TITLE:
Experimental and Data Analysis Workflow for Soft Matter Nanoindentation

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SUPPLEMENTARY PROTOCOL:

Part 1. Preparation of substrates / cells for nanoindentation measurements

1   Preparation of PAAm hydrogels

NOTE: For a detailed protocol on the preparation of elastic PAAm hydrogels of controlled Young's Modulus *(E),* refer to the work by Tse and Engler[1].

1.1   Preparation of hydrophobic glass slides and acryl-silanized coverslips

1.1.1   Submerge the required number of glass slides, for 5 min, in a commercially available hydrophobic-coating solution (see **Table of Materials**). Then, rinse with purified water and let it air dry.

NOTE: The hydrophobic glass slides will be used as the substrate where hydrogels are polymerized.

1.1.2 Clean the coverslips of the desired size (12 mm in diameter and 130-160 µm thickness) by sonication, first in purified water and then in absolute ethanol. Wait until the coverslips are fully dry.

CAUTION: Perform steps 1.1.3 and 1.1.4 in a fume hood, and make sure air flow is switched on.

1.1.3   Place the coverslips in a vacuum pump desiccator. For this protocol, an oil-less piston vacuum pump was used to achieve a maximum vacuum pressure of 60 Torr. Place a glass slide with enough acrylsilane to cover all coverslips (50 µL per 12 mm coverslip).

1.1.4   Turn on the vacuum pump and let the reaction take place for a minimum of 3 h.

1.1.5   Remove the coverslips from the vacuum chamber and cure them for 1 h at 60°C in an oven.

1.2   Preparation of flat PAAm hydrogels

CAUTION: Perform PAAm hydrogels preparation in a fume hood and make sure air flow is switched on.

1.2.1 Mix 40% (w/v) acrylamide and 2% (w/v) bis-acrylamide to the appropriate final concentrations[1] in purified water in a reaction tube. The exact volume depends on how many hydrogels are to be tested.

1.2.3   For hydrogel polymerization, add 10% (w/v) ammonium persulfate (APS) (1:100 total volume) and tetramethylethylenediamine (TEMED) (1:1000 total volume) to the acrylamide mixture.

1.2.4   Vortex for 5 s and quickly move on to the next step to prevent polymerization of the gel in the reaction tube.

1.2.5   Pipette the desired volume onto a hydrophobic glass slide. Pipette 20 µL of the hydrogel solution to create a ~200 µm thick hydrogel (**Figure 2a**, left – main text).

1.2.6   To flatten the hydrogel, place the silane treated coverslip on top of the pipetted drop of the hydrogel and wait ~ 20 min for polymerization to take place (**Figure 2a**, left– main text).

1.2.7   Gently peel the coverslip off the hydrophobic glass slide to find a coverslip-gel composite (**Figure 2a**, center).

1.2.8   Place the coverslip-gel composite in purified water overnight for the hydrogels to swell. The hydrogels can be stored at 4 °C until mechanical characterization.

NOTE: 5–6 hydrogels can by polymerized on the same glass slide.

1.3   PAAm sample preparation for mechanical characterization

1.3.1 For mechanical characterization, the material must be stably attached to a surface. To achieve this, attach the coverslip-gel composite to a Petri dish by using super glue or thin double-sided tape. Handle the coverslip-gel composite with tweezers and submerge it in purified water before testing to avoid drying (**Figure 2a**, right).

2   Preparation of cells

NOTE: To perform single cell indentation measurements, culture cells in the appropriate media in treated tissue culture dishes. For Human Embryonic Kidney 293T (HEK293T) cells, follow the steps outlined below.

2.1   Thaw and split cells to culture them at a density of 6,000 cells/cm^2 in a cell culture treated 35 mm dish. Keep cells in a high glucose DMEM containing sodium Pyruvate, L-Glutamine, 10% fetal bovine serum (FBS) and 1% Penicillin/streptomycin (P/S).

2.2   Leave cells to adhere for a minimum of 4h to perform nanoindentation measurements. Cells can be cultured until reaching 70% confluence for performing single cell measurements, as it may become challenging to indent single cells once a confluent monolayer starts to form (**Figure 2b** – main text).

NOTE: Cell cycle might influence the mechanical properties of cells[2]. To reduce variability within the same cell population, cells can be serum starved in 1% FBS media overnight, to achieve cell cycle synchronization prior to performing measurements[3].

Part 2. Starting up the device, probe choice and probe calibration

1   Starting the system

1.1   Switch on the nanoindentation device and associated optical microscope.

1.2 Switch on the computer associated with the device.

1.3 Switch on the interferometer.

1.4 Switch on the controller box.

1.5 Switch on the inverted optical microscope associated with the nanoindenter. The specific instructions depend on the microscope's manufacturer, and the imaging modality used.

1.6 If applicable, switch on the air compressor of the anti-vibration table on which the microscope and nanoindentation device are mounted on. Wait for the compressor's pressure indicator to reach the desired level. Check that the anti-vibration table is working by gently tapping on it. The table should quickly stabilize back to its original position.

1.7 Open the nanoindentation software (version 3.4.1 is used in this protocol) on the computer by double clicking on the associated desktop icon. Wait for all hardware to be ready, which means they will show as **idle** in the status bar of the pop-up window on the screen.

1.8 A second pop-up window will appear asking to home the stage. Answering **Yes** will move the nanoindentation stage to the front left corner setting both *x* and *y* coordinates to 0, and move to the last *(x,y)* point in the matrix scan parameters. Answering **No** will not

move the arm. It is suggested to click on **Yes**, however care must be taken that the arm will not bump into anything while automatically moving to the front left corner.

2   Go back to the main **Protocol** for steps on how to select the appropriate nanoindentation probe.

3   Mount the nanoindentation probe as follows

3.1 Carefully remove the chosen probe from the housing box by holding the plastic probe holder with one hand and the connector with the other hand. Take extra care when handling the probe as accidental contact with the cantilever or bending of the fiber wire will most likely break it.

3.2 Insert the plastic probe holder in the dedicated housing on the nanoindenter's arm. Make sure the probe is mounted in the correct way, with the spherical tip pointing downwards. Carefully connect the green optical fiber connector in the appropriate socket. Take extra care not to pull or bend the optical fiber.

3.3 Bring the probe in the center of the microscope's field of view by gently moving the nanoindenter's arm by hand or using the digital *x/y* control arrows in software's main window. Do not focus the *z* position of the probe at this point, as this will change both during the calibration procedure and the actual experiment.

4 Go back to the main **Protocol** for steps on how to calibrate the probe.

Part 3: Measuring the Young's Modulus of soft materials

1   Cleaning the probe and switching off the instrument

1.1   After completing all measurements, discard or store the sample away.

1.2   Clean the probe with isopropanol followed by water using a Pasteur pipette.

1.3   Once the probe has been cleaned, make sure the probe is free of any remaining residue with the aid of the microscope before carefully storing it away in the same position it was shipped in (the box should provide the appropriate grooves to prevent the probe from breaking whilst stored). The probe will dry whilst securely stored.

1.4   If the probe has some remaining sample residue, carefully clean it with a kimwipe making sure it only touches the debris and not the probe, or repeat the cleaning step. If the cantilever is bent, return it to its original position with the aid of a kimwipe. If the probe is wet, the cantilever may be more prone to bending, especially when it is soft. In this case, wait for the probe to dry and for the cantilever to return to its horizontal position on its own rather than trying to unbend it whilst wet, as this can lead to probe damage.

1.5   Once the probe is stored away, turn off the equipment in the reverse order compared to when switched on: i.e., software, microscope, controller, interferometer and optical

microscope.

Part 4: Data analysis

1   Downloading and installing the software

NOTE: The data analysis of *F-z* data is performed through an open-source software programmed in Python and equipped with a user-friendly graphical user interface (GUI). The software consists of two GUIs, one advised to be installed on the lab computer (data preparation) and one advised to be installed on the computer where the formal data analysis will be performed (data analysis). This is optional, and both GUIs can be installed on the same computer.

1.1   The software is programmed in Python, and the interpreter for this language must be installed on computers where the software is intended to be used. Navigate to www.python.org and install the latest stable release of Python 3 for your operating system. Older versions of Python 2 are not supported. Further instructions on how to install Python can be found on www.python.org.

1.2   Navigate to https://github.com/CellMechLab/NanoPrepare/releases/tag/v0.1.1[4] and download the **Source code (zip).** Rename the downloaded folder **NanoPrepare**. Navigate to https://github.com/CellMechLab/nanoindentation/releases/tag/v1.0.0[5] and download the **Source code (zip)**. Rename the downloaded folder **NanoAnalysis**.

1.3   Both software require a number of dependencies in order to run, including: SciPy[6], NumPy[7], PyQt5[8] and PyQtGraph[9]. The easiest way to install those dependencies is to use the PIP utility, which can be called from the command line. For example, to install SciPy[6], run the command: **pip install scipy** (windows) or **pip3 install scipy** (MacOS). Further information on how to install packages can be found at: https://packaging.python.org/tutorials/installing-packages/. Install the latest stable versions for all packages.

2. Go back to the main **Protocol** for instructions on how to use the software for analysis of nanoindentation data.

Supplementary References

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6. Virtanen, P. *et al.* SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nature Methods*. **17** (3), 261–272, doi: 10.1038/s41592-019-0686-2 (2020).

7. Harris, C.R. *et al.* Array programming with NumPy. *Nature*. **585** (7825), 357–362, doi: 10.1038/s41586-020-2649-2 (2020).

8. Riverbank Computing PyQt5. at <https://pypi.org/project/PyQt5/> (2021).

9. PyQtGraph. at <https://www.pyqtgraph.org/>.