Fluorescence Microscopy to Aanalyze Lignin

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Lignin is one of the most studied and analyzed materials due to its importance in cell structure and in lignocellulosic biomass. Because lignin exhibits autofluorescence, fluorescence microscopy methods have been developed that allow it to be analyzed and characterized directly in plant tissue and in samples of lignocellulose fibers. Compared to destructive and costly analytical techniques, fluorescence microscopy presents suitable alternatives for the analysis of lignin autofluorescence. The existing qualitative methods are Epifluorescence and Confocal Laser Scanning Microscopy; however, other semi-qualitative methods have been developed that allow fluorescence measurements and to quantify the differences in the structural composition of lignin. The methods are fluorescence lifetime spectroscopy, two-photon microscopy, Föster resonance energy transfer, fluorescence recovery after photobleaching, total internal reflection fluorescence, and stimulated emission depletion. With these methods, it is possible to analyze the transport and polymerization of lignin monomers, distribution of lignin of the syringyl or guaiacyl type in the tissues of various plant species, and changes in the degradation of wood by pulping and biopulping treatments as well as identify the purity of cellulose nanofibers through lignocellulosic biomass.

fluorescence microscopy | lignin | epifluorescence | Confocal laser scanning microscopy | Fluorescence analytical methods

1. Introduction

Lignin is one of the main components of the plant cell wall, as it provides structural rigidity to withstand differences in water pressure in vascular tissue [1]; in addition, lignin accumulates in specialized cells for support and storage, such as fibers, sclerenchyma, and parenchyma [2], and in the epidermis and cortex in conjunction with suberin and cutin [3]. Lignin in the cell wall and in the lignocellulosic biomass is a limiting factor in the use of plant biomass because lignin is the restrictive barrier for the penetration of cellulase enzymes used for the degradation of cellulose [4]; therefore, lignin is primarily responsible for the recalcitrance of lignocellulose [5].

Analyzing the structure of lignin in plant tissues and biomass is important because it provides information on the quality of cellulosic materials by identifying the presence of lignin in cellulose nanofibers, which affects the quality of production [6]. In addition, based on the percentage of lignin present in the biomass, the potential for degradation of celluloses and hemicelluloses for fermentation and biofuel production can be identified [4]; and finally, the chemical composition of lignin allows identifying the possibility of hydrolyzing and using lignin for the production of biofuels [7].

The presence of autofluorescent monomeric structures in lignin is used in different fluorescence microscopy techniques to qualitatively and semi-qualitatively analyze plant cell walls and lignocellulosic biomass. The main advantage of fluorescence microscopy methods is the possibility of observing and analyzing the samples without the need to carry out a staining method in addition to the fact that it is not necessary to degrade or modify the samples as in other analytical methods [8].

2. Structure and Autofluorescence of Lignin

Lignin is a heteropolymer composed of three main monomers, namely *p*-coumaryl, coniferyl, and sinapyl alcohol [9]; once synthesized in the lignin molecule, the name of the monomers are *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively (**Figure 1**) [10].

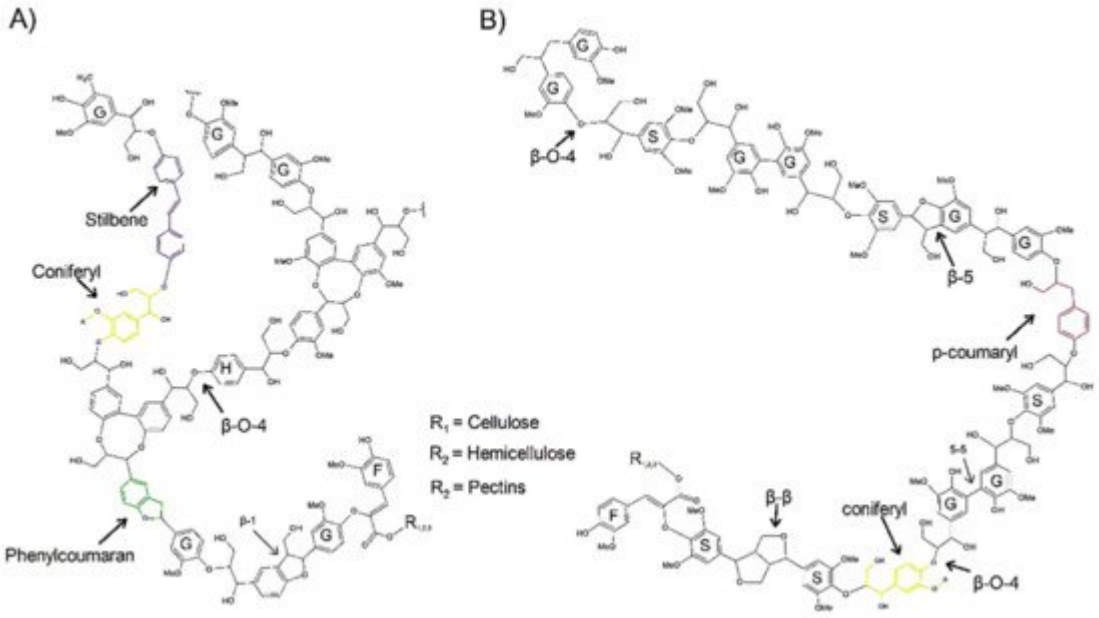

Figure 1. Molecular structure of lignin. (**A**) G-type lignin with the presence of fluorophores. (**B**) S-type lignin with the presence of fluorophores. F, ferulates; H, *p*-hydroxyphenyl; G, guaiacyl; S, syringyl.

One of the properties of lignin is the presence of autofluorescence generated due to the presence of fluorophores in the lignin structure. Fluorophores are molecules that have the ability to absorb energy at a certain excitation wavelength that causes an excited electronic singlet state; during this short period (few nanoseconds), electron undergoes energy dissipation that is emitted in the form of light when the fluorophore returns to the ground state with a longer emission wavelength [11]. For each photon absorbed, there is a photon emitted, so the intensity of the fluorescence emission is directly proportional to the intensity of excitation [12].

For lignin, phenylcoumarans and stilbenes have been identified as the main chromophores [13] (**Figure 1**) although coniferyl alcohol, biphenyl [14], and to dibenzodioxocins [15] are also considered as lignin chromophores. The presence of different side chains on the benzene ring and substitutions at various positions on the ring or side chain are what produce variations in the emission of fluorophores [16]. The lifetime of fluorescence is lower in

fluorophores bound to lignin due to the fact that lignin structure presents random and chaotic bonds as well as branches compared to fluorophores bound to other structural components, such as cellulose [17]. In addition, the intensity of fluorescence varies based on pH; the more alkaline (pH 9), the greater the intensity if visible light is used, while for UV excitation, there is no change in intensity [18]. Most fluorescence microscopy techniques take advantage of lignin autofluorescence to characterize and analyze lignin.

3. Fluorescence Microscopy Methods

There are several methods for lignin analysis, such as those used to obtain qualitative images: epifluorescence microscopy (Epi) and confocal laser scanning microscopy (CLSM). In addition, there are methods that allow semi-quantitative analysis, such as fluorescence lifetime imaging microscopy (FLIM), total internal fluorescence reflection (TIRF), fluorescence recovery after photobleaching (FRAP), Föster resonance energy transfer (FRET), microscopy of two-photon excitation (TPM), and stimulated emission depletion (STED).

A characteristic that the different techniques share is that they work at similar wavelengths, both to excite the sample and to detect their fluorescence. In general, the range goes from 400 to 640 nm. However, some methods such as excitation using UV ranges from 300 nm of excitation, while some lasers reach up to 820 nm of excitation [19]. Based on the intensity and wavelength in which the compounds are detected, whether, in the blue, green, or red channel, some of the structural components present in the samples can be identified. In the case of lignin, the range in which fluorophores emit fluorescence is wide, so their presence can be detected in different channels [20], depending on the wavelength in which fluorophores are excited and detected, in addition to the treatments or dyes that the sample receives [19].

3.1. Wield-field fluorescence or Epifluorescence (Epi)

Epi involves the simultaneous illumination and detection of the entire field of view with low doses of photons and the rapid acquisition of the image to avoid photobleaching. However, the main disadvantage is that Epi collects quite out-of- focus light (Figure 2), so a deconvolution procedure can be applied to the image to reduce the amount of out-of-focus light and obtain a better quality image [21].

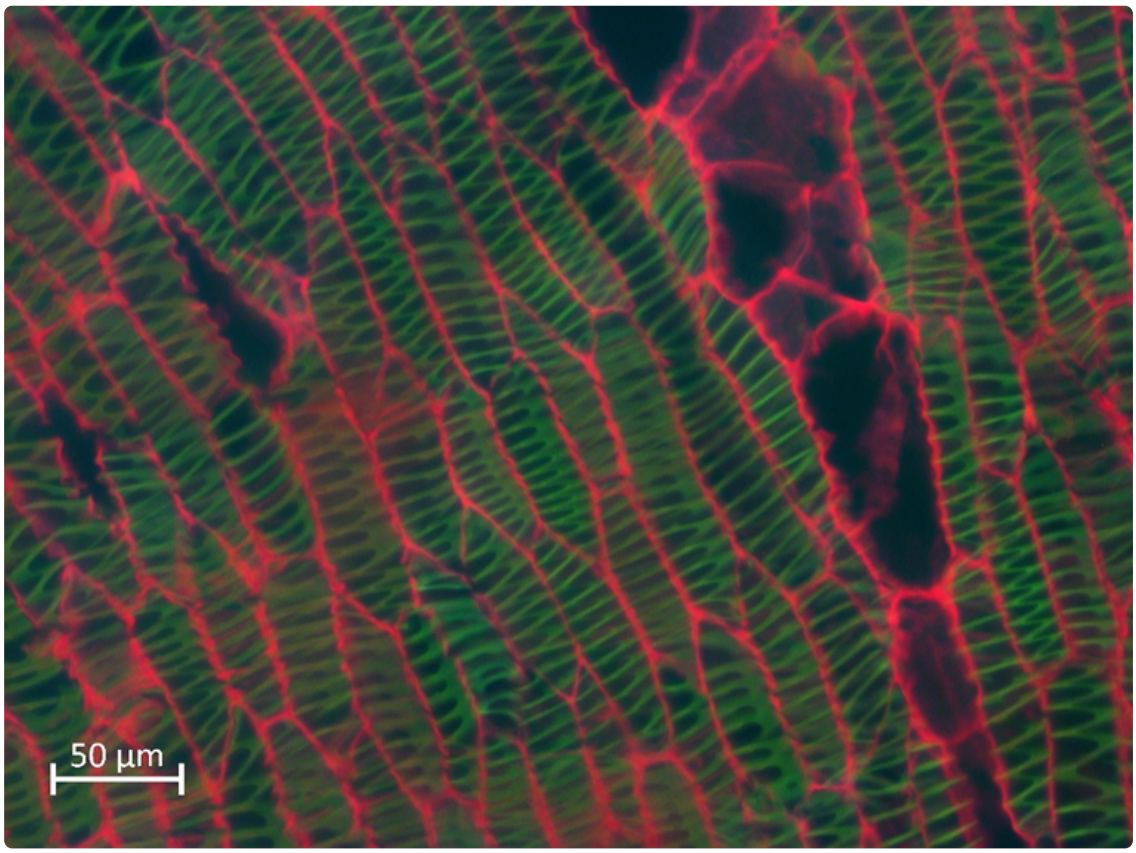

Figure 2. Epifluorescence image from a *Lophocereus marginatus* secondary xylem stained with safranin - fast green, excited at 365 nm (blue), 470 nm (green) and 546 nm (red) at the same time. Scale bar: 20 µm. Image taken from personal file

3.2. Confocal Laser Scanning Microscopy (CLSM)

Similar to Epi, CLSM uses a focused laser at a defined point and at a specific depth, and performs a transverse and axial scan, to collect all the emitted fluorescence information by a point detector consisting of a pinhole. The pinhole eliminates most of the light outside the focal plane, so better quality images can be obtained compared to Epi [21]. The use of this method precedes and is the basis of other techniques because the image is first taken of the area to be analyzed and later analyzes are carried out with techniques such as FRET [22][23].

3.3. Fluorescence Lifetime Spectroscopy (FLIM)

FLIM is a technique that makes it possible to identify the time that elapses between the excitation of the fluorophore, the emission of fluorescence, and its decay, which is measured in picoseconds or nanoseconds, and values obtained can be statistically analyzed [24]. However, FLIM analysis can only be used to infer the chemical changes in the sample but does not give information on what causes the changes, which may even be due to changes in the environment such as pH [25]. FLIM measurement is done by single-photon or multiple photons [20].

A variant of FLIM is Fluorescence spectral and lifetime measurement (SLiM) proposed by Terryn et al. to analyze the interaction between lignin-degrading enzymes and lignocellulose directly in plant tissues [26]. The difference

with respect to FLIM that obtains the fluorescence lifetime and the CLSM image in a single wavelength range, with SLiM the range from 455 nm to 655 nm is divided into 16 spectral channels so that both the image as the fluorescence lifetime are obtained for each channel.

3.4. Two-Photon Microscopy (TPM)

With this technique, thick samples are analyzed so 3D images can be generated. However, unlike the confocal microscope, there is no photobleaching or phototoxicity above or below the plane in focus, which is why TPM has been used to characterize lignocellulose biomass samples [27]. TPM uses a double excitation by means of two photons simultaneously (Figure 3). Each photon has half the energy of the single-photon excitation event, so the energy of a photon is inversely proportional to its wavelength. In a two-photon excitation, the photons must have a wavelength approximately twice the wavelength of the photons needed to achieve an equivalent transition under one-photon exposure [28].

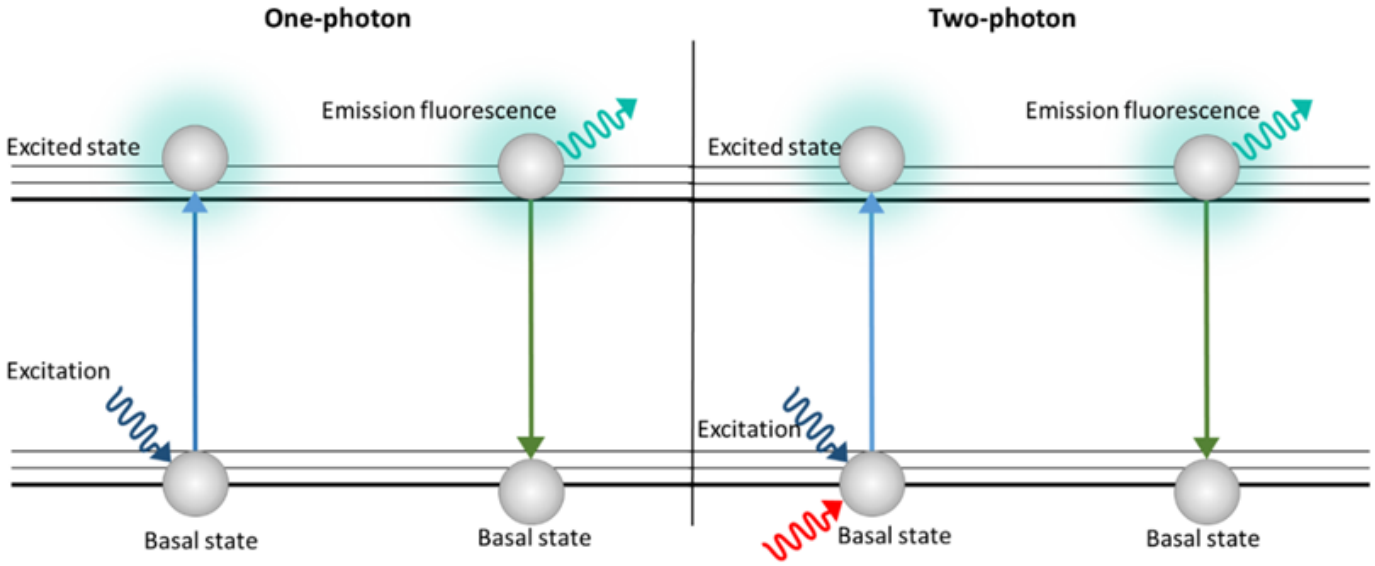

Figure 3. Schematic image of excitation with one photon (Epi, CLSM) and two photons (TPM)

This type of microscopy has been used to analyze the structural morphology of sugarcane bagasse before and after a hydrolysis and bleaching treatment, because when penetrating the sample, better quality details of the structure are obtained compared to confocal microscopy [29].

3.5. Förster Resonance Energy Transfer (FRET)

This method consists of measuring the molecular interaction between two fluorophores by superimposing the emission and excitation spectra [30]. The process occurs when a donor fluorophore and an acceptor fluorophore are within 10 nm, making it possible for a non-radiative transfer of excitation energy from donor to acceptor to occur [12]. When the distance between donor and acceptor increases, the efficiency of FRET decreases to the sixth power of the distance [26]. Measurement with FRET occurs by determining the changes in fluorescence intensity of

the donor and acceptor or by the change in fluorescence over the lifetime of the donor in the presence or absence of the acceptor [20].

3.6. Fluorescence Recovery After Photobleaching (FRAP)

FRAP consists of three steps: the first is to mark a region of interest (ROI) in the sample and record the fluorescence intensity prior to photobleaching, then, the ROI is photobleached with a high-power laser beam so that the fluorophores in ROIs are destroyed and irreversibly stop fluorescing (Figure 4). Subsequently, the area around the fluorescent molecules that can freely distribute in the ROI increases and thus increases the ROI fluorescence, so that the fluorescence recovery reaches a plateau. The scanning fraction that is exchanged between the unbleached area and the bleached area is called the mobile fraction, while the fraction that cannot be exchanged is called the immobile fraction. What is measured is the intensity of fluorescence and the time it takes to recover after photobleaching [12].

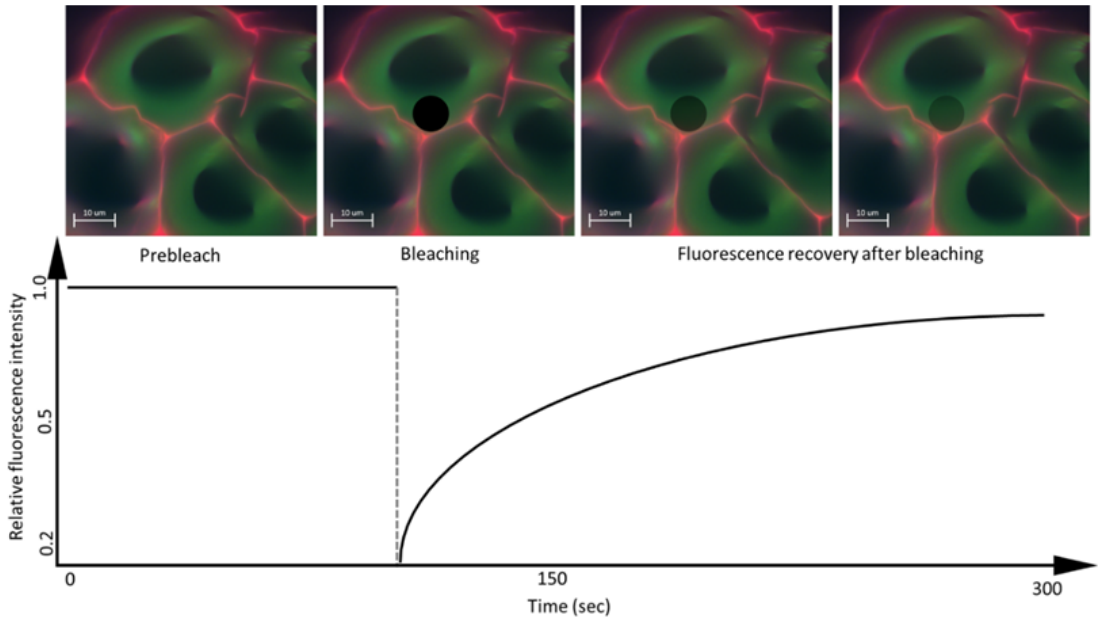

Figure 4. Schematic image of the FRAP method. Image taken from personal file, graphic based in [31].

3.7. Total Internal Reflection Fluorescence (TIRF)

TIRF uses specific optics to produce illumination light only in the range of 50 to 100 nm at the interface [21], which drastically reduces the light from the bulb and improves the ability to detect fluorescent molecules only on the surface of the sample. TIRF uses the evanescent wave generated when the incident light undergoes total internal reflection, or a highly inclined laminated optical sheet (HILO) to illuminate only a partial volume smaller than 200 nm, obtaining the dynamic behavior of a single fluorescent molecule [31]; unlike CLSM which detects the fluorescence emission of the sample from the inner part of the sample. Due to better resolution images, TIRF can be used in conjunction with epifluorescence to characterize lignified fibers and cellular structures [32].

3.8. Stimulated Emission Depletion (STED)

STED is a super-resolution microscopy method based on confocal microscopy, in which images are acquired by scanning a point of light focused on a ROI, and fluorescence is collected sequentially pixel by pixel [33]. The system combines blue excitation lasers with red depletion lasers that pass through a phase plate to be patterned into a donut shape in the focal plane. The resulting excitation is a superposition of the two beams, leading to a high-resolution probe scanning the sample, whereby the resulting effective detected fluorescence emission is collected with high spatial and axial resolutions on the nanometer scale, similar to what obtained with transmission electron microscopy (TEM) [34].

4. Conclusion and Future Perspectives

Most studies take advantage of the autofluorescence of lignin and the dyes they use are mainly to dye cellulosic or hemicellulosic structures because they do not emit autofluorescence, especially in the FRET method, where lignin and Rhodamine dye interact for the fluorescence emission. On the other hand, although the selected articles were the most recent, the FRET, FRAP, TPM FLIM, STED, and TIRF techniques have recently been applied to analyze the structure and composition of lignin. The data obtained can be semi-quantitative and they allow comparisons to be made between the samples analyzed quickly, at low cost, and with non-destructive and super-resolution procedures, as in the case of STED.

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