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A detailed guideline for the fabrication of single bacterial probes used for atomic force spectroscopy

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Abstract. The atomic force microscope (AFM) evolved as a standard device in modern microbiological research. However, its capability as a sophisticated force sensor is not used to its full capacity. The AFM turns into a unique tool for quantitative adhesion research in bacteriology by using 'bacterial probes'. Thereby, bacterial probes are AFM cantilevers that provide a single bacterium or a cluster of bacteria as the contact-forming object. We present a step-by-step protocol for preparing bacterial probes, performing force spectroscopy experiments and processing force spectroscopy data. Additionally, we provide a general insight into the field of bacterial cell force spectroscopy.

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1 Introduction

Infectious biofilms on implants or catheters cause serious medical problems that may lead to major medical intervention [1,2]. One key step in the development of a biofilm is the adhesion of bacteria to these medical devices. However, a fundamental understanding of the basic processes governing bacterial adhesion is still lacking [3]. Atomic force microscope (AFM) used in force spectroscopy mode is a promising technique to close this gap in knowledge. By attaching a single bacterium or a cluster of bacteria to an AFM cantilever, so called 'bacterial probes' can be prepared, which allow studying the adhesion process of bacteria with nanometer spatial and piconewton force resolution [4–7].

This paper details a protocol for the fabrication of bacterial probes, both with a cluster of bacteria ('bacterial cluster probe', Fig. 1a) or one single bacterium ('single bacterial probe', Fig. 1b). Further, a detailed description of how to measure and process force spectroscopy data ('force/distance curves', Fig. 1c) with bacterial probes will be given.

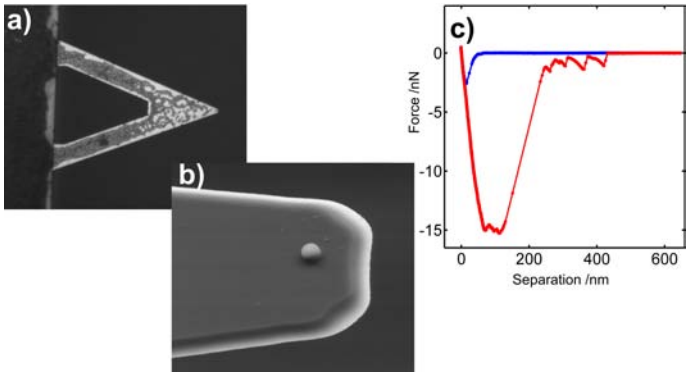

Fig. 1. a) 'Bacterial cluster probe', tipless cantilever covered with a large number of bacteria, b) 'single bacterial probe', tipless cantilever with one single bacterial cell attached. c) representative force/distance curve taken with a single *S. aureus* cell adhering to a hydrophobized Si wafer in PBS buffer (for preparation of the hydrophobic substrate see ref. [8]). Approach (retraction) curve in blue (red).

2 Bacterial cell force spectroscopy

Single cell force spectroscopy (SCFS) is a well-established method for the characterization of adhesive properties of eukaryotic cells [9–12]. The concept of bacterial cell force spectroscopy is the logical continuation of SCFC to prokaryotic cells.

To perform AFM force spectroscopy experiments with bacterial probes, a single bacterium or a cluster of bacteria has to be immobilized on an AFM cantilever. For the immobilization, two parameters are of major importance, namely the geometry of the AFM tip and the selection of an appropriate glue, i.e. a glue that binds the bacteria strong enough to the cantilever to perform force measurements, without changing the properties of the bacterial cell.

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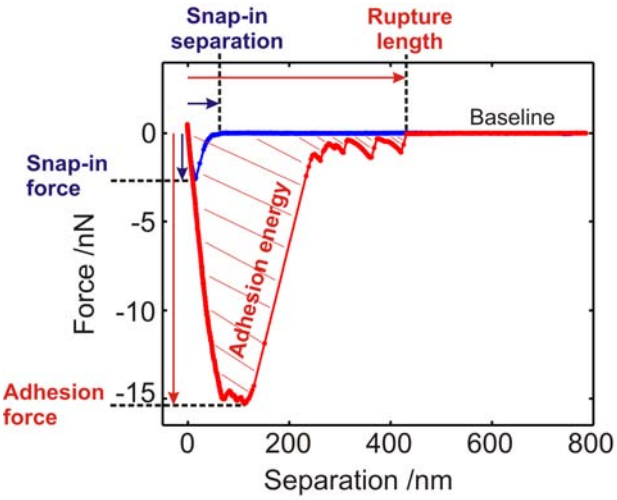

Fig. 2. Representative force/distance curve taken with a single *S. aureus* cell adhering on a hydrophobized Si wafer in PBS buffer. Approach (retraction) curve in blue (red). Fundamental measurands that characterize the bacterial adhesion process are highlighted.

The outcome of AFM force spectroscopy measurements with bacterial probes are called force/distance curves. Thereby, the force acting on the bacterium (attached to an AFM cantilever), is monitored as the cantilever (and the bacterium) is approached to the surface, pressed onto it with a certain maximum force (called 'force trigger'), and retracted from the surface. Fig. 1c and Fig. 2 show a representative force/distance curve taken with a *Staphylococcus aureus* single bacterial probe; the approach and retraction curves are shown in blue and red, respectively. Force/distance curves allow to quantify bacterial adhesion by several means (cf. Fig. 2): The range of attractive forces upon approach can be measured ('snap-in separation'), additionally the 'snap-in force' is a measure for the strength of the attractive forces. During retraction, the lowest point of the curve determines the adhesion force of the bacterium and the distance where the adhesive contact is lost defines the 'rupture length'. Integrating over the area above the retraction curve provides the adhesion energy. Further quantitative parameters such as the separation of the adhesion peak, or the number and depth of secondary peaks can be evaluated depending on the experimental goal. Thus, AFM force spectroscopy with bacterial probes is an unique tool to gain access into bacterial adhesion in a quantitative manner.

2.1 Tip geometries

Various tip geometries can be used as a basis for bacterial probes. To implement experiments enabling both, a large number of repetitions required in biological experiments as well as a large degree of control of experimental parameters, the number of immobilized bacteria needs to be controlled while keeping the preparation procedure as simple as possible. The most advanced bacterial probes feature one single immobilized bacterium (single bacterial probe) as this is the most precise way to characterize bacterial adhesion [7,13]. However, bacterial probes with a larger number of immobilized bacteria (bacterial cluster probes) may be used, as their preparation is less complex and time consuming [14,6]. When using bacterial cluster probes, on the one hand only measurements with the same bacterial probe are comparable, on the other hand the overall larger adhesion force and the averaging over many individual adhesion events can lead to better statistics of the measurements.

The most common tip geometry is the absence of a tip [5,15–18]. These so-called tipless cantilevers feature a large and accessible contact area. Functionalization with a glue and fixation of bacteria are straightforward in the case of tipless cantilevers.

Further typical tip geometries utilized as a basis for bacterial probes are spherical probes [19–21] and pyramidal tips [22–24]. Both spherical probes and pyramidal tips offer only a small contact area to the bacteria due to the curved and pointed geometry. Therefore, a high adhesive strength of the glue holding the bacteria onto the cantilevers is necessary. Moreover, it is challenging to place single bacteria at a specific spot, the apex of the tip or the topmost part of the sphere.

Here we detail a protocol using tipless cantilevers which possesses ease of use and, while using 'single bacterial probes', ensures the comparability of different bacterial probes.

2.2 Immobilization methods

As mentioned above, the selection of the best suited glue is challenging due to two major requirements: On the one hand, bacteria have to be attached to the cantilever by a force that exceeds the adhesion force to the substrate under study. On the other hand, the viability and the properties of the bacterial cell wall that is not in contact with the cantilever should not be affected.

Various types of glues based on different binding mechanisms have been presented in the literature:

- Positively charged polymer coatings such as polyethyleneimine (PEI) [22,25] and poly-L-lysine (PLL) [16, 26] can be used, since the surfaces of both the bacterium and the cantilever, are negatively charged at a physiological pH. However, the effectivity of the electrostatic immobilization may decrease depending on the concentration of electrolytes.
- By using aminosilanes, -thiols [20,27], or (poly)dopamine (PDA) [28,5], the cantilevers can be functionalized with amino groups that can form strong, unspecific, covalent bonds with carboxyl groups that are accessible in the bacterial cell wall. Covalent binding between carboxyl and amino groups may be enhanced by 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) treatment [29,30].
- Specific linkage can be achieved by coating the cantilevers with proteins serving as ligands for compo-

nents of the bacterial cell-wall (i.e. fibronectin-fibronectin binding proteins) [31,32]. Similarly, a commercially available cell adhesive protein derived from *Mytilus edulisi* (Cell-Tak^{TM}) was reported to be a suitable glue to immobilize bacterial cells [30,33].

Although approaches such as the use of regular glue (e.g. glass adhesive) have also been reported in the literature [15,34], a satisfactory fulfillment of the second requirement, the prevention of any alteration of the bacterium, is highly doubtful. The same is true for procedures involving a crosslinking via glutaraldehyde or formaldehyde [25,22], which are known to have an effect on the surface properties of the entire bacterium [35,36].

To immobilize bacterial cells, our protocol utilizes a polydopamin coating of AFM cantilevers that is inspired by a work by Lee et. al. [37]. This coating combines ease of use with biological compatibility and durability [5,7,13].

2.3 Bacterial cluster force spectroscopy

The small size of bacterial cells makes their handling challenging, yet measuring the adhesion of bacterial cell clusters using 'bacterial cluster probes' circumvents this problem [14,6]: These bacterial probes are much easier to produce, but lead to less controlled and less quantitative experimental results as the number of bacteria and the area of contact interacting with the respective surface is largely unknown. When comparing the adhesion to different surfaces, this problem can be handled by performing consecutive measurements on the surfaces of interest with the identical bacterial probe. The result, however, will always be a relative one, since absolute force values cannot be measured with this kind of probe. A comparison between different bacterial probes or different bacterial species is not possible [6]. Bacterial cluster probes are usually based on tipless cantilevers [18,6], some studies, however, describe the use of spherical tips [19] or even normal cantilevers, where the tip is covered with bacteria [23,14]. Another interesting approach is the application of an entire bacterial biofilm to a glass sphere attached to an AFM cantilever [38,39]. Regardless of the exact procedure, all of these bacterial cluster probes lack a certain level of control.

2.4 Single bacterial cell force spectroscopy

The problem of measuring adhesion in an absolute manner can be solved by controlling the number of adhering bacteria, at best by using only one bacterium ('single bacterial probes'). However, the accurate attachment of a single bacterium to an AFM cantilever is fairly impossible without adequate technical equipment, e.g. an AFM with an integrated inverted microscope [7,21] or a micromanipulation system [5,13]. The use of a single bacterial probe results in a highly controlled experiment in terms of the load applied to the bacterium and the measured quantities, in particular the adhesion force. In addition, the viability of the characterized single bacterium can be checked by subsequent live/dead staining.

3 Experimental protocol

3.1 Fundamentals

3.1.1 AFM

We use a Bioscope Catalyst (Bruker-Nano, Santa Barbara, Ca, USA) for AFM bacterial cell force spectroscopy. Yet, the protocol detailed here does not require any special AFM model, except for the possibility of recording force/distance curves.

The following components are part of our AFM system:

1. BioScope Catalyst head ('head')
2. Nanoscope V controller ('controller')
3. BioScope Catalyst Electronics Interface Box ('E-Box")
4. BioScope Catalyst baseplate with sample holder plate ('sample holder')
5. EasyAlign for infrared laser alignment ('alignment station')
6. Joystick for controlling x,y,z motors
7. Nanoscope Software (version 8.15) ('software')
8. Probe holder for measurement in liquid
9. Mount for the probe holder while changing cantilever
10. Magnetic sample substrate clamps

3.1.2 Micromanipulation system

The components of our micromanipulation system are:

1. Inverted fluorescence light microscope Leica DMIL LED Flou
2. Micromanipulator Narishige MOM 202D
3. A homemade aluminum arm with a hole on its upper end (Fig. 3a)
4. A small cross of PMMA that can be inserted into the aluminum arm (Fig. 3b)
5. Double-sided adhesive tape

3.1.3 Cantilevers

The adhesion forces of bacteria can vary over a huge range of forces, from below 100 pN to several tenths of nN. Hence, some experience is necessary to identify the right cantilever spring constant, since stiff cantilevers allow the measurement of higher forces but reduce the experimental resolution in terms of force. We use spring constants between 0.03 and 0.5 N/m depending on the expected forces during the adhesion process.

3.2 The protocol

3.2.1 Functionalization of the cantilever

This method of cantilever-functionalization is inspired by a publication by Lee et al. [37].

1. Take out as many cantilevers as you plan to use in today's experiment, and put them into a clean glass petri dish. The cantilever coating should be freshly prepared each day.
2. Cantilevers are cleaned in an air-plasma for 30 seconds, to get rid of any organic residues.
3. From now on, we perform every step under class 100 (less than 100 particles/ft^3) clean room conditions to reduce the risk of cantilever contamination (clean room conditions are helpful, yet might not be necessary).
4. Prepare a solution of 4 mg/ml dopamin hydrochloride (Sigma-Aldrich) in TRIS/HCL-Buffer (Sigma-Aldrich, 10 mMolar, pH 7.9 at 22 °C).
5. Dip the cantilevers vertically into the dopamin solution and store them for about one hour in the refrigerator.
6. Take the cantilevers out of the solution and rinse them carefully with ultrapure water (0.055 μS/cm at 26 °C).
7. Dry the cantilevers under vacuum (approx. 1 mbar) for about 15 minutes or under a laminar flow bench for at least one hour.
8. Proceed with the calibration of the cantilever.

3.2.2 Preparation of the substratum

The surface preparation very much depends on the type of substratum. Therefore, this issue is not detailed here. However, some fundamentals have to be obeyed irrespective of the exact surface: The surface has to be clean and inert concerning the used buffer solution and it must be fixed within the liquid cell (a petri dish or something similar) in order to prevent unwanted motion of the substratum. If using glue for fixation, it must not contaminate the buffer and should not dissolve. We use polystyrene of high molecular weight (780 kg/mol) dissolved in chloroform (in a concentration of 40-50 mg/ml), which works well in combination with polystyrene petri dishes. The chloroform evaporates fast and the residual polymer melt is a ideal non-dissolving glue.

3.2.3 Calibration of the cantilever

The calibration of the cantilever is a crucial step in AFM force spectroscopy. In order to be able to apply exact force values, calibration should be done before bacterial force spectroscopy measurements. If the single bacterium is attached using the AFM piezo drive, no difficulties will occur during this step (the protocol is the same)[21]. However, this protocol describes the attachment of a single bacterium via an external micromanipulation system. Therefore, the cantilever has to be removed from the AFM head after calibration. This step could result in change of the deflection sensitivity since the laser spot has to be refocused after the reassembly of the cantilever into the AFM head. Yet, experience has shown that the deflection sensitivity does not change significantly if (i) the laser position on the cantilever matches the position during calibration, which should be controlled by eye and (ii) the laser sum is almost identical to before. In the following, we give step-by-step instructions for calibrating an AFM cantilever:

1. Check that AFM, computer, controller, and other AFM electronic devices as well as all necessary components of the optical microscope are turned on. Depending on the instruments, it may take a significant amount of time until e.g. thermal drifts have equilibrated.
2. Prepare everything for a contact mode experiment in liquid.
3. For the calibration of the cantilever, a hard (indeformable) sample should be used to determine the deflection sensitivity.
4. Insert the functionalized cantilever carefully into the cantilever holder that enables measurements in liquid and cover it with a droplet of liquid (e.g. PBS) to avoid contamination.
5. Integrate the cantilever holder into the AFM head.
6. Align the laser spot on the back of the cantilever maximising the sum of the voltage signal on the photodiode.
7. Place the cantilever over the hard sample surface to calibrate it.
8. Approach the surface to a distance of about 100 μm manually.
9. Give the AFM/cantilever some time to equilibrate, until a constant signal on the photodiode is reached.
10. Enter the deflection setpoint to approach the surface.
11. Start the approach.
12. As soon as the cantilever reaches the surface, change into ramp mode.
13. Give values for ramp rate (ramp size). Common values are between 0.5 and 1.5 Hz (600 to 1000 nm).
14. Enter an approximate spring constant to perform the calibration force/distance curve. Usually, the value indicated by the manufacturer is sufficient at this point.
15. Enter a force trigger of 3-8 nN (depending on the cantilever stiffness) and record one single force/distance curve.
16. To get a reliable value for the deflection sensitivity, a large, undisturbed, linear part in the contact regime of the force/distance curve is necessary. If the force curve does not exhibit an appropriate linear part, try a larger force trigger.
17. The deflection sensitivity is determined by calculating the inverse of the slope of the force/distance curve in the contact regime (this is usually implemented in the AFM software).
18. Update the deflection sensitivity.
19. Retract the cantilever from the surface.
20. To prepare for thermal tune, the influence of the surface must be excluded. Therefore, enlarge the distance between the surface and the cantilever. It should be at least 50 μm.

21. Perform a thermal tune to determine the cantilever spring constant (details should be checked in the user manual of the respective AFM).
22. Update the spring constant and retract the cantilever completely.
23. Remove the cantilever holder (with cantilever) from the AFM. Care should be taken to maintain a small amount of liquid on the cantilever holder covering the cantilever to avoid contamination of the cantilever.
24. Go on with 'attachment of bacteria'.

3.2.4 Attachment of bacteria

In the following, we describe two different methods for attaching bacteria to a functionalized cantilever: i) A rather simple method to produce a bacterial probe with a cluster of attached bacteria, and ii) a more complex preparation for the attachment of a single bacterial cell to a tipless cantilever is detailed.

Bacterial cluster probe

1. Place the functionalized tipless cantilever on a hydrophobic surface. The side intended to carry the bacteria faces upwards.
2. Cover the cantilever with a droplet of bacterial solution ($\approx 60\,\mu$l) and leave it in the refrigerator (to reduce Brownian motion) for at least one hour.
3. Remove the bacterial solution and rinse the cantilever carefully with PBS buffer to get rid of poorly attached bacteria.
4. By optical microscopy, verify that bacteria are attached close enough to the free end of the cantilever (not further away than roughly three bacterial diameters[1] to safely exclude cantilever/substrate interactions), while the cantilever stays in liquid the whole time. Ideally, this step is done using reflection optical microscopy. Alternatively, a transmission optical microscope can be used after integrating the cantilever into the cantilever holder for measurements in liquid and using a set-up similar to the one described in the section "single bacterial probe".
5. Integrate the cantilever into the probe holder for measurements in liquid and mount it to the AFM head.
6. Cover the cantilever immediately with a droplet of PBS to avoid drying.

Single bacterial probe

For the attachment of a single bacterium to a functionalized cantilever, a micromanipulation system is used. Stress due to capillary forces or drying should be avoided by maintaining bacterium as well as cantilever in liquid/buffer during the entire preparation procedure.

[1] The cantilever is usually tilted by an angle α in the AFM, the upper limit of the distance l between the bacteria (with diameter d) and the free end of the cantilever can be calculated as $l = d/sin(\alpha)$.

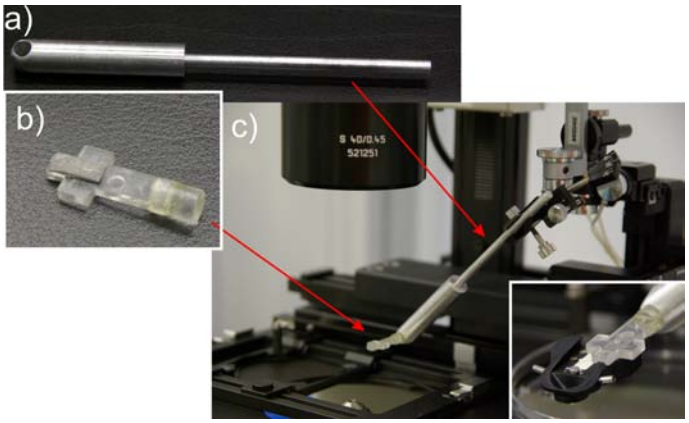

Fig. 3. a) Aluminium micromanipulation arm with a hole to insert the PMMA cross, b) PMMA cross with a small piece of double-sided adhesive tape, c) manipulation arm with PMMA cross inserted into the micromanipulator. The inset shows the cantilever holder attached to the PMMA cross.

1. Put a plastic petri dish on the microscope of the mircomanipulator.
2. Place a tiny droplet ($\approx 1\,\mu$l) of bacterial solution on the petri dish.
3. Give the bacteria some minutes to sediment on the petri dish, without complete drying.
4. Insert the manipulation arm into the micromanipulator (cf. Figs. 3a and c).
5. Put a small piece of the double-sided adhesive tape on the PMMA-cross (cf. Fig. 3b).
6. Fix the cantilever holder with the cantilever and the covering droplet (resulting from the calibration step) on the PMMA-cross (cf. inset to Fig. 3c)[2].
7. Insert the PMMA-cross with the cantilever holder into the aluminum arm (cf. Fig. 3c).
8. Place a droplet of PBS-buffer ($\approx 20\,\mu$l) on the tiny droplet covering the pre-attached bacteria.
9. Use a 10x objective/10x eyepiece of the microscope to bring the cantilever directly over the droplet and lower it into the droplet of bacterial solution.
10. Focus onto the bacteria lying on the petri dish and approach the cantilever to the surface until it is almost in focus.
11. Change to 40x or higher objective and use the precision control to place the cantilever straight above a single bacterium.
12. Lower the cantilever onto the single bacterial cell and press it gently (the pressure can be controlled by watching the light reflection off the cantilever, which should change only slightly) onto the bacterium.
13. Pull the cantilever immediately away from the surface again. Focus onto the cantilever to confirm the attachment of a single bacterial cell close enough to the free end of the cantilever (cf. bacterial cluster probe).
14. If the bacterium did not attach, repeat step 10 to 13.

[2] The exact procedure of integrating the cantilever holder into the micromanipulator might differ, depending on type and design of the cantilever holder.

15. Retract the cantilever from the surface and out of the droplet. Ensure that a small amount of buffer remains at the probe holder covering the cantilever. As the cantilever has to be covered by liquid the whole time, some more liquid might be added if the cantilever starts drying out.
16. Confirm again the attachment of the single bacterium close enough to the free end of the cantilever (see above). If the bacterium has detached, start again with step 8.
17. Remove the probe holder from the micromanipulator and insert it into the AFM head.
18. Go on with section 'Force distance measurements with a bacterial probe'.

3.3 Force distance measurements with a bacterial probe

1. Place the bacterial probe right above the surface on which the adhesion will be measured. At best, the droplet covering the cantilever and the liquid covering the surface do not touch at this step.
2. Use the AFM step motor to lower the cantilever towards the surface. Stop the movement about 100 μm above the surface. Crashing the cantilever into the surface will at best only detach bacteria, but may destroy the cantilever.
3. Check the experimental parameters: AFM in 'contact mode', set scan size to zero, choose a 'deflection setpoint' that ensures a force of less than 1 nN.
4. Start the approach.
5. As soon as the approach is finished, change into force spectroscopy mode, this will retract the bacterium from the surface.
6. Set the parameter values for the force/distance measurements:
   - Define the total distance the piezo moves during the force/distance curve (this value may be called 'ramp size'). The value for the ramp size depends on the expected rupture length (see above), common values for the ramp size are around 1 μm.
   - Define the number of data points while approaching/retracting, which constitutes - in combination with the ramp size - the z-resolution of the curve. The z-resolution should be at least one point per nm.
   - Define the number of full force/distance curves per second ('ramp rate'). In combination with the ramp size, the ramp rate defines the tip velocity. Typical values are between 0.5 and 1.5 Hz.
   - Define the speed of the piezo movement in z-direction. This defines in combination with the ramp size - the ramp rate.
   - Define the so called 'trigger threshold' (this is the force value at which the cantilever/bacterium approach is stopped). Typical values are less than 0.5 nN.
   - Define a time span between stopping the approach and starting the retraction of the cantilever, i.e. a time of contact between bacterium and surface (this value is called 'surface delay').
   - A second timespan may be defined that delays the start of a force/distance curve after full retraction of the preceding one.
7. Perform one single force/distance curve with the above-defined parameters.
8. Investigating the shape of the force/distance curve will help to decide whether the bacterium is still attached to the cantilever (cf. Ref. [13] Fig. 3) or not. However, as the shape of the force/distance curve depends on the combination of surface and bacterium, this may require some experience. If the bacterium becomes detached, attach a new one.
9. Run a number of force/distance measurements with one set of parameters. Take care that the same spot of the substratum is not probed twice to exclude influences of potential residues originating from preceding approaches. Some AFM offer an automatic realization of a number of force/distance curves on different spots.
10. Conduct additional sets of force/distance curves while changing the experimental parameters according to the respective experimental goal.
11. Take care that the last series of force/distance curves for a bacterial probe reproduce the parameters of the first series. That way, changes of the bacterial adhesion properties due to the force measurements can be identified. The number of maximum force/distance curves per bacterial probe is usually limited due to fading effects (i. e. the adhesion strength of the bacterium may decrease due to repeated pull-off events, possibly by loosing surface adhesins) or loss of the bacterium/bacteria. The influence of fading effects depends on the respective bacterium/surface combination, however, at least 100-150 curves per bacterial probe are usually possible.
12. If all measurements are finished, retract the cantilever from the surface.
13. In the case of single cell measurements, the existence of the single bacterium can be confirmed optically.
14. If the AFM is linked to an integrated inverse microscope, the presence of the single bacterium on the cantilever can be checked directly. Otherwise, the cantilever has to be removed from the AFM and reintegrated into the inverse microscope linked to the micromanipulator:
    (a) Use the motors to retract the cantilever as far as possible from the surface.
    (b) Remove the cantilever holder (with cantilever) from the AFM. It is important that some liquid (buffer) remains that covers the bacterial probe. This avoids losing the bacterium by capillary forces and prevents it from drying out. If the droplet covering the cantilever is tiny, add a small amount of buffer ($\approx 10\,\mu$l) with the pipette.
    (c) Insert holder and cantilever into the microscope set-up (specified in paragraph 'Single bacterial probe').

(d) Use the microscope to confirm the attachment of the single bacterium.

15. The cantilever may be reused by detaching the single bacterium and attaching a new one:
    (a) Put a plastic petri dish on the inverted microscope linked to the micromanipulator.
    (b) Use the 10x objective to approach the cantilever with the single bacterium towards the surface of the petri dish and stop a few micrometers before.
    (c) Use the 40x objective and carefully press the bacterium onto the surface until a slight deflection of the cantilever can be seen by a change in the cantilever light reflection.
    (d) Pulling the cantilever backwards over the surface (in the xy plane) will shear the bacterium off the cantilever.
    (e) Retract the cantilever from the surface. Make sure that a small amount of liquid remains on the petri dish covering the bacterium. The viability of this bacterium can then be checked by a live/dead stain in the following.
    (f) Repeat the 'single bacterium probe' steps to attach a new bacterium.

3.4 Viability of bacteria

The viability of the bacterium/bacteria either attached to the cantilever or the one sheared off on a petri dish (see previous paragraph) can be checked via a live/dead stain. However, as the shearing process may harm sensitive bacterial cell types, we recommend testing the viability directly on the cantilever.

1. Focus the fluorescence microscope on the bacterium lying on the petri dish or fixed to the cantilever.
2. Add a small amount of live/dead stain (e.g. Life Technologies GmbH, Germany) (about 20 μl) to the buffer covering the bacterium.
3. Shade all the surrounding light to avoid photo-bleaching and wait for ten minutes.
4. Verify the viability of the bacterium used in the force measurements by means of its color (cf. Fig. 4).

3.5 Data calibration

The basic data recorded by the AFM during a force spectroscopy experiment are the voltage applied to the piezo controlling the movement in z-direction (z-piezo) and the voltage signal on the photodiode, quantifying the shift of the laser spot reflected from the back of the cantilever. A 'height sensor' may give a second measure for the z-position of the cantilever. Based on the calibration, these outputs are then presented as a force vs. z-position curve. This is usually done automatically, nevertheless, we will go through it here:

1. The AFM internal calibration of the z-piezo converts the applied voltage into the dilatation of the piezo. However, users should be aware that the z-position is always a relative measure between the starting point and the actual position.
2. Two steps are performed to convert the voltage signal from the photodiode into the actual force exerted on the cantilever:
   – The deflection of the cantilever 'd' in nm can be obtained by multiplying the voltage signal by the deflection sensitivity (see calibration of cantilever).
   – The force on the cantilever 'F' in nN can be calculated by applying Hooke's law ($F = k \cdot d$), with the spring constant 'k' of the cantilever (see calibration of cantilever).
3. Since the output of the photodiode is a relative measure, the baseline of the force distance curve - representing the zero-force part before/after contact - is often shifted along the y-axis. By applying an offset correction, the baseline can be brought in line with the x-axis (cf. Fig. 5)

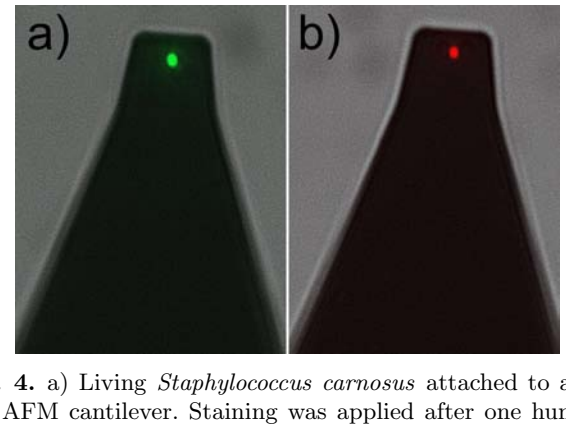

Fig. 4. a) Living *Staphylococcus carnosus* attached to a tipless AFM cantilever. Staining was applied after one hundred force/distance curves. b) Ethanol-killed *S. carnosus* cell attached to a tipless AFM cantilever. Live/dead staining was performed as described in chapter 3.4.

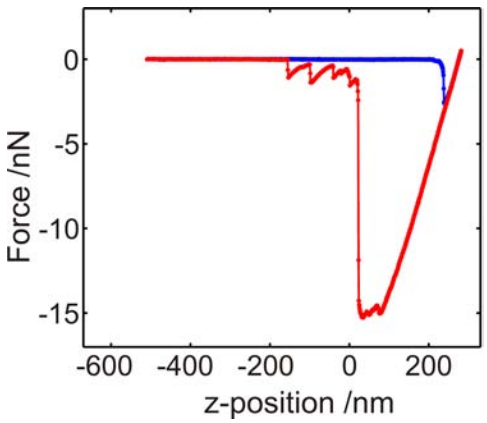

Fig. 5. Force/distance curve plotted as force vs. z-position after baseline-correction. Approach (retraction) curve is shown in blue (red).

These three steps result in a calibrated force/distance curve in the form of a force vs. z-position plot (cf. Fig. 5). For most subsequent analysis steps, however, the force vs. z-position representation is not ideal and rather a force 'F' vs. separation 's' plot is required. One of the main disadvantages of AFM in general is that it lacks the ability to directly measure the separation between probe and surface since the system basically reports solely the z-position of the cantilever mount. Yet, if the point of zero separation i.e. the point of contact 'z_0' between probe and surface can be accurately determined in the force/distance curve, it is possible to convert the z-position to the actual separation. In the case of a bacterium adhering to a hard surface, the point of contact can be assumed to be the point at which force is again zero after the snap-in event (cf. Fig. 6a). To then convert the force vs. z-position plot then into a force vs. separation plot, the following two steps are required (cf. Fig. 6):

1. Define the contact point z_0, in our case this is the point of zero force after the snap-in (cf. Fig. 6a). Shift the force/distance curve along the x-axis by calculating $z' = z_0 - z$ (cf. Fig. 6b).
2. The separation between the bacterium and the substrate surface is calculated by adding the deflection d to the shifted z-position z' (cf. Fig. 6c).

The calculation can be done simultaneously for both approach and retraction part of the force/distance curve.

4 Conclusion

Here we present a simple and reproducible procedure to fabricate viable bacterial probes and to perform bacterial cell force spectroscopy measurements. The protocols presented describe the fabrication of both bacterial cluster probes, as well as single bacterial probes, in detail. Our approach allows for measurements with high precision and high throughput and features a simplicity with regards to applicability and equipment availability, which may pave the way for bacterial cell force spectroscopy as a standard technique in modern bacterial adhesion research.

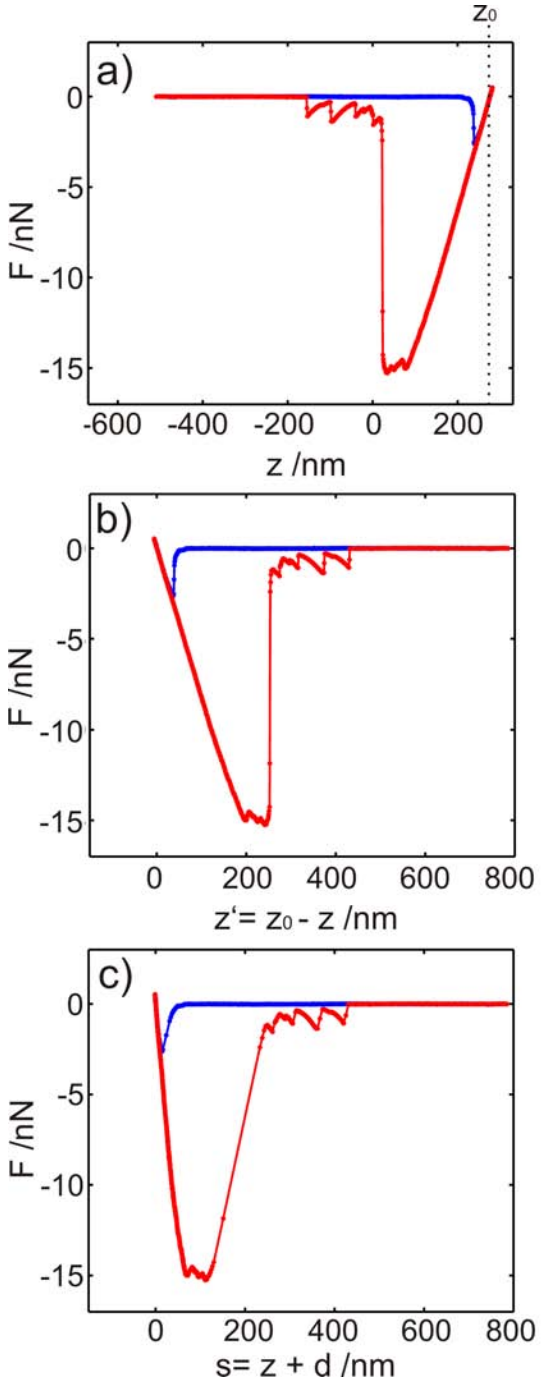

Fig. 6. Work steps for calculating a force (F) vs. separation (s) curve. a) Starting with a baseline-corrected force (F) vs. z-position (z) curve, b) a force (F) vs. z-position (z') curve with the respective point of contact is calculated and c) subsequently transformed into the force (F) vs. separation (s) curve.

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