3

Tools for the Characterization of Biomass at the Nanometer Scale

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3.1 Introduction

To take advantage of nanoscale features in plant cell walls and create our own nanostructures based on plant biomass, we must make reliable measurements at the nanoscale. Although nanoscale measurement methods have expanded in recent years, not all these techniques are useful for soft, hydrophilic, nonconducting biomass specimens. Here we discuss those methods with the potential to be particularly useful in studying nanoscale properties of plant biomass.

In contrast to most engineering materials, plant biomass structure changes with water availability. Water swells biomass, creating pores that transport enzymes and reagents into and out of the cell wall during processing. Therefore we begin with a description of basic interactions of water and biomass. Nanoscale accessibility and reactivity of the cell wall are often critical to bioprocessing, so we discuss several methods of evaluating these properties. This chapter also describes methods to measure cellulose crystallinity, because crystallinity affects properties and crystallites are an interesting material in themselves. Finally the chapter reviews microscopic and spectroscopic methods useful for the study of biomass at the nanoscale.

3.2 Water in Biomass

Water has a profound effect on the nanoscale structure of plant cell walls. Nanoscale pores in wet spruce wood, a representative biomass, commonly contain 0.3 g water.
per gram of biomass (1, 2). When dried normally, wood typically contains only ca. 2% void space (3), a typical value for amorphous polymers. Clearly water is acting as a plasticizer, forcing itself between the molecular chains of the polymer, causing swelling and softening. This ‘bound water’ acting as biomass plasticizer has higher density, lower freezing temperature, and lower vapor pressure than water at standard temperature and pressure (1). Other solvents swell wood also, but hydrogen bond donors are the most effective (4). Not all components of wood have the same moisture affinity: lignin, cellulose, and hemicellulose absorb 0.60, 0.92, and 1.56 times as much water, respectively, as an equal weight of dry wood (2), and no water enters the interior of cellulose crystals.

Fiber saturation point (FSP) is the point where biomass becomes saturated with bound water. Below FSP, any water removed must come from between polymer chains within the cell wall. This creates enormous surface tension forces, which collapse the hydrated layers. Compared to never-dried biomass, air-dried biomass has high density, nonporous cell walls, and modified molecular conformations.

Mechanical action and chemical treatments can change FSP. Breaking chemical crosslinks within the fiber makes it easier for water to push into the spaces between polymer chains and generally results in higher FSP. Because water cannot enter cellulose crystals, increasing cellulose crystallinity leads to lower FSP. Extractives and lignin tend to displace water in the cell wall, so removing these usually increases FSP. Hysteresis is also evident: the FSP of never-dried wood is 10% to 20% higher than after the first drying (1, 5).

3.3 Measurement of Specific Biomass Properties

3.3.1 Pore Structure and Accessibility

Processes for utilizing biomass often depend on mass transfer into and out of cell walls and the accessibility of cell wall components. Bioconversion of lignocellulose through enzymatic saccharification depends on enzyme accessibility to cellulose. Pores within cell walls provide a route for enzymes to access cellulose inside the cell wall. As a result, porosimetry, or determining pore size and structure, of biomass is important. This section reviews several techniques developed for this task.

3.3.1.1 Porosimetry by Solute Exclusion

The solute exclusion technique was initially created to determine the accessibility of macromolecules to fibrous substrates in water-swollen state (6). Later the technique was applied to wood and wood pulp (7–11). The technique is based on the accessibility of solute molecules (probe molecules) to the substrate pores of different sizes. The pore is considered accessible when the pore is connected to the bulk water and large enough to hold the probe molecule (Figure 3.1). Solute exclusion experiments can be conducted using the following procedure. A known volume and concentration of a probe molecule solution is added into a swollen substrate immersed in an excess of water. After thorough mixing, the probe molecule solution is diluted by the water contained in the initial substrate. If all pores are accessible to the probe molecule, then all water in the
Figure 3.1  Pore water in biomass substrate is inaccessible when the probe molecule cannot enter the pore.

Initial substrate will contribute to the dilution. The water present in the pores that is not accessible to the probe molecules will not contribute to dilution. As a result, the measured concentration of the probe molecule in the final substrate mixture depends on the pore size and volume distribution. Using a set of different solute (probe molecule) solutions with various molecule sizes, the substrate pore size and volume distribution can be determined by

\[ c_f = \frac{w}{w + w_{ac}}, \quad c_i = \frac{w}{w + (q - w_{in})} \cdot c_i \]  \hspace{1cm} (3.1)

where

- \( c_f \) and \( c_i \) are the final and initial probe concentrations,
- \( w \) is the weight of the probe molecule solution,
- \( w_{ac} \) and \( w_{in} \) are the weight of water accessible and inaccessible to the probe molecule in the wet pulp, respectively, and
- \( q \) is the weight of water in the wet pulp (\( w_{ac} = q - w_{in} \)).

Therefore, \( w_{in} \), the pore water per gram of biomass that is inaccessible to a probe molecule, is

\[ W_{in} = \frac{w_{in}}{p} = \frac{w + q}{p} \left[ 1 - \frac{w}{w + q} \times \frac{c_i}{c_f} \right] \]  \hspace{1cm} (3.2)

where \( p \) is the weight of dry biomass sampled.
Accurate concentration measurement is critical when using the solute exclusion technique. Probe molecule concentrations have been measured by interference refractometer (8), spectropolarimeter (12, 13), and HPLC using a refractive index detector (14).

The validity and accuracy of the solute exclusion technique for pore size and volume distribution measurements are based on two assumptions: (1) the concentration of a probe molecule in the accessible pores is the same as in bulk solution surrounding the pulp specimen, and (2) probe molecules can fully penetrate the pore to get full access to the pore water. To meet these two assumptions, the probe molecules should not adsorb on nor chemically react with the substrate. They should be spherical and must be available in a large variety of highly monodisperse molecular sizes. Cross-linked dextran (10) and poly(ethylene glycol)s (11) were originally proposed. Both of these probe types have seen continued use (12–15). Gel permeation chromatography indicated that complete penetration of a pore is not possible (16). The concentration of the probe molecule in pores is shown both theoretically (17, 18) and experimentally (19–21) to vary with pore shape and relative size of the pore and probe molecule. Probe molecules with low hydrogen bonding capability have limited potential to access water in pores, and electrostatic charge on the probe can effect the ability to penetrate pores (22).

Other problems with determining pore size and volume distribution with the solute exclusion technique are the ‘ink-bottle’ effect and osmotic pressure (23) (Figure 3.1). Probe molecules can be excluded from the water in pores with a narrow opening but wider space inside the pore. If the pore substrate contains ionized groups, even nonionic solutes can be excluded from pores by osmotic pressure. In this case, using molecules that interact and adsorb on fibers has been suggested as they can be forced to enter pores (24). Cell walls appear to have a lamellar structure (25), and the simple slit model for cell wall pores is a reasonable assumption (8, 10).

In view of the discussion above, we should emphasize the terms ‘effective pore size’ and ‘accessible water’ when measured by the solute exclusion technique. The solute exclusion technique is a valuable tool for determining the total pore volume accessible to a probe molecule of given size. It can also quantify relative changes in the porous structure from various treatments and between different substrates. Solute exclusion is not an acceptable tool for the determination of absolute pore size and volume distribution. In this sense this technique fits the needs for practical applications in bionanosensing, such as determining enzyme accessibility to substrates and comparing the effectiveness of various mechanical and chemical pretreatment processes and substrates for lignocellulosic biocconversion.

Despite the shortcoming of the solute exclusion technique, it is very useful for understanding the effects of molecular size on accessibility (26, 27). In addition, a very useful pore structure model is based on the results of the method (10).

### 3.3.1.2 Porosimetry by Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) technique for pore size distribution measurements is based on the principle that water contained inside pores has a lower freezing point than that of bulk water. This technique, called thermporosimetry, was initially developed for measuring pore size distribution in other materials (28–30) but has been
successfully applied to pulp fibers (31, 32). The relations between the specific melting enthalpy and pore size are described by the Gibbs-Thompson equation:

\[
    r = \frac{-V_m\sigma_{ls}}{\Delta H_m \ln \frac{T}{T_0}} \tag{3.3}
\]

where

- \( r \) is the radius of cylindrical pore,
- \( V_m \) is the specific molar volume of ice,
- \( \Delta H_m \) is the specific melting enthalpy of water,
- \( \sigma_{ls} \) is the surface energy at the ice-water interface,
- \( T_0 \) is the melting temperature of water at normal pressure, and
- \( T \) is the melting temperature corresponding to \( \Delta H_m \).

The pore volume \( V_r \) corresponding to the pore size at a measured melting enthalpy is the volume occupied by the frozen water melted in DSC measurements:

\[
    V_r = \left( \frac{\Delta H_I}{\Delta H_m \cdot \rho_w} \right) \tag{3.4}
\]

where \( \Delta H_I \) is the energy absorbed when the ice in a frozen specimen is completely melted (the area under dynamic endothermic curve obtained from DSC measurement) and \( \rho_w \) is the water density at the melting temperature.

One problem with thermoporosimetry is the hysteresis observed when the freezing and melting cycle is repeated several times. The following thermal sequence has been developed to minimize hysteresis: cooling to \(-45^\circ C\), heating at \(5^\circ C/min\) to \(-30^\circ C\) and holding for 5 min, then cooling to \(-45^\circ C\) (31). The cycle was repeated with the isothermal melting set point at \(-8, -5, -3, -2, -1, -0.6, -0.4, -0.2, -0.1\), and \(0^\circ C\) for the calibration specimen. The final temperature scan was a dynamic measurement from \(-30^\circ C\) to \(+15^\circ C\) used to determine \( \Delta H_l \). The specimen is entirely frozen and melted only twice in this sequence. Other thermoporosimetry issues to keep in mind are that osmotic pressure can also cause melting point depression and that the pore size distribution may change with freezing or with raising temperature to where enzyme accessibility to lignocellulose substrate is of great interest. Furthermore, some enclosed pores contribute to the measured pore volume but cannot be accessed by enzymes (33).

### 3.3.1.3 Porosimetry by NMR

Water inside the cell wall has a much slower diffusion rate than free water. In nuclear magnetic resonance (NMR) measurements, this diffusion rate influences the T2 relaxation time. Therefore, NMR T2 relaxation time is useful for quantifying the amount of water in different environments such as the cell wall and cell lumen. This technique has been used to characterize pore water in various cellulosic fibers (34–36), native wood (37), pretreated corn stover (38), enzymatically hydrolyzed cellulose (39), and model plant cell walls (40).
3.3.1.4 Porosimetry by Gas Absorption

The gas absorption technique for pore measurement is well established and sound. It requires dry specimens so is useful for dry biomass applications but has limited utility for understanding wet processing such as enzymatic hydrolysis of lignocellulose in biorefining. The technique is based on the Brunauer-Emmett-Teller (BET) equation (41), where the volume of gas absorbed in a monolayer, \( v_m \), is related to the ratio of the equilibrium (\( P \)) and saturation (\( P_0 \)) pressures and the total gas volume absorbed, \( v \):

\[
\frac{1}{v[(P_0/P) - 1]} = \frac{c - 1}{v_m c} + \frac{P}{P_0} + \frac{1}{v_m c}
\]  

(3.5)

Therefore \( v_m \) and \( c \) (BET constant) can be obtained from fitting Equation (3.5) to the experimental data of \( P/P_0 \) and \( v \). The specific surface is then evaluated using

\[
S_{\text{BET, total}} = \frac{(v_m N s)}{V}
\]  

(3.6)

where \( N \) is Avogadro’s constant, \( s \) is adsorption cross section (area per adsorbed molecule), and \( V \) is molar volume of the adsorption gas. Water sorption on cellulose powder was studied by comparing the adsorption of water and nitrogen with BET theory (42).

3.3.1.5 Enzymatic Hydrolysis Rate

The rate of enzymatic degradation of cellulose is especially important for those attempting to use biomass-derived glucose for fermentation. This assay typically applies a standard cellulase to biomass and measures the rate of cellulose depolymerization. Reducing aldehydes produced by the depolymerization are commonly detected by color reactions with reagents such as Cu(II) or ferrocyanide. For further discussion, we suggest a recent review (43) because there are too many variations on this method to discuss here. Access of the enzymes to the substrate is critical to hydrolysis, and this accessibility can be elegantly assayed (43). Caution should be used when comparing cellulase activities because the size of the substrate, in addition to porosity, can influence observed cellulase activity when diffusion is limiting (27).

3.3.2 Cellulose Crystallinity

Cellulose crystallinity is often measured because cellulose crystals (crystallites, whiskers) have a slow degradation rate, high strength, high aspect ratio, and ability to form chiral nematic liquid crystals. Assessing the mass fraction and size of crystals is commonly accomplished by NMR or X-ray diffraction, but Raman and infrared (IR) methods can also be used. Crystal size can be increased by allowing structures to relax at high temperature and humidity, such as during kiln drying of wood, and decreased by mechanical damage, such as ball milling or swelling. Although we discuss cellulose crystals, a more proper term might be ‘ordered domains’ because they do not technically meet all the requirements of a perfect crystal. Not only is there a large number of defects in the directions perpendicular to the cellulose chain, but there appears to be a slow twist to
the entire structure. The structure of cellulose, the various crystalline forms, and the issue of crystallinity are discussed in Chapter 6.

Spectroscopic methods of measuring cellulose crystallinity (NMR, Raman, IR) are typically used with pure cellulose samples because hemicellulose and amorphous cellulose produce very similar signals, whereas lignin is much different. The X-ray diffraction method, by contrast, can be used on native biomass samples because the lignin, hemicellulose, and amorphous cellulose all contribute to a broad background peak, whereas the cellulose crystals produce a sharper peak.

3.3.2.1 X-ray

Cellulose crystallinity index (CrI), or percentage by mass of crystalline cellulose in a sample, is determined by diffracting or reflecting Cu Kα X-rays off of randomly oriented, relatively pure cellulose specimens. The original method is still widely used. In this method, the peak intensity of the 002 peak ($I_{002}$, $2\Theta = 22.6^\circ$) diffracted from the crystalline cellulose is compared with the intensity of the reflection from amorphous cellulose, ($I_{amorph}$), which has a very broad peak at $18^\circ$ (44).

$$CrI = \frac{I_{002} - I_{amorph}}{I_{002}} \times 100 \quad (3.7)$$

Segal (44) also discusses the effect of specimen packing, density, orientation, and other factors that must be controlled to get reproducible results.

Crystal width can be calculated using the Scherrer equation on the peak assigned to (002) planes:

$$D = K\lambda(B \cos \Theta)$$

where $D$ is crystal thickness, $\lambda$ the radiation wavelength, $\Theta$ the diffraction angle, and $B$ the full width of the diffraction peak measured at half-maximum height. The correction factor, $K$, is typically set at 0.9 (45).

Because of the small crystal size and subsequent broad peaks, numerous efforts have been made to improve quantification. These range from simply taking areas rather than heights for the crystalline and amorphous reflections (46) to modeling the entire diffraction pattern (47). If treatments partially dissolve cellulose, then different crystal forms, such as cellulose II, and their reflections are likely to appear upon solvent removal. The exact peak positions are reported to vary by more than 0.5°.

3.3.2.2 $^{13}$C NMR

Solid-state $^{13}$C NMR is often used to assay the amount of cellulose in crystalline vs. amorphous form. The anhydroglucose carbon 4 (C4) peak is shifted 89 ppm when inside the crystal and 86 ppm when on the crystal surface or in amorphous cellulose and hemicellulose (45, 48). In highly crystalline specimens, the C6 peak at 65 ppm can be compared with the amorphous peak at 60–62 ppm. Band assignments have been made for all the components (49), and the effect of some factors such as residual hemicellulose and crystal polymorph ($I_\alpha$ vs. $I_\beta$) on the observed crystallinity has been described (50, 51).
3.3.2.3 Raman and Infrared Spectroscopy

Raman and IR spectroscopy have both been suggested as ways to determine the ratio of amorphous to crystalline cellulose I. In Raman, a linear correlation has been observed between X-ray crystallinity and $I_{1481}/(I_{1481} + I_{1462})$, where the peaks at 1481 cm$^{-1}$ and 1462 cm$^{-1}$ are assigned to crystalline and amorphous cellulose, respectively (52). Using IR, the height or area of the crystalline band at 1280 cm$^{-1}$ is compared with the relatively constant band at 1200 cm$^{-1}$ (53). In both methods the intensity of the bands need to be determined through mathematical deconvolution of the spectra because of overlap. The correspondence of Raman-derived values and X-ray crystallinity values is better when the Raman crystallinity is calibrated on a contiguous set of cellulose samples rather than cellulose from widely varying sources. Also, there is some indication that the Raman method is not sensitive above crystallinity index of 75. The FTIR method does not account for any potential changes in the ratio of cellulose $I_\alpha$ to $I_\beta$ but otherwise is a reasonable method for routine characterization.

3.4 Microscopy and Spectroscopy

So far we have described a variety of techniques for measuring specific attributes of biomass. The remainder of this chapter will give an overview of microscopic and spectroscopic techniques useful in generating images and chemical information, respectively, from biomass samples.

3.4.1 Specimen Preparation

Biomass has several characteristics that can make it difficult to analyze. We have already discussed water interactions and how the methods of freezing or removing water from biomass can alter nanostructure. The modest pyrolysis temperature of 300$^\circ$C (54) for cellulose, as well as long experience with organic substrate damage by electrons and x-rays shows that biomass constituents are susceptible to change during observation by high energy probes. While the aromatic nature of lignin distinguishes it from carbohydrates, cellulose and hemicellulose are chemically very similar and so are difficult to differentiate.

The obvious approach to analyzing biomass structure by selectively removing particular components has serious problems. Removing one polymer from a plant cell wall almost always causes chemical and physical changes in the polymer removed, as well as the residual material. For example, the relatively gentle acid chlorite delignification procedure only removes about half of lignin before damage to hemicellulose becomes apparent (55). Amorphous cellulose and hemicellulose are so chemically similar that it is very difficult to dissolve one without disturbing the natural state of the other. In addition, chemical crosslinks (lignin-carbohydrate complexes, or LCC) between lignin and hemicellulose are well documented and hinder the removal of hemicellulose (56). These chemical considerations, as well as the interpenetrating nature of the cellulose, hemicellulose, and lignin polymer networks, makes it very difficult to analyze the native structure of any one of the wood components by itself.
Many techniques require preparing dry, flat or thin specimens for characterization. Preparing these without introducing artifacts is a challenge because biomass is naturally wet and fibrous. Confirmation from multiple independent techniques is often necessary before one can be confident that the observation is not an artifact of specimen preparation. We suggest an excellent review of microscopic techniques used to probe cell wall structure (57).

### 3.4.1.1 Drying Issues

Although drying invariably changes biomass structure, understanding the nature of those changes can minimize the impact and guide interpretation of results. Analysis of biomass in the wet state is usually preferred, but some analytical techniques require high vacuum. Even the simple task of getting the dry weight of a specimen can ruin the specimen for other analyses, as the act of drying causes irreversible changes in cell wall nanostructure.

Several methods have been used to preserve structures during water removal, and many books are devoted to the techniques, as biological specimens for electron microscopy have always faced this problem (58, 59). Two principal mechanisms cause specimen alteration during drying: changing solvent properties and surface tension. Although surface tension can be avoided, biomass structures surrounded by air or a different solvent have a different energy, and so have different stability, than in water. A stable molecular conformation in water could be unstable after water removal. Therefore, even the best water removal methods have the potential to modify specimens.

The simplest drying strategy is simply to let water evaporate, for example in an oven at 105°C. This is an easy method but causes the pores to collapse from surface tension. To avoid this, specimens are either frozen while wet or the solvent is exchanged. Solvent exchange is often followed by critical point drying or embedding.

In freeze drying, specimens are frozen while wet and placed under vacuum so that ice sublimes, completely avoiding surface tension. Standard freeze-drying techniques cause ice crystal formation that partially dehydrates the specimen, causing some fiber shrinkage, collapse, and artifacts from crystal formation (60). Because of the interaction with wood polymers, the freezing point of some bound water is as low as $-30^\circ$C (31), confirming empirical observations that specimens must be kept extremely cold throughout the entire freeze-drying process. Partial freeze drying is also common, especially in electron microscopies with a cold stage. In this technique, some surface water is allowed to sublime by heating the specimen to ca. $-80^\circ$C, and then the specimen is cooled again to prevent further water loss during imaging.

Water can also be removed by deep freezing followed by flooding the specimen with dry acetone or ethanol (59, 61). This is often followed by embedding procedures, carried out at low temperature up to the point of polymerizing the embedding medium.

Under proper conditions, damage by ice crystal formation can be avoided by producing vitreous ice (ice without crystals). Vitreous ice is formed when cooling rates approach $10^6 \, ^\circ$C/s, and the water is diluted with 10–15% of a solute such as sucrose (62, 63). Samples kept at high pressure during freezing can have vitreous layers ca. 10 times thicker than samples frozen at atmospheric pressure (200+ vs. 20 µm) (62). Fast freezing is achieved by placing thin specimens in contact with liquids or plates cooled.
by liquid nitrogen (59, 61, 62). While this kind of super-fast cooling is essential for live cells, this method is rarely used when preparing biomass samples, even though there is evidence that slow or normal freezing changes pore structure (31).

Another approach to avoiding liquid:vapor surface tension during drying is to use critical point drying (CPD). In this method, water is replaced by a transitional solvent (ethanol or acetone), and then CO₂. When CO₂ is pressurized to the critical point, there is no surface tension between the liquid and gas phases, allowing evaporation of all the liquid with no surface tension (64). If proteins and lipids are present, fixation before CPD is suggested.

The primary problem with either CPD or low-temperature embedding is the need to change solvent. Besides the obvious problem of dissolving cellular components, changing solvents changes the stability of solvated structures, resulting in bulk swelling and shrinking and unknown changes to nanostructures. The extent of biomass swelling in a few typical solvents is given in Table 3.1.

Exposing wood to a solvent may change some aspects of the nanostructure, but it can also be useful in revealing nanoscale features. Removing extractives (resins, waxes, gums, fats) by acetone or ethanol, or ethanol/toluene is common as these extractives can interfere with chemical analysis. Tokareva showed how various specimen preparation procedures, including extraction by acetone or critical point drying, improved their ability to see fine structure such as cellulose macrofibrils (66).

### 3.4.1.2 Microtoming

Preparing 20- to 30-nm cross sections of homogeneous materials with a microtome is technically challenging, but the heterogeneity and fibrous nature of biomass makes producing these specimens from biomass even more difficult. The microtome knife is really initiating a crack that will grow along the path of least resistance. Biomass is difficult to microtome without distortion because fibrous structures and very nonuniform mechanical properties of different cell wall components redirect the progress of the crack.

Distortions introduced by microtoming have been systematically examined (67), and means to minimize distortion with diamond and glass knives have been reported (68, 69). H. Sitte has extensively studied microtomy and directed the design of commercial instruments and has thoroughly reviewed microtomy practice (70).

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### Table 3.1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Liquid-holding capacity of α-Cellulose (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Liquid-holding capacity of sulfite pulp (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tangential swelling of spruce wood (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>85.0</td>
<td>403.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>60.5</td>
<td>253.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>41.7</td>
<td>109.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Acetone</td>
<td>13.4</td>
<td>47.6</td>
<td>5.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>α-Cellulose is pure cellulose.

<sup>b</sup>Sulfite pulp contains some hemicellulose and lignin residues; cellulose is less crystalline than α-Cellulose.

<sup>c</sup>Tangential swelling is relative to oven dry.
3.4.1.3 Focused Ion Beam Cutting

Focused ion beams (FIBs) are often used in the semiconductor electronics industry, but the process may be especially useful for cutting specimens with phases of very different hardness, which is a particular problem when preparing plant cell walls. Typically FIB cuts specimens with a 100-nm wide-beam of 30-keV gallium ions. In the usual process, stair-stepped depressions are carved into the specimen with a rastered ion beam on either side of a thin (~100 nm) section to be studied. Finally, the edges of the thin section are cut free from the specimen, which is recovered and mounted on a transmission electron microscopy (TEM) grid (71–73).

While cutting the specimen, these ions also cause a number of artifacts from ion implantation and heat (74). Specific results depend upon material composition and ion energy, but the ion implantation depth is usually about 20 nm and atom displacements are confined to about 30 nm for gallium ions in low atomic number materials. The depth of the damage layer is strongly dependent upon ion accelerating voltage and the incident ion milling angle (75). Because almost all the ion kinetic energy is converted to heat (76), organic and polymeric materials can experience temperatures up to 500 °C at the etching site. However, FIB cutting does not necessarily overheat specimens; vitreously frozen water has been prepared without heat-induced ice crystal formation (77). New developments in cluster ions, discussed later under ‘Secondary Ion Mass Spectrometry,’ promise to produce even less damage in biomass specimens.

Most published examples of FIB involve inorganic semiconductor materials (78, 79) or inorganic composites (80), but use with soft materials is also possible. Human hair and housefly eye (81) represent biological specimens. Photographic film (79) and a toner particle (82) have also been successfully sectioned. The toner particle is an important example because contrary to microtomy sections, filler within the toner was not damaged or displaced in FIB sections. FIB is also used to decompose gases such as W(CO)₆ to tungsten metal at the specimen surface for protection, to minimize charge accumulation, or to weld a section to a micromanipulator.

3.4.2 Scanning Probe Microscopies

Scanning probe microscopy (SPM) techniques, especially tapping mode atomic force microscopy (AFM), are the most frequently used techniques for characterizing nanometer-scale structures. These techniques move a probe along the specimen surface to determine topography, material properties, or chemical structure. SPM techniques are reported in well over 10,000 research papers each year, but the results must be interpreted with caution. Like all microscopy techniques, with enough images it is possible to find exactly what is of interest, even if it is an artifact of specimen preparation or not representative.

The early use of AFM by Hanley and Gray et al. to describe wood cell structure illustrates some of the specimen preparation problems and limitations (83, 84). Cell structures were subjected to physical and chemical treatment in the preparation process. Although such methods are commonly used, whether the resulting specimen is an accurate representation of native plant morphology is questionable. The AFM images in the paper illustrate a problem common to all SPM: the observed surface is a convolution of the actual surface and the shape of the probe tip.
Interpretation of high-resolution SPM images requires a thorough understanding of probe/specimen interactions. Quantitative modeling of the contrast mechanism is encouraged. The use of other microscopy techniques along with SPM is beneficial in two ways: (1) examination at larger scale will establish a context for high-resolution studies and help to select representative fields and (2) other imaging methods create different types of artifacts and so can be used to confirm observations.

Recent reviews of scanning probe microscopies abound (85, 86). One excellent and comprehensive review by an SPM pioneer offers a good place to begin learning of the promise and pitfalls of SPM (87). This review begins with scanning tunneling microscopy (STM), follows its evolution, and concludes with the measurement of mechanical properties utilizing nanoindentation methods.

3.4.2.1 Atomic Force Microscopy

Atomic force microscopy (AFM) is easy to use and is the most frequently used SPM method for describing molecular solids or biological specimens. In its original mode, contact AFM, the stylus tip was maintained in contact (or near contact) with the specimen as in a miniature profilometer. To minimize specimen damage, most current work uses tapping mode, where the stylus and supporting cantilever are set into vibration near their resonant bending frequency (nominally ~100 kHz).

In tapping mode, the AFM tip makes only intermittent contact with the specimen surface, but the tip/specimen interactions alter the amplitude, resonance frequency, and phase angle of the oscillating cantilever. Amplitude modulation mode (AM-AFM) is an excellent mode for specimens in air or liquids. In AM-AFM, the oscillation frequency of the tip is kept constant, while the amplitude of vibration reveals topography and the phase shift between driving force and oscillation reveals interaction forces dependent on specimen viscoelastic properties (primarily stiffness) and adhesion between the tip and specimen. The phase information in AFM has been used to measure material properties of specimens such as elastic modulus, hardness, and material boundaries (88). A good understanding of the mechanisms involved is important because the interpretation of phase information is not always clear (87, 89). An exhaustive review of dynamic AFM including theory and operation is available (90).

Interactions between the AFM tip and specimen arise from many different mechanisms, including van der Waals attraction, electrostatic, friction, viscoelastic, and wetting forces. Which of these forces are dominant is not always clear. One frequent effect that is not always anticipated is the condensation of water on the specimen surface about the tip. This results in a capillary force large enough to dominate the probe/specimen interaction. Purging the sample chamber with nitrogen gas diminishes this condensation effect enough to image most samples but does not eliminate it. The most frequent practice for high-resolution studies is to work in ultrahigh vacuum (usual for atomic solids) or under fluids (usual for biological specimens). Static charges on tip or specimen can be avoided by placing an ionization source (an alpha particle emitter, for example) near the specimen.

A review of the 20-year history of developments in atomic force microscopy along with the physics involved is recommended (91). This work also views scanning tunneling microscopy, subatomic imaging, atom manipulation, and future projections. An excellent
guide to AFM for organic and biological materials has been written by pioneers in this field (92). This book was written for biologists but contains detailed practical information useful to chemists and material scientists working with soft and molecular materials.

A few examples of AFM studies of nanostructure in biomass are given here. Two related papers treat the subject of specimen roughness and its effect on pull-off force and the use of soft colloidal probes to minimize the effect of roughness on force measurement (93, 94). The van der Waals forces between regenerated cellulose surfaces in an aqueous environment with low pH and high ionic strength to suppress charge effects is described by Notley (95), while Zauscher studied cellulose surface interactions in various electrolyte concentrations (96).

A critical comparison of the AM (amplitude modulation) tapping mode and the FM (frequency modulation) noncontact tapping mode for imaging soft matter is available (97). In the FM mode, the tip exerts a very gentle force on soft materials and provides height measurement close to the true value. In the AM mode, the tip typically exerts a stronger force on soft materials and causes their deformation, especially in the liquid environment.

### 3.4.2.2 Force Spectroscopy

In addition to the topographic image, force spectroscopy can provide chemical information about a specimen from the forces that occur as the AFM probe approaches and retracts from the surface (98, 99). Leite and Herrmann provide an introduction and extensive review of this subject with a particular emphasis on adhesion phenomena (100). Force curves can be recorded at many locations on the specimen surface. These experiments can measure attractive and adhesion forces, the location and area of contact, and the mechanical properties (modulus and plasticity) of the specimen.

Chemical force microscopy (CFM) uses chemically modified AFM tips to expand the range of possible interactions. Once demonstrated (101), CFM has been applied to characterize cell surfaces (102–104) and intermolecular interactions of cellulose surfaces (96, 105). A thorough and readable introduction to CFM, along with many applications, is suggested (106).

Nanotribology, or nanoscale friction, can also be studied using SPM (107, 108). An in-depth review of friction force measurement applied mostly to atomic solids was prepared by Carpick and Salmeron (109). The nonvertical component of stylus motion is often attributed to friction or viscosity, but as Carpick points out, ‘If the sample surface is not flat, the surface normal force will have a component directed laterally and will result in contrast in the lateral force image.’ Despite these problems, valuable information on friction at cellulose surfaces has been obtained with AFM (105, 110, 111).

A novel AFM design called torsional harmonic cantilever (THC) allows the mapping of interaction forces and topology across a surface in a short time (112). Previous methods make time-consuming point-by-point measurements by approaching and retracting the tip. By placing the tip off-center on the cantilever, every time the tip taps the surface, the cantilever experiences a torque. The torsional oscillations are at a much higher frequency than the flexural resonance of the cantilever, allowing them to be analyzed separately to determine a force. As a demonstration of the capabilities of this approach, Sahin et al. measured the mechanical properties of blend of poly(methyl methacrylate)
and polystyrene as it was heated (112). These rigid polymers become rubbery and flexible above their glass transition temperatures. The different phases were readily imaged while the Young’s moduli of the components were measured. The loading forces are estimated at a modest 10 nN with a few nanometer indentation of the specimen.

3.4.2.3 Probe Shape

In all scanning probe microscopies, the shape and size of the probe tip is important because the SPM image is a convolution of the probe tip shape and the morphology of the specimen. The tip of the probe is often described as a hemisphere having a particular radius, though real probe tips are more complex and change by wear during the experiment. Therefore characterizing the tip is an important step when obtaining nanoscale morphological information (113).

Carbon nanotubes with diameters near 1 nm have been suggested as the ultimate high-resolution probe. These probes are currently very expensive and fragile, and the data can be difficult to analyze when the nanotube is not precisely perpendicular to the specimen surface (114). Multiwalled carbon nanotubes used as probes in tapping mode AFM are more rugged and therefore easier to use than the single-walled nanotubes, but data interpretation is more difficult because of the complex mechanics of multiwalled nanotubes (115).

3.4.2.4 Near-Field Scanning Optical Microscopy

The spatial resolution attainable with conventional optical techniques is limited to about half the wavelength of the light source used. For visible radiation, this results in a theoretical resolution limit of 200–300 nm. Higher resolution can be obtained with near-field scanning optical microscopy (NSOM) by illuminating a specimen through a small aperture (approximately 50 nm) positioned within one aperture diameter of the specimen. An extensive and detailed review of NSOM is recommended (116).

NSOM tips are typically used in tapping AFM mode by synchronizing the detection and tip vibration. Many examples involve illuminating specimens with NSOM and recording fluorescent emission from the specimen. Spectra from single molecules have been measured using this approach. One of the current limitations of NSOM is the high temperature (up to 500 °C) developed at the end of the scanning tip because most of the radiant energy is absorbed by the conductive coating that defines the aperture.

3.4.2.5 Nanoindentation

Although AFM has been used to measure material properties of specimens, such as elastic modulus, hardness, and the identification of phases, the method has serious limitations. More rigorous and quantitative material property measurements can be obtained at a sacrifice of speed and some spatial resolution using a nanoindenter (87, 117). With a nanoindenter, the force on the probe and the position of the probe are measured independently. This is not the case with an AFM probe, for which the force on the stylus point is determined by the deflection of the cantilever, which is related to the position relative to the specimen. The nanoindenter probe moves only vertically, whereas an AFM stylus is always tilted from the vertical and often twisted by lateral force.
However, nanoindenter probes are on the order of 50 nm in tip radius, compared with approximately 10 nm for an AFM. A review of the relevant contact mechanics has been published (118), emphasizing the assumptions underlying and restricting the application of most commonly used models and their implications for nanoscale force measurements.

Nanoindentation offers an excellent way to transition between the micron scale of optical microscopy and the nanometer scale of AFM. It has been used to quantitatively measure static (119, 120) and dynamic (121) mechanical properties on the nanometer scale. One of the more difficult aspects of nanoindentation experiments is preparing a relatively smooth surface free of artifacts.

The earliest applications of nanoindentation in biomass compared the hardness and Young’s modulus of spruce tracheid secondary walls (122) with the lignin-rich cell corner middle lamella (123). Since then, nanoindentation has been used to characterize cell wall development (124), the effect of anisotropy (125), microfibril angle (126), and pyrolysis (127) on mechanical properties. Nanoindentation has also been useful in understanding adhesive–wood interactions (128–130).

The original nanoindentation theory, which was used in the previously mentioned studies, was derived assuming the indentation of an isotropic elastic half-space. Almost all biological specimens will violate this assumption. For example, a 1-μm-diameter nanoindent placed on the transverse plane of a cell wall of wood will be in relatively close proximity to structural heterogeneities, such as the empty lumen or middle lamella, because the cell wall is typically only 5 μm wide. Previous researchers minimized this effect by filling the lumina with epoxy to support the cell walls during testing, but whether epoxy changes the properties of the cell walls is not certain. Methods to prepare wood specimens without any embedment and to account for structural compliances that result from nearby structural heterogeneities have since been developed (131, 132).

3.4.2.6 Scanning Thermal Microscopy

AFM and nanoindenter tips can also be used to measure thermal properties of materials. An electrically heated AFM probe with a tip radius of about 20 nm can be used to simultaneously heat and image the specimen surface. When the material below the tip reaches a phase transition, it softens and the probe penetrates the specimen. This provides the nanoscale equivalent of a bulk thermomechanical analysis experiment, where phase transition temperatures \( T_g \) or \( T_m \) are measured (133, 134). The thermal conductivity and surface temperature of the specimen can be measured as well. Nanothermal analysis has been used to investigate adhesive penetration and modification in wood cell walls (135).

3.4.3 Focused Beam Microscopies

Focused beam microscopies differ from SPM in that a beam of particles or rays, rather than solid objects, are scanned across the specimen. In most cases, the absorption or scattering of the incident beam is monitored, but particles or radiation generated by the specimen can also be analyzed. The major experimental artifact generated by focused beams is specimen damage. Scientists must be aware that the beams used almost always have enough energy to break chemical bonds in biological specimens, commonly resulting in mass loss and chemical modification.
3.4.3.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) provides high-resolution topographic information with little specimen preparation. SEM cannot differentiate carbon, nitrogen, and oxygen so provides little chemical information about biomass unless higher atomic weight elements are present in the specimen. In SEM, a narrow beam of electrons is scanned across a specimen while the intensity of reflected or ejected electrons provides the image. SEMs require a vacuum and some means to dissipate the charge of electrons that lodge in the specimen. Traditionally, charge was dissipated by coating specimens with conductive materials. Microscopes that tolerate higher pressure, called variable pressure or ‘atmospheric’ SEMs, were developed to image specimens in a more natural state, and have the added advantage that ionized gas surrounding the specimen often carries away accumulated charge. With a tiny aperture over the specimen to limit evaporation rate, some variable pressure SEMs can image wet specimens at room temperature.

Another option that allows SEM of wet biomass is the cold stage, which keeps the specimen very cold (down to $-120\,^\circ$C). At this temperature, ice-filled specimens can be imaged under high vacuum, and the temperature, pressure, and time on the stage can be adjusted to allow a controlled amount of freeze drying of the specimen surface. Additionally, specimens analyzed at cryogenic temperatures typically show less evidence of beam damage (136).

Biomass specimens may benefit from low voltage (0.5–5 keV) SEM operation (LVSEM), which often affords good image contrast on uncoated specimens and minimizes charging and damage (137). LVSEM can also be used to optimize the difference in secondary electron emission between polymeric materials of different composition (138). LVSEM images represent only a shallow surface layer because the penetration depth of these electrons is limited (137).

Energy dispersive X-ray analysis (EDX) uses X-rays stimulated by SEM electrons to make maps of elemental distribution. Resolution is typically a few microns, and sensitivity is far better for heavy atoms than for oxygen and carbon (137). Gases in the specimen chamber also scatter electrons, so variable pressure or ‘environmental’ EDX analyses almost always has more background than measurements at high vacuum.

3.4.3.2 Helium Ion Microscopy

A helium ion microscope is analogous to an SEM except that helium ions, rather than electrons, bombard the specimen. Helium ion microscopes provides a brighter imaging beam with better spatial resolution and different elemental sensitivity than SEM (139). Spatial resolution (approximately 0.5 nm) is partly the result of a much smaller beam/substrate interaction volume for helium than for electrons. Image contrast mechanisms are different than in SEM, which can provide new opportunities for image enhancement.

Although high-atomic-number ions (such as cesium ions) can damage specimens by sputtering, the low-atomic-number helium ions have low sputtering probability. As with an SEM, images can be generated from backscattered ions, whose probability of
backscattering is dependent upon atomic number. Helium ions can penetrate deeply into materials, effectively creating another contrast mechanism. To date no studies of biomass have been reported using a helium ion microscope.

### 3.4.3.3 X-ray Beam Probes

Scanning transmission X-ray microscopy (STXM) measures the absorption or deflection of an X-ray beam by a specimen. STXM uses a synchrotron X-ray source to generate up to 10-nm, 0.3-eV resolution images of specimens 100–150 nm thick. Though specimen degradation is still an issue, X-rays are less damaging than are the electrons used in transmission electron microscopy. Contrast is developed by selecting X-rays of different wavelengths. This technique has been used to describe the morphology of polymer composites (140). Developing contrast between cellulose and hemicellulose may be difficult, though a recent study of mixtures of ethylene–butene copolymer with ethylene–octene copolymer distinguished the separate phases of these polymers differing only in the length of the side chain (141).

X-ray photoelectron spectroscopy (XPS), originally known as ESCA, is a common technique for measuring the presence and oxidation state of atoms at the specimen surface. XPS uses a monochromatic X-ray to eject an electron in the specimen, whose kinetic energy is determined by the difference between X-ray energy and binding energy of the electron. Ejected electrons rarely escape from more than 10 nm below the surface. XPS is an ultrahigh-vacuum technique that typically probes approximately a square centimeter of surface but can have up to 20 nm resolution when coupled to a focused X-ray source, such as a synchrotron (142). This method is sensitive enough to determine the oxidation state of carbon, so it can distinguish lignin from carbohydrate, and has been applied extensively in probing chemical modification of surfaces. XPS data should be interpreted cautiously because the escape depth, and therefore sensitivity, may change with chemical composition of the surface, and the incident X-rays and ejected electrons may cause chemical reactions in the organic substrate. Also, localized areas can become charged and therefore eject electrons with different energy than the rest of the specimen.

### 3.4.3.4 Secondary Ion Mass Spectrometry

Secondary ion mass spectrometry (SIMS) produces good chemical specificity with a resolution of 50 nm to 1 μm. A focused ion beam is directed to a solid surface, removing material in the form of neutral and ionized atoms and molecules. The ions are accelerated into a mass spectrometer and separated according to their mass-to-charge ratio. This is inherently a surface probe with 1- to 10-nm depth resolution. The sensitivity to different chemical species varies greatly because only ions are detected, whereas most of the products of sputtering are neutral fragments (143). SIMS is inherently an ultrahigh vacuum technique requiring flat specimens and some means to eliminate charge accumulation.

The use of cluster ions, that is, ions of high molecular weight such as C₃₅₇, SF₅, C₆₀, and Auₙ, to bombard the specimen generally produces better SIMS spectra for molecular solids than the original SIMS method, which uses gallium ions (144, 145).
Cluster ions produce greater useful signal intensity and sputter rate while limiting damage and penetration depth. Cluster ions may be used for analysis at low ion current (static SIMS) or can systematically remove layers from the specimen at higher ion currents than gallium, as shown on poly(methyl methacrylate) (146). Because many fragments have no charge, a second ion beam can be used to ionize fragments (144). Winograd reviewed the recent development of cluster ion mass spectrometry (147). Some focused ion guns allow SIMS imaging. An Au$_3$ ion source provides a high-brightness beam with a 200-nm spot size, whereas C$_{60}$ and gallium ions have been focused to about 2 μm and 50 nm, respectively.

Specimen bombardment with C$_{60}$ may produce a larger spot size than other ions but has many desirable properties. C$_{60}$ produces little roughening during erosion experiments and has larger usable mass range and sensitivity than do gallium ion beams. C$_{60}$ ions have increased secondary ion yields and fewer low-mass fragments but no increase in damage with ion energy up to 120 keV (148). Langmuir-Blodgett films sputtered with C$_{60}$ ions produced close to two orders of magnitude more characteristic secondary ions than gallium (149), apparently from increased sputter yield. Carbohydrate films doped with peptides bombarded with C$_{60}$ ions produced high-quality time-of-flight secondary ion mass spectra, even with ion doses 100 times greater than those used for gallium bombardment (150).

The combination of SIMS and AFM is useful because SIMS produces chemical images and AFM provides topology and other material properties. Zhu used this combination to examine penetration of gold atoms through alkanethiolate self-assembled monolayers (151). Wucher used this pair of techniques to produce a three-dimensional representation of a 300-nm-thick peptide-doped carbohydrate film with a combination of imaging and etching (152).

### 3.4.4 Transmission Electron Microscopy

Transmission electron microscopy (TEM) is analogous to transmission light microscopy except that electrons, rather than photons, are passed through the specimen. TEM can provide images with subnanometer resolution and so has been extensively used by biologists for nearly 50 years. The most serious limitations of TEM are that specimens must resist electron damage and usually have thickness less than approximately 100 nm so that only single-electron scattering events are likely.

Carbon replicas have long been a standard means of studying biomass with TEM (153–155). For example, the highest resolution images of cellulose fibril morphology were produced by carbon replicas of developing wood cells at a stage prior to the incorporation of lignin (156). In this example, cells were frozen quickly to avoid ice crystal formation and cleaved at −150°C. The exposed surfaces were coated with carbon and shadowed with platinum; then the biomass was dissolved with concentrated sulfuric acid and the replica examined by TEM.

TEM specimens are also made by cutting 30- to 60-nm sections of material. Before cutting, samples are embedded in resin, which holds the specimen together. Of all specimen preparation techniques, embedding in ice by fast freezing and keeping at −100°C throughout specimen preparation and imaging produces TEM specimens most similar to the original biomass.
One problem with TEM is that the electron scattering cross section shows little difference, and hence contrast, between carbon, oxygen, and nitrogen. One approach to developing image contrast is to preferentially label a component with stains containing high-atomic-number elements. Lignin has been labeled using bromine, potassium permanganate, and osmium tetroxide (57). Our current knowledge of hemicellulose deposition is from TEM studies where hemicellulose has been labeled with gold-tagged antibodies (157). Use of antibodies tagged with nanometer-sized gold particles is routine in biological microscopy (158).

Another problem of TEM is damage from the electron beam. Embedding medium, a model organic substrate, shrank 5% laterally and 25% in thickness during the first 5 min of TEM exposure, or 13,000 electrons per nm$^2$ (159). Shrinkage and degradation of specimens should be considered whenever evaluating TEM images of biomass.

The electron energy loss spectrum (EELS) is commonly used to determine the valence state of atoms in TEM specimens. Electrons passing through a specimen lose energy by ionization of specimen atoms, and these quantized losses are characteristic of different elements. Thus electrons interacting with carbon and oxygen lose 285 eV and 532 eV, respectively (160). The valence state of the atom can be determined by small shifts in this value.

3.4.4.1 Electron Tomography

In contrast to conventional two-dimensional TEM, electron tomography (ET) using brightfield TEM produces a three-dimensional rendering of a volume. ET has been extensively used to study cell structure (161, 162). To determine a unique three-dimensional representation, approximately 100 images must be obtained using $1^\circ$ to $5^\circ$ tilt increments over a large angular range, such as ±70°.

The large number of images needed could subject a specimen to severe electron damage, so several techniques have been adopted to minimize electron exposure (see Transmission Electron Microscopy, above). Low-dose microscopy techniques use automatic focusing and drift correction on a specimen field adjacent to the one being imaged. Because many images will be summed to make the final composite, each image can tolerate slightly higher noise than standard images and therefore less electron exposure. A case has been made that the total electron exposure needed for a tilt series is about the same as that for a single two-dimensional image (163). In any case, the electron flux should be kept within a few thousand electrons per square nanometer (159). Another method of minimizing damage from electron exposure is to maintain the specimen at cryogenic temperature (136). Cryo-electron tomography has been useful in describing cellular structure and the structure of cell components (164–166).

Electron tomography has revealed the nanometer-level organization of cellulose microfibrils in the S2 layer of the cell wall and the arrangement of some residual lignin and hemicellulose about the cellulose microfibrils in radiata pine (167). The cell sections were partly delignified with peracetic acid, dehydrated, treated with multiple heavy metal stains, and embedded in Spurr’s epoxy. Although some structural distortion may have been introduced by the treatment, the results are a major step toward understanding wood cell wall structure.
3.4.4.2 Ultrafast Electron Microscopy

Nobel laureate Ahmed H. Zewail and associates developed ultrafast electron microscopy (UEM) which enables imaging of kinetic processes at atomic resolution (168). A conventional TEM was modified so that a femtosecond pulsed laser stimulated one electron emission per laser pulse. A version capable of imaging wet specimens is under construction (169). Although the resolution is the same as for conventional TEM, the time resolution provided by this new technology promises to shed new light on dynamic processes.

3.5 Summary

Biomass is a difficult substrate to analyze at the nanoscale. It is a nanoscale, interpenetrating, lightly crosslinked network of three different types of polymer. Compared with most inorganics, the polymers of biomass are strongly affected by water, unstable, and difficult to distinguish from each other.

Because the natural state of biomass is in water and because of the strong interaction with water, native structures should be characterized in water when possible. Removing water from biomass causes structural changes that are only partially reversible and not well understood. The nanostructure of biomass will be different depending on the amount of water present during analysis and how water has been removed (i.e., its history).

Because biomass is organic, many analytical techniques, particularly focused beam microscopies, can easily damage specimens. Most particle or electromagnetic beams with high resolution have the potential to vaporize or initiate chemical changes in the biomass substrate. The probe beam can also cause accumulation of charge on the nonconducting biomass substrate, which can directly effect measurements in some methods, as well as cause further chemical changes.

Atomic force microscopes are the most common scanning probe microscopes, because they are so versatile and easy to use. They were originally developed to measure nanoscale topography, but a variety of techniques now provide information on chemical and material properties as well. More specialized instruments, such as nanoindenters and near-field scanning optical microscopes, may be less versatile but generally provide ‘cleaner’ information that requires the user to make fewer assumptions during analysis.

In short, the characterization of nanostructure in biomass is challenging. Like most scientific problems, however, proper choice of analytical techniques and specimen preparation, as well as a skeptical approach to data interpretation, continue to provide new understanding of this fascinating system.

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