Automation of Bio-Atomic Force Microscope Measurements on Hundreds of C. albicans Cells

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TITLE:

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SUMMARY:

This protocol aims at automatizing AFM measurements on hundreds of microbial cells. In the first step, microbes are immobilized into PDMS stamp microstructures while in the second step, force spectroscopy measurements are performed automatically on hundreds of cells immobilized in step one.

ABSTRACT:

The method presented in this paper aim at automatizing Bio-AFM experiments and especially the recording of force curves. Using this method, it is possible to record, in 4 hours, forces curves, on 1000 cells, automatically. To maintain a 4 hours analysis time, the number of force curves per cell is reduced down to 9 or 16. The method combines a Jython based program and a strategy for assembling cells on defined patterns. The program, implemented on a commercial Bio-AFM, is able to center the tip on the first cell of the array and then to move, automatically, from cell to cell while recording force curves on each cell. Using this methodology, it is possible to access the biophysical parameters of the cells such as their rigidity, their adhesive properties etc. Thanks to the automation and the large number of cells analyzed, one can access the

behavior of the cells population. That is a breakthrough in the Bio-AFM field where data have, so far, been recorded on only a few tens of cells.

INTRODUCTION:

The main purpose of this work is to provide a methodology to perform automatic force measurements on hundreds of living cells using an atomic force microscope (AFM). It also provides a method to immobilize microbes on a PDMS microstructured stamp that is compatible with AFM experiments conducted in liquid environment.

Bio-AFM is a highly specialized technology especially conceived for applications in biology and then to study living cells. It requires a trained engineer able to analyze one cell at the time. In these conditions the number of different cells that can be analyzed is rather small, typical 5 to 10 cells in 4-5 hours. However, the quantity of force measurements recorded on a single cell are usually very high and can easily reach 1000. Thus, the current paradigm of AFM force measurements on living cells is to record hundreds of force curves (FCs) but on a limited number of cells.

Statistically, this approach is questionable, and raises the issue of the representativeness of the sample. Indeed, it is difficult, for example, to evaluate the heterogeneity of a cell population by measuring only a few cells, even if hundreds of measurements are recorded on these few cells. However, it is on the basis of this paradigm that major advances have been made in biophysics, microbiology and nanomedicine[1–3]. Indeed, nanometer analysis at the scale of single cells has provided new information for example on cellular nanomechanics, on the organization of transmembrane proteins, or the action mechanism of antimicrobial or anticancer drugs[4–7]. However, during these last years, several high throughput biomechanical tests conducted on cells have emerged[8], showing the scientific community interest in changing this paradigm and to access the cell population heterogeneity. These tests all rely on microfluidic systems to deform cells and optically measure their deformation under stress to obtain an indirect measure of their overall surface elasticity[8]. However, an important issue with these methods is that they are mono-parametric: only cell elasticity can be probed. Moreover, they do not allow the measurement of the mechanical parameters of adherent cells, which can be limiting for the studies of non-circulating mammalian cells or biofilms for example.

Approaches involving AFM have been developed by the teams of S. Scheuring[9] and M. Favre[10]. The first team immobilized cells on fibronectin patterns[9], forcing individual cells to take the shape of the pattern. Then this team mapped the mechanical properties of a few cells to define average data, representative of 14 to 18 cells. The development carried out by the 2nd team[10] aimed at multiplexing the measurements by parallelizing the AFM cantilevers. To our knowledge, this work in the multiplexing direction has not led to measurements on living cells.

An interesting approach proposed by Dujardin's team presents an automated AFM capable of identifying cells and imaging them at the bottom of custom-made wells. Although this method does not allow for the analysis of a large population of cells it allows the automatic testing of different conditions in each well[11].

Our objective in this work was more ambitious since we wanted to measure at least 1000 cells to access not an average cell, but, on the contrary, the heterogeneity between cells.

The strategy that we developed here to access cell population heterogeneity using AFM is based on the analysis of hundreds of cells on which a limited number of force curves are recorded. Compared to the "classical" approach of recording a large number of force curves on a limited number of cells, this approach should be considered as complementary since it does not provide the same information. Indeed, while the typical method allows one to probe individual cell surface heterogeneity, using our approach, we are able to access the entire cell population heterogeneity. To achieve this objective, we have combined a method consisting in directly immobilizing microbes (here the yeast species *Candida albicans*) into the wells of a PDMS microstructured stamp[12], and developing an original program for moving the AFM tip, automatically, from cell to cell[13] and measuring the mechanical properties of each cell.

PROTOCOL

1. Microbial cell culture

1.1. Revivify cells from a glycerol stock.

NOTE: *C. albicans* are stored at -80 °C in glycerol stocks, on marbles.

1.1.1. Pick a marble in the -80 °C stock and rub it on yeast peptone dextrose (YPD) agar. Grow the cells for 2 days at 30 °C, before liquid cultivation.

1.2. Prepare liquid cultures.

1.2.1. Fill a culture tube with 5 mL of sterile YPD broth and add a single colony of *C. albicans* cells, grown on the YPD agar plate.

1.2.2. Grow the culture in static conditions at 30 °C for 20 h before harvesting by centrifugation (4000 x *g*, 5 min). Discard the supernatant and eliminate as biohazard waste.

1.2.3. Wash the pellets 2x with 10 mL of acetate buffer (8 mM sodium acetate, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH = 5.2). Centrifuge (4000xg, 5 min) in between washings.

1.2.4. Resuspend the pellet in 2 mL of acetate buffer and use this solution for cell immobilization on the PDMS stamp.

NOTE: This suspension cannot be stored and should be prepared fresh for section 3. ~~must be used extemporaneously in section 3 (sample preparation).~~

2. PDMS stamp preparation

2.1. Silicon master mold preparation

2.1.1. Draw, using computer assisted design (CAD) software, the desired microstructures.

2.1.2. If a clean room is available follow steps 2 to 12 of the previously published protol[12.] Otherwise, silicon master mold can be acquired from commercial clean room facilities.

2.2. PDMS stamp molding

2.2.1. Prepare 55 g of PDMS prepolymer solution containing a mixture of 10 to 1, mass ratio, of PDMS oligomers and curing agent ~~cross-linking agent~~ (**Table of Materials**).

2.2.2. Mix and degas this solution under vacuum (in the range of 10^{-1}, 10^{-2} bars) until all trapped bubbles are removed from the PDMS solution (5−10 min).

2.2.3. Pour 20 g of the degassed solution on the silicon master mold and degas again (in the range of 10^{-1}, 10^{-2} bars).

NOTE: The stamp thickness should be around 2−3 mm.

2.2.4. When all bubbles are removed, reticulate the PDMS at 80 °C during 1 h.

2.2.5. Cut the PDMS microstructured stamp with a scalpel (0.5 x 1.5 cm^2) in a direction parallel to the visible microstructure arrays.

2.2.6. Peel the stamp from the silicon master mold.

2.2.7. Return the stamp to exhibit the microstructures on its upper side and deposit it on a glass slide. Make sure to have the microstructures facing up away from the glass slide. Align the microstructures that can be seen on the stamp with the side of the glass slide, which will later serve as a reference for the AFM automation procedure.

NOTE: At this stage, the PDMS stamp is ready for cell immobilization. The PDMS stamps can be stored on the silicon master mold for several months. When all the PDMS is removed from the master mold, a new PDMS stamp can be casted again on the master mold (to keep the master mold safe, it is possible to replicate it in polyurethane)[14].

3. Sample preparation

3.1. Cell immobilization

3.1.1. Centrifuge (500 x g, 5 min) 600 µL of the resuspended cell solution to separate the buffer from the cells.

3.1.2. Pipet 200 µL of the supernatant from step 3.1.1 onto the PDMS stamp, and degas under vacuum (in the range of 10^{-1}, 10^{-2} bars) for about 40 min.

NOTE: This step is important to improve the cell immobilization inside the wells. Molecules from the yeast cell wall, present in the supernatant, are probably deposited on the PDMS surface during this pre-wetting step. These molecules, most probably, enhance the adhesion of the cells and contribute to the increase in the stamp filling rate.

3.1.3. After 40 min, with a pipet, remove the buffer from the PDMS surface and deposit, with a pipet, 200 µL of the cell solution from step 1.2.4 for 15 min at room temperature.

~~3.1.4.~~ The cells are then placed into the microstructures of the stamp by convective/capillary assembly. For that, manually spread 200 µL of cells suspension across the stamp using a glass slide in both direction with an angle comprised between 30 and 50°.It may be necessary to pass the glass slide several times on the stamp to achieve a high filling rate. ~~Several passes may be necessary to reach a high filling rate.~~

NOTE: A full description of this method is available in refernce[13].

3.1.5. Remove the cell suspension with a pipet. Wash the stamp 3x with 1 mL of acetate buffer, pH = 5.2 to remove the cells that were not trapped.

3.1.6. Dry the back of the stamp using nitrogen flow, in order to ensure that the stamp will adhere to the dry Petri dish.

3.1.7. Finally deposit the PDMS stamp filled with cells in a Petri dish (**Table of Materials**) and fill it with 2 mL of acetate buffer to maintain the cells in liquid medium.

3.2. Setting the stamp on the AFM stage

3.2.1. Center the stage at 0;0 when starting AFM operations.

3.2.2. Calibrate sensitivity and spring constant of the cantilever on glass and in water as described in Unsay et al.[15]

3.2.3. Take the Petri dish with the stamp and place it in the AFM Petri dish holder.

3.2.4. Align the stamp edge perpendicular to the Petri dish holder Y axis.

NOTE: An acceptable tilt angle is under 5° as illustrated in **Figure 1**.

3.2.5. Place the AFM head onto the stage and be careful that the stepper motors are sufficiently extended to avoid the tip to crash on the stamp.

4. Running the AFM program

NOTE: The AFM program is provided as a **Supplementary Material (**AutomatipSfotware2019.pdf). It requires a JPK-Bruker AFM Nanowizard II or III equipped with a motorized stage and JPK desktop software version 4.3. The program has been developed under Jython (version based on python 2.7)

4.1. Data acquisition

4.1.1. Center the AFM tip on top of the left corner of the 4.5 x 4.5 μm^2 wells (corresponding to the cell size) using the AFM optical microscope. If another well size is needed, center on top left corner of the desired wells.

4.1.2. Perform a 64 x 64 force map (Z range = 4 µm, tip velocity = 90 $\mu m \cdot s^{-1}$, applied force 3 to 5 nN) over a 100 x 100 μm^2 area. Select Force Mapping mode from the Measurement mode drop-down box. In the force control mapping panel input the following paramteres: Rel. Setpoint = 3 to 5 nN; z length 4 µm; Z movement: constant duration; extend time: 0.01s; ext. delay:0; Retr delay: 0, Delay mode: Constant Force, Sample rate 2048 Hz; Z closed loop uncheck; Grid: check Square image, Fast 100 µm, slow: 100µm, X offset: 0 µm; Y offset: 0 µm; grid angle: 0 degree; Pixels: 64x64; pixel ratio: 1:1

NOTE: A typical resulting image is shown in **Figure 2**. This image will help measure and verify the pitch between two wells.

4.1.3. Note the coordinates of the center of the top left well (W1) and of the bottom left well (referred as W2 on **Figure 2**). To do so, make a square box around the well. The coordinate of the center of the box appears on the left panel of the AFM software in x,y coordinates boxes.

4.1.4. To Open the automation software (Automatip_scan.py): in the JPK desktop software click on advance in the top bar menu and select open script. In the window that opens select the path toward the script file provided in **Supplementary Data (**Automatip_scan.py).

4.1.5. Implement W1 and W2 coordinate values in the Inputs box section of the Jython script (Erreur ! Source du renvoi introuvable.**3**). Input the W1 coordinates in the P1 variable line 239 of the script and the W2 coordinates in the P2 variable line 241.

NOTE: The wells selected as initial coordinates (W1 and W2) should not be too close from the scanning area edge. Otherwise the centering algorithm would not execute correctly because it needs to measure the height on the PDMS surface on each side of the well. For an exemple see **Figure 4.**

4.1.6. Attribute the pitch value to the pitch variable line 245 of the script.

4.1.7. Input the well dimension in the *Ws* variable line 248 This is known from the design of the well patterns and can be checked on the same image as the one used to verify the pitch (**Figure 2**).

4.1.8. Write the path to the saving directory in line 251 to save the data at the desired place.

4.1.9. Set the *totalArea* variable line 254 to the desire multiple "*n*" of 100 µm (that is the maximum scan area of the AFM used). The total number of wells that will be probed can be calculated using this value and the pitch: maximum scan area/pitch*n^2.

NOTE: In the example of Figure 3, 9 areas of 100 x 100 µm^2 will be analyzed.

4.1.10. Set the force curves matrix, row and column (3, 3 or 4, 4), recorded per well in the *numScans* variable line 257.

NOTE: In the example of Erreur ! Source du renvoi introuvable.**3**, a matrix of 3 x 3 = 9 FCs will be recorded for each well.

4.1.11. Run the program, click on the start button.

NOTE: The program first automatically executes a centering algorithm to better determine the center of W1 and W2 wells (step 1). It then automatically acquires the Force Curves (FCs) matrix on each well of the first scanning area (step 2). When all the wells of that area are probed, the script automatically moves the AFM tip to the first well of the next scanning area. The tip is retracted, the microscope stage moves to the next area, the tip is again approached on the stamp and the centering algorithm is executed again to re-center automatically on the first well (1') of that area (step 3). The first area is defined by the user, the second one, is on the right etc. till n is reached. n+1 area is underneath n, n+2 on the left of n+1 etc. till 2n is reached. 2n+1 is underneath 2n, and 2n+2 is on the right on 2n etc. Globally, the tip serpentine through the total area. Step 2 and 3 are repeated automatically until the total number "n^2" of scanning areas have been probed. **Figure 5** presents the flowchart of the program.
It takes ~4 h to complete the program.
~~Come back 4 hours later and start data analysis. Data can also be analyzed later.~~

4.2. Data analysis

4.2.1. Execute the "Copy files" python script (**Copy_files_L.py**, provided in **Supplementary Data**) to organize the FCs files into one folder. This script was developed with Pyhton 2.7 and the SciPy module. Use Video Studio Code software to open the python script. Input path to the general folder (line 34 of the script provided in supplementary data) and where it will be stored (line 37).

4.2.2. Open the AFM manufacturer data processing software to analyze the force curves. In the

top menu ‘File’ select open ‘batch of spectroscopy curves’.

4.2.3. In the batch processing window, select the process provided in **Supplementary Data (StiffnessProcess.jpk-proc-force)**. Select the last step of the process and click on ‘keep and apply to all'. All force curves will receive the same treatment.

NOTE: The process uses the calibration from the FCs files to convert the deflection curves into force curves calibrated in Newton; a data smoothing algorithm is applied (average of 3 consecutives points); the baseline is translated to rest on the zero axis; the contact point is extrapolated and the FC is offset to place the contact point at coordinate (0,0); the bending of the cantilever is subtracted to the FCs, the retract slope is fitted. At the end of the data treatment, the software generates a file that contains a table giving for each FCs: its name, Young Modulus, contact point, adhesion force, slopes, etc.

4.2.4. Repeat steps 4.2.1 to 4.2.3 for all experiments. Be careful to save the data in different folders (i.e.: “…\TREATED\” and “…\UNTREATED\”)

4.2.5. Use the R script provided in **Supplementary Data** to plot histograms and box plots and perform ANOVA statistical treatments.

4.2.5.1. To open the R script (**DataAnalisys.R**), use R studio software and load the files containing the information extracted with the data processing software (.tsv).

4.2.5.2. On the environment window use the “Import Dataset” button, from the list displayed select “from text (readr)” and in the new window select the Browser button and find the .tsv file.

4.2.5.3. Once the file has loaded, select the columns (stiffness and adhesion) to be included for the analysis. To run all the code, press ctrl+alt+r.

NOTE: The script works with 4 datasets, consider two experiments both having untreated and treated cells. It is possible to execute blocks of the script and see how the variables change according to the functions executed.

REPRESENTATIVE RESULTS:

We used the described protocol to analyze the effect of caspofungin on the biophysical properties of the opportunistic human pathogen *C. albicans* in its yeast form. Caspofungin is a last chance antifungal molecule used when other drugs are ineffective because of resistance mechanisms cells develops towards antifungals. Its mechanism of action is based on the inhibition of the subunit Fks2 of the complex fks1/Fks2 responsible for the ß glucan synthesis. As ß glucans are a major component of the fungal cell wall[16, 17] we expected modification of the biophysical properties of the cell wall: rigidity and adhesion.

Figure 6 presents typical histograms obtained when all the protocol presented above is applied.

The red histogram represents the stiffness repartition recorded on 957 native cells and the blue one on 574 caspofungin treated cells. The first interesting observation is that both histograms demonstrate a bimodal distribution of the values. This observation is possible only because we measured hundreds of cells. On smaller samples, researchers usually observe a single distribution and miss the population heterogeneity.[17, 18]

The second observation concerns the effect of caspofungin. It globally reduces the stiffness of the cells while still 2 subpopulations exist.
In a last step the proposed protocol provides an ANOVA comparison of the native and treated cells as presented in Figure 7. It demonstrates that the 2 conditions have a different stiffness and that this difference is highly significant (pvalue < 0.001). This value is reached thanks to the large number of cells analyzed and provides a greater confidence in the obtained results.

The adhesion has also been extracted from the automatically recorded data and we found that the adhesion force between the bare tip and native cells was of 0.64 +/- 0.6 nN. In this case also 2 subpopulations were found: the first one has a mean adhesion force of 0.7 +/- 1.4 nN while the second of 4.5 +/- 1.5 nN. The treatment with caspofungin had unpredictable effects on the adhesion. In one experiment no effect was observed, but in another experiment, caspofungin induced a decrease in the adhesion to the tip and a reduction of the population adhesion heterogeneity. These results are extracted from Proa et al.[13], where they are presented in totality.

FIGURE LEGENDS:

Figure 1: Acceptable position of the micro structured stamp on the AFM stage. The tilt angle on the left pictures (up to 5°) can be handled by the program but the tilt on the right is too important (10°). This Figure has been modified from[13].

Figure 2: Typical AFM image of a filled PDMS stamps showing the initial coordinates as W1 and W2, the size of the scanning area (Δ2), the tilt angle (θ). *This Figure has been modified from*[13].

Figure 3: User input section of the script. P1 and P2 refers to the coordinates of well 1 (W1) and well 2 (W2) of Figure 2. The other parameters are the pitch in meter, the well size in meter (Ws), the directory path for saving the data, the total square area that will be probed by the automated AFM (totalArea is the length in meter of the side of the total square area) and the number of force-curves per wells (numScans). All units are in meters.

Figure 4: Optical image providing an example of valuable (green dots) initial wells. The black square represents the scanning area and the red spots, initial wells that should better be discarded. This Figure has been modified from[13].

Figure 5: Program flowchart showing the 5 steps automatically executed by the AFM.

Figure 6: Histograms of the median stiffness values. A and B: Show the median results per cell for native and caspofungin treated cell. This Figure has been modified from[13]..

Figure 7: Box plots comparing native and treated with caspofungin cells. The 3 stars represent a significativity of $p<0.001$. The box represents 90% of the results, the central line is the median value and the vertical bars represent the range of all the data. This Figure has been modified from[13].

Figure 8: Time-position dependency of values. Histograms in the center are the original data which is divided into the different subgroups corresponding to the subpopulations founded (cyan/green). A and B: Show the presence of the two sub-populations at every hour in the experiment. C and D: show the positions of indentation; on each position it is possible to see the presence of the subpopulations (cyan/green). Subgroup organization was done using the k-means algorithm. This Figure has been modified from[13].

Figure 9: The safe area. An area, inside the PDMS well, has been defined as the area where the pyramidal tip does not touche the well edge while reaching the well bottom (in the cas of an empty well). This Figure has been modified from[13].

DISCUSSION

The main improvement provided by this methodology is a significative increase in the number of measured cells in a determined amount of time. The counterpart is a reduction of the number of points measured per cell. It means that our method is not designed to provide a detailed analyzis of a single cell.

The method only applies to cells that can fit in the wells of our PDMS stamp. The stamp is quite versatile, while it contains wells of 1.5x1.5 µm2 up to 6x6 µm2. Still it is impossible to immobilize bacillus or much bigger cells. The stamp and capillary convective deposition cannot be used to immobilize mammalian cells that are much bigger (around 100µm in length).

In this context, Peric et al.[19] developed a smart microfluidic device to immobilize bacillus like *Escherichia coli* and *bacillus subtilis.* This device makes it possible to immobilize, at defined positions and under physiological conditions, bacillus. It would be very interesting to adapt our software to the particular size of this device.

Tip contamination can also be a problem in this automated system. In the case of microbial cells, it is not so pregnant but it is of high importance in the case of mammalian cells. Dujardin et al.[11] addressed this issue by adding, in their automated protocol, a cleaning step. This step consists in checking the laser sum and to activate the cleaning procedure if the sum is too low. The clean step consists in immersing the tip in a well filled with water or ethanol.

A question that systematically arises from this automation work has been: "does the heterogeneity comes from the evolution of the cells during the experiment?". To answer this question, we plotted the stiffness results as a function of time as presented in Figure 8 A and B. It clearly demonstrates that heterogeneous stiffness values are recorded at any time during the experiment.

In the same context the question of the tip position during the measure emerged. It actually could be possible that force curves recorded on the edge of a cell would have a different stiffness from FC recorded on the top of the cells. To avoid this inconvenient we defined what we called the safe area. It is depicted in Figure 9 A and B and represents an area inside the wells where the tip will not touch the well edges during force measurement. Using this "safe area" we were sure to record FC only on cells and at the top of them. As shown in figure 8 C and D the tip position within the safe area is not responsible for the heterogeneity of the results; as we found both phenotypes for each position of the tip, in the safe area.

To make sure that the values recorded at each position are homogeneous we plotted the stiffness values as a function of the position as presented in

Figure 9 C and D. It shows that heterogeneous stiffness values are recorded on each position in the well which means that the observed heterogeneity is not an artifact due to the tip position in the wells.

The protocol presented in this article represents a conceptual and methodological breakthrough in the field of AFM applied in life science. The large amounts of data generated are compatible with automatic analysis which will undoubtedly allow the classification of cell populations according to new criteria. The application of this protocol to protein or sugar arrays is entirely feasible and requires only a few adaptations to consider the spacing between areas of interest.
It is therefore a versatile protocol that is the result of strong interdisciplinary collaboration.

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DISCLOSURES:
The authors have nothing to disclose.

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