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

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1 **TITLE:**
2 **Automation of Bio-Atomic Force Microscope Measurements on Hundreds of *C. albicans* Cells**

3
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24

25 **KEYWORDS:**

26 atomic force microscopy, *C. albicans*, automation, cell nanomechanics, mechanobiology, force
27 curve analysis, cell adhesion, AFM automation, Jython, JPK experiment planner

28

29 **SUMMARY:**

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

34

35 **ABSTRACT:**

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

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

47

48 **INTRODUCTION:**

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

53

54 Bio-AFM is a highly specialized technology especially conceived for applications in biology and
55 then to study living cells. It requires a trained engineer able to analyze one cell at the time. In
56 these conditions the number of different cells that can be analyzed is rather small, typical 5 to
57 10 cells in 4-5 hours. However, the quantity of force measurements recorded on a single cell
58 are usually very high and can easily reach 1000. Thus, the current paradigm of AFM force
59 measurements on living cells is to record hundreds of force curves (FCs) but on a limited
60 number of cells.

61

62 Statistically, this approach is questionable, and raises the issue of the representativeness of the
63 sample. Indeed, it is difficult, for example, to evaluate the heterogeneity of a cell population by
64 measuring only a few cells, even if hundreds of measurements are recorded on these few cells.
65 However, it is on the basis of this paradigm that major advances have been made in biophysics,
66 microbiology and nanomedicine¹⁻³. Indeed, nanometer analysis at the scale of single cells has
67 provided new information for example on cellular nanomechanics, on the organization of
68 transmembrane proteins, or the action mechanism of antimicrobial or anticancer drugs⁴⁻⁷.
69 However, during these last years, several high throughput biomechanical tests conducted on
70 cells have emerged⁸, showing the scientific community interest in changing this paradigm and
71 to access the cell population heterogeneity. These tests all rely on microfluidic systems to
72 deform cells and optically measure their deformation under stress to obtain an indirect
73 measure of their overall surface elasticity⁸. However, an important issue with these methods is
74 that they are mono-parametric: only cell elasticity can be probed. Moreover, they do not allow
75 the measurement of the mechanical parameters of adherent cells, which can be limiting for the
76 studies of non-circulating mammalian cells or biofilms for example.

77

78 Approaches involving AFM have been developed by the teams of S. Scheuring⁹ and M. Favre¹⁰.
79 The first team immobilized cells on fibronectin patterns⁹, forcing individual cells to take the
80 shape of the pattern. Then this team mapped the mechanical properties of a few cells to define
81 average data, representative of 14 to 18 cells. The development carried out by the 2nd team¹⁰
82 aimed at multiplexing the measurements by parallelizing the AFM cantilevers. To our
83 knowledge, this work in the multiplexing direction has not led to measurements on living cells.

84

85 An interesting approach proposed by Dujardin's team presents an automated AFM capable of
86 identifying cells and imaging them at the bottom of custom-made wells. Although this method
87 does not allow for the analysis of a large population of cells it allows the automatic testing of
88 different conditions in each well¹¹.

89

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

92

93 The strategy that we developed here to access cell population heterogeneity using AFM is
94 based on the analysis of hundreds of cells on which a limited number of force curves are
95 recorded. Compared to the “classical” approach of recording a large number of force curves on
96 a limited number of cells, this approach should be considered as complementary since it does
97 not provide the same information. Indeed, while the typical method allows one to probe
98 individual cell surface heterogeneity, using our approach, we are able to access the entire cell
99 population heterogeneity. To achieve this objective, we have combined a method consisting in
100 directly immobilizing microbes (here the yeast species *Candida albicans*) into the wells of a
101 PDMS microstructured stamp¹², and developing an original program for moving the AFM tip,
102 automatically, from cell to cell¹³ and measuring the mechanical properties of each cell.

103

104 **PROTOCOL**

105

106 **1. Microbial cell culture**

107

108 1.1. Revivify cells from a glycerol stock.

109

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

111

112 1.1.1. Pick a marble in the -80 °C stock and rub it on yeast peptone dextrose (YPD) agar. Grow
113 the cells for 2 days at 30 °C, before liquid cultivation.

114

115 1.2. Prepare liquid cultures.

116

117 1.2.1. Fill a culture tube with 5 mL of sterile YPD broth and add a single colony of *C. albicans*
118 cells, grown on the YPD agar plate.

119

120 1.2.2. Grow the culture in static conditions at 30 °C for 20 h before harvesting by centrifugation
121 (4000 x *g*, 5 min). Discard the supernatant and eliminate as biohazard waste.

122

123 1.2.3. Wash the pellets 2x with 10 mL of acetate buffer (8 mM sodium acetate, 1 mM CaCl₂, 1
124 mM MnCl₂, pH = 5.2). Centrifuge (4000xg, 5 min) in between washings.

125

126 1.2.4. Resuspend the pellet in 2 mL of acetate buffer and use this solution for cell
127 immobilization on the PDMS stamp.

128

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

131

132 **2. PDMS stamp preparation**

133

134 2.1. Silicon master mold preparation

135

136 2.1.1. Draw, using computer assisted design (CAD) software, the desired microstructures.

137 2.1.2. If a clean room is available follow steps 2 to 12 of the previously published protocol¹².

138 Otherwise, silicon master mold can be acquired from commercial clean room facilities.

139

140

141 2.2. PDMS stamp molding

142

143 2.2.1. Prepare 55 g of PDMS prepolymer solution containing a mixture of 10 to 1, mass ratio, of
144 PDMS oligomers and curing agent ~~cross-linking agent~~ (**Table of Materials**).

145

146 2.2.2. Mix and degas this solution under vacuum (in the range of 10^{-1} , 10^{-2} bars) until all trapped
147 bubbles are removed from the PDMS solution (5–10 min).

148

149 2.2.3. Pour 20 g of the degassed solution on the silicon master mold and degas again (in the
150 range of 10^{-1} , 10^{-2} bars).

151

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

153

154 2.2.4. When all bubbles are removed, reticulate the PDMS at 80 °C during 1 h.

155

156 2.2.5. Cut the PDMS microstructured stamp with a scalpel ($0.5 \times 1.5 \text{ cm}^2$) in a direction parallel
157 to the visible microstructure arrays.

158

159 2.2.6. Peel the stamp from the silicon master mold.

160

161 2.2.7. Return the stamp to exhibit the microstructures on its upper side and deposit it on a glass
162 slide. Make sure to have the microstructures facing up away from the glass slide. Align the
163 microstructures that can be seen on the stamp with the side of the glass slide, which will later
164 serve as a reference for the AFM automation procedure.

165

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

170

171 3. Sample preparation

172

173 3.1. Cell immobilization

174

175 3.1.1. Centrifuge (500 x g, 5 min) 600 μL of the resuspended cell solution to separate the buffer
176 from the cells.

177

178 3.1.2. Pipet 200 μL of the supernatant from step 3.1.1 onto the PDMS stamp, and degas under
179 vacuum (in the range of 10^{-1} , 10^{-2} bars) for about 40 min.

180

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

185

186 3.1.3. After 40 min, with a pipet, remove the buffer from the PDMS surface and deposit, with a
187 pipet, 200 μL of the cell solution from step 1.2.4 for 15 min at room temperature.

188

189 ~~3.1.4.~~ The cells are then placed into the microstructures of the stamp by convective/capillary
190 assembly. For that, manually spread 200 μL of cells suspension across the stamp using a glass
191 slide in both direction with an angle comprised between 30 and 50°. It may be necessary to pass
192 the glass slide several times on the stamp to achieve a high filling rate. ~~Several passes may be~~
193 ~~necessary to reach a high filling rate.~~

194

195 NOTE: A full description of this method is available in reference¹³.

196

197 3.1.5. Remove the cell suspension with a pipet. Wash the stamp 3x with 1 mL of acetate buffer,
198 pH = 5.2 to remove the cells that were not trapped.

199

200 3.1.6. Dry the back of the stamp using nitrogen flow, in order to ensure that the stamp will
201 adhere to the dry Petri dish.

202

203 3.1.7. Finally deposit the PDMS stamp filled with cells in a Petri dish (**Table of Materials**) and fill
204 it with 2 mL of acetate buffer to maintain the cells in liquid medium.

205

206 3.2. Setting the stamp on the AFM stage

207

208 3.2.1. Center the stage at 0;0 when starting AFM operations.

209

210 3.2.2. Calibrate sensitivity and spring constant of the cantilever on glass and in water as
211 described in Unsay et al.¹⁵

212

213 3.2.3. Take the Petri dish with the stamp and place it in the AFM Petri dish holder.

214

215 3.2.4. Align the stamp edge perpendicular to the Petri dish holder Y axis.

216

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

218

219 3.2.5. Place the AFM head onto the stage and be careful that the stepper motors are
220 sufficiently extended to avoid the tip to crash on the stamp.

221

222 4. Running the AFM program

223

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

228

229 4.1. Data acquisition

230

231 4.1.1. Center the AFM tip on top of the left corner of the $4.5 \times 4.5 \mu\text{m}^2$ wells (corresponding to
232 the cell size) using the AFM optical microscope. If another well size is needed, center on top left
233 corner of the desired wells.

234

235 4.1.2. Perform a 64×64 force map (Z range = $4 \mu\text{m}$, tip velocity = $90 \mu\text{m}\cdot\text{s}^{-1}$, applied force 3 to 5
236 nN) over a $100 \times 100 \mu\text{m}^2$ area. Select Force Mapping mode from the Measurement mode drop-
237 down box. In the force control mapping panel input the following paramteres: Rel. Setpoint = 3
238 to 5 nN; z length $4 \mu\text{m}$; Z movement: constant duration; extend time: 0.01s; ext. delay:0; Retr
239 delay: 0, Delay mode: Constant Force, Sample rate 2048 Hz; Z closed loop uncheck; Grid: check
240 Square image, Fast $100 \mu\text{m}$, slow: $100\mu\text{m}$, X offset: $0 \mu\text{m}$; Y offset: $0 \mu\text{m}$; grid angle: 0 degree;
241 Pixels: 64×64 ; pixel ratio: 1:1

242

243

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

246

247 4.1.3. Note the coordinates of the center of the top left well (W1) and of the bottom left well
248 (referred as W2 on **Figure 2**). To do so, make a square box around the well. The coordinate of
249 the center of the box appears on the left panel of the AFM software in x,y coordinates boxes.

250

251 4.1.4. To Open the automation software (**Automatip_scan.py**): in the JPK desktop software click
252 on advance in the top bar menu and select open script. In the window that opens select the
253 path toward the script file provided in **Supplementary Data (Automatip_scan.py)**.

254

255 4.1.5. Implement W1 and W2 coordinate values in the Inputs box section of the Jython script
256 (**Erreur ! Source du renvoi introuvable.3**). Input the W1 coordinates in the P1 variable line 239
257 of the script and the W2 coordinates in the P2 variable line 241.

258

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

263

264 4.1.6. Attribute the pitch value to the pitch variable line 245 of the script.

265
266 4.1.7. Input the well dimension in the *Ws* variable line 248 This is known from the design of the
267 well patterns and can be checked on the same image as the one used to verify the pitch (**Figure**
268 **2**).

269
270 4.1.8. Write the path to the saving directory in line 251 to save the data at the desired place.

271
272 4.1.9. Set the *totalArea* variable line 254 to the desire multiple "*n*" of 100 μm (that is the
273 maximum scan area of the AFM used). The total number of wells that will be probed can be
274 calculated using this value and the pitch: $\text{maximum scan area/pitch} * n^2$.

275
276 NOTE: In the example of Figure 3, 9 areas of $100 \times 100 \mu\text{m}^2$ will be analyzed.

277
278 4.1.10. Set the force curves matrix, row and column (3, 3 or 4, 4), recorded per well in the
279 *numScans* variable line 257.

280
281 NOTE: In the example of Erreur ! Source du renvoi introuvable.3, a matrix of $3 \times 3 = 9$ FCs will be
282 recorded for each well.

283
284 4.1.11. Run the program, click on the start button.

285
286 NOTE: The program first automatically executes a centering algorithm to better determine the
287 center of W1 and W2 wells (step 1). It then automatically acquires the Force Curves (FCs) matrix
288 on each well of the first scanning area (step 2). When all the wells of that area are probed, the
289 script automatically moves the AFM tip to the first well of the next scanning area. The tip is
290 retracted, the microscope stage moves to the next area, the tip is again approached on the
291 stamp and the centering algorithm is executed again to re-center automatically on the first well
292 (1') of that area (step 3). The first area is defined by the user, the second one, is on the right
293 etc. till *n* is reached. *n*+1 area is underneath *n*, *n*+2 on the left of *n*+1 etc. till 2*n* is reached. 2*n*+1
294 is underneath 2*n*, and 2*n*+2 is on the right on 2*n* etc. Globally, the tip serpentine through the
295 total area. Step 2 and 3 are repeated automatically until the total number "*n*²" of scanning
296 areas have been probed. **Figure 5** presents the flowchart of the program.

297 It takes ~4 h to complete the program.

298 ~~Come back 4 hours later and start data analysis. Data can also be analyzed later.~~

299
300 4.2. Data analysis

301
302 4.2.1. Execute the "Copy files" python script (**Copy_files_L.py**, provided in **Supplementary**
303 **Data**) to organize the FCs files into one folder. This script was developed with Python 2.7 and
304 the SciPy module. Use Video Studio Code software to open the python script. Input path to the
305 general folder (line 34 of the script provided in supplementary data) and where it will be stored
306 (line 37).

307
308 4.2.2. Open the AFM manufacturer data processing software to analyze the force curves. In the

309 top menu 'File' select open 'batch of spectroscopy curves'.

310

311 4.2.3. In the batch processing window, select the process provided in **Supplementary Data**
312 **(StiffnessProcess.jpk-proc-force)**. Select the last step of the process and click on 'keep and
313 apply to all'. All force curves will receive the same treatment.

314

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

322

323 4.2.4. Repeat steps 4.2.1 to 4.2.3 for all experiments. Be careful to save the data in different
324 folders (i.e.: "...\\TREATED\\" and "...\\UNTREATED\\")

325

326 4.2.5. Use the R script provided in **Supplementary Data** to plot histograms and box plots and
327 perform ANOVA statistical treatments.

328

329 4.2.5.1. To open the R script (**DataAnalysys.R**), use R studio software and load the files
330 containing the information extracted with the data processing software (.tsv).

331

332 4.2.5.2. On the environment window use the "Import Dataset" button, from the list displayed
333 select "from text (readr)" and in the new window select the Browser button and find the .tsv
334 file.

335

336 4.2.5.3. Once the file has loaded, select the columns (stiffness and adhesion) to be included for
337 the analysis. To run all the code, press ctrl+alt+r.

338

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

342

343 **REPRESENTATIVE RESULTS:**

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

351

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

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

358
359 The second observation concerns the effect of caspofungin. It globally reduces the stiffness of
360 the cells while still 2 subpopulations exist.

361 In a last step the proposed protocol provides an ANOVA comparison of the native and treated
362 cells as presented in Figure 7. It demonstrates that the 2 conditions have a different stiffness
363 and that this difference is highly significant (pvalue < 0.001). This value is reached thanks to the
364 large number of cells analyzed and provides a greater confidence in the obtained results.

365
366
367 The adhesion has also been extracted from the automatically recorded data and we found that
368 the adhesion force between the bare tip and native cells was of 0.64 +/- 0.6 nN. In this case also
369 2 subpopulations were found: the first one has a mean adhesion force of 0.7 +/- 1.4 nN while
370 the second of 4.5 +/- 1.5 nN. The treatment with caspofungin had unpredictable effects on the
371 adhesion. In one experiment no effect was observed, but in another experiment, caspofungin
372 induced a decrease in the adhesion to the tip and a reduction of the population adhesion
373 heterogeneity. These results are extracted from Proa et al.¹³, where they are presented in
374 totality.

375
376 **FIGURE LEGENDS:**

377 **Figure 1: Acceptable position of the micro structured stamp on the AFM stage.** The tilt angle
378 on the left pictures (up to 5°) can be handled by the program but the tilt on the right is too
379 important (10°). This Figure has been modified from¹³.

380
381 **Figure 2: Typical AFM image of a filled PDMS stamps showing the initial coordinates as W1**
382 **and W2, the size of the scanning area (Δ), the tilt angle (θ).** *This Figure has been modified*
383 *from¹³.*

384
385 **Figure 3:** User input section of the script. P1 and P2 refers to the coordinates of well 1 (W1) and
386 well 2 (W2) of Figure 2. The other parameters are the pitch in meter, the well size in meter
387 (Ws), the directory path for saving the data, the total square area that will be probed by the
388 automated AFM (totalArea is the length in meter of the side of the total square area) and the
389 number of force-curves per wells (numScans). All units are in meters.

390
391 **Figure 4:** Optical image providing an example of valuable (green dots) initial wells. The black
392 square represents the scanning area and the red spots, initial wells that should better be
393 discarded. This Figure has been modified from¹³.

394
395 **Figure 5:** Program flowchart showing the 5 steps automatically executed by the AFM.

396

397 **Figure 6:** Histograms of the median stiffness values. A and B: Show the median results per cell
398 for native and caspofungin treated cell. This Figure has been modified from¹³..

399

400 **Figure 7:** Box plots comparing native and treated with caspofungin cells. The 3 stars represent a
401 significance of $p < 0.001$. The box represents 90% of the results, the central line is the median
402 value and the vertical bars represent the range of all the data. This Figure has been modified
403 from¹³.

404

405 **Figure 8:** Time-position dependency of values. Histograms in the center are the original data
406 which is divided into the different subgroups corresponding to the subpopulations founded
407 (cyan/green). A and B: Show the presence of the two sub-populations at every hour in the
408 experiment. C and D: show the positions of indentation; on each position it is possible to see
409 the presence of the subpopulations (cyan/green). Subgroup organization was done using the k-
410 means algorithm. This Figure has been modified from¹³.

411

412 **Figure 9:** The safe area. An area, inside the PDMS well, has been defined as the area where the
413 pyramidal tip does not touche the well edge while reaching the well bottom (in the cas of an
414 empty well). This Figure has been modified from¹³.

415

416 **DISCUSSION**

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

421 The method only applies to cells that can fit in the wells of our PDMS stamp. The stamp is quite
422 versatile, while it contains wells of $1.5 \times 1.5 \mu\text{m}^2$ up to $6 \times 6 \mu\text{m}^2$. Still it is impossible to
423 immobilize bacillus or much bigger cells. The stamp and capillary convective deposition cannot
424 be used to immobilize mammalian cells that are much bigger (around $100 \mu\text{m}$ in length).

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

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

434

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

440

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

449

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

452

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

456

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

463 It is therefore a versatile protocol that is the result of strong interdisciplinary collaboration.

464

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475 The authors have nothing to disclose.

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