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RAPID COMMUNICATION

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Fluorescence lifetime imaging microscopy study of wood fibers

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Introduction

In this study, we analyzed the fluorescence lifetime distributions in wood fibers using a novel application of fluorescence lifetime imaging microscopy (FLIM). Lignin is a major structural polymer in the fiber cell walls of all higher plants. However, the absolute structure of native solid lignin within the plant cell wall (i.e., protolignin) has not been fully elucidated, because unaltered native lignin can be neither completely quantitatively nor qualitatively isolated from the plant fiber. The lignin substructures contribute the color and fluorescent properties of lignocellulose (e.g., wood fibers).[1–4] By analyzing the fluorescence of synthesized lignin model compounds and isolated milled wood lignin fragments, several structural elements responsible for the lignin fluorescent emission have been identified.[4–6] However, the structural complexity of lignin with its chemical and conformational variations has kept the nature of native lignin fluorescence obscure to date.

FLIM is a technique that allows the analysis of the spatial distribution of fluorescence lifetimes of fluorophores using microscopy,[7–11] thereby combining chemical and spatial information. Lifetime analysis of fluorescence benefits from the fact that lifetime is independent of fluorophore concentration, light path, and orientation of fluorochrome structures. Moreover, multifrequency FLIM allows the use of relatively inexpensive light-emitting diodes (LEDs) as the light source and the analysis can be performed using a conventional wide-field fluorescence microscope fitted with a frequency-domain FLIM system, a modulated LED light source, and a modulated intensified CCD camera. Because the lifetime of the fluorophore electrons in the excited state is affected by the chemical and physical environment, FLIM may provide a sensitive and selective means to probe the lignocellulosic autofluorescence for structural and chemical changes with spatial resolution. Therefore, in this study, the application of multifrequency domain FLIM was demonstrated on solid wood (Norway spruce) and thermomechanical pulp (TMP) paper and the lifetime components and their distributions were successfully explored.

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Experimental

Materials

As a source of a homogenous lignocellulose fiber material of uniform thickness and density (45 g m^{-2}), commercial unbleached newsprint of thermomechanical softwood pulp (Norway spruce, *Picea abies*) was used ("Standard News," from SCA, Sundsvall, Sweden). The paper sheets consisted of 100% lignocellulose (about 28% lignin) and were sufficiently thin for light to pass through for analyses in a wide-field fluorescence microscope. Samples from sapwood of commercial Norway spruce (*Picea abies*) boards were wetted, cut in 20-μm cross sections using a sledge microtome, and thereafter dried on glass slides and analyzed without prior sample treatments. Photobleached TMP was obtained using the commercial unbleached newsprint of thermomechanical softwood pulp, which was exposed to irradiation (filter EX 545/30) at maximum intensity for 5 min in the wide-field fluorescence microscope using a Nikon Intensilight as the light source.

Fluorescence lifetime imaging microscopy

The lignocellulose autofluorescence was analyzed using a Nikon TE2000 microscope equipped with the frequency-

domain FLIM system LIFA (Lambert, Roden, the Netherlands), consisting of a modulated LED light source and a modulated intensified CCD camera. The lifetime from phase (τ_φ) and modulation (τ_M) were determined from the phase shift and decreased modulation of the emission relative to the excitation light using a sinusoidally modulated 3 W 451 nm LED at 40 MHz under epi-illumination. The separate fluorescence component lifetimes were measured using multifrequency measurements with a 3 W 448 nm LED at 20 modulation frequencies from 10 to 70 MHz. Fluorescein was used as a lifetime reference standard. The samples were imaged with a NA 1.40 objective using filter set EX 436/20; DM 455; BA 480/30. The fluorescence lifetimes (τ) were determined using the LI FLIM software package by recording a sequence of images of 12 different phase steps. The images presenting lifetime distributions were composed of the measured lifetimes (pixel-by-pixel) superimposed on a fluorescence intensity image.

Results and discussion

Fluorescence lifetimes and relative component contributions to the emission can be resolved by FLIM in individual pixels by measuring the phase shift and decrease in modulation depth at different frequencies using a sinusoidally modulated LED light source and a modulated and intensified CCD camera. The emission (φ_{em}) and excitation (φ_{ex}) phases, as well as the modulation depths of the emission (M_{em}) and excitation (M_{ex}), can be used to determine the fluorescence lifetimes (ω is the angular frequency used for the modulations, $2\pi f$). In Fig. 1, the phase-shift-based lifetime (τ_φ) described in Eq. 1 and modulation (τ_M) in Eq. 2 are defined.[9]

$$\tau_\varphi = \frac{\tan(\varphi_{em} - \varphi_{ex})}{\omega} \quad (1)$$

$$\tau_M = \frac{1}{\omega}\sqrt{\frac{1}{\left(\frac{M_{em}}{M_{ex}}\right)^2 - 1}} \quad (2)$$

Lignocellulose fluorescence lifetime analyses

Initial single frequency FLIM analyses of the lignocellulose showed that the lifetime from phase (τ_φ = 1.84 ± 0.13 ns) differed from the lifetime from modulation (τ_M = 2.87 ± 0.21 ns). In monoexponential decays, phase and modulation show the same lifetime, but when τ_M and τ_φ differ the fluorescence contains different lifetime components.[9–11] Subsequently, multifrequency-domain FLIM (20 modulation frequencies from 10 to 70 MHz) was performed to determine the number of fluorescent components and their respective lifetimes. Multiple lifetime components and their relative contributions to the emission can be resolved in individual pixels by measuring the phase shift and decrease in modulation depth at several different modulation frequencies. The Levenberg-Marquardt nonlinear least-squares algorithm was used to vary the fraction and lifetime parameters of the multiexponential decay model to arrive at the best fit between the modeled and measured phase and modulation data.[11] The correctness of the model can be judged from the value of the goodness-of-fit parameter χ^2 (Eq. 3). The best model was selected by observing the value of χ^2 while increasing the number of lifetime components to fit for. Adding an extra component was justified only when it resulted in at least a factor of 2 decrease in the value of χ^2.

$$\chi^2 = \frac{1}{\nu}\sum_\omega\left(\frac{\varphi_\omega - \varphi_{c\omega}}{\Delta\varphi}\right)^2 + \frac{1}{\nu}\sum_\omega\left(\frac{m_\omega - m_{c\omega}}{\Delta m}\right)^2 \quad (3)$$

where ω is the angular frequency $2\pi f$, ν is the number of degrees of freedom, φ_ω is the measured phase shift at frequency ω, $\varphi_{c\omega}$ is the calculated phase shift (given a set of lifetimes and fractions), $\Delta\varphi$ is the error in phase shift (estimated), m_ω is the measured decrease in modulation depth, $m_{c\omega}$ is the calculated decrease in modulation depth (given a set of lifetimes and fractions), Δm is the error in decrease in modulation depth (estimated).

Distribution of fluorophores and lifetimes

The wood fibers of Norway spruce showed a best overall fit for two main lifetime components at λ_{ex} 436/20 and λ_{em} 480/30. When analyzing the lifetime distributions of component 1 and 2 individually, the component distributed relatively evenly within the images of TMP paper sheets (Fig. 2a, b). The same analysis was performed on a cross section of wood to show the applicability of the FLIM technique on native wood (Fig. 2c, d). The lifetime component distributions clearly correlated with the wood structure. The shorter (blue) and longer (yellow/red) lifetime components were distributed all over the wood material and the hexagonal features of cross sections of wood could be seen. The empty fiber lumen can be identified as "black holes" and the more intensely colored horizontal bands of the images are ray cells. The spatial distributions of lifetimes in the cross sections of wood show a noteworthy difference in the lifetime distributions in cell walls and cell corners, which corroborates the applicability of FLIM on wood, considering that lignin heterogeneity has been indicated previously (e.g., more condensed-type lignin in cell corners).[12] The TMP paper sheet consists of fibers and fiber/fine fractions stacked onto each other, equalizing the fluorescence of the inherently heterogeneous wood fiber, and was therefore used for more detailed analyses of the fluorescence lifetime components. The TMP paper sample used in this study was previously characterized by wide-field fluorescence microscopy and electron microscopy and displayed a relatively smooth and uniform structure.[13,14] The average fluorescence lifetimes (of all pixels) for both components, shown in Fig. 2a,b, were analyzed using the software. Samples analyzed in triplicate gave a lifetime of 0.74 ± 0.07 ns for component 1 and 4.29 ± 0.78 ns for component 2.

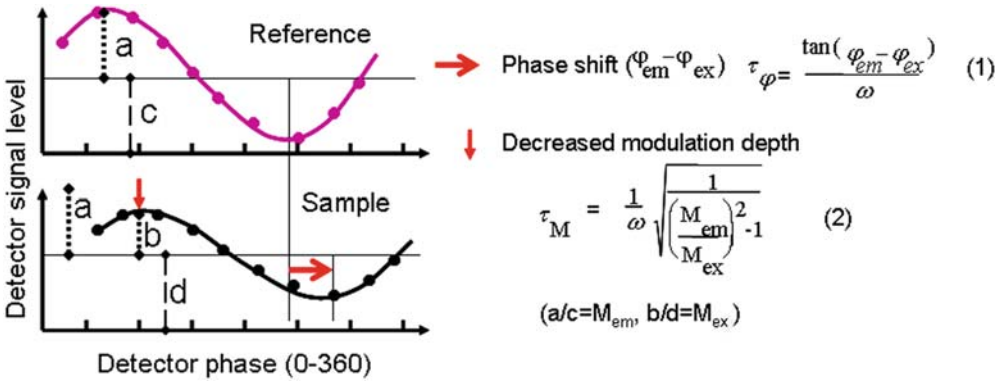

Fig. 1. Determination of frequency-domain fluorescence lifetimes from phase and modulation measurements

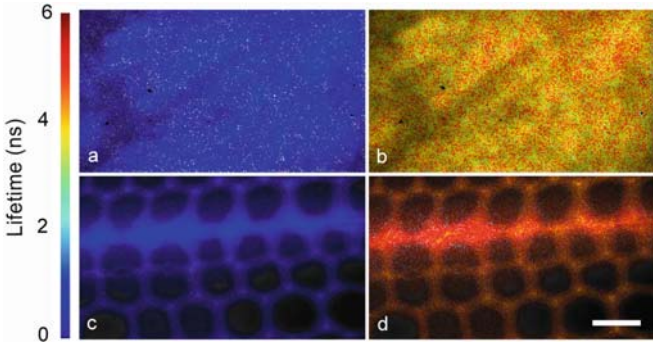

Fig. 2a–d. Fluorescent lifetimes of 0–6 ns mapped pixel by pixel for **a** thermomechanical paper sheet (component 1), **b** thermomechanical paper sheet (component 2), **c** cross section of solid wood (component 1), and **d** cross section of solid wood (component 2). *Color bar* indicates the lifetime in nanoseconds (ns). *Bar* 30 µm

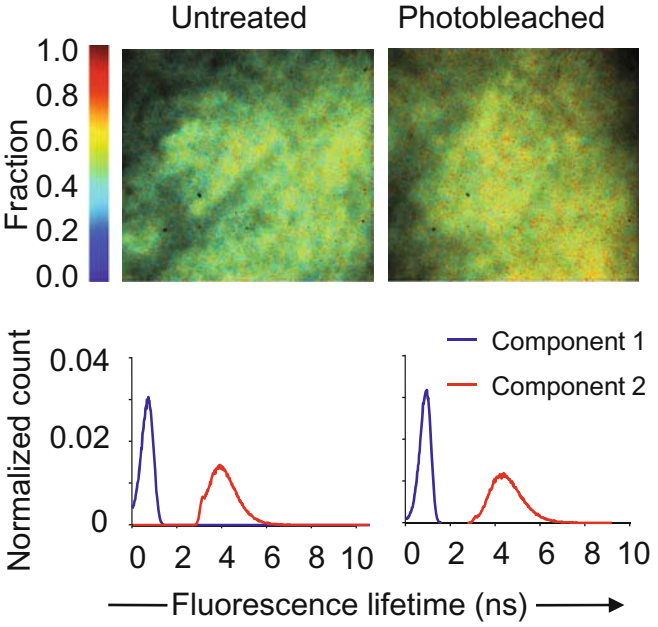

Fig. 3. The relative contributions of component 1 and 2 to the emissions. Spatial distributions (*above*) and histograms (*below*) of normal untreated and photobleached thermomechanical pulp

Relative fluorophore contributions to emission

The fractional contributions of each fluorophore to the emission can be obtained according to Eq. 4, where α_i and τ_i are the pre-exponential factor and decay time, respectively. The α_i values were normalized in the LI FLIM software. The relative contributions to the emissions in Fig. 2a,b were 0.53 for component 1 and 0.47 for component 2.

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (4)$$

We used one range of excitation wavelengths, commonly used in fluorescence microscopy, in order to exemplify the visualization of lifetime distributions in wood and wood fibers. Fluorophore groups, such as 7-hydroxyflavone, 8-hydroxy-3,7-dimethoxydibenzofuran-1,4-quinone, and dehydrodivanillin, may contribute to emissions around 500 nm and above; coniferaldehyde, stilbenes, dibenzodioxocins, and phenylcoumarone and biphenyl structures have shown to contribute to the emission range of 300–450 nm.[4–6] In previous studies on lignin excitation and emission spectra, using different techniques on wood, isolated lignin fragments, and lignin model compounds, different fluorescence lifetime components have been explored.[3,4,6,15] Because wood contains different fluorophore groups with different excitation wavelength maxima, the light source and optical filters need to be chosen to match the corresponding excitations and emission ranges of interest.

Photobleaching of TMP

In Fig. 3, the effect of photobleaching of TMP paper is shown. The photoinduced effect on the fluorophores was clearly visualized by FLIM and resulted in longer lifetimes for both components. Thus, the lifetimes are affected by photoirradiation, which is of importance for high-yield pulps in the context of brightening and yellowing. Photooxidative degradation of coniferaldehyde structures and conversion of quinones to colorless structures have been discussed regarding the photobleaching effect.[16] However, additional studies are needed to reveal the relationships between native lignin fluorophore structures, irradiation effects, and fluorescence intensity and lifetimes, especially at visible wavelengths.

Applications of FLIM on wood fibers

The fluorescence intensity depends on fluorophore concentration, orientation, and quantum yield, and, in some natural materials like plant fibers, there may be different fluorophores of unknown local concentrations displaying overlapping excitation and emission spectra. While such mixtures result in complex and not easily resolved emission spectra, the fluorescence lifetime is independent of the concentrations of the fluorophore, light path, and orientation of fluorochrome structures. Therefore, pixel-by-pixel analyses of fluorescence lifetimes may allow for good contrast between fluorophore groups with different excitation and emission kinetics. FLIM applications are previously developed for spatially resolved analyses of calcium ions,[17] pH,[18] and molecular interactions[8] in living cells. Because intensity and lifetime are affected by the fluorophore's nanoenvironment, it is plausible that the nanostructure of wood fibers also affects the properties of the lignocellulose fluorescence. This means that FLIM can be used as a powerful new tool for spatially resolved studies of cell-wall synthesis, lignin degradation, pulping, and for localization of fluorescent markers used for immunolabelling of wood or wood fibers.

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