REVEALING ORGANIZATION OF CELLULOSE IN WOOD CELL WALLS BY RAMAN IMAGING

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Abstract

Anisotropy of cellulose organization in mature black spruce wood cell wall was investigated by Raman imaging using a 1-µm lateral-resolution capable confocal Raman microscope. In these studies, wood cross sections (CS) and radial longitudinal sections (LS) that were partially delignified by acid chlorite treatment were used. In the case of CS where latewood cells were examined, Raman images of S2 and S1 regions, produced using cellulose contribution at 1098 and 2773-3045 cm^{-1}, showed significant differences. While the spectra associated with the S2 images indicated the microfibrillar orientation there to be longitudinal, in contrast, the spectra corresponding to the predominantly S1 images indicated that the net alignment of cellulose microfibrils in this region was transverse such that their long fibril axis made a small angle (~30°) to the S1 wall surface. Or in other words, cellulose in S1 was oriented approximately 60° to the expanding direction of the growing cells. In the CC regions, although as expected the intensities of the spectral contributions were quite low, they were due to cellulose. Additionally, results of the radial LS studies provided evidence that corroborated findings from the CS.

Keywords: black spruce, wood, cell wall, cellulose, microscopy, Raman, imaging, orientation

Introduction

The wood cell wall is a heterogeneous natural nano-composite of cellulose, lignin, and hemicelluloses. Cellulose, the major component of the cell wall, is 50-60% crystalline[1] and is assembled from cellulose microfibrils to provide a rigid reinforcing layer around the cell membranes in trees. The cell wall is organized in several layers. The primary wall (P), secondary wall (S1, S2, and S3), and middle lamella (mL) have different structures. Likewise, within the secondary wall, S1, S2, and S3 differ with respect to the orientation of cellulose microfibrils. The middle lamella surrounds the cell and holds the neighboring cells together. An understanding of cell wall architecture is important for both research and technological purposes. Basic information is needed to comprehend how growth and development factors influence cell wall formation. For example, such information will be useful for understanding how the cell wall structure is modified in response to environmental stimuli. Additionally, from the technological perspective, structural insights are important to understand the mechanical, chemical, and biological behaviors of wood and wood containing materials.

Several techniques have been applied to the study of subcellular distribution and organization of wood components[2-12]. Cellulose has been more difficult to analyze than lignin. A number of traditional methods require isolation of morphologically distinct tissue or sectioning of tissue by embedding the samples. Such methods are enormously disruptive to native-state structures. Moreover, methods such as optical and electron microscopy describe only cell wall morphology and do not provide information on molecular structure. Additionally, for lignin analysis using electron microscopy–energy dispersive x-ray analysis (EM–EDXA), chemical treatment by Br_2 or Hg is indicated[4,12]. None of these limitations exists in Raman imaging because sampling is done directly. Besides, both lignin and cellulose can be mapped simultaneously by selecting Raman bands that are specific to these cell wall components.

Raman microscopy was previously used to analyze cell walls *in situ*[13,14]; the research showed that information on both cellulose and lignin can be obtained. However, at that time progress towards detailed cell wall investigations was hindered due to the limitations driven by both sampling and instrumentation. Sample fluorescence and a lignin band that was less than fully stable made Raman investigations difficult. In addition, a highly efficient Raman microprobe equipped with an automated high-resolution x,y stage was not available. The latest Raman microprobes incorporate these advances and are well suited to investigate lignin and cellulose organization in the cell walls of woody tissue. These capabilities permit compositional mapping of the woody tissue with chosen lateral and axial spatial resolutions. Recently state-of the-art-confocal Raman microscopy has been applied to investigations of woody tissue[2,15.16] and new useful information has been generated.

The current study focuses on investigating the organization of cellulose microfibrils which is known to vary as a function of morphological region in the tissue. The intent, using the microprobe, was to study

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the anisotropy of the cellulose organization which is likely to result from the stress exerted during the wood cell growth.

Experimental

Sample Preparation

A 55- to 57-year-old black spruce [P. mariana (Miller) B.S.P.] tree was harvested from northern Wisconsin. The samples were taken from the main stem and no compression wood was present in the area of the samples. Transverse and radial longitudinal sections (CS and LS, respectively), 1 cm by 0.5 cm by 30 μm thick, were prepared by microtome from segments between the 30th and 40th annual rings. Some of the cross sections were delignified for 2 hours at 70° C using acid chlorite treatment. Sampling in D_2O was performed using the previously published procedure[17,18].

Raman Analysis

A Raman microprobe spectrometer, LabRam HR 800 (Horiba Jobin Yvon, Edison, NJ) was used in the present study. This spectrometer is equipped with a confocal microscope (Olympus BX40), a piezoelectric x,y stage, and a CCD detector. This instrument provides ultra-high spectroscopic resolution and unique polarized 633-nm laser (20-mW) excitation, which enables the system for woody tissue investigations. Woody tissue spectra from various morphological regions were obtained by using the serial (raster) mapping technique. An Olympus 100X water immersion objective was used for the Raman studies. Further details were the same as published earlier[2].

The lateral resolution of the confocal Raman microscope was ~1 μm. Considering the lateral resolution, only the S2 and CC areas of the distinct cell wall regions could be sampled by individually. Other investigated areas had contributions from two or more layers. For example, sampling in the mL region incorporated the P walls of the adjoining cells (mL + P, called the CmL region), but there also may have been some contribution from S1 depending on the thickness of the CmL region. For the purposes of the present study, the total mapped area was analyzed in terms of the CC, CmL, S2, S2-S1 (predominantly S1, called S1), and lumen regions. LabSpec 4.08 software was used to obtain Raman spectra in specific spectral ranges (250 to 3200 cm^{-1}). This software was used to perform all spectrometer operations, process collected data, and generate Raman images. Such images were based on component-specific spectral regions and represented distribution and organization of the cell wall components. Unless stated otherwise, peak height of the Raman band at 1098 cm^{-1} and band area of region 2773–3045 cm^{-1} were used for investigating cellulose. For the later region, area intensity was calculated by the sloping baseline method. Spectral data and Raman images were not processed or manipulated in any way. Experimental error was close to 10%. This calculation was based on spectra obtained sequentially at different times from the same sample location.

Results and Discussion

Recently, in our laboratory, cell walls of black spruce wood have been studied using a Raman microscope[2]. It was found that bands at 330, 380, 1098, 1120, 2890, and 2940 cm^{-1} were useful in investigating the distribution and orientation of cellulose. It is known from the literature[19] that in polarized Raman spectroscopy the bands at 330, 1098 and 2890 cm^{-1} are particularly sensitive to cellulose fibril orientation.

A bright field image of a latewood cell that was selected for the imaging work is shown in Fig. 1. This Fig also shows the sampling grid. The Raman image produced using the peak intensity of the1098 cm^{-1} band is shown in Fig. 2.

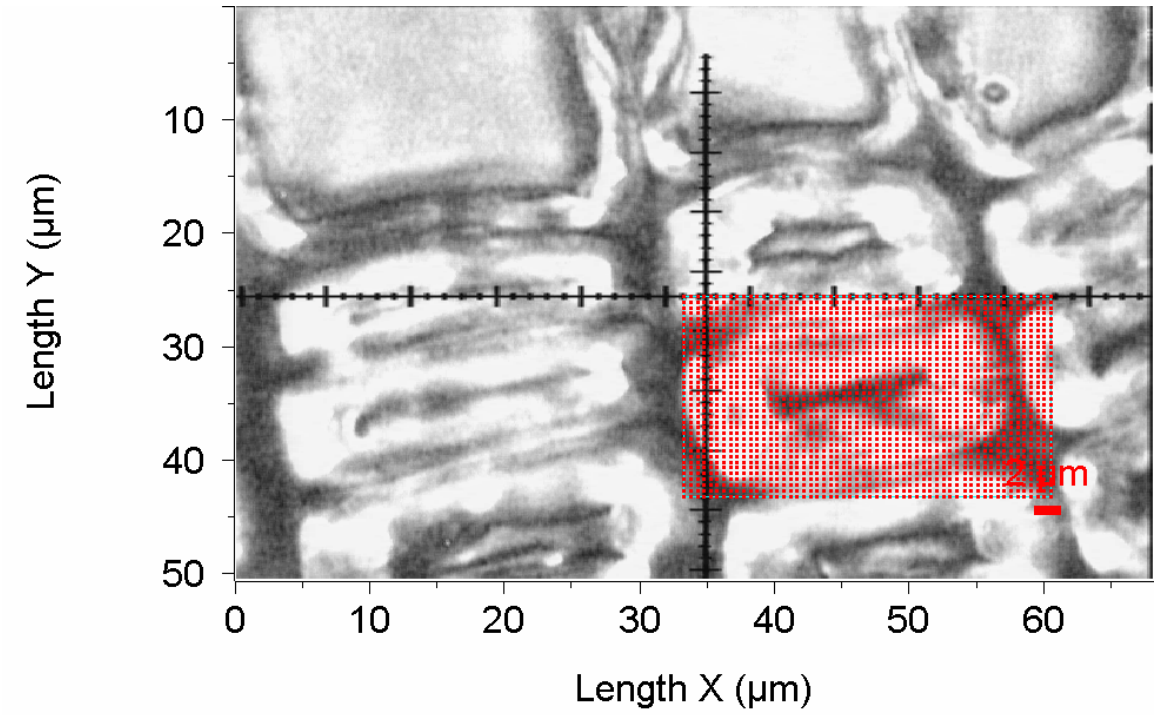

Figure 1—Bright field image of partially delignified woody tissue with superimposed sampling grid pattern (27.3 by 17.7 µm at 0.5 m resolution, 1932 points). Spectra were obtained sequentially from a series of positions using point-by-point scanning.

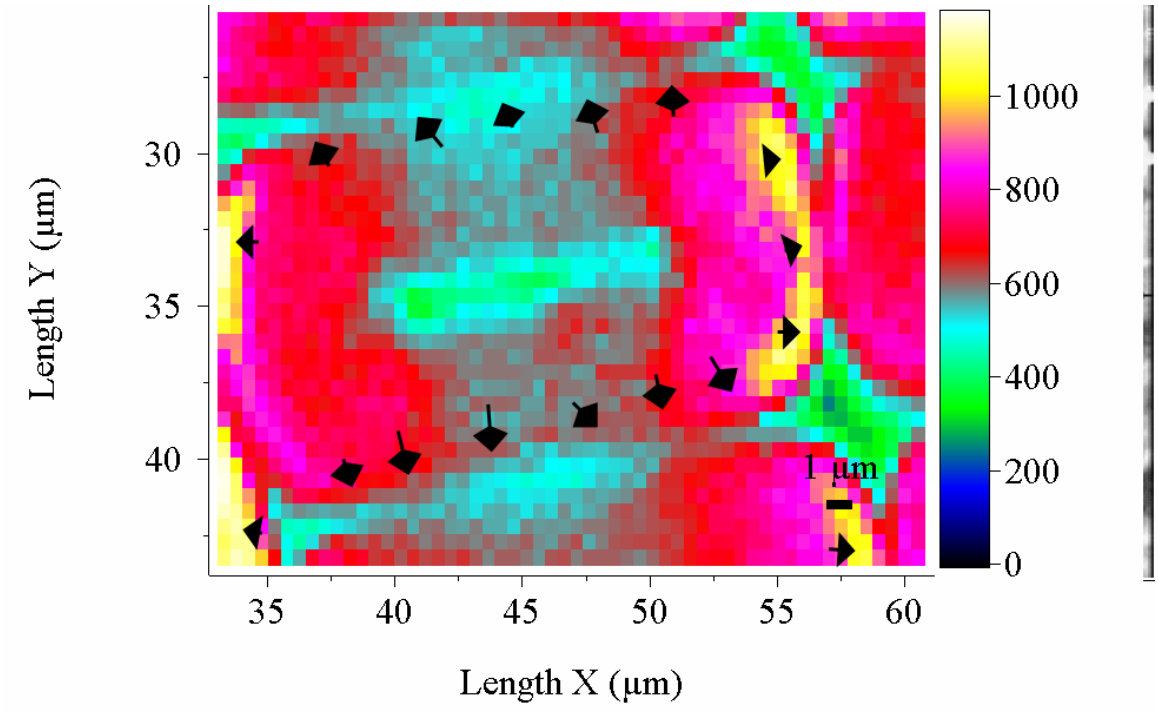

Figure 2—Raman image (false color) of cellulose spatial orientation and/or distribution in the cell wall area selected in Fig. 1; Image was generated using the 1098 cm^{-1} peak intensity. Bright white/yellow locations indicate high cellulose image intensity; light blue/green regions indicate very low intensity, e.g., lumen area. Intensity scale appears on the right. The electric vector direction of the laser was in the Y direction.

Considering that the 1098 cm^{-1} band is sensitive to cellulose chain orientation, its peak intensity was chosen to study alignment of microfibrils. By choosing the peak height

method, the contribution of other neighboring overlapping bands, present in the skeletal vibrational region of cellulose, was minimized. Another point to note in Fig. 2 is that the CC and lumen regions do not display zero intensity because although the Raman band is quite weak in these locations, there remains an underlying contribution from the residual fluorescence signal at 1098 cm^{-1}.

Intensity variation in S1

Compared to most regions in the S2, the image has several locations in S1 (annotated by triangular arrows) where the intensity is significantly higher (≥ 1000 intensity). Upon closer examination these locations were found to be associated with those S1 locations where the angle between the direction of the S1 and the laser electric vector (ev) was small. To obtain information on how microfibrils were oriented, first the dependence of band intensity ratios on the angle between the cellulose chain axis and the ev direction needed to be calculated. This was accomplished, for radial LS, by varying the angle between 0° and 90°. Fig. 3 shows the results of this investigation where Raman intensity ratios 330/380, 1098/1120, and 2890/2940 are plotted against the angle. Comparing the 1098/1120 ratios in the spectra of the intense S1 regions (triangular arrows in Fig. 2) to the data in Fig. 3 indicated that in these locations microfibrils made an angle of about 30° with the ev. Or in other words, the microfibrils in S1 were transversely aligned about 60° from the cell axis. Additionally, this observation accounted for the weaker image intensity (Fig. 2) in some other S1 locations (diamond shaped arrows in Fig. 2). Because in these spots the angle between the transversely aligned cellulose fibrils and the ev was about 60°.

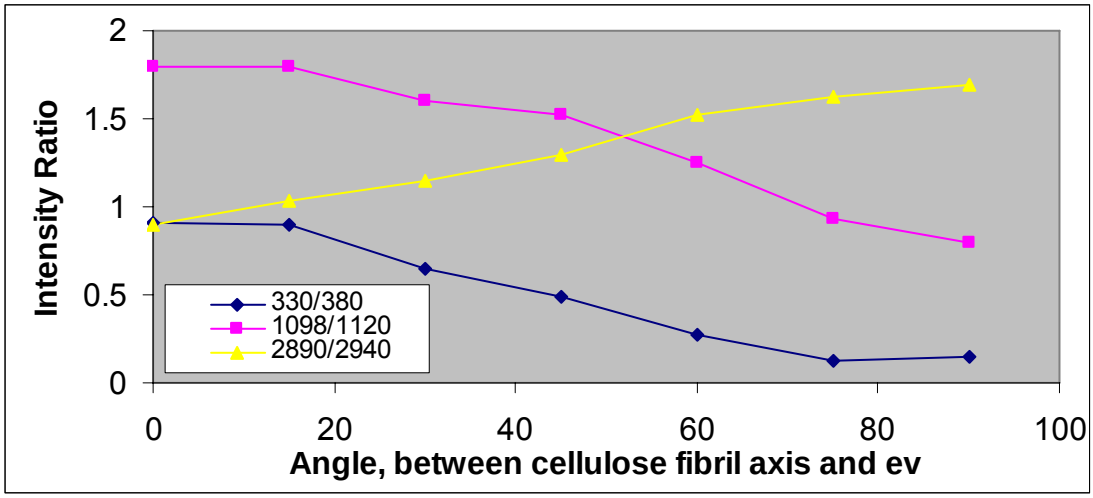

Figure 3—Variation of 330/380, 1098/1120, and 2890/2940 intensity ratios when, in a radial LS, the cell's longitudinal axis is rotated with respect to the ev direction. These plots were used to determine the orientation of the microfibrils in S1 and S2 regions.

Another image from the same cell wall area was generated using the 2890 cm^{-1} band region (C-H stretch) of cellulose (Fig. 4). Earlier, in polarized Raman spectroscopy analysis of cellulose containing materials where cellulose is oriented it has been reported[19] bands at 1098 and 2890 cm^{-1} showed opposite band intensity patterns when the angle is changed from 0° to 90°. When the intensity of the former is high, the intensity of the latter is low and *vice versa*. This is an outcome of the interaction of differently oriented Raman modes (tensorial in nature) with the polarized laser light used for sample excitation.

As can be noted from Fig. 4, compared to the regions in S2 (yellow, magenta, and red) those image regions in S1 which had high intensity in Fig. 2 now have significantly low intensity (Fig. 4, green or light-blue). A low 2890 cm^{-1} band region intensity is indicative of cellulose chain orientation making a small angle to the ev direction (Y axis). This supports the finding mentioned in the above paragraph that in S1 region the microfibrils are transversely aligned.

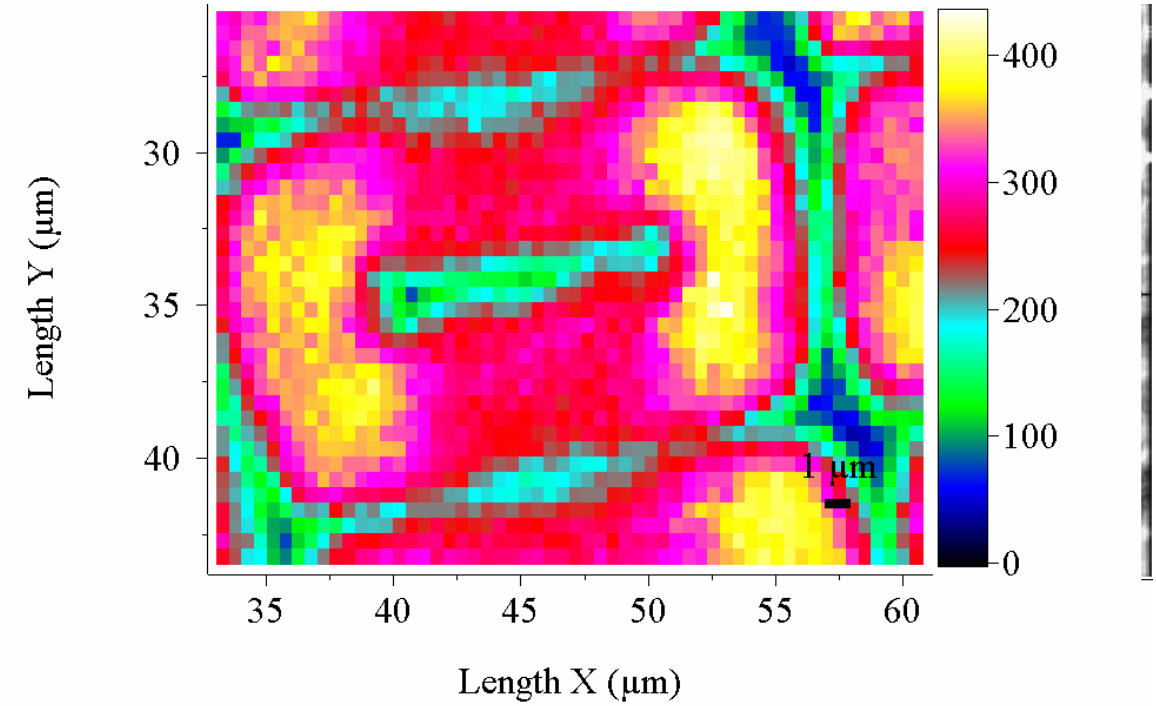

Figure 4—Raman image (false color) of cellulose spatial orientation and/or distribution in the cell wall area selected in Fig. 1; Image was generated using the 2890 cm^{-1} band area intensity. Bright white/yellow locations indicate high cellulose concentration; dark blue/green regions indicate very low concentration, e.g., lumen area. Intensity scale appears on the right. The electric vector direction of the laser was in the Y direction.

Intensity variation in S2

Although image intensity in S2 region varies from 550 to 850 units (Fig. 2), the variation is largely due to the compositional difference. This interpretation is based on the observation that when the ratio of the 1098-to-1120 cm^{-1} band is compared from various locations in S2, the ratio is about same which argues against any significant change in the alignment of the microfibrils. It is known that in S2 the orientation of the microfibrils is longitudinal which makes the cellulose chain axis orthogonal to the ev direction (Y axis). Once again, the 2890 cm^{-1} region image plot showed such compositional variation in S2 region and supported these observations (Fig. 4). The reason for this compositional difference within S2 is presently not clear.

CC and CmL regions

Both Fig. 2 and Fig. 4 indicated that as expected there is hardly any cellulose in the CC regions of the partly delignified CS [image colors, respectively, green (Fig.2) and dark blue/green (Fig. 4)]. The higher intensity in Fig. 2 compared to Fig. 4 can be rationalized by the way these images were generated. In the case of Fig. 2 the fluorescence background intensity was included (because peak height was used) whereas for Fig. 4 a sloping baseline used to calculate area intensity rejected most of the background contribution.

Before one can discuss cellulose image intensity in the CmL region a couple of points need to be considered. The total width of the CmL region is estimated to be about 0.5 µm although it can vary from region to region. Because Raman micro-sampling lateral resolution is 1 µm at any given location the sampling will involve some contribution from the region neighboring that CmL location. Second and more importantly, as the tissue is delignified it swells further in water (imaging experiments were carried out in D_2O) until the cells can expand no more – a limitation due to geometric/spatial considerations. From our Raman results (Fig. 2) it appears that in some areas there are no CmL domains left and the S1 regions of the neighboring cells are in contact. Contrarily, in some other locations (e.g., longer side of the cell in Fig. 2 and Fig. 4; top and bottom), such regions are retained. The regions where S1s from two neighboring cells make a contact, no significant change in cellulose-intensity-based images could be detected (Fig. 2 and Fig. 4) implying that the contact was good. On the other hand, evidence of poor contact due to retaining of the original CmL regions is borne out by the observations that not only these regions are cellulose intensity poor but also that there are

no spectral changes that will support significant changes in cellulose orientation.. Such image regions are clearly visible in both Fig. 2 and Fig. 4.

Images of radial LS

An area selected for imaging the radial LS is shown in Fig. 5. The area included the double cell wall and the CmL region.

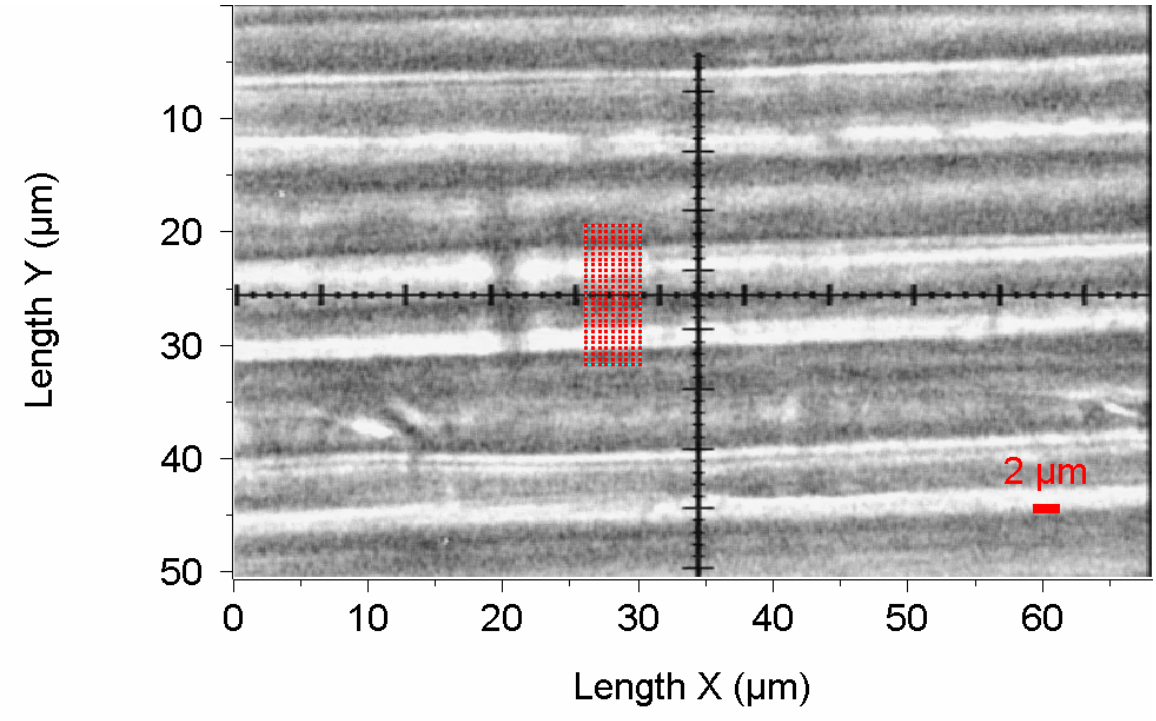

Figure 5—Bright field image of partially delignified radial LS with superimposed sampling grid pattern (234 points at 0.5 m resolution). Spectra were obtained sequentially from a series of positions using point-by-point scanning.

Using the 2890 cm^{-1} band region an area intensity image was generated (Fig. 6) and it can be noted that the images of the double cell wall regions are roughly equally intense. This indicates that perhaps both the composition and orientation in these regions are similar. When representative spectra of selected locations in the regions were extracted and compared the results indicated that indeed this was the case. The incident laser ev was parallel to Y direction and perpendicular to the cell axis (X-direction). In the CmL region (center of the image), the magenta color indicated that there was a decline in the intensity of the image, which upon further analysis was found to be due to a reduction in the concentration of cellulose. Only a small reduction is expected because although the entire CmL region was sampled, given the 1 µm lateral-resolution limit, some of the neighboring S1 regions were also sampled. This explains why there was not a bigger intensity drop in the CmL regions. Additionally, in a delignified section, effect of swelling on the dimensional change of the CmL is to be considered.

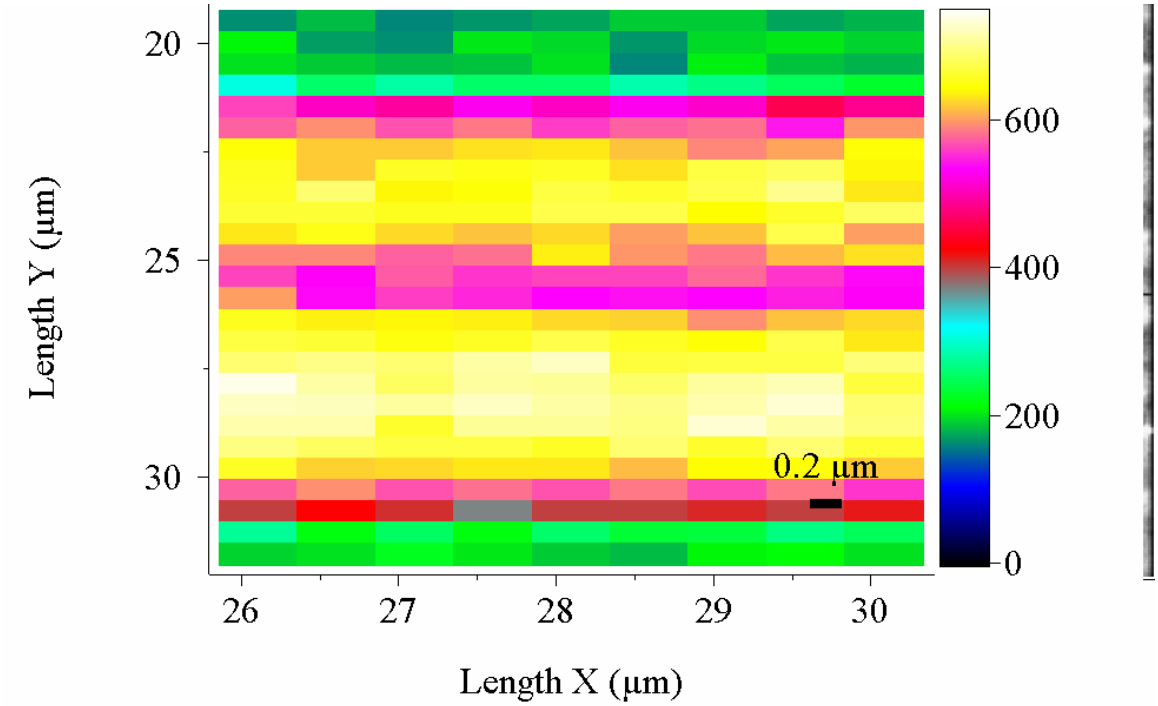

Figure 6—Raman image (false color) of cellulose spatial orientation and/or distribution in the cell wall area selected in Fig. 5; Image was generated using the 2890 cm^{-1} band area intensity. Bright white/yellow locations indicate high cellulose concentration; light blue/green regions indicate very low concentration, e.g., lumen area. Intensity scale appears on the right. The electric vector direction of the laser was in the Y direction.

Conclusions

Cellulose organization in black spruce cell walls was investigated using Raman imaging. The images of S2 and S1 regions in the CS showed major differences and these were found to be related to the manner in which cellulose microfibrils were oriented in these regions. In CS, image intensity variations in S2 were mostly due to changes in cellulose concentration. The study also indicated that small amount of cellulose was present the CC regions.

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