

Fluorescence microscopy utilization for lignin detection in wooden cell walls in spruce. A technical note.

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Introduction

Softwood structure is very simple. It only consists of tracheids (aprox. 90 % of the volume) and parenchyma cells (Panshin & de Zeeuw 1980). Therefore, softwood is very suitable for the xylogenesis (i.e. wood formation process) analysis. There are three zones in a newly forming softwood tree-ring: the radial enlarging zone, the maturation zone and the zone of matured cells (Wodzicki 1971). Tracheids in the radial enlarging zone have cell content, their radial diameters are increasing and their cell walls consist of a middle lamella and a primary cell wall. When they move to the maturation zone their radial diameter remains constant and the process of the secondary thickening (the secondary cell wall formation) starts; the cell wall corners are rounding and the cell walls are gradually lignifying. Meanwhile, the cell content is gradually consumed. In the third zone, the matured cells zone, tracheids have their final cell wall diameter and they may be considered as fully differentiated. In this zone tracheids have no cell content and they are completely dead.

It is necessary to distinguish non-lignified and lignified tracheids to identify the individual zones of a tree-ring. A frequently used method for this purpose is double staining with safranin and fast green (Bamber 1961, Horáček et al. 1999). This method is based on the fact that stained wood cell elements are different in colour of their cell walls. The non-lignified cell walls are green in colour and the lignified ones are red. Subsequently, microscopic wood slices have to be mounted to Canada balsam (or other mounting medium) to make permanent microscopic specimens. The described method of double staining and permanent specimen making is quite difficult and time demanding. Therefore, a new method was suggested as a replacement of double staining. The new method of using fluorescence microscopy is based on autofluorescence of lignin.

Some materials and objects are able to absorb electromagnetic radiation (excitation radiation) and contemporarily emit radiation of different wavelengths. The term used for this phenomenon is photoluminescence. If the light emission persists for up to a few seconds after the excitation source is withdrawn, the phenomenon is known as phosphorescence. Fluorescence, on the other hand, describes light emission which continues only during the absorption of the excitation light. The time interval between the absorption of excitation radiation and the emission of re-radiated light in fluorescence is of an extraordinarily short duration, usually less than a millionth of a second (Rost 1992). The main initiator of fluorescence is irradiation of fluorochrome (i.e. material that can emit fluorescence radiation) molecules by specific wavelength electromagnetic radiation. This causes collisions of high-frequency photons and fluorochrome atoms. A fluorochrome molecule absorbs a part of the incident photon energy. If the absorbed energy is sufficient, an electron in the fluorochrome molecule is boosted up to a higher energy level of the excited state, i.e. the electron is excited. The excited electrons may lose some vibrational energy and return to the "lowest excited singlet state". From the lowest excited singlet state, the electrons "drop back" to the ground state with simultaneous emission of fluorescent light. Light emitted at fluorescence is called fluorescent light (Valeur 2002).

Fluorescence microscopy is an excellent method for the kind of substances that can be forced to emit fluorescence radiation by their own nature (primary fluorescence or autofluorescence) or by staining (secondary fluorescence) with specific fluorescent chemicals – fluorochromes. The emission spectrum of a fluorochrome is always shifted into longer wavelengths (lower energy) in

relation to the absorption spectrum. This shift is called Stoke's shift (Rost 1992) and it makes it possible to separate the excitation and the emission radiation using optical filters. An optical filter consists of an excitation filter, a dichroic mirror and a barrier filter. The excitation filter only transmits the radiation of specific wavelengths (excitation radiation) to the investigated sample. The barrier filter prevents the excitation radiation to reach the microscope ocular and transmits only a specific part of wavelengths of emitted fluorescent radiation. The dichroic mirror is a specialized filter designed to reflect the excitation radiation and to transmit the radiation emitted by the investigated sample. It is possible to observe the emitted light in the microscope ocular or capture it using a camera. Besides cellulose, lignin is the most abundant and important polymeric organic substance in wood. Lignin impregnates polysaccharides of wood cell walls. There are chemical bonds between lignin and polysaccharides and together they form lignopolysaccharidic complexes. The lignin amount in wood varies from 20 to 40 % of wood weight in dependence on the species (Fengel & Wegener 1989). Lignin may be considered a native fluorochrome, i.e. a substance that can autofluorescence (Donaldson 2001), and the new method for lignin detection for purposes of distinguishing zones in a newly forming ring is based on this phenomenon.

Material and Methods

40 µm thick microscopic specimens of wood of Norway spruce (*Picea abies* (L.) Karst.) were sectioned on the microtome. Wood samples were taken from 6 trees at the height of 1.3 m from the ground. Both non-permanent specimens (microscopic sections in a drop of water) and permanent specimens (sections mounted in the Canada balsam) were prepared. The number of non-permanent specimens was 84, the number of permanent specimens was 5. A mercury lamp was used as the source of radiation of a wide range of wavelengths (250–700 nm) to induce autofluorescence of lignin. The radiation emitted by the lamp went through an optical filter block. Nikon Optihot-2 microscope with a trinocular was equipped with three Nikon optical filters blocks that were being tested (Tab. 1).

Table 1: Tested Nikon optical filters specifications.

Optical filter block type	G-2A	B-2A	DAPI
EX – Excitation wavelength (nm)	510–560	450–490	340–380
DM – Dichroic mirror – border wavelength (nm)	575	505	400
BA – Barrier filter – minimal wavelength of radiation transmitted to the ocular (nm)	590	520	435–485

PlanFluo 20 and PlanFluo 40 objectives suitable for fluorescence microscopy were used for observations. CCD camera Hitachi HV-C20 was attached to the microscope for digitalization of the observed picture. Image analysis software Lucia G was used for picture processing.

The excitation radiation falls upon the investigated sample through the lens, and the emitted radiation goes through the same lens to the ocular (episcopic fluorescence microscopy).

It was supposed that there was no need of transparent specimens in contrast to fluorescence microscopy with transmitted light. That is why the “non-microscopic” wood samples (10 × 10 × 20 mm) with surface “smoothed” by the microtome were prepared (20 pcs).

Results

There were problems with the microscopic sections mounted in Canada balsam due to its slight fluorescence properties. It made lignin autofluorescence observations impossible. On the other hand, the microscopic sections that were in water only (non-permanent specimens) were suitable for lignin autofluorescence observations. Each of the available optical filter block was tested for its suitability for fluorescence microscopic observation of the non-permanent specimens. The

autofluorescence of lignin occurred at $\lambda_{\text{exc}} = 510\text{--}560$ nm (G-2A filter) and at $\lambda_{\text{exc}} = 340\text{--}380$ nm (DAPI filter). There was no fluorescence light observed at $\lambda_{\text{exc}} = 450\text{--}490$ nm (B-2A filter). The best image quality was observed at $\lambda_{\text{exc}} = 510\text{--}560$ nm (green light) and the emitted fluorescence light was red (According to printing requirements all pictures were transformed from 24bit mode to greyscale mode. (Fig. 1). But the fading effect (reduction of the emitted light intensity) occurred.

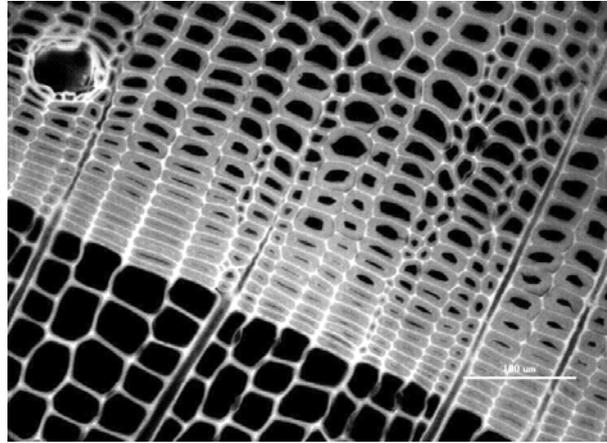


Figure 1: Lignin autofluorescence in Norway spruce at $\lambda_{\text{exc}} = 510\text{--}560$ nm (transformed to greyscale for printing)

When the DAPI filter was used, i.e. $\lambda_{\text{exc}} = 340\text{--}380$ nm (UV-A), the emitted light was blue. The intensity of the emitted light gradually increased.

It was possible to observe gradual lignification of cell walls of anatomical elements in a forming ring thanks to periodically sampled wood samples (Fig. 2). The emitted light indicates the areas of lignin occurrence in the specimens. Other areas of specimens, i.e. areas without lignified cells did not emit any light and they were black in the observed picture.

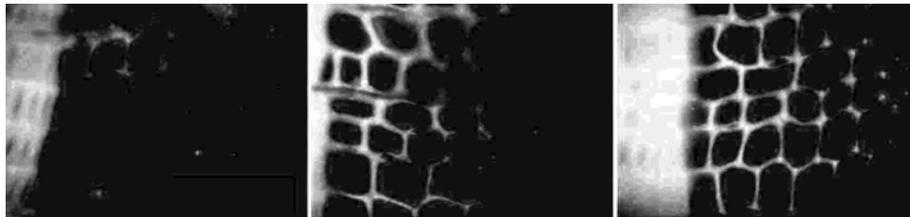


Figure 2: The sample taken on June 2nd (left) – no lignification. The sample taken on June 16th (middle) – two cells of each radial row are lignified, the third and the fourth cells are lignified only in corners. The sample taken on June 30th (right) – at least four cells are lignified. Fluorescence microscopy (transformed to greyscale for printing).

It was found useful to combine a fluorescent picture and a picture acquired by common light microscopic technique for the same observed area of the specimen. Such a kind of picture makes it easy to distinguish the fully lignified, the partly lignified and the non-lignified cell walls. It is supposed that the emitted light intensity is related to lignin concentration in the cell wall. Most lignin concentration was observed in the corners of the middle lamella.

The lignification process began in the cell wall corners in the middle lamella, then it continued in the radial cell walls and finally in the tangential cell walls.

Because of the incident (reflected light or episcopic) illumination principle of used microscopic technique it was not necessary to use thin slices of wood. The picture acquired from the surface of the “non-microscopic” wood sample observation is presented in figure 3.

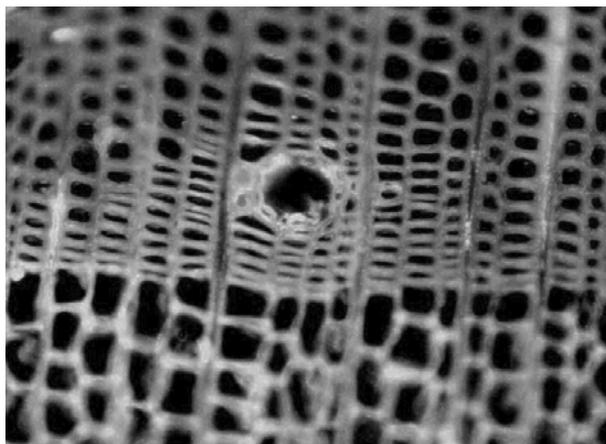


Figure 3: Transverse section surface of non-microscopic sample of wood of Norway spruce. Fluorescence microscopy at $\lambda_{exc} = 340\text{--}380\text{ nm}$ (transformed to greyscale for printing).

Discussion

The method of fluorescence microscopy based on lignin autofluorescence was proved as suitable for lignin detection in the cell walls of wood of Norway spruce. In other words, it makes lignin visible. The advantage of this method is that the double staining procedure is not necessary and it does not require samples mounted in Canada balsam. It saves the time needed for specimen preparation. The use of non-permanent specimens is an indisputable advantage. On the other hand, the disadvantages are the necessity to process the image of non-permanent specimens immediately and the impossibility of their storing. However, it is possible to archive all specimens as digital pictures, which eliminates this disadvantage. The combined pictures created as a result of the logical sum of fluorescent and common light pictures can fully substitute pictures acquired by the double staining method. For lignin, the maximum fluorescence, as measured with a fluorescence spectrophotometer, occurs at $\lambda_{exc} = 335\text{ nm}$ (Kolpak et al. 1983). This wavelength was very close to the excitation wavelengths of the DAPI filter ($\lambda_{exc} = 340\text{--}380\text{ nm}$). Donaldson (2001) observed lignin autofluorescence at $\lambda_{exc} = 530\text{ nm}$. This wavelength is in the range of excitation wavelengths of the G-2A filter ($\lambda_{exc} = 510\text{--}560\text{ nm}$). Kolpak et al. (1983) reported that no significant fluorescence was observed at $\lambda_{exc} = 633\text{ nm}$. No such optical filter was available. The lignin autofluorescence that occurred with DAPI and G-2A optical filter blocks is in accordance with the results in the studies mentioned above. No lignin autofluorescence was achieved using the B-2A optical filter block. It was probably caused by the mercury arc lamp emission spectra. The mercury arc lamp showed very small relative spectral radiation at the wavelength range corresponding to B-2A optical filter excitation wavelengths.

The fading effect occurred at $\lambda_{exc} = 510\text{--}560\text{ nm}$. This effect was described by Rost (1992). On the other hand, the fluorescent light intensity was detected to increase at $\lambda_{exc} = 340\text{--}380\text{ nm}$. This phenomenon was not described in the available studies by other authors. It was probably caused by high energy (short wavelength) of incident radiation, which was able to penetrate deeper, which means it was possible to excite electrons of more molecules.

The result of the observation of lignification is in accordance with the previous studies (Wodzicky 1971, Antonova & Stasova 1997, Horáček et al. 1996); lignification begins in the middle lamella and continues in the radial cell walls and finally in the tangential cell walls.

The quality of the fluorescent picture acquired directly from the surface of non-microscopic piece of wood sample (i. e. without microscopic slices) depends on the quality of the observed sample surface, especially on its flatness. It was found suitable to smooth the surface by a few trims on the microtome. All experiments were carried out using the wood of Norway spruce but it is supposed that similar results should be obtained even with other European softwood species.

Conclusions

The method for lignin detection using fluorescence microscopy is a full-value replacement of the double staining method. The new method includes a few steps:

1. Sectioning the wood samples on the microtome.
2. Making non-permanent specimens out of microscopic sections.
3. Specimen observation using light microscopy.
4. Digitalisation of the observed specimen area (saving as a picture file).
5. Digitalisation of the same specimen area (without moving) with fluorescence microscopy at $\lambda_{\text{exc}} = 510\text{--}560$ nm (red fluorescence) or at $\lambda_{\text{exc}} = 340\text{--}380$ nm (blue fluorescence).
6. Logical sum of both pictured using image analysis software (e.g. Lucia G).

The method can be recommended for purposes of xylem growth analysis as was described in the Introduction. Lignin autofluorescence was observed at both 340–380 nm and 510–560 nm excitation wavelengths.

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