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Utilization of Light Microscopy and FFT for MFA Measurement from Unstained Sections of Red Pine (*Pinus Densiflora*)*1

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ABSTRACT

This study demonstrates the utilization of light microscopy and Fast Fourier Transform-Peak Finding (FPF) method for microfibril angle (MFA) measurement from unstained sections of red pine (*Pinus densiflora*). To obtain an image with optimal contrast and resolution for MFA measurement, effects of numerical aperture (NA) of condenser lens and color filters were investigated. About 60% of NA of the maximum condenser NA produced an image with optimal contrast, but a color filter with short wavelength range (DAPI) created images with improved resolution. Manual angle measurement and the FPF method were applied to the image with optimal contrast for MFA measurement. The experimental results from the FPF method were considered to be more repeatable and less subjective than those from the manual angle measurement.

Keywords : microfibril angle (MFA), light microscopy, brightfield, condenser NA, FFT, peak finding

1. INTRODUCTION

Microfibril Angle (MFA) is a key indicator causing differences in physical properties of wood cell wall. Last few decades various measurement methods such as X-ray diffraction (XRD), Wide-Angle X-ray Scattering (WAXS) and Small-Angle X-ray Scattering (SAXS), Near Infra-Red (NIR), Polarization Light Microscopy (PLM), Differential Interference Contrast (DIC), Confocal Laser Scanning Microscopy (CLSM), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) have been used to determine MFA from thin or bulk samples of wood. For further details, there are several extensive reviews regarding MFA related subjects (Andersson *et al.*, 2000 Barnett and Bonham, 2004 Abe and Funada, 2005 Donaldson, 2008).

Various transmitted light microscopic methods such as PLM and DIC have been used for MFA measurement. PLM method utilizes birefringent property of cellulose crystalline to obtain orientation information of microfibrils in

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the cell wall (Leney, 1981 Oldenbourg, 1999 Abraham and Elbaum, 2013). A birefringent material rotates the polarization plane of light. Thus different MFA shows different degree of rotation. It is useful method for single cell wall in the longitudinal direction or thin cross sections of the cell wall. For thicker sections containing more than single cell wall, great variation of MFA caused by anatomical structures such as pits and ray parenchyma cells and double cell wall makes MFA determination difficult. The DIC technique (Peter *et al.*, 2003) effectively creates direct image of microfibrils in the cell wall of single fiber.

CLSM methods have advantages by utilization of laser that has limited wavelength and coherent illumination. Polarization of laser and optimized optics in CLSM also enhance visualization of any forms of crystals in the cell wall. Polarization CLSM method (Bergander *et al.*, 2002) utilizes the fact that amount of light from cell walls with different MF is changed by rotation of the angle of incident polarization. Reflectance CLSM visualizes iodine crystals precipitated in micro-cavities along cellulose crystal chains in the cell wall (Donaldson, 2008 Donaldson and Frankland, 2004). CLSM was also used to study non-uniformity of MFA within normal and compression wood tracheids (Sedighi-Gilani *et al.*, 2005).

Biological treatment such as soft rot fungi was applied to improve visualization of microfibril orientation in the cell wall. Since soft rot fungi creates passage in the S2 layer of the cell wall, the decayed space by soft rot fungi is easily visible under light microscope (Anagnost *et al.*, 2002, 2000). This technique reveals microfibrils of the S2 layer in radial and tangential cell walls as well as the tip of fibers. However, this method requires considerable preparation time to grow soft rot fungi in wooden specimen.

This study demonstrates that combination of a simple optical adjustment and sample preparation produces images of microfibrils for MFA measurement. Contrast enhancement in bright-field (BF) that is the most conventional light microscopic modality was adopted to generate micrographs with improved contrast of microfibrils from unstained sections of red pine. For MFA measurement, Fast Fourier Transform (FFT) and peak finding software were utilized for objective and fast angle measurement from the micrographs. Theoretical and practical aspects of the contrast enhancement and the objective angle measurement methods were also discussed.

2. MATERIAL and METHODS

2.1. Materials

Red pine (*Pinus densiflora*) was obtained from Sogwang-ri, Seo-myeon, Uljin-gun, Gyeongsangbuk-do, Korea. Discs of about 5.8 cm thick were taken at 3.6 m above the soil surface.

2.2. Methods

2.2.1. Specimen Preparation

Small blocks (1 cm^3 in volume) were collected from the discs and softened in autoclave for an hour. They were stored in solution of ethanol (99 %), distilled water and glycerine (1:1:1, v/v/v).

From the small blocks, radial sections of 20 μm thick were prepared by a sliding microtome (HM 430, Thermo Scientific Microm). Unstained sections were mounted on a slide glass using double-distilled water as medium with cover slip.

2.2.2. Light Microscopy and Image Acquisition

An inverted light microscope, Axio Observer. Z1 (Carl Zeiss) equipped with Axio Cam MRm

Rev.3, monochrome CCD camera (Carl Zeiss), a 40x water immersion objective with 1.2 NA, condenser lens of 0.55 NA, and color filters (DAPI, GFP and Rhodamine) was used for image acquisition.

From the thin sections of red pine specimen, various combinations of NA of the condenser lens and the color filters were tested to generate microfibril images with sufficient contrast and resolution for MFA measurement.

1) Variation of NA of the condenser lens

NA of the condenser lens was varied from 0.1 to 0.55 with interval of 0.05 NA.

2) Restriction of illumination wavelength by DAPI, GFP and Rhodamine filters.

The wavelength ranges of the color filters are as following:

a. DAPI : 420 nm~470 nm
b. GFP : 500 nm~550 nm
c. Rhodamine : 575 nm~640 nm

2.2.3. Determination of Optimal Condenser NA and Color Filter

Changes in contrast and spatial resolution of an image can be described by the concept of Modulation transfer function (MTF). By definition, the MTF can be calculated by the equation:

MTF = image modulation/object modulation

This quantity is an expression of the contrast alteration observed in the image of a sinusoidal object as a function of spatial frequency.

The image modulation is determined by collecting I_{max} and I_{min} from each micrograph.

$$\text{Image modulation} = (I_{max} - I_{min}) / (I_{max} + I_{min})$$

The object modulation is calculated from a sample with known frequency of repeating structure such as a periodic grating target. In this study, microfibrils with parallel alignment play a role as a periodic grating target. The frequency of microfibrils is not known, but it is fixed for a given section. This means that the object modulation is a fixed constant value. Therefore, relative changes in MTF dependent on NA of the condenser lens and the color filters can be examined by only with the image modulation calculated from the micrographs acquired at different combination of NA of the condenser lens and the color filters.

2.2.4. MFA Measurement by FPF Method

FFT and peak finding from the FFT result were utilized to measure MFA from an image of microfibrils. Images of microfibrils with certain spacing and orientation from the fiber axis in spatial domain are transformed into positions at certain coordinates in frequency domain. Spacing and orientation of the microfibrils can be calculated from the coordinates in frequency domain. However, the spacing calculated from the coordinates in frequency domain is not actual spacing of the microfibrils because the real spacing between the microfibrils is much smaller than that in the micrographs generated by light microscope that has the diffraction-limited resolution (~200 nm).

On the other hand, the orientation or angle of the microfibrils still can be captured from micrographs with low resolution. The low resolution image reveals microfibrils as thicker line and wider spacing than the original microfibrils. The differences of microfibrils in between high and low resolution images are mostly in the thickness of microfibrils, not in orientation. Thus, it is possible to measure MFA of the microfibrils from the low resolution micrographs.

The angle measurement was performed by utilizing ImageJ (Schneider *et al.* 2012) and its FFT functionality. ImageJ displays angle value

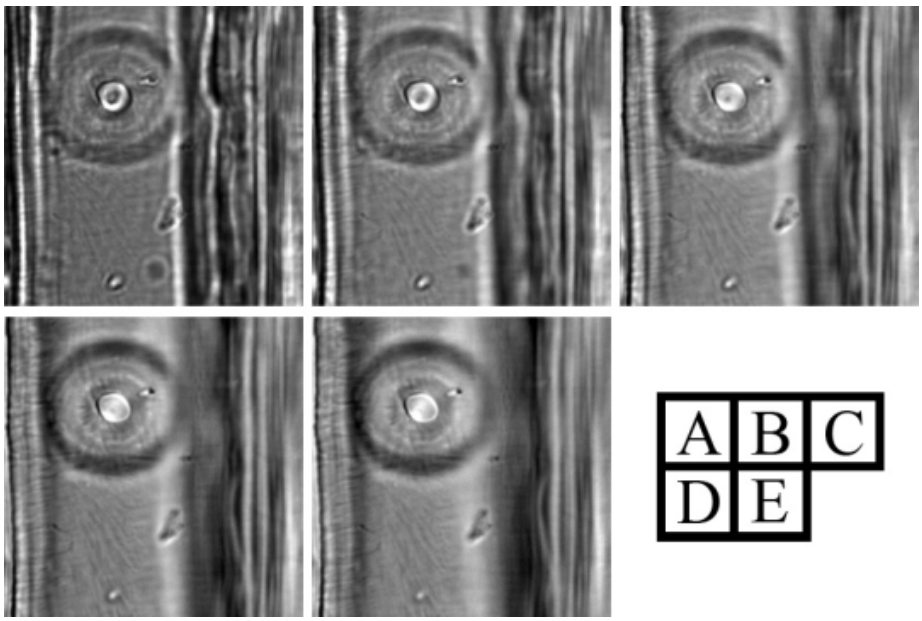

Fig. 1. Micrographs at different NA of the condenser lens. A) NA = 0.1, B) NA = 0.2, C) NA = 0.3, D) NA = 0.4 and E) NA = 0.5.

at certain position from the FFT result window.

3. Resultsand discussion

3.1. Effect of NA of Condenser Lens and Color Filter

NA value of the condenser lens was varied by image acquisition software for the light microscope. Series of micrographs at different NA level was acquired (Fig. 1). From the acquired images, I_{max} and I_{min} were collected by examining minimum and maximum intensity of the images. The image modulation was calculated from I_{max} and I_{min} and plotted on the graph with NA of the condenser lens in the x axis and the image modulation in the y axis (Fig. 2).

The image modulation corresponding to NA value of the condenser lens was continuously decreased by increase of NA values (Fig. 2). Decrease of modulation means decrease of image contrast under investigation. Among the color filters, DAPI filter showed the highest image modulation in 0.3~0.5 NA of the condenser lens. The higher modulation means better contrast and thus 0.3 NA with DAPI filter was determined to be the optimal NA value for providing sufficient spatial resolution as well as contrast.

Interpretation of the effect of NA of the condenser lens and the color filters in light microscopy requires fundamental understandings of roles NA and illumination wavelength on image contrast and resolution. Optical resolution is reduced by polychromatic illumination, scattered light, and reduction in the degree of coherence of the contributing waves. A color (or bandpass) filter can be used to restrict illumination to a limited range of wavelengths. Additionally, partially closing the condenser diaphragm, which reduces effective NA of the condenser lens, produces considerable improvement in the qual-

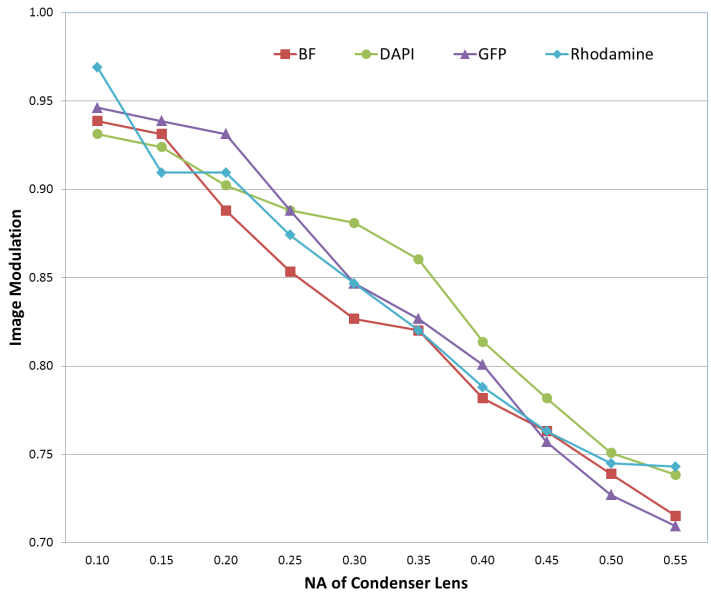

Fig. 2. Variation of modulation at different NA of the condenser lens and range of illumination wavelength. BF = Brightfield, white light; DAPI = 420~470 nm; GFP 500~550 nm; Rhodamine = 575~640 nm.

ity of the image.

If monochromatic light is used as illumination, resolution can be improved because chromatic aberration is eliminated and unit diffraction spots in the image are all of uniform size. With white light, a color filter creates monochromatic light. If a laser is available as illumination, it sharpens the image and increases contrast, particularly for objects with inherently low contrast.

From the understanding of fundamental roles of NA and illumination wavelength, it is possible to explain why reduction of condenser NA increases the contrast and improves the visibility of microfibrils in the cell wall:

1) Reduction of stray light

The stray light is reflected and scattered at the periphery of the lens. This creates blurring and higher background intensity. Thus, reduction of stray light decreases blurriness but increases contrast.

2) Increase of the coherence of light

By selecting a smaller portion of the illumination, the phase relationships among diffracted rays are more defined, and interference in the image plane results in higher-amplitude differences. Thus, contrast of the image is increased.

3) Thicker lines and edges

Thickening of lines and edges in the image is created by the bigger unit diffraction spots. The thicker lines and edges cover a greater number of photoreceptor cells on the retina or CCD units. Thus, it increases visibility of the lines and edges.

However, if the condenser NA is reduced too far (Fig. 1A and 1B), the image loses significant spatial resolution and the dark diffraction edges around objects become objectionable (Fig. 1A).

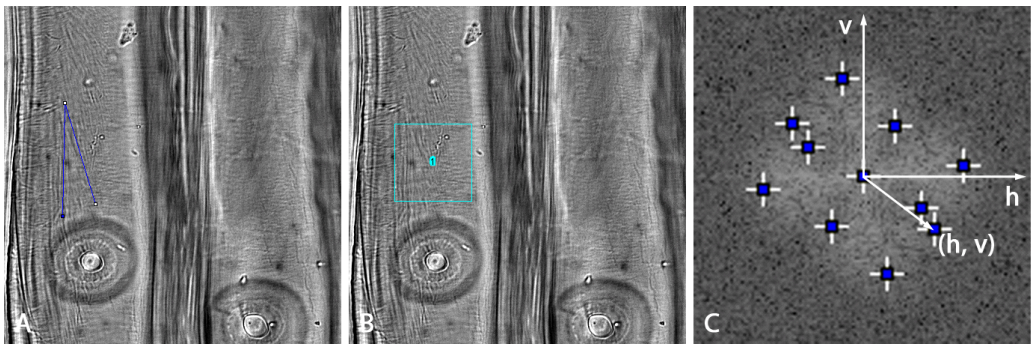

Fig. 3. MFA measurement in spatial and frequency domain. A) manual angle measurement, B) a ROI for FFT and C) FFT result of the ROI in B h = horizontal axis in frequency domain = y axis in spatial domain; v = vertical axis in frequency domain = x axis in spatial domain (h, v) = a coordinate in frequency domain.

3.2. MFA Measurement by FFT

From micrographs by an optimal condenser NA and color filter, MFA was measured by manual angle measurement and FFT-peak finding (FPF) method. Angle measurement tool in ImageJ was used to perform manual angle measurement. FPF method utilizes FFT functionality of ImageJ and a peak finding plugin for ImageJ.

A ROI was set for a region to be measured (Fig. 3B) and performed FFT (Fig. 3C). Peak finding was applied to the FFT window (Fig. 3C). The radius (R in Fig. 3C) can be calculated from (h, v), but it is meaningless due to the diffraction-limited resolution of light microscope. The angle calculated from (h, v) is displayed in the status bar of ImageJ main window. The displayed value was recorded and compared to the manual angle measurement on the micrographs for the same ROI (Fig. 3A).

MFA measurement by manual angle measurement and FPF method is compared in Table 1. For the MFA from the same ROIs, manual angle measurement and FPF method showed significant difference. No definite similarity or trend between MFA by the manual and FPF method was found. The MFA result by manual angle measurement was considered to be affected by visual judgment of beginning and end positions as well as thickness of microfibrils. There are no well-defined criteria for which part of microfibrils under measurement should be considered for the angle measurement. Thus, the manual angle measurement was thought to be subjective dependent on person who performs the measurement.

Table 1. MFA measurement by manual angle measurement and FFT-peak finding method

Region	Manual	FPF 1.3*	FPF 2.0**
ROI 1	28.42	16.57	32.0
ROI 2	13.13	16.55	21.0
ROI 3	10.32	3.90	13.0

* Peak finding parameter: Gaussian blur = 1.3
** Peak finding parameter: Gaussian blur = 2.0

FPF provides more objective approach than the manual angle measurement. However, an optimal value for a parameter such as Gaussian blur is required to be determined. The Gaussian blur was used to smoothing out small differences among peaks in FFT results. Number of peaks in the FFT window is reduced by the smoothing and more dominant periodic struc-

tures with similar spacing and orientation are selected by the peak finding plugin. This is why FPF with greater Gaussian blur showed greater MFA than FPF with less Gaussian blur did. More blurring tends to smoothing out more peaks in FFT window, and peak positions after Gaussian blur in the FFT window move or disappear depending on frequency distribution in the FFT window, which is representation of the structural compositions in the real image.

MFA results from FPF method with appropriate Gaussian blur seemed to provide us more faithful than that from manual angle measurement. However, a method of determination of optimal parameters for the peak finding plugin is necessary to be investigated in the future.

4. Conclusions

A simple adjustment of NA of condenser lens and restriction of illumination light allows us to investigate MFA from unstained thin section of red pine. Stopping down the aperture at the front focal plane of the condenser lens increases contrast of image while decreases resolution. The optimal effective NA of the condenser lens was determined by choosing NA value of the condenser lens at maximum modulation from series of micrographs without significant loss of resolution and dark diffraction edges.

Restriction of illumination wavelength was achieved by inserting color filters with different bandwidth. A color filter with short wavelength range increases contrast as well as resolution.

FPF method with appropriate Gaussian blur seemed to produce objective and faithful results of MFA determination. However, it is necessary to determine an optimal Gaussian blur for proper peak finding results by objective and systematic approach.

Utilizing the simple optical adjustment investigated in this study, light microscope was shown to be useful instrument to investigate MFA distribution from thin sections of wooden specimen. Sample preparation is even simpler because there is no additional step such as staining, biological, chemical and physical treatment. As a result, it would promote investigation of MFA distribution and its effects on physical properties of wooden samples.

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