SHADING CORRECTION METHODS FOR DIGITAL IMAGE ANALYSIS OF CONFOCAL WOOD IMAGES

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SUMMARY

Confocal fluorescence microscopy provides a rapid method for acquiring high quality optically thin section images of wood suitable for measurement of cell dimensions. Single optical slice images of wood may occasionally contain artefacts due to differential light absorption caused by variation in the distance between the sample surface and the imaging plane across the field of view. Regional brightness variations, which we call shading, may cause problems when such images are used for wood cell measurements using digital image analysis, affecting the accuracy of wood cell dimensions. We have compared various shading correction methods for confocal microscope images and investigated the effect of shading on both the classification of cell wall pixels and the resulting cell dimension measurements. Severe shading results in significant errors for measurement of cell wall area, but smaller errors for cell wall thickness and lumen diameter. Some shading correction methods have unwanted effects on pixel classification and cell dimensions, while more effective methods remove the shading without introducing further artefacts. The effect of shading is influenced by choice of thresholding method.

Key words: Segmentation, image analysis, confocal microscopy, cell wall thickness, lumen diameter, Pinus radiata.

INTRODUCTION

Wood cell dimensions are important determinants of wood properties. For example, tracheid wall thickness and lumen diameter determine wood density and influence related properties such as stiffness and hardness, as well as papermaking properties (Cown & Kibblewhite 1980; Kibblewhite 1980; Panshin & DeZeeuw 1980; Bamber & Burley 1983; Evans 1994). Cell dimensions are typically measured using digital image analysis (McMillin 1982; Gasson 1985; Lee & Rosen 1985; Peachey & Osborne 1990; González & Woods 1992; Diao et al. 1996, 1997; Jun et al. 2000; Moell & Borgefors 2001) and with the development of fast personal computers this has become routine in many laboratories (Evans 1994; Xu et al. 1997; Donaldson & Lausberg 1998; Moell & Donaldson 2001).

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Confocal fluorescence microscopy provides a method for the rapid acquisition of high quality, thin optical section images of wood suitable for measurement of cell dimensions without the need for tedious embedding (Donaldson & Lausberg 1998). In confocal microscopy, an image is acquired as a planar optical section at a specific depth in the sample. The attenuation of the light is dependent on the distance traveled within the sample (Donaldson & Lausberg 1998). For samples with an uneven surface resulting from either sectioning artefacts or shrinkage distortion during drying, thicker regions (further from the local surface) appear darker and thinner regions (closer to the local surface) appear brighter. We refer to this brightness variation as shading, which can significantly affect the image quality of a confocal slice. Shading can be a problem particularly when imaging dry wood, as the wood slice may become distorted as a result of shrinkage. Although it is possible to remove the shading effects by projecting a series of optical sections, this has the effect of reducing the spatial resolution as the thickness of the projected image is increased (Moell & Donaldson 2001). Making projection images from a series of slices is also more laborious, time consuming and thus expensive. Finally, in conventional light microscopy, where shading artefacts may occur as a result of defects in the optics, a useful correction method is background subtraction [demonstrated in Model & Burkhardt (2001)]. This technique cannot be used in confocal fluorescence microscopy, since only the object is illuminated, and thus no background is available.

Classifying a picture element (pixel) as either lumen or cell wall, a process known as segmentation, significantly affects the accuracy of wood cell measurements (Moell & Donaldson 2001). Segmentation can be influenced by both overall image brightness and by shading caused, for example, by slight differences in staining, optical defects in the microscope, or sample distortion. Many fast and simple segmentation methods assume good contrast images, i.e. distinct classes of lumen and cell-wall pixels. Therefore, such methods may not be suitable to apply directly to images containing shading artefacts, because the optimum segmentation threshold varies in different parts of the image (Russ 1995). In order to obtain accurate data from such images, shading correction methods suitable for confocal microscopy are required.

We have investigated the use of shading correction methods to improve segmentation for confocal slice images containing shading artefacts. Shading correction can be a pre-processing method aimed at improving the image before the segmentation process, or it can be part of the segmentation process itself. Our aim was to find a suitable shading correction method for single slice confocal microscope images of wood, to reduce the demands on sample preparation and to allow us to extract accurate information, even from the occasional samples that produce suboptimal images.

MATERIALS AND METHODS

Sample preparation and imaging

Samples of radiata pine (Pinus radiata D. Don) wood were saturated in water and fixed in formalin aceto-alcohol (FAA). Using a sledge microtome, the samples were sectioned in the transverse plane at a thickness of 60 µm. Sections were stained with 1%
safranine (aq.) for 2–3 minutes and washed in water (Donaldson & Bond 2005). Sections were then briefly oven dried at 100 °C, mounted in immersion oil (N_o = 1.515, 25 °C) and examined on a Leica TCS/NT confocal microscope using a ×16 objective lens. An Ar/Kr laser was used for illumination. Confocal pinhole size was set to the optimum value for the objective lens used (1 airy disk). All images examined were acquired as single confocal optical sections (approximately 4 μm thick for the objective lens and excitation wavelength used) using fluorescence from near the surface of the section. The images were collected using an excitation wavelength of 568 nm and an emission wavelength of 600 nm. The optical resolution of the objective lens (×16 NA = 0.5) was 450 nm. All images were acquired at a size of 1024 × 1024 pixels, which yields a resolution of 0.6 μm/pixel and a total field of view of 625 × 625 μm. The resolution in grey scale was 8 bits (grey levels were in the range 0–255).

**Shading measure**

In order to measure the amount of shading, we calculate a coefficient as follows. Each 1024 × 1024 grey scale image was divided into 16 sectors of 256 × 256 pixels, numbered 1 to 16 from the top left corner to the bottom right. To investigate the uniformity of the brightness distribution, we used the brightness information in the odd numbered sectors, evenly spread across the field of view. For each of these eight sectors, the average of all grey levels greater than 5 was calculated (average brightness - Moell & Donaldson 2001). The reason for processing only pixels with a grey level greater than 5 was to exclude the lumens from the calculation. In confocal fluorescence images, the lumen pixels mainly contain brightness values of 0. The average brightness for the whole image of all grey levels greater than 5 was also calculated. The absolute value of the difference between the sector average and the whole image average was calculated for each sector. We call the average of these absolute values of the eight sectors the shading coefficient in units of grey level, which is our measure of brightness variation in an image:

\[ S = \sum (|B_s - B_i|) / 8 \]

where \( S \) is the shading coefficient, \( B_s \) is the sector brightness and \( B_i \) is the image brightness.

**Shading correction methods**

We have compared the following shading correction methods.

1) Planar shading correction

A multiplicative model of the shading effect is assumed as follows:

\[ o(x, y) = t(x, y)s(x, y) \]

where \( o \) is the observed image, \( t \) the true image, and \( s \) is the shading component. In confocal microscopy it is not possible to explicitly obtain the shading image \( s \). Therefore, the image \( s \) is estimated. Here, we assume that the shading component is of planar form, i.e.

\[ s(x, y) = ax + by + c \]
where \(a, b,\) and \(c\) are real-valued constants. We assume the variations of brightness in the true image \(t\) to be small compared to variations of the shading component \(s\). This allows a pixel-wise least-mean-square fit of \(\hat{s}(x, y)\) to \(o(x, y)\), which yields an estimate of the constants \(a, b,\) and \(c\), and thus an estimate of \(s(x, y)\). The estimate of the true image, \(t\), is then obtained by simply multiplying \(1/s\) by \(o\).

In this study, we have used the implementation of planar shading correction provided by Digital Optics V++ software.

2) Weighted average method

This method estimates the shading by intensively blurring the original image using a weighted average smoothing filter to isolate brightness information from morphological information to create an estimate of the shading image (Russ 1995). A multiplicative model of the shading effects is assumed as follows:

\[ o(x, y) = t(x, y)s(x, y) \]

where \(o\) is the observed image, \(t\) the true image, and \(s\) is the shading component. The shading component is estimated by applying a weighted average filter using the following coefficients

\[
\begin{array}{ccc}
1 & 2 & 1 \\
2 & 4 & 2 \\
1 & 2 & 1 \\
\end{array}
\]

with a scaling factor of 16. The filter is applied five times in succession. The estimate of the true image, \(t\), is then obtained by simply multiplying \(1/s\) by \(o\).

3) Spline fitting

Here, we have used the shading correction described by Lindblad and Bengtsson (2001). A multiplicative model of the shading effects was assumed.

\[ o(x, y) = t(x, y)s(x, y) \]

The shading component is assumed to be smooth and without edges, and cubic splines are used to model it, as described below. The model of the shading component is

\[
\hat{s}(x, y) = \sum_{i=0}^{n_x-1} \sum_{j=0}^{n_y-1} \tilde{p}_{ij} B_i^x(x) B_j^y(y)
\]

where, \(n_x\) and \(n_y\) are the number of control points in the horizontal and vertical direction, \(\tilde{p}_{ij}\) are the control points, and \(B_i^x(x)\) and \(B_j^y(y)\) are cubic B-spline functions (Lancaster & Šalkauskas 1986).

The control points of the B-spline are distributed on a regular mesh. In this study, \(5 \times 5\) and \(8 \times 8\) mesh sizes were used. Estimates of the control points are found by minimising the mean-least-square expression \(\sum_{x,y \in \Psi} (\hat{s}(x, y) - o(x, y))^2\). The set \(\Psi\) is iteratively refined to match the true foreground set, as described in Lindblad and Bengtsson (2001). Note that \(\Psi\) in our application is the set of foreground pixels, \(i.e.\) cell-wall pixels. The initial estimate of \(\Psi\) was the entire image for all sample images.
4) Local segmentation

A simplified variation of the dynamic threshold method used for determining fibre distribution by Xu et al. (1997) was developed. Our implementation involves dividing the image into 16 \(256 \times 256\) pixel sectors (other sector sizes can be used if required). Each sector is segmented separately using the average brightness method (Moell & Donaldson 2001), i.e.

\[ T = k \bar{x} \]

where \(T\) is the threshold, \(k\) is a constant and \(\bar{x}\) is the average brightness of pixels in the image with grey level intensity \(> 5\). For this study \(k = 1\) was used but the implementation allows the user to change this value in order to further optimise the threshold.

Segmentation without shading correction

To study the effect of shading artefacts on pixel classification as either cell wall or lumen, the sample images were processed initially by average brightness, confirmed in a previous study to give near-optimum segmentation results on wood images free from shading effects (Moell & Donaldson 2001). In addition, the shaded images were also processed using average brightness optimised for the bright or dark regions of the cell wall, and by manual thresholding. Optimised thresholding was done by measuring the optimum threshold in a selected bright or dark part of the image and then applying this threshold to the entire image. Manual thresholding was done in such a way as to avoid blank areas in the image due to poor segmentation of cell wall pixels in the shaded areas.

Segmentation with shading correction

The sample images were pre-processed using the different shading correction methods, and the resulting images were then segmented using revised thresholds calculated as above. Shaded images were also segmented by the local implementation of average brightness using \(256 \times 256\) pixel sectors.

Comparison of shading correction methods

The effectiveness of shading correction was determined by measuring the shading coefficient before and after processing. The XOR logic function (Russ 1995) finds the number of different pixels between two binary images. We made an XOR comparison of shaded and unshaded images using the spline-corrected images, which had the least amount of shading, as a reference point for comparison. This value was expressed as a percentage of total image pixels, or of the cell wall pixels in the reference image.

To study the effect of shading on cell dimension assessment, we manually measured the tangential wall thickness and radial lumen diameter of 50 tracheids in each image before and after shading removal by placing two cursor points on either side of each object to be measured and determining the Euclidean distance using Digital Optics V++ software. Double cell wall thickness was subsequently divided by 2 to give cell wall thickness. We also measured the average cell wall thickness and radial lumen diameter using our routine image analysis software (Donaldson & Lausberg 1998) before and after shading correction. This provides a measure of the effect of shading correction, and its interaction with thresholding method, on the accuracy of cell dimensions.
RESULTS AND DISCUSSION

For the purposes of this study, we chose ten example images ranging in quality from good, with relatively little shading, to poor, containing significant shading artefacts (Fig. 1). Thus, some preparations had fairly flat section topography, while others had surfaces of more varying height due to shrinkage distortion. Variation in thickness, which effectively produces an uneven surface with respect to the planar optical slice, results either from uneven sectioning or from distortion and shrinkage of the section during preparation, as shown in Figure 2. The origin of shading effects from section topography was confirmed by making an x-z section (Fig. 2B) (a vertical slice through the section) through an extended volume, which includes the section surface. The section surface appears as the brightest region in the x-z section and can be seen to be lower on the left and higher towards the right (Fig. 2B).

Shading correction

The amount of shading was reduced by all of the correction methods, with planar correction being least effective (shading often still visible), and spline 8 x 8 the most effective (shading no longer visible) (Table 1, Fig. 3). Since the two spline corrections were very similar, we only report further on results for the 8 x 8 method. The shading

Figure 1. Examples of confocal images (single optical slices) with mild shading (top) and more severe shading (bottom). – A: Image 9, S = 8.6. – B: Image 10, S = 8.3. – C: Image 5, S = 21.3. – D: Image 7, S = 22.3. — 625 × 625 μm.
values for the local method are not available, since this involves direct segmentation to produce a binary image without any intermediate grey level image. Because it is based on the assumption of linear shading trends, the planar technique is most effective when the actual shading is also approximately linear. For example, the method works well when one side of the image is shaded, but it will produce a compromise when the centre of the image is shaded and there is no trend from one side of the image to the other.

Figure 2. Demonstration of the principal cause of shading effects in confocal wood images. – A: A single optical slice showing shading on the left side of the image (625 × 625 µm). – B: An xz slice through the sampled volume (in this case from left to right at the centre of the image and 56 µm thick) showing the sloping surface of the section (the brightest area). Note also the decline in intensity with increasing depth. An optical section through this volume in the transverse plane will thus have light and dark areas determined by light absorption related to the varying mass of cell wall between the plane of the optical section and the surface of the sample (625 × 56 µm).

Table 1. Values of the shading coefficient for the shaded and shading corrected images.

<table>
<thead>
<tr>
<th>Image</th>
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**XOR comparison**

The XOR logic function compares the pixel differences between binary images (Russ 1995; Moëll & Donaldson 2001). Because they have the least shading, we use the spline-corrected images as positive controls in comparisons of the shaded and corrected images. Comparisons are shown as a proportion of all image pixels and as a proportion of the cell wall pixels in the spline-corrected images. We also compare the optimised thresholds for bright and dark regions, and manual thresholding in the shaded images (Fig. 4).

Shading results in a classification error of 4% of pixels on average relative to the whole image, and 11% relative to cell wall pixels using average brightness thresholding (Table 2). Using the optimised thresholds for bright and dark regions, the classification errors are slightly worse than automated average brightness, with 6% of pixels being misclassified relative to the whole image, and 17% relative to cell wall pixels. For individual highly shaded images, the classification errors may be as high as 37% (Table 2). Planar correction results in a slight reduction in classification error, while weighted average correction results in more than 20% of cell wall pixels being misclassified. Local thresholding results in the smallest classification error in comparison to spline correction, with only 2% of pixels being misclassified (Table 2, Fig. 5). This result confirms that local thresholding gives a very similar improvement to spline correction (Fig. 5). Since local thresholding is easier and faster to implement than spline-based shading correction, this is our preferred method.

Figure 3. Examples of a single confocal image (single optical slice) after correction of shading using different methods. – A: The shaded image. – B: Planar correction. – C: Weighted average correction. – D: Spline correction. The weighted average and spline corrected images contain no visible shading in this example (625 x 625 μm).
Figure 4. A comparison of segmented binary images using a shaded image. The images on the left are the binary images and those on the right are the XOR comparison images compared with the spline-corrected image. The first two segmentations are examples of under-thresholding, while the second pair are examples of over-thresholding. – A: Automated average brightness. – B: Optimised for the bright areas using average brightness. – C: Optimised for the dark areas using average brightness. – D: Manual threshold (625 × 625 μm).
Table 2. XOR values expressed as a percentage of all image pixels or as a percentage of cell wall pixels. The XOR comparison is for shaded or corrected images compared to the spline corrected images, which had the least amount of shading. The comparison includes automated average brightness thresholding as well as optimised thresholding using the same algorithm but optimised for bright or dark cell wall regions. Manual thresholding is also shown for comparison.

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The XOR comparisons confirm that there are significant pixel classification errors that occur as a result of shading and that these errors vary among images and with the thresholding technique used (Fig. 4). These errors can be reduced to negligible levels by appropriate shading correction, either spline-based correction or local thresholding. The optimised thresholds produce greater classification errors with large regions of the images often being poorly segmented (Fig. 4). This will cause significant problems for image analysis systems that measure cell wall area across the entire image because of blank areas where the cell walls are not detected (Fig. 4). Image analysis systems that measure cell wall thickness directly on a cell by cell basis will not be so affected although there will be fewer cells available to be measured. Lumen diameter measurements will contain errors unless the image analysis system can recognise and exclude non-typical measurements. For example, if the image is under-thresholded there will be
one or more blank areas in the image that will appear as very large cell lumen objects (Fig. 4). Such errors can be excluded by size or shape filters. The software we use applies both types of filter so the effect of segmentation errors will be minimised.

**Manual measurement of cell dimensions**

Manual measurement is inherently susceptible to bias and is also tedious and time consuming. Manual measurement of tangential cell wall thickness and radial lumen diameter is expected to be independent of shading, as no segmentation is involved. The results for tangential wall thickness shown in Figure 6 indicate that cell dimensions are essentially the same in both shaded and corrected images, confirming that manual measurement is unaffected by shading. Radial lumen diameter showed the same result. Manual measurements of cell wall thickness are similar to automated measurements (3.5 μm cf. 3.0 μm using average brightness). Because the automated measurement system which we have used determines wall thickness from wall area in the entire image, and wall area is dependent on both wall thickness and on other features, such as the number and size of rays, these two measurements are not entirely comparable. However, the difference is comparable to the optical and pixel resolutions of our measurement system and can thus be regarded as negligible.

Figure 5. A comparison of segmented binary images showing A: automated local thresholding, B: spline-corrected average brightness threshold, and C: the XOR comparison of these two images (625 x 625 μm).
Figure 6. Manual measurements of tangential wall thickness comparing shaded images with three shading correction methods and with local thresholding. The values shown are the average of 50 measurements from 10 images.

**Weighted average correction**

Among the shading correction methods, it is apparent that the weighted average correction results in a consistent overestimation of cell wall thickness, confirming the results from XOR analysis and indicating that this correction method alters the cell dimensions and is thus unsuited to this application. The reason for this distortion is related to changes in the brightness distribution of pixels in the image (Fig. 3). The weighted average correction increases the relative brightness of the intermediate pixels that lie between the cell wall and the lumen resulting in these pixels being added to the cell wall classification. It should be possible to correct this error by calculating a different threshold. We have only compared similar thresholds to avoid introducing too many comparisons but the weighted average correction does reduce shading (Fig. 3), so it may be a useful technique in some applications.

**Effect of shading on cell dimensions**

Shading is expected to result in an image with different thresholds for bright and dark areas of the image (Fig. 4). Measurements indicate that for shaded images, cell wall thickness is weakly correlated to the shading coefficient (r = 0.68, p = 0.03) while for corrected images this correlation does not exist (r = 0.05). Thus shading correction can reduce any bias due to shading, and potentially reduce the among image variance thereby allowing more sensitive detection of differences between treatments.

The effect of shading on measurement of cell dimensions is surprisingly small, even in the most severely shaded images. Automated thresholding based on the entire image produces a compromise threshold. For shaded images, this compromise results in overestimations of cell wall thickness in the bright areas, and underestimations in the
Table 3. Comparison of automated measurements of cell wall thickness and radial lumen diameter comparing automated average brightness and a threshold optimised for the bright or dark areas of the image. Manual thresholding is shown for comparison. The confidence interval for average values is 0.2 $\mu$m for wall thickness and 1.3 $\mu$m for radial lumen diameter. For individual images, differences in cell wall thickness among treatments larger than 0.6 $\mu$m (the pixel size) are considered real effects.

<table>
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</table>

**Optimised average brightness**

| Wall thickness ($\mu$m) | Bright | 2.6 | 2.5 | 2.7 | 2.8 | 1.8 | 2.1 | 2.1 | 2.8 | 2.8 | 2.8 | 2.5 |
| Dark | 3.5 | 3.0 | 3.0 | 3.2 | 3.7 | 3.3 | 4.1 | 3.4 | 3.6 | 3.3 | 3.4 |

| Radial lumen diameter ($\mu$m) | Bright | 36.8 | 29.4 | 35.3 | 32.7 | 32.2 | 33.8 | 30.9 | 33.3 | 33.9 | 31.2 | 32.9 |
| Dark | 31.4 | 26.9 | 29.9 | 31.3 | 30.1 | 26.8 | 32.7 | 31.7 | 32.5 | 32.3 | 30.6 |

| Manual | Wall thickness ($\mu$m) | 3.8 | 3.1 | 3.2 | 3.5 | 3.6 | 3.8 | 4.1 | 3.5 | 3.7 | 3.5 | 3.6 |
| Radial lumen diameter ($\mu$m) | 30.8 | 26.5 | 28.2 | 30.4 | 30.1 | 28.9 | 33.0 | 32.0 | 32.3 | 32.8 | 30.5 |

dark areas, thereby resulting in fairly accurate approximate average values, even for the most severely shaded images (Table 3). Automated thresholding based on average brightness is thus a robust method. However, for optimised or manual thresholding, the more severely shaded images produce significant errors in cell wall thickness of 1–2 $\mu$m. In contrast, shading has little effect on measurement of lumen diameter, mainly because lumens are measured individually and hence any poorly segmented lumens can be excluded in a robust image analysis system using shape or size filters. Lumen measurements are also less affected because they are relatively large compared to the
pixel size and because the grey level of lumens is very uniform making them insensitive to even quite large segmentation errors.

There are several characteristics of wood images that contribute to their insensitivity to segmentation errors. First, wall thickness is proportional to the square root of wall area. Furthermore, the error in wall thickness is also being spread across a relatively large number of cells. Finally, the images we have used contain only earlywood tracheids and hence are very uniform. In other applications where shading has been found to be a major problem, the images were less uniform (Model & Burkhardt 2001). Hence, if all the large objects tend to be in the shaded region, the overall result will be greatly affected. The same situation could occur with wood images containing a mixture of earlywood and latewood, for example. Despite the relatively small effects on cell wall thickness, shading will have a significant effect for applications such as high resolution measurements related to shrinkage, where very high accuracy is needed.

All of the shading correction methods we have tested employ a multiplicative implementation as opposed to an additive implementation. For confocal images of wood where the shading is only present in the cell wall pixels, a multiplicative shading correction will avoid any alteration to the unshaded lumen pixels, which mainly have values of zero. Additive shading corrections will change the lumen pixels and will therefore be less effective, removing the shading from the cell wall pixels but adding new shading to the lumen pixels.

Alternatives to shading correction

Images containing shading artefacts could simply be replaced by new images, but shading effects can be difficult to avoid when samples are prone to shrinkage distortion. If drying the sections can be avoided, then shading is much less of a problem, but cell dimensions from wet cell walls are poorly correlated to properties such as basic density. Drying the sections under constraint by clamping them between two microscope slides during drying can produce flatter sections and thus reduce shading effects. Other factors, such as spherical aberration due to refractive index mismatch between the mounting medium and the immersion medium of the objective lens, uneven staining, or the presence of mobile air-bubbles drifting into the field of view during image acquisition, can also produce shading effects. It is, however, relatively easy to avoid these problems by adequate sample preparation.

Optical effects on shading

Two optical effects have an influence on shading. The numerical aperture (NA) of the objective lens on the microscope affects the optical thickness of confocal slice images. The larger the numerical aperture, the thinner the optical slice thickness and hence the greater the shading effect. Using an objective lens with a lower numerical aperture should reduce shading effects, but it also reduces the optical resolution. The size of the confocal aperture also affects the optical thickness (confocality) so opening this aperture will reduce shading but it will also decrease resolution and contrast.
CONCLUSIONS

Shading can produce significant errors in cell dimension measurement for individual images but this also depends on segmentation method (automated, optimised or manual) and analysis method (object-wise or global). Errors due to shading can sometimes be more than 1 µm, which represents more than 30% of a typical softwood earlywood tracheid wall thickness (3 µm). Manual thresholding is susceptible to shading errors, but automated segmentation using average brightness produces a compromise threshold which tends to hide the effects of shading in average values. Because shading errors may still exist for individual images, it is still important to remove the shading to minimise the among-image variance and avoid any bias from individual badly shaded images. Because measurement of cell wall thickness is influenced by resolution limitations, any procedure that removes a potential source of error is likely to be beneficial. Based on comparison of cell dimensions, the spline and local methods are most effective at removing the shading effects. Because local thresholding is easier to implement it is our preferred method.

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