Z-AXIS CALIBRATION IN OPTICAL SECTIONING FROM XYLEM CROSS SECTIONS FOR GRAIN ANGLE MEASUREMENT

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SUMMARY
Optical sectioning using confocal microscopy may be problematic under some conditions due to contamination with light from outside the focal plane and resulting z-axis compression. These problems can affect quantitative wood anatomy, such as grain angle measurement. In the present report, the exact surface of xylem sections, z-axis scaling, and available scanning depth with confocal microscopy were determined in xylem transverse sections of Japanese cedar (Cryptomeria japonica D. Don). The optical section containing the sample surface was determined using power spectral analysis to find the sharpest image. Image cross-correlation analysis in serial transverse optical sections revealed that the optical sections above the sample surface showed no tangential shift with that of the surface, indicating the non-focal cell wall information. Optical sections using an oil immersion lens with oil and a dry lens without oil were compared. Optical sections with an oil lens were relatively precise while those with a dry lens showed a z-axis distortion of about ×1.5 due to the mismatch of refractive index. Therefore, the exact cell orientation angle without oil can be obtained by the two-thirds multiplication. Adequate cell wall information was available up to c. 80 μm deep.

Key words: Optical sectioning, confocal microscopy, z-axis scaling, image cross-correlation, Fourier analysis, wood grain angle, Cryptomeria japonica.

INTRODUCTION
In confocal microscopy, light from a specimen is condensed and passes through a pinhole to effectively eliminate the stray light from non-focal planes and to obtain higher horizontal (xy) resolution and higher contrast than those obtained with ordinary light microscopy (Wilson 1990; Pawley 1990). Especially, axial (z) discrimination is available. Wilson (1990) mentioned that xy and z resolution are 0.20 μm and 0.34 μm for dry lens of 0.95 in numerical aperture (N.A.), respectively, 0.14 μm and 0.23 μm for oil immersion lens of 1.4 in N.A., respectively. Z resolution is, in general, inferior to xy resolution (Wilson 1990; Hasegawa 1995). A confocal laser scanning microscope that uses lasers for illumination to efficiently condense the light is available. When sufficient fluorescence from a specimen is detected, an optical section with focal plane infor-
mation is effectively obtained. In contrast, low fluorescence detection requires increased sensitivity of the imaging elements and larger confocal aperture, and this results in increased thermal noise and decreased signal-to-noise ratio.

Confocal microscopy has been applied to wood anatomical studies (Donaldson & Lausberg 1998; Kitin et al. 1999; Kitin et al. 2000; Ogata & Fujita 2005). Fluorescence from lignin in the cell wall or from stain reagents can be used to observe xylem tissue. Safranin has been used to stain xylem tissue for confocal microscopy (Donaldson & Lausberg 1998; Kitin et al. 2000). In particular, Kitin et al. (2000) reported detailed verification of wood anatomy from optical tangential sections of xylem tissue. Optical sectioning in xylem transverse sections, however, has been described in only a few reports (Donaldson & Lausberg 1998), as has comparison between fluorescence from safranin and lignin, although Kitin et al. (2000) reported that auto-fluorescence from lignin could not be sufficiently detected.

The quality of an optical section obtained from the surface of the original section is influenced by surface undulations due to its acquisition from a narrower z-axis range (Donaldson & Lausberg 1998). Donaldson and Lausberg (1998) described that optical transverse sections in radiata pine are adequate to a depth of 30 to 100 μm, although those close to the surface had unequal intensity. In the present paper, power spectral analysis was applied to identify the surface of the sample which was used to calibrate z-axis image compression for optical sectioning. This analysis can reveal the frequency information of an image and thus identify the sample surface which is the most distinct and the sharpest, based on sufficient high-frequency information.

In order to calibrate z-axis image compression, cell orientation was measured using image cross-correlation analysis of the optical sections. Cross-correlation analysis reveals a shift between two different data series (ex., time series data). In dendrochronology, a floating chronology is dated using cross-correlation analysis. Image cross-correlation analysis uses a two-dimensional cross-correlation function between two images and it is thus useful to know their horizontal shift. Template matching using this function is well developed to detect image strain.

Ogata and Fujita (2005) measured the tangential shift between two optical cross sections from different depths in 8-year-old *Hopea odorata* Roxb., a tropical tree, to estimate radial variation in its interlocked grain. Although computing two-dimensional cross-correlation function in large images requires complex calculations, Ogata and Fujita (2005) achieved a more rapid calculation of the cross-correlation function using the fast Fourier transform, by applying the description by Hino (1977) to a two-dimensional image. This technique enabled detection of changes in the wood grain using a less-destructive transverse section of *Hopea odorata*, without using the radial splitting method, which is conventional for measuring wood grain angle and requires a relatively larger wood portion (Ogata & Fujita 2005).

There was, however, a problem with the reliability of the cell wall information in the deeper optical cross sections where cell wall information from the non-focal plane was introduced when the sensitivity of the imaging element was increased. When using a dry objective lens without immersion oil, the refraction of fluorescence from the specimen results in a z-axis image compression (Carlsson 1991; Hell et al. 1993; Grey et al. 1999; Bucher et al. 2000). Grey et al. (1999) and Bucher et al. (2000) observed
z-axis compression of their specimen images without immersion oil and thus applied calibration of the z-axis scaling based on Carlsson (1991) and Hell et al. (1993). Investigation of cell orientation in transverse sections with known tilt angles using confocal microscopy is useful to evaluate the z-axis range available for adequate optical sectioning in the xylem tissue.

In the present research, image cross-correlation analysis was applied to transverse sections of Cryptomeria japonica, in which the wood grain is very straight, i.e., approximately parallel to the stem axis (Ohkura 1958). These sections were adjusted to produce different cell orientation angles to the stem axis.

**MATERIALS AND METHODS**

**Preparation for optical sectioning**

A wood block approximately parallel to the stem axis and four other blocks with differently inclined angles to the axis were cut from a wood collection of Cryptomeria japonica and named WB1 to WB5 in ascending angle order, respectively. Transverse sections of 30 μm thick were sliced from WB1 to WB4 and mounted using Canada balsam (refractive index: 1.519) and a cover glass (c. 0.17 mm thick; refractive index: 1.5255 ± 0.0015) without staining. Transverse sections of 30 and 100 μm were sliced from WB5 and mounted using Canada balsam and a cover glass with and without safranin staining.

Transverse optical sections were scanned using an FV300 confocal microscope system (Olympus Corporation, Tokyo, Japan). An argon-ion laser (wave length: 488 nm) and a high-pass filter (> 465 nm) were selected for the optical sectioning. Kitin et al. (2000) used a helium-neon laser (wave length: 543.5 nm) as the illumination for detection of the safranin fluorescence in the xylem tissue. Preliminary tests showed that while both the helium-neon laser and the argon-ion laser produced sufficient energy to induce fluorescence from safranin, the helium-neon laser was insufficient for the detection of autofluorescence from lignin. Therefore the argon-ion laser was used in the present research to compare the suitability for image cross-correlation analyses between samples with and without safranin. Two different ×40 objective lenses (UniPlanApo, Olympus Corp.) with N.A. of 1.00 and 0.85 for oil and dry, respectively, were used, except for optical tangential sectioning in which a ×20 objective lens (UniPlanApo, Olympus Corp.) was used. The ×40 oil immersion lens with 1.00 in N.A. was used in the ‘oil condition’ and the ×40 dry lens with 0.85 in N.A. was used in the ‘dry condition’ in the present report, respectively. In the oil condition, immersion oil (refractive index: 1.404, Olympus Corp.) was used. Regions of 2048 × 2048 pixels, corresponding to 350 × 350 μm², were selected as regions of interest (ROI) in these transverse sections. Using ×40 objectives, each pixel size was 0.173 μm, smaller than 0.38 μm of the theoretical resolution power of the objectives (Wilson 1990). After slicing transverse sections, tangential 30 μm thick sections were sliced at both the cambial and bark sides, including the plane of transverse sectioning. Image analyses in the present research were executed using software which was programmed using Microsoft Visual Basic 6.0 (Microsoft Company, Ltd, Tokyo, Japan).
**Relation between laser scanning and fluorescence attenuation (Analysis 1)**

To determine the relation between repeated laser scanning and fluorescence attenuation, an arbitrary tangent within the regions in sections with and without safranin staining was defined. Optical sectioning on the tangent at the same depth was repeated 1000 times to sum the intensity of each scanned tangent image. Relative fluorescence intensity was calculated as the percentage of each image intensity sum to the first image intensity. This attenuation data was used for the research of the latter section.

**Evaluation of cell orientation angle (COA) on tangential sections (Analysis 2)**

Three arbitrary ROIs including the transverse-sectioning plane were selected in each of the tangential sections that originated from all wood blocks. Tangential optical sections were analysed using azimuthal power spectral analysis (Nagao 1983; Tanaka 1989; Maekawa *et al.* 1993; Ogata *et al.* 2003). From this analysis, azimuthal angles of cell orientation and the transverse sectioning plane within optical sections were evaluated and the difference between both azimuthal angles was calculated as cell orientation angle to the plane (COA). The COA was calculated to within 0.1 degree of precision by interpolating the data.

**Determination of the sample surface position along the optical axis (Analysis 3)**

ROIs in this analysis were selected in earlywood of the transverse sections in WB1, at approximate right angles to the stem axis, without staining in the oil condition. The regions included approximately 140 cells, traversed at the cell bodies. Forty-one optical sections at 1-μm z-axis steps, including the surface of the transverse sections, were scanned and named OS1 to OS41 in descending z-axis order. The intensity sum of each optical section was calculated and related to scanning depth (Analysis 3-1). Then, the intensity sum of each optical section was equalized with each other to fairly apply the frequency power spectral analysis (Analysis 3-2). Relative intensity in this analysis was adjusted in each frequency region, i.e., extremely low- and low- (1–64), middle- (65–128), high-frequency (180–360), to clearly show the intensity difference between optical sections. The fluorescence from the deeper section might be the less detectable due to the interference by the cell wall above and it is possible that the optical section with the maximum intensity is obtained from the surface. There are, however, some reports that the cell wall information from the deeper optical section is less distinct (Wilson 1990; Pawley 1990; Donaldson & Lausberg 1998; Grey *et al.* 1999; Ogata & Fujita 2005). Supposing the sample surface is perfectly flat, an optical section from the surface shows the sharpest and the brightest. However, the actual sample surface is more or less undulating and the brightest optical section cannot be correspondent with the sharpest one. In this analysis, the optical section that includes the average height of the whole surface within ROI was defined as that from the sample surface rather than the section that includes the lowest surface within ROI. The defined optical section from the sample surface was determined as the section with sufficient high-frequency information, i.e., showing distinct and sharp cell wall borders in this analysis.
Relation of COA between image cross-correlation analysis and power spectral analysis on tangential sections (Analysis 4)

If the cells are oblique to the stem axis or the exact sample surface, the locations of cells between two different depth level are different. Xylem cells can be, in general, tangentially inclined due to wood grain and thus two optical sections with different depth level show tangential shift. In this analysis, three regions of each of the cambial and pith sides corresponding to the regions in Analysis 2 were selected in the transverse sections of each block. In each region, two optical sections at 3 and 13 μm depth from the surface were scanned under dry and oil conditions. The tangential shift of the lower optical section to the upper section was computed using image cross-correlation analysis (Ogata & Fujita 2005). The shift was converted into COA (degree) according to the following equation:

\[
COA = \tan^{-1}\left(\frac{TL}{DD}\right)
\]

where \(TL (\mu m)\) and \(DD (\mu m)\) indicate tangential lag and depth difference, i.e., 10 μm, respectively (Ogata & Fujita 2005). COA using the present analysis and those in tangential sections (in Analysis 2) were compared (Analysis 4-1). The mismatch in the refractive index in the dry condition possibly leads to the z-axis lag of the mechanically estimated depth to the actual depth in optical sections (Carlsson 1991; Hell et al. 1993; Grey et al. 1999; Bucher et al. 2000). Based on Analysis 4-1, therefore, the z-axis lag of the mechanical z-axis movement to the actual depth difference was evaluated and a correction of COA under dry and oil conditions was performed.

To visualise cell orientation from the surface downward, a tangent was set within an arbitrary ROI and a series of tangent images scanned at 0.2-μm z-axis steps on the tangent was reconstructed an optical tangential section (Analysis 4-2). These z-axis steps are smaller than z resolution in this confocal microscope system.

Determination of z-axis range available for COA measurement (Analysis 5)

In transverse sections of 30 and 100 μm thick in WB5 that had the maximum COA, optical sections in the same ROI were scanned under oil and dry conditions. The z-axis ranges for optical sectioning ranged from 10 μm above to 35 μm below the surface at 1-μm z-axis steps for 30 μm thick sections and from 15 μm above to 110 μm below at 5-μm z-axis steps for 100 μm thick sections, respectively. The optical section obtained from the sample transverse section surface was used as the reference optical section for image cross-correlation and the tangential lag between the reference section and each optical section was computed. The lags were converted into COA and compared with the COA in Analysis 2. Based on the z-axis range of adequate COA determined in this analysis and the comparison with that obtained in Analysis 2, the relation between mechanical z-axis movement and actual scanning depth are discussed below.

RESULTS AND DISCUSSION

Relation between laser scanning and fluorescence attenuation (Analysis 1)

The relation between laser scanning and fluorescence detection in this analysis is shown in Figure 1. The figure indicates that the detectable amount of auto-fluorescence...
from lignin decreased by 10% and 20% after scanning 60 and 200 times, respectively (black line in Fig. 1). In contrast, the detectable amount of safranin fluorescence decreased by 10% and 20% after 10 and 25 times, respectively (gray line in Fig. 1). This finding suggests that safranin fluorescence is more rapidly attenuated than lignin auto-fluorescence although safranin has a much higher fluorescence emission. Although the attenuation in the gray line in Figure 1 includes that of lignin auto-fluorescence, the main attenuation depends on that of safranin. This relation could vary, however, based on the laser emission level, the selection of detecting wavelength range, stainability, etc. It is, thus, necessary to determine the relation between each confocal microscope system and each specimen. In summing the intensity of optical sections in the present report, the intensity was corrected using the relation estimated in this analysis. Image cross-correlation analysis could be executed using only two optical sections in the same ROI (Ogata & Fujita 2005), and thus the attenuation of fluorescence would have little impact on the analysis.

**Evaluation of COA on tangential sections (Analysis 2)**

Table 1 shows the COA in each tangential section of cambial and pith sides of each wood block. One example of a tangential section (Fig. 2a) and its azimuthal power

Table 1. The values of COA (degree) estimated using azimuthal power spectral analysis in tangential sections from cambial and pith sides of each wood block (Analysis 2). WB1 to WB5 are the blocks with different COA in ascending angle order. These data were used in Figures 6, 8–11 and 13–16.

<table>
<thead>
<tr>
<th>Side</th>
<th>No.</th>
<th>WB1</th>
<th>WB2</th>
<th>WB3</th>
<th>WB4</th>
<th>WB5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambium</td>
<td>1</td>
<td>2.6</td>
<td>7.2</td>
<td>12.4</td>
<td>21.0</td>
<td>26.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.7</td>
<td>7.8</td>
<td>10.5</td>
<td>19.6</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.4</td>
<td>4.8</td>
<td>4.0</td>
<td>18.6</td>
<td>24.9</td>
</tr>
<tr>
<td>Pith</td>
<td>1</td>
<td>4.9</td>
<td>8.4</td>
<td>15.2</td>
<td>21.9</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.8</td>
<td>7.5</td>
<td>14.9</td>
<td>21.3</td>
<td>22.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.5</td>
<td>8.7</td>
<td>14.0</td>
<td>22.1</td>
<td>24.5</td>
</tr>
</tbody>
</table>

Fig. 1. Relation between relative fluorescence intensity and laser scanning during 1000 scans in a non-stained section (black line) and a safranin-stained section (gray line).
Fig. 2. Measurement of COA using power spectral analysis in a tangential section of WB5. – a: tangential section for this analysis. – b: azimuthal power spectral analysis in a, indicating the three peaks of 13 degrees for fiber orientation (F), 81 degrees for cross-sectioning plane (C), and 90 degrees for intensity change at the horizontal edges of the optical section (W).

Fig. 3. Summary of serial optical transverse sections in Analysis 3. – a–d: Optical sections of OS11, OS13, OS16, OS20, respectively. OS13 (b) provided sufficient high-frequency information and was thus determined to be from the surface of the original section. OS16 (c) had the maximum intensity among the series of optical sections. — Scale bar = 100 μm.
Grain angle measurement spectral analysis (Fig. 2b) shows three peaks at 13, 81, and 90 degrees. These peaks indicate distinct periodic features toward the azimuthal angle (Nagao 1983; Tanaka 1989; Maekawa et al. 1993; Ogata et al. 2003), e.g., the peaks at 13, 81, and 90 degrees represent fiber orientation (F in Fig. 2b), the cross-sectioning plane (C in Fig. 2b), and intensity change at the horizontal edges (or window) of the optical section (W in Fig. 2b), respectively (Ogata et al. 2003).

**Determination of the surface position along optical axis (Analysis 3)**

A selection of optical sections from Analysis 3 series is shown in Figure 3. Analysis 3-1 revealed that OS16 had the maximum fluorescence intensity among the series (Fig. 3c & 4). The amount of detectable auto-fluorescence from lignin decreased by half at OS32, 16 µm deeper than in OS16.

The result of Analysis 3-2, frequency power spectral analysis in each optical section is shown in Figure 5. In a power spectrum, the reciprocal of actual distance \( R \) is expressed as the distance from the center of the spectrum \( F \) in Equation 2:

\[
F = \frac{1}{R} \quad \text{(Equation 2)}
\]

In digital discrete Fourier Transform, Equation 3 is applied:

\[
F_D = \frac{S}{R_D} \quad \text{(Equation 3)}
\]

where \( F_D \) (pixel), \( R_D \) (pixel), \( S \) (pixel) represent the length from the center of power spectrum (pixel), the actual distance (pixel), the image size (pixel), respectively. In other words, an ROI includes \( S \times S \) pixels. In this analysis, \( S \) was 2048 pixels and each pixel size is 0.173 (µm/pixel). If the thickness of a single cell wall in Cryptomeria japonica is 1 to 2 µm, then \( R_D \) (pixel) is calculated as the thickness (µm) over 0.173 (µm/pixel) of each pixel size and \( F_D \) (pixel) is 180 to 360 pixels. Extremely-low- \((F: 1 \text{ to } 16)\) and low- \((F: 17 \text{ to } 64)\) frequency information of cell intervals (Fig. 5a) were sufficiently detectable even in OS20, the deeper optical sections (lighter gray line in Fig. 5a). Middle-frequency information \((F: 65 \text{ to } 128)\) includes double cell wall periodicity and was similar in each optical section (Fig. 5b). High-frequency information \((F: 180 \text{ to } 360)\) shows...
single cell walls and the edges of cell walls and was sufficiently detected in OS13 (Fig. 3b and black line in Fig. 5c). In other words, these optical sections provided more distinct cell wall information than OS16 (darker gray line in Fig. 5c), which had the maximum intensity. Based on these findings, OS13 had the most distinct cell wall information and was determined to be the optical section obtained from the surface of the original transverse section. In Figure 4, an optical section from slightly below the surface had the maximum intensity.
Relation in COA between image cross-correlation analysis and power spectral analysis on tangential sections (Analysis 4)

In Analysis 4-1, COA based on image cross-correlation analysis using optical sections from non-stained transverse sections in the dry condition were distinctly larger than COA using tangential sections in Analysis 2 (filled marker in Fig. 6). In contrast, COA in the oil condition was similar to that in Analysis 2 (open marker in Fig. 6). This finding indicates that a mismatch in the refractive index led to optical sectioning that seemed deeper than the mechanical z-axis movement in the dry condition, while in the oil condition the fluorescence would pass approximately straight due to the similar refractive index of the glass (1.515) and immersion oil (1.404). Figure 7 shows the reconstructed tangential (xz) plane in Analysis 4-2, in which COAs in both conditions were respectively similar to those in Analysis 4-1.

Fig. 7. Tangential (xz) optical section reconstructed from serial optical transverse sections in the dry (a) and oil (b) condition. Bright oblique lines represent cell walls and the top and the bottom of the lines indicate the top and the bottom of the original section. In the dry condition (a), cell orientation is steeper and the thickness of the section is narrower than in oil condition (b). — Scale bar = 50 μm.

To correct the overestimation of COA in the dry condition, linear regression was applied to both data series (Analysis 4-1) in Figure 6, resulting in Equation 4 (dashed line).
black line in Fig. 6) and Equation 5 (black line in Fig. 6) in the oil and dry conditions, respectively:

\[
\theta_O = 0.99 \theta_T \quad \text{(Equation 4)}
\]

\[
\theta_D = 1.51 \theta_T \quad \text{(Equation 5)}
\]

where \(\theta_T\), \(\theta_O\), and \(\theta_D\) represent COA in tangential sections (Analysis 2), in the oil and dry condition, respectively. Based on Equation 4, it is not necessary to correct COA in the oil condition. It is, however, necessary to correct the COA in the dry condition according to the following equation:

\[
\theta_T = 0.66 \theta_D \quad \text{(Equation 6)}
\]

Thus, the COA in the dry condition in the present research should be multiplied by approximately two-thirds.

This correction might differ with species, intra-ring location, and optical system. Therefore, the COA measured in transverse sections must be compared with that in corresponding regions in tangential sections to determine the appropriate correction similar to Equation 6. In the present research, an average of approximately three image cross-correlation analyses in a similar ROI provided an adequate COA. Also, the comparison of COA in image cross-correlation analysis with that in tangential sections in three ROIs with a different COA led to an applicable corrective equation. Equation 6 might be a corrective equation specific to the situation of the present research and thus the coefficient of the equation (e.g., 0.66 in Equation 6) should be verified for each research condition.

**Determination of z-axis range available for COA measurement (Analysis 5)**

Figures 8–11 show tangential lags of each optical section to the reference optical section obtained from 30 μm thick transverse sections along the z-axis. Cell wall information sufficient for an estimation of the periodicity in cell alignment could be available in the optical sections from 5 μm above the surface (Fig. 12). In Figures 8–11, however, tangential lags of such optical sections to the reference section were approximately zero, different from the actual cell orientation, indicating that the cell wall image in such sections provide non-focal plane information from around the surface.

Below the surface, the series of tangential lags along the z-axis were aligned linearly in both the dry and oil conditions and the lags were not available in the sections deeper than 30 μm from the surface in both conditions (Fig. 8–11). In the oil condition, the estimated linear cell orientation could be determined up to a depth of 25–30 μm (lines in Fig. 8 & 10) and COA in this condition was similar to COA in tangential sections, indicating an adequate thickness of the transverse sections and adequate cell wall information (dashed lines in Fig. 8, & 10). On the other hand, the estimated linear cell orientation could be determined only to a maximum depth of 20 μm (lines in Fig. 9 & 11) in the dry condition, i.e., the thickness of the sections was underestimated and the COA was distinctly larger than that in tangential sections (dashed lines in Fig. 9 & 11). These facts support the deduction that COA is overestimated due to compression of the images along the z-axis verifies Equations 4 and 5 in Analysis 4. Bucher et al. (2000) discussed...
a similar z-axis compression in their dry condition and corrected the compression using a coefficient of 1.581 in their optical condition. In the present research, a coefficient of 1.51 was estimated (Equation 5).

Fig. 8–11. Cell orientation estimated using image cross-correlation analysis of a non-stained 30-μm section in the oil (8) and dry conditions (9), a safranin-stained section in the oil (10) and dry conditions (11). Dots indicate the tangential lag data from image cross-correlation analysis and lines indicate their regression. Dashed lines indicate cell orientation in tangential sections (Analysis 2). Above the surface, the cell orientations were estimated to be straight, different from the actual cell orientation. Under the oil condition (8 & 10), adequate section thickness and COAs were estimated, while under the dry condition (9 & 11), the thicknesses were underestimated and the cell orientations were more inclined than the actual orientation.
Fig. 12. An optical section 5 μm above the surface of the original transverse section, showing out-of-focus cell wall information from the vicinity of the surface plane. — Scale bar = 100 μm.

Fig. 13–16. Cell orientation estimated using image cross-correlation analysis of a non-stained 100-μm section in the oil (13) and dry conditions (14), a safranin-stained section in the oil (15) and dry conditions (16). Arrows in Figures 13 and 15 indicate inadequate cell orientation due to the insufficient working distance.
The analyses of the 100 μm thick transverse sections (Fig. 13–16) were similar to those in the sections of 30 μm thick, although inadequate cell orientation was estimated in the deeper optical sections in the oil condition (arrows in Fig. 13 & 15). This finding suggests that the working distance of the objective lens in the oil condition (120 μm) was insufficient for the combined thickness of the 100 μm section and the Canada balsam above the section, and thus the lens was too close to the cover glass. This can be resolved by making the distance between the cover glass and the specimen as small as possible or by observing without a cover glass. By correcting the COA using image cross-correlation analysis in the dry condition with Equation 6, it is possible to perform image cross-correlation analysis in optical sections 100 μm deep.

Based on the intensity sum of optical sections, an intensity sufficient for measurement of the COA was obtained from top to bottom in transverse sections of both thicknesses. It is, however, possible that the intensity sum of the 100 μm deep optical section with a slightly inclined cell orientation was less than those from the optical section of this analysis due to fluorescence attenuation by passing through the cell wall above. Also, considering that a sufficient correlation coefficient is obtained in image cross-correlation analysis (over 0.2 at least), adequate cell wall information is obtained to a depth of approximately 80 μm, which supports the findings of Donaldson and Lausberg (1998). Additionally, cell wall thickness should be estimated in a quite shallow region with sufficient high-frequency information, due to the difficulty in demarcating between the cell wall and cell lumen in the deeper optical sections.

REFERENCES


