

Raman imaging of lignin and cellulose distribution in black spruce wood (*Picea mariana*) cell walls

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ABSTRACT

A detailed understanding of wood cell wall structure and organization is important from both fundamental and practical point of views. A state-of-the-art 633-nm laser based confocal Raman microscope was used *in situ* to investigate the cell wall organization of black spruce wood. Chemical information on lignin and cellulose from morphologically distinct cell wall regions was obtained and Raman images of their distribution were generated. The initial findings were that lignin and cellulose concentration in tracheid cell walls differed significantly both within and between the distinct morphological regions. For instance, secondary wall region of some cells showed lignin concentration variation by as much as 100%. Although, normally the concentration of lignin was higher in the compound middle lamella region (CmL) compared to secondary wall (S2 and S3), there were numerous instances when the magnitudes of the two concentrations were similar or were even reversed. Some of the true cell corner middle lamella (CCmL) regions had the highest lignin concentration; however, even in this morphological region, the lignin concentration varied. Compared to lignin, cellulose distribution was more uniform; nevertheless, in specific limited areas, cellulose concentration varied significantly. At the present time the intensity maps of lignin and cellulose distributions are interpreted solely in terms of concentration differences but effect of orientation needs to be carefully considered in order to truly reveal the organization of the wood cell walls.

INTRODUCTION

Wood is a heterogeneous natural polymer composite of cellulose, lignin, and hemicelluloses [1]. To develop an improved understanding of various properties of wood, wood pulp, and paper products, it is important that the structure, organization, and function of lignocellulosic materials be understood [2-6].

In the case of woody tissue, numerous techniques have been applied to study the sub-cellular distribution and organization of wood components [7-17]. For lignin, different techniques provided results that were different and reconciling such differences has not been easy. The intent of the work presented here was to carry out Raman microanalysis of woody tissue and generate information on the organization and distribution of the cell wall polymers. Raman microscopy was previously used to analyze cell walls *in situ* and it was shown that information on both cellulose and lignin can be obtained [17-20]. However, at that time progress towards detailed cell wall investigations was hindered due to the limitations that were both sample and instrument driven. Whereas on one hand, sample fluorescence and a less-than-fully-stable lignin band were causes of concern, on the other hand, non-availability of a

highly efficient Raman microprobe equipped with an automated high-resolution X-Y stage was a limiting factor. Fortunately, In 1990s, new technologies like introduction of holographic notch filter and availability of CCD (charge-coupled devices) multichannel detectors decreased the acquisition times by more than an order of magnitude [21]. Additionally, rugged, air cooled lasers (e.g., He-Ne 633 nm) simplified utility requirements and provided additional beam pointing stability over water-cooled lasers. The latest Raman microprobes incorporate these advances and are well suited to investigate lignin and cellulose distributions in the cell walls of woody tissue. These capabilities permit compositional mapping of the woody tissue with chosen lateral and axial spatial resolutions. Thousands of spectra can now be obtained in a realistic time frame to generate component-specific Raman images to study wood ultrastructure. A recent report applied this Raman approach to study the tissue of beech wood [22]. The objective here is to study the heterogeneity of black spruce cell wall composition and organization by using a 633 nm based confocal Raman microprobe.

EXPERIMENTAL

Sample Preparation

From a northern Wisconsin location a black spruce [*P. mariana* (miller) B.S.P.] tree was harvested. The samples were taken from the main stem. The transverse-sections were prepared by microtome from segments between the 30 and 40 annual rings and were 10 μm thick. The sections were stored in 95% ethanol. Prior to sampling, the selected section were sequentially extracted with acetone:water, toluene:ethanol (9:1), and methanol. The extracted and dried section was affixed to one side of a double-stick carbon tape disc which was readied for the section adhesion by punching a hole through its center. The hole was needed for the light to pass through the disc so that the section could be imaged in the bright field mode. The other side of the tape disc was fixed to the microscopic glass slide.

Raman Analysis

A Raman microprobe spectrometer, LabRam HR 800 (Horiba Jobin Yvon, Edison, NJ) was used in the present study. The spectrometer is equipped with a confocal microscope, a piezoelectric x, y stage, and a CCD detector. This instrument provides ultra high spectroscopic resolution and unique polarized 633 nm excitation that enable the system to be well suited to woody tissue research. The woody tissue spectra from various morphological regions were obtained by using the serial (raster) mapping technique. Woody tissue was placed on an automated piezoelectric x, y mapping stage, and Raman spectra are obtained at different points in the tissue by moving it equivalently under the microscopic objective. For mapping in x, y plane at chosen spatial resolutions, the specimen is moved in two spatial dimensions (x and y) with a spectrum being recorded at each (x, y) position. The vertical z displacement was controlled with the manual micrometer adjustment. The confocal pinhole diameter was set to 100 μm for all experiments. The spectral and spatial information was recorded using a two-dimensional CCD detector. LabSpec 4.02 software was used to obtain Raman spectra in

specific spectral ranges (between 250 to 3200 cm^{-1}). Further, the software was used to perform all spectrometer operations, process collected data, and generate Raman images. Such images based on component specific spectral regions, represented distribution of the cell wall components.

An Olympus 100X metallurgical objective, NA 0.90, was used for Raman studies. The different acquisition times were used depending upon the signal-to-noise desired and duration of the experiment. Similarly, experiments with higher x, y resolutions were carried out depending upon the nature of the information desired. Confocal aperture was set at 100 μm in all experiments. The lateral resolution of the confocal Raman microscope was $\sim 1\mu\text{m}$ (as reported by the vendor).

RESULTS AND DISCUSSION

Raman contributions of cellulose, lignin and hemicelluloses have been identified [23]. Earlier Raman studies (both visible- and near-IR-excited) of black spruce wood have indicated that spectral contributions of the 2 main components, cellulose and lignin, are detected in regions that for the most part do not overlap. However, the contribution of hemicelluloses was found to be masked by the bands of cellulose due to the fact that the former is chemically similar to cellulose and is non-crystalline. The latter characteristic of hemicelluloses makes their Raman contributions broad and diffuse.

Raman Spectrum

A Raman spectrum obtained from an S2 location is shown in Fig. 1. The bands of lignin and cellulose that were used in generating Raman images have been annotated.

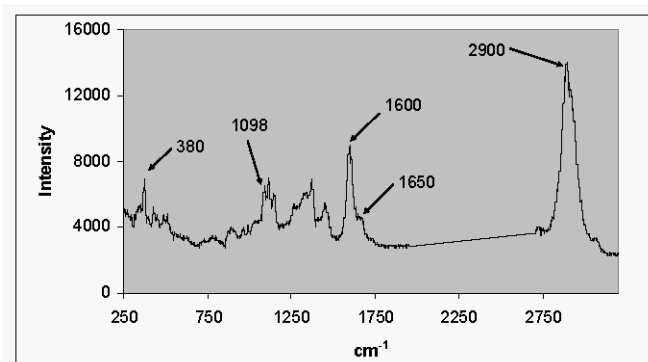


Fig.1: Raman spectrum of S2 in the transverse-section of black spruce wood. The bands at 380, 1098 and 2900 cm^{-1} are due to cellulose. Lignin contributions are present at 1600 and 1650 cm^{-1} .

Lignin Stability

As reported in earlier [17, 19, 20] Raman microprobe work, lignin's 1600 cm^{-1} band showed some decline when woody tissue was sampled in water and exposed to intense laser beam (514.5 nm and 647 nm excitations). Although "water-sampling" was not used here, it was considered prudent to check for any 1600 cm^{-1} band instability. This was done by obtaining a series of sequential spectra from the same location in both S2 secondary wall and cell corner middle lamella (CCmL) regions. The results

for S2-spot are shown in Fig. 2 where the integrated band intensity is plotted against time. It is clear from the data in Fig. 2 that the band intensity remained constant (within experimental error). Similar results were obtained from the spot in a CCmL region.

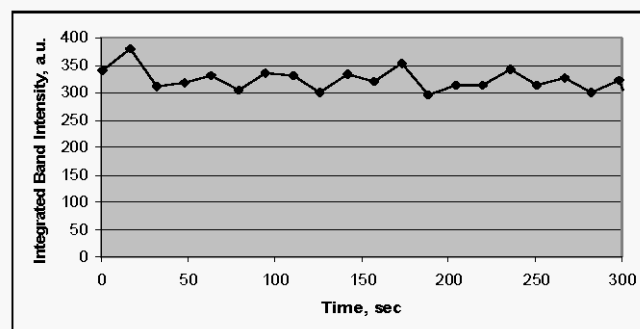


Fig. 2: Change in the area-intensity of the 1600 cm^{-1} band with time. The sampled spot was located in S2 and had not been previously exposed to the laser beam. Each point is a 15s acquisition.

Measured Depth Profile

The reported depth resolution for the 100 μm confocal hole, based on the silicon (standard) phonon at 520 cm^{-1} band is $\sim 4\mu\text{m}$. Because the spectra were obtained from the surface of the spruce section, it can be assumed that the axial resolution, as measured for silicon (4 μm), and the depth resolution at the surface of the woody section are the same. However, considering that woody tissue's refraction and reflection properties are significantly different compared to silicon, it was decided to measure the depth of focus by generating a z-scan intensity-depth profile and measuring the full-width-at-half-maximum (FWHM). The z-direction depth profile for dry woody tissue transverse section is shown in Fig. 3. The apparent thickness 10 μm (based on the FWHM) and the true thickness of the section matched well indicating that under the experimental conditions used no significant deterioration of the axial resolution took place as a function of sampling depth.

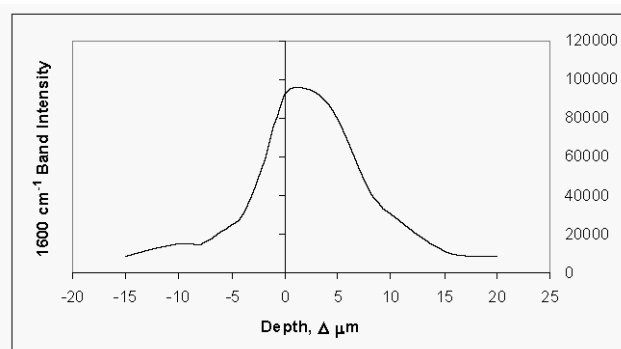


Fig. 3: Experimentally observed z-direction intensity-depth profile for the 100 X objective and a 10 μm thick black spruce transverse-section. Section thickness is correctly estimated ($\sim 10\mu\text{m}$). In the z-direction scan, the spectra were obtained at steps of 2 μm

Whole Cell

The lignin and cellulose distribution in various cell wall morphological regions across a whole latewood cell was studied using two Raman band regions 1519-1712 and 978-1178 cm^{-1} , respectively for lignin and cellulose. The lignin band at 1600 cm^{-1} and cellulose's contribution at 1098 cm^{-1} lie in these spectral regions. In the selected area of mapping (Fig. 4), adjoining regions from the neighboring cells were included as well. In Fig. 4, the 30 X 20 μm^2 area of investigation is included within a red rectangle. This area was mapped at 0.5 μm x, y resolution and 2400 spectra (in each region) were obtained to generate the Raman images shown in Fig. 5. Lignin distribution is shown in Fig. 5a whereas cellulose distribution is shown in Fig. 5b

Lignin distribution

In the Raman spectrum of black spruce, the most intense contribution from lignin is seen at 1600 cm^{-1} (Fig. 1). This band was therefore selected to study lignin distribution. Raman image of the distribution was generated in two ways - using only the 1600 cm^{-1} band and using both the 1600 and 1650 cm^{-1} bands. The 1650 cm^{-1} contribution was included not only because it is a lignin feature but also due to its partial overlap with the band at 1600 cm^{-1} . However, the Raman images generated in these two different ways were almost identical with the exception that the signal-to-noise was slightly improved for the two-band case. Therefore, it was decided that Raman image of lignin distribution will be generated using the combined band region 1519-1712 cm^{-1} . Fig. 5a shows such an image of the area selected in Fig. 4. In this 2-D plot, lignin concentration is highest (600-550 counts, white/yellow) in areas that correspond to CCmL (Fig. 4 and Fig. 5a). Next highest lignin concentration (450-400 counts, magenta/red) was in regions that are outside cores of the CCmL followed by compound middle lamella (CmL) areas (400-350 counts). Considering the lateral resolution of the confocal spot ($\sim 1 \mu\text{m}$), it is likely that CmL areas contained some contributions from the S2. Lignin distribution in S2 and S3 showed a more complex behavior. In some places it was as high as the CmL value (400-350 counts) whereas in other locations it was significantly lower (300-200 counts). In one region (top left, towards the center, Fig. 5a), where the CmL seems to have bifurcated, the locations in the center of the splitting showed low lignin intensity (150 counts). The results for the CCmL regions are same as expected from earlier knowledge. However, in the Raman image (Fig. 5a), lignin is non-uniformly distributed in the S2 and S3 areas and that the detected band intensity differences may have been a reflection of lignin's orientation – lignin orientation was previously detected by Raman microscopy [18, 19]. Although here the He:Ne laser was polarized in the x-direction, the sample could not be rotated to produce the data that would have addressed the issue of the effect of orientation on lignin band intensity. It is hoped that a future study specifically targeted to look into this aspect of the findings will reveal the extent to which the intensity variations can be interpreted in terms orientation and concentration effects.

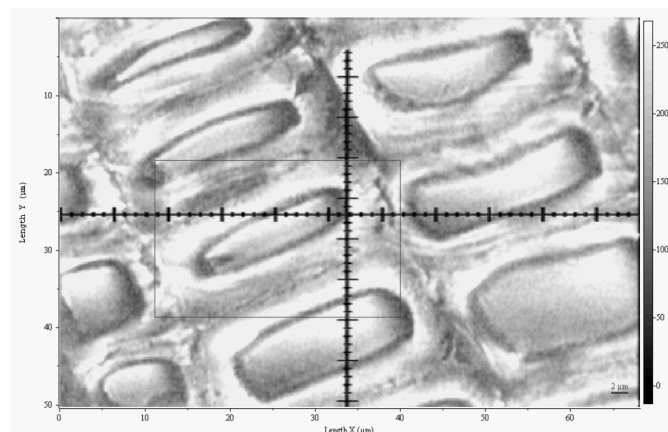
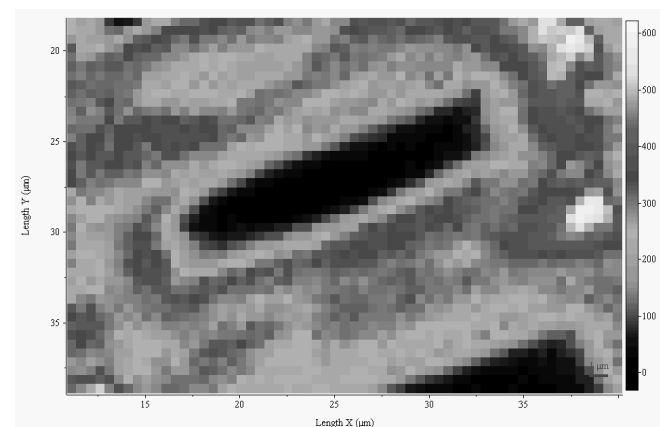
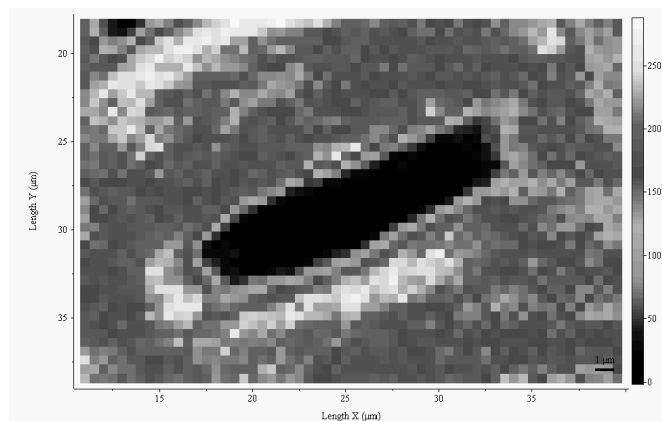


Fig. 4 White light (bright field) image of the spruce section showing the area (in red) investigated by the Raman microscopy. Raman images of the area are shown below in Fig. 5a (lignin) and Fig. 5b (cellulose).



(Fig. 5a)



(Fig. 5b)

Fig. 5: Raman images (false color) of lignin (Fig. 5a) and cellulose (Fig. 5b) spatial distribution in the area shown in Fig. 4. The intensity scale is shown on the right hand side. Although the absolute intensity values in the two intensity scales are different, the colors indicate the same relative scale. Bright whitelyellow locations indicate high concentration of lignin (Fig. 5a) and cellulose (Fig. 5b). Dark blue/black regions indicate very low concentration; e.g., lumen area where neither of the 2 components is to be found. See electronic document for images in color.

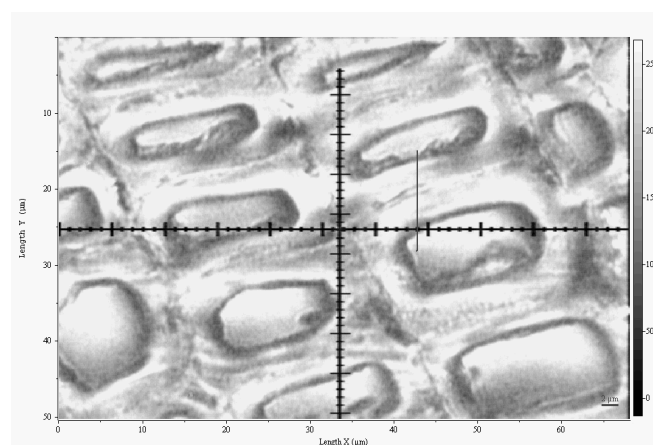
In most-S2 and CCmL areas, lignin's 1600 cm^{-1} band intensity varied by a factor that was less than 2.4 (ratio of highest to lowest 600/250). Nevertheless, an average value of the S2 lignin-intensity will be significantly higher because there are many more spots where intensity was greater than 250. This provides support for the previously obtained results [24] where CCmL concentration of lignin was found to be no more 2 times that of the S2 in sodium borohydride treated black spruce sections. Lastly, the lignin band was quite weak near the interface of the S3 and lumen. This is to be expected due the fact that at the interface sampled spot contains some of the empty volume of the lumen.

Cellulose distribution

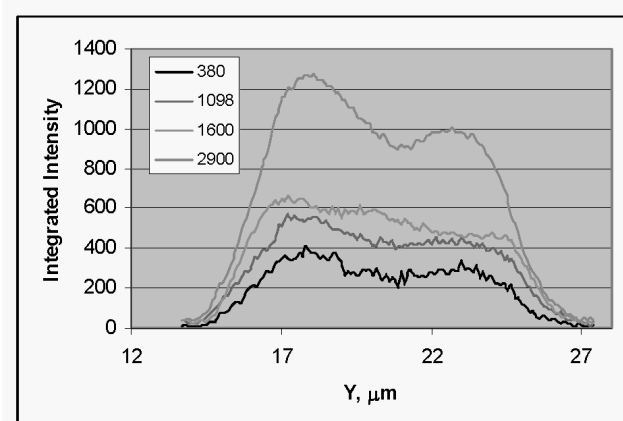
Compared to lignin, cellulose distribution was much more uniform (mostly red/magenta, Fig. 5b). Nevertheless, there were locations of high (white/yellow) and low (green/sky blue) concentrations. As expected, regions of highest lignin concentration, CCmLs, were now locations of low cellulose concentration (125-100 counts). An interesting area was found to be the bifurcated area (see above lignin distribution) where both lignin and cellulose intensities were low indicating that neither of the components was strongly present. High concentration (300-250 counts) cellulose spots were mostly confined to S2 and S3 regions. Considering that cellulose is a crystalline polymer and is highly oriented (chain-axis largely perpendicular to the transverse section), it is likely that such intensity variation may be a reflection of cellulose orientation. This is likely to become clear when, as was the case for lignin, further studies focusing on the effect of orientation on the band intensity are carried out. Lumen-S3 interface once again produced weak cellulose concentration and was the consequence of the fact that at such interfaces sampling included contribution from the lumen.

Double Cell Wall Line Scan

A double cell wall line scan (Fig. 6) was carried out using the four different Raman band regions (380 , 1098 , 1600 , and 2900 cm^{-1}). In addition to obtaining information on how cellulose and lignin concentrations varied across the two neighboring cell walls, the intent was to see if there were any differences among the plots generated using the different bands of cellulose (380 , 1098 , and 2900 cm^{-1}). A $13.7\text{ }\mu\text{m}$ -long line scan was carried out in the y-direction with a y spatial resolution of $0.1\text{ }\mu\text{m}$. The reason for such a high y resolution was to see if one was able to identify areas of sudden concentration change, if any, near the CmL region. Considering that the lateral resolution of the Raman probe is significantly larger than the true mL width, this approach was thought to be one possible way to delineate concentration differences.



(Fig. 6a)



(Fig. 6b)

Fig 6: White light image (Fig. 6a) of the spruce section showing the location of the line scan (red) in the y-direction. From the $13.7\text{ }\mu\text{m}$ long scan, 138 spectra in each of the 4 spectral regions were obtained. Raman intensity variation for the line scan is shown in Fig. 6b for lignin (1600) and for cellulose (380 , 1098 , and 2900). The Figs. are better seen in color in the electronic document.

All 3 cellulose band profiles showed a similar pattern (Fig. 6b) although signal-to-noise was greatly enhanced in the 2900 cm^{-1} plot. This was due to the fact that the latter is the most intense Raman band in the spectrum of the transverse section (Fig. 1). Similarity of the 3 band profiles indicated that any small contribution by lignin in the spectral regions of the 1098 and 2900 cm^{-1} band profiles did not distort the results to any significant degree. It ought to be noted here that of the 3 spectral regions only 380 cm^{-1} band region ($309\text{-}399\text{ cm}^{-1}$) has contribution from only cellulose, the other two - 1098 and 2900 cm^{-1} regions (respectively, $978\text{-}1178$ and $2773\text{-}3045\text{ cm}^{-1}$) - have definite but small contributions from lignin and/or other hemicelluloses [23]. The advantage of using only one cellulose region of course is to reduce experiment time and if one uses the 2900 cm^{-1} region, the additional advantage is of higher signal-to-noise in the images. The latter translates to having more confidence in the analysis. On the basis of these findings, for cellulose, the 2900 cm^{-1} region results were considered better compared to the 380 and 1098 cm^{-1} regions.

From Fig. 6b it is very clear that upon moving from the top lumen side the cellulose concentration quickly rises to high value in S2, then slowly declines to a minimum value in the CmL region, and then rises again to a high value in the S2 of the lower cell wall, before falling again as the scan moves to the lower lumen. The cellulose band intensities show slightly higher intensity maximum on the left hand side of the plot and the reason for this is not known. This could be real or could be an aspect associated with the experiment. For instance, it is possible that the underlying fluorescence played a role although the intensity was calculated using the base line method. It is known that woody tissue fluorescence decays with time. By the time lower cell wall was sampled, the latter area has been exposed to the diffuse 633 nm light for quite some time. And any residual fluorescence contribution to the band intensity is bound to be lower compared to the contribution from the upper cell wall.

Lignin intensity profile, shown by the 1600 cm^{-1} curve in Fig. 6b showed an additional intensity-hump in the center of the curve. Additionally, compared to the cellulose profiles, the profile is broader on the S3 side indicating that lignin concentration is high in both S2 and S3. It declines a little bit as the scanning approaches the CmL area then increases somewhat and then declines again slightly. As the scan continues into the lower cell wall, the declining lignin intensity rises again in S2 and S3, prior to falling monotonously towards the lumen. Contrary to expectation, no significant intensity enhancement in the CmL region, that was not already present in S2 and S3, was seen in the double cell wall scan. This may have to do either with laser spot size (as pointed out before) or the fact that this area is a low lignin concentration mL region (There are several such areas in Fig. 5a.). As additional data becomes available this will become more clear.

Highly Resolved CCmL

To study, at high resolution, how lignin and cellulose distribution changed in the CCmL and vicinal areas another such region in the latewood area of the section was selected (Fig. 7). Cellulose and Raman distribution images obtained from this area are shown in Fig. 8.

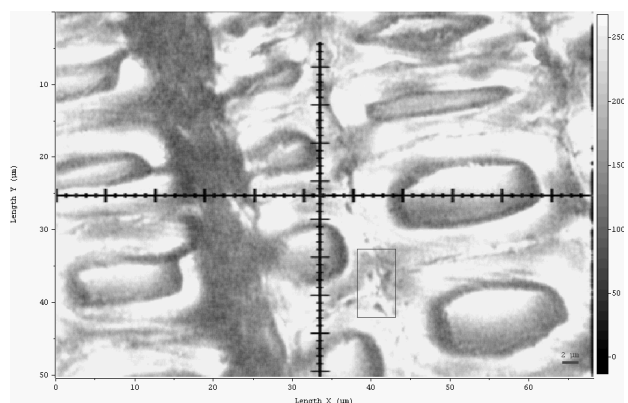
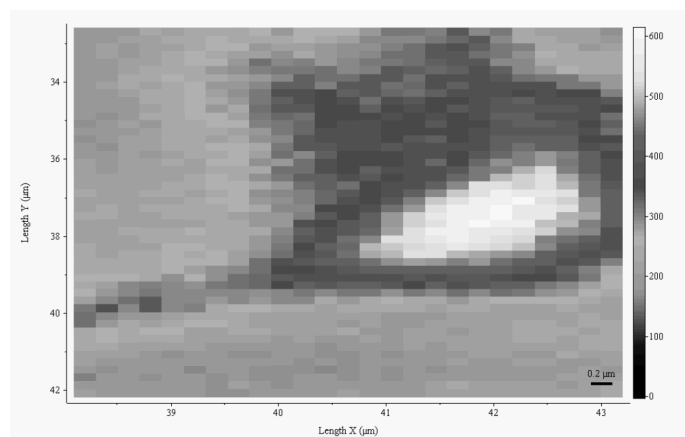
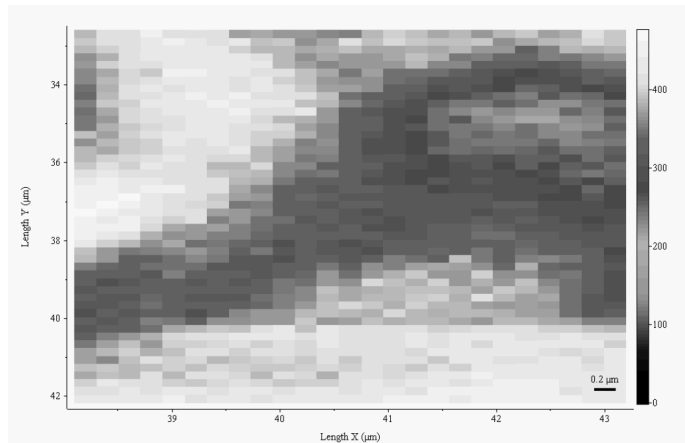


Fig. 7: White light image of the spruce section showing the CCmL area selected for high resolution study. Raman images of the area are shown in Fig. 8a (lignin) and Fig. 8b (cellulose, 2900 cm^{-1} band).



(Fig. 8a)



(Fig. 8b)

Fig. 8: Raman images (false color) of lignin (Fig. 8a) and cellulose (Fig. 8b) spatial distribution in the area shown in Fig. 7. The intensity scale is shown on the right hand side. Although the absolute intensity values in the two intensity scales are different, the colors indicate the same relative scale. Bright white/yellow locations indicate high concentration of lignin (Fig. 8a) and cellulose (Fig. 8b).

Lignin distribution in Fig. 8a suggests that compared to immediate neighboring S2 areas its concentration in the core CCmL areas were about 3 times higher. Earlier, in this paper, we have found it to be 2.4, this indicated that the lignin concentration ratio of the CCmL-to-S2 could be as high as 3 and is not constant. As expected, the lignin concentration declined (red/sky blue) as the mapped locations moved out of the CCmL into the mL, P, S1 and S2 areas. Once pure S2 areas could be sampled the lignin concentration declined significantly (green, Fig. 8a).

In the case of the cellulose, the distribution image (Fig. 8b) showed the lowest intensity at a location that was different than the area of the maximum lignin intensity (CCmL). Nevertheless, the area, just above the CCmL location, was still in the general region where the top 2 mLs merged (Fig. 7). Areas of highest cellulose concentration (white/yellow) were mostly in S2 regions whereas the mL and primary wall regions had medium cellulose concentration.

Earlywood

To assess how the earlywood distributions of lignin and cellulose were different from that of latewood an area was selected (Fig. 9) and Raman images were generated

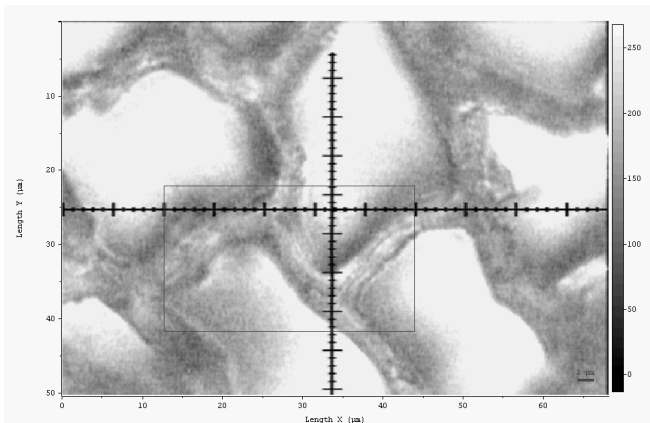
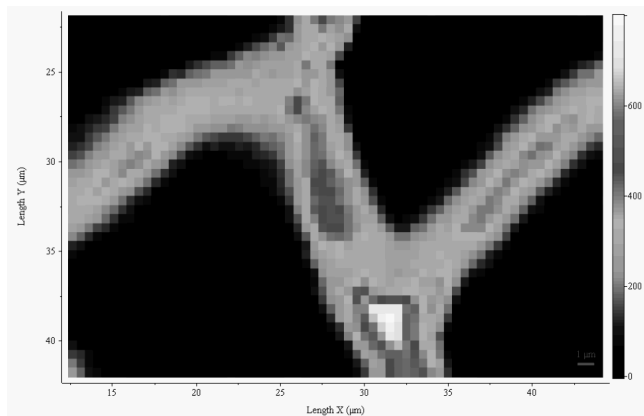
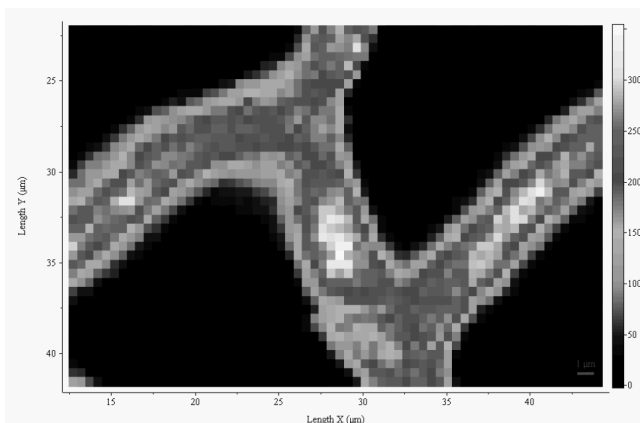


Fig. 9: White light image of the earlywood showing the selected area (enclosed in red rectangle) for the comparative study.



(Fig. 10a)



(Fig. 10b)

Fig. 10: Raman images (false color) of lignin (Fig. 10a) and cellulose (Fig. 10b) spatial distribution in the area selected in Fig. 9. The intensity scale is shown on the right hand side. Although the absolute intensity values in the two intensity scales are different, the colors indicate the same relative scale. Bright whitelyellow locations indicate high concentration of lignin (Fig. 10a) and cellulose (Fig. 10b).

Lignin distribution

The lignin image was generated using the same band region as for the latewood ($1519-1712\text{ cm}^{-1}$). The images of the two distributions are shown in Fig. 10. As expected, highest lignin concentration was found in one of the two CCmL areas (lower center, Fig. 9 and 10a). The concentration here was higher (800 counts) compared to CCmLs in the latewood (Fig. 5a and Fig. 8a). This may be a reflection of the often reported fact that compared to the latewood earlywood is more enriched in lignin. However, noteworthy is also the observation that the 2nd earlywood CCmL's (Fig. 9) lignin concentration was significantly lower (400 counts, Fig. 10b) implying that not all CCmL regions have the same lignin concentration. This further supported our earlier results [17] which had indicated that in black spruce CCmL the concentration of lignin was not uniform. Some additional comments can be made from the lignin distribution in Fig. 10a. Not only the lignin concentration in the mL regions was lower than the highest CCmL concentration, it also was heterogeneous. Some mL locations had the same intensity as the neighboring S2 locations. In the remainder of the earlywood wall regions (S2, S3) lignin concentration declined the further away the location was from the mL region. In general, compared to S2, S3 regions were less enriched in lignin (Fig. 10a).

Cellulose distribution

The earlywood cellulose distribution map is represented in Fig. 10b and was produced using the 1098 cm^{-1} band region ($978-1178\text{ cm}^{-1}$). Compared to lignin the distribution is different but there are some areas of overlap - in these locations where both cellulose and lignin are highly concentrated. For instance, lower yellow area in the center section of the plot. Some of the locations in the common area seem to be in the mL region. Although, in the present study we have assumed that the $978-1178\text{ cm}^{-1}$ region represents only cellulose, contribution from other polysaccharides (e.g., pectin or hemicelluloses) can not be ruled out. Other earlywood cell wall areas of high cellulose content were located in S2 regions. The concentration in S3 was found to be lower than in S2. One area where low cellulose concentration was detected was the CCmL area of the highest lignin concentration (Fig. 10a and Fig. 10b). However, this is to be expected because in CCmL areas the cellulose concentration is known to be significantly reduced compared to S2 and S3.

Multiple Transition-wood Cells

Lignin distribution

To visualize lignin and cellulose distribution over a larger region, a single annual ring transition-wood area containing 7 cells was selected (Fig. 11). The distribution image was generated at $0.5\text{ }\mu\text{m}$ x, y resolution (Fig. 12). Once again, evidence supporting lignin concentration variation was detected both within the morphological regions as well as between the regions. The lignin concentration variation order from highest to lowest was CCmL > CmL > S2 > S3. However, some CmL regions were not as lignified as the rest of them (Fig. 12). Additionally, specific areas within S2 and CCmL regions existed that showed exception to this order.

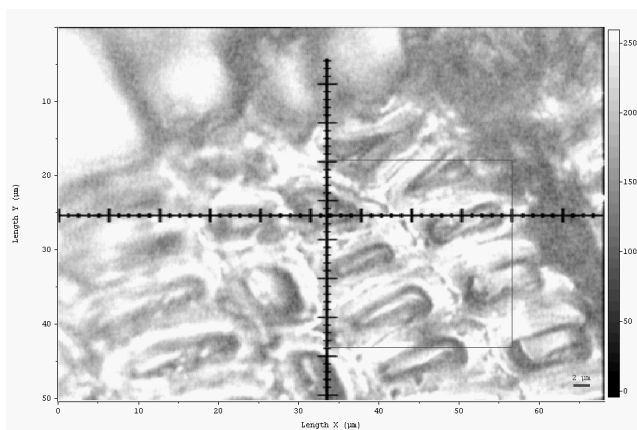


Fig. 11: White light image of the transition-wood area containing cell walls of at least 7 cells. The distribution of lignin was studied in the selected area (enclosed in red rectangle)

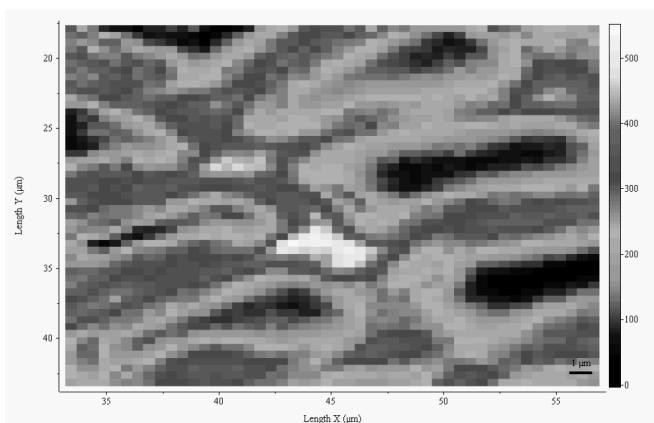


Fig. 12: Raman image (false color) of lignin distribution in the area selected in Fig. 11. The intensity scale is shown on the right hand side.

Cellulose distribution

Cellulose distribution was studied from a slightly smaller area (Fig. 13) compared to that chosen for lignin analysis. The cellulose experiment was performed after data acquisition for lignin was complete. Therefore, lignin and cellulose images were obtained sequentially and not simultaneously. Nevertheless, considering that all of the cellulose area in Fig. 13 consisted of the area that was part of lignin investigation (Fig. 11), all area-of-analysis in Fig. 13 is common between the two sets of experiments.

The cellulose distribution image was generated using the 1098 cm^{-1} band region ($978\text{--}1178\text{ cm}^{-1}$). The results were consistent with the prior findings and evidence supporting cellulose concentration variation was detected. Although most of the areas in Fig. 14 were red/magenta (200-150 counts), there were pockets of different color (yellow and green/blue areas; respectively, high and low intensity) indicating variability in cellulose distribution and/or organization.

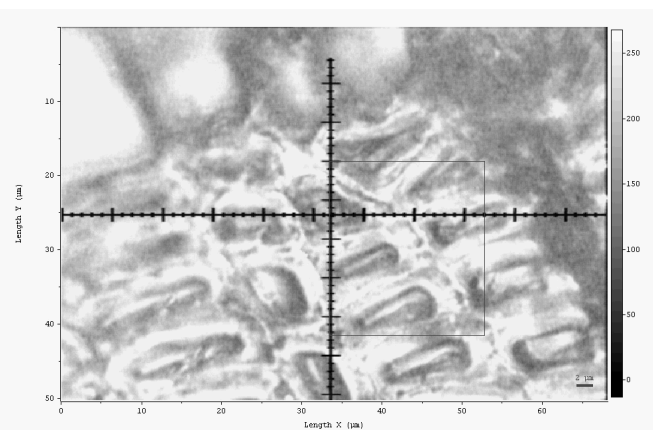


Fig. 13: White light image of the transition-wood area containing cell walls of at least 7 cells. The distribution of cellulose was studied in the selected area (enclosed in red rectangle). This area was slightly smaller compared to the area investigated in Fig. 11.

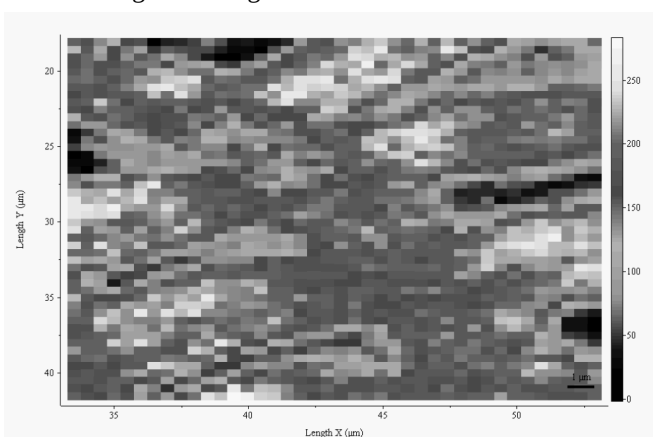


Fig. 14: Raman image (false color) of cellulose distribution in the area selected in Fig. 13. The intensity scale is shown on the right hand side.

CONCLUSIONS

The distribution of lignin and cellulose in the cell walls of black spruce wood was studied using a confocal Raman microscope. The Raman images, specific to lignin and cellulose distribution, indicated that at the microscopic level the concentrations of both cellulose and lignin varied extensively both within and between the distinct morphological regions. Such variability was present even within the group of a few transition-wood cells that were part of a single annual ring. Both earlywood and latewood regions were analyzed. One of the many observations made on the basis of the intensity maps was that when the highest lignin containing CCmL regions of earlywood and latewood were compared, the lignin concentration in the former was about 33% higher.

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In: Proceedings of the 59th APPITA Annual Conference and Exhibition incorporating the 13th ISWFPC (International Symposium on Wood, Fibre, and Pulping Chemistry), held in Auckland, New Zealand (May 16-19, 2005). [Carlton, Victoria, Australia]: APPITA, c2005. Both print and CD versions are available. pp. 377-384.