Force Scanning with the MFP-3D™ AFMs: Two Capabilities In One

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Atomic force microscopy (AFM) is able to reveal many properties about a material. Most commonly, it is used to obtain topographical information, but it can also probe mechanical stiffness, electrical conductance, resistivity, and magnetism. Researchers have used it to study interactions between enzymes and their substrates[1], structural changes in injured or diseased tissue[2], macromolecular interactions between lipids[3] and analysis of nucleic acid organization and structure[4], to name a few applications. AFM performs analyses on a micro and nanoscale, allowing it to quantify phenomena as miniscule as van der Waals forces, electrostatic interactions, and molecular bonds[5]. AFM is also able to produce high-resolution, detailed images of sample surfaces, displaying micro and nanoscale properties of materials as flat as cleaved mica or as non-uniform as a cell. An interesting aspect to AFM is its ability to measure multiple micro- and nanoscale properties in a single test on samples that are unfixed, unstained, and alive. Of particular use in many fields is the simultaneous measurement of topographical features and mechanical properties.

Traditional light microscopy is able to reveal a wealth of information about a sample, especially a biological one. Light microscopy can tell investigators the shape of a cell, localization of subcellular structures within the cell, and even organization of cellular infrastructure, among many other parameters. But a limitation with these optical data is that we are unable to measure, in a directly quantifiable way, the mechanical properties of that cell; these properties give investigators important information about the cell's cytoskeletal organiza-

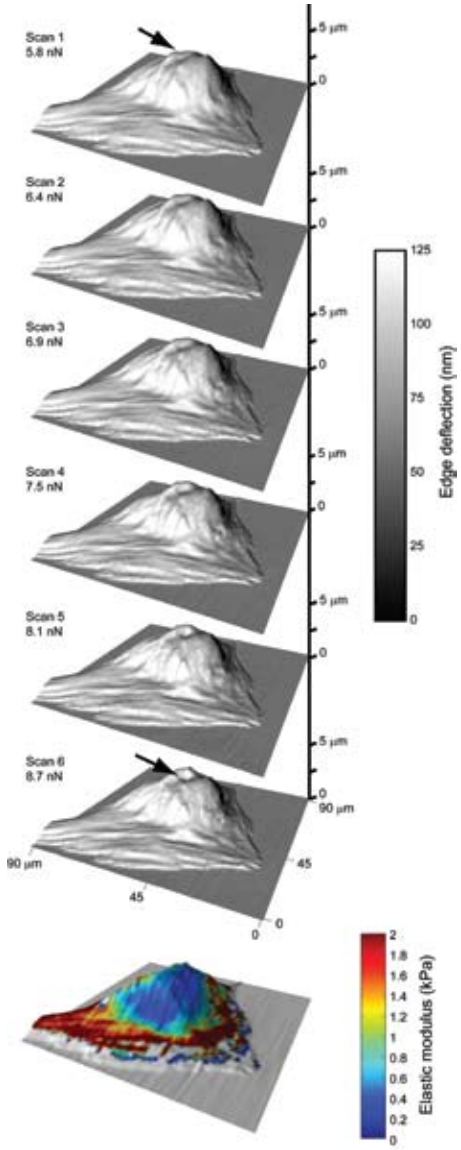

Figure 1: An incremental series of quick, contact-mode scans can be stacked to capture force-indentation curves for all points in an image. Elastic moduli can then be extrapolated from these data, producing high-resolution, spatial modulus maps. The arrow points to a region of greater compliancy located over the cell nucleus.

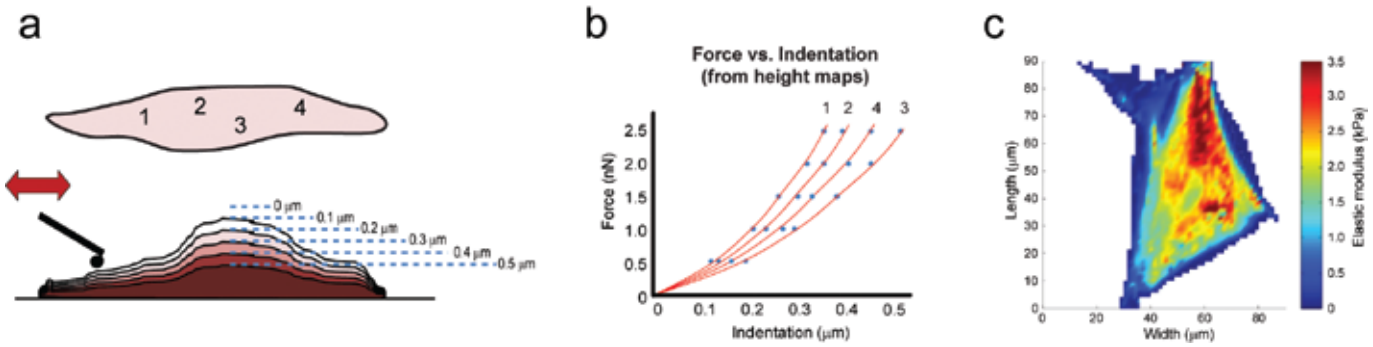

Figure 2: Force scanning involves combining a series of contact-mode scans that deform a material with incrementally larger setpoint forces (a). Using height and setpoint data, force vs. indentation curves can be constructed for all points across a sample, which are then fit with mathematical models to obtain mechanical properties (b). Overlaying this information on the sample can reveal structure-property relationships (c).

tion and phenotype. The cell's stiffness, quantified by measuring the elastic modulus of the cell, is different at various points across its surface; cells tend to be softer over the cytoplasm and stiffer over cytoskeletal structures. Generally speaking, AFM is able to assess both mechanical and topographical properties of any material, including cells, simultaneously in a single assay.

Since the 1990s, AFM researchers have used force mapping to simultaneously provide nanoscale topographical and mechanical information about a substrate. Force mapping involves generating individual force curves at discrete points on a material, which are then used to calculate stiffness and height values[6,7]. This workhorse technique is simple and straightforward, making it easy to implement with any AFM setup. However, it can also be described as a relatively slow procedure with low lateral resolution, which is non-ideal for many biological applications.

We developed a novel technique, termed "force scanning," to generate both structural and functional information about a material of interest through a much quicker process[8] (Figure 1). This AFM-based application rapidly captures high-resolution topographical images of a substrate (a resolution of 32x32 points takes only seconds when using scanning frequencies between 2-5Hz) which are then used to quantify location-specific, mechanical properties during post-processing. Force scanning is a simple to execute, broadly applicable approach to analyze compliant materials. The technique can be used for any deformable material, from agarose gels to cartilage to living cells. It has been shown to precisely map gel surfaces, quantify cell-cell interactions, and characterize living cells and extracellular matrices at high resolutions[7], which is an improvement from previous attempts[6]. Force scanning requires no special cantilevers, equipment, or modifications to standard AFM technology. So long as a clear contact-mode image can be obtained, force scanning is a viable option.

To demonstrate the versatility of force scanning, a variety of samples were assessed using the Asylum Research MFP-3D-BIO™ Atomic Force Microscope, which is mounted onto an inverted optical microscope. Cantilevers were modified with 5µm borosilicate glass beads to ensure well-defined, conformal contact with the sample surface. Indentation with a spherical indenter allows modeling of the mechanical properties of the substrate via the Hertz contact model, which states that when a spherical indenter presses into an elastic surface, the surface area of contact increases and the applied force can be related to the displacement caused by the indenter. Spring constants were calculated from the power spectral density of thermal noise fluctuations prior to each experiment[9]. For data collection, an Asylum Research function, 'LongTermScanning.ipf' (available through the AR Forum), was modified to rapidly collect a series of contact-mode scans. This function was originally designed to collect multiple high-resolution, adjacent scans and stitch them together to create a single, very high-resolution scan that adjusts for voltage drift over time. For the force scanning application, this function was altered so that a single region was scanned repeatedly but with incrementally higher setpoints to

create a stack of topographical images. Scans were done at a 90 degree angle, recording z-piezo movement and normal/lateral deflections. While using a 0 degree scan angle is an option, this can increase the possibility of sample damage and makes it difficult to account for frictional forces, which can be measured using data from the cantilever's lateral deflection. For the examples shown here, however, lateral forces were found to have little effect on the measured mechanical properties because the applied, normal forces were much greater in magnitude, effectively swamping any lateral contributions. While not explored as part of this application note, it would be theoretically possible to choose specific testing parameters (e.g. normal/lateral stiffness of the cantilever, tip geometry, scan rate, setpoint force) that allow for concurrent measurement of frictional properties, surface topography, and material stiffness. Regardless of approach, all recorded data are post-processed and analyzed using custom MATLAB scripts incorporating a Hertz contact model. These scripts correct for any recorded drifts during the test (e.g. z-piezo or laser) and calculate effective indentation forces from the normal and lateral cantilever deflections. Proper calibration of the cantilever is necessary to convert voltage signals into applied force. It should be noted that the contact mechanics involved in this application are complex, and it is recommended that a control material of known stiffness be used to verify the accuracy of the procedure for a specific equipment setup.

Force scanning itself relies on the AFM performing a raster scan across a surface while maintaining a (roughly) constant applied downward force (i.e. *setpoint*). The force causes the cantilever to indent into the surface while it scans, effectively tracing the material of interest and producing an accurate topographical map, albeit slightly deformed in the vertical direction due to the setpoint force. Multiple scans are performed with incrementally increasing setpoint forces, indenting the surface more and more with each iteration (Figure 2). Typically, 5-7 scans are performed per sample. Appropriate setpoint values can vary based on the indenter geometry (e.g. 5µm sphere) and sample characteristics (stiff vs. soft), both of which should be determined prior to force scanning. A few indentation curves on the sample should be sufficient to determine an appropriate range of values (e.g. 4-12nN for agarose, 0.5-4.5nN for cells, 150-500nN for cartilage). The amount of deflection and indentation will vary with the stiffness of

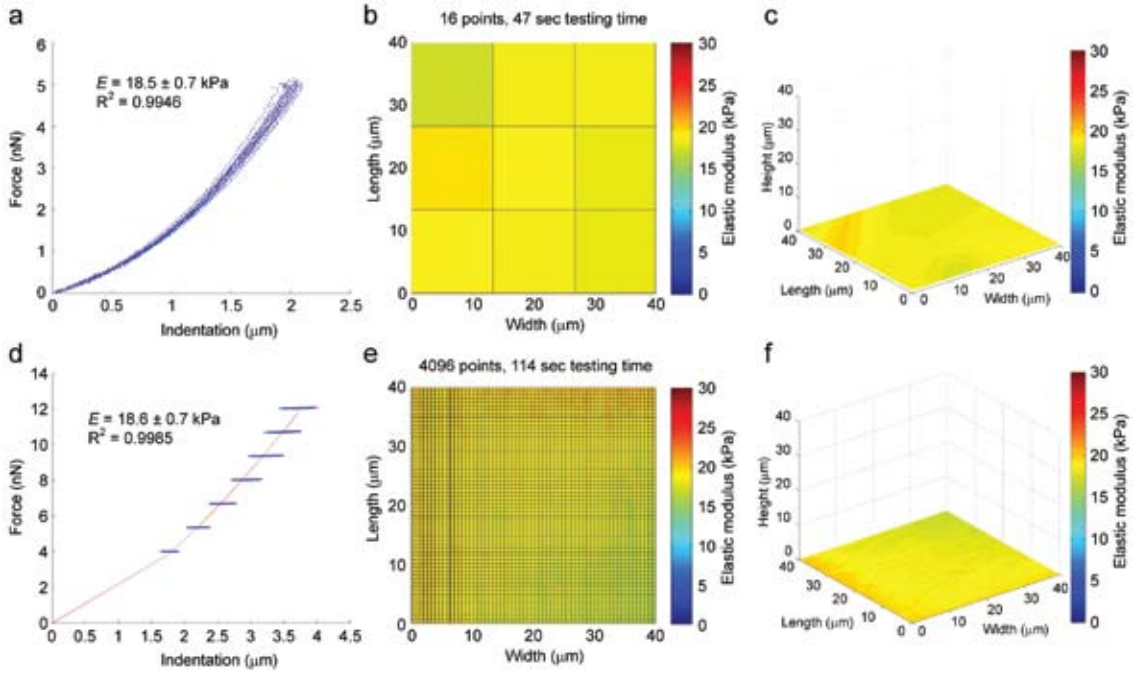

Figure 3: Validation of the force scanning technique was done in part using a soft agarose gel. The standard approach, force mapping, involves applying a series of single indentations to obtain mechanical and topographical data, although at relatively low lateral resolution (a, b, c). Force scanning of the same sample can achieve much more detailed images by sacrificing force curve resolution (d, e, f). The mechanical characteristics of the sample are virtually identical.

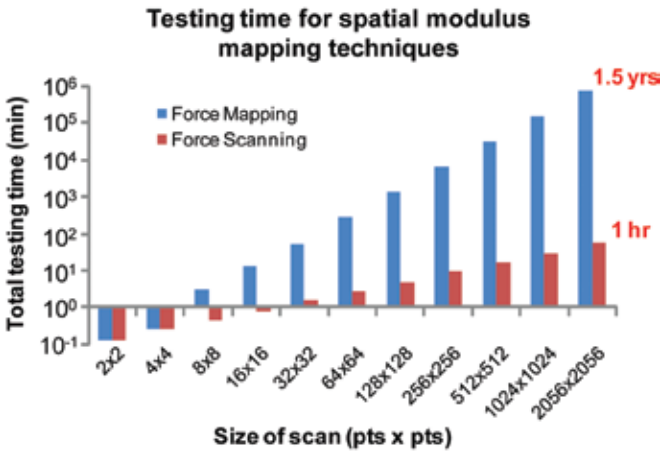

Figure 4: High-resolution scanning takes prohibitively long using standard techniques. Based on temporal trends observed during testing, very high-resolution images could only be achieved using force scanning within a reasonable time frame. For example, a scan size of 2056 x 2056 points would require over a year to complete using force mapping, whereas force scanning cuts that time down to just an hour.[8]

the substrate; thus, the setpoint range maxima and minima are determined by performing single force indentation curves on each material. A setpoint that is too low will not produce consistent deflection of the cantilever, while a setpoint that is too high will violate the mathematical model or damage a living sample. Each scan provides raw data for heights, normal deflection, and lateral deflection, which can be easily transformed into applied force and indentation. The relationship between these two values describes the elastic modulus of a material.

Direct comparisons of force mapping and force scanning are difficult to do since force scanning does not apply discrete force curves at specific points on a substrate but instead generates them from the data. However, results for the two methods on the same sample are remarkably similar; validation tests on agarose gels showed that measured elastic moduli did not vary significantly (Figure 3). The major benefit of using force scanning is the simplicity and speed with which the tests are completed. Surface images can be done using a range of scan rates that can shorten or lengthen to overall process. The examples presented here typically took scans at 1-5Hz (20-450µm/s scan rate, depending on region size), which provided a good balance between short testing times and good image quality. The other factor involved in force scanning duration is the number of setpoints used to generate the force curve, with each one requiring a complete scan. At least 4-5 are recommended for fitting the Hertz model, but more can be taken if desired. Force mapping, on the other hand, requires time to take not only the individual force curves (typically done at 0.5-15µm/s) but also time to move from point to point. This process can be lengthened if the relative height of the sample varies greatly, since full force curves (e.g. 5-20µm in length) are necessary to maintain tip-sample separation before indentation, and account for any artificial changes in the cantilever deflection during approach. This translates into a much slower process than for force scanning. Based on empirically determined testing times, if a scan size of 2056 x 2056 points is desired, it would take about a year and a half using force mapping, whereas force scanning cuts that time down to just sixty minutes (Figure 4). While these testing times can vary depending on the sample characteristics and the scanning parameters used, generally force

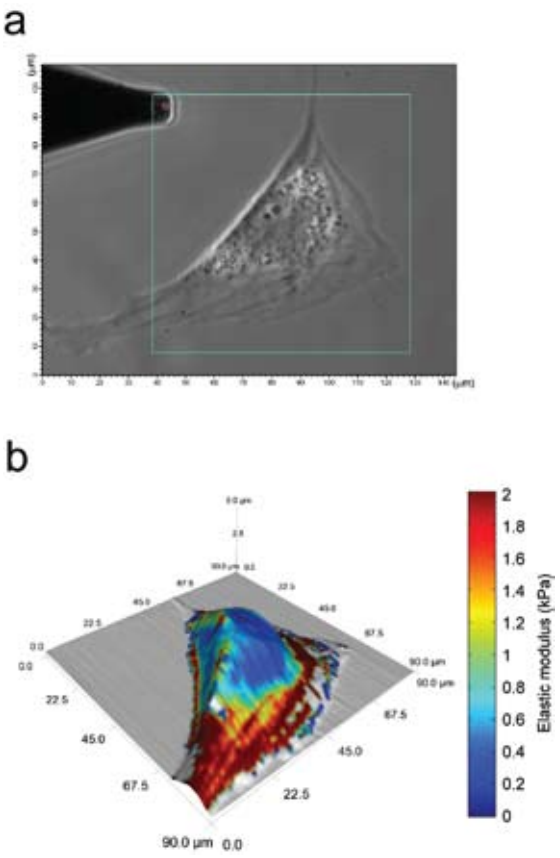

Figure 5: Living, adipose-derived stem cells (ASC), viewed using phase contrast imaging, can be assessed for mechanical property variations across their surface (a). A 128x128 point scan reveals lower moduli in the perinuclear area and higher moduli over cytoskeletal actin filaments (b). This detailed, topographical and mechanical property information was obtained from a six minute scan.[8]

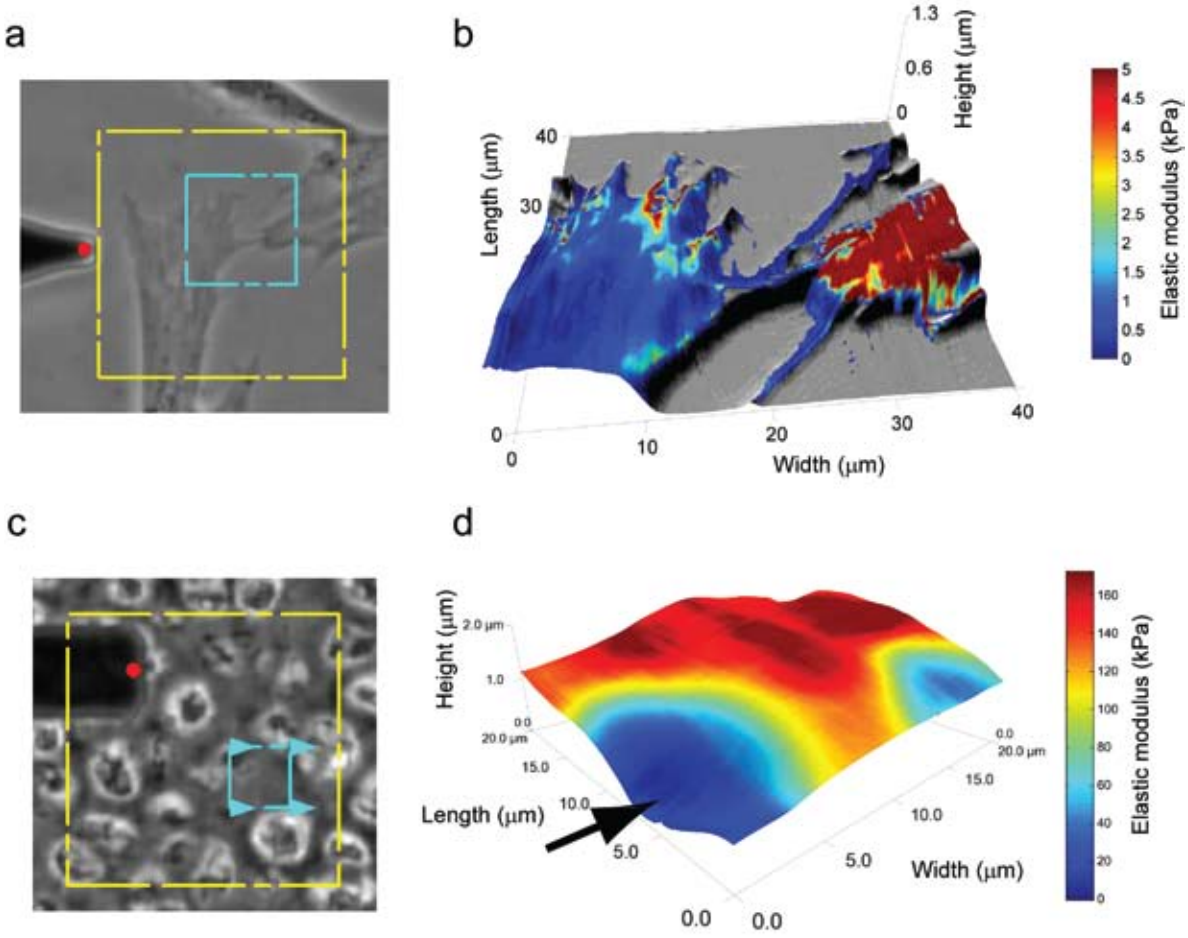

Figure 6: Spatial modulus maps can be used to investigate cell-cell interactions (a-b). Variation in elastic moduli between the two cells is clear, with one cell being much stiffer than the other. Furthermore, filopodial extensions are shown to be much softer than the cell body, although the measurement accuracy for these extremely thin regions is still under investigation. Force scanning also shows excellent applicability for probing the mechanical properties of biological tissues, like the extracellular and pericellular matrices of articular cartilage (c-d). In the rotated force scanning image, the arrow denotes where a cell would be in the native tissue, and the tissue gets progressively stiffer moving outward from the cell edge to the pericellular matrix to the extracellular matrix. The dotted, blue box in (a) and (c) defines the scanned regions.

scanning ends up being faster by trading off force curve resolution in exchange for speed and overall image resolution. Test duration is important for many samples and is absolutely critical for living materials like cells. Force scanning is sufficiently fast that tests conducted on living samples have the possibility of being completed before major conformational changes take place, a capability lacking in conventional force mapping.

Force scanning reveals a wealth of information when used on biological samples that might not be possible if using a slower technique like force mapping. Subcellular features can be distinguished topographically and mechanically, the cell nucleus in particular. The difference in elastic modulus between the cell cytoplasm and the cell nucleus is clearly visible during a force scan, as seen by the variation in elastic modulus between the perinuclear and cytoplasmic regions of the cell in Figure 5. Spatial modulus mapping can also reveal the mechanical characteristics for stress fiber distributions and lamellipodial extensions, composing a detailed and informative image of the cell under analysis. The force scanning technique has also been used to investigate cell-cell interactions and the mechanical characteristics of biological tissues (Figure 6). While the elastic moduli associated with these two materials are orders of magnitude different, the force scanning application can accurately quantify the mechanical properties of either by using the appropriate cantilever and scanning parameters.

Force scanning is also widely adaptable and applicable to a variety of fields; although it works particularly well with soft biological samples and

biomaterials, it has applications in many other fields and areas of research as well. This method can easily be used to measure the mechanical and cosmetic features of any deformable material that can be assessed using contact-mode AFM. Force scanning can be done in fluid or air environments, on stiff or soft materials, without need for modification of standard AFM technologies. Post-processing of the data is necessary to calculate mechanical properties, but this can be done using a variety of available software packages (e.g. MATLAB).

Though this technical note focuses on using force scanning to quantify mechanical and topographical features, adaptations to the technique open many other possibilities for analysis. For example, functionalization of the cantilever tip could yield data on frictional properties or adhesion forces in comparison to data collected using a non-functionalized tip. Likewise, the frictional properties of a surface can be quantified using lateral force data. Even without these new adaptations, being able to rapidly quantify the mechanical characteristics of biological samples in relation to physical structures allows for the study of many phenomena. Mechanotransduction, in which cells translate mechanical signals into biological responses, requires knowledge of the mechanical properties of the cell and environment. Cell attachment and movement are other processes that involve mechanical changes at the subcellular level which can be investigated using this technique. Force scanning is quick, simple, and easy to implement, paving the way for higher throughput analyses with greater resolution and detail. With this application, researchers can better investigate cytoskeletal organization, cell phenotype, and microenvironment materials under physiological conditions, which allows for the study of healthy, living samples.

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