Fluorescence detected linear dichroism of wood cell walls in juvenile Serbian spruce. Estimation of compression wood severity

Brief title: Fluorescence detected linear dichroism of wood

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Abstract

Fluorescence detected linear dichroism (FDLD) microscopy provides observation of structural order in a microscopic sample and its expression in numerical terms, enabling both quantitative and qualitative comparison among different samples. We applied FDLD microscopy to compare the distribution and alignment of cellulose fibrils in cell walls of compression wood (CW) and normal wood (NW) on stem cross sections of juvenile *Picea omorika* trees. Our data indicate decrease in cellulose fibril order in CW compared to NW. Radial and tangential walls differ considerably in both NW and CW. In radial walls, cellulose fibril order shows a gradual decrease from NW to severe CW, in line with the increase in compression wood severity. This indicates FDLD analysis of cellulose fibril order in radial cell walls as a valuable method for estimation of compression wood severity.

Key words: cell wall, cellulose fibrils, compression wood severity, Fluorescence detected linear dichroism microscopy, juvenile wood, *Picea omorika* (Pančić) Purkyně.

Introduction

Wood tracheid cell walls are composed of several layers containing an ordered array of cellulose fibrils, organized in microfibrils, as reinforcing material, embedded in a matrix of polysaccharides such as pectin, hemicellulose, and lignin (Harris 2006). The distribution and orientation of cellulose microfibrils (MFs) in wood cell walls is determined by both, genetic and abiotic factors. Genetic factors include: cell wall layer (primary wall, S1, S2, S3 layer of secondary cell wall), position (radial or tangential cell wall), plant age (juvenile or mature wood), and season of maturation within the growth ring (early and late wood); while abiotic factors include mechanical stress resulting from wind and stem lean. This is why, one of the most frequently measured ultrastructural variables in the wood cell wall is microfibril (MF)

angle – the angle between the tracheid axis and the cellulose microfibrils as they coil around the cell (Barnett & Bonham, 2004).

The outermost layer of the cell wall, the middle lamella, does not contain cellulose MFs, and is hard to distinguish from the primary cell wall which contains several layers of randomly arranged MFs. The secondary cell wall is divided into S1, S2, and S3 layers (Timell, 1986) with MFs aligned in an ordered parallel organisation, characteristic for each layer (Donaldson & Xu, 2005).

In conifer juvenile wood, MF's are oriented at high angles to the tracheid axis, while in the mature wood they are oriented at smaller angles (Plomion et al., 2001, Donaldson 2004, 2008). MF angle also differs in layers of cells within a growth ring, so that the trend from early to late wood is a gradual decline in MF angle.

In conifers, leaning stems develop reaction wood known as compression wood (CW) (Timell, 1986). Wood opposite to the CW in the same growth ring is termed opposite wood while wood from growth rings that do not contain any compression wood is termed normal wood (NW) (Timell 1986). CW occurs in a range of gradations from mild to severe. Juvenile trees growing in an open or wind-exposed environment produce large amounts of randomly distributed mild CW (Donaldson et al. 2004), so NW is often uncommon in juvenile trees. Severe CW is characterized by increased wall thickness, reduced lumen diameter, reduced tracheid length, rounder cell cross-sectional profile, the absence of S3 layer, and the presence of intercellular spaces (Yumoto et al. 1983, Donaldson 2004, Plomion et al., 2001). CW contains higher amounts of lignin and lower amounts of cellulose (Nanayakkara et al. 2009).

Picea omorika (Pančić) Purkyně, Serbian spruce, is a Balkan endemic coniferous species and Tertiary relict of the European flora. This conifer has been legally protected since 1964. Nowadays its natural habitat is reduced to the middle and upper courses of the Drina River. It inhabits open habitats, such as cliffs, forest clearings and vegetation gaps. *P.*

omorika habitats on Tara mountain are characterized by strong northerly wind. Despite its limited natural range, *P. omorika* is considered to be one of the most adaptable spruces. It belongs to slow growing conifer species, in which compression wood typically occurs in a severe form (Donaldson et al., 2004; Timell 1986). These two features make Serbian spruce a challenging model plant for studying cell wall structure and organization.

Some previous studies have described imaging of cell wall organisation and MF orientation/angle in plant cell walls by using polarized light/fluorescence microscopy, such as polarization confocal microscopy (Verbelen & Stickens 1995; Jang 1998; Thomas et al. 2012), Raman imaging acquired with linear polarized laser light and combined with modeling (Gierlinger et al. 2010). Fluorescence detected linear dichroism (FDLD) microscopy represents a unique technique providing observation of structural order in a microscopic sample, expressing this in numerical terms due to the pixel-by-pixel measurement of the fluorescence under modulated polarized excitation. This enables a quantitative comparison among different samples (Steinbach et al. 2009). Such an approach provides examination of the finest orientation properties of the material. An example of application of this method to a plant sample is *Convallaria majalis* root sections, where it has been used to map the optical anisotropy of the cell walls corresponding to the structural arrangements of the polymers (Steinbach et al. 2008, Steinbach et al. 2014). We applied FDLD microscopy to compare cellulose fibril distribution and alignment in radial and tangential cell walls of CW and NW in stem cross sections of juvenile *P. omorika* trees. We measured linear dichroism of the cell walls in sections stained by Congo Red, which predominantly binds to cellulose. Since linear dichroism distribution reflects relative orientation of cellulose fibrils, on the basis of these results we estimated the structural order in the corresponding cell walls. Mapping linear dichroism on the wood cross sections enables quantitative comparison of linear dichroism distribution among different wood samples.

Materials and methods

Plant growth and sample collection

Four-year-old *Picea omorika* trees were grown in plastic pots (20 x 20 x 20 cm) outdoor in Belgrade, Serbia (44° 49' N, 20° 29' E). Plants were about 70 cm tall. Static bending stress was applied at the end of the growing season, in October 2009. Bending was performed at 37 cm from the base of the stem by wiring (the bending angle was about 90 degrees), as described in Mitrović et al. (2015). Stem samples (about 3 cm long) were taken at about 15 cm from the base of the stem in July 2010, debarked, air dried (for FDLD method) or stored in formalin aceto-alcohol (FAA) (for FESEM and confocal fluorescence microscopy).

As compression wood samples for microscopy analysis (Fig. 1) we selected the last growth ring from plants exposed to static bending by wiring. Since in juvenile *P. omorika* trees normal wood is rare, we selected a growth ring with the lowest amount of CW (Fig. 1) to provide a normal wood sample for comparison.

Scanning electron microscopy

Samples of wood stored in FAA were sectioned in the transverse plane with a sledge microtome. Sections were air dried, coated with chromium, and examined with a JEOL 6700 field emission scanning electron microscope.

Confocal fluorescence microscopy

Small 2x3mm blocks of normal and compression wood were embedded in LR White resin and sectioned with a diamond knife at a thickness of 700 nm. Sections were stained for lignin using 0.0001% acriflavin for 3 mins, washed in distilled water, dried for 1h at 50 ^{o}C

and mounted in immersion oil. Samples were imaged using a Leica SP5 II confocal microscope using 476 nm excitation and emission from 490-600 nm for lignin fluorescence to confirm the presence of compression wood, with simultaneous polarized transmitted-light imaging to show the birefringence of cell wall layers.

FDLD measurements and image processing

Cross sections of air dried stem samples, 100 μm thick were prepared using a microtome and stained for two hours in Congo Red 1 mM solution (from Sigma) and then washed with deionized water until the all excess dye was removed.

The FDLD imaging was performed using a Zeiss LSM 410 confocal microscope equipped with a differential polarization (DP) extension (DP-LSM). Confocal images were acquired at a resolution of 512x512 pixels, covering the area of 64x64 μm. Each image consists of two channels, an FDLD channel and a fluorescence emission channel. FDLD values for dipoles fully oriented along the Y-axis correspond to 1, while the values of dipoles fully oriented along the X axis correspond to -1. The FDLD values are theoretically in the range from -1 to +1 due to the definition: FDLD = $(I_1-I_2)-(I_1+I_2)$. (I_1 fluorescence intensity was measured with vertically polarized excitation, whereas I_2 was measured with horizontally polarized excitation). In order to avoid division by zero before the FDLD calculation an intensity thresholding was performed. The pixels with lower intensity than 5 were excluded from the FDLD imaging.The basic principle of operation of the DP-LSM unit is essentially identical to the operation of modern dichrographs. The polarization state of the excitation beam is modulated by a photoelastic modulator (PEM) between two orthogonal polarization states. The frequency of the modulation is 100 kHz (twice the mechanical resonance of the PEM). A lock-in amplifier is used for demodulation of the amplitude of the fluorescence emission, the signal for demodulation arrives from the photomultiplier tube (PMT) of the

fluorescence channel. The modulation and the demodulation fit in the dwell time. The relative orientation of the fluorophores can be determined by measurements of the difference between the fluorescence intensities elicited by vertically and horizontally linearly polarized excitation light beams. The FDLD signal is calculated pixel-by-pixel and imaged by the microscope as an additional channel. Using the runtime calculation the FDLD imaging needs only a single scan and it avoids all the artefacts from the multiple scans (sample and light intensity stability, bleaching…). The provided pixel values are the average of 20 cycles of modulation.

The measurements were done approximately 5µm below the surface of the sections. Excitation was performed using an Ar-ion laser at 488 nm, fluorescence emission was detected above 560 nm.

Image processing was performed using ImageJ program with macros developed for this analysis. The 8 bit TIFF images were set to have a color scale from blue (#0000FF) to yellow (#FFFF00). The ImageJ macro accepted rectangular selections. They were marked on the images for further presentation and the content was exported for numerical analysis. The different areas: vertically (radial cell walls) and horizontally (tangential cell walls) ordered cellulose fibrils were marked by yellow and blue rectangles, respectively. The histograms represent the distribution of the values of all the rectangles associated with each group.

Results and discussion

Figure 3 shows main differences between NW and severe CW of juvenile *P. omorika,* on confocal fluorescence microscopy and field-emission scanning electron microscopy (FESEM) images. Acriflavin stained lignin fluorescence on confocal images in fluorescence and polarization modes, confirm the presence of compression wood and show the birefringence of cell wall layers. The different profile of NW and CW tracheids is apparent. The CW tracheids are round in shape, with thick cell walls. The NW tracheids are rectangular

in shape, with thin cell walls. Such differences in shape and thickness of cell walls of NW and CW in conifers were previously defined and reviewed (Donaldson 2004, Plomion et al. 2001). The thickest S2 layer is clearly distinguishable from the outer layers. The high brightness of fluorescence in the S2L layer of CW confirms the highest lignification of this layer. The innermost S3 layer of secondary cell wall is present in NW, while in CW it is absent (Fig. 3), as one of the main characteristics of CW tracheid cell walls (Timell, 1986).

Cellulose fibrils, as reinforcing material, in wood cell walls determine tracheid cell wall properties and wood quality. Here we observed the relative order of cellulose fibrils in cell walls in the X-Y plane using FDLD microscopy. We showed the potential of FDLD microscopy for fast screening of the main differences between NW and CW in cellulose fibril order in the X-Y plane, on stem cross sections of juvenile conifer trees.

In our measurements FDLD microscopy exploits fluorescence originating from cellulose fibrils stained specifically by Congo red. Different colours represent a different orientation of cellulose fibrils in the X-Y plane of the cross section. Thus the blue colour represents dipoles (cellulose fibrils) that absorb light polarized predominantly parallel with the X-axis. The yellow colour indicates dipoles (cellulose fibrils) oriented predominantly parallel to the Y-axis. As a consequence, a grey colour represents fibrils orientated at about 45° with the X-axis. That is why analysed stem cross sections were oriented in such a way that each observed tracheid tangential wall is oriented parallel to the X-axis, while the radial wall is oriented parallel to the Y-axis. Hence, the tracheid axis corresponds to the Z-axis of this coordinate system (Fig. 4 R, S). As cellulose fibrils coil around the cell (Barnett & Bonham, 2004), blue, yellow and grey colours are located in tangential, radial walls and cell corners, respectively, i.e. they are mainly determined by the shape of the cells. All three colours indicate cellulose fibrils transversally oriented, i.e. at a high angle in regard to the tracheid axis (Z-axis). Axially oriented cellulose fibrils relative to the tracheid axis (Z-axis)

would also be grey. But, in conifer juvenile wood, fibrils in all cell wall layers are oriented transversally i.e. at high angles to tracheid axis, with angles increased in CW (Plomion et al., 2001, Donaldson 2004, 2008). On the cross sections of juvenile *P. omorika* stems, a grey colour unambiguously represents cellulose fibrils oriented at about 45° to the reference line (tracheid X-axis).

On the FDLD images, a blue colour (cellulose fibrils more parallel to X-axis), in NW (Fig. 4 A) corresponds to the entire tangential cell walls of adjacent cells, excluding cell corners. This indicates that in tangential walls of NW, cellulose fibrils are aligned mostly in parallel to the X-axis in all cell wall layers (Fig. 4 A). In contrast, in cell walls of severe CW the blue colour (Fig. 4 I, M), corresponds only to the outer cell wall layers of adjacent cells. This indicates that, in difference to NW, in tangential walls of severe CW cellulose fibrils are aligned mostly in parallel to the X-axis only in the outer cell wall layers, while in innermost part of S2 layer (Fig. 4 E, I, M) cellulose fibrils are more disordered. Similar difference between NW and severe CW holds for the yellow colour (cellulose fibrils in parallel to Y-axis) in radial walls (Fig. 4 A, I, M), although much less pronounced. This is in line with the main severe CW characteristics: reduced level of cellulose deposition in the S2 layer, and the absence of a S3 layer (Timell, 1986). In the sample of mild CW (Fig. 4 E), differences in cellulose fibril alignment/order compared to NW are, as expected, slightly less expressed in both radial and tangential walls.

Applying ImageJ macros on DP images the differences in FDLD distribution and thus relative distribution and order of cellulose fibrils within tangential and radial walls in NW and CW is evaluated and quantified (Fig. 4 T, U). In NW, FDLD distributions are narrow in both tangential and radial walls, with maxima (Fig. 4 C, D) positioned in blue (fibrils predominantly more parallel to X-axis) and yellow (fibrils predominantly more parallel to Y-axis), respectively. In CW there is a widening of FDLD distribution and shift of pixels to grey

(meaning an increasing number of fibrils are disorientated), in both tangential (Fig. 4 G, K, P) and radial walls (Fig. 4 H, L, Q) compared to NW (Fig. 4 C, D). This clearly shows the decrease in order/alignment of cellulose fibrils in CW compared to NW, as well as the decrease in order/alignment of cellulose fibrils with compression wood severity.

Most investigations of cellulose fibril orientation have been carried out on radial cell walls (Donaldson, 2008). It has been shown that radial and tangential walls can differ considerably, regarding cellulose microfibrilar angle as the most commonly measured feature of wood cell wall (Gorišek & Torelli 1999, Khalili et al., 2001, Anagnost et al., 2005).

FDLD microscopy of cross sections of wood samples allows screening and quantification of the main differences between radial and tangential cell walls of a single tracheid regarding distribution and order of cellulose fibrils. We show that in NW (Fig. 4 A) and CW (Fig. 4 E, I, M), the FDLD distributions in radial walls are narrower than in tangential walls. This indicates that in radial walls there is a higher order of cellulose fibrils than in tangential walls and this phenomenon is maintained regardless of compression wood severity. At the same time, in both the tangential and the radial walls of different samples (Fig. 4 A, E, I, M) aligned from NW to mild and severe CW (increasing severity), FDLD distributions show gradual shifts toward grey (an increasing number of disorientated fibrils, Fig. 4 T, U). FDLD values of radial walls (Fig. 3 D, H, L and Q) for samples shown on Fifue 4 A, E, I and M respectively, show gradual decrease in relation to CW severity (Fig 4 U), suggesting sample on Fig 4. M as a sample with severest form of CW. On the contrary, in tangential walls there is a notable difference between FDLD values for NW sample (Fig. 4 C) and CW samples (Fig. 4 G, K, P), the values for all CW samples, from mild to severe CW, being very close to each other. Therefore, we show that radial and tangential walls in *P. omorika* juvenile trees differ considerably regarding cellulose fibril order in the X-Y plane,

and that FDLD of radial cell walls could be suggested as a measure of compression wood severity (Fig. 4 T, U).

These results show that this method, based on FDLD mapping and quantification of cellulose fibril order, could be used for a rapid estimation of compression wood severity, which may be unobservable applying other methods based on anatomical features (Yumoto et al. 1983), microfibrillar angle (Donaldson et al. 2004), lignin fluorescence spectroscopy (Altaner et al. 2009, Chen et al. 2007, Donaldson et al. 2010), or chemical composition (Nanayakkara et al. 2009).

Conclusion

Presented measurements confirm that DPLSM is a useful and easily applicable technique for rapid screening of cell wall structural order in wood samples, by mapping the linear dichroism of cellulose fibrils. Our data shows a considerable difference in cellulose fibril order between NW and CW, as well as between radial and tangential walls in *P. omorika* juvenile wood. The results indicate FDLD analysis of cellulose fibril order in radial cell walls as a valuable method for estimation of compression wood severity.

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References

ALTANER, C.M., TOKAREVA, E.N., WONG, J.C.T., ADRIAN, I., HAPCA, A.I., MCLEAN, J.P. & JARVIS, M.C. (2009). Measuring compression wood severity in spruce. *Wood Sci Technol* **43**, 279-290

ANAGNOST, S.E., MARK R.E. & HANNA R.B. (2005). S2 orientation of microfibrils in softwood tracheids and hardwood fibres. *IAWA J* **26**, 325–338.

BARNET, J.R. & BONHAM, V.A. (2004). Cellulose microfibril angle in the cell wall of wood fibres. *Biol. Rev* **79**, 461–472.

CHEN, Q-M., HU, Z., CHANG, H-M. & LI, B. (2007). Micro analytical methods for determination of compression wood content in loblolly pine. *J Wood Chem Technol* **27**, 169-178.

DONALDSON, L.A., GRACE, J.C. & DOWNES G. (2004). Within tree variation in anatomical properties of compression wood in radiata pine. *IAWA J* **25**, 253–271

DONALDSON, L.A. & XU, P. (2005). Microfibril orientation across the secondary cell wall of radiata pine tracheids. *Trees* **19**, 644-653.

DONALDSON, L. (2008). Microfibril angle: measurement, variation and relationships – a review. *IAWA J* **29**, 345–386.

DONALDSON, L.A., RADOTIĆ, K., KALAUZI, A., DJIKANOVIĆ, D. & JEREMIĆ, M. (2010). Quantification of compression wood severity in tracheids of *Pinus radiata* D. Don using confocal fluorescence imaging and spectral deconvolution. *J Struct Biol* **169**, 106-115.

GIERLINGER, N., LUSS, S., KÖNIG, C., KONNERTH, J., EDER, M. & FRATZI, P. (2010). Cellulose microfibril orientation of Picea abies and its variability at the micron-level determined by Raman imaging. Journal of Experimental Botany **61**, 587-595.

GORIŠEK, Ž. & TORELLI, N. (1999). Microfibril angle in juvenile, adult and compression wood of spruce and silver fir. PHYTON **39**, 129-132.

HARRIS, P.J. (2006). Primary and secondary plant cell walls: A comparative overview. *NZ J For Sci* **36**, 36–53.

JANG, H.F. (1998). Measurement of fibril angle in wood fibres with polarization confocal microscopy. *J Pulp & Paper Sci* **24**, 224–230.

KHALILI, S., NILSSON, T. & DANIEL, G. (2001). The use of soft rot fungi for determining the microfibrillar orientation in the S2 layer of pine tracheids. *Holz als Roh und Werkstoff* **58**, 439–447.

MITROVIĆ, A., DONALDSON, L.A., DJIKANOVIĆ, D., BOGDANOVIĆ PRISTOV, J., SIMONOVIĆ, J., MUTAVDŽIĆ, D., KALAUZI, A., MAKSIMOVIĆ, V., NANAYAKKARA, B. & RADOTIĆ, K. (2015). Analysis of static bending-induced compression wood formation in juvenile *Picea omorika* (Pančić) Purkyně. *Trees – Struct Funct* **5**, 1533-1543.

NANAYAKKARA, B., MANLEY-HARRIS, M., SUCKLING, I.D. & DONALDSON, L.A. (2009). Quantitative chemical indicators to assess the gradation of compression wood. *Holzforschung* **63,** 431-439.

PLOMION, C., LE PROVOST, G. & STOKES, A. (2001). Wood formation in trees. *Plant Physiology* **127**, 1513–1523.

STEINBACH, G., POMOZI,I., ZSIROS, O., PÁY, A., HORVÁTH, G.V. & GARAB, G. (2008). Imaging Fluorescence Detected Linear Dichroism of Plant Cell Walls in Laser Scanning Confocal Microscope. *Cytometry* **73A**, 202-208.

STEINBACH, G., POMOZI, I., ZSIROS, O., MENCZEL, L. & GARAB, G. (2009). Imaging anisotropy using differential polarization laser scanning confocal microscopy. *Acta Histochem*. **111**, 316–325.

STEINBACH, G., PAWLAK, K., POMOZI, I., TÓTH, E.A., MOLNÁR, A., MATKÓ, J. & GARAB, G. (2014). Mapping microscopic order in plant and mammalian cells and tissues: novel differential polarization attachment for new generation confocal microscopes (DP-LSM). *Methods App Fluoresc* **2**, 015005 (9pp).

THOMAS, J., INGERFELD, M., NAIR, H., CHAUHAN, S.S., DAVID A. & COLLINGS, D.A. (2012). Pontamine fast scarlet 4B: a new fluorescent dye for visualising cell wall organisation in radiata pine tracheids. *Wood Sci Technol* **47**, 59-75.

TIMELL, T.E. (1986). Compression wood in gymnosperms. Springer-Verlag, Heidelberg.

VERBELEN, J.P. & STICKENS, D. (1995). *In vivo* determination of fibril orientation in plant cell walls with polarization CSLM. *J Microscopy* **177**, 16.

XU, P., HUAWU LIU, H., DONALDSON, L. & ZHANG, Y. (2011). Mechanical performance and cellulose microfibrils in wood with high S2 microfibril angles. *J Mater Sci* **46**, 534–540.

YUMOTO, M.,. ISHIDA, S. & FUKAZAWA, K. (1983). Studies on the formation and structure of compression wood cells induced by artificial inclination in young trees of *Picea glauca.* IV. Gradation of the severity of compression wood tracheids. *Res Bull Coll Exp For Hokkaido Univ* **40**, 409–454.

Figure 1. Stem cross section of *P. omorika* tree exposed to static bending by wiring for 9 month; arrows indicate areas selected for analysis.

Figure 2. Scheme of full equipped DP-LSM configuration. The original components of the LSM are blue, the DP attachments are shown in red. Thick, thin and double lines belong to the light path, the electronic signal and the PEM controller functions, respectively. (Steinbach et al. 2009) In the present case, for the FDLD imaging PEM1 was placed into the excitation beam, PEM2, depolarizer and analysers were not used; PEM – photoelastic modulator, DB – dichroic beamsplitter, PMT – photomultiplier tube, ADC – analogue digital converter, detector – photodiode transmission detector of the Zeiss CLSM Fig. 3 Confocal fluorescence (acriflavin stained lignin), transmitted light polarisation, and field emission scanning electron microscopy of normal and compression wood in juvenile *P. omorika* stems. S1, S2, S3 – secondary cell wall layers, S2L – outer S2 layer, ML – middle lamella. Scale bars = 30 µm for light microscopy and 10 µm for electron microscopy.

Fig. 4. FDLD images of *P. omorika* sections and corresponding anisotropy histograms. A) normal wood; E) mild compression wood; I, M) severe compression wood; B, F, J, O) Pixel values were collected in the marked areas, in tangential walls (blue boxes) and in radial walls (yellow boxes), and used to obtain anisotropy distributions; number of analysed areas in radial and tangential walls is about 20 per image; C, G, K, P) tangential walls FDLD distributions (FDLD values: -0.16 (σ: 0.11), -0.04 (σ: 0.17), -0.05 (σ: 0.15) and -0.02 (σ: 0.16), respectively); D, H, L, Q) radial walls FDLD distributions (FDLD values: 0.28 (σ: 0.07), 0.26 (σ: 0.1), 0.22 (σ: 0.1) and 0.20 (σ: 0.15), respectively); T) overlaid distributions C, G, K, P with black lines representing corresponding Gaussian fits (white arrow represents gradual shifts toward grey – increasing number of disorientated fibrils); U) overlaid distributions D, H, L, Q with black lines representing corresponding Gaussian fits (white arrows represents

gradual shifts toward grey – increasing number of disorientated fibrils); R, S) schemes of NW and CW tracheid sections, respectively. Excitation at 488 nm, emission above 560 nm; image size is 64x64 μm. Colour scale from blue (FDLD=0.6) to yellow (FDLD=0.6) is labelled on X-axis of histograms. The blue and yellow colours of coordinate axes, shown in the schematic presentation of tracheid sections of NW and CW, correspond to the orientation of tangential and radial walls on the images, respectively.