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Method Article

Atomic force microscopy nano-characterization of 3D collagen gels with tunable stiffness

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A B S T R A C T

As extracellular matrix (ECM) nano-characteristics play a crucial role in cell behavior, including cancer development and metastasis, several ECM in vitro models have been used in order to study cells behavior under different biochemical and mechanical conditions. Among the ECM constituents, collagen (especially collagen type I) has been extensively used as an essential component of ECM models, since it is one of the most abundant ECM protein. Use of three-dimensional (3D) collagen gels provides the advantage of allowing the cells to grow in a 3D environment that bears strong similarities to their natural, in vivo setting. Thus, the ability to form collagen gels with tunable stiffness and well defined naturally occurring nano-characteristics is crucial for these studies. Atomic Force Microscopy (AFM) is a unique tool that is ideal for the complete characterization of such models, in terms of morphology and mechanical properties without destroying the collagen fiber structure. In this protocol, the development and the AFM nano-scale characterization of 3D collagen type I gels is presented. The protocol includes:

- The formation of 3D collagen type I gels with tunable stiffness
- The preparation of histological sections from collagen gels
- The AFM-based morphological and mechanical nano-characterization of the gels

A R T I C L E I N F O

Method name: AFM for 3D collagen gels

Keywords: Collagen gel histological sections, D-band periodicity, Stiffness, Young's modulus

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Specifications Table

Subject area	*Materials Science*
More specific subject area	*Nano-characterization of biomaterials*
Method name	*AFM for 3D collagen gels*

Formation of collagen gels with tunable stiffness

For the formation of 3D collagen I gels of tunable concentration and stiffness, we use a modification of a previously published protocol [1]. A high concentration collagen I solution (8.34 mg/ml, rat tail collagen, 354249, Corning) is carefully chosen, as not all commercially-available collagen solutions are appropriate for formation of 3D collagen gels. Then, collagen solution concentration and pH is adjusted, accordingly. The required materials and equipment include: high concentration collagen (e.g. rat tail collagen Corning 354249), Minimal Essential Medium (MEM, 10x) (e.g. MEM 10x, 21430-020, Gibco - Life Technologies), Human insulin solution (e.g. I9278, Sigma-Aldrich), distilled water (e.g. dH_2O, DNAse/RNase Free, 10977-035, Gibco by Life Technologies), 1 N NaOH, glass cover slips or petri dishes depending on the experimental procedure, falcon tubes, pipettes and pipette tips and sterile cell culture hood. The following table includes all products used in all steps of the protocol (Table 1).

Detailed protocol for collagen gel formation

(1) Add the appropriate amount of collagen so as to get the desired final concentration in a solution containing 10% 10x MEM, 1% human insulin solution and dH_2O. For the validation of this procedure we used a collagen solution with initial concentration of 8.34 mg/ml and we generated gels of 0.5, 1.0 and 3.0 mg/ml. Table 2 presents the exact amounts used for the formation of 1 ml of each one of the designated collagen gels. More specifically, this step requires to:

a Add MEM, insulin and dH_2O to a mixing tube
b Add collagen to mixing tube
c Mix solution gently by inverting several times:
(2) *Adjust the pH to approximately 7.4 by adding 1 N NaOH.*
d Add appropriate amount of 1 N NaOH and mix solution gently by inverting the tube several times until the solution becomes pink.

Table 1
List of products employed for the protocol.

Product	Product Numbers
Collagen	Rat Tail Collagen, 354249, Corning
Minimal Essential Medium	MEM 10x, 21430-020, Gibco - Life Technologies
Human Insulin Solution	I9278, Sigma-Aldrich
Distilled Water	dH_2O, DNAse/RNase Free, 10977-035, Gibco - Life Technologies
Finder Grid	Copper finder grid, G2761C, Agar Scientific
Tissues Embedding Medium –optimal cutting temperature compound (OCT)	OCT Compound, 4583, Tissue-Tek SAKURA
Phosphate Buffered Saline (PBS)	PBS, LM-S2041, Biosera
Cryomolds	Cryomolds, 4728, Tissue-Tek SAKURA
Plastics Molds	Weighing boats, 30321, Sterlin
Contact mode AFM probes	SICON, Applied Nanostructures
Tapping mode AFM probes	ACT, Applied Nanostructures
Mechanical properties characterization AFM probes	PNP-TR, Nanoworld

Table 2
Amount of gel ingredients used for the formation of 1 ml of collagen gel of different concentrations.

	0.5 mg/ml	1.0 mg/ml	3.0 mg/ml
MEM 10x (μl)	100	100	100
Insulin (μl)	10	10	10
Collagen (μl)	60	120	360
dH_2O (μl)	830	770	530

NOTE: All manipulations following pH adjustment should take the minimum amount of time as collagen solutions tend to solidify soon after pH adjustment.

(3) *Preparation of collagen gels*:

e *Add the* solution prepared from the table *on a cover slip or petri dish and incubate in a cell incubator at 37 °C for at least 30 min.*

f *For AFM studies using glass cover slips, 200 μl of collagen solution are added on a cover slip (of 12 mm diameter). Special attention should be paid to cover the entire surface of the cover slip with collagen and avoid formation of bubbles.*

g *Remove samples from the incubator and carefully attach each cover slip to the AFM disc using double-sided adhesive tape.*

h *Let the samples dry in air at room temperature overnight.*

i *Mount coverslips on the AFM sample disc/stage.*

j *For AFM studies under liquid conditions add 2 ml of the solution in a 6-well plate* (Fig. 1) *or a 35 mm petri dish (depending on the AFM modality that you are using) and attach them directly in the AFM sample disc/stage. Add in the well or plate 1 ml of phosphate-buffered saline (PBS, LM-S2041, Biosera) or cell culturing media.*

(4) Preparation of samples for AFM imaging:

k *Mount a small piece of a double-sided tape or (double-sided conductive taps (you can also use epoxy glue) on an AFM metal specimen disk.*

l *Attach a finder grid (e.g. Copper finder grid, G2761C, Agar Scientific, Essex, UK) on the tape.*

m *Then firmly mount the sample with the collagen gel* (*see*Fig.2).

n *Mount the sections on AFM sample plates for AFM imaging.*

- The use of finder/locator grids help the AFM user to map the surface [2]. It should be noted that more than one finder grids can be used to map a larger proportion of the sample's surface. The finder grids can be applied both for imaging different areas of the collagen gel and for indentifying the same region of interest or even the same collagen fiber at a later stage [3–6].

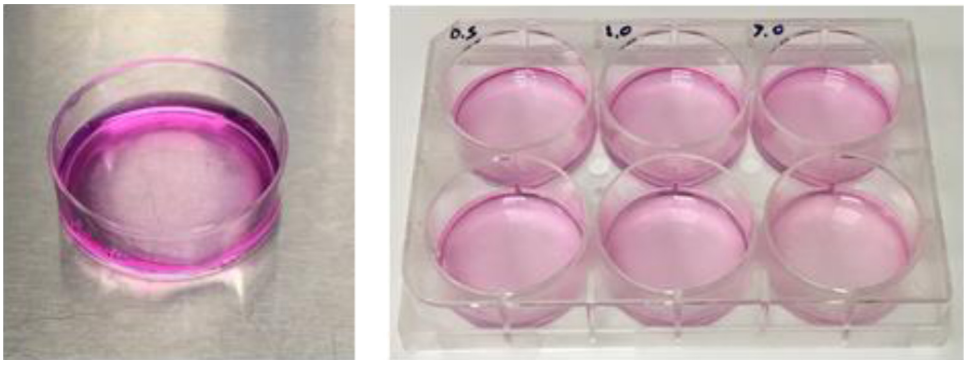

Fig. 1. Collagen gels with different concentration in a petri dish and 6-well plate.

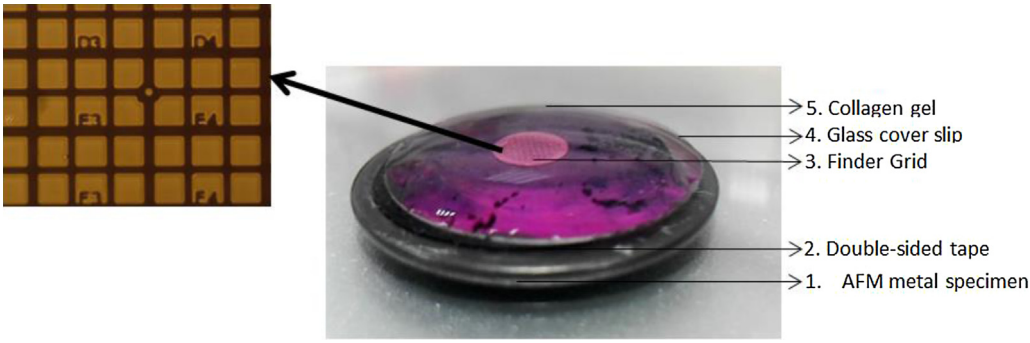

Fig. 2. The use of finder grids to map the collagen surface. Left: Optical microscope (10x) of the locator grid. Right: The placement of the finder grid between the AFM metal specimen dick and the sample.

Preparation of histological sections from collagen gels

For the preparation of histological sections [7], the following materials and instruments are required: plastics molds, 4% paraformaldehyde (PFA), PBS, Tissues Embedding Medium –optimal cutting temperature compound (OCT, OCT Compound, 4583, Tissue-Tek SAKURA), liquid nitrogen, cryostat, microtome blades, coverslips or positively charged microscope slides and ultrapure water.

a Prepare collagen gels according to the previously-mentioned protocol in cryomolds (4728, Tissue-Tek SAKURA) or plastics molds (Weighing boats, 30321, Sterlin) (Fig.3).
b Fix the gels by the adding 0.5 ml of 4% PFA for 30 min [7].
c Wash gels three times with PBS.
d Cover the gels with OCT and incubate overnight at 4 °C.
e Snap-freeze the plastic molds in liquid nitrogen (Fig. 4).
f Gently remove the gels from the plastic molds and cut them into 40 μm thick sections using a cryostat.
g Mount tissue sections on coverslips or positively charged microscope slides.
h Wash the sections with ultrapure water to remove excess OCT and let the samples dry in air at room temperature.
i *Mount the sections on* AFM sample plates for imaging.
j Start AFM imaging (Fig. 5).

Fig. 3. Collagen gels in cryomolds and plastic molds of different shapes.

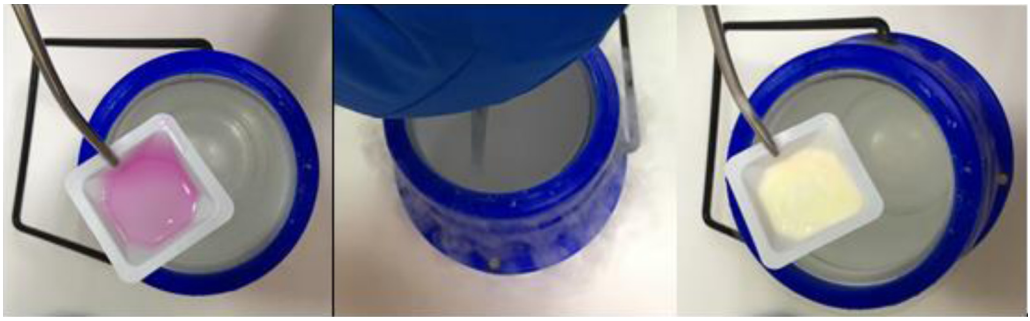

Fig. 4. Snap-freezing of collagen gels in plastic molds.

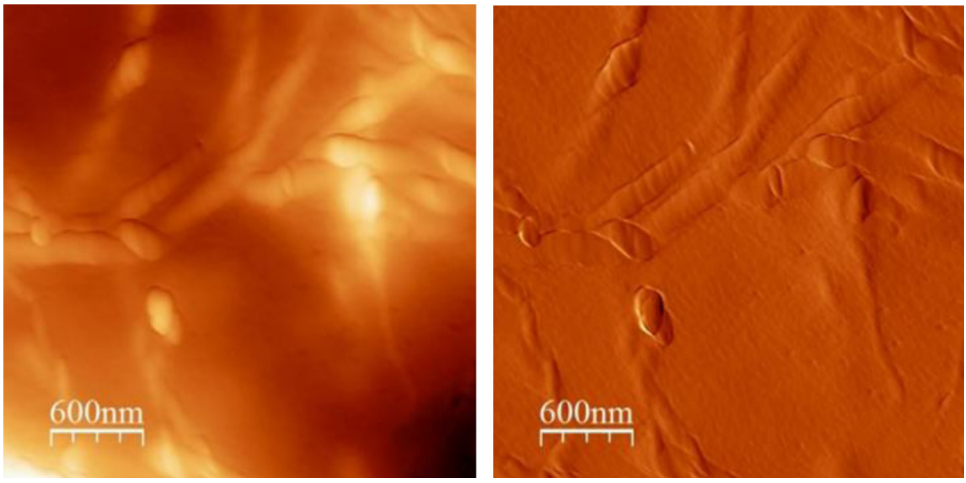

Fig. 5. AFM imaging of collagen section. Contact mode topography (Left) and deflection (Right) image of a section of a collagen gel.

Atomic force microscopy nano-characterization

Atomic Force Microscope (AFM) is a unique tool that can operate in several different modes under different environmental conditions (e.g. air and liquid) [8]. The most frequently used modes are contact and intermittent contact (also known as tapping) mode.

Contact mode is the first developed mode. In that mode, the AFM tip is in full contact with the sample's surface. As a result, this mode can be quite destructive, but it is preferred for combining imaging with mechanical characterization measurements. On the other hand, tapping mode is preferred for imaging biological specimens as it is a more “gentle” method since the tip oscillates and touches the sample surface only for a short period of time. Both modes can be used for the nano-characterization of collagen gels, but appropriate AFM probes should be selected. For instance, the probes appropriate for contact mode are characterized by lower resonance frequency and softer cantilevers than those for tapping mode.

Required equipment and software

Atomic Force Microscope, AFM tips, Image processing software. For the validation of the methodology we used: the PicoPlus (currently known as 5500 Atomic Force Microscope, Keysight

Technologies) AFM system, the software that accompanies the AFM system PicoView, the freeware available software WsxM [9] for image analysis and AtomicJ for Young's modulus calculations. Other commercial or freeware available software, such as OpenFovea [10], Gwyddion [11], SPIP™-Image Metrology, can be used. Also for the validation of the protocol we used the following AFM probes: SICON (Applied Nanostructures, for Contact Mode), ACT (Applied Nanostructures, AC-Tapping Mode) and PNP-TR (Nanoworld, for Contact mode and Material property characterization).

AFM surface nano-characterization

The protocol for AFM imaging characterization of the sample depends both on the used AFM system and the sample's characteristics. Here we briefly present the steps for imaging collagen gels using the PicoPlus AFM system both in contact and tapping mode [12]. The steps can be appropriately modified for other AFM systems.

System power up

Turn on all the AFM parts, boot up the computer and start the PicoView software that accompanies the AFM system. Then, in the menu *"Mode"* select either *"Contact" or "AC AFM"* depending if you will image in contact or tapping mode.

Loading the cantilever

After inserting the nose into the scanner, mount an appropriate probe and insert the scanner in the microscope. Then align the laser spot on the cantilever and insert and align the photo-detector.

Tune the tip

This step is required only for tapping mode. In the *"Controls"* menu choose *"AC Mode Tune"* and select the *"AC Mode Tune button"*. Then in the *"Auto"* panel set ▶Start (kHz): 50, ▶ End (kHz): 500, ▶Peak Amplitude (V): 5 and ▶Off Peak (kHz): -0.10. After that, click on the *"Auto Tune"* button so as to get the Cantilever Tune Graph.

Set the gain

In the *"Servo"* panel you will find make the *"I Gain"* and *"P Gain"*. In the contact mode both to 10%, while in tapping mode set *"I Gain (%)"* to 1 and *"P Gain (%)"* to 20.

Loading the sample

Prepare the sample according to the previous mentioned protocols. For samples on glass cover slips, use double face tape to mount them on AFM metal specimen discs. Then mount the sample on the plates. For imaging under liquid conditions place the liquid cell on the sample plate, insert your sample and add some PBS into the liquid cell. Finally insert the sample in the system. For samples on petri dish directly mount them on the appropriate sample plate and place them in the AFM system.

Motor cantilever toward sample

Use the *"Close"* switch to raise the sample but keep enough space between the tip and the sample surface to avoid damaging the AFM tip. Then click on the *"Approach"* button so that the system will make an auto-approach to sample's surface.

Start the imaging and parameter tuning

Select the *"Scan"* tab in the *"Scan and Motor"* window. Set a scan *"Speed* [Lines/Second (ln/s)] between *1–2* ln/s. Select a resolution from the list, for final images we recommend a 512 resolution, although for initial imaging 256 can also be used. In the *"Scan and Motor"* panel, click either the *"Down"* or *"Up"* blue arrow to initiate the scan starting at the top of the grid or from the bottom, respectively. In the "*Realtime Images*" window the images will begin to be acquired. During imaging, the user will need to re-adjust Gains and speed in order to achieve the best possible image. For collagen samples recommended values for the I and P gains are 7% and 14%, respectively and for the velocity 1 ln/s works very well for the specific system. In case you want to select a different location, press the "*Withdraw*"

bottom in order to detach the tip from the surface. Then locate the new region of interest on the specimen by manually moving the X/Y stage control micrometers while watching the video window. Subsequently, follow the same procedure, as mentioned previously, to approach the specimen's surface and start imaging.

Analysis of AFM images

The analysis of the AFM images can be performed using several commercial or freeware software. Here, we present few functions that can be used for analyzing collagen gels with the open access freeware software WsXM (5.0, develop 8) [9]. The user can extend the image analysis depending on the scope of the performed research.

Load files

Open the WsXm software, select *"File"* and then *"Open"*. Set *"All Files (*.*)"* in the "File Type" and select the file in the folder you want to analyze. From the pop-up window select the *"Extract&Load"* option to form a subfolder with the images and load the images in the software. When the file is loaded a number of different images will be loaded. For instance, in the case of the tapping mode, Height image and Phase image might open. The number of the different images that will open depends on the initial image acquisition and which images the user sets to the system to be acquired.

The first image is the Height image, which is always acquired during AFM imaging. This type of image is the most widely-used and is the most appropriate to be used in order to measure distances or height values. Click on this image to select it.

Add scale bar

In the "Display", click on "Show Scale Bar" to add a scale bar to the image. The position of the scale bar can also change (Fig. 6, Left).

Brightness and contrast

Select the *"Palette settings"* in the *"Screen"* option from the main tool bar. The brightness and contrast of the image can be adjusted by the Pallet settings window (Fig. 8, Right).

Height profile

Select the *"Process"* from the main tool bar and click *"Profile"*. With the mouse drag a line on the figure so as to form a height profile of the line.

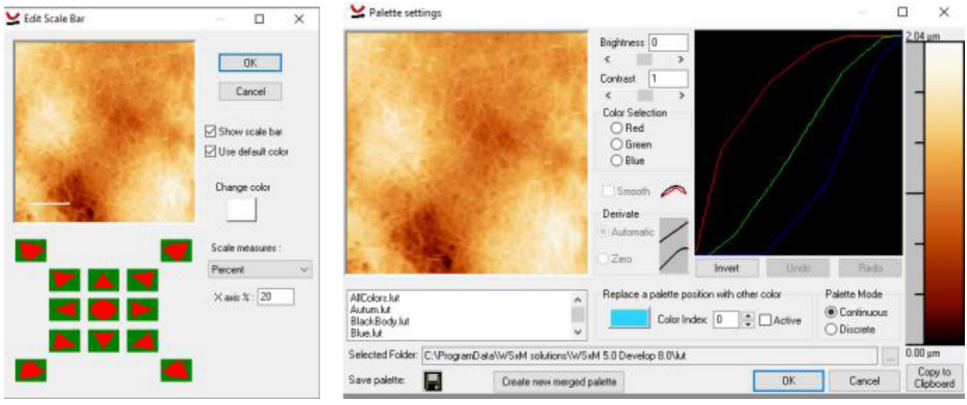

Fig. 6. Add scale bar and palette settings.

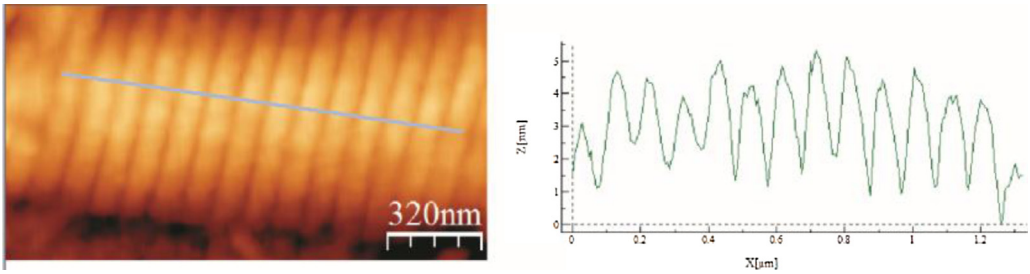

Fig. 7. D-band measurement. Left: Collagen fiber with the D-band periodicity and Right: height profile of the blue marker line on the collagen fiber.

This feature can be also used for measuring the characteristic D-band periodicity of a collagen fiber. Use the "Profile" to drag a line parallel to the main direction of the collagen fiber so that the height profile to demonstrate the D-band periodicity (Fig. 7).

Roughness analysis

Select the *"Roughness Analysis"* in the *"Process"* selection. In the *"Roughness Analysis"* panel a number of different values will be automatically calculated, such as RMS Roughness, Roughness Average and Average height (Fig. 8). By clicking *"OK"* a *"Histogram"* of the surface will appear.

Mechanical characterization – stiffness measurements

The procedure for the AFM mechanical characterization is quite similar with the imaging techniques but some calibration is required for the quantification of the measurements.

Preparation of the system

Prepare your system for Contact Mode as it was presented in the previous section, following the steps of "System Power Up", "Loading the Cantilever" and "Loading the Sample". For calibration of the system, a "hard" sample is initially loaded, such as a fresh cleaved mica disk, a glass slide or a petri dish.

Deflection sensitivity calibration

First you have to set the Deflection Sensitivity. This parameter termed "Deflection Sens.", can be found in the "Advanced" tab in the "Spectroscopy" window. Then in the Servo dialog box, set the following parameters: ▶"I Gain": 8%, ▶ "P Gain": 8%, ▶ "Setpoint": 0 V, ▶"Range": The maximize

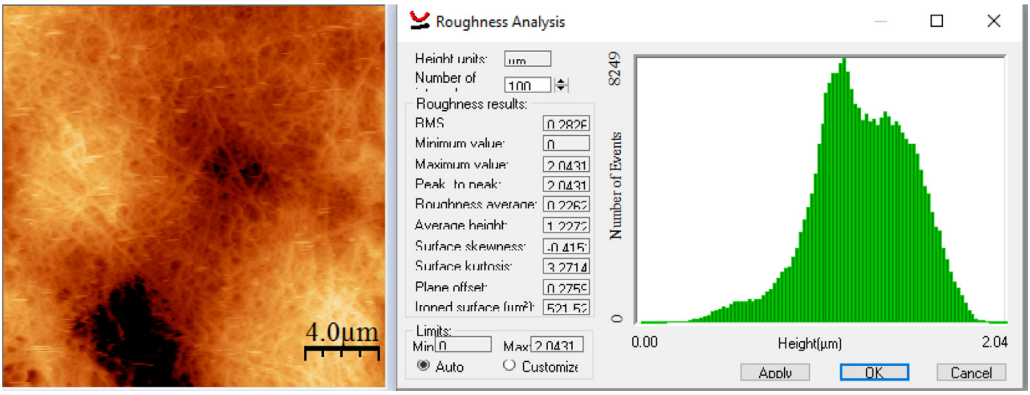

Fig. 8. Roughness analysis of collagen gels.

possible value. For instance for the scanner that we used, this value was 7.895 μm. To set the maximum possible value enter a very large number (e.g., 999) and the software (PicoView) will adjust to the maximum value for the scanner. After this click on the "Approach" button to allow the tip to approach on the sample's surface. Click on the "Spectroscopy" toolbar button to open the Spectroscopy function window. In the *Basic"* tab, set the following values: ▶ *"Sweeps"*: *1*, ▶ *"Duration"*:*1* and ▶ *"Data Points"*: 200. Maximize the *"Start"* and minimize the *"End"* values by entering a very large number and a large negative number, respectively. Finally, click on the play button "▶", which is located on the "Basic" panel of the Spectroscopy window, to acquire a force vs. distance curve.

In the force vs. distance curve, right click on the curve so as to add a *"Ruler"* to the linear part of the plot, in this part the tip is in hard contact with the sample. Set the end points of the "Ruler" in order to cover a large proportion of the linear part of the curve. After that, Right click on the ruler and select *"Deflection Sensitivity"* so that the software will automatically calculate and set the Deflection Sensitivity in *"Deflection Sens."* option.

Spring constant calibration- thermal tuning

After the "Deflection Sensitivity calibration" procedure (that was presented previously) click on the "Withdraw" button to withdraw the tip from the sample surface. Open the "Thermal K" plot window by clicking on the *"Thermal K"* button which is located in the *"Advanced"* tab of the "Spectroscopy" window. Then set the following parameters: ▶"Acquisition time":1 s and ▶"Averages": 10. Then select the *"Plot"* and click and drag the red and green circles to define the frequency range in which the resonance frequency should be found, according to the probe manufacture. Click the *"Compute"* button and the *"Apply"* button in the *"Thermal K*" window. The spring constant is now set.

Acquisition of mechanical data from collagen gels

Given that the mechanical characterization of the gels is performed under liquid conditions, the calibration procedure should be performed under liquid (PBS) conditions too. Also, the measurements of the collagen gels should be performed either in a petri dish in the presence of PBS or in a glass cover slip with the use of the liquid cell in the presence of PBS.

After the "Deflection Sensitivity Calibration" and the "Spring Constant Calibration" click the "Withdraw" bottom to detach the tip from the surface and use the "Open" bottom to remove the tip in a safe distance from the sample. Remove the sample that you used for the calibration procedure and mount the collagen sample by following the "Loading the Sample" procedure that has been previously presented. Select a region of interest and follow the "Motor Cantilever Toward Sample" step so that the tip to come in contact with the sample's surface. Perform a contact mode imaging and change areas of imaging until you find an area that you want to acquire stiffness measurements.

When you select the area of interests you can proceed to the Force Scanning Procedure. In the Servo dialog box set: ▶ "I Gain": 13% and ▶ "P Gain": 7% (you should tune appropriately these gains according to your system and sample so as to acquire better results), ▶ "Setpoint": *0 V*, ▶ "Sweeps": *1*, ▶ "Duration": *1*, ▶ "Data Points": 200, ▶"Start": maximum and ▶ "End": minimum (to set the maximum and minimum values enter a very large number (e.g., 999) and a large negative number for the (e.g., -999), respectively).

In order to acquire a force vs distance curve click on the play button "▶" on the *"Basic"* panel of the *"Spectroscopy"* window. Adjust the sweep range as needed using the Start/End parameters in the "Setup" tab so as to fine tune the force distance curve. In the Spectroscopy window select the "*Volume"* tab, set the resolution to 512 and Turn On the Toggle Auto Full Scale. Begin the sweep of the selected area by clicking the *"Scan Up"* or *"Scan Down"* buttons.

Young's modulus calculation and Young's modulus maps

For the extraction of valuable information from the data that were obtained with volume spectroscopy the open source software AtomicJ [13] is used. Of course other freeware or commercial software (WsXm [9], OpenFovea [10], Gwyddion [11], SPIP^{TM}-Image Metrology) can be employed.

Open the AtomicJ software and in the *"Gear icon"* click on the *"Process force curve and maps"* function. Click on *"Add"* and select the file with force volume data and then select the *"Next"*. In the *"General"* panel

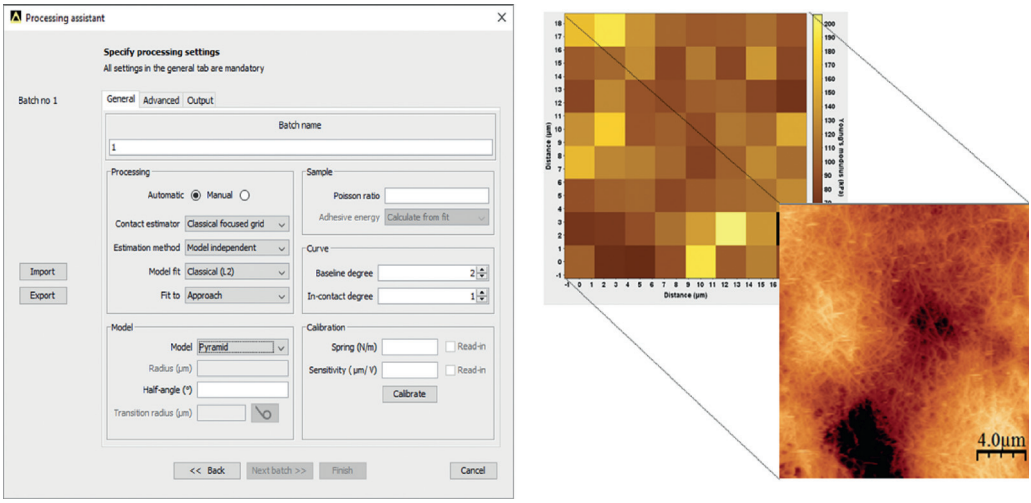

Fig. 9. Young's modulus maps formation. Left: The processing assistant window and Right: Young's Modulus Map and the corresponding area as illustrated from an AFM Height image.

of the *"Processing Assistant"* window (see Fig.9) that will open set the following parameters (these parameters is for pure collagen gels, with PNP-TR tips and by applying the Hertz model): ▶Model: *"Pyramid)"*, ▶Radius (μm): "0.01″ (It is provided by the probe manufacturer), ▶Poisson ratio: "0.3″, ▶Spring (N/m): use the value from the calibration procedure (for the used tip is ∼"0.32″), and ▶Sensitivity (μm/V): use the value from the sensitivity calibration procedure that you follow for this specific probe. Then click *"Finish"* so as the *"Graphical results"* and the *"Maps"* windows will open. In the "Maps" window you will find the several tabs including the Young's modulus map. In the Young's Modulus map each square represent different Young's modulus values of the sample with a different pseudocolor. A final useful function is the formation of a histogram presenting the Young's modulus values. In the *"Maps"* window select the "Draw Histogram" icon and the *"Histogram Assistant"* panel will appear. Then click on the *"Young's modulus"* and select *"Next"*. In the new panel select *"Square Root"* in the *"Method"*, check the *"Add Fit"* and click finish. The Histogram will be formed.

Conclusions

In this paper we presented a protocol describing the formation of 3D collagen type I gels with tunable stiffness, the preparation of histological sections from collagen gels and the AFM-based morphological and mechanical nano-characterization of the gels. We demonstrated that AFM offers a complete non-destructive characterization of such collagen-based culture models, in terms of morphology and mechanical properties. The formation of collagen gels with tunable stiffness and the characterization of such models offer the advantage of providing a more physiologically-relevant environment for culturing cells that also takes into account cell-ECM interactions and growth in 3D, fundamental parameters that are often neglected in standard *in vitro* studies. Hence, this protocol is useful for studying cell behavior under different mechanical conditions.

Acknowledgements

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