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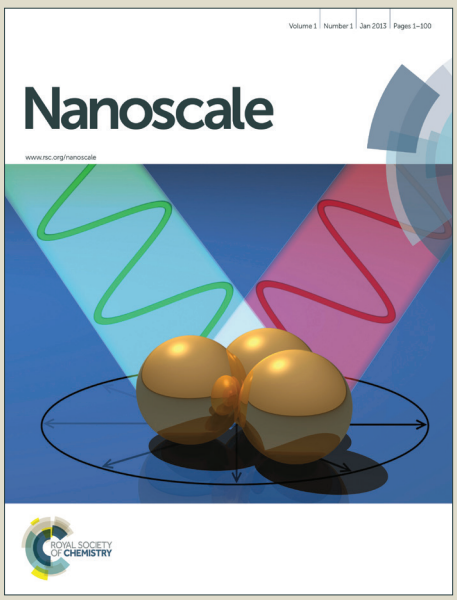

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Comment on "The Ultrastructure of Type I Collagen at Nanoscale: Large or Small D-Spacing Distribution?"

It was with great interest that we read the recent work of Su and colleagues (1). As they note, D-spacing is an important structural feature of collagen which we believe is related to the internal assembly of fibrils as well as enzymatic and non-enzymatic cross-links. Importantly, measuring D-spacing and its distribution in tissues may provide nanoscale information related to disease states and their impact on the assembly of collagen molecules and fibrils. Our previous studies have reproducibly shown that distributions exist in all Type I collagen based tissues, and these distributions shift under various conditions including disease, pharmacological treatment and mechanical stimulation.

One highlighted finding in the paper by Su and colleagues was that collagen D-spacing values measured with AFM have a small distribution of 2.5 nm centered at 67 nm. The authors suggest that previously reported larger ranges in distribution are an artifact of thermal drift during imaging. Our lab has studied thermal drift and many other assumptions and limitations of AFM assessment of collagen (2). To investigate the impacts of thermal drift, a single 3.5 μm × 3.5 μm region in bone was scanned up and down continuously over 1.5 h at 2 Hz (20 scans). The D-spacing of single collagen fibrils at various angles was monitored. In this exaggerated case of what might happen during a normal scan (which would typically take just over 8.5 minutes when scanning at 1 Hz and 512 lines as noted by the authors), the variation in D spacing of individual fibrils was less than 2 nm. During a single scan, even at a slower rate of 0.5 Hz, this drift would have a negligible effect on D-spacing. As further evidence that the scan direction and drift do not significantly affect our D spacing measurements or distributions, we found no relationship between D-spacing and the angle of measured fibrils relative to the fast scan direction. Together, these data suggest that systematic errors which may arise from thermal drift and scanning angle do not impact D-spacing measurements. The same publication investigated tendon fibrils in historical SEM images (2), a technique that is not impacted by thermal drift. A similar distribution of D spacing was noted, with a width of nearly 8 nm from a limited sampling of 13 fibrils. These data support that the presence of relatively wide distributions is real and not caused by AFM imaging artifacts.

Su and colleagues also reported that the value at the center of their distribution was 67 nm. It should be noted that in order for measurements of D-spacing made using AFM to have accuracy and precision, proper attention must be paid to system calibration and the method of analysis used. These issues have also been address in detail in the aforementioned publication (2). When making absolute distance measurements, accuracy requires proper piezo calibration. Su and colleagues indicate that prior to imaging, their AFM was calibrated according the manufacturer's instructions. Bruker suggests using a 10 μm pitch calibration standard and operating at the full x-y range of the AFM. We found that following this procedure led to an absolute error of over 9% in the fast scan direction when scan size and surface feature size were reduced to a size comparable to the D-spacing of collagen. This uncertainty would mean an absolute error of over 6 nm on a 67 nm measurement in a 3.5 μm × 3.5 μm image, suggesting that a true 67 nm feature could measure anywhere between 67 and 73 nm. In our work we have found best results by calibration of the AFM using a much finer 100 nm standard to within 1.5% in both x and y directions, using the same probes and scan parameters used in our experimental tissue samples.

Finally, the author's indicate that D-spacing was measured within the manufacturer's software, suggesting that analysis by line scans was employed. This technique suffers from limited lateral resolution due to pixel size of the image (~7 nm in a 3.5 μm images at 512 x 512 pixels) and the radius of curvature of the imaging probe. In addition, the user must draw a line along a fibril which is orthogonal to the fibril's D-spacing. We found that a 5° deviation away from normal can alter the measured value by as much as 8% (2). To bypass these limitations, analysis using 2 dimensions fast Fourier transforms (2D FFTs) in third party software is preferred.

We applaud the work of Su and colleagues as it highlights the important role of collagen ultrastructure. However we believe it is premature to suggest that collagen distributions are simply artifact and encourage more work in this area to gain insight into the nanoscale mechanisms which underlie a tissue's response to a variety of conditions.

Joseph M. Wallace, Ph.D.
Assistant Professor
Department of Biomedical Engineering
Indiana University-Purdue University Indianapolis

Department of Orthopaedic Surgery (adjunct)
Indiana University School of Medicine

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