Atomic Force Microscopy
elasticity measurements on living / fixed cells
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Practical Output

Cells

Cell type : Adherents carcinoma cells of the cervix (HeLa).
Obtained from : ATCC

Dish

Glass bottom Willco wells GWSt-5040

HeLa cell culture

Medium : MEM + 10% FBS (Gibco)
+ L-Glutamine : 2mM (Gibco)
+ Pen / Strep 1:100

From cells stored in liquid nitrogen : (vial 1 ml : 50% FCS; 40 % medium; 10 % DMSO)
Unfreeze the vial at 37°C in water bath during 1 to 2 mn
Get back the cell (1 ml) with 9 ml medium
Centrifuge 5 min at 1000 rpm, at room temperature (RT)
Discard the surpernatant
Resuspend cells with 8 ml complete growth medium
Deliver into flask 25 cm2
Incubate at 37°C
Change medium day after

Subculturing :
Discard culture medium
Wash with 6 ml (flask25); 10ml (plate100mm); 12 ml(flask75) of sterile PBS 1X
Discard PBS
Add 2 ml(F25) or 3ml(F75) of Trypsin-EDTA (Gibco) to culture dish and observe cells under an inverted microscope until cell layer is dispersed
Incubate at 37°C 5 mn to facilitate dispersal.
Add 6 to 8 ml of complete growth medium and aspirate cells by gently pipetting (trypsin inactivation)
Add appropriate aliquots of the cell suspension to new culture dish or flask.
Dilution ratio: 1/5 to 1/10 is recommended.

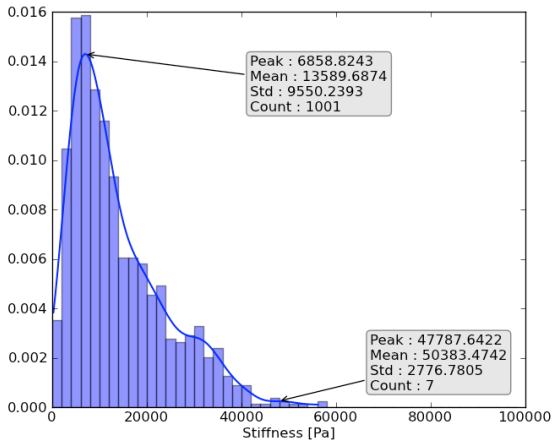
Day 1 - Monday 29th : Live cells and Glutaraldehyde Fixation

Glutaraldehyde fixation protocole (from H. Obertleithner 's lab):
- Culture cells (kidney, gut, lungs, brain, etc) in an incubator on solid supports (plastic, glass, coated glass, filter membranes, etc, whatever your cells like).
- Get glutaraldehyde as a fixative (sold by SERVA; 25% stock solution; Catalogue No:23114) and predilute it with your respective culture medium to 5%. You can also predilute it with HEPES buffer (in mM: NaCl 140, KCl 5, MgCl2 1, CaCl2 1, HEPES 10 mM; pH 7.4).
- Transfer cells from the incubator to the hood and pipet an adequate volume of your 5% glutaraldehyde solution (fixative solution) into the medium of the culture dish where the cells are. Achieve a final concentration of 0.5% glutaraldehyde in your dish. This means, e.g., that you have to add 300 µl of the fixative solution to 3 ml of culture medium that is already in the culture dish. Give the dish a 2 times "rotating swing" so that the fixative distributes well.
- Let it sit for about 1 hour.
- Remove the medium and add Hepes buffer. Be careful that the cells are always covered by some fluid.
- Seal the dish with parafilm and store the sample at 4 - 8°C.
- In my experience, you can use this sample immediately but also store it for at least 2 weeks (frig).
- Perform AFM experiments in fluid (e.g. in HEPES buffer; see above).
- In case you want to work on an air-dried sample, remove the fluid after fixation (after point 4), wash the sample with H2O and let it sit in air. Store (dust-free) in air at room temperature.

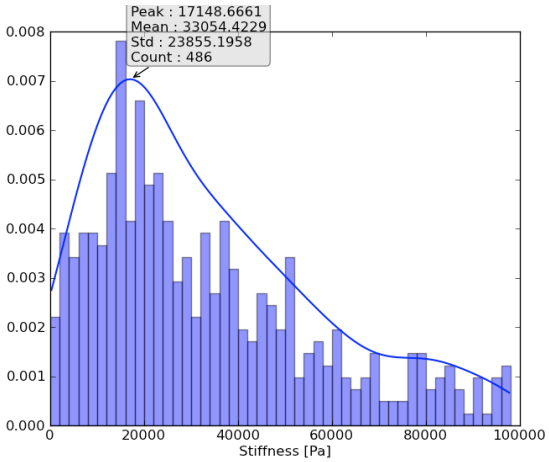
Experiments were conducted at RT, living cells were analyzed first while another batch of cells were fixed.

Output :
Young modulii were calculated at 100nm indentation into the cell, with Poisson ratio=0,5 and tip diameter of 40nm (Hertz sphere model).
We got several scans on 2 or 3 cells, which is not sufficient to have any statistical significance. We here give an indication on how the Young modulus is affected by fixation but for definitive statement much more data should be obtained.

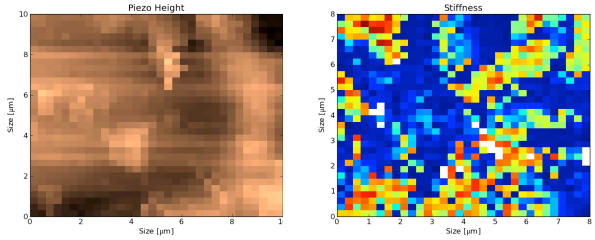
Histogram for living cells
Peak @ 6.8 kPa



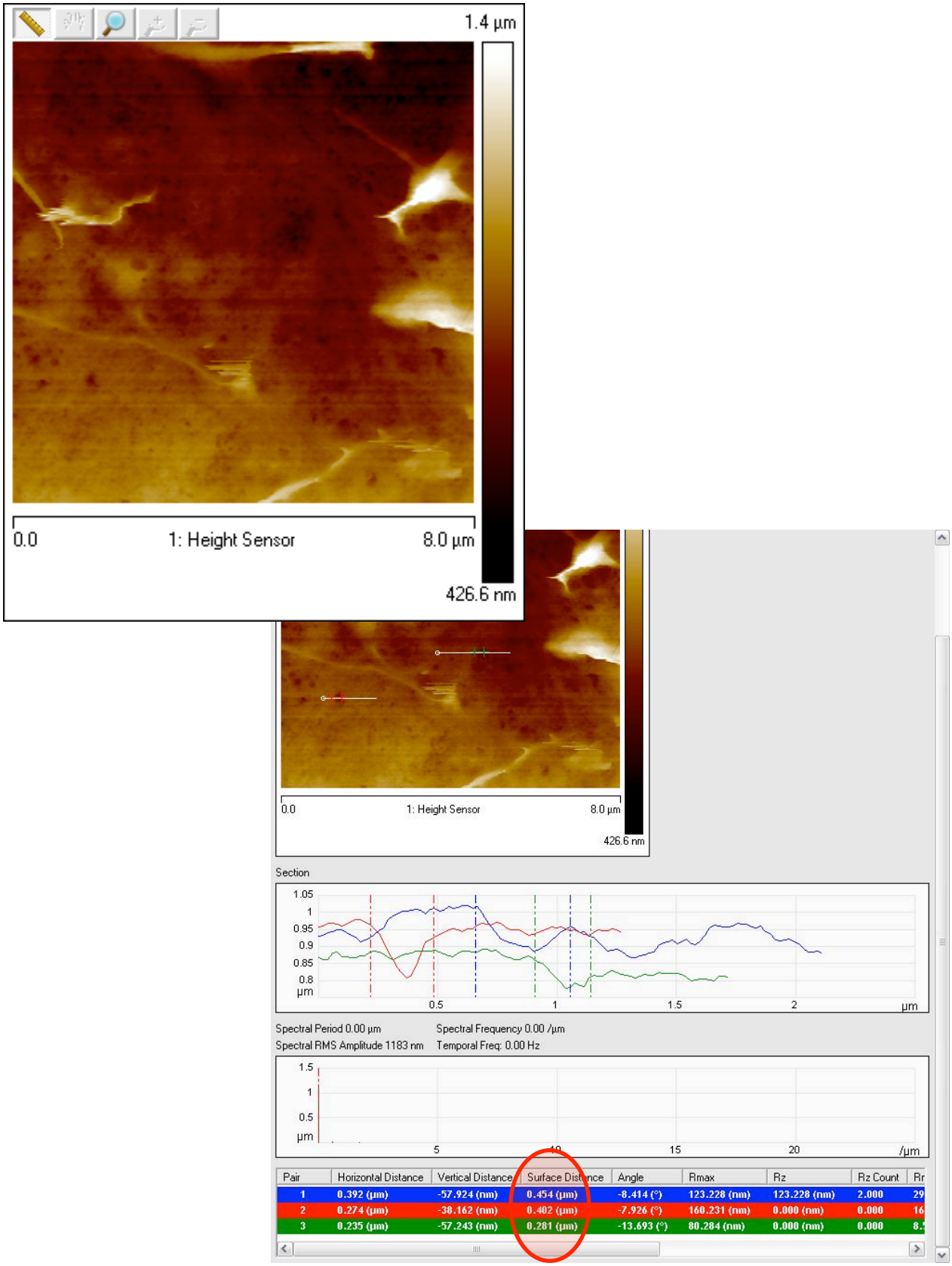
Histogram for gulta-fixed cells
Broad peak starting @ 17 kPa



There is a sharp increase in elasticity. So sharp that at some positions we didn't even indent as far as 100nm to make the calculation (we should have increased the trigger threshold...) See on the right some high positions on the cell that appear very hard. We also see some well defined areas with very different elasticities.



We also performed some Peakforce Tapping imaging overnight. One can observe pores in the plasma membrane probably due to fixation. Variations were observed for diameter sizes: in the rage of hundreds of nm wide and tens of nm height (it is required to take tip/sample interactions into account). Also, fixation results in topography modification:

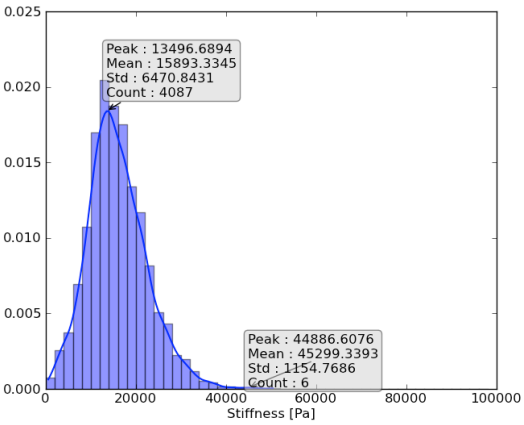


Day 2 - Tuesday 30th : Live cells and PFA Fixation

PFA protocol as performed in routine for immunofluorescence experiments:
Discard culture medium
Add 37°C Paraformaldehyde 4% in PBS (PFA4%) and incubate for 15 min
Rinse 3 times with PBS
Incubate 5mn with 50mM NH_4Cl (quenching of aldehydes)
Rinse 3 times with PBS and keep in PBS buffer

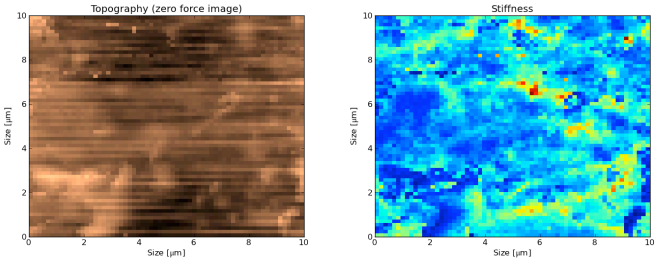
Experiments were conducted at RT, living cells first while fixing the other batch of cells.

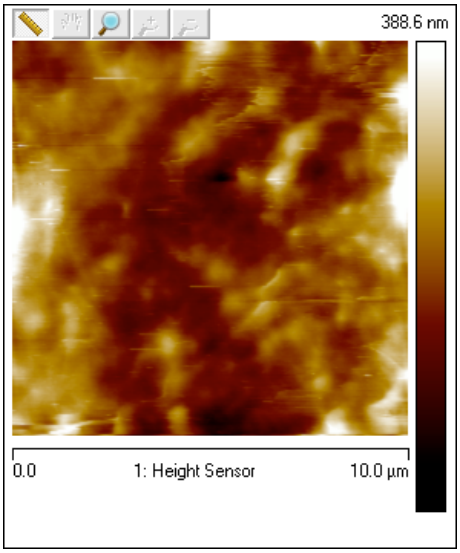
Output :
Young modulii were calculated at 100nm indentation into the cell, with Poisson ratio=0,5 and tip diameter of 40nm (sphere model).
We got several scans on 2 or 3 cells, which is not sufficient to have any statistical significance. We here give an indication on how the Young modulus is affected by fixation but for definitive statement much more data should be obtained.



Indentation was easily sufficient to determine elasticity @ 100nm. This «cortical» elasticity nearly doubled between live and fixed cells (from 6.8 kPa to 13.5 kPa)

We could see some hard cytoskeleton parts underlying, sometimes completely uncoupled from the topography :





The PFA fixation did not affect the plasma membrane with pores like it did with glutaraldehyde.

Sébastien JANEL

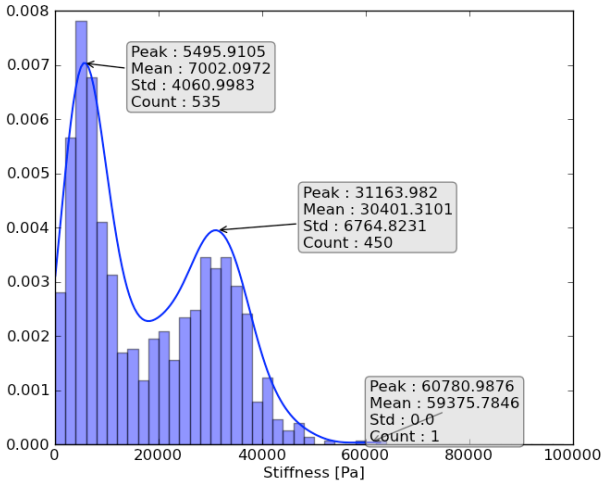
Day 3 - Wednesday 31th : CytochalasinD treatement, Live cells and PFA Fixation

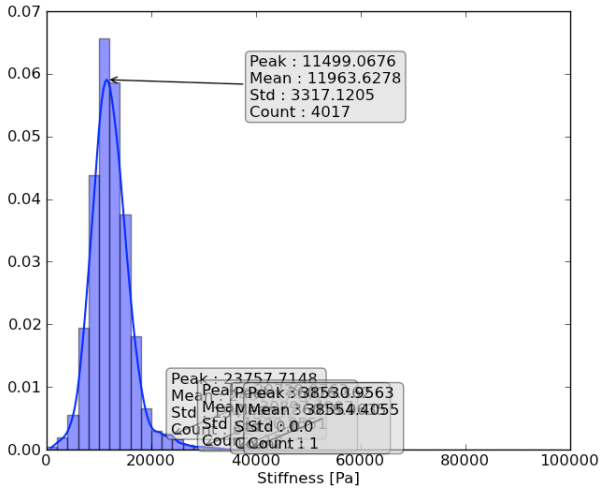
Output :
Young modulii were calculated at 100nm indentation into the cell, with Poisson ratio=0,5 and tip diameter of 40nm (sphere model).
We got several scans on 2 or 3 cells, which is not sufficient to have any statistical significance. We here give only an indication on how the Young modulus is affected by fixation but for definitive statement much more data should be obtained.

We treated cells with cytochalasin D (CytD) to disrupt the actin cytoskeleton. Treatment was at least 10mn (the longer, the more pronounced effect).

As compared to living cells, we see a decrease in the elasticity, peak goes from 6.8 kPa to 5.4 kPa (in agreement with previouswork published in the litterature).

We see another peak at 31kPa which is likely due to an artefact because the scan was obtained on the edge of cells. These data should be discarded but serve as a «bad» example for the practical :)





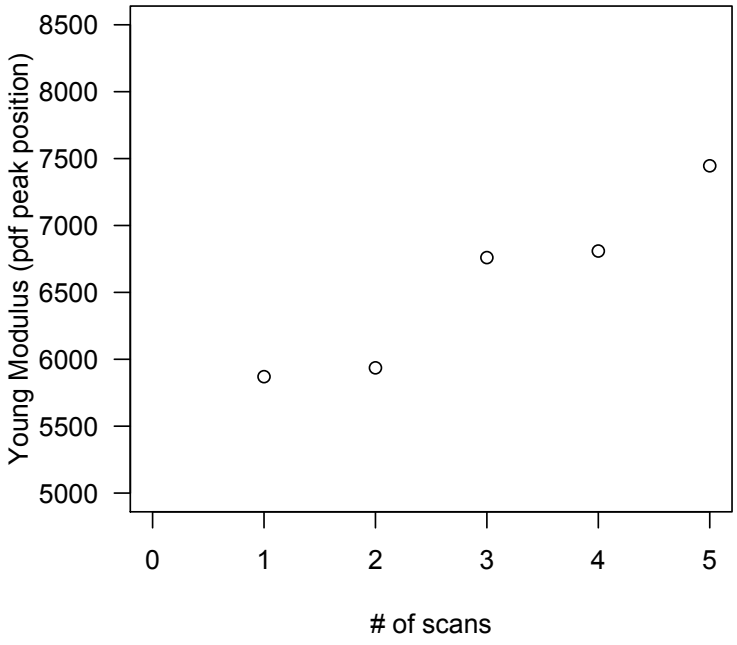
We also scanned PFA-fixed CytD-treated cells. We see here 2 things :
- The elasticity seems much higher as compared as to normal treated cells, the fixation as a big effect
- The elasticity decreased as compared to control PFA-treated cells (peak varies from 13.5 to 11.5 kPa)

Hence, given this small set of data, it appears difficult to analyze the effect of actin destabilizing drug comparing fixed and living cells.

Day 4 - Thursday 1st : Kinetics of PFA fixation

We used the Bruker's flow cell to performed force-distance curves (force-volume) in realtime during PFA fixation. Unfortunately, we encountered technical problems :
- The flow itself induced perturbations (that could be possibly solved by decreasing the flow rate)
- Cell debris moving under the flow perturbed the measurements (dead cells were also moving...)

Even though the data set was not satisfactory, we were able to observe a mean increase in the young modulus :



This is just an indication and further analysis would be definitively required for definitive statement...

Data analysis

Data were processed using OpenFovea software which has been written in G. Dietler/S. Kasas lab by Charles Roduit. You can find more information here :
http://www.freesbi.ch/fr/openfovea

It should be released soon as a freeware with possibilities of implementing add-ons.

For further information, do not hesitate to contact me @ sebastien.janel@ibl.fr