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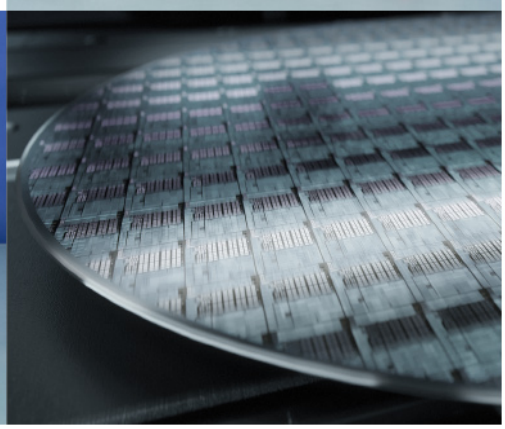

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Using imaging ToF-SIMS data to determine the cell wall thickness of fibers in wood

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Here, we demonstrate the use of time-of-flight secondary ion mass spectrometry (ToF-SIMS) imaging data for estimation of cell wall thickness in wood samples. Current research in forest biotechnology focuses on transgenic trees with wood properties tailored to specific applications. Appropriate analytical methods to characterize the very heterogeneous wood material are constantly being developed and improved. Chemical imaging of wood by ToF-SIMS represents an interesting tool for this purpose with many applications. In addition to wood chemistry, the impact of specific genetic modifications on wood anatomy needs to be assessed. Cell wall thickness is an important anatomical parameter that among others is used for assessing biomass accumulation. We developed a strategy to estimate cell wall thickness from ToF-SIMS images and implemented it in the open source programming language 'R'. In brief, random lines are projected over the black and white mask of a ToF-SIMS image, and length values of all line sections that cut across a cell wall are collected. After enough iteration, the shortest values of the obtained count distribution represent the crossing sections normal to the cell walls, hence cell wall thickness. Compared with conventional light microscopy image analysis, TOF-SIMS data offers many advantages such as submicron resolution and additional spectral information for automated annotation of distinct anatomical features. This work underlines the importance of SIMS imaging for studies of wood chemistry and anatomy and provides a new approach to obtain an important wood anatomical parameter from ToF-SIMS data.
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Keywords: ToF-SIMS; image analysis; wood analysis

Introduction

In recent years, a number of labs have established methodology to apply time-of-flight secondary ion mass spectrometry (ToF-SIMS) for wood analysis by studying sample preparation, compound identifications, and the use of various instrumental setups and conditions.[1–3] An important reason for the increasing popularity of ToF-SIMS in wood analysis is its sensitivity toward the lignin polymer, one of the major components of wood. Combined with the high spatial resolution, ToF-SIMS allows microanalytical studies of morphological details within single cell walls.[4]

The most common techniques to obtain images for cell wall thickness determination are light microscopy (LM), confocal laser scanning microscopy and scanning electron microscopy.[5] Among those, LM is probably the most readily used technique as a result of its widespread availability. ToF-SIMS is superior to many other techniques for its imaging capability; however, there are to our knowledge very few studies where the hyperspectral images have been used to calculate anatomical features of the sample by image analysis. In a biological respect, wood anatomical parameters are of large importance: Genetic modification, an approach widely used to study gene function, can result in anatomical and/or chemical effects.[6] As a change in anatomy will usually also affect results of chemical analysis, it is important to separate between the two effects. In this paper, we show a method to determine cell wall thickness on wood samples by the use of ToF-SIMS image data. Using ToF-SIMS to determine cell wall thickness is an extension of applications for ToF-SIMS when the data has already been recorded for chemical analysis.

Determination of cell wall thickness can be done manually by using software tools such as ImageJ or generic drawing software. This is, however, very tedious and usually not feasible for a large number of samples. A number of automated protocols using image-processing algorithms have also been used to determine cell wall thickness batch wise.[5,7] Many of them are based on 'watershed' segmentation, 'Voronoi diagrams' and similar algorithms. Here, we have implemented a novel approach to estimate cell wall thickness from ToF-SIMS data.

Experimental

Images of wood tissue sections, fixed to the sample stage by double-sided tape, were recorded on a TOF.SIMS 5 (ION-TOF GmbH, Münster, Germany). Positive spectra were obtained using a 25 keV

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Bi_3^+ bismuth ion gun at a current of 0.3 pA, in 'high current bunched' mode. Surface charge compensation was accomplished with an electron flood gun. The primary ion dose was 1.91×10^{12} ions/cm^2. Images were recorded in the positive ion mode. Each image was of the dimension 512×512 pixels corresponding to 256×256 μm. Five images were recorded from just outside of growth ring three in the 'late wood', wood that has been synthesized in the end of the growing season.

Sample material

Field-grown 5-year-old poplar trees were harvested (summer 2010) and transported on dry ice to the lab where they were kept at −20 °C until further processing. Transversal sections of 20-μm thickness obtained using a cryostat at −20 °C with a steel knife (HM 505E, Microm Intl. GmbH, Walldorf, Germany) were freeze dried overnight prior to ToF-SIMS analysis.

Applied methods

Data export, import, pre-processing

The recorded raw data was peak picked using the 'Surface Lab 6' (Version 6.3, ION-TOF GmbH, Münster, Germany) software and exported to the proprietary binary format, BIF6. For all further data processing and analysis, 'The R Project for Statistical Computing' (R, version 3.0.2)[8] was used. BIF6 files were first imported to R by an in-house script; then, the first component of a principal component analysis (PCA) was calculated to create an image with increased contrast between cell wall material and cell lumen. The local minima in the multimodal distribution function, calculated from the PCA score intensities, were used for thresholding to create black and white masks so that the cell wall fraction became foreground and lumen area background. Vessel lumens were selected using a cutoff size chosen by a local minima in the size distribution function of cell lumens and converted to foreground. The R library 'EBImage'[9] was used for all aforementioned image processing steps.

Estimation of cell wall thickness

A number of lines with random position and angle were projected on the black and white ToF-SIMS image masks. The length of the sections that cut over cell walls was then calculated and stored in a variable. Assuming that the distribution of cell wall thickness within one sample is rather narrow, the shortest sections are those that cut normal to the cell wall direction; hence, they represent cell wall thickness. Fiber/vessel cell walls differ both in size and chemistry from fiber/fiber walls. Therefore, they are to be excluded in fiber cell wall thickness determinations. As indicated in the previous paragraph, we achieved this by converting vessels to the same color coding as cell wall material. Consequently, line sections cutting over vessels are much longer than reasonable length measures of a cell wall. Hence, those sections can be excluded by a cutoff size.

Results and discussion

Verification of the method with simulated data

To test our method, we created a regular black and white mask with known dimensions to imitate wood structure. The cell wall was set to 8 pixels and both lumen length and width to 10 pixels. One pixel corresponds to 0.5 μm in the ToF-SIMS images. Figure 1a shows a visual example of applying the described method to such an 'artificial cell wall' mask. The resulting counts for distribution of length values from sections that cut the cell wall is shown in Fig. 1b. Sections that extend to the border of the image are excluded, as they are potentially too short. The regular pattern of the artificial cell wall masks yields a count distribution with several distinct peaks (Fig. 1b). The shortest sections will not have the highest counts, as there are many more possibilities for sections not normal to the cell wall direction. For simplicity, we used the local maxima of a fitted distribution curve ('*density()*' function, R, Gaussian Kernel, default parameters) as a cell wall thickness metric, which yields values slightly higher than the true values. The sufficient number of random lines was determined to be 5000 by subsequently increasing the number of random lines until the result converged beyond the precision of the ToF-SIMS measurement. Calculated cell wall thickness for simulated data with true cell wall thickness of 8 pixels was 8.7 pixels or 4.35 μm.

Application of the method to real samples

Figure 2a–e shows the total ion count images after peak picking. Black and white masks, after thresholding are shown in Fig. 2f–j, whereas Fig. 2k–o shows the corresponding section length count

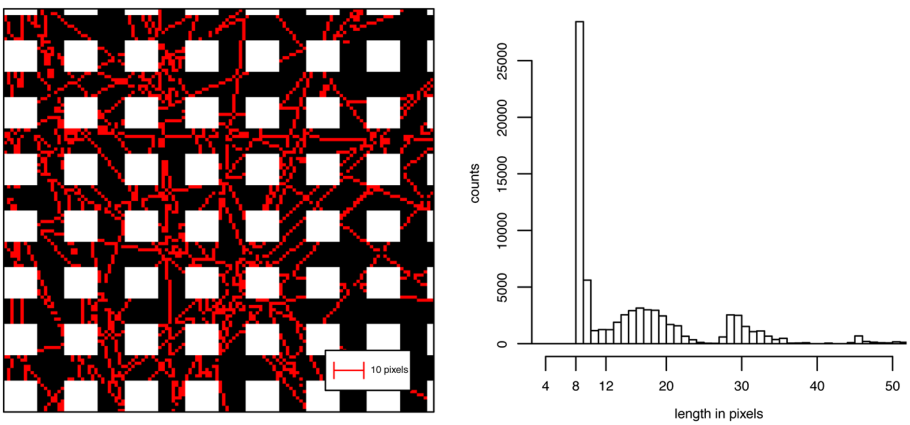

Figure 1. a An artificially created cell wall mask with an 8-pixel cell wall thickness and 10-pixel square lumen size. Random lines were projected onto the mask, and the length values of sections that cut the cell wall were collected and arranged in a histogram shown in Fig. 1b. This is the histogram of length values when cutting 5000 random lines with the artificial cell wall mask.

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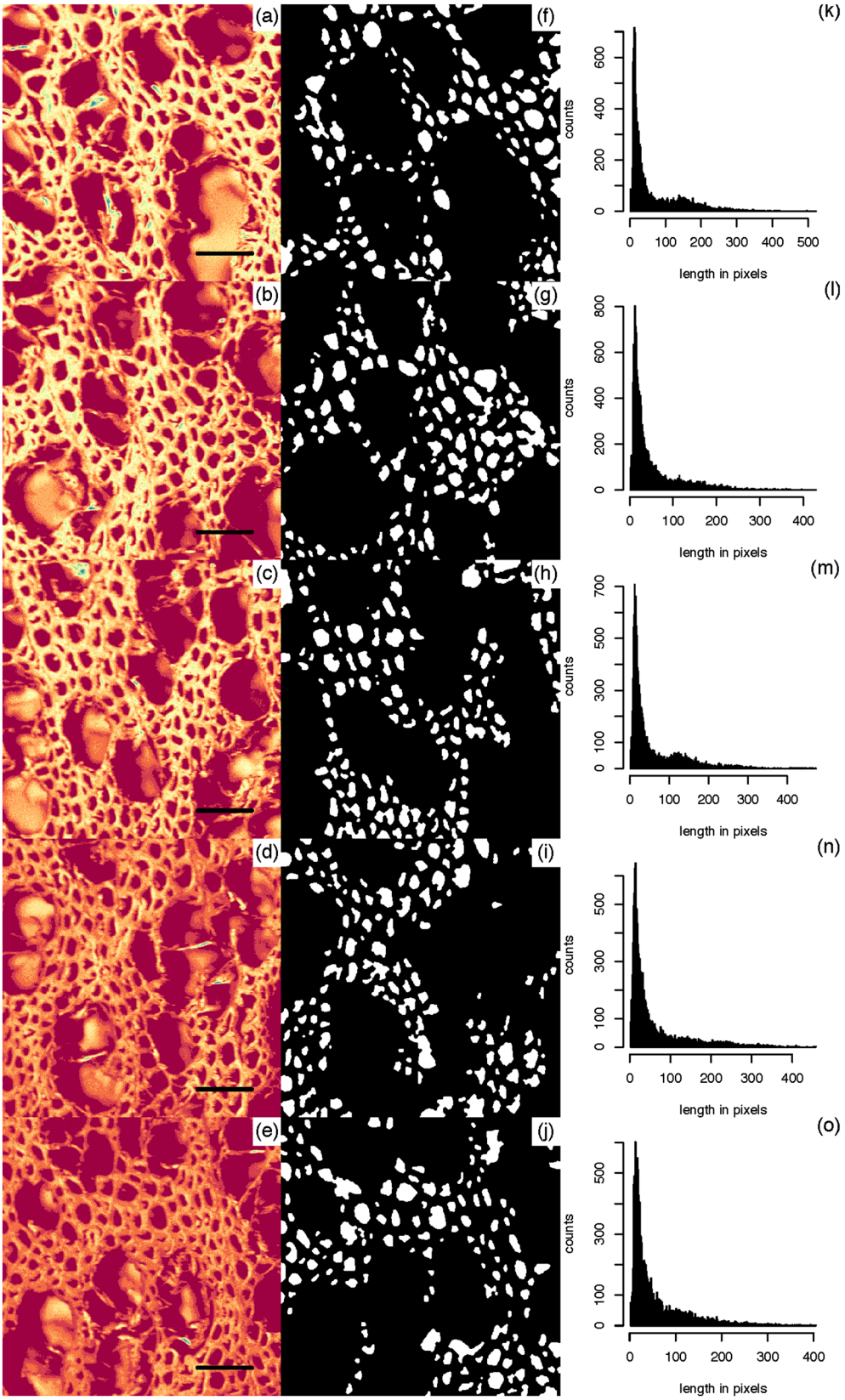

Figure 2. Panels (a–e) show the total ion images of ToF-SIMS data collected on poplar wood sections. Panels (f–j) show the binarized mask where cell wall plus vessel lumen area are foreground and fiber lumen are background. Panels k–o show the resulting length distribution when cutting 5000 random lines with the respective cell wall masks shown in panels (f–j).

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distributions. Compared with the count distribution in Fig. 1b from simulated data, those from real images show far less well-separated subdistributions. This is expected and can be explained by the large cell wall thickness variations within a real sample. Further, count distributions from real images contain more counts for very short values. This is also an effect of the large variation in biological samples compared with simulated data. The side length of the square images was 512 pixels, corresponding to 256 μm. The determined cell wall thickness values for the samples were 8.4, 7.0, 7.6, 7.8 and 7.8 μm, resulting in an average cell wall thickness of 7.6 ± 0.4 μm. An average value from literature is 5.4 μm.[10] Our estimated value seems reasonable, considering that late wood fiber cell walls are known to be thicker than average.[10]

Conclusion

We have used ToF-SIMS images as a basis to automatically estimate the anatomic parameter 'cell wall thickness' in wood samples. The method was first tested on simulated data and then applied to real samples. In the future, we are planning to use this method for discrimination analysis between wild type and genetically modified wood samples.

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