Electronic Supplementary Material (ESI) for Nanoscale.
This journal is © The Royal Society of Chemistry 2023

Annex 1: Protocol for measuring cell mechanics

Sandra Pérez-Domínguez[a], Shruti G. Kulkarni[a], Joanna Pabijan[b], Kajangi Gnanachandran[b], Hatice Holuigue[c], Mar Eroles[d], Ewelina Lorenc[c], Massimiliano Berardi[e,f], Nelda Antonovaite[f], Maria Luisa Marini[g], Javier Lopez Alonso[g] Lorena Redonto-Morata[g], Vincent Dupres[g], Sebastien Janel[g], Sovon Acharya[h], Jorge Otero[i], Daniel Navajas[i], Kevin Bielawski[f], Hermann Schillers[h], Frank Lafont[g], Felix Rico[d], Alessandro Podestà[c], Manfred Radmacher*[a], Małgorzata Lekka*[b]*

[a.] *Institute of Biophysics, University of Bremen, 28359, Bremen, Germany*
[b.] *Department of Biophysical Microstructures, Institute of Nuclear Physics, Polish Academy of Sciences, PL-31342 Kraków, Poland*
[c.] *Department of Physics "Aldo Pontremoli" and CIMAINA, University of Milano, via Celoria 16, 20133 Milano, Italy*
[d.] *Aix-Marseille Univ, CNRS, INSERM, LAI, Turing centre for living systems, Marseille, France*
[e.] *Laserlab, Department of Physics and Astronomy, Vrije Universiteit Amsterdam, De Boelelaan 1081, 1081 HV, Amsterdam, The Netherlands*
[f.] *Optics11 life, Hettenheuvelweg 37-39, 1101 BM, Amsterdam, The Netherlands*
[g.] *Université de Lille, CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, U1019-UMR9017, CIIL—Center for Infection and Immunity of Lille, F-59000 Lille, France*
[h.] *Institute of Physiology II, University Muenster, Robert-Koch-Str. 27b, 48149 Muenster, Germany*
[i.] *Institute for Bioengineering of Catalonia and Universitat de Barcelona, Barcelona, Spain. CIBER de Enfermedades Respiratorias, Madrid, Spain.*

*Corresponding authors:

Malgorzata.Lekka@ifj.edu.pl, alessandro.podesta@unimi.it, radmacher@uni-bremen.de

Protocol sheet for measuring cell mechanics

TOC

0. General Information
1. Shipment of cells
2. Cell cultures
3. Preparing cells for AFM measurements
4. Calibration of the AFM
5. AFM measurements on cells
6. Analysing force data.
7. Additional info on cells

0. General Information

Materials needed:

- two 25 cm^2 culture flasks (#1, #2) with living cells (here, the protocol was tested for pancreatic cancer PANC-1 cells);

Note 1: PANC-1 cells were established from a human pancreatic cancer, isolated from a pancreatic carcinoma of ductal cell origin of a 56-year-old male (Lieber et al. International Journal of Cancer. 15 (1975) 741–747, doi:10.1002/ijc.2910150505). These cells have an epithelial morphology and are adherent in cell culture flasks; however, they tend to clump. PANC-1 cells can metastasize, but they have poor differentiation abilities.

- four empty culture flasks (sterile)
- two 50 ml tubes of culture medium (DMEM + 10% FBS) with antibiotics (1%) - (sterile);
- one 50 ml tube of PBS (sterile)
- one 15 ml tube with trypsin solution (sterile);
- one 15 ml tube of freezing medium: DMEM + 5 % DMSO (sterile)
- one 2 ml tube of 1 M HEPES (sterile)

Cell Culture Dish, 35x10mm – TPP cat no. 93040
Tissue Culture Flasks 25 cm2 – TPP cat no. 90025
DMEM - ATCC cat no. 30-2002
FBS - SIGMA cat no. F9665
Trypsin-EDTA solution (10x) - SIGMA cat no. T4174
Dimethyl sulfoxide (DMSO) – SIGMA cat no. D2438
HEPES buffer (1 M) – SIGMA cat no. H0887
PBS – SIGMA cat no. P4417
Antibiotics (Penicillin − Streptomycin − Neomycin Solution Stabilized) – SIGMA, cat no P4083

Note 2: if it is planned to use other cell lines, please adjust culture conditions accordingly. Importantly, for the SOP, use of a cell monolayer is strongly advised.

1. Shipment of cells

Living cells can be shipped at 50-60% confluency; culture flasks should be filled with a maximum amount of culture medium (for PANC-1 cells: DMEM + 10% FBS + 1% antibiotics).

1. Document sample and shipping details:

Culture flask number: _____

Prepared by: _____

Preparation date: _____

Sent data: _____

Sent to (location) _____

Arrival date & time: _____

Flask 1 Passaging date & time _____

Flask 2 Passaging date & time _____

2. Cell culture

Work under sterile conditions (under the laminar flow chamber):

1. After arrival, remove half of the culture medium and exchange the bottle stopper (take a new one from an empty culture flask)

2. Take the culture flasks and make a passage according to cell **protocol #1**.

3. Leave cells **overnight** in a CO_2 incubator.

Protocol #1 (cell passaging, preparation of cells for AFM measurements):

1. Remove and discard the culture medium.
2. Briefly rinse the cell layer with 1 mL PBS solution to remove all serum traces containing trypsin inhibitor.
3. Add 2.0 ml of pre-warmed 0.25% Trypsin/PBS solution to the culture flask and place the flask for 3 minutes in the CO_2 incubator. Observe cells under an inverted microscope until the cell layer is dispersed (usually, it takes 5 to 15 minutes).

Note: To avoid clumping, do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach.

4. When ≥ 90% of the cells have detached, tilt the vessel for a minimal time to allow the cells to settle.

5. Transfer the cells to a 15-ml conical tube, add 3mL of culture medium (DMEM +10% FBS + 1% antibiotics), and centrifuge them at 1800 rpm for 4 minutes.
6. Remove the supernatant – a cell pellet is located at the bottom of the conical centrifuge tube.

Note: At this step (if cells are not needed, e.g., cells from culture flask #2 can be frozen), according to protocol #2

7. Add 2 ml of DMEM with 10% fetal bovine serum and dissolve the cell pellet by gently pipetting up and down.
8. Count the cells in the cell suspension using a Bürker chamber or similar. Dilute cell suspension to have 150 000 cells/mL (in DMEM + 10% FBS).

Note: In case of no cell counter chamber, take 400 ul of cell suspension and place it into a Petri dish or 24 well-plate, and fill up 2 ml with culture medium.

9. Prepare two culture flasks and 4 Petri dishes.
10. Take 2 mL of cell suspension into one Petri dish with an internal diameter of 34 mm (TPP Petri dish surface is 9.2 cm^2). The cell volume (or number) has to be adjusted for a different size Petri dish. Let the cells grow in a CO_2 incubator for 48h prior to AFM measurements.
11. The remaining cell suspension should be divided and put into new culture flasks (not provided). Then add culture medium (4 mL) and place culture flasks into the CO_2 incubator.

Note: The passage ratio for these cells is: 1:2 or 1:4 (we recommend using 1:3).

Protocol #2 (freezing cells):

7. Conduct steps 1 to 6 from procedure #1.
8. Add 3 mL of freezing medium (10% DMSO dissolved in culture medium, here DMEM), mix, and aliquot the cell suspension into a cryogenic vial (typically, 1 mL per one vial).
9. Vials must be frozen at -80°C for 4 hours and, later on, placed in liquid nitrogen.

Note: Own protocol to freeze cells can be applied, too.

3. Cell Preparation for AFM measurements

1. Take one Petri dish prepared for AFM measurements (see section 2, protocol #1)
2. Exchange medium with a fresh culture medium containing 10 mM of HEPES.

Note: Take 10 mL of culture medium (DMEM + 10% FBS +1 % antibiotics) and add 100 uL of 1 M HEPES to reach the concentration of 10 mM) – use this solution in the AFM measurements.

Note: If the mechanical properties of cells depend on the number of passages, this must be verified before the experiments, or the number of passages must be kept similar.

4. Calibration of the AFM

Note: The proposed AFM measurements were conducted at 37°C. For room temperature measurements, adapt accordingly.

Note: The cantilevers chosen for the study were hemispherical MLCT-SPH-DC E, precalibrated by the manufacturer. It is advisable to use spherical probes with relatively large radii and spring constant above 0.1 N/m.

Note: The calibration of deflection sensitivity *S* must be performed within a similar range of the cantilever deflections.

2. Document the experiment:

Experimenter:	_____
Date:	_____
Manufacturer	_____
Instrument:	_____
Software	_____
Cantilever Type	MLCT-SPH DC E
Spring Constant (k_{if})	_____
Tip Radius	_____
Kappa:	_____
deflection sensitivity:	_____

3. Mount the cantilever and the sample in the AFM. Immerse the cantilever in the culture medium, adjust the laser beam to the cantilever probe, and adjust the photo-detector position.

Note: A bare Petri dish (without cells) to calibrate without time pressure is recommended.

4. Apply the SNAP procedure for deflection sensitivity calibration

Note: SNAP procedure is described in detail in an open-access journal *Scientific Reports* (Schillers et al. *Scientific Reports* 7 (2017) 5117 (doi: 10.1038/s41598-017-05383-0).

5. Set the kappa factor to a value of 1.11.

Note: The kappa factor is the ratio of the dynamic (as during the thermal oscillation) to the static deflection sensitivity (as during the static loading of the cantilever against a stiff substrate).

6. Calibration of the deflection sensitivity S is the most critical part of setting up the instrument. Thermal spectra should be of high quality; thus, sample and average data for at least 20s are recommended. Here we suggest two contactless ways of deflection sensitivity calibration, both requiring the knowledge of the cantilever spring constant (for example through interferometry):

i. If the AFM instrument software allows for a contactless calibration, then acquire a thermal noise spectrum, enter the spring constant value k_{if} (for example the precalibrated value given by the manufacturer, or a previously accurately measured value) and let the software calculate the deflection sensitivity.

ii. Alternatively, the SNAP procedure can be applied.:

- type in a reasonable deflection sensitivity value, $S_{measured}$
- record a thermal noise spectrum, analyze it to get a force constant k_{th}
- change the deflection sensitivity by multiplying it by $\sqrt{\frac{k_{th}}{k_{if}}}$:
- $S_{corrected} = S_{measured} \cdot \sqrt{\frac{k_{th}}{k_{if}}}$

Note: After calibration is done, save the last recorded thermal, export it as text, and take a screenshot of the thermal in case of trouble with its importing.

1. Document the obtained deflection sensitivity:

Filename of thermal: _____

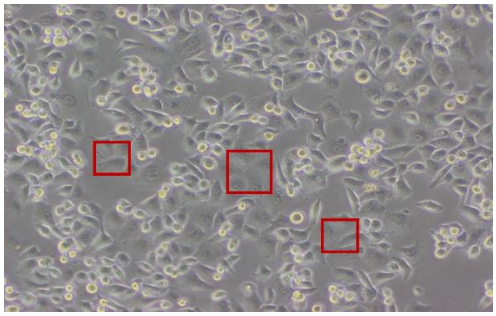
Filename of screenshot: _____

5. AFM measurements on cells

1. Move the cantilever above a center of a cell.
2. Take an optical image of the place chosen to be measured.

Note: If the maximum time of 2 hours is exceeded, change the sample (use another Petri dish)

Note: Choose flat cells as it is indicated in the image of PANC-1 cells:



3. Record force volumes with the following parameters:
   z-travel distance: 5 µm
   number of points in the force curve: possibly 1 point/nm (i.e. n > 5000)
   tip velocity: 5µm/s
   force scan rate 0.5 curves per second
   trigger point (the point at which approach ends): 7 nN
   number of curves per volume 10 curves x 10 curves
   area of force volume 50 µm
   z-close loop: ON, or alternatively also save the z-sensor channel

Note: The area of the force volume could be adjusted to a single cell diameter or to the size of a cluster containing few cells.

4. Measure 10 different locations separated by at least 200 microns. Locate cellular islets/regions with the features indicated in the attached optical image.

5. Document the force volume collected:
   Filename of force volume 1: _____
   Filename of force volume 2: _____
   Filename of force volume 3: _____
   Filename of force volume 4: _____
   Filename of force volume 5: _____
   Filename of force volume 6: _____
   Filename of force volume 7: _____
   Filename of force volume 8: _____
   Filename of force volume 9: _____
   Filename of force volume 10: _____

6. Analysing force data using local software

1. Analyze force volume data using the Hertz model for a spherical (parabolic) tip by fitting the whole data range.
2. Use the radius of the probing sphere provided by the manufacturer with the cantilevers.
3. Use a Poisson ratio of 0.5 and disable tilt correction.
4. Calculate for each force volume the median and standard deviation of elastic modulus values:

   Force volume 1: avg: _____ Pa sdv: _____ Pa

   Force volume 2: avg: _____ Pa sdv: _____ Pa

   Force volume 3: avg: _____ Pa sdv: _____ Pa

   Force volume 4: avg: _____ Pa sdv: _____ Pa

   Force volume 5: avg: _____ Pa sdv: _____ Pa

   Force volume 6: avg: _____ Pa sdv: _____ Pa

Force volume 7: avg: _____ Pa sdv: _____ Pa

Force volume 8: avg: _____ Pa sdv: _____ Pa

Force volume 9: avg: _____ Pa sdv: _____ Pa

Force volume 10: avg: _____ Pa sdv: _____ Pa