Measuring the fibril angle of fibres using confocal microscopy

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A new technique for measuring the S2 fibril angle in wood fibres using a confocal microscope in reflectance mode is presented. Polarized laser light is reflected from the fibre and passed through an analyser polaroid crossed with the polarization of the illuminating laser beam. The measured intensity goes through a minimum whenever the orientation of the fibrils matches the polarization of either the incoming laser light or the analyser. As the confocal optics of the microscope allows detection of only light scattered from on or around the focal plane of the microscope, optical interference from the second fibre wall is eliminated. Thus the fibril angle can be measured without extensive sample preparation. A number of examples of the new method are presented.

A wood fibre wall is composed of a number of layers. The most important of these in determining the mechanical properties of the fibre is the S2 layer which typically takes up about 80% of the cell wall. The structure of the S2 layer consists of helically wound microfibrils, with the angle of the helix with respect to the fibre axis being known as the fibril or microfibril angle.

Page et. al. *(1)* have briefly reviewed attempts to examine the effect of species, fibre type, pulping process, yield, chemical composition and other variables on fibre tensile strength. They noted that these studies showed little or no agreement with each other and proposed that a critical factor influencing fibre tensile strength is the fibril angle. They showed that, when the large variation in fibre strength due to fibre defects is accounted for, fibres having the same fibril angle have similar breaking stresses independent of fibre type (springwood or summerwood) and species (black or white spruce). Theoretical and experimental work *(1,2,3)* have shown that while the elastic modulus and strength of fibres are approximately constant at very low fibril angles, they fall rapidly for fibril angles beyond 5 or 10°. At fibril angles of 40° and above, the tensile strength is reduced to about a third *(1)* and the stiffness is reduced to about a fifth *(2)* compared to fibres with angles between 0 and 10°.

As consideration is given to selecting or engineering fibres to make paper with a desired set of mechanical properties, it is necessary to know the distribution of fibre strength and elastic modulus. However making sufficient single fibre measurements to establish these distributions remains too time consuming for routine use. Thus it is vital to have a fast and accurate method for measuring the fibril angle as this is the single factor that most strongly influences the mechanical properties of fibres. According to Crosby and Mark *(4)*, there are five criteria for an ideal fibril angle measurement technique: specific to an individual fibre; characteristic of the S2 layer only; accurate; non-destructive; and designed to avoid wall structures which distort local fibril orientation.

As the fibrils in the S2 layer are tightly packed and the S2 layer itself is not exposed, it is not possible to observe directly the fibril angle except in a limited number of special cases (e.g. softwood compression wood). A number of different techniques have been tried to overcome this problem. These include: x-ray diffraction techniques on single fibres and whole wood samples *(5,6,7)*; etching and iodine staining to reveal the fibrils; phase contrast microscopy under near ultraviolet illumination *(4)*; and the observation of cracks and pits in the fibre wall. The most common methods fall into the category of polarized microscopy techniques. Comparison of results obtained by some of these methods has been made by Meylan *(5)* and Crosby and Mark *(4)*.

Polarized microscopy techniques take advantage of the natural birefringence of cellulose. To measure the fibril angle by polarized microscopy it has been necessary to isolate a single cell wall, since if light passes through opposite cell walls then the effect on the light polarization from the first cell wall is cancelled as the light passes through the second. This isolation of a single cell wall is achieved either mechanically, by slicing the fibre along its length *(8)*, or by impregnating the fibre lumen with mercury *(9)*. The sample is then illuminated with polarized light and the reflected (mercury impregnation) or transmitted (single fibre wall) light is passed through an analyser crossed with the polarization direction of the incident light. The fibre is rotated until maximum extinction occurs. The angle between the fibre axis at maximum extinction and the polarization axis of the polarizer is taken as the fibril angle. Using either form of the technique, the light must pass through both the S1 and the S3 layers as well as the S2 layer. The relative and total thickness of all three layers are known to affect the measured extinction position. El-Hosseiny and Page have made an optical analysis of the system and have shown that in most cases the deviation in the measured extinction position from the true fibril angle value is relatively minor, although under some circumstances large deviations can occur *(10)*.

A variation of this technique that does not require isolation of a single fibre wall has recently been published *(11,12)*. The fibre is placed with its axis at 45° to the polarization of the incident light. The intensity of light transmitted through an analyser is then measured as the analyser is rotated. The fibril angle can then be determined either by a least squares

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regression on a complete 180° set of data or analytically by taking fewer measurements but over multiple wavelengths. In neither case can the fibril angle be determined by visual inspection of the data.

Considerable work has also been performed on the determination of the fibril angle using fluorescent dyes. Some of these dyes, for example Congo Red, are known to produce fluorescent light, with an intensity dependent on the orientation of the fibrils with respect to the direction of polarization of the incident laser beam. Verbelen and Stickens *(13)* have reported using fluorescent dyes with a confocal scanning laser microscope (CLSM) to measure the fibrillar orientation in tobacco plant leaves. The advantage of the CLSM is that the depth resolution capability of the instrument allows the fibrillar orientation in a well defined position to be determined. Confocal microscopy was also used in work reported recently in which the fibril angle was determined using Raman spectroscopy *(14)*. The advantage of using the confocal system to collect the Raman signal again lies in the high level of depth resolution that can be obtained.

NEW TECHNIQUE

In this paper a new technique is reported for measuring the fibril angle that allows the fibril angle to be measured quickly with minimum sample preparation. The technique is based on the classical crossed polaroid method but takes advantage of the powerful depth resolution capabilities of the confocal microscope. By operating the confocal microscope in reflection mode a signal is generated from light reflected only from the focal plane of the instrument. Thus it is not necessary to physically isolate one cell wall to avoid optical interference from the second cell wall. The reflected light is passed through an analyser polaroid crossed with the polarization of the illuminating laser beam and the measured intensity goes through a minimum whenever the fibril angle matches the polarization of either the laser light source or the analyser.

In practice the depth resolution for any confocal microscope and lens system is finite with the intensity contributed by scattering from a plane given by a Gaussian like curve which is generally characterized by a full width at half maximum (FWHM). For the configuration in use in these experiments the FWHM of the z-depth resolution function was 1.6 μm. This is substantially smaller than the estimated fibre wall thickness of softwoods such as radiata pine *(15)* and somewhat smaller than average wall thickness for mixed eucalypt kraft *(16)*. Thus even in the thinner fibres the detected back-scattered light will come almost entirely only from one cell wall, provided the microscope is focussed in the middle of the cell wall.

Some of the advantages of this technique compared to other techniques for measuring the fibril angle are:

- Nothing needs to be done to the fibres to prepare them for testing. This reduces the time required to test fibres, and is also of importance if the fibres are to be used in other tests.
- Rapidity and ease of measurement. Very rapid measurement of fibril angle can be obtained from visual estimation of the extinction position with a typical accuracy of ±4°, while even more accurate measurements can be performed by measuring the intensity as a function of angle and then fitting the data to estimate the position of the intensity minimum.
- The optical path length for the scattered light through the sample depends on the depth of focus rather than the thickness of the sample. Thus errors in the crossed polaroids technique associated with varying sample thickness can be controlled.
- Both fibres and solid wood can be measured.

EXPERIMENTAL PROCEDURE

A confocal laser scanning microscope[1] was used with an SPLAN APO 40 x 0.95 N.A. dry objective. The FWHM of the depth resolution function of the lens was determined using a technique developed by Cogswell et. al. *(17)*. Argon ion laser light with a wavelength of 488 nm was used. An analysing polaroid crossed with the polarization of the laser beam was placed in the detector beam path. Neutral density filters were used to obtain a plot of relative intensity versus grey scale pixel values. The nonlinearity of the grey scale with intensity was rectified by application of a gamma correction.

Samples to be measured were centred in the field of view of the microscope and images were obtained by backscattering from the sample without the use of dyes. The images were 128x128 pixels, 0 to 255 grey scale, collected in a single scan requiring one eighth of a second to complete. The brightness and contrast were adjusted using the photomultiplier tube voltage and the dark level control so that the maximum intensity did not saturate the detector.

Images were collected over a range of at least 180° in steps of 10°. A macro was written to determine the image intensity by (a) gamma correcting the grey scale (b) subscribing a circular region of interest 128 pixels in diameter about the centre of the image to allow measurement on a constant fibre length and (c) calculating the relative intensity by summing the grey scale values in the region of interest.

The intensity values, I, were then plotted against the orientation of the fibre axis with respect to the polarization direction, θ. The theoretical crossed Polaroid equation $I = A \cdot \sin^2[2(\theta-\delta)] + C$ was then fitted to the data to obtain the fibril angle. In this equation A is the amplitude of intensity variation, C is a constant background intensity and δ is the fibril angle.

RESULTS AND DISCUSSION

Results are presented from measurements on three different types of fibres: rayon, *Eucalyptus globulus* and *Pinus radiata* compression wood. A set of measurements on a *E. nitens* chip was also made.

Figure 1 shows a set of intensity measurements made on a radiata pine compression wood fibre. It can be seen that the measured intensity goes through a series of maxima and minima with an approximate periodicity of 90°. It can also be seen that the intensity of the two maxima at -90 and 90° are not as large as the intensity of the central maximum. This is probably caused by a slight variation in the position of the focal plane in the fibre during rotation. Some random fluctuations in the measured intensity are

[1] Optiscan F900E personal confocal system (Optiscan Pty. Ltd., Australia) with Olympus BH2 biological research microscope

also observed which appear to come from noise in the system. After fitting a single $\sin^2$ curve to the set of data (solid line), the fibril angle was determined to be 41±2°. This corresponds to the position of the minimum in the curve on the right hand side of the figure. Since pine compression wood fibres have the orientation of fibrils visible as striations on the surface of the fibre it was also possible to visually determine the fibril angle for this particular fibre as 38±4°, in excellent agreement with the value measured in the confocal microscope.

Figure 2 shows intensity measurements taken from a eucalypt chip. For this sample the fibril angle was determined from the fit to be 5°. We do not have any independent confirmation of the average fibril angle within the chip to compare this value with. It is noted however, that this value is well within the expected range of fibril angles for *E. nitens (14)*. Very similar curves were obtained for a single eucalypt fibre (measured fibril angle of 5±2°) and for a rayon fibre (2±2°). The rayon fibre result is a particularly good confirmation since rayon fibres are known to have an average cellulose chain orientation which is either 0° or very close to 0°.

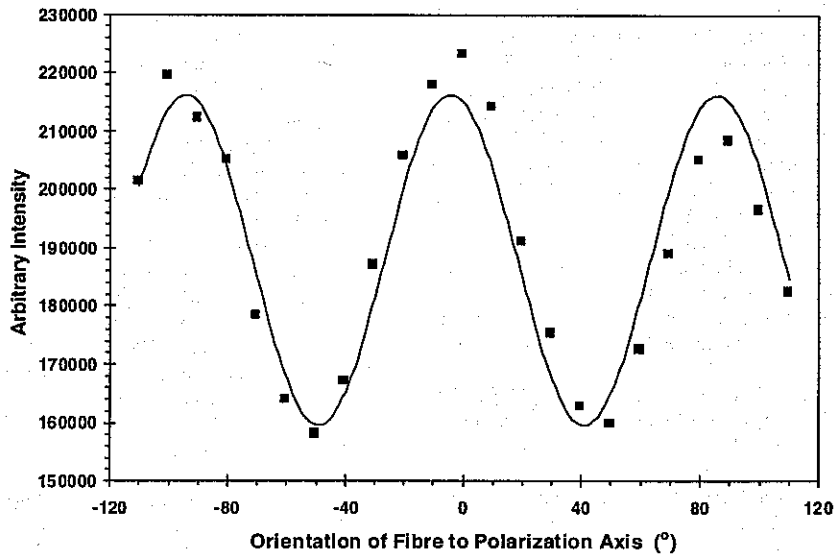

Fig. 1 Variation of reflected intensity with fibre orientation to incident light polarization direction for a radiata pine compression wood fibre. The measured intensity values (■) are the sum of gamma corrected grey scale values.The solid line is the fit to the data (r^2 = 0.91) giving a fibril angle of 41±2°.

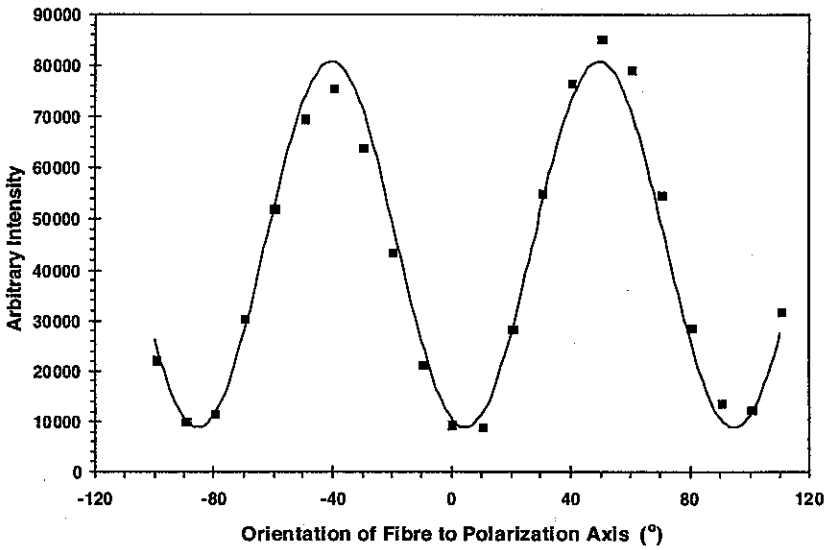

Fig. 2 Variation of reflected intensity with average fibre orientation to incident light polarization direction for a *E. nitens* wood chip. The measured intensity values (■) are the sum of gamma corrected grey scale values. The solid line is the fit to the data (r^2 = 0.98) giving an average fibril angle of 5±1°.

CONCLUSIONS

A new technique has been developed for measuring the fibril angle of wood fibres. The method is a modification of the classical crossed polarizer and analyser technique and uses the depth resolution capabilities of a confocal microscope to measure light scattered from a single cell wall. It has the advantage over other techniques of being extremely rapid and requiring no sample preparation. Measurements on compression wood fibres have shown good agreement between the observed fibril angle and the fibril angle determined by the new technique.

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