

Two-photon autofluorescence spectral imaging applied to probe process-effects in thermomechanical pulp refining

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SUMMARY: Norway spruce wood pulps were produced in full industrial scale trials at different thermomechanical pulp refining conditions, such as plate gap, housing pressures and energy consumption levels. To investigate the effects of these refining conditions on the lingo-cellulosic matrix in fines and fibers, we applied high-resolution spectral imaging in a two-photon fluorescence microscope and compared with conventional pulp and paper analyses (strength, freeness etc.). The fluorescence spectra of lignin in native wood and fibers- and fines from pulps showed that spatial- and spectral heterogeneities can be observed using two-photon autofluorescence spectral imaging, and successfully probed on a microscopic level. Moreover, it was shown that wood autofluorescence depends on fiber morphology and becomes red-shifted by increased temperature, but the fluorescence spectrum of TMP long fiber fraction shifted towards blue by increased refining pressure.

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In thermomechanical pulp (TMP) production, wet solid wood chips are mechanically disintegrated by a rotating refiner disc at elevated temperature and pressure. The properties of the resulting pulp fiber's matrix and surface depend on the structure and chemistry of the raw material and the process conditions. Since TMP production is relatively energy demanding, the development of more energy efficient processes and products is of interest. In this work, we have studied thermomechanical pulps (TMP) that have been produced at Holmen Paper, Sweden, using the pulp mill's full industrial scale primary stage refining system, at different refiner housing pressures, plate gaps and at different levels of energy input. The produced pulps have been

analyzed for general physical properties. But, also analysis of process induced differences in the pulp fibers on microstructural level is important since specific and localized fiber properties, such as the fiber surface-chemistry and ultrastructure, affect the surface interactions (i.e. fiber-to-fiber bond properties) and subsequently the paper quality. Therefore, microscopically resolved information about the wood pulps is of great interest. Fibers can be straightforwardly imaged using fluorescence microscopy of lignin (Donaldson et al. 1999; Hafrén 2007). Also, fluorescence spectroscopic analysis has been performed on wood and fibers; it is successfully used in, for example, bleaching and yellowing studies of mechanical pulp and on kraft pulp (Lundquist et al. 1978; Castellán, Davidson 1994; Davidson et al. 1991; Olmstead, Gray 1997; Liukko et al. 2007). Also, lignin autofluorescence was used to quantify compression wood severity (Donaldson et al. 2010). It was suggested in recent literature that long wavelength emission of lignin is due to 5 conjugated structures composed of coniferyl alcohol or a mixture of guaiacyl and p-hydroxyphenyl units (Donaldson et al. 2010). Chemical modifications of lignin may alter the conjugated systems of the complexes; this means that these modifications can be probed by the lignin spectral shape and give information on pulp fluorescent properties. We performed a spectroscopic analysis of lignin autofluorescence in wood and the pulps produced after wood refining.

To analyze the fluorescence of lignin on a sub-fiber level, we combined autofluorescence spectral detection with two-photon excitation fluorescence microscopy. In single-photon confocal scanning laser microscopy, the excitation wavelength is matched with the absorption spectrum of the fluorophore of interest. In two-photon excitation microscopy, energies of two photons are combined to excite one fluorophore in one quantum event; for example, two photons of 800 nm are used to excite at 400 nm. Two-photon absorption is a nonlinear process that depends on the square of the excitation intensity and the absorption of two photons occurs almost simultaneously ($<10^{-15}$ s); therefore, a femtosecond pulsed laser is usually employed. Due to the non-linear behavior, two-photon excitation outside the focal plane is a rare event and out-of-focus blur is minimized and mainly the fluorophores at the focal area of the beam are excited. Multi-

photon excitation (Denk et al. 1990) may be useful on wood since wood (similar to most bio-materials) has a low optical density in the near IR region that is used for excitation. Consequently, one-photon absorption is low and the excitation light may penetrate deep into the material. In this study, the scanning of the multiphoton microscope is synchronized with the spectral acquisition of a CCD camera based spectrograph (Palero et al. 2006; Palero et al. 2007). This way, full fluorescence emission spectra (128 wavelength channels) were recorded pixel-by-pixel at very short acquisition times.

The aim of this study was to describe the effects of refiner conditions on pulp properties using conventional pulp and paper tests and by probing lignin modifications in the pulps using two-photon spectral imaging. Spectral imaging may be used as a fast method for visualization of spectral variations in the lignocellulose matrix, and may also be applied to image any fluorescent marker used on wood or plant samples.

Materials and Methods

The wood samples were Norway spruce (*Picea abies*) cut in 1 μm cross sections using a slide microtome from air dried blocks of normal sapwood. Heat treated samples were produced using an autoclave, in which wood sections were placed and heated for 1 h, at about 121°C.

TMP production and samples

The TMP was produced from Norway spruce wood chips, at Holmen Paper's pulp mill (at Braviken, Sweden). The pulp were sampled from the primary stage refiner (counter rotating RGP68DD double disc refiners from Metso), operated at 4.4, 6.4 and 7.1 bar housing pressure, with different refiner plate gaps and at different energy consumption levels. The temperatures (166-177°C) in the refiner zone were controlled by feed and housing pressures (4.4, 6.4 and 7.1 bar). The samples were tested for physical properties as pulps and handsheets, produced using rapid Köthen (ISO standard 5269-2:2004). The pulp samples for autofluorescence spectral imaging were fractionated for size, and Bauer-McNett fractions mesh 30 and 200 were used representing long fiber- and fine fractions, respectively. The physical properties and the pulping procedures for these pulps have recently been described in more detail by Muhić et al. (2010).

Analyses of the pulp and paper

The pulp and paper properties were analyzed according to respective standardized test method, tensile index (ISO standard, 1924-2:1994),

consistency (ISO standard, 4119:1995), bulk density (EN standard, 20534:1994), freeness (ISO standard, 5267-2:2001) and brightness (ISO standard, 2470-2:2008).

Nonlinear spectral imaging

The nonlinear spectral imaging microscope is home built and optimized for high detection sensitivity over a broad wavelength range (Bader et al. 2011; Palero et al. 2006; Palero et al. 2007). It consists of a mode-locked Titanium Sapphire laser (Tsunami, Spectra-Physics, Sunnyvale, CA) generating 100 fs pulses of 1 W average output power at a repetition rate of 82 MHz. In this study an average excitation power of 5 mW at the specimen was used. The laser light (excitation $\lambda = 720\text{-}800\text{ nm}$) was attenuated by a dual ND filter wheel (Model 5254, New Focus, CA, USA). The computer controlled laser-scanning head consisted of an XY scanning mirror, a piezo driven focusing element (Nano-F200, MadCityLabs, Madison, USA), and a microscope objective (Fluor 40X/0.80 water immersion, 2.0 mm working distance, Nikon). Light collected by the objective was filtered by a dichroic mirror and a laser blocking multiphoton Emission Filter (FF01-680/SP-25, SemRock, Rochester, NY USA). The spectrograph consisted of one fused silica (Suprasil) dispersion prisms and an emCCD camera (Cascade128+, Photometrics, Tuscon, USA). Fluorescence spectra can be recorded at a maximum rate of 8000 emission spectra per second at a nominal spectral resolution of 2.7 nm from 350 nm to 650 nm (100 channels). The fluorescence spectral images (224 x 224 pixels, 128 channels/spectrum) were acquired at 0.128 ms per pixel.

The obtained spectra were corrected for the wavelength-dependent sensitivity of the system. To visualize the three-dimensional (i.e., x and y dimensions and wavelength channel) images, they were transformed into RGB images. Here, the RGB color represents the 'real' color of the autofluorescence. Furthermore, for regions of interest the average autofluorescence spectrum was extracted.

Statistical analyses

The spectra were compared using Student's t-test, mean p -value of all data points over indicated wavelength range.

Results and Discussion

Refiner conditions and pulp and paper properties

Temperature-induced softening of lignin is known to facilitate mechanical wood-fiber separation (Becker et al. 1977; Salmén 1984); and a reduction in specific energy demand when refining at elevated temperature and pressure is obtained (Höglund et al.

1997). The industrial TMP line used to produce the samples used in this study has a relatively new counter rotating double disc refiner system equipped to run at relatively high temperatures and pressures. Using the counter rotating double disc refiner, wood chips have been pulped at three refiner house pressures (4.4, 6.4 and 7.1 bar) to various specific energy consumption levels.

In Fig 1a, tensile indexes for pulps produced with energy input of about 1500-1900 kWh/adt (air-dried ton) are shown. The general trends show an improved tensile strength is obtained with increased pressure and energy input. In order to more specifically compare the pulps using spectral imaging, five pulps were selected for more detailed studies (Fig 1b); three pulp samples were produced at the same pressure (4.4 bar) but to three different freeness levels, and three of the pulp samples were refined to similar freeness levels (about CSF 150) but at three different pressures.

Table 1 shows a summary of the pulping conditions and pulp properties of the five pulp samples. The pulps clearly showed the expected relations between higher energy input and lower freeness level and lower shive content. In Table 2, the properties of paper sheets (made from the pulps in Table 1) are shown and the sheet density clearly correlates positively with the energy input. It is shown that increased refiner house pressure results in higher paper strengths at a certain specific energy level (Muhić et al. 2010); an increased housing pressure (from 4.6 to 7.1 bar) resulted in a reduced energy consumption (80-150 kWh/adt) to a given tensile index, and that the energy saving effect was larger at lower specific energy input. Also, a lower freeness value was obtained at a given level of

energy input when applying higher pressure. Higher pressure may shorten the wood material residence time in the refiner, and cause more intense defibration (Muhić et al. 2010).

Wood fiber morphology and spectral imaging

Tensile index is an important pulp parameter when comparing pulps, pulping and fiber treatments and the tensile strength can be partially correlated with fiber-to-fiber bonds strength, which in turn depends on e.g. the morphological and chemical properties of the fiber surface. Two- and multi-photon fluorescence microscopy has previously been useful linking sample morphology with autofluorescence (Huang et al. 2002; Masters et al. 1997; Palero et al. 2006; Palero et al. 2007; Bader et al. 2011). We have recorded the autofluorescence spectra of the wood fibers, by nonlinear microscopy. Lignin is probed here simply as a direct indicator of the lignocellulose material since its autofluorescence and heterogeneous abundance can be probed directly using spectral imaging, without extraneous stains or chemical derivatizations.

Spectral imaging of wood

Modifications to the aromatic part of the lignin ultrastructure result in alterations of lignin autofluorescence. The fluorescence of lignin may be described as being composed of 5 discrete emission bands (Donaldson et al. 2010). The difference between the emission maxima of these components is smaller than the width of the spectra, so they cannot be readily distinguished as separate peaks.

The excitations of the autofluorescent components in lignin of solid wood (Norway spruce) are wave-length dependent.

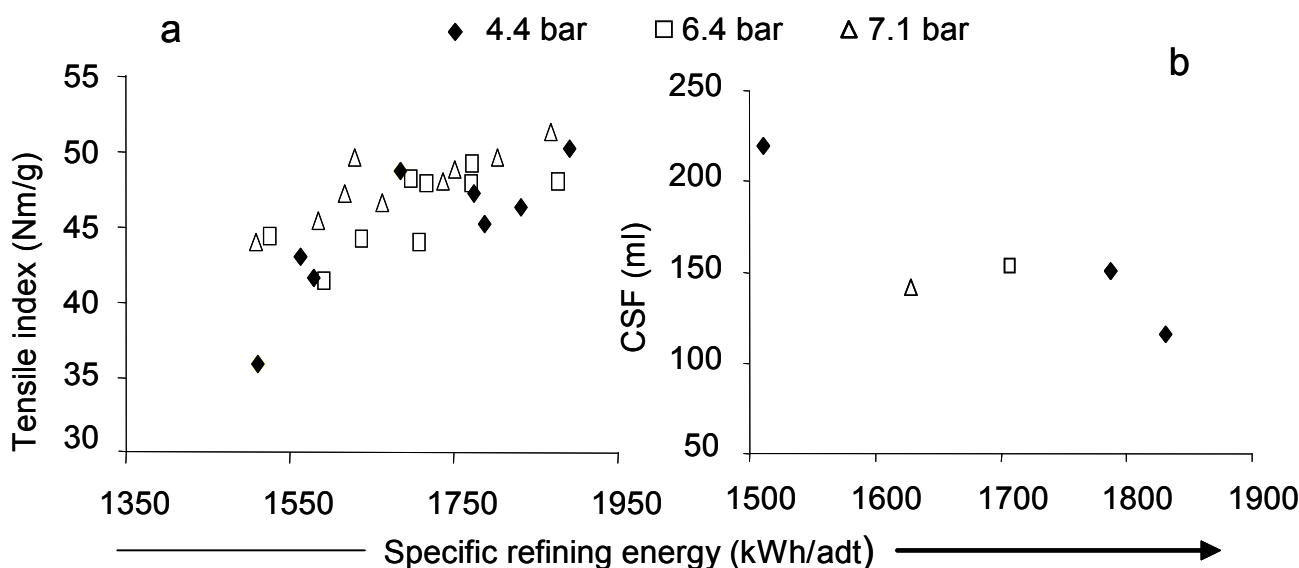


Fig 1. Specific refiner energy, kWh/adt (air-dried ton) and tensile strength (a) and Canadian Standard Freenes (CSF, b).

Table 1. Refining conditions and pulp properties.

Sample	Temp ^a (°C)	Pressure ^b (bar)	Energy (kWh/ton)	Consistency (%)	CSF ^c (ml)	Shive (sum/g)	Plate gap (mm)
1	166	4.4	1511	33.5	220	572	1.07
2	168	4.4	1788	36.8	151	314	0.92
3	170	4.4	1832	40.1	115	272	0.81
4	174	6.4	1708	37.3	153	378	0.82
5	177	7.1	1629	36.3	142	189	0.85

^a Measured temperature in the refining zone, ^b Measured housing pressure, ^c Canadian standard freeness

Table 2. Properties of paper sheets; made of the pulps from table 1.

Paper Sample	Tensile index ^a	Density (kg/m ³)	Brightness
P1	35.9	438	60.92
P2	45.2	460	60.84
P3	46.3	495	62.34
P4	44	481	61.62
P5	49.6	491	60.04

^a Strength index (Nm/g)

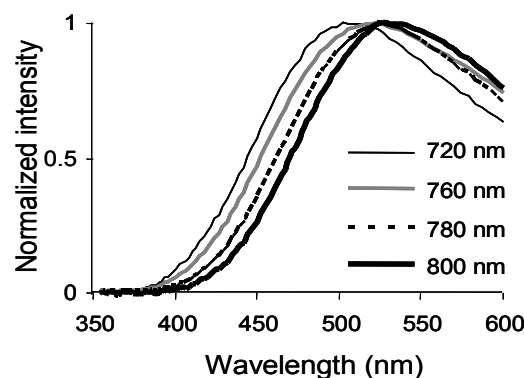


Fig 2. Autofluorescence emission spectra of wood, excited at different wavelengths (two-photon excitation).

In Fig 2, excitation wavelengths of $\lambda = 720\text{--}800\text{ nm}$ (absorption at $\lambda = 360\text{--}400\text{ nm}$) was used. The lignin spectra excited at 720 nm and 800 nm appear relatively more narrow; presumably containing relatively more of components IV ($\lambda_{\text{max}} = 500\text{ nm}$) and V ($\lambda_{\text{max}} = 535\text{ nm}$) (nomenclature of Donaldson et al. 2010). The spectra excited at 760 nm and 780 nm seem to contain more of a mixture of fluorescent components.

Next, local variations in the lignin composition were investigated. Autofluorescence spectra were collected from two regions of interest in Fig 3b. The respective averaged spectra of ray cell-fiber and fiber-fiber cell walls displayed different wavelength distributions; lignin in-between a fiber tracheid and ray cell displayed a more red-shifted emission spectrum than between the two wood fibers. Apparently, fiber tracheids-ray cell walls have a different lignin composition, i.e. they seem enriched in component V. Higher concentration of the red edge components was also observed in compression wood (Donaldson et al. 2010). Note that elongation of the π -electron system typically results in a red shift (Valeur 2002). Since elongated aromatic systems are also more rigid, it is plausible the increased rigidity may affect some strength properties. Indeed, it has been proposed that relatively higher lignin content and p-hydroxyphenylpropane units, which can crosslink, increase the fiber cell-wall resistance to compression failure (Gindl 2002). We hereby established that two-

photon spectral imaging can discriminate between morphological structures in wood lignocellulose.

We also investigated the effect of thermal treatment of the wood. TMP is produced at elevated temperatures, and the temperature has shown to affect wavelength- and intensity distributions of the fluorescence of lignin rich pulps (Chupka et al. 1995; Forsskål et al. 2000). Samples subjected to pre-heating displayed a narrower spectrum compared to untreated wood, as shown by the emission spectrum (Fig 3a and b). The more narrow spectrum observed here suggests that one component (IV) is relatively more abundant and red shifted. Possibly, the red shift is the result of chemical rearrangements extending conjugated structures, and/or physical rearrangements in the nano-environment facilitating energy transfers between fluorophores.

Spectral imaging of pulps

After analyzing the fluorescence properties of native wood by two-photon microscopy, we analyzed the five selected pulps from Table 1. The morphological specificity displayed in Fig 3 was also shown in spectral images of the pulps, where different fractions of fibers and fines were present; the spectra of different areas of the images displayed variability in their wavelength distributions. Therefore, in order to get more homogeneous samples fractionated pulp samples were used and the long fiber- and fine samples were used separately for spectral imaging analyses.

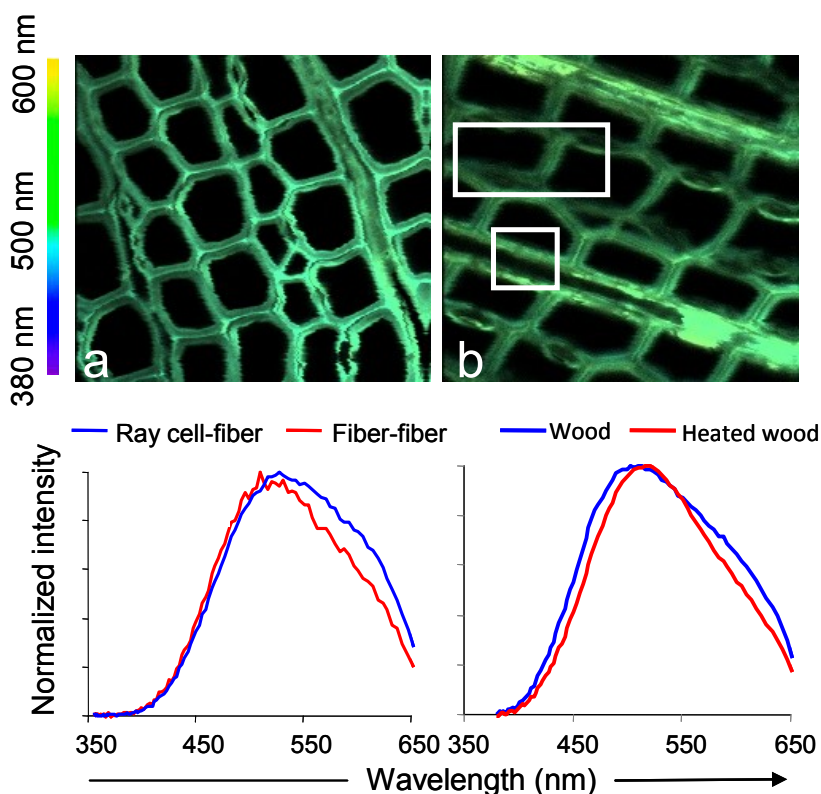


Fig 3. Autofluorescence spectral images of wood (image size $75 \times 75 \mu\text{m}$), at excitation 760 nm. Image 3a shows a cross section of native spruce. Image 3b shows the cross section of heat treated spruce. A fiber wall section between fiber tracheids has been marked by a rectangular box, and a wall section between fiber tracheid and ray cell has been marked by a square box. The relative wavelength distributions of the autofluorescence within the respective regions of interest are shown in the spectra below to the left. The spectra to the right show the averaged wavelength distributions of all pixels in the whole images 3a and b, respectively.

Fractionation is a mesh-based size exclusion procedure that results in a relative enrichment of different fraction of the pulp material and we used mesh fraction 30 (long fibers) and 200 (fines). Still, after fractionation the pulp material is not completely uniform, however, the spectral imaging technique allows for direct visualization of the sample's features and spatially optimized sampling on microscopic level for the autofluorescence spectral analyses. Thus, in the images of fine fractions we identified and analyzed "fiber free" regions of interest, in order to specifically obtain spectra of smaller and more amorphous objects.

The five refined pulp samples were analyzed with two-photon spectral imaging. The respective sample spectrum, containing the sum of all spectra from an image, was used for comparing spectra between different samples. In all cases the whole pulp fluorescence was blue shifted in emission compared to the wood ($\lambda_{em,pulp} \approx 495 \text{ nm}$, $\lambda_{em,wood} \approx 505 \text{ nm}$). This shift is the opposite of what has been observed for heated wood. Apparently, the pulp refining has a different effect on the fiber autofluorescence than what increased temperature, alone, had on wood (Fig 4).

Several factors affect the lignin, and consequently also its autofluorescence, like irradiation and chemical treatments (Davidson et al. 1991; Olmstead, Gray 1997; Liukko et al. 2007). One interesting question is which aspect of mechanical pulping is responsible for the fluorescence spectral alterations. In Fig 5, the pulp fibers and fines from samples 1-3 were compared by their emissions spectra. These samples are produced using the same refiner housing pressure, but they differ in specific energy input (increases from sample 1 to 3).

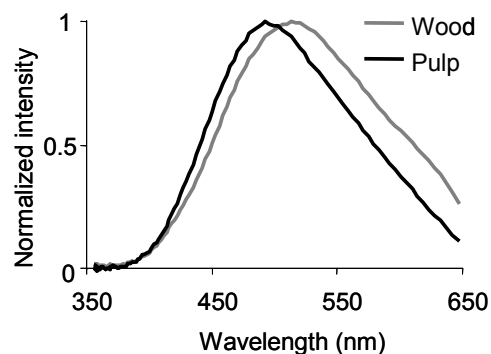


Fig 4. Averaged autofluorescence emission spectra of native wood (Wood) and whole pulp at low pressure and energy input (Pulp).

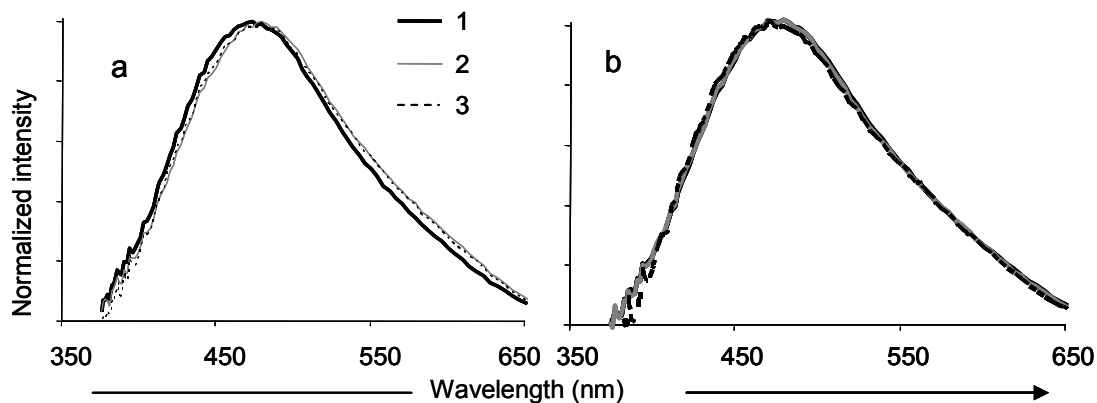


Fig. 5 Autofluorescence spectra of fines (a) and fiber (b) fraction of pulp samples 1, 2 and 3 (see Table 1), at excitation 760 nm.

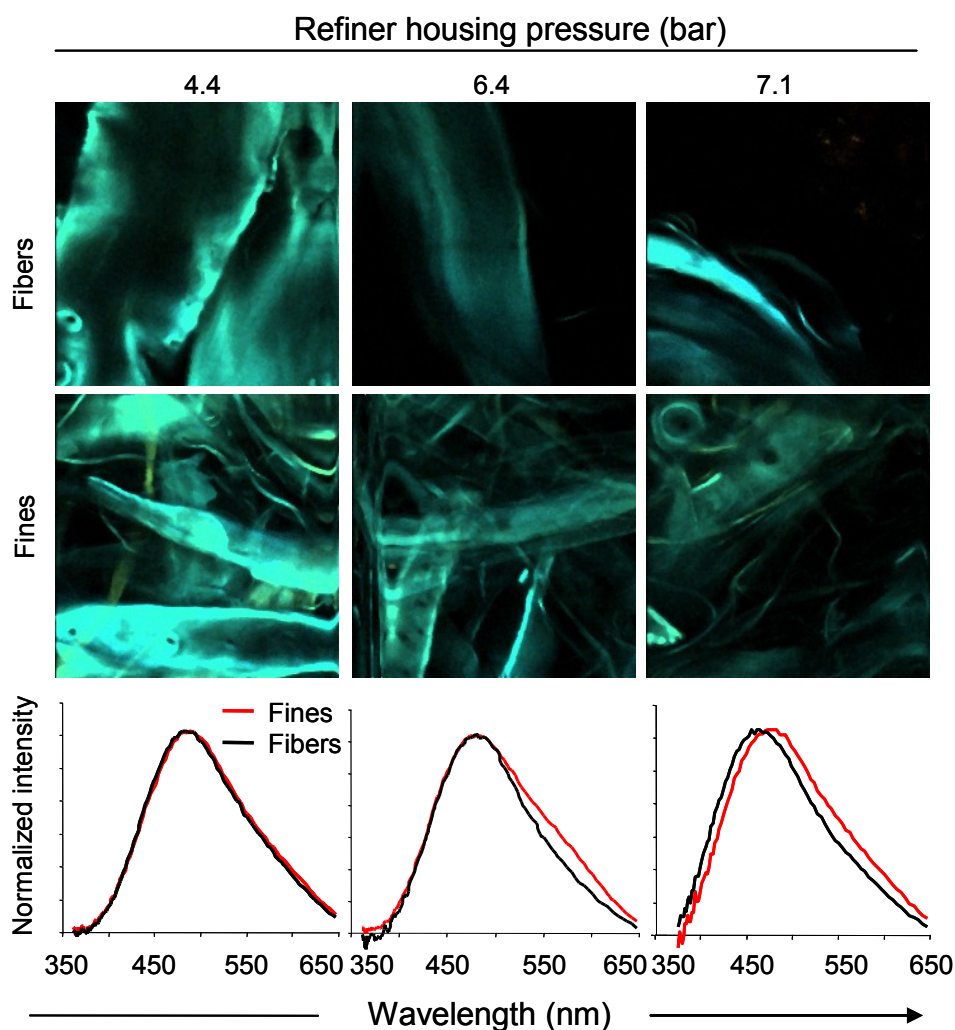


Fig 6. Autofluorescence images and spectra of fibers and fines from pulp samples 2, 4 and 5 (image size $75 \times 75 \mu\text{m}$), at excitation 760 nm.

Relative to sample 1 there was a slightly increased fluorescence at the longer wavelengths in sample 2 and 3 for the fine fractions and no significant differences for the fiber fractions. We therefore conclude that a higher energy input alone does not much affect the relative autofluorescence properties of lignin.

In Fig 6, the images and spectra of the fines and fibers in samples 2, 4 and 5 are shown. Here, the

effect of increasing refining housing pressure was studied. Especially for the long fiber fractions, the increasing pressure induced a relative blue shift of the spectra emission maxima (4.4 bar $\lambda_{\text{em}} \approx 480 \text{ nm}$, 6.4 bar $\lambda_{\text{em}} \approx 470 \text{ nm}$ and at 7.1 bar $\lambda_{\text{em}} \approx 460 \text{ nm}$); the fines were less affected. The spectral imaging showed a difference in the RGB color for the samples, with more blue for fibers processed at high pressure. At 6.4 bar the spectra

were significantly different over 500-650 nm ($p < 0.01$). The spectra of the fibers showed a relative decreased fluorescence at longer wavelength. At 7.1 bar, the whole fluorescence spectrum of fibers displayed a blue shifted maxima relative to the fines resulting in a difference ($p < 0.05$). The more fibers are refined, the more material is broken off the fibers and shredded off the fiber surface and turned into fines. Thus, some fines produced from separated fibers represent the fiber's former surface, and the new fiber surface exposed is made up from underlying cell wall layers. There is also a lignin spatial- and chemical heterogeneity throughout the cell wall layers- and middle lamella of the wood fiber (Terashima et al. 1993). In addition, process parameters, like rotational speed of the refiner has previously shown to affect the energy consumption and resulting fiber morphology, such as fiber-length and splitting (Kure et al. 1999). The effects on the fiber surface development in this study presumably also derived from temperature, cell wall thinning, surface delamination and fiber splitting, factors that in turn also depend on the intrinsic structural and chemical properties of the raw material (i.e. middle lamellae, cell-walls and layers of the native wood fiber structure) and process parameters.

Conclusions

In this study we described the effect of different TMP refiner conditions on the pulp and paper properties and probed the fiber surfaces and fines using two-photon excitation microscopy, which together with conventional pulp and paper tests showed a pressure/temperature depended patterns on the pulp material. By analyzing the pulps autofluorescence, refining-induced fluorescence spectral shifts and spatial heterogeneities were shown in the pulps. Thus, fast-probing nonlinear spectral imaging enables visualization of spectral variations in the fines and fibers, and may also be applicable on any plant derived fluorescence-labeled or autofluorescent sample benefiting from fast acquisition times and low photo-bleaching.

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