Ultrastructure and Force Property Measurements of Wheat Grain Tissues Using HarmoniX Mode in Fluid

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INTRODUCTION

It is of great importance to the food industry to advance the fundamental understanding of how the structure and morphology of grains influence food processing behavior. Atomic force microscopy can be used to increase this understanding. In this research we demonstrate how recent advances in technology, namely the combination of the unique aspects of HarmoniX™ Nanoscale Material Property Mapping mode with fluorescence microscopy, can probe the surface characteristics of tissues isolated from the wheat grain. The resulting data provides new insights into this essential ingredient in human nutrition.

BACKGROUND

The atomic force microscope (AFM) has been developed to extract an increasing variety of signals from the physical interaction between a scanning probe and a sample of interest.[1] During the last decade, AFM has emerged as the tool of choice for the investigation of biological materials, especially cells, both for imaging[2, 3] and force spectroscopy[4, 6] modes. Recently, Veeco's BioScope™ family of AFMs have coupled AFM data with optical measurements, providing key information regarding cell dynamics and architecture.[7-12]

The widely used AFM TappingMode™ technique allows quantitative mapping of such sample properties as adhesion, force peak, dissipation, and stiffness. The new HarmoniX mode[13] extends this fundamental capability, utilizing special cantilevers and probe tip geometries to record both flexural and torsional motions of the lever while scanning over the sample of interest. This revolutionary mode has been tested successfully on a wide variety of samples with very different Young's moduli, ranging from 10 MPa to 10 GPa, while combining the benefits of both TappingMode and force spectroscopy with high-resolution imaging and relatively fast data acquisition.

The study of the ultrastructure of the wheat grain and its components is of key interest when considering how cereals are processed. The resulting product properties and qualities are dictated by the nanometer-scaled organization of the component biopolymers.[14-16] The ultrastructure of the wheat grain then has a correspondingly strong impact on cereal technology and its derived products.[17] Therefore, any new investigative technique that allows the recognition of the different wheat components may lead to improved understanding of the architecture of the wheat grain, and relate that architecture to the macroscopic properties of the corresponding products.

The outermost tissue of a wheat grain, associated with the coat and endosperm, is of a great interest as it contains proteins, polysaccharides, and lipids, or a combination of these, arranged with a high degree of natural organization.[18] The coat and endosperm are formed by the association of specific biopolymer-rich zones. The coat contains the pericarp, the seed coat (testa) and the nucellus epidermis tissue; the endosperm is composed of the aleurone layer, the sub-aleurone layer, and the starchy endosperm. Each region is characterized by homogeneous

assemblies of polysaccharides, lipids, and proteins. For example, arabinoxylans are the main non-starch polysaccharides that constitute the longitudinal assemblies in the aleurone and pericarp cell wall of wheat.

Numerous AFM studies have been reported on the structural characterization of the different biopolymer components of food products or plant materials after they have been extracted and purified.[19-21] One such example is the imaging of starch granules extracted before AFM observation.[22-24] Few studies, however, have been made from whole zones of the wheat grain in which the biopolymer structures are kept in their natural environment.[25-27]

This note supplies an overview of how the latest advances in AFM technology and techniques provide a better understanding of the biopolymer interaction and organization in wheat grain, starting from intact tissues. The ease and suitability of combining fluorescence with AFM is also demonstrated, highlighting its abiltity to provide quick identification of the grain structure, acquire higher resolution magnification, and clearly elucidate the ultrastructure of different wheat grain tissues.

SAMPLE PREPARATION

High-pressure freezing followed by freeze substitution has been shown to provide superior preservation of the ultrastructure of plant tissues.[28] This protocol was used to prepare thin sections through wheat grains for investigation by AFM and fluorescence. Small pieces of the peripheral zone of winter wheat grains (pericarp, testa, nucellus epidermis, aleurone layer and starchy endosperm) were placed inside a copper platelet and frozen at high pressure using an EMPACT freezer (Leica-Microsystems) followed by freeze substitution in acetone/osmium and embedding in London Resin White- (LRW), as adapted from Studer et al.[29] Semithin (1µm) or ultrathin sections (100nm, 500nm) were cut using a Microm MT-7000 ultramicrotome and collected on glass supports for further staining and/or AFM examination. Structural staining was conducted with fluorescent dyes: Nile blue (0.01% in water, excitation at 488nm), which stains lipid phases; Calcofluor (0.01% in 1/1 by vol. ethanol/water, excitation at 400nm), which stains cell wall β-glucan-based polysaccharides; Acid fuchsin (0.01% in 1% acetic acid, excitation at 453nm), which stains protein phases, and Acridine orange (0.02% in phosphate buffer, excitation at 488nm), which stains metachromatically a variety of components. Unstained sections of wheat tissue also exhibit auto-fluorescence, a response of cell wall phenolic compounds.[30]

As an empirical rule, ultrathin sections exhibit the lowest fluorescence intensity but are remarkably flat, whereas thicker sections have a more significant roughness with a higher intensity of fluorescence.

The samples were imaged with fully integrated BioScope II and BioScope Catalyst™ AFMs (Veeco Instruments) equipped with an AxioObserver (Zeiss). Most of the images were done in fluid to preserve the structure of the cuts. Optical and fluorescent views were acquired with an AxioCam MRm (Zeiss). Different cantilevers (Veeco Instruments) were used to scan samples under varied conditions (nominal values are given): OTESPA (k = 2N/m, f_0 = 70kHz) for tapping in air, MLCT (k = 0.5N/m, f_0 = 120kHz) for tapping in liquid, MLCT (k = 0.01N/m) for contact in liquid, and HMX (f_0 = 65kHz) and HMXS (f_0 = 30–36kHz) for HarmoniX in air and in liquid, respectively. In addition, for especially rough specimens with very tall features, topographic images were acquired using SC-EH cantilevers (Team Nanotec) that have a tip height greater than 20µm and a small cone angle (<25°).

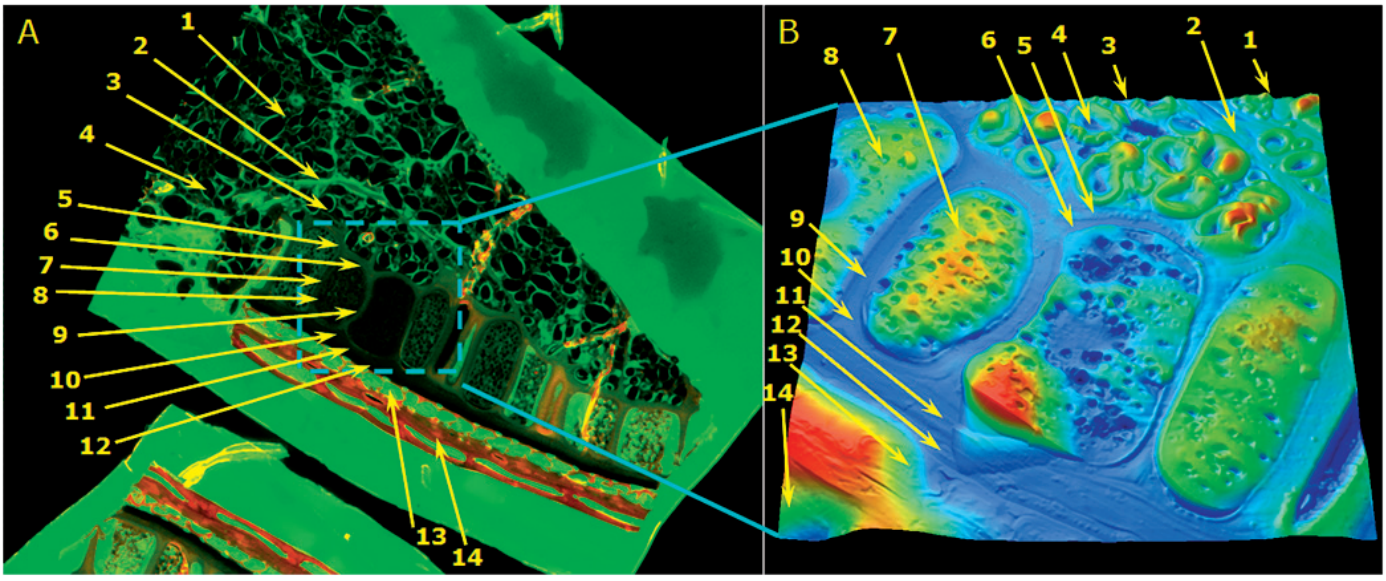

Figure 1. Correlation between epifluorescence (A) and AFM height (B) images. 1 = Starchy endosperm; 2 = Starchy endosperm wall; 3 = Sub-aleurone layer; 4 = Starch granule; 5 = Sub-aleurone wall; 6 = Aleurone cell wall; 7 = Aleurone cell from the aleurone layer; 8 = Aleurone protein body; 9 = Aleurone cell wall between two aleurone cell; 10 = Cellular junction; 11 = Nucellus epidermis; 12 = Tube cells; 13 = Cross cells; 14 = External pericarp. Optical image was acquired using a 10X dry objective. AFM image (110 x 110 x 5µm) was generated by contact mode in air.

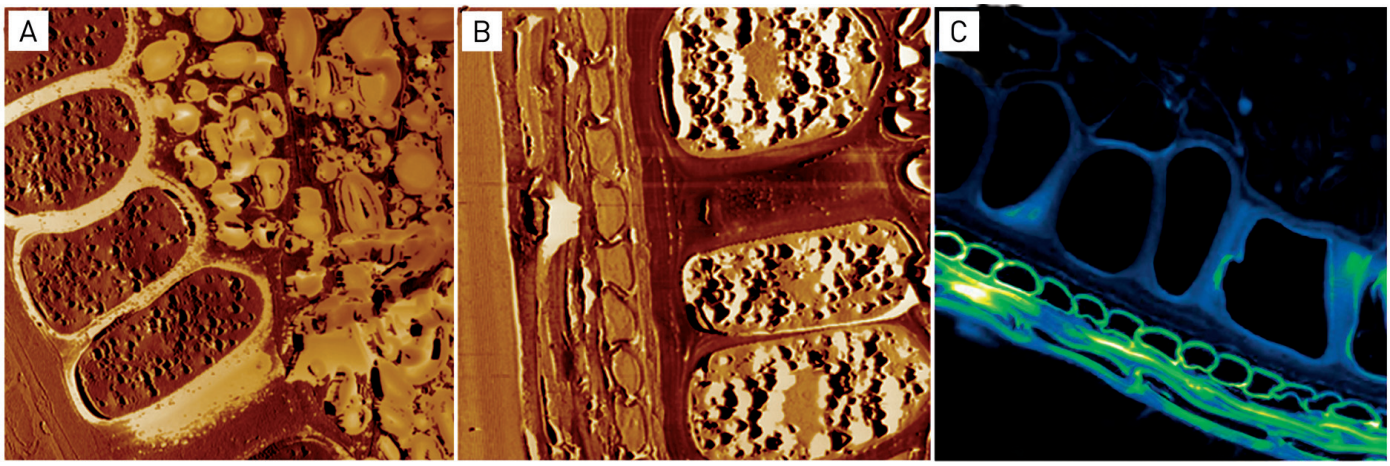

Figure 2. Phase images (150 x 150µm x 100°) (panel A) and (panel B) were acquired by TappingMode in liquid. Several rsp were tested to investigate surface and volume properties of the wheat cuts. In light tapping conditions (A), the phase signal is more likely to reflect adhesion forces, whereas in moderate/hard tapping conditions (B), it is more sensitive to viscoelastic behaviors. The optical correlation allowed identifications of the components in the cut, for instance, labeling with calcofluor (panel C) reveals the β–glucans of the coat (epifluorescence 20X).

All the experiments were carried out using the same procedures. First identification of the cell types and components was made using bright field (BF), digital interference contrast (DIC) and epifluorescence (EF). Then the probes were navigated to the area of interest for subsequent AFM imaging. Finally, we made a comparison of optical and AFM data. Vision® software (Veeco Instruments) was used for off-line rendering of AFM height data.

EASY CORRELATION OF AFM AND OPTICAL DATA

Preliminary experiments were carried out on five different wheat sections stained for fluorescent imaging. The fluorescence image was then used to easily navigate to a region of interest for subsequent AFM. All of this was achieved using Veeco's new MIRO (Microscope Imaging Registration and Overlay) software feature, which is unique to the BioScope II and Catalyst AFMs. After a quick calibration of the sample and the tip, any optical image can be imported into NanoScope® as a background for a whole series of AFM experiments. The location of several AFM scans can be selected on the optical image by drawing a box. After each scan, each AFM image is automatically inserted into the optical image. One sample is featured in figure 1. When stained with acridine orange, a metachromatic dye, different regions of the ultrastructure appear stained with different colors. In panel A, green zones correspond to the cell walls and the surrounding resin, whereas pericarp layers appear with a red-shift emission.[31] The blue square represents the location of the AFM scan. Panel B shows the AFM topographic or height data as a 3D representation. The easy correlation between the fluorescent and AFM images using the Catalyst AFM and MIRO allowed rapid identification of the different cell types and components. This was the key requirement for the remainder of the study.

TAPPING AND CONTACT MODE ANALYSES

We then focused on deriving better insight into the largely undocumented mechanical properties of the wheat samples. The phase signal is known to correspond to surface (adhesion forces) and volume (viscosity, elasticity) properties when scanning parameters are set properly.[32] In phase images, the brighter signal is interpreted to mean that more energy is dissipated between the tip and the sample. Phase contrast data can be critically dependent upon the force of tapping. This force can be modulated by adjusting the ratio setpoint (rsp), which is defined as the ratio between the amplitude setpoint and the free amplitude of oscillation. Depending on this value, we can refer to soft (high rsp) or hard (low rsp) tapping.

Initial tests were carried out using TappingMode in air to monitor the phase signal. Under these conditions, the phase contrast was rather weak and predominately related to topography. We thus decided to switch to liquid environment for further analysis. These data yielded much more relevant results. The principle of tapping in liquid studies is summarized in figure 2. If images are acquired in light tapping conditions (A), where the phase signal is more sensitive to surface forces, a clear contrast can be observed between different cell types. Focusing on the aleurone layer, the phase signal is particularly dark inside the aleurone cells, including proteins and lipid bodies (dark granules), which indicates low adhesion. On the other hand, the aleurone cell wall with middle lamellae (the interstitial layer between aleurone cells) and starch grains appear much brighter. All other compartments like the nucellus epidermis or the pericarp exhibit no phase contrast.

If images are acquired using much "harder" tapping conditions (B) where phase is more sensitive to volume than surface properties, like elasticity and viscosity, the phase contrast between aleurone cells and their surrounding neighborhood is inverted. Furthermore, it is now much easier to distinguish between the different components of the pericarp. Once again, immediate correlation of the fluorescence image with the AFM image allowed for easy identification of the different components.

Further experiments were done using contact mode in liquid, utilizing the "Point & Shoot" features of NanoScope V (see figure 3). A topographic image was recorded after the spring constant of the

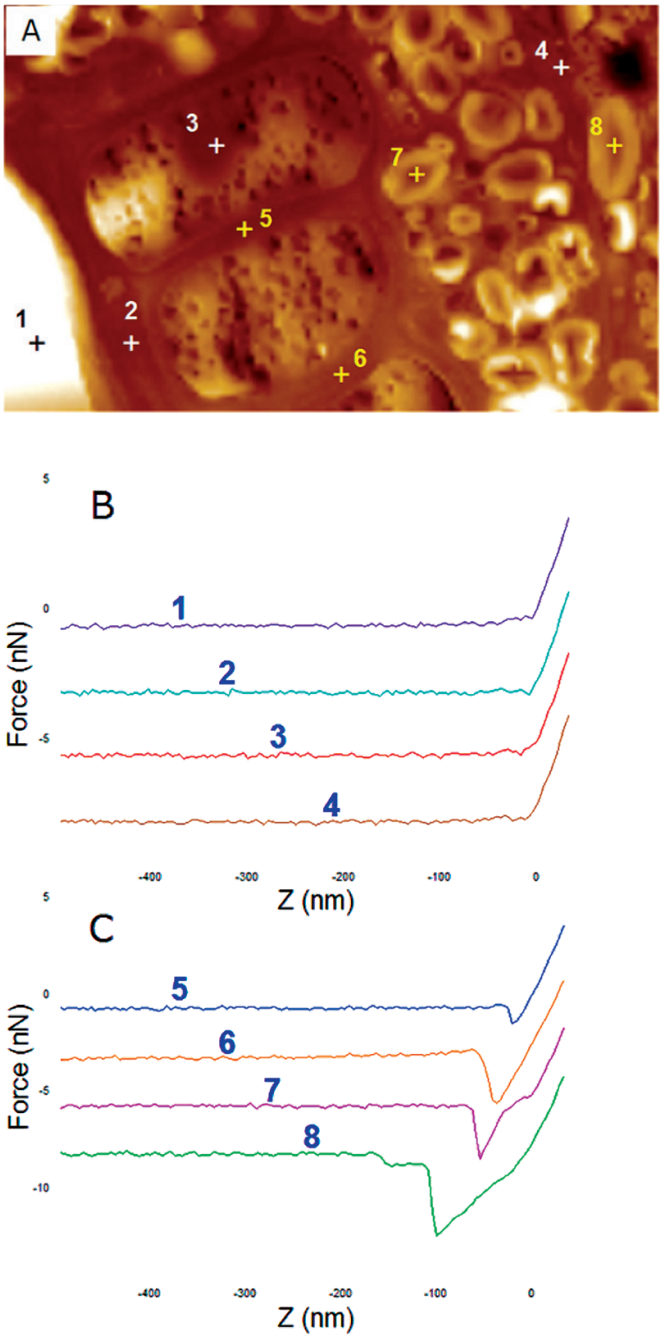

Figure 3. Point & Shoot experiments on wheat cuts. After capturing a height image (A: 140 x 85 x 5µm), several spots of interest were targeted, allowing classification of areas exhibiting low (B) or high (C) adhesion forces.

cantilever was determined. The "Point & Shoot" feature offers the option to draw a line or a box on such an image, define a number of points, and then the AFM tip will automatically move to those locations to capture one approach-retract curve at each point. For our studies, it was more interesting to choose "manually" a few spots of interest, selected at a variety of regions on the sample. As explained previously,[33] the approach curve reveals the stiffness/elasticity, whereas the retract curve reflects any adhesion forces between the tip and the sample. In figure 3, the retract curves are displayed, that is, the curves associated with adhesion forces, for the eight points of interest (A). The weakest adhesion forces (B) were found to be focused in the pericarp (curve 1) and nucellus epidermis (curve 2), the aleurone cells (curve 3) and the starchy endosperm wall (curve 4); whereas the aleurone cell wall (curve 5), middle lamella (curve 6) and starch granules (curves 7 and 8) exhibit very strong adhesion events.

Although the sample preparation used a resin-embedding step, which may influence the approach-retract curves, these observations correlate very well with the phase contrast observed in figure 2A. All experiments were done using a plain tip but these results certainly open the field of much more relevant investigations using functionalized tips. We also compared approach curves to probe the elasticity of the sample. A clear contrast could be observed between the different components. The highest Young's moduli were found in aleurone cells (but not the protein/lipid bodies), internal pericarp, and cross cells. Force volume experiments were also carried out with similar conclusions.

HARMONIX: OPEN ACCESS TO ALL PARAMETERS SIMULTANEOUSLY

Finally, the samples were investigated using HarmoniX mode. This mode, enabled by the NanoScope V controller, allows for non-destructive and high-resolution imaging of samples using forces equivalent to TappingMode, as well as real-time acquisition of data and quantification of various sample properties, such as stiffness, dissipation, force, etc.

First, the specialty HMX and HMX-S tips (Veeco Instruments) were calibrated on a well-known polymer sample to achieve quantitative values. Then the HarmoniX mode was operated in liquid on different types of wheat cuts using both hard and soft probes. The best contrast was obtained using the soft probes, as shown in figure 4. This wheat cut plane was obtained using a different angle of cutting that was more parallel to the aleurone layer. This explains why the aleurone cells cover 80% of the optical image. HarmoniX was mainly used in this area, but also on pericarp areas (not visible here) and starch areas, which are visible in the bottom left and top right corners of the optical image (for clarity, only the AFM images in the aleurone area are shown here).

In HarmoniX mode, up to eight channels can be recorded simultaneously, though only five are shown in figure 4. On the phase image, the contrast is very similar to what was shown in figure 2B, which figures since HarmoniX is operated in "hard" tapping conditions. On the adhesion channel, the lowest adhesion values are recorded inside the aleurone cells, whereas the surrounding aleurone cell walls are much more adhesive, corresponding to the force volume experiments (figure 3). Peak force and elasticity values are much higher inside the aleurone cells (except inside protein and lipid inclusions, which are known to be very soft) than in the surrounding aleurone cell walls. This again agrees with the previous force volume experiments. Finally, much more energy is lost in the cells than in the cell walls. As the tip was calibrated, the mean Young's moduli could be extracted from the stiffness channel using the bearing analysis function of the

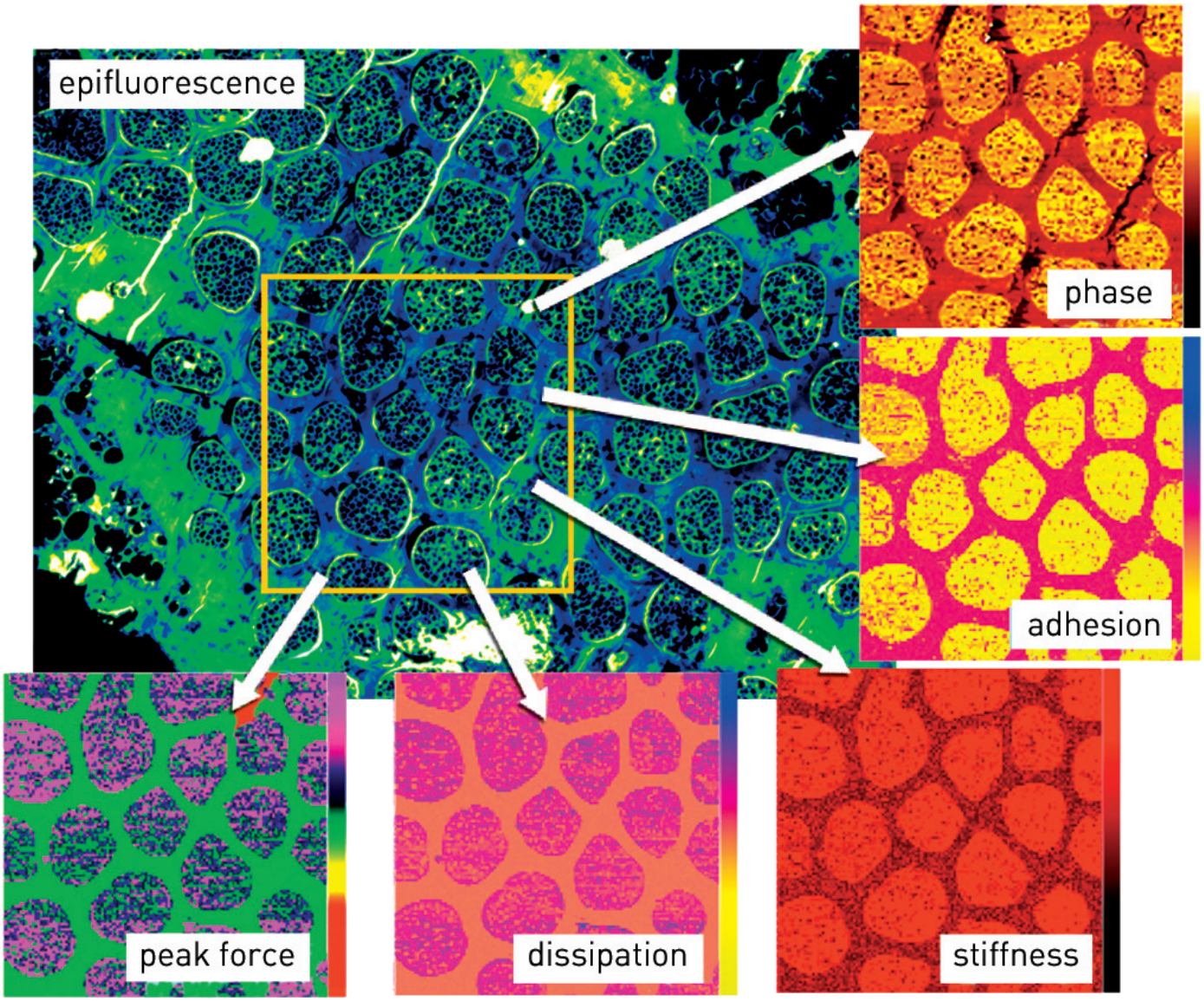

Figure 4. HarmoniX imaging in liquid environment from an ultrathin section of a resin-embedded wheat aleurone tissue. The epifluorescence image shows the major part of the cut consists in aleurone cells surrounded by starchy zones seen in bottom left and top right corners of the image. The yellow frame represents the AFM scan location where HarmoniX measurement was performed after calibration of the tip on a polymer sample. Five of the eight AFM channels that can be recorded simultaneously are shown on this image; phase (z-scale = 30°), adhesion (40nm), peak force (200nm), dissipation (arbitrary units) and stiffness (z-scale = 5GPa). The information displayed on these five channels is in total agreement with both the data found in literature and the results of the experiments carried out on similar samples in regular TappingMode and force mode (see figures 2 and 3).

NanoScope V. Example values for the calibration were 4.6GPa for the aleurone cells, and 2.4GPa for the surrounding aleurone walls.

Similar comparisons and calculations were achieved with other areas (e.g., pericarp and starchy endosperm zones), and confirmed the relevance of comparing the results of different AFM modes.

CONCLUSION

The combination of AFM and fluorescence imaging using the BioScope II and Catalyst AFMs provided useful insight into the ultrastructure of intact wheat grain tissues. These fully integrated tools allowed the simultaneous recording of both fluorescent staining of specific components and AFM signals. The unique potential of HarmoniX mode, enabled by the NanoScope V controller, was also shown to be very useful to probe the surface and volume properties of a plant tissue. Working in fluid environment was essential to allow preserving the structure of the cut and would not have been possible on a native sample. The next obvious step is to map more precisely the different HarmoniX data as a function of the cell layers and components of a variety of resin-embedded or native grain samples. This new emerging technology opens a wide range of applications in food research and plant tissue investigation.

REFERENCES

1. G. Binnig, C.F. Quate, and G. Gerber. 1986. "Atomic Force Microscope." *Phys. Rev. Lett.* 56: 930-33.

2. L. Kreplak, H. Wang, U. Aebi, and X. P. Kong. 2007. "Atomic Force Microscopy of Mammalian Urothelial Surface." *J. Mol. Biol.* 374: 365-73.

3. S.G. Kaminskij and T.E. Dahms. 2007. "High Spatial Resolution Surface Imaging and Analysis of Fungal Cells Using SEM and AFM. *Micron* (in press).

4. M. Radmacher, M. Fritz, C.M. Kacher, J.P. Cleveland, and P.K. Hansma. 1996. "Measuring the Viscoelastic Properties of Human Platelets with the Atomic Force Microscope." *Biophys. J.* 70: 556-67.

5. V. Dupres, C. Verbelen, and Y. Dufrene. 2007. "Probing Molecular Recognition Sites on Biosurfaces Using AFM." *Biomaterials* 28: 2393-402.

6. T.G. Kuznetsova, M.N. Starodubtseva, N.I. Yegorenkov, S.A. Chizhik, and R.I. Zhdanov. 2007. "Atomic Force Microscopy Probing of Cell Elasticity." *Micron* 38: 824-33.

7. N. Almqvist, R. Bhatia, G. Primbs, N. Desai, S. Banerjee, and R. Laly. 2004. "Elasticity and Adhesion Force Mapping Reveals Real-Time Clustering of Growth Factor Receptors and Associated Changes in Local Cellular Rheological Properties." *Biophys. J.* 86: 1753-62.

8. D. Pesen and J.H. Hoh. 2005. "Micromechanical Architecture of the Endothelial Cell Cortex." *Biophys. J.* 88: 670-79.

9. A. E. Pelling, Y. Li, W. Shi, and J.K. Gimzewski. 2005. "Nanoscale Visualization and Characterization of Myxococcus xanthus Cells with Atomic Force Microscopy." *Proc. Nat. Acad. Sci. U.S.A* 102: 6484-89.

10. A. Trache and G.A. Meininger. 2005. "Atomic Force-Multi-Optical Imaging Integrated Microscope for Monitoring Molecular Dynamics in Live Cells." *J. Biomed. Opt.* 10: 064023/1-064023/12.

11. A. Berquand, A. Holloschis, S. Ritz, M. Hafner, and P. Kioshis. 2008. "AFM and Neurodegenerative Diseases (part I): Correlating Atomic Force Microscopy (AFM) and Fluorescence Microscopy to Detect Changes in Cell Morphology Caused by Protein Aggregates of Mutant Huntingtin." *AN117*, Veeco Instruments.

12. A. Berquand, A. Holloschis, S. Ritz, M. Hafner, and P. Kioshis. 2008. "AFM and Neurodegenerative Diseases (part II): Correlating Atomic Force Microscopy (AFM) and Fluorescence Microscopy to Study the Interaction between Dopamine and the D1-Receptor in SH-SY5Y Cells." *AN118*, Veeco Instruments.

13. O. Sahin abd N. Erina. 2008. "High-Resolution and Large Dynamic Range Nanomechanical Mapping in Tapping-Mode Atomic Force Microscopy." *Nanotechnology* 19: 445717-26.

14. T. Evers and S. Millar. 2002. "Cereal Grain Structure and Development: Some Implications for Quality." *Journal of Cereal Science* 36: 261-84.

15. J.M. Aguilera, D.W. Stanley, and K.W. Baker. 2000. "New Dimensions in Microstructure of Food Products." *Trends in Food Science & Technology* 11: 3-9.

16. M. Kalab, Miloslav, P. Allan-Wojtas, and S.S. Miller. 1995. „Microscopy, and Other Imaging Techniques in Food Structure Analysis." *Trends in Food Science & Technology* 6: 177-86.

17. G.B. Fincher and B.A. Stone. 1986. "Cell walls and Their Components in Cereal Grain Technology." Advances in Cereal Science and Technology, vol. VIII, Y. Pomeranz (ed.), *American Association of Cereal Chemists Inc.*, St Paul, MN, 207-95.

18. D.H. Simmonds and T.P. O'Brien. 1981. "Morphological and Biochemical Development of the Wheat Endosperm." Advances in Cereal Science and Technology, vol. IV, Y. Pomeranz (ed.), *American Association of Cereal Chemists*, St. Paul, MN, 5-70.

19. H. Yang, Y. Wang, S. Lai, H. An, Y. Li, and F. Chen. 2007. „Application of Atomic Force Microscopy as a Nanotechnology Tool in Food Science." *Journal of Food Science* 72: R65-75.

20. V.J. Morris, A.P. Gunning, A.R. Kirby, A. Round, K. Waldron, and A. Ng. 1997. Atomic Force Microscopy of Plant Cell Walls, Plant Cell Wall Polysaccharides and Gels International." *Journal of Biological Macromolecules* 21: 61-66.

21. A.P. Gunning, A.R. Mackie, A.R. Kirby, P. Kroon, G. Williamson, and V.J. Morris. 2000. "Motion of a Cell Wall Polysaccharide Observed by Atomic Force Microscopy." *Macromolecules* 33: 5680-85.

22. P.M. Baldwin, J. Adler, M.C. Davies, and C.D. Melia. 1998. "High Resolution Imaging of Starch Granule Surfaces by Atomic Force Microscopy." *Journal of Cereal Science* 27: 255-65.

23. J.M.C. Dang, F. Braet, and L. Copeland. 2006. "Nanostructural Analysis of Starch Components by Atomic Force Microscopy." *Journal of Microscopy* 224: 181–86.

24. P. Mongondry, C. Gaillard, P. Colonna, and V. Planchot. 2008. "Role of Amylase Content in the Structure of Potato Starch Granule Revealed by AFM." Submitted to *Biomacromolecules*.

25. S. Neethirajan, D.J. Thomson, D.S. Jayas, and N.D. White. 2008. "Characterization of the Surface Morphology of Durum Wheat Starch Granules Using Atomic Force Microscopy." *Microscopy Research and Technique* 71: 334.

26. H. Yu, R. Liu, D. Shen, Z. Wu, and Y. Huang. 2008. "Arrangement of Cellulose Microfibrils in the Wheat Straw Cell Wall." *Carbohydrate Polymers* 72: 122-27.

27. L. Yan, W. Li, J. Yang, and Q. Zhu. 2004. "Direct Visualization of Straw Cell Walls by AFM." *Macromolecular Bioscience* 4: 112-18.

28. D. Studer, M. Michel, and M. Muller. 1989. "High Pressure Freezing Comes of Age." *Scanning Microsc.* 3: 253-69.

29. D. Studer, W.Graber, A. Al-amoudi, and P. Eggli. 2001. "A New Approach for Cryofixation by High Pressure Freezing." *J. Microsc.* 203: 285–94.

30. R.G. Fulcher 1982. Fluorescence microscopy of cereals. *Food Microstructure* 1: 167-175.

31. S. Krishnan and P. Dayanandan. 2003. "Structural and Histochemical Studies on Grain-Filling in the Caryopsis of Rice (Oryza sativa L.)." *J. Biosci.* 28: 455–69.

32. A. Berquand, P.E. Mazeran, and J.M. Laval. 2003. "Influence of Volume and Surface Properties on Phase Contrast in Tapping Mode Atomic Force Microscopy." *Surf. Sci.* 523: 125-30.

33. C. Roduit, F. Gisou, van der Goot, P. De Los Rios, A. Yersin, P. Steiner, G. Dietler, S. Catsicas, F. Lafont, and S. Kasas. 2008. "Elastic Membrane Heterogeneity of Living Cells Revealed by Stiff Nanoscale Membrane Domains." *Biophys. J.* 94: 1521-32.