However, such images cannot be measured accurately because the pits are often recognized as gaps and then two cells connected through a pit will be identified as one big cell. This problem does not occur when using wood blocks for imaging with the CLSM system.

Image analysis with WinCELL

Currently, three image analysis software packages (WinCELL, ROXAS and ImageJ) are commonly used in quantitative wood anatomical studies (Regent Instruments Ins., Canada; Abramoff et al., 2004; von Arx and Dietz, 2005). For this technical note the image analysis is described for WinCELL only, but it needs to be pointed out that other software packages are also capable of conducting such analyses.

The image analysis software WinCELL 2011 (Regent Instruments Ins., Canada) is specifically designed for wood cell analysis. It focuses on parameters such as cell size, cell wall thickness and cell lumen area. Before the measurements can be conducted the WinCELL system is calibrated using objects of known dimensions. Wood structures such as resin ducts, ray cells and gaps are excluded manually to avoid measurement mistakes. Furthermore, potential measurement noise is automatically removed by setting reasonable filters (area, length, width, form, length to width ratio) for excluding large or small morphological features.

A special type of analysis procedure is provided in WinCELL 2011 to analyse the cell structures in individual tree rings. The tree-ring analysis process is conducted semi-automatically, that is, the ring boundaries are first identified visually, the boundaries traced and the years entered by the operator. Once all ring boundaries have been identified, WinCELL will create analysis regions in each tree ring by closing the regions delimited by the ring boundary paths. The result is one analysed region per tree ring and the cell measurements are stored individually for each tree ring (Fig. 6).

Fig. 6 Example for measurements based on the CLSM image of *Pinus sylvestris*. Note the dashed line is the identified tree-ring boundary (white arrow); right image: zoom of left image.

WinCELL stores all data in a format that can be opened by XLCell, a special add-in to Microsoft Excel supplied by Regent Instruments Ins., Canada. XLCell separates the measurement data into total lumen area (TLA), cell number (CN), average lumen area (ALA), average lumen diameter (ALD) for each individual ring into different sheets for one or many images. Finally, the measurements of all subdivided core samples are merged into one data sheet in order to conduct the time series analysis on the entire data set. Long series of various histometric parameters such as TLA, ALA, ALD are easily derived from the new data set and can be used for multi-proxy studies of various environmental signals. Additional parameters such as cell wall thickness (CWT), the first 30% average lumen area (30ALA) and the 30 maximum lumen area (30MAX) can be derived from XLCell files (Fig. 7).

Fig. 7. Example of a diagram illustrating cell parameters total lumen area (TLA), cell number (CN), average lumen area (ALA), average lumen diameter (ALD), cell wall thickness (CWT), the first 30% average lumen area (30ALA), and the 30 maximum lumen area (30MAX) in comparison to tree-ring width (TRW) and earlywood width (EWW).

Conclusions and outlook

Here, we have presented a new procedure in the field of quantitative wood anatomy which meets the requirements of an efficient and accurate histometric analysis in an innovative and contemporary way. The new method has several advantages which has the potential to facilitate the development of long time series of conifer wood cell measurements. The procedure swiftly measures various cell structures of conifer wood which so far has been relied on laborious thin-sectioning. Additional staining is necessary only occasionally if shading occurs. But under normal circumstances core samples are used without further treatments. Since the images are the results of a laser scanning device no geometrical distortions appear and thus an easy and error-free merging of the images is possible. The monochrome nature of the images optimises the contrast between cell lumen and cell wall and thus facilitates easier analyses with digital imagery software such as WinCELL. Furthermore, the usage of core samples avoids that gaps in the cell walls due to pit structures occur in the cross-sectional images which often happens when using thin sections. Such gaps will result in measurement mistakes during the analysis with software packages such as WinCELL.

While this technical note concentrates on conifer wood, it is important to note that the technique presented here has good potential for the analysis of diffuse and semi-diffuse porous wood of angiosperm species as well. From a methodological point of view, the analysis of diffuse and semi-diffuse porous wood is quite demanding and thus quantitative wood anatomical studies on this wood are scarce (Sass and Eckstein, 1995; Schume et al., 2004; Campelo et al., 2010). First results from work in progress on vessel features of beech (*Fagus sylvatica* L.) indicate that a particular good contrast between vessels and the rest of the wood can easily be achieved with our method.

The combination of the WSL core microtome, the Olympus CLSM, cellB software and WinCELL worked well in our approach but other equivalent combinations may lead to similar results. The

273 procedure now needs to be applied to various sample material. Long and well-replicated
274 chronologies of cell structures need to be developed. New questions will arise, e.g., how should
275 raw values of cell measurements of several hundred years length be handled? Do they have
276 any age trends or other trends which need to be considered more carefully? Do we have to
277 detrend them? Before we can start to tackle such questions we first need long histometric
278 chronologies and our new procedure seems to be well suited to build them in an efficient way.

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