Recent developments in atomic force microscopy for underwater imaging of biological composite

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The study of biological samples is one of the most attractive and innovative fields of application of atomic force microscopy AFM. Recent breakthroughs in software and hardware have revolutionized this field and this paper reports on recent trends and describes examples of applications on biological samples. Originally developed for high-resolution imaging purposes, the AFM also has unique capabilities as a nano-indentor to probe the dynamic visco-elastic material properties of living cells in culture. In particular, AFM elastography combines imaging and indentation modalities to map the spatial distribution of cell mechanical properties, which in turn reflect the structure and function of the underlying structure. This paper describes the progress and development of atomic force microscopy as applied to animal and plant cell structures.

**Keywords**: atomic force microscopy; biological cells, aqueous medium, tapping mode

1. Background

Recently there has been enhanced interest in using atomic force microscopy (AFM), to study the properties of biological materials, including plant cell walls. AFM provides a critical imaging ability to investigate cell walls at the nanometer scale with no complex sample preparation or perturbation. In 1997, Baker et al. first reported AFM measurements of the surfaces of the Valonia cellulose crystals [1]. Kirby et al. performed AFM measurements on several different plant cell wall materials [2]. They demonstrated the feasibility of using AFM to image hydrated plant cell. The results showed that cell wall consisted of different layers of fibers orientated in different directions, as polylaminate structures. Davis and Harris did AFM measurements on onion and Arabidopsis thaliana cell walls and observed tightly interwoven microfibrils [3]. Ding and Himmel reported AFM imaging of the maize parenchyma cell wall surface, which showed similar random orientation of the microfibrils reported by Davis and Harris [4]. AFM has proved to be a powerful tool that can provide high topographic resolution with plant cell walls. However most of those work reported were performed with dehydrated or partially dehydrated plant cell walls in air. Since primary wall contains much water, up to 80% in the matrix and water plays an important role in the physical properties of the wall. Dehydrated or partially dehydrated cell walls may show different properties from the natural cell walls. In this work, fresh cell walls will be studied in liquid or different solvent to reveal the native cell wall properties.

With its pico-newton force sensitivity and nanometer displacement accuracy, the AFM has been recognized as a useful tool measuring the elastic moduli of biological samples [5-9]. The nanoindentation with an AFM has been recently applied to living plant cells in conditions close to natural. Mechanical properties of suspended grapevine cells cultured in liquid medium, the primary cell wall of shoot apical meristems, rosette leaves, tomato cells and epidermal cells of living roots of Arabidopsis thaliana were measured using AFM based nanoindentation [9-13]. The indentation depth is much smaller (about 100 nm) than the conventional nanoindentation. In this paper we will review some recent AFM imaging results of hydrated celery and onion epidermis walls structures and nanoindentation results of single layered onion epidermical cell walls.

2. AFM images of hydrated plant cell walls

The structure of primary celery (Apium graveolens L.) epidermis cell walls was characterized at the nano-scale using the AFM in the Peak Force Tapping Mode after simple treatments [14]. In Fig.1, the images show that the micro-fibrils are well separated with spacing of up to almost 50 nm and could identify and evaluate 5 layers in terms of fiber thickness, angular orientation and spacing. It can be concluded that the micro-fibril structure is weakly anisotropic. The results are significant in that they provide information about cell wall characteristics below the surface [14].

The outer and inner scales of onion could represent various developing stages of primary cell walls, with inner scales being younger and outer scales being older. The outer and inner scales of onion could represent various developing stages of primary cell walls, with inner scales being younger and outer scales being older. Furthermore, to investigate cellulose microfibril orientations at various developmental stages, the outer and inner scales of onion were chosen to be examined, with inner scales being younger and outer scales being older. Abaxial epidermal layers from the 2nd, 5th, 8th, and 11th scales were studied and AFM images were shown in Fig. 2. The onion epidermis cell walls of the cellulose microfibril orientation were found to vary gradually from the dispersed arrangement in the inner scales to the...
transverse orientation in the outer scales [15]. These results are very important to study how cellulose microfibrils develop in intact cell walls and will provide insight for mechanical properties and growth mechanism study of plant cell walls.

The capability to determine the average orientation of cellulose microfibrils in intact cell walls will be useful to study how cellulose microfibril orientation is related to biomechanical properties and the growth mechanism of plant cell walls.

**Fig. 1** 500 nm scan of the celery fibrils, left image is Topography and right image is Peak Force Error Signal [14].
3. AFM indentation of hydrated plant cell walls

Based on the previous observation, we assume that the cellulose microfibril packing and orientation may play important roles in the cell wall mechanical properties. Alternatively, it is also possible that the matrix component such as pectin may have strong impacts on the elastic modulus of cell walls. To verify our assumptions, we applied AFM based nanoindentations to measure the elastic modulus of intact single layer cell walls.

The mechanical property measurement with AFM nanoindentation is based on force-distance (F-D) curve analysis. Figure 3 shows a representative F-D curve obtained on onion epidermis cell wall in liquid. F-D curves can vary with different samples. In our experiments, because the cell wall polymers are hydrophilic (except cuticles in the outside layer), the adhesion between cell walls and clean SiO2 tip surface is very small in water. Thus, a simple Hertz model can be used, especially when the applied load is much larger than the adhesion force.

Hertz model were chosen for F-D curve analysis to obtain the elastic modulus E. The Hertz model assumes that the sample is isotropic, elastic and occupies infinite half space. It also assumes that the indenter is not deformable and there is no additional interaction between the tip and sample [16]. Because the cell wall polymers are hydrophilic, the adhesion between cell walls and clean SiO2 tip surface in water is very small. Thus, a simple Hertz model can be used for F-D curve analysis to obtain the elastic modulus E.

As shown in Fig. 4, when using the AFM tip to indent the sample, the recorded piezo displacement D from the free standing position is comprised of two parts: the tip cantilever bending x and the sample indentation δ.

\[ D = x + \delta \] (1)

According to Hertz model, the force measured with a four-sided pyramid can be represented as.

\[ F = \frac{E}{1 - \nu^2} \frac{\tan \alpha}{\sqrt{2}} \delta^2 \] (2)

where F is the recorded force, E is sample’s elastic modulus, \( \nu \) is samples’s Poisson ratio, \( \alpha \) is the geometry angle of the indenter. In our analysis, \( \nu \) can be assumed to be 0.3 and \( \alpha \) is estimated as 13° according to the parameters given by the manufacturer.

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**Fig. 3** A representative F-D curve (approach and retract) obtained in the experiment on onion epidermis cell wall in liquid. The y axis is the recorded force in nano-newtons and the x axis is the relative piezo displacement in nm.

**Fig. 4** Sketch of indentation distance and tip geometry.
Knowing that \( x = \frac{F}{k} \), where \( k \) is the cantilever spring constant, from the above two equations, we can easily obtain relationship between recorded force \( F \) and recorded piezo displacement \( D \):

\[
F = \frac{E}{1 - \nu^2} \frac{\tan \alpha}{\sqrt{2}} (D - \frac{F}{k})^2
\]

(3)

By fitting the experimental F-D retrace curve, we can get the elastic modulus \( E \).

Elastic moduli of the abaxial epidermis walls from four scales (11th, 8th, 5th and 2nd scales) in different onions were measured by AFM nanoindentation (see Fig. 5). We found that unlike the cellulose microfibril alignment trend, the indentation modulus shows no definitive trend from younger/inner to older/outer scales. [16]

3.1 EDTA effect

In order to partially remove the EDTA-soluble pectin from the epidermis walls, 100 mM EDTA was added onto the cell wall sample taken from the 5th onion scale. Topography images were generated in the same area in water and after adding the EDTA solution. 80 F-D curves were probed in the sample area. Topography images and elastic moduli results are shown in Fig. 6. [16]

Topography images became clearer with time after adding in 100 mM EDTA solution. Elastic modulus decreased slightly and remained stable with time after adding EDTA solution. The variation is statistically significant (in student’s t-test, \( p=0.04 \)). Elastic modulus decrease with EDTA treatment is probably due to the removal of EDTA-soluble pectin.

3.2 Calcium ion effect

100 mM CaCl₂ solution was added onto the cell wall removed from the 5th onion scale. Topography images were generated in the same area in water and after adding CaCl₂ solution. 120 F-D curves were generated in the same area. Topography images and elastic moduli results are shown in Fig. 7.

Control experiments on pectin network on the other hand showed that the pectin can substantially impact the indentation modulus. The AFM based nanoindentation can effectively track variations in cell wall properties, demonstrating the feasibility of this technique as a tool to characterize intact plant cell walls in their fully hydrated state.

4. Discussion and future work

The study of biological samples is one of the most attractive and innovative fields of application of atomic force microscopy AFM. Recent breakthroughs in software and hardware have revolutionized this field and this paper reports on recent trends and describes examples of applications on biological samples. Originally developed for high-resolution imaging purposes, the AFM also has unique capabilities as a nano-indentor to probe the material properties of living cells in culture. In particular, AFM combines imaging and indentation modalities to map the spatial distribution of cell mechanical properties, which in turn reflect the structure and function of the underlying structure.
To investigate cellulose microfibril orientations at various developmental stages, the outer and inner scales of onion were chosen to be examined, with inner scales being younger and outer scales being older. Abaxial epidermal layers from the 2nd, 5th, 8th, and 11th scales were studied by the AFM. The onion epidermis cell walls of the cellulose microfibril orientation were found to vary gradually from the dispersed arrangement in the inner scales to the transverse orientation in the outer scales. The use of AFM imaging technique for the study of cellulose microfibril orientation in abaxial epidermal walls of onion scales was also verified by SFG spectroscopic technique of a college. [15] For abaxial epidermis walls of an onion bulb, the net orientation of cellulose microfibrils across the entire wall thickness seems to vary gradually from the dispersed arrangement in the inner scales to the transverse orientation in the outer scales. The similar trend is observed for the orientations of cellulose microfibrils exposed at the newly deposited cell wall surface. These results are very important to study how cellulose microfibrils develop in intact cell walls and will provide insight for mechanical properties and growth mechanism study of plant cell walls.

Based on the previous observation, we assume that the cellulose microfibril packing and orientation may play important roles in the cell wall mechanical properties. Alternatively, it is also possible that the matrix component such as pectin may have strong impacts on the elastic modulus of cell walls. To verify our assumptions, an atomic force microscopy (AFM) based nanoindentation method was employed to study how the structure of cellulose microfibril packing and matrix polymers affect elastic modulus of fully-hydrated primary plant cell walls. The isolated, single-layered abaxial epidermis cell wall of an onion bulb was used as a test system since the cellulose microfibril packing in this cell wall is known to vary systematically from inside to outside scales and the most abundant matrix polymer, pectin, can easily be altered through simple chemical treatments such as ethylenediaminetetraacetic acid (EDTA) and

**Fig. 6** Topography images of onion 5th scale in a) water, b) 20 minutes after dipping in 100 mM EDTA, c) 40 minutes after dipping in 100 mM EDTA, d) 100 minutes after dipping in 100 mM EDTA. e) Elastic moduli measured in water and after adding EDTA solution in the same area of cell wall shown in topography images. The error bar shows sample standard deviation [16].
calcium ions. Experimental results showed that the pectin network variation has significant impacts on the cell wall modulus, and not the cellulose microfibril packing. The AFM based nanoindentation can effectively track variations in cell wall properties, demonstrating the feasibility of this technique as a tool to characterize intact plant cell walls in their fully hydrated state.

![Image of topography images](image)

**Fig 7.** Topography images of 5th scale in a) water, b) 20 minutes after adding 100 mM CaCl₂, c) 30 minutes dipping in CaCl₂ solution, d) 40 minutes in CaCl₂ solution, e) 50 minutes in CaCl₂ solution and f) 60 minutes in CaCl₂ solution. g) Elastic moduli of onion cell wall from 5th scale in water and in CaCl₂ solution with time. The increase is statistically significant. [16]

The mechanical properties of plant cell walls in their natural hydrated state are critical in understanding cell wall structure and cell growth. Indentation technique can provide valuable information in this regard. However, in indentation tests, the indentation depth needs to be less than 10% of the sample thickness so that the substrate effect becomes negligible. Our thickness measurements of onion epidermis cell wall indicated that the wall thickness in younger scales can be as thin as around 2 μm. This means accurate measurements would require the indentation depth to be 200 nm or less, making the conventional indentation technique unsuitable. We therefore conducted nanoindentation tests with AFM, allowing the indentation depth to be controlled below 150 nm. The AFM based nanoindentation also provided the ability to operate in liquid environment so that the cell walls can be studied in their natural, fully hydrated state.

The elastic modulus of single layered, fully hydrated abaxial epidermis walls from four scales (11th, 8th, 5th and 2nd scales) in four onions were measured by AFM nanoindentation. The moduli increase slightly from inner to outer scale for two onions in experiments but not in the other two. From the view of composite mechanics, the indentation was
measured along the direction normal to the microfibril layers and caused very small lateral displacement. Thus, the cellulose microfibril orientation would influence more the in-plane tensile stress/strain anisotropy than the out-of-plane modulus. Although the net orientation of cellulose microfibrils vary gradually from the dispersed arrangement in the inner scales to the transverse orientation in the outer scales as shown in our previous study [15], the nanoindentation moduli of cell walls do not show substantial change that can be correlated with such variation. This indicates that, for the onion epidermis cell walls, the cellulose microfibril orientation alone may not be the dominating factor determining the out of plane modulus. Other factors such as the local density of microfibrils and pectin network status could make more significant contribution to the nanoindentation modulus.

Pectin forms a gel phase structure that holds cellulose-hemicelluloses frame in cell walls. From the mechanical point of view, pectin serves as a matrix material in the cell wall biopolymer composite and has long been suspected of playing an important role in cell wall mechanics. Since our AFM based nanoindentation was operated in liquid, the sample chemical environment can be controlled and modified. This allowed us to investigate the effect of pectin network structure on wall mechanical property. Two methods were used to modify the pectin network: EDTA to partial remove and Ca2+ to cross-link the pectin network.

These two methods caused different effects on the cell wall property. In particular, Ca2+ treatment caused the formation of cross-linked pectin gelation structures, which can be clearly observed in the topography images. The elastic moduli were found to increase dramatically as a result. On the other hand, the topography images after the EDTA treatment became clearer as a result of surface pectin removal. Correspondingly, the moduli decreased, indicating the weakening of pectin structure. This verifies that the pectin network has significant impact on cell wall property. In plant, the pectin network could be altered by various factors such as the environment, age, etc., which would substantially affect the cell wall mechanical property. This is an interesting topic that warrants future studies.

References