Nanomechanical mapping of hydrated rat tail tendon Collagen I fibrils Supplement

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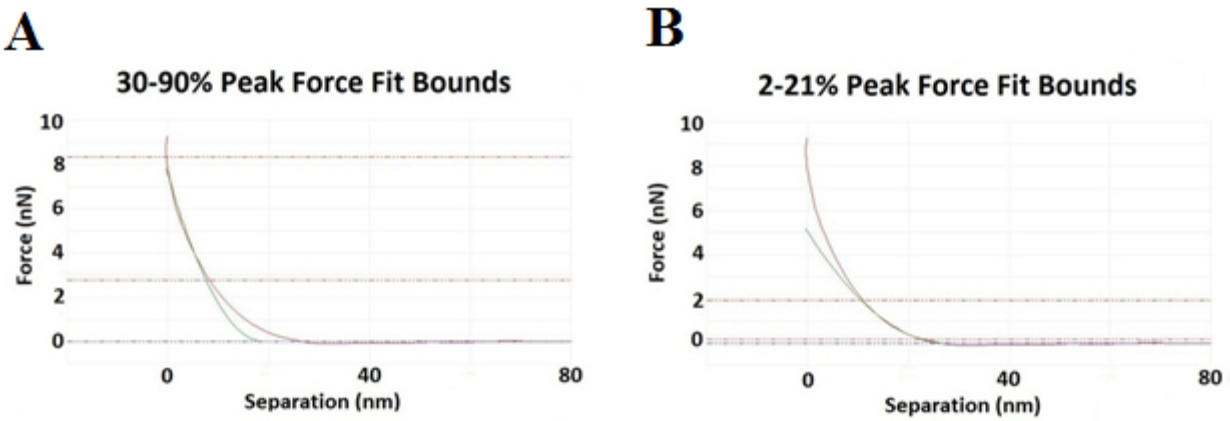
Modulus distribution

A simple methodology to extract modulus distributions over an entire segment of a fibril that takes into account the effect of the underlying substrate and the shape of the fibril was developed. Fitting different regions of the FD curves has a dramatic impact on the obtained modulus distribution. As an example we extract the modulus distribution of a collagen fibril using two different methods. We either fit the upper part of the FD curves between 30 and 90% of the applied peak force (Fig. S1 A) or we fit the lower part of the FD curves up to an indentation depth corresponding to 10% of the average "zero force" height of the fibril (Fig. S1 B) in accordance with Bueckle's rule.(1, 2) The corresponding images and histograms are very different with an average modulus of 63.5±0.3 and 15.5±0.1 MPa, respectively (Fig. S2 A,B, C and D). This fourfold overestimation of the modulus in the first method is attributable to the presence of the glass substrate underneath the fibril and highlights the importance of only fitting the FD curves according to Bueckle's rule.(1, 2)However, it is noticeable that in both methods the modulus distribution has a tail on the left side of the main peak (Fig. S2 C and D).



(Figure S1: *Regions of fit.* Two different sneddon modulus fits of a force-seperation curve acquired from the apex of a collagen fibril. A) 30-90% peak force fit bounds. B) 2-21% peak force fit bounds corresponding to an indentation depth of 10% of the average "zero force" height of the fibril.

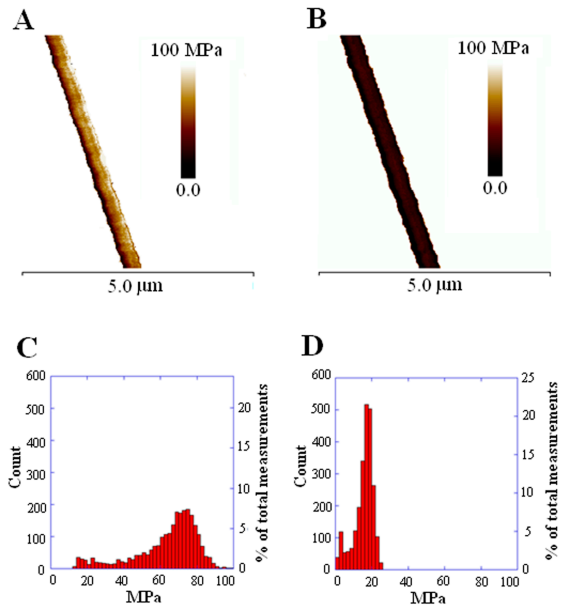


Figure S2: *Effect of the substrate on the modulus distribution*. Modulus maps of a collagen fibril. A) Fitting the FD curves in the region between 30 and 90% of the applied peak force. B) Fitting the FD curves up to an indentation depth corresponding to 10% of the average "zero force" height of the fibril, 20 nm in this case. C) (n = 2508) and D) (n = 2387) Corresponding histograms of modulus distribution. Notice that the first method provides a fourfold overestimate of the modulus compared to the second method.

In order to understand the origin of this tail we look at the systematic variations in peak force associated with the response time of the feedback loop observed in AFM tapping modes (1.7)(Fig. S3). The image obtained from the peak force error channel always shows a dark and a bright edge on each side of the fibril (Fig. S3 A). Inverting the scanning direction exchanges the bright and dark edges. One possible explanation is that the response time of the feedback loop is too long to accommodate the rapid change in topography on the sides of the fibril. Another explanation is that the bright edge occurs due to coupled cantilever torsion and deflection away from the fibril. This results in an applied force in excess of the set point visible as a spike on the

right side of the peak force error profile across the fibril (Fig. S3 C). As the tip scans across the fibril the peak force error first (1.7) goes through zero as it passes the apex and then spikes negatively as it slips of the side of the fibril (Fig. S3 C), giving rise to a dark edge on the image (Fig. S3 B).

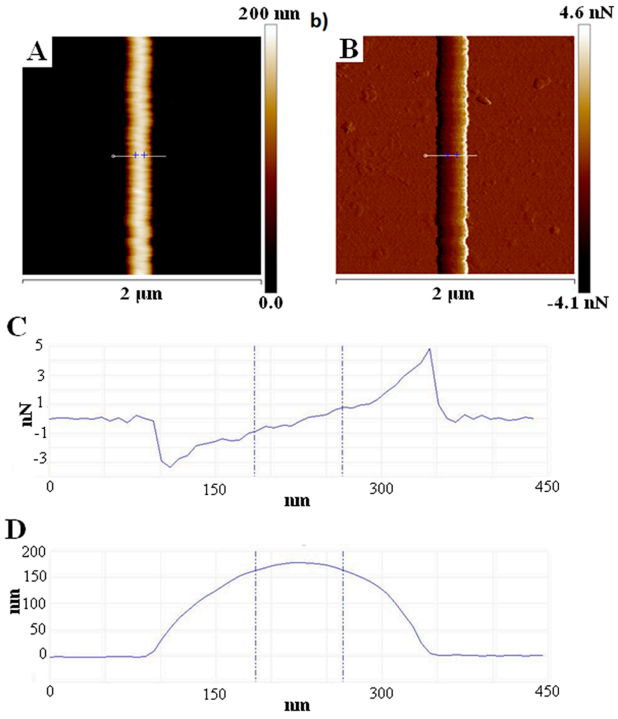


Figure S3: *Variations in applied peak force correlate with sample topography*. Height and peak force error images of a collagen fibril, A) and B), respectively. The peak force set point was 10 nN and the scanning direction was right to left. C) and D) are corresponding line profiles across the fibril. The region where the peak force error is ±10% of the peak force set point (broken lines) corresponds to the apex of the fibril.

Due to the systematic nature of the peak force error profile across a fibril, it is possible to select different regions across the fibril by selecting different ranges of peak force error (see Supplement S5). Force curves were sorted into 0.1 nN bins to allow accurate fitting parameters to be applied to the sorted force curves due to the uniformity of the applied force within each sorted bin. This becomes relevant when considering the fitting parameters within the analysis software are based on percent peak force per curve and not on specific force values.

The modulus distributions extracted from a 5 by 5 μm image are shown for an entire fibril (Fig. S4 A), the left side corresponding to negative peak force error (Fig. S4 B), the right side corresponding to positive peak force error (Fig. S4 C), and the apex corresponding to minimal peak force error (Fig.S4 D). The tail in the distribution obtained for the full fibril is associated only with the left side of the collagen fibril (Fig. S4 B) whereas the positive peak force error on first contact is responsible for a broadening of the main peak (Fig. S4 C). The distribution obtained from the apex of the fibril is Gaussian with a standard deviation of 2.5 MPa giving rise to an estimate of the modulus with an accuracy better than 1% at 18.63±0.07 MPa (Fig. S4 D). This is not the absolute accuracy of the measurement since the cantilever spring constant calibration is only 10% accurate, but it shows the potential of the approach to detect fluctuations in modulus along the length of a fibril or before and after modifications of a fibril by an external factor such as temperature or pH.

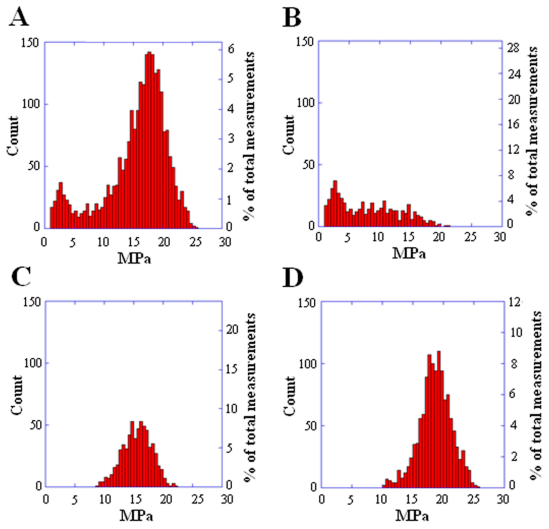


Figure S4: *Selecting the apex of the fibril using the peak force error*. Modulus distributions extracted from a 5 by 5 μm image of a collagen fibril according to Bueckle's rule (ref). A) Entire fibril, peak force error ranging from -5 nN to +5 nN. B) Region where the tip slip on the fibril side, peak force error ranging from -5 nN to -1 nN. C) Region where the tip first contacts the fibril, peak force error ranging from +1 nN to +5 nN. D) Apex of the fibril, peak force error ranging from -1 nN to +1 nN. The peak force setpoint was 10 nN as in Figure 2. The total number of force curves (n) of each region ,a-d, are 2297,515, 632, and 1250 respectively.

Supplement S5

Sorting Algorithm (MATLAB v7.10.0)

```
DataSourceFolder =('C:\Users\Sam\Desktop\before\');%make sure it ends
with a \
DataSortedFolder =('C:\Users\Sam\Desktop\after\'); %make sure it ends
with a \

LowBound =(0)
HighBound = (22)
SEnN = (.1)  %Sorting by this many nN  MAKE SURE THAT FOLDERS
GENERATED ARE ALWAYS A FACTOR OF AN INTEGER.
SD = (1/SEnN)  %Sorting Denominator


LowerBound= (LowBound);
UpperBound= (HighBound*SD);
BoundRange=[LowerBound:UpperBound];

%Making Destination Folders

mkdir(DataSortedFolder)
for x=(BoundRange)
    str = num2str(x/SD);
    MakeFolders = strcat(DataSortedFolder,str);
    mkdir(MakeFolders)
end
UBoundFolder=strcat(DataSortedFolder,'Above Upper Bound');
mkdir(UBoundFolder)
LBoundFolder=strcat(DataSortedFolder,'Below Lower Bound');
mkdir(LBoundFolder)
```

```
%Defining Nanoscope utilities
NS = NSMatlabUtilities();

F = dir(DataSourceFolder);
F = F(~[F.isdir]);
NumberOfFiles = length(F);


%CHECK TO SEE I AF NEEDS TO UPDATE WITH EACH LOOP AKA IT LOOKS FOR
(1,1)
%and its GONE after first go
for p=[1:NumberOfFiles]
    FN = {F.name}; %FileNames=FN
    AF = FN(1,p); %ActiveFile=AF
    AF = AF{1};  %Reads Cell element as a string
    TAF = strcat(DataSourceFolder,AF);%TAF= total active file path
    NS.Open(TAF)
    [xTrace, xRetrace, yTrace, yRetrace, xLabel, yLabel] =
NS.CreateForceZPlot(1, NS.FORCE, 0);
    PF = max(yTrace);
    for x=(BoundRange);
        if ((x/SD)> PF+SEnN)
                break;
        end
        if ((x/SD)>PF)
            XS=num2str(x/SD);
            TargetFolder = strcat(DataSortedFolder,XS);
            movefile(TAF,TargetFolder)% EX if moved to folder 9 the PF
is between 8 and 9
            %WHEN USEING FITTING SOFTWARE USE %BOUNDS AJUSTED FOR THE
            %MIDDLE OF THE RANGE EX 6-7 BOUNDS ARE AT 6.5nN
        elseif (PF>UpperBound)
            movefile(UBoundFolder)
```

```
        elseif (PF<LowerBound)
            movefile(LBoundFolder)
        end

    end
end
```

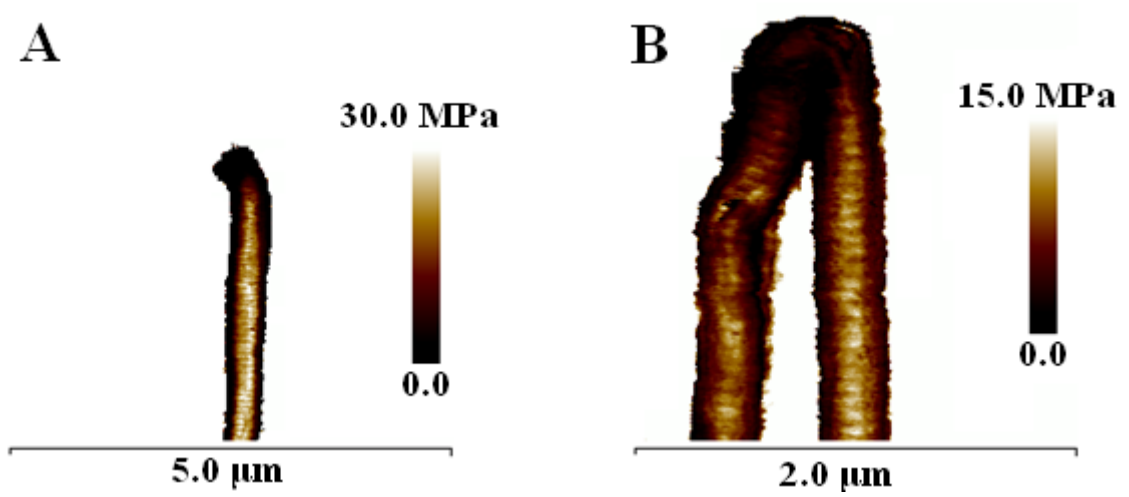


Figure S6: *Modulus maps of mechanical alterations*. A cleaved collagen fibril (a) and a sharp bend (b). The D-period is observed in the surrounding regions demonstrating a decrease in average modulus, but not at the localized site of alteration.

Collagen assembly *in vitro*.

In vitro collagen fibrils were synthesized from rat tail collagen I purchased from (Sigma, St. Louis, MO). The tropocollagen molecules were diluted in PBS to a concentration of .2mg/ml. Such solutions were incubated ate 37°C for 1 hour after which 0.5 ml of solution was deposited onto a glass bottom dish. After 1 hour the samples were trice rinsed with deionized water to remove residual salts. During the washing process careful technique ensured the sample remained hydrated. The hydrated sample underwent mechanical measurement as previously described or was stored at 4°C until use. The fibrils formed were polydisperse in their dimensions, while only those with a zero force height greater than 200nm were used for the acquisition of figure S8. The fibrils displayed no D-period when hydrated, but it appeared upon dehydration. Interestingly the *in vitro* fibrils displayed a twisting structure in the modulus channel similar to that of *ex vivo* fibrils after temperature exposure.

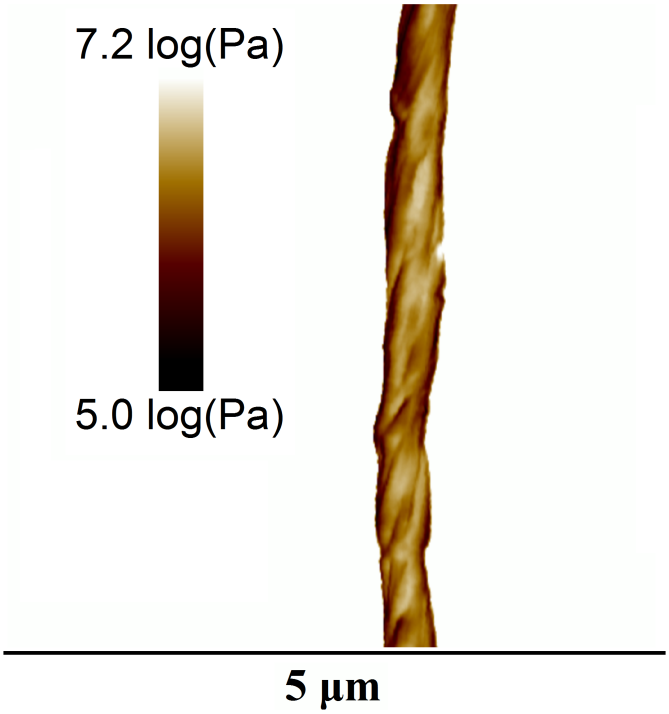


Figure S7: *Twisted structure of in vitro fibrils in modulus.* The log sneddon modulus map of an in vitro fibril acquired at 1200 µm/s.

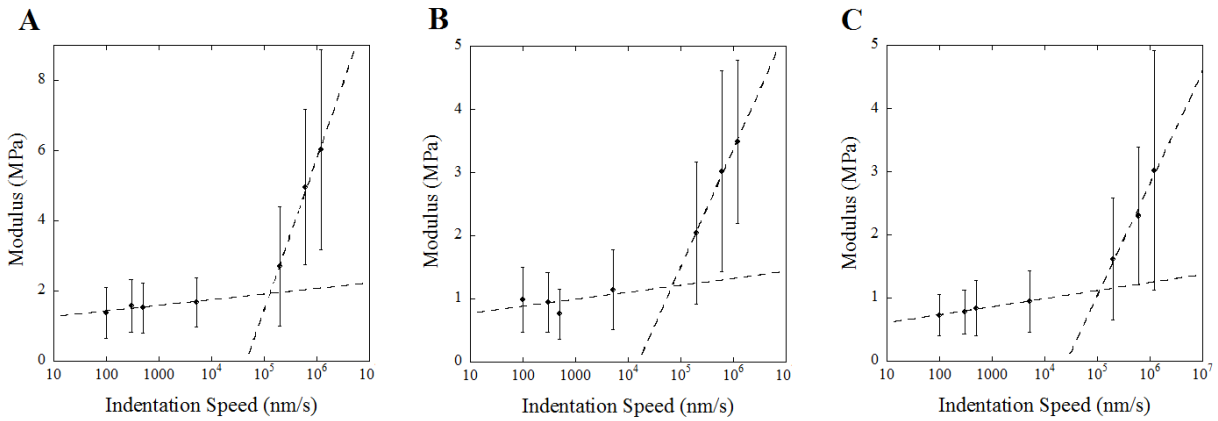


Figure S8: *Dependence of the modulus of in vitro fibrils with indentation speed*. The modulus of three, 5µm segments of different *in vitro* assembled collagen fibrils as a function of indentation speed. Logarithmic least square fits of the data highlights the presence of two distinct regimes.

Supporting references

1. Persch, G., C. Born, and B. Utesch. 1994. Nano-Hardness Investigations of Thin-Films by an Atomic-Force Microscope. Microelectronic Engineering 24:113-121.
2. Bueckle, H. 1973. The Science of Hardness Testing and its Research Applications. American Society for Metals, Ohio.