Microscopy and computerized image analysis of wood pulp fibres multi-scale structures

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Wood pulp fibres have multiscale characteristics. Roughly, the length and width of fibres vary from 1-3 mm and 10-50 µm, respectively. The fibre wall structure is mainly composed of well-organized fibrils with diameters in the nano-scale. The multi-scale structures of cellulose fibres require dedicated microscopy techniques for assessment of their characteristic details. In this work, a description of several microscopy techniques is given. The surface and bulk, 2D and 3D, structures are assessed with light microscopy, X-ray microtomography, conventional scanning electron microscopy and modern field-emission scanning electron microscopy. Conventional microscopy is extended to quantitative microscopy, aided by the increasing development of the ImageJ program and its distribution packages, which are in the public domain and freely available. Several case studies are presented based on specific plugins, which are described in detail.

Keywords Microscopy; image analysis; surface analysis; texture; metrology; paper; wood pulp fibres; ImageJ.

1. Introduction

Wood pulp fibres are gaining increasing interest within several industry sectors. From being used conventionally as the major component in paper, fibres are presently being utilized as reinforcement in bio-degradable composites and as a source of sugar for bio-energy production. Wood pulp fibres can be manufactured by e.g. thermo-mechanical, chemi-thermo-mechanical and chemical pulping. Thermo-mechanical pulping (TMP) disrupts the fibre wall structure mechanically. Chemical pulping removes the lignin and preserves the cellulose. During the recent years, chemical pulp fibres have been applied as the raw material for the production of nanofibrils, which have enormous potential in several applications [1]. However, a successful utilization of cellulose fibres requires a comprehensive understanding of their structure and how their morphologies are affected by a given production procedure and treatment.

Microscopy has a long history within the pulp and paper industry. Light microscopy (LM), confocal laser scanning microscopy (CLSM), atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been most useful techniques for quantification of fibre structural details [2-7]. In addition, modern techniques such as X-ray microtomography (X-µCT) for 3D characterisation [8,9] and field-emission SEM (FE-SEM) for nanostructural assessment of fibre surfaces [10-12] are contributing to the understanding of fibre structures and properties.

Modern techniques for image acquisition expose details unattainable by conventional microscopy. However, in order to exploit the potential of modern image acquisition devices, effective image processing and analysis techniques are required to extract and quantify relevant information. Techniques for image processing, analysis and visualization of digital data have had a tremendous development during the last two decades. This is specially the case with ImageJ, a program in the public domain, which has proved to be a most suitable package for processing, analysis and visualization of 2D and 3D data.

This work will demonstrate several preparation techniques, image acquisition devices and the corresponding computerized image analysis, applied to the study of wood pulp fibres.

2. ImageJ – image processing, analysis and visualization

ImageJ is a public domain program developed by Wayne Rashband at the National Institute of Mental Health, Bethesda, Maryland, USA. The program has been available since 1997 and has had a continuous development through the years. The Imagej program is based on Java and thus available for Windows, Mac and Linux operative systems. Presently, ImageJ has comprehensive capabilities for image processing, analysis and visualization.

The ImageJ program has an open architecture. The capabilities of the program can thus be extended for specific purposes. Specialized procedures can be developed mainly at three levels, i.e. 1) modifying the freely available source code, 2) developing plugins and 3) developing macros or Java scripts. The complexity of each level varies from an experienced programmer (level 1) to a motivated user (level 3). The open architecture provided by ImageJ has facilitated the development of a comprehensive library of plugins and macros, which have been created by Rasband and by multidisciplinary developers worldwide [13]. Plugins and macros are available for several applications, spanning from simple image processing to interactive 3D visualization and quantification.
3. Assessment of the structure of wood pulp fibres

Wood pulp fibres have several important characteristics, which determine their structural and mechanical properties. The fibre wall thickness, the fibre collapsibility and the degree of fibrillation are just a few examples of the characteristics that describe a fibre structure and behaviour [5,14-16]. Proper quantification of the mentioned structural details requires an adequate image quality of the assessed fibre material. Roughly, the quantification depends on i) the device applied for image acquisition, ii) the pre-processing steps and iii) the segmentation of the fibres from the background.

3.1 Image acquisition devices

In the next sections relevant 2D and 3D analyses of fibre micro and nano-structures will be described. Although several additional image acquisition devices have been applied to the study of wood pulp fibres (CLSM, AFM, TEM), the analyses described in this work are based on X-ray microtomography (X-µCT), light microscopy (LM), scanning electron microscopy (SEM) and field-emission SEM (FE-SEM) (Table 1). The acquisition of relevant images of fibre structures and their corresponding computerized image analysis will be demonstrated.

<table>
<thead>
<tr>
<th>Image acquisition device</th>
<th>Effective resolution (µm)</th>
<th>Advantages</th>
</tr>
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<tbody>
<tr>
<td>X-µCT</td>
<td>&lt; 1.0</td>
<td>3D visualization and quantification</td>
</tr>
<tr>
<td>LM</td>
<td>0.2</td>
<td>Colour images</td>
</tr>
<tr>
<td>SEM</td>
<td>0.01</td>
<td>Surface and cross-sectional quantification</td>
</tr>
<tr>
<td>FE-SEM</td>
<td>0.001</td>
<td>Versatile and high-resolution device</td>
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3.2 X-ray microtomography

Since its introduction as a research tool for the pulp and paper industry [8], X-ray microtomography (X-µCT) has been evolving considerably. Presently, desktop devices are capable of achieving sub-micrometer resolutions, which is most suitable for quantification of the fibre micro-structural details. In addition, modern synchrotron radiation X-µCT expands the effective resolutions of 3D volume images. Sub-micrometre and nano-resolutions may be achievable.

A typical size of a 3D volume image is e.g. 1024x1024x1024 voxels (volume elements). No mechanical sectioning is necessary, thus giving a smooth transition between consecutive slices. This improves a given 3D reconstruction (Fig. 1). The voxels are isotropic, i.e. they have the same dimension in all directions. 3D volume images demand effective methods for processing and analysis. In addition to ImageJ [13], the Fiji program [17] offers several plugins for processing 3D images. Processes such as smooth, median, FFT and distance transforms can be performed in 3D. In addition, Fiji is distributed with several plugin packages organized in a well-structured manner.

Provided good image quality, sufficient resolution and good contrast between the fibres and the surroundings, the process for reconstructing a fibre structure is relatively easy. 1) The images for reconstructing the fibre fragment exemplified in Fig. 1 were acquired with a SkyScan SEM-microCT system. 2) The fibres were segmented applying a fixed threshold. 3) The resulting binary images were smoothed (Fiji/Plugins/Process/Smooth 3D). 4) The size of the volume image was increased in the x, y and z directions (Fiji/Adjust/Size...). 5) The smoothed and resized binary 3D images were visualized with the 3D Viewer plugin (Fiji/Plugins/3D Viewer). It is worth to mention that in addition to the 3D Viewer there are alternatives ImageJ plugins that are most effective for 3D visualization of surface and volume images [18] and quantification [19]. X-µCT is most useful for 3D assessment. Details such as fibre morphology, spatial distribution and fibre orientation in composite materials can be assessed. However, X-µCT has a limitation with respect to its effective resolution. Complementary microscopy techniques are thus necessary for describing the various scales of a fibre structure.

![Fig. 1. X-ray microtomography (X-µCT). 3D reconstruction of part of a cellulose fibre. Note the semi-collapsed shape of the fibres, which is typical of fibres processed chemically. The volume is approximately 42 µm x 42 µm x 42 µm.](image-url)
3.3 Light microscopy

Light microscopy (LM) has been one of the most applied techniques for characterising fibre structures [3,14]. Light microscopy has several advantages, e.g. relatively easy, rapid image acquisition and images can be acquired in colour. LM is a good technique for inspecting wood pulp fibre structures. However, LM has a narrow depth of field (Fig. 2). The Extended depth of field is an ImageJ plugin for increasing the capabilities of a light microscope [20]. In addition to create a composite image (Fig. 2d), the Extended depth of field creates a topology image, which also may be applied to perform topography analysis of a given surface. The method is based on acquiring images at several depths and combining them into a single in-focus composite image [20].

Fig. 2. Light microscopy images of wood pulp fibres, acquired at 3 planes of focus (a-c). A composite in-focus image (d), which has been created by combining some 20 LM images acquired at increasing depth.

Serial sectioning for LM can be applied for 3D reconstruction and analysis [21]. However, LM has an effective resolution that might be considered low for assessing some fibre structural details. According to Nanko et al. [4], transmission electron microscopy (TEM) gives a detailed visualization of the fibrils on fibre surfaces and how they interact in fibre-fibre contacts. However, TEM is demanding and time-consuming. The introduction of SEM bridged a gap between LM and TEM, thus complementing structural analyses [22], as we will see in the next sections.

3.4 Scanning electron microscopy

Scanning electron microscopy (SEM) has been an important technique for assessment of fibre structures since its introduction [2]. SEM has been applied for exploring surface structures in secondary electron mode. In addition, backscattered electron imaging (BEI) mode has been most suitable for quantification of cross-sectional dimensions (Fig. 3). Cross-sectional analysis requires a careful sample preparation and distortion-free surfaces. Compared to microtoming [23], grinding and polishing [24] is capable of producing surfaces most suitable for fibre cross-sectional analyses in SEM-BEI mode [16]. In addition, techniques such as Br2 and KMnO4 staining has expanded the assessment of fibre structures [6]. Lignin-rich structures can thus be easily differentiated and quantified. SEM has thus been an important tool for revealing the effect of industrial processes on the morphology of wood pulp fibres [25].

Proper assessment of the morphology of a fibre population requires the quantification of a large number of cross-sectional fibres [16]. Such process may be tedious and time-consuming. However, computerized image analysis has advanced the automatic quantification of wood pulp fibres [15,16,26,27]. Fig. 3 presents a SEM cross-sectional image of Pinus radiata kraft pulp fibres, acquired in BEI-mode. The fibres were freeze-dried, embedded in epoxy resin, ground and polished [16]. The contrast between the fibres and the background is caused by the difference in average atomic number between the organic cellulose fibres and the embedding epoxy resin [28]. The inset in the middle exemplifies a binary version of a local region. Note the fibre no. 3, where the fibre wall has been converted into a distance map. Distance transforms convert a binary image into a greylevel image, where all pixels have a value corresponding to the distance to the nearest feature pixel [29]. Distance maps have thus been applied for quantification of fibre wall thickness and fibre wall thickness variation [16]. In addition to cross-sectional dimensions, some shape statistics of the binary cross-sections (fibres 1 to 5) can be quantified with ImageJ and the Shape descriptor plugin, as exemplified in Table 2.

Fig. 3. Scanning electron microscopy of kraft pulp fibres. Inset corresponds to a binarized region. Fibre number 3 exemplifies the distance map applied to quantify the fibre wall thickness. The numbers indicate the fibres assessed in Table 2.
Table 2  Some cross-sectional dimensions and shapes of the fibres indicated in Fig. 3.

<table>
<thead>
<tr>
<th>Fibre no.</th>
<th>Area ($\mu$m²)</th>
<th>Form factor</th>
<th>Feret ($\mu$m)</th>
<th>Solidity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area without lumen</td>
<td>$\frac{4\pi Area}{perimeter^2}$</td>
<td>Longest axis</td>
<td>Area Convex area</td>
</tr>
<tr>
<td>1</td>
<td>186</td>
<td>0.3</td>
<td>32.4</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>0.1</td>
<td>34.1</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>0.4</td>
<td>26.4</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>169</td>
<td>0.3</td>
<td>23.3</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>196</td>
<td>0.5</td>
<td>27.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

SEM has been proposed for acquiring 3D images of fibre and paper structures [30,31]. The method is time-consuming, however, images with sub-micrometre resolution can be obtained. Due to the serial sectioning and the constant removal of the specimen from the vacuum chamber, the consecutive images may not be aligned properly for 3D reconstruction. In this respect procedures for alignment are most useful. For easing the alignment of serial sections, Ugelstad beads [32] with a known size have been embedded with the samples [31]. Such beads can be used as fiducial marks for improving the alignment process (Fig. 4).

ImageJ has several options for alignment of pair of images. The TurboReg and StackReg plugins are available [33]. The plugins perform five types of alignments, i.e. Translation, Rigid body, Scaled rotation, Affine and Bilinear. The alignment can be performed manually and automatically. Fig. 4 shows two consecutive cross-sectional images of a fibre. The images were acquired in low-vacuum mode [31]. A fibre and some Ugelstad beads (d=10 $\mu$m) are observed in the images. In this case an Affine alignment is selected. Three markers, which appear on the pair of images, are applied for selecting the appropriate fiducial marks (Fig 4A and 4B). In addition, the TurboReg plugin has an automatic option, which may be suitable for large stacks of serial sections. However, care should be taken as the serial sections may be aligned wrongly (Fig. 5). This is especially the case with fibres where the structure is not straight, but curled. In this respect, beads used as fiducial marks are suitable for verifying the adequacy of the alignment procedure. Such Ugelstad beads may be useful even with modern automatic systems for in-situ serial sectioning [34].

![Fig. 4. Alignment of consecutive serial sections. Two consecutive sections (a,b) and the aligned section c).](image)

![Fig. 5. Alignment of several serial sections. a) Original images. b) Automatic aligned images. c) Manual aligned images. Note that the automatic option (b) aligns the sections with respect to the fibre, which is in this case wrong.](image)
Irrespective of the image acquisition device, digital images may contain random noise. Such noise may obscure the structures to be quantified. There are several methods for removing noise, e.g. smooth and Gaussian blur filters. These filters are low-pass filters, which reduce the sharp transition from a fibre wall edge to the background. Low-pass filters may thus be a source of error during the quantification of fibre dimensions. There are some alternative ImageJ plugins for edge-preserving filtering. Some of the filters are e.g. Anisotropic diffusion, Kuwahara, A trous and SUSAN. Fig. 6 gives an overview of the effect that some filters have on the representation of a fibre cross-section.

![Fig. 6. Some edge-preserving filters. a) Original images. b) Median filter. c) Anisotropic diffusion filter. d) Kuwahara filter. e) A trous filter. f) SUSAN filter. Note the differences on the fibre edges and on the “+” sign. The images have been modified to enhance the contrast. The corresponding grey level histograms are given in the lower row.](image)

It is worth to notice the capability of some of the filters to remove noise while the edges are preserved. This is especially the case with the SUSAN filter, which yields an image with a smooth grey level representation within each local structure and preserves the edges. The potential of the filter is also exemplified in the lower-right Ugelsstad bead, where the “+”-sign is completely preserved. A minor disadvantage with the tested filters is that several parameters must be adjusted to find the most suitable settings for filtering. Doing this manually may be tedious and time consuming for inexperienced users. In this respect, one of the major advantages of the ImageJ program is most useful, i.e. the capability of writing simple, yet effective macros. The macro applied to find adequate settings for filtering is exemplified in Fig. 7. The macro filters a given image with several settings and save the images into a given directory. Once the images have been filtered, the user can import the images into a stack and select the settings that are suitable for the required purposes.

![Fig. 7. Macro for finding the best settings applied to filter the images presented in Fig. 6.](image)

SEM has proved to be a suitable technique for the assessment of several fibre sub-micrometre structural details. SEM combined with adequate preparation and image analysis techniques have provided information that has been unattainable by other conventional methods. In addition, during the last years we have experienced major advances in the development of modern electron microscopy techniques. Field-Emission SEM is a clear example, which expands the potential of a SEM for revealing structures at the nano-scale.

3.5 Field-emission scanning electron microscopy

FE-SEM offers a detailed description of the fibre structures at the nano-level. The FE-SEM has thus been a valuable tool for increasing our understanding of fibre structures and their nano-components. Being applied for surface analysis, FE-SEM has revealed the fibre wall structure effectively. However, proper assessment of the fibre wall structure requires adequate preparation techniques. There are several methods that have been proposed for preparation of fibres, e.g. freeze-drying, cryofixation and critical point drying. According to de Silveira et al. [35], freeze-drying may cause a
certain degree of fibre shrinkage. Duchesne and Daniel [10] also mentioned some macrofibrillar shrinkage effects on the fibre surface structure due to the freeze-drying and critical point drying methods. Irrespective of the presumptive limitations, the freeze-drying method is relatively simple to perform and has proved to be a suitable technique for preparation of fibres for surface analysis [12].

Some pulp fibres of *Pinus radiata* were freeze dried as described by Reme et al. [16]. The pulp fibres were gently placed on an aluminium plate and coated with a layer of gold. The gold sputtering time was 45 sec in an Agar auto sputter coater. The microscope was a Zeiss Ultra FE-SEM, operated in the secondary electron (SE) mode. The working distance, magnification and acceleration voltage were 5 mm, 5000x and 5 kV, respectively. The surface structure of a *Pinus radiata* fibre is exemplified in Fig. 8. Note the detailed visualization of the fibre surface, which reveals several characteristic details of pulp fibres, i.e. wrinkles of the fibre wall, the fibrils on the fibre surface and the organized fibrils forming the surface of the two pits in a circular arrangement.

![Fig. 8](image)
a) FE-SEM of wood pulp fibre surfaces. b) Ellipses representing the local orientations and anisotropies have been superimposed on the images.

In addition to offering high-resolution, the versatility of a FE-SEM is a major advantage. Images can quickly be visualized at low (e.g. 50x) and high magnifications (e.g. 100000x), giving a unique opportunity to explore fibre surfaces rapidly and effectively. However, the potential of FE-SEM is not exploited totally without having appropriate image analysis routines for structural quantification. Chinga-Carrasco et al. [12] proposed a gradient analysis for quantification of the fibre surface texture. Roughly, the texture may be affected by the wrinkles, which are typical of chemical pulp samples, and by the orientation of fibrils, which are exposed at the different layers of the fibre wall. A gradient analysis yields the orientation and orientation anisotropy of a given local area. Such surface descriptors may be applied for quantifying the effects of a given treatment on the morphology of the fibres.

The SurfCharJ plugin [36] was implemented for quantification of surface topography. In addition to quantifying roughness values, the SurfCharJ plugin also performs a gradient analysis. Local orientations and anisotropies can be estimated. The local orientations represent the orientations of the local facets of a given feature. The orientation of the feature is thus 90 degrees relative to the orientation of the generated polar plot. Fig. 8a shows a high-resolution image of a fibre surface with two pits. Local ellipses have been generated and superimposed on the image by applying a combination of the SurfCharJ, Stack Maker and Analyze particles plugins (Fig. 8b). Note how the polar plots follow the local feature orientations. The higher the aspect ratio of the local polar plots, the higher the degree of orientation in a given direction. The image exemplifies the potential of gradient analysis for estimation of local orientation and anisotropy (Fig. 8b). Such capabilities have also been implemented in a tailor-made plugin for automatic assessment of fibre surface orientation [12].

An image of a surface structure acquired at high magnification is presented in Fig. 9a. Details such as the well-organized network of fibrils covering the fibre surface can easily be visualized. Such structures correspond probably to rests of the primary wall of the fibre. The surface underneath the network of fibrils is presumptively the secondary wall layer (S1). In order to shed more light on the structure of the assessed cellulose fibres a cross-sectional analysis was performed. The fibres were embedded in LR white and sectioned with a diamond knife mounted in a microtome (Microm Zeiss Stemi SR HM 350, Microm GmbH, Sandhausen, Germany). The thickness of the cross-sections was 5 µm. The sections were collected with copper wire loops and placed on a clean slide. The samples were visualized in a Zeiss Ultra FE-SEM, applying the inlens capability. The working distance, magnification and acceleration voltage was < 1 mm, 12000x and 0.5 kV, respectively. Note that the layers of the fibre wall (S1, S2 and S3) are easily observed in the cross-section (Fig. 9b). Although not shown, the arrangement of fibrils (diameters < approximately 20 nm) is different in the various layers of the fibre wall. The S2 layer is the thickest layer and has fibrils oriented in a helical organization. Part of the fibril structure that may be encountered in S2 layers are visualized in a fracture area of a fibre (Fig. 9c).
During the last years major research activities have been performed for developing efficient procedures for fibrillating cellulose fibres into single fibrils. Since its introduction in the 80’s, several applications have been foreseen for the fibrillated material [1]. Presently, the fibrillated material may be manufactured with a wide range of sizes, from sub-micrometre structures to nanofibrils with sizes of roughly 3-100 nm (Fig. 10) [37-39].

The production of nanofibrils requires dedicated procedures for quality control. Model films are usually manufactured for testing the quality of the nanofibrillated material [39]. Films may be made from a suspension of nanofibrils by e.g. free-drying or on a supporting wire/filter paper. The image exemplified in Fig. 10 corresponds to a small surface of a model film. The image has been acquired by FE-SEM from an area without a conductive metallic layer. Proper visualization of non-conducting areas requires the in-lens capability of a modern FE-SEM. The images can be acquired applying a short working distance (<1 mm) and low acceleration voltage (<1 kV). The surface nanostructure may thus be visualized [40]. Note the pores, which are formed in the surface layers. It seems that the pores formed during drying of the nanofibril films are not continuous, but defined by several single layers in the film z-direction (Fig. 10b). It is worth to notice that the pore structures observed in Fig. 10 are not revealed when applying a metallic surface coating. Even AFM may be challenged by the particular pore structures of nanofibril film surfaces. In addition, several computerized image analysis methods have been proposed for characterisation of model film structures [40], which are valuable for quality control of a given material.

4. Final remarks

Wood pulp fibres are bio-degradable and are potentially an important raw material in a wide range of application areas. The utilization of cellulose fibres in a given application requires a comprehensive understanding of their properties and how they are affected by production variables. Modern microscopy provides valuable tools for detailed exploration of a given structure. However, the full potential of a given microscopy technique is not totally exploited without having adequate computerized image analysis methods for extraction of relevant data. Quantitative microscopy is a major advantage, as several structural characteristics details can be quantified efficiently and objectively.

Some selected microscopy techniques for assessing wood pulp fibres have been described. The techniques exemplify the advances with respect to visualization and quantification of a given structure. In addition, advances are expected from a microscopy and image analysis point of view. In the long run, such advances will provide a comprehensive understanding of fibre and fibril structures and thus contribute to the development of novel bio-based materials.

Acknowledgements. My sincere thanks to Prof. Torbjørn Helle (NTNU) for revising the original manuscript.

References


