



TECHNICAL NOTE

Using steam to reduce artifacts in micro sections prepared with corn starch

David Montwé^{a,*}, Miriam Isaac-Renton^b, Heinrich Spiecker^a, Andreas Hamann^b^a Chair of Forest Growth, Albert-Ludwigs-Universität Freiburg, 79106 Freiburg, Germany^b Department of Renewable Resources, University of Alberta, Edmonton, AB T6G 2H1, Canada

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ABSTRACT

Preparation of micro sections to measure cell wall thickness and lumen diameter is a widely used method in the fields of dendroecology, dendroclimatology and tree physiology. Efficient sample preparation and image analysis are critical for studies with long time series and large sample sizes. Recently, there have been substantial improvements in micro section preparation techniques, including a corn starch-based non-Newtonian fluid treatment. This method reduces cell wall damage during cutting with a microtome, which in turn decreases artifacts during image analysis. Although this procedure does in fact improve sample quality, we found starch grains sometimes to be difficult to remove and to cause artifacts during image analysis. This technical note outlines a simple, fast and effective steam treatment that causes starch gelatinization and a reduction in the number of starch grain artifacts.

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1. Introduction

Measuring cell wall thickness and lumen diameter in xylem cells requires the preparation of micro sections, typically 10–20 μm thick. Measurements of these microscopic structures are important in dendroecology, dendroclimatology, and tree physiology research (Fonti et al., 2010). In tree physiology, cell wall thickness and cell lumen diameter are closely correlated with cavitation resistance (Hacke et al., 2001), and can therefore be used to infer drought resistance of tree species or populations. In dendroecology, this information enables the assessment of tree species' abilities to acclimate to changing environmental conditions and their overall suitability to increased drought severity under climate change (Bryukhanova and Fonti, 2013). In dendroclimatology, time series of cell parameters can serve as proxies for climate reconstructions (Wimmer, 2002). The proportion of cell walls to cell lumen is closely related to wood density (Wassenberg et al., 2014), which is an important proxy for summer temperatures (Briffa et al., 2004).

Preparing micro sections from large stem diameters, representing long dendrochronological time series is a challenging and labor-intensive task, however. These obstacles have led to the development of alternative preparation techniques, including

the use of high-precision diamond-fly-cutters for reflected light microscopy (Spiecker et al., 2000), but also to advances in microtome techniques. Sliding microtomes using common cutter blades have emerged as being capable of producing high-quality micro sections, while also being much easier to maintain than earlier alternatives (Gärtner et al., 2014; 2015b). These instruments are intended to develop long anatomical time series, with a single micro section covering multiple tree-rings (Gärtner et al., 2015a, b).

One remaining issue, however, is that secondary cell walls of tracheids are sensitive to pressure induced by the cutting blade, often causing them to be pushed into the cell lumen (Schneider and Gärtner, 2013). Such damage to the cell structure can create problems for automatic image analysis, which are time-consuming to manually remove. Until recently, this problem was addressed by embedding the sample in paraffin wax, for example (Feder and O'Brien, 1968). However, this procedure is time-consuming and the wax must be removed before staining (Schneider and Gärtner, 2013).

As an alternative method to reduce cutting artifacts in the development of long time series of anatomical properties in tree-rings, Schneider and Gärtner (2013) proposed using a non-Newtonian fluid to stabilize the cell structure. A fluid mixture of corn starch and water is applied to the surface of the sample prior to the microtome cut. The effectiveness comes from the fluid's non-Newtonian properties. While being applied with a brush, the mixture is liquid, and

* Corresponding author.

E-mail address: david.montwe@iww.uni-freiburg.de (D. Montwé).

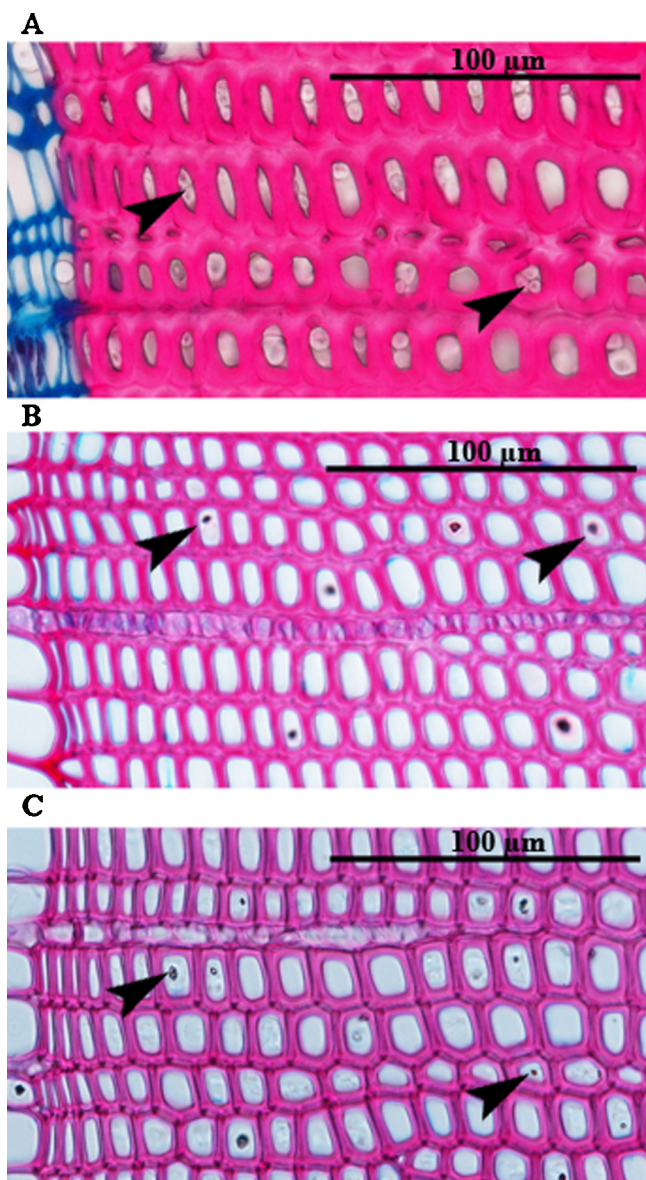


Fig. 1. Three examples of micro sections of stained xylem cells made from lodgepole pine samples (*Pinus contorta* Douglas ex Loudon). Two black arrows per panel highlight examples of starch grains that remained after rinsing, staining and dehydration. The center of the grains appears darker, especially in panels B and C. The starch grains sometimes absorbed Safranin during the staining stage, as seen in Panel B.

flows into the empty cell lumen. Under pressure, the starch grains form a solid structure, stabilizing the cell walls. As a result, artifacts due to deformed cell walls can be mitigated. We applied this method to lodgepole pine (*Pinus contorta* Douglas ex Loudon) samples. The corn starch mixture worked as expected and did indeed minimize the aforementioned artifacts. We found the corn starch solution efficient, easy to use, and to improve accuracy of the image analysis.

However, during our sample preparation, we sometimes found it difficult to completely wash the corn starch grains out of the cells. We found that the starch grains were more easily removed from the larger earlywood cells, leading to good contrast between cell walls and cell lumen, but the removal could be more problematic in the narrower latewood cells. Here, the grains were held in place by friction and adhesion, and even long and intensive rinsing could not always overcome these forces. Examples of remaining starch grains are shown in Fig. 1 and become especially visible under

higher magnification. The dark, crystalline centers of the grains are especially visible. Sometimes, it appears that the grains also take up Safranin stain, which could lead to poorer contrast in the image analysis stage. We found these grains to interfere with the automatic detection of WinCELL Version 2013a (Regent Instruments Canada Inc., 2013), despite trying to eliminate the starch by setting appropriate filters. Often, the grains had to be removed by manual image manipulation. Although more sophisticated tools for image manipulation may better filter the remaining starch grains, we found their complete removal, without altering other structures in the image, to be laborious.

Starch gelatinization by inducing heat is a common procedure in the food industry (Bauer and Knorr, 2005). By adding water and applying heat, the intermolecular bonds of the starch are broken and more water molecules can link to hydrogen bonding sites. Therefore, the starch grains change their crystalline structures to a gelatinous, viscous solution (Zobel, 1984). This reaction may be useful when starch grains need to be completely removed from the micro sections. In this technical note, we describe how a steaming procedure can be used to dissolve and wash out the starch before staining. This presents an additional step to the methodology proposed by Schneider and Gärtner (2013). This procedure could be useful, where starch grains remain abundant after rinsing and interfere with image analysis, and where image processing to remove starch grains may cause other inaccuracies.

2. Materials and methods

Stem disks of lodgepole pine were collected in a 40-year-old provenance trial located in the vicinity of Kamloops, British Columbia, Canada, at an elevation of approximately 1400 m a.s.l. (Illingworth, 1978). The air-dry disks were already sanded to allow for measuring of tree-ring width and latewood proportions. In preparation of the wood anatomical measurements, 1 cm wide, diagonal cross-sections were marked on the surface of the disks. Subsequently, the cross sections were sawed from the disks using a standard circular saw. The resulting samples were then trimmed to a thickness of 1.5 cm with a small circular saw. The saw cuts were also made perpendicular to the fibers, to ensure a vertical orientation of the tracheids (Gärtner and Schweingruber, 2013). These samples were split at the pith, yielding two pieces per disk, between 4 cm and 7 cm long.

Prior to cutting micro sections with the microtome, the corn starch solution was prepared as a mixture of corn starch, water and glycerol in the ratio of 10g:8 ml:7 g (Schneider and Gärtner, 2013). Following this step, the sample was placed in the object holder of a GSL1-microtome (Gärtner et al., 2014), was softened by applying water with a brush, and then transverse micro sections were cut. The first few cuts were needed to remove the top layer of fibers damaged by the saw. We then changed the blade (A170, NT-Cutter, Japan) and applied the corn starch solution with a brush to the surface of the sample (Schneider and Gärtner, 2013; Gärtner et al., 2015b). We aimed to produce sections of a thickness of approximately 10–20 µm, which were subsequently placed on a glass slide. To wash out the starch grains, the section was rinsed for 4 min by pumping water through the sample with a pipette (Gärtner and Schweingruber, 2013) and subsequently stained as described below.

Our simple laboratory set-up for steaming the micro sections is illustrated in Fig. 2. A lab grade, 2.5l stainless steel container is placed on a hot plate and is filled with about 500 ml of distilled water. A metal rack is placed in the bottom of the container so that the top sits above water level. It is important that the glass slide remains well above water to prevent the sample from being washed off the slide and damaged by the boiling water. A lid is

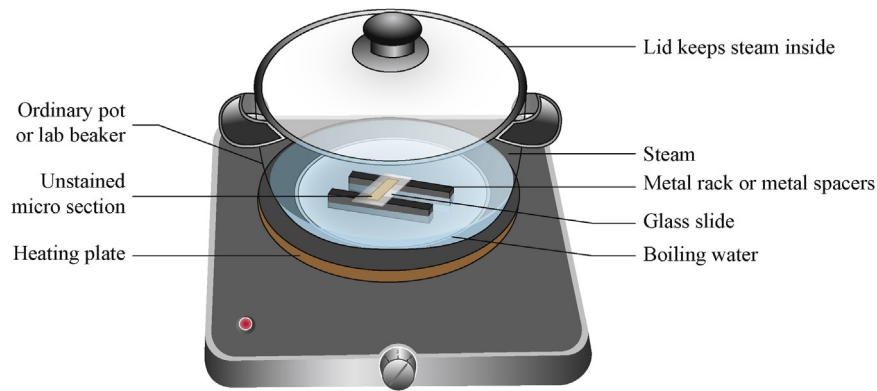


Fig. 2. Schematic view of steaming a micro section. A pot or beaker is placed on a heating plate, a metal rack or a substitute placed inside. The water level in the container should not exceed the height of the rack. After the water is boiling, wet micro sections on glass slides can be placed on top of the rack. The lid helps to keep the steam inside.

used to close the container and the water is heated to boiling. Once boiling, the glass slide supporting the micro section can be placed on the rack with laboratory tweezers or forceps. The sample remains in the container for 1 min before being removed. The hot steam acts upon the sample by causing the starch grains to lose their crystalline structures and to form a liquid solution. To save time, multiple samples can be steamed together. When fresh micro sections cannot be steamed immediately, they can be temporarily embedded in glycerol to prevent drying out as suggested by Gärtner and Schweingruber (2013). Before steaming, the glycerol should be washed out, because it increases the gelatinization onset temperature (Tan et al., 2004).

After steaming, the samples were rinsed with water to remove the gelatinized starch liquid. Subsequently, the samples can be stained and dehydrated according to standard procedure (Gärtner and Schweingruber, 2013). A few drops of a 1:1 mixture of Safranin and Astra Blue were applied with a pipette. After 5 min, the staining solution was washed away with water, and the micro sections were dehydrated in an ascending series of ethanol 70%, 96% and 99% to remove the rest of the surplus stain (Gärtner and Schweingruber,

2013). Excess ethanol was removed with a paper towel, and micro sections were then embedded in glycerol and water.

Digital photos were obtained using a Nikon Eclipse Ni-E upright microscope with a motorized stage, automatic Z-focus and a color camera with a resolution of 5 megapixels and 12-bit color depth (Nikon DS-Fi2). Micrographs were taken at 200 \times magnification and automatically merged into a large image with the stitching function in the NIS-Elements software (Version 4.20.01, Nikon Corporation, 2014). For illustrating the problem and the effectiveness of the proposed treatment, we also show how the samples appear in the widely used software WinCELL.

3. Results and discussion

Two micro sections from the same lodgepole pine wood sample are contrasted in Fig. 3. Both were treated with corn starch during cutting with the microtome, but only one had an additional steaming treatment before both were stained. As seen in the two left panels in Fig. 3(A) and (C), several starch grains remained present after the rinsing, staining and dehydration stages. The smaller cells

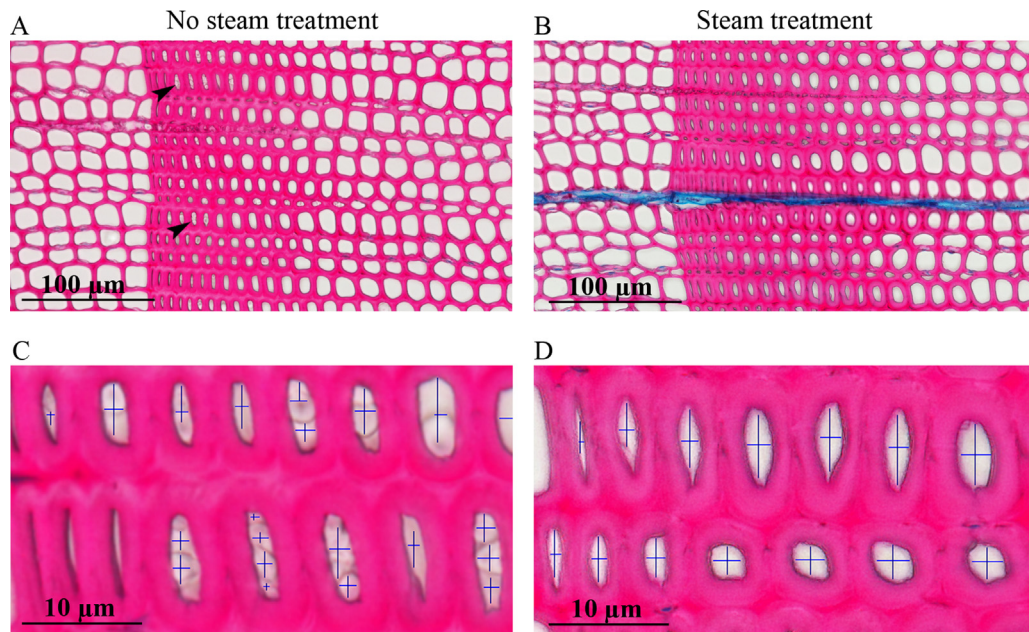


Fig. 3. Panels A and C show transverse sections of tree-rings from the same wood sample close to the border between latewood and earlywood photographed at 200 \times magnification. Images on the left (A and C) correspond to samples that were rinsed, stained and dehydrated and did not undergo a steam treatment. The arrows in panel A highlight two examples of remaining starch grains. The images on the right (B and D) display samples that underwent an additional 1-min steam treatment. The lower panels are zoomed versions of the top panels and show the cell lumen detection in WinCELL (Version 2013a).

in the latewood, or cells cut toward their tapered end, seemed to be primarily affected. These cells were measured incorrectly in WinCELL, meaning the complete analysis of a year's growth would not be possible without manual correction. Although WinCELL allows the image contrast to be increased and filters to be applied, we found these functions to be insufficient in removing the image artifacts and to consistently help in correctly identifying cell lumen and cell wall thickness. We found that steaming of samples was less time-consuming than manual removal in image analysis software. Efforts to use image manipulation to select and remove artifacts with various settings often discarded either too little or too much from the cell wall, which biased cell wall measurements. Since every micro section and resulting image might have slightly different properties, the parameters may also need to be adjusted from slide to slide, making it difficult to standardize. In contrast, the images on the right side of Fig. 3(B) and (D) correspond to the steam treatment and contain fewer obvious artifacts. Under very high magnification, some gelatinized starch was visible, but did not cause severe artifacts during image analysis.

The first step to reducing the presence of starch grains is rinsing the sample thoroughly with water after cutting with the microtome (Tardif and Conciatori, in press). We tested several rinsing methods, including a spray from a typical laboratory squirt bottle, ordinary tap-rinsing, or rinsing and/or soaking in a Petri-dish. The most effective method for our samples, however, involves placing a pipette on top of the section and pumping water through the sample, as recommended by Gärtner and Schweingruber (2013). Although starch grains can be reduced to a low number after several minutes of rinsing, we found that more than 4 min did not further reduce the abundance of grains. This may vary for other species or samples with a different proportion of latewood. We should also note that in the case of the steaming treatment, it is important to again rinse the sample with water. This reduces the presence of gelatin which could reduce the quality of the micro section.

Although steaming is reported to increase the crystallinity of cellulose (Ito et al., 1998), and heat can plasticize lignin and hemicelluloses (Inoue et al., 1993), we found no indication that the short steam treatment damaged the cellular structure. Prolonged heat treatment at high temperatures can distort wood, as shown in an 8-h heat treatment at 200 °C by Priadi and Hiziroglu (2013). Steam treatments are, however, also used to soften wood specimens prior to cutting with a microtome (Tardif and Conciatori, in press). It appears unlikely that brief steam treatments under normal atmospheric pressures will compromise the cell structure of an un-restrained micro section. That said, it may still be generally advisable to treat all samples consistently and to thoroughly document the sample preparation to avoid potential bias in subsequent measurements.

We conclude that the additional steam treatment for xylem micro section preparation only requires a minor investment in time and equipment. We consider cellular damage by the presented steaming method to be of low concern. Instead, steaming leads to improved section quality and more accurate image analysis without the need for image manipulation that may lead to other artifacts. This gelatinization procedure may therefore be a useful, additional step to the method introduced by Schneider and Gärtner (2013).

Conflict of interest

The authors declare no conflict of interest.

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