Toward imaging live, competent *Acinetobacter baylyi* with Liquid Cell Atomic Force Microscopy

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By

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Abstract

Imaging live bacteria with an atomic force microscope (AFM) is a challenging process, taking into account the lateral forces exerted by the probe on cells that must be immobilized but still immersed in liquid. The Lang-Lostroh labs use AFM to examine *Acinetobacter baylyi* cells that are competent (able to take up DNA from the environment). In order to image cells while they are competent, it is necessary to find an effective combination of liquid media and sample preparation method while maintaining the cells alive, competent, and attached to the AFM pucks with porcine gelatin. Many combinations were tested using different washing and gelatin immobilization media (distilled deionized water, and phosphate-buffer saline), as well as using different media (distilled deionized water, phosphate-buffer saline, and diverse dilutions of Luria-Bertani broth with and without sodium chloride) during imaging itself. The combinations that presented the most satisfying images of immobilized cells with AFM were submitted to membrane integrity and competence assays. Washing and immobilizing the cells with distilled deionized water and imaging them with 50% Luria-Bertani broth without sodium chloride was the most successful combination. Even though the cells did not show detectable competence ability, they were alive yet inactive since after the imaging procedure they were still able to form colonies on plain Luria-Bertani agar plates. It provides insight on a viability spectrum that must be taken into account when distinguishing cell viability. Further research should focus on testing smoother media transitions with access to food source to avoid osmotic pressure stress and starvation.

Introduction

Since its invention and introduction in 1986 (Binnig et al., 1986), atomic force microscopy (AFM) has been a vital imaging tool in both scientific and industrial settings. Microbiology easily incorporated this scanning probe microscopy into research, as it can generate three-dimensional maps at nanoscale resolution and measure surface physical properties (Dufrêne, 2004; Muller and Dufrêne, 2008). The possible applications and developments of AFM can enhance our understanding of bacteria, providing different types of images not possible using other types of microscopy commonly used in microbiology (optical and electron).

AFM combines the best of both optical and electron microscopy, since it can image in ambient air or liquid environment at a very high resolution, however at a slower pace. AFM overcomes some of the major limitations of optical and electron microscopy. Firstly, optical microscopy limits the resolution at approximately 0.2 μm, only a fifth of the usual bacterial diameters (1 μm), which is unhelpful to study their macromolecular structures. Secondly, electron microscopy can also achieve nanoscale resolution, but the expensive, long sample preparation method and the need of a vacuum environment to operate irreversibly damage the cells. In contrast, AFM requires minimal sample preparation as it can be performed in air (dry cells) or liquid media, reducing deterioration of biological structures. Furthermore scanning electron microscopy only provides a pseudo-three dimensional image, while images taken by AFM are truly three-dimensional so that measurements can

be taken in all three dimensions. AFM imaging in air does require that the cells adhere onto the substrate while desiccating and consequently killing them. Nonetheless, it is possible to visualize and study macromolecular structures as small as approximately 10 nm that were present on the outside of the cell before drying out, such as appendages (Jonas et al., 2007; Chao and Zhang, 2011). Even though AFM imaging in liquid media does not yield the same image quality and resolution (Allison et al., 2011; Kuyukina et al., 2014), it enables the opportunity to study live bacteria in their natural environment and also under laboratory conditions.

AFM is a scanning probe type of microscopy that can provide a true three-dimensional map. The microscope relies on the back-and-forth movement of a cantilever over an area of a selected size (Fig. 1). A highly sensitive photodiode (light sensor) detects the position of a laser beam that is being reflected on top of the cantilever. The cantilever has a tip facing downwards that will contact the surface of the sample. When the tip encounters an obstacle, it will be displace the cantilever thus displacing the laser beam from the center of the detector. A computerized system will compensate that movement by bringing back the laser beam to the center of the detector, thus allowing the microscope to truthfully display minute differences in height. The cantilever will scan the same line twice laterally, for a previously set number of lines on a given area. The nature of this probing microscopy creates small lateral forces on the sample, which may affect imaging live bacteria in liquid.

Imaging bacteria in a liquid environment with AFM is a challenging process, taking into account the lateral forces exerted by the probe on cells that must be

immobilized onto the substrate. There are several methods of cell immobilization available, each with their advantages and disadvantages (Meyer et al., 2010; Kuyukina et al., 2014). These include physical entrapment in pre-manufactured microwells (Kailas et al., 2010; Francius et al., 2008), which allows greater flexibility of imaging media, but it may physically obstruct the probing action at the cell surface periphery in contact with the microwell. Another common adherence approach takes advantage of the chemical interactions between the negatively charged cell surface with positively charged gelatin (Doktycz et al., 2003; Allison et al., 2011), poly-L-lysine (Lonergan et al., 2014), or others. On one hand, the cell surface-gelatin electrostatic interaction limits the use of ionic media because charges in the media can disrupt the immobilization. On the other hand, immobilization with gelatin is applicable to cells of all sizes and shapes (Meyer et al., 2010). Due to the variability of cell surface components across bacteria, the immobilization method must reflect the specimen's characteristics as well as the research focus and needs to be optimized for every different species examined and for use in the particular research application.

In this study, the objective was to image *Acinetobacter baylyi* ADP1 (BD413) in liquid media, while ensuring the cells' viability throughout the scanning process, which has not been performed before. *A. baylyi* is a Gram-negative, strictly aerobic, soil bacterium, with a remarkably high rate of competence for natural transformation (Juni and Janik, 1969). Natural transformation is a bacterial process where extracellular DNA is taken up by the cells from the environment and incorporated into the genome with the possibility of using that new genetic

information to synthesize proteins that might be advantageous if the appropriate conditions are met. A fresh cell culture can reach the stationary phase within 8 hours, facilitating laboratory research (Barbe et al., 2004) due to a 30-minute generation time. As *A. baylyi* undergoes morphological changes throughout the 8-hour period until reaching stationary phase (Lang et al. unpublished data), it is not desirable to use a physical entrapment method for immobilization. Doing so may exert foreign forces on the cells, compromising their natural growth (Lonergan et al., 2014). Within the chemical interaction methods, the use of gelatin is preferred as it is less likely to perturb the cell surface morphology and composition than poly-L-lysine (Kuyukina et al., 2014). Gelatin immobilization method was previously applied to study pathogenic Gram-negative bacteria, such as *Acinetobacter baumannii* (Soon et al., 20011), *Escherichia coli* (Beckmann et al., 2006), and *Pseudomonas aeruginosa* (Mortensen et al., 2009). Furthermore, porcine gelatin has been more effective at firmly immobilizing most bacterial cells than bovine gelatin (Doktycz et al., 2003; Allison et al., 2011).

The crucial component of this study is the ability to determine whether the cells being imaged are alive because the long-term goal is to observe cells during the process of natural transformation. The definition of life has been a controversial point in the field, but it can be generally characterized by the ability to reproduce, grow and sustain metabolic activity, as well as the presence of structure (cell) and dynamic genetic information (Joux and Lebaron, 2000). In this study, membrane integrity and competence assays were used to determine the viability of the cells prepared for AFM liquid imaging. Living cells have intact membranes that exclude

certain fluorescent dyes, while dead cells do not; only living cells can be naturally transformed with DNA encoding resistance to an antibiotic. Testing for transformation involves requiring the cells to form a colony on solid media after being exposed to DNA; cells that could form colonies on media lacking the antibiotic were alive during the imagine procedure, while cells that could form colonies on media containing the antibiotic were transformed by the DNA.

Membrane integrity has been commonly used to determine cells viability after being submitted to the AFM sample preparation methods (Meyer et al., 2010; Soon et al., 2011; Lonergan et al., 2014). A membrane breach disrupts the electrochemical potential between the inside and the outside of the plasma membrane. The potential across the membrane is responsible for various metabolic processes, such as ATP synthesis, internal pH regulation, active transport, etc. (Joux and Lebaron, 2000). The membrane integrity assay uses fluorescent microscopy to identify nucleic acids stained green and red with the dyes SYTO9 and propidium iodide (PI), respectively. While bacterial cytoplasmic membranes allow the passive diffusion of SYTO9, they are impermeable to PI (Boulos et al., 1999). It implies that cells solely fluorescing green present normal levels of permeability, whereas the ones that also stained red are considered not viable due to reduced membrane integrity. This assay disregards the fact that cells with an intact membrane (which only stain green) may be metabolically inactive under stressful conditions, or that some bacteria can restore membrane integrity (Kaprelyants et al., 1993). A more complete approach requires further testing their ability to reproduce and to uptake DNA from the environment, as it is common to happen with *A. baylyi*.

Natural transformation is a process exclusive to bacteria that allows the cell to uptake of extracellular DNA, incorporate it into the genome, and express the newly encoded genes. The cells must be in a state of competence triggered by environmental conditions to express the sixteen genes required for transformation (Averhoff and Graf, 2008). This metabolically costly mechanism can be used to confirm cell viability through a liquid transformation test. Firstly, if the cells grow on nutrient growth agar after being submitted to the AFM sample preparation methods, there is evidence of viability because the cells had to reproduce to form a colony. Secondly, the cells were provided with DNA encoding resistance to an antibiotic, so if the cells have become resistant to the antibiotic as detected by reproducing on nutrient agar containing that antibiotic, evidence of expensive metabolic activity is provided.

This study investigates which working combination of liquid media and AFM sample preparation method allows *A. baylyi* ADP1 to be imaged, while keeping the cells viable, and attached to the gelatin. The combination was tested with different washing and immobilization media: distilled deionized water (ddH_2O) and phosphate-buffer saline (PBS). Several imaging media were investigated: ddH_2O and diverse dilutions of Luria-Bertani broth (LB) with and without sodium chloride. The successful methods were assayed for membrane integrity with fluorescence microscopy, and for competence ability with liquid transformation test, in order to determine the viability of the cells.

Methods

Cell culture

Strain.

All experiments used two strains of wild-type *Acinetobacter baylyi* ADP1 (BD413). Both were expected to behave similarly, since they share the same genome. Specifically, strain CCL2056 was originally frozen down in 2014, while strain CCL2201 was originally frozen in the summer of 2015. In the past, cells stored at -70^{o}C for more than one year have exhibited lower appendage formation, so the model bacterium switched to CCL2201 once the new cells had become available.

Culture preparation.

Frozen *A. baylyi* was streaked in *Acinetobacter* minimal succinate agar and incubated at 37^{o} C for 24 hours, to use over the following 6 days only, in case cells incubated for long periods of time lose their competence as cells passaged multiple times do (Bacher et al., 2006). A single colony of the streaked plate was inoculated into a test tube with 3 mL of LB (10 g tryptone, 5 g yeast extract, 10 g of sodium chloride per L). The test tube was incubated at 37 ^{o}C with high aeration overnight. At time t=0, 1 mL of the overnight culture was transferred into a baffled Erlenmeyer flask with 24 mL of LB to create a 1:25 dilution. The flask was incubated at 37 ^{o}C with high aeration, and at time t=3 hours the 25-mL dilution was centrifuged for 5 minutes at 3000 rcf.

Atomic Force Microscopy

Gelatin coated puck preparation.

Pucks with cleaved mica on one side were coated with 0.5% porcine skin gelatin (Sigma, G1890) solution. It was prepared by adding 0.5 g of the gelatin into 100 mL of boiling deionized water in a small beaker while swirling. After cooling the solution to approximately 60 ^{o}C, the pucks were quickly submerged only on the side with cleaved mica, and let dry in the upright position overnight against a microfuge tube rack on top of a Kimwipe to collect and absorb the excess. The gelatin solution was stored at 4 ^{o}C, and reused over the month following its production by heating it up to 60 ^{o}C. Gelatin coated pucks were utilized up until 2 weeks after preparing them (Allison et al., 2011).

Sample preparation.

Upon centrifuging at time t=3 hours and discarding the supernatant, the cells were re-suspended, washed, and pelleted at 3000 rcf for a minute twice, each with sterile 750 µL of ddH_2O or PBS (8 g sodium chloride, 0.2 g potassium chloride, 1.44 g monosodium phosphate, 0.24 g monopotassium phosphate per L). The cells were re-suspended in 50, 60, or 100 µL of the respective previous wash media to verify the most appropriate concentration of cells for successful immobilization and imaging. A 30 µL drop of the final suspension was placed in the center of the porcine gelatin coated puck. It was let sit over 10 or 30 minutes to allow attachment of the cells to the gelatin. Upon the immobilization period, the puck was rinsed with 200 or 600 µL of the same media used before (ddH_2O or PBS). Finally, 90 µL and 30 µL drops of

ddH_2O, PBS, LB, or LB without salts at different concentrations (10%, 50%, or 100%) were respectively placed on the puck and on the cantilever in the liquid cell chamber.

AFM imaging.

All imaging was performed with a Bruker MultiMode atomic force microscope and a NSV Controller 64-BIT MOD-B, using a Tapping Mode Fluid Cell Model V2, also from Bruker. The liquid cell was used at ambient conditions, with the previously mentioned imaging media. The liquid cell chamber held a Bruker's ScanAsyst-Fluid+ cantilever with a silicon tip on nitride lever, resonance range of 100-200 kHz, spring constant of 0.35-1.4 N/m, and a nominal tip radius of 20 nm. All images were acquired using Peak Force Tapping Mode, with full or partial aid of Bruker's ScanAsyst software. Most parameters controlled by ScanAsyst (peak force amplitude, peak force setpoint, scan rate) were manually changed to provide gentler probing of the cells, and avoid their dislodging from the gelatin while imaging, for example the reduction of the peak force setpoint from 0.150 V to 0.100 V. The images dimensions ranged from 10 μm - 50 μm, and 30 μm x 30 μm when repeatedly scanning the same area looking for cells that have been dislodged. All images contained 512 samples per line, at a scan rate ranging from 0.301 Hz to 0.997 Hz.

AFM image analysis.

Observation of the differences between several scans over the same area provided evidence on whether the mounting substance, such as the gelatin, and the sample preparation allowed a good cell immobilization method (all cells were present through all scans). A poor cell immobilization compromised of cells that were at first in the image but subsequently disappeared out of the frame, or piled up on the edge of the image due to the probe action.

Membrane Integrity Assay

Gelatin coated slides preparation.

Glass slides were coated with 0.5% porcine gelatin solution on one side to perform the live/dead bacterial assay with fluorescent microscopy. The gelatin was prepared by adding 0.5 g of the gelatin into 100 mL of boiling deionized water in a small beaker while swirling. Upon cooling the solution to approximately 60 ^{o}C, the gelatin was poured directly onto one side of the glass slide, and let dry in the upright position overnight against a microfuge tube rack on top of a Kimwipe to collect and absorb the excess.

Sample preparation.

The LIVE/DEAD *Bac*Light™ Bacterial Viability Kit L7012 allowed determining the AFM sample preparation method under which the cells preserved membrane integrity while attached to the gelatin. According to manufacturer directions, the cells must undergo an incubating period of 1 hour in 0.85% saline or

70% isopropanol to keep them alive or kill them, respectively. After washing and re-suspending both live and dead cells with 0.85% saline, the dyes are mixed and transferred to the dead or live test solutions (3 μL of dye mixture for each mL of the bacterial suspension). The cells are ready for observation after a 15-minute incubation period in the dark, by pipetting 4 μL onto the glass slide.

It was necessary to adapt these instructions to the sample preparation methods that have yielded satisfying images of immobilized cells. Upon centrifuging at time t=3 hours and discarding the supernatant, the cells were re-suspended in 2 mL of 1% saline solution. Half of the bacterial suspension (experimental group) was transferred to a microfuge tube, and washed twice with 750 μL of ddH_2O. Finally, the cells were re-suspended into 100 μL of the different imaging media (ddH_2O, LB without salts at different dilutions), alongside 0.3 μL of the mixed dyes solution. It required 15 minutes of dark incubation in the microfuge, if it were tested on a bare glass slide. To use the gelatin coated glass slides, it was necessary to pipette 4 μL of the bacterial solution with the dyes onto the gelatin coated slide and let incubate for 30 minutes in the dark before imaging. The other half of the initial bacterial suspension in 1% saline was transferred into 9 mL of 70% isopropanol, rupturing the cell membrane and killing the cells as a control group (suggested by the manufacturer directions). After centrifuging the solution with dead *A. baylyi* at 3000 rcf for 5 minutes at room temperature, they were provided the same treatment as the experimental group.

Fluorescence microscopy.

All images were taken with Zeiss Axio Imager Microscope equipped with a Zeiss HBO 100 Mercury Arc Lamp with green and red filters to detect SYTO9 and PI, respectively. The digital images were obtained using a Zeiss Axiocam HRc and the software Zeiss ZEN. Locations randomly chosen in the glass slide sample were imaged with both filters (one at a time) at a magnification of 10X, 20X, 40X, and/or 100X. The presence of SYTO9 was expected in all cells, while PI could only show up if the cell's membrane were disrupted. Therefore, the membrane integrity assay compromised of comparing images of the same location and magnification with the different filters: the presence and frequency of cells with deficient membrane integrity (red cells) with all cells (green cells).

Competence test

Sample preparation.

The competence test was only performed with the sample preparation methods that have yielded satisfying images of *A. baylyi* in liquid. Upon centrifuging at time t=3 hours and discarding the supernatant, the cells were re-suspended, washed, and pelleted at 3000 rcf for a minute twice, each with 750 µL of ddH_2O. The final re-suspension was performed with 750 µL of ddH_2O or 50% LB without salts, and then separated 100 µL into 4 labeled (0-3 hours) microfuge tubes each. To replicate the required time for gelatin immobilization, there was a 30 minutes period of no activity. In only one of the microfuge tubes, the cells were provided with 5 µL of chromosomal DNA isolated from a streptomycin resistant donor; the

DNA was at a concentration of approximately 1 μg/mL. After 30 minutes incubation at room temperature to allow the DNA uptake, 5 μL of DNAse I and 45 μL of DNAse buffer were transferred into the tube, followed by 15 minutes incubation at 37 ^{o}C with high aeration to give the DNase enzyme time to work. Ten-fold dilutions were then performed with a 96-well plate and 1% sterile saline. LB (control) and LB-strp^{20} (20 μg/mL streptomycin) agar plates were divided into 8 sections to plate the dilutions 10^{0}-10^{7}. Three 10 μL droplets of each dilution were platted into the correspondent section. The plates were inverted and incubated at 37 ^{o}C overnight after the puddles had soaked in. The experiment was repeated after 1, 2, and 3 hours with the remaining microfuge tubes after the immobilization period to ensure the cells' competence ability over the time required for imaging.

Competence analysis.

After 24 hours incubating at 37 ^{o}C, the number of countable colonies formed from each puddle was determined. The colony-forming unit per milliliter (CFU/mL) was calculated dividing the average of the total countable colonies of the smallest dilution factor possible by the puddle volume, to finally normalize it with the respective dilution factor, in both the LB and LB-strp^{20} plates. The transformation efficiency was obtained from the ratio of the growth on LB-strp^{20} and the growth on LB CFU/mL in the same time period. Statistically significant test were used to compare the average transformation efficiency rates across conditions at the same time (2-tailed t-test assuming unequal variance), and across time within the same

condition (one-way ANOVA test with http://turner.faculty.swau.edu/mathematics/math241/materials/anova/).

Results

Atomic Force Microscopy

The first goal was to identify the conditions under which *Acinetobacter baylyi* ADP1 can be immobilized while imaged in liquid.

30 minute incubation allows better cell immobilization than shorter incubation

As porcine gelatin has been previously used in other studies (Doktycz et al., 2003; Allison et al., 2011), it was chosen as the immobilizing agent. Successful immobilization requires time for the cells to sink from the medium bubble onto the mounting substrate, where the electrostatic interaction occurs. To test this, *A. baylyi* was imaged in ddH_2O full treatment, allowing the cells attaching to the gelatin for 10 or 30 minutes. The latter condition allowed a much greater number of cells to be immobilized (Fig. 2). It suggests that the cells require approximately 30 minutes to be able to electrostatically attach onto the porcine gelatin with success.

Full treatment with ddH2O allows better cell immobilization than full treatment with PBS

The next question investigated whether full treatment with ionic media can affect the immobilization process. To test this, the cells were washed, immobilized,

rinsed and imaged fully in ddH_2O or PBS. Most cells were dislodged and disappeared from the scan area with the latter treatment (Fig. 3). As mentioned above, the full treatment with ddH_2O allowed good immobilization (Fig. 2.a). It follows the idea that high ionic solutions may disrupt the electrostatic interaction of the cells with the porcine gelatin (Doktycz et al., 2003).

Imaging with LB (regardless of its ionic content) dislodges cells

Cells are likely to be alive and metabolically active in LB, therefore it was used as the imaging medium. To test this, the cells were washed, immobilized, and rinsed with ddH_2O, followed by imaging with LB with or without sodium chloride. In either case, the totality of the cells did not adhere to the gelatin (Table 1), being dislodged and dragged to the side of the scan frame by the cantilever tip. It suggests that the tryptone and/or yeast extract components of LB are affecting the immobilization of cells.

Decreasing LB concentration allows better cell immobilization

As it is desirable to image metabolically active cells, the concentration of tryptone and yeast extract in LB were reduced to verify if that would permit successful adherence. To test this, the cells were given the same treatment as with full concentration LB (no salts), except that they were imaged with a 50% dilution. The cells were present throughout several scans, but it is unclear whether some cells were initially moved to the side due to the amount of space in between the frame sides (Fig. 4.a). The doubling effect on the cells may be due to cantilever tip

contamination, most likely caused by the broth and/or the cells that were dislodged. It suggests a malfunction with the rinsing process and/or the lateral probing forces exerted on the cells. The former may be too powerful to the extent of disrupting the gelatin-cell electrostatic interaction, or too weak so that not all the poorly immobilized cells are removed. The lateral probing forces may be too invasive on these poorly attached cells, because the cantilever tip can physically sweep the cells outside of the framing area, which may also be a cell detacher factor by itself.

Gentler rinsing and probing allows better cell immobilization with 50% LB (no salts)

As all cells were not properly attached to the gelatin in the previous experiments, it became necessary to verify the factors that could be directly affecting the cell-gelatin interaction, such as the rinsing process and the probing settings. To test this, the cells were given the same treatment as above, except that they were rinsed with 200 μL, while until now that had been done with approximately 600 μL. Furthermore, the probing settings were gentler on approach and scanning by reducing the peak force amplitude and approach set point. Even though there were less cells on the second scan compared to the first one, the ones that were attached remained so on the following scan and the image quality improved (Fig. 4.b). This suggests that the tip may have removed the poorly immobilized cells in a much gentler manner, allowing the other cells to remain attached even in the center of the scanned area. Furthermore, the less dramatic dragging lines that decreased with time suggest that tip contamination (e.g. cells attached to the cantilever tip) decreased with more scans in the same area. Perhaps

it is due to the up-and-down movement of the cantilever in the peak force mode that allowed the detachment of cells from the tip, as well as the controlled liquid flow around the cantilever that drifted detached cells away from the scanned area.

Optimal LB (no salts) concentration for imaging is inconclusive

As previously mentioned, decreasing the concentration of tryptone and yeast extract by half allowed successful cell adherence. Perhaps lowering those concentrations even more could improve the cell-gelatin attachment further, as well as the image quality (less contamination). To test this, the cells were given the same gentler treatment as above described with 10% LB (no salts) instead. There were no significant improvements compared to imaging with 50% LB (no salts) in the same conditions. Less cells moved from the first to the second scan, but the images had lower quality: more dragging lines and the image was less crisp (Fig. 4.c). In fact, the image quality seems to be the greatest when imaging with ddH_2O (Fig. 2.a), which suggests that imaging with any LB derivative enhances contamination of the tip as well as the laser reflective area of the cantilever.

Membrane Integrity Assay

The second goal was to determine whether the sample preparation methods that rendered successful images allowed the cells to preserve membrane integrity over the time required for scanning.

Membrane integrity is not preserved with full ddH_2O treatment

After successfully immobilizing and imaging *A. baylyi* with full ddH_2O treatment, it was important to verify the cells' membranes integrity to see if they were still viable. To test this, the same AFM sample preparation method with ddH_2O was applied except that in some cases, after the initial pelleting, the cells were incubated in 1% saline and centrifuged with the same settings as the original bacterial suspension at t=3 hours. The saline incubation period was reduced from 1 hour (manufacturer instruction) to 15 minutes, 5 minutes (while centrifuging), or no incubation at all as an adaptation process to the AFM imaging protocol. Furthermore, some were also immobilized in gelatin coated glass slides. Only the cells that were not incubated in saline did not preserve membrane integrity (Table 2). It suggests that the osmotic stress of the cells being directly transferred from LB (ion and carbon rich medium) to ddH_2O does not permit the viability of the cells as previously observed when submitted to the full ddH_2O treatment.

Membrane integrity is preserved when imaged with 50% LB (no salts)

Similar to the full ddH_2O treatment, the sample preparation method that allowed imaging cells with 50% LB (no salts) was also tested for the membrane integrity of the cells. To test this, the same AFM imaging sample preparation method was applied on gelatin coated or uncoated glass slides. In some cases, the previously mentioned extra incubation period in 1% saline for 15 minutes was administered (Table 2). The positive control provides an insight on how the cells are supposed to look when their membrane has been ruptured: bright red (Fig. 5.f). Most viability

assays with LB presented cells with two different shades of red: a bright one (similar to positive control), and a much fainter one in most of the remaining cells (Fig. 5.a). It suggests that some of the fluorescent PI dye is reaching the inside of cells in a different fashion than the direct passage through a ruptured membrane. In any case, the positive control allows a clear distinction between the two shades of red in the experimental settings to the extent of confirming membrane integrity.

Cells keep membrane integrity when imaged with 50% LB (no salts) over time

Imaging with AFM requires some time, therefore the cells must keep their membrane integrity throughout the scanning process. To test this, the cells were submitted to the same treatment without saline incubation as mentioned above, over a time period of 2-hours (similarly to AFM imaging time). They were tested every 30 minutes, starting after a 30-minute inactivity period to reproduce the time taken for the cells to adhere onto the gelatin. The cells mostly presented the same faint red shade as previously mentioned in all the time intervals, and occasional bright red cells similar to the positive control (Fig. 5.a-e). It enhances the idea that most cells kept membrane integrity since they are not labeled in the same fashion as the positive control, even over a two-hour window.

Competence test

Finally, the third goal was to determine whether the successful imaging conditions of cells that preserved membrane integrity also allow them to be competent over the time required for scanning.

Imaging in 50% LB (no salts) does not allow significant competence over time

As imaging with 50% LB (no salts) yielded images of *A. baylyi* that kept membrane integrity, the next metabolic viability confirmation is the ability to uptake DNA. To test this, the cells were washed with ddH_2O and re-suspended in that same medium or in 50% LB (no salts). After a 30-minute rest period to simulate the time required for gelatin immobilization, liquid transformation tests were performed for both media. In both cases, cells grew on the plain LB agar plates, suggesting that they are inactive until provided the appropriate conditions to grow and reproduce. There was no evidence of competence at any time when treated solely with ddH_2O (Table 3), which goes along the fact that the cells did not preserve membrane integrity. When testing with 50% LB (no salts), very few colonies grew in the LB-strp^{20} agar (Table 3), but not enough to achieve a statistical significant difference from the ddH_2O treatment. It suggests that access to food source enhances, but only very slightly, the cells' survival. There was also no significant difference between the average transformation efficiency rates over time (one-way ANOVA, $p=0.5957$). It suggests that the very slight improvement achieved by providing a carbon source only affects very few cells to become active, even over time.

Discussion

Washing, immobilizing, and rinsing the cells with ddH_2O, followed by imaging with 50% LB (no salts) was the most successful AFM sample preparation method to image *Acinetobacter baylyi* in liquid. Even though the cells kept membrane integrity, they displayed very poor competence as their transformation efficiency was not significantly different from no competence at all. Furthermore, there was no significant difference of transformation efficiency over a 3-hour period. The full treatment with ddH_2O yielded better images (less cell detachment and tip contamination), but it did not demonstrate membrane integrity or competence. Interestingly, it was possible to achieve membrane integrity when adding incubation in 1% saline before washing.

The immobilization process with porcine gelatin limits the preparation methods and media due to the nature of cell surface-gelatin electrostatic interactions. While it was relatively easy to immobilize the cells with ddH_2O, no cells attached when imaged in PBS or LB with and without salts. It confirms the inability of using high ionic media (Meyer et al, 2010), as well as full growth media (Doktycz et al., 2003) regardless of the ionic content, as they compete with bacteria for the positively-charged gelatin. The same way diluting PBS was a successful adaptation to image *Escherichi coli* (Allison et al., 2011), diluting LB without salts also ensured attachment of *A. baylyi*. Even though it enhances the contamination of the cantilever tip and laser reflection surface, it provides a food source increasing their possibility of survival and advanced metabolic activity. Taking into account the flexible nature

of *A. baylyi* to metabolize different carbon sources (Barbe et al., 2004), it may allow the use of other growth media that would not interact with the gelatin.

The preparation method that yielded the best images (only ddH_2O) did not maintain viability, as the cells lost membrane integrity. The rough transition from LB to ddH_2O may cause osmotic stress, leading to membrane breach induced by excessive turgor pressure. However, it is necessary to use ddH_2O to wash the ions from the immobilization bacterial suspension to ensure the cell-gelatin electrostatic interaction. Interestingly, when the cells were submitted to the 1% saline incubation period suggested by LIVE/DEAD *Bac*Light™, their membrane integrity was not disrupted which is identical to the salt concentration in LB. Future research should include smoother transitions between the sample preparation media similar to the saline incubation, and ultimately the imaging broth: washing the cells with increasingly diluted ionic media, as previously performed with *E. coli* and PBS (Allison et al., 2011).

Contrastingly, imaging with 50% LB without salt kept membrane integrity, regardless of the saline incubation period. As previously described, all cells under this treatment stained red with PI, which would imply they lost membrane integrity. However, there were two shades of red (bright and faint) suggesting that the dye targeted the inside of the cells in a different fashion. PI stains nucleic acids, under the assumption that the cytoplasmic membrane is impermeable to it, and not to SYTO9. LB (no salts) is composed by tryptone and yeast extracts, and consequently nucleic acid molecules. Possibly, the cells could have taken in nucleic acids already stained with PI, thus presenting a much fainter red color, which did not happen in

ddH_2O. Furthermore, purposely breached cells (positive control) only presented the bright red stain. Further research should provide more evidence on the possibility of PI staining nucleic acids present in food sources. This could be tested by repeating this experiment with an *A. baylyi* mutant that can repress its ability of taking in nucleic acids inductively. Ultimately, this staining procedure may be expanded to study competence or other mechanisms involving the intake of nucleic acids.

Membrane integrity recovery is an important factor to take into account when transitioning cells between different media. As previously mentioned, the extra 1% saline incubation step preserved membrane integrity when fully treating the cells with ddH_2O. Moreover, they kept their membrane integrity with 50% LB (no salts) even without the saline incubation. It suggests one way that the damage caused by the direct LB-ddH_2O transition can be recovered is if the cells have access to a carbon source. Therefore, the combination of smoother media transition (e.g. LB-saline-ddH_2O) and access to food source while imaging or washing the cells may improve their viability. For example, previous studies on *E. coli* spheroplasts (Sullivan et al., 2005) and *P. aeruginosa* cells (Mortensen et al., 2009) have added 0.25 M sucrose to the sample preparation media to counter the osmotic stress.

As membrane integrity is not the only sign of metabolically inactive cells, the ability to uptake environmental DNA was used to further confirm *A. baylyi* viability. There was no competence at all when the cells were submitted to the full ddH_2O treatment, and the transformation efficiency rates were not significantly different to null when testing them with 50% LB (no salts) over time. It suggests that regardless of their membrane integrity, both treatments do not yield fully viable cells.

However, there was still growth on LB-strp^{20} agar plates when treated with 50% LB (no salts), and the cells kept their membrane integrity. In contrast, when treated solely with ddH_2O there was no growth nor membrane integrity. Furthermore, the growth observed in plain LB agar for the competence test is evidence that the cells are not dead because they are able to grow and reproduce in a favorable environment. It implies that likewise *Micrococcus luteus* (Votyakova et al., 1994), *A. baylyi* can also restore membrane integrity and viability given the appropriate conditions. Cell viability may be restored by creating less stress for the cells with the previously suggested combination of smoother media transitions and access to food source.

Thus, the idea of a viability spectrum arises. It includes different levels of cell inactivity, which at any point can be reversed provided the appropriate conditions. This has not been accounted for in other studies that use membrane integrity as their sole measure of cell viability (Soon et al., 2011; Meyer et al., 2010). The bacteria investigated in those studies may have different responses to stress, giving them their own unique viability spectrum not allowing for direct comparison with *A. baylyi*. Membrane integrity assay is an incomplete assessment of their viability that should be complemented with other factors, such as growth and reproduction, especially taking into account the resilient nature of bacteria. Further methodological research on imaging live bacteria with AFM should present a holistic approach to determine the cells' viability.

Overall, the ultimate goal of the experiment was not achieved as no method ensured adhesion to the gelatin and full viability of *A. baylyi*. However, the cells were

not dead, but instead inactive due to stress. Future research should not only attempt to decrease the stress using the previously suggested combination of smoother media transition and access to food source, but also try different mounting substrates and their effects on cell viability. Eventually, if any combination is successful, it will be possible to further the understanding of several complex mechanisms in their natural liquid environment, such as visualizing the competence machinery of *A. baylyi* as it uptakes DNA. Furthermore, it will also be possible to use specialized cantilever tips with a DNA probe to compare and measure competence levels under different conditions or different mutants. Similar applications have been developed to study colistin resistance in genetically similar pathogens (Barbe at al., 2004), such as *A. baumannii* (Soon et al., 2011) and *P. aeruginosas* (Mortensen et al., 2009). Any further developments in liquid AFM should be pursued, as it has an immense potential for microbiology studies in natural environments

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Works cited

Allison, D.P., Sullivan, C.J.; Mortensen, N.P. ReOerer, S.T., Doktycz, M.J. 2011. Bacterial immobilization for imaging by atomic force microscopy. Journal of Visualized Experiments 54:e2880

Averhoff, B. and Graf, I. 2008. The Natural Transformation System of *Acinetobacter baylyi* ADP1: A Unique DNA Transport Machinery. *Acinetobacter* Molecular Biology: 120-139.

Bacher, J.M., Metzgar, D., de Crécy-Lagard, V. 2006. Rapid evolution of diminished transformability in *Acinetobacter baylyi*. Journal of bacteriology 188(24): 8534-8542.

Barbe, V. et al. 2004. Unique features revealed by the genome sequence of *Acinetobacter* sp ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Research 32(19):5766-5779.

Beckmann, M.A. et al. 2006. Measuring cell surface elasticity on enteroaggregative *Escherichia coli* wild type and dispersin mutant by AFM. Ultramicroscopy 106(8-9):695-702.

Binnig, G., Quate, C.F., Gerber, C. 1986. Atomic Force Microscope. Physical Review Letters 56(9):930-934.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., Desjardins, R. 1999. LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. Journal of Microbiological Methods 37(1):77-86.

Chao, Y. and Zhang, T. 2011. Optimization of fixation methods for observation of bacterial cell morphology and surface ultrastructures by atomic force microscopy. Applied Microbiology and Biotechnology 92:381-392.

Doktycz, M.J., Sullivan, C.J., Hoyt, P.R., PelleBer, D.A., Wu, S., Allison, D.P. 2003. AFM imaging of bacteria in liquid media immobilized on gelatin coated mica surfaces. Ultramicroscopy 97:209-216.

Dufrêne, Y.F. 2004. Using nanotechniques to explore microbial surfaces. Nature Reviews Microbiology 2(6):451-460.

Francius, G., Domenech, O., Mingeot-Leclerq, M.P., Dufrêne, Y.F. 2008. Direct Observation of *Staphylococcus aureus* Cell Wall Digestion by Lysostaphin. Journal of Bacteriology 190(24):7904-7909.

Jonas, K., Tomenius, H., Kader, A., Normark, S., Römling, U., Belova, L.M., Melefors, O. 2007. Roles of curli, cellulose and BapA in *Salmonella* biofilm morphology studied by atomic force microscopy. BMC Microbiology 7:70.

Joux, F. and Lebaron, P. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. Microbes and Infection 2:1523-1535.

Juni, E. and Janik, A. 1969. Transformation of *Acinetobacter calco-aceticus* (*Bacterium anitratum*). Journal of Bacteriology 98(1):281-288.

Kailas, L., Ratcliffe, E.C., Hayhurst, E.J., Walker, M.G., Foster, S.J., Hobbs, J.K. 2009. Immobilizing live bacteria for AFM imaging of cellular processes. Ultramicroscopy 109:775-780.

Kaprelyants, A.S., Gottschal, J.C., Kell, D.B. 1993. Dormancy in non-sporulating bacteria. FEMS Microbiology Letters 104(3-4):271-285.

Kuyukina, M.S., Korshunova, I.O., Rubtsova, E.V., Ivshina, I.B. 2014. Methods of microorganism immobilization for dynamic atomic-force studies (review). Applied Biochemistry and Microbiology 50:1-9.

Lonergan, N.E., Britt, L.D., Sullivan, C.J. 2014. Immobilizing live *Escherichia coli* for AFM studies of surface dynamics. Ultramicroscopy 137:30-39.

Lostroh et al. 2014. Bacterial Morphological Adaptation Spanning the Transition from Exponential to Stationary Phase. Unpublished data

Meyer, R., Zhou, X., Tang, L., Arpanaei, A., KingshoO, P., Besenbacher, F. 2010. Immobilisation of living bacteria for AFM imaging under physiological conditions. Ultramicroscopy 110:1349-1357.

Mortensen, N.P. et al. 2009. Effects of Colistin on Surface Ultrastructure and Nanomechanics of *Pseudomonas aeruginosa* Cells. Langmuir 25(6):3728-3733.

Muller, D.J. and Dufrêne, Y.F. 2008. Atomic force microscopy as a multifunctional molecular toolbox in nanobiotechnology. Nature Nanotechnology 3(5):261-269.

Porstendörfer, D., Gohl, O., Mayer, F., Averhoff, B. 2000. ComP, a Pilin-Like Protein Essential for Natural Competence in *Acinetobacter* sp. Strain BD413: Regulation, Modification, and Cellular Localization. Journal of Bacteriology 182(13):3673-3680.

Soon, R., NaBon, R., Harper, M., Adler, B., Boyce, J., Tan, C., Li, J., Larson, I. 2011. Effect of colistin exposure and growth phase on the surface properties of live Acinetobacter baumannii cells examined by atomic force microscopy. International Journal of Antimicrobial Agents 38(6):493-501.

Votyakova, T.V., Kaprelyants, A.S., Kell, D.B. 1994. Influence of Viable Cells on the Resuscitation of Dormant Cells in *Micrococcus luteus* Cultures Held in an Extended Stationary Phase: the Population Effect. Applied and Environmental Microbiology 60(9):3284-3291.

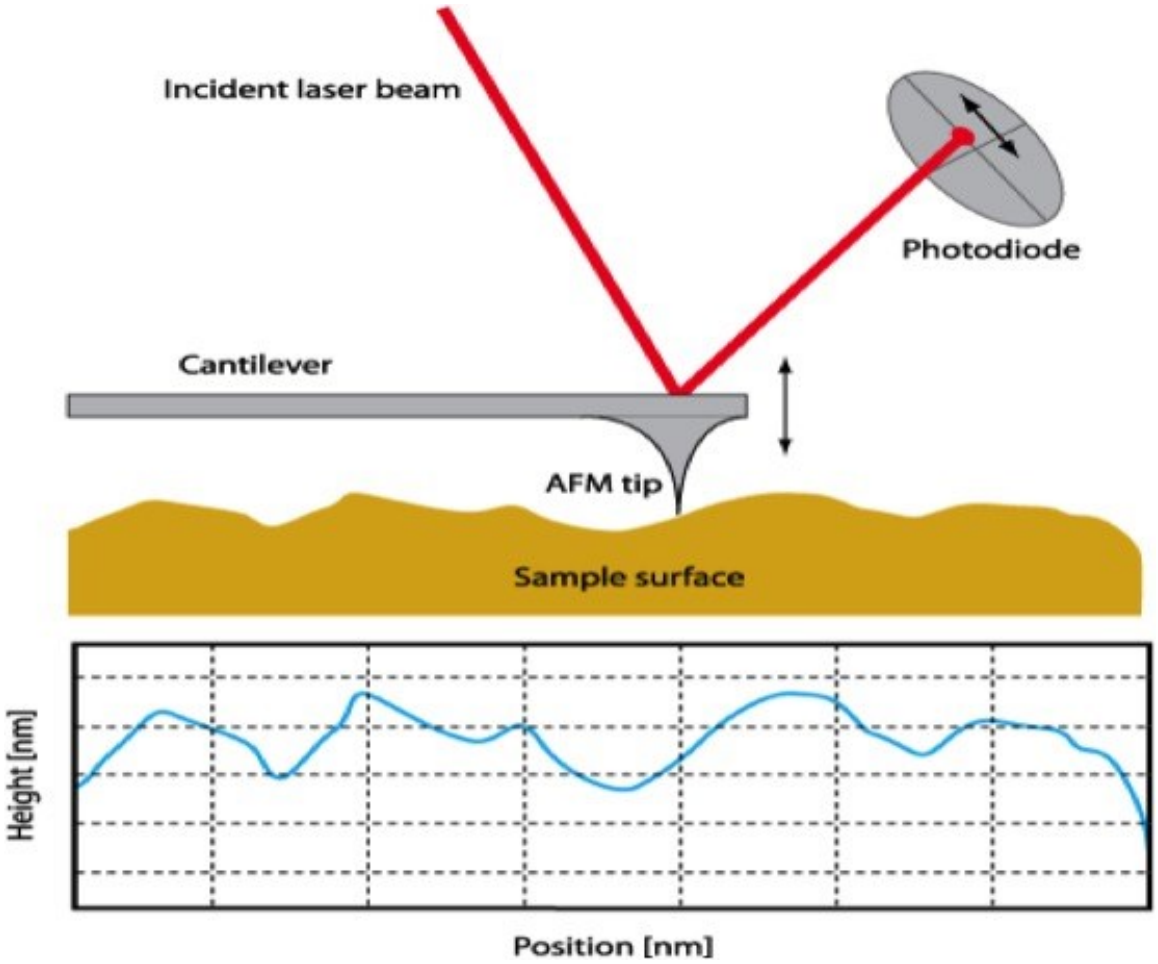

Figure 1. Diagram of the AFM probing mechanism. Each line is scanned twice in each direction before moving to the next one. The combination of all the traces will form the desired image. Image of AFM diagram quoted from http://scienceinyoureyes.com/techniques/atomic.html.

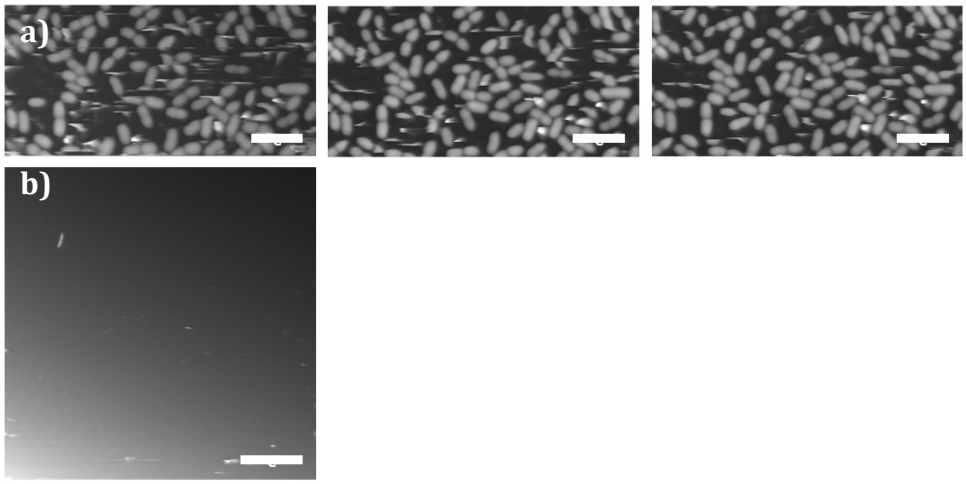

Figure 2. AFM images of *Acinetobacter baylyi* with full ddH_2O treatment with different immobilization times. The cells were left for 30 **(a)** or 10 **(b)** minutes incubating on the gelatin. **(a)** provides a chronological account of scans in the same area from left to right (30 µm X 14.5 µm, 512 X 248 lines, 0.501 Hz). The scale bars represent 5 µm. The size of image **(b)** was 50 µm X 50 µm, its pixilation was 512 X 512 lines at a scan rate of 0.977 Hz. The scale bar represents 10 µm.

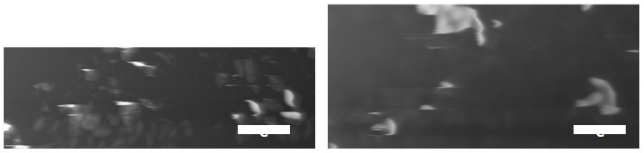

Figure 3. AFM images of *Acinetobacter baylyi* with full PBS treatment. Both images are from the same scanning area with 30 μm X 10.02 μm **(left)**, and 30 μm X 13.95 μm **(right)**. The pixilation was 512 X 167 lines **(left)** and 512 X 238 lines **(right)**. Both images were scanned at 0.501Hz. The scale bars represent 5 μm.

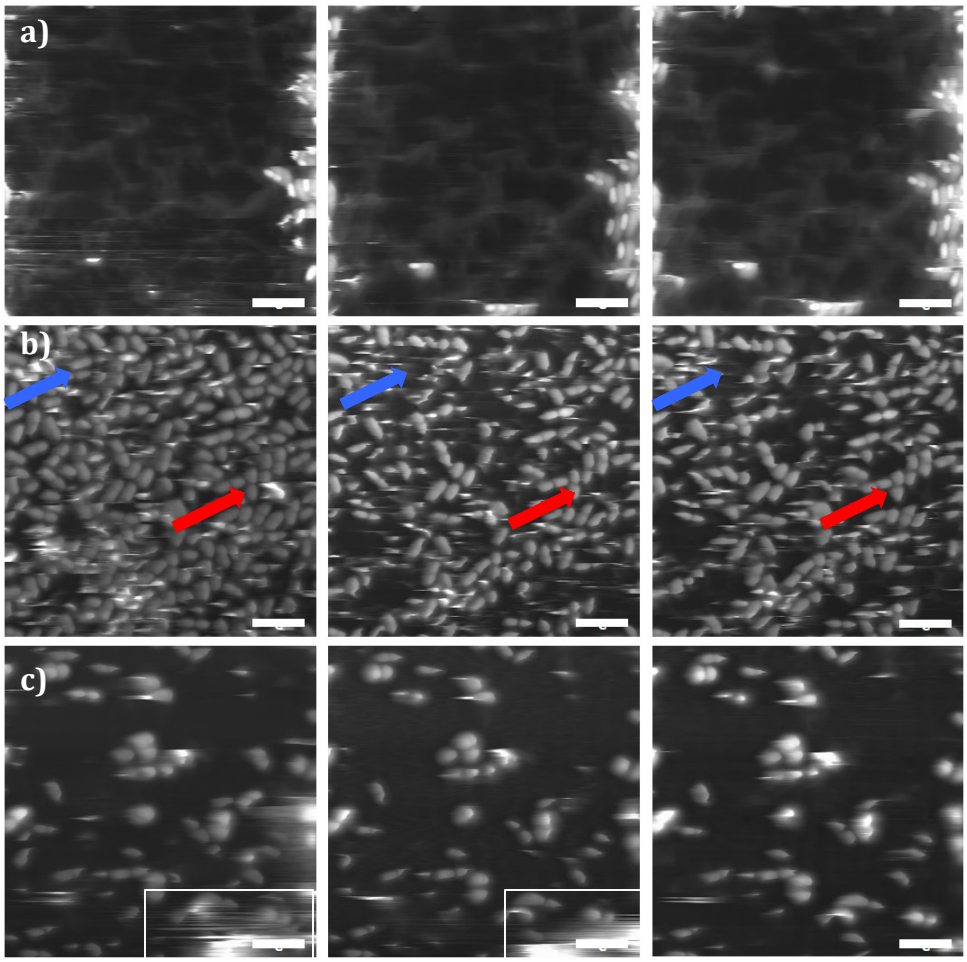

Figure 4. AFM images of *Acinetobacter baylyi* that held to the mounting sample with 50% and 10% of LB (no salts). All samples were washed, rinsed and immobilized with ddH2O, and then imaged with 50% **(a-b)** or 10% **(c)** LB (no salts). The rinsing and probing processes were gentler in **(b-c)**. All images were 30 µm X 30 µm, and the pixilation was 512 X 512 lines. **(a-b)** were scanned at 0.301 Hz, and **(c)** at 0.399 Hz. The scale bars represent 5 µm. The scans from the same area are chronologically placed from left to right. The blue arrows indicate the area where a cell that has been displaced, whereas the red arrows indicate cells that have been successfully immobilized with gelatin. The white box highlights dragging lines that decreased with time.

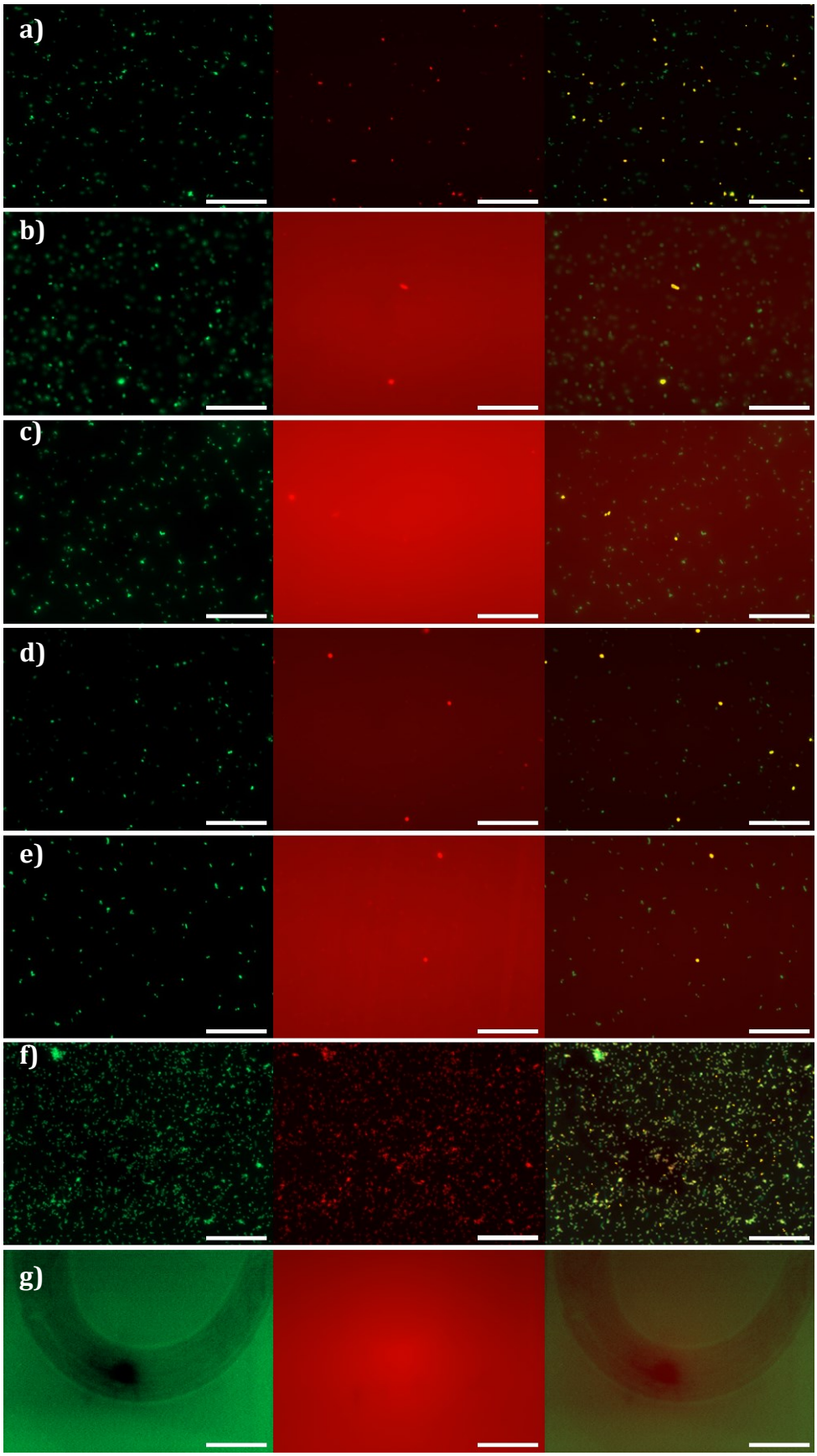
a)
b)
c)
d)
e)
f)
g)

Figure 5. Viability assay over a 2-hour period post immobilization when imaging *Acinetobacter baylyi* with 50% LB (no salts) and no gelatin. The cells were assayed in increments of 30 minutes, from time t=0 hours **(a)** through t=2 hours **(e)**. Positive control **(f)** compromises solely of dead cells, while the negative control **(g)** verifies the effect of the medium with the dyes. All cells are stained green (left column), while only the cells with compromised membrane integrity are stained red (middle column), The cells with ruptured membrane are colored in yellow in the merged image (right column). The scale bar represents 50 μm.

Table 1. Cells immobilization survey with different sample preparation methods and imaging media.

Media				Immobilized cells*	Comments
Washing	**Immobilizing**	**Rinsing**	**Imaging**		
PBS	PBS	PBS	PBS	N	See Fig. 3
ddH_2O	ddH_2O	ddH_2O	ddH_2O	Y	See Fig. 2 (a)
ddH_2O	ddH_2O	ddH_2O	LB	N	
ddH_2O	ddH_2O	ddH_2O	LB (no salts)	N	
ddH_2O	ddH_2O	ddH_2O**	LB (no salts)	N	
ddH_2O	ddH_2O	ddH_2O	50% LB (no salts)	Y	See Fig. 4 (a)
ddH_2O	ddH_2O	ddH_2O**	50% LB (no salts)	Y	See Fig. 4 (b)
ddH_2O	ddH_2O	ddH_2O**	10% LB (no salts)	Y	See Fig. 4 (c)

* No (N), yes (Y)
**Gentler rinsing and scanning.

Table 2. Membrane integrity of cells imaged with ddH_2O or 50% LB (no salts) in the presence or absence of gelatin and 1% saline.

Media	Saline incubation (min)	Gelatin*	Membrane integrity*
ddH_2O	15	N	Y
	5	N	Y
	15	Y	Y
	5	Y	Y
	0	N	N
50% LB (no salts)	15	N	Y
	15	Y	Y
	0	N	Y

* No (N), yes (Y).

Table 3. Transformation efficiency of streptomycin antibiotic resistance on cells imaged with ddH_2O (n=2) or 50% LB without salts (n=3).

	Transformation Efficiency (CFU mL^{-1})	
Time (h)	**50% LB (no salts)***	**ddH_2O**
0	3.75E-08	0
1	9.86E-07	0
2	3.83E-07	0
3	7.58E-07	0

* No significant difference found between both conditions at the same scanning time (t-test), nor overtime within the same condition (ANOVA).

Appendix A: Imaging live *Acinetobacter baylyi* with AFM in liquid

Puck preparation:

1. Clean the pucks by putting them in a clean beaker, adding 2-propanol until they are covered and ultrasounding for 5 minutes. Let the pucks dry.
2. Put double-sided adhesive on one side of a clean puck.
3. Stick mica onto the adhesive on the puck, and press down firmly with finger.
4. Hold the puck with tweezers and place the puck on its side and cleave a layer of mica using a scalpel so that the mica layer is totally smooth.

Gelatin solution (can be used for up to 1 month):

1. Boil 100 mL of **___a___** water (microwave). *deionized/ddH2O*
2. Add 0.5 g of porcine gelatin and swirl until dissolved.
3. Cool to 60-70^{o} C.
4. Place 15 mL in small beaker for coating the mica.
5. After use, store in fridge at 4^{o} C.
6. To reuse, reheat to 60-70^{o} C in microwave/waterbath.

Gelatin coated mica (can be used for up to 2 weeks):

1. Tip the small beaker with the gelatin (60-70^{o} C) in it to about 45 degrees, and then, using tweezers, lay the side of the puck with the mica onto the surface of the gelatin. Ensure the entirety of the mica is covered in the gelatin, but that the back of the puck does not touch the gelatin.
2. Overnight, dry it vertically on paper towel/Kimpwipe, with the back edge resting against a microfuge rack.
3. The same is applied to prepare gelatina coated slides.

Cell preparation:

1. Streak ADP1 (BD413) strain CCL2056 or strain CCL2201 on minimal succinate agar and grow at 37 ^{o}C for 24 hours.
2. Make an overnight broth
   i) Put **___b___** mL of LB broth in a test tube. Use a toothpick to inoculate the broth with one colony of ADP1. *6.2/3*
   ii) OR flame a sterile loop, scoop up colony (be sure its cool) and swish around in broth to inoculate
   iii) Grow 18-24 hours @ 37C with shaking (make sure tube is tilted in the incubator for maximal aeration)
3. Inoculate **___c___** mL overnight to **___d___** mL LB broth in a **___e___** mL baffled Erlenmeyer flask, to yield a 1:25 dilution. *6/1 ; 144/24 ; 1000/125*
4. Let this grow **___f___** hours at 37C with shaking. *3/6.5*
5. Remove **___g___** mL of the broth culture from flask and put in a **___h___**-mL blue cap tube. *5/25 ; 15/50*
6. Pellet the whole thing in the giant centrifuge @3000rcf for 5 mins at **___i___.** *room temperature/4 ^{o}C*
7. Remove supernatant by inverting and dumping into a waste container

8. Add 750 uL of **___j___** to the pellet, mix, and transfer to a 1.5mL microfuge tube. *ddH2O/PBS*
9. Pellet this again @3000 rcf for 1 min, in a normal size microcentrifuge.
10. Repeat the wash/pellet/resuspend for a total of 3X
11. Suspend the final pellet in **__k__** uL **___l___** *50/60/100, ddH20/PBS/LB*

Transferring the Cells to the Puck:

1. Apply **___m___** uL of cell suspension to gelatin coated mica on a puck (see "Gelatin coated mica"), and spread with pipette tip without touching the mica. *20/30*
2. Let sit for **___n___** mins. *10/30*
3. With a **___o___,** pipette **___p___** uL of **___q___** on top of the puck to rinse it. *Plastic pipette/Eppendorf pipette ; 1200/600/200* ; *ddH2O/PBS/LB*
4. Apply 90 uL drop of **___r___ ___s___** on the puck while mounted on the AFM, and 30 uL on the liquid cell chamber (cantilever). *ddH2O/LB/PBS ; with/without DNA*

Cell's viability assay (Soon et al.):

1. Apply green fluorescent DNA-binding SYTO 9 (viable) and red fluorescent DNA-binding stain propidium iodide (non-viable) in 1 mL of ddH2O (1.5 uL of each stain)
2. Apply 300 uL in cells stuck to gelatin and incubate in a dark room for 30 min.
3. Verify and quantify amount of fluorescence of each color (they have special software for this)

1st attempt:

Puck preparation:

1. Put adhesive on one side of a clean puck.
2. Stick gelatin-coated mica (see below) into the puck, and press down on the edges of the mica with tweezers ensuring to avoid touching the center of the mica.

Gelatin coated mica (lasts 2 weeks):

1. Hold a mica sheet vertically, and cut on the middle to obtain two sheets with one cleaved side each.
2. Quickly submerge and withdraw the cleaved mica (60-70^{o} C)
3. Overnight, dry it vertically on paper towel/Kimpwipe, with the edge against a microfuge rack.

a) deionized
b) 6.2
c) 6
d) 144
e) 1000
f) 3 and 6.5*
g) 5
h) 15
i) RT
j) ddH2O
k) 100
l) ddH2O
m) 20 and 30*
n) 10 and 30*
o) plastic pipette
p) 1200 and 600*
q) ddH2O
r) ddH2O
s) without DNA

*respectively for f)

2nd[st] attempt:

To check for same concentration.

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) 4 °C
j) ddH2O
k) 50
l) ddH2O
m) 30
n) 30
o) plastic pipette
p) 600
q) ddH2O
r) ddH2O
s) without DNA

3[rd] attempt:

To check for imaging in LB.

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) 4 °C
j) ddH2O
k) 100
l) ddH2O
m) 30
n) 30
o) plastic pipette
p) 600
q) ddH2O
r) LB
s) without DNA

4[th] attempt:

To try preparing everything in PBS

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) 4 °C
j) PBS
k) 60
l) PBS
m) 30
n) 30
o) plastic pipette
p) 600
q) PBS
r) PBS
s) without DNA

5th attempt:

To try to observe appendages in the presence of DNA. To verify if it is possible to image with these conditions:

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) 4 °C
j) PBS (0.2% succ)
k) 100
l) PBS (0.2% succ)
m) 30
n) 30
o) Eppendorf pipette
p) 600 uL
q) PBS (0.2% succ)
r) LB w/out salts
s) with 50uL of DNA

6th attempt:

Is ddH2O necessary to image the cells without dislodging them?

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) RT
j) PBS (0.2% succ)
k) 100
l) PBS (0.2% succ)
m) 30
n) 30
o) Eppendorf pipette
p) 600 uL
q) ddH2O
r) ddH2O
s) without DNA

7th attempt:

Is the presence of salts in LB the reason why imaging in LB doesn't work?

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) 4 °C
j) ddH2O
k) 100
l) ddH2O
m) 30
n) 30
o) Eppendorf pipette
p) 600 uL
q) ddH2O
r) 100% LB w/out salts
s) without DNA

8th attempt:

Is the LB concentration the reason why imaging in LB doesn't work?

a) deionized
b) 3
c) 1
d) 24
e) 125
f) 3
g) 25
h) 50
i) RT
j) ddH2O
k) 100
l) ddH2O
m) 30
n) 30
o) Eppendorf pipette
p) 600 uL
q) ddH2O
r) 50% LB w/out salts
s) without DNA

9th attempt:

Is the rinsing process too strong? Is the peak force setpoint too strong?

Same as attempt 8, except for *p) 200 uL*, and *low peakforce setpoint (0.100)*.

10th attempt:

Is the LB concentration too strong?

Same as 9th attempt, except for *r) 10% LB w/out salts*.

11th attempt:

Same as 7th attempt, except for *i) RT* and *p) 200 uL,* and *low peakforce setpoint (0.100)*.

Appendix B: Membrane integrity assay of the conditions that allowed cell immobilization and imaging.

1. Prepare the cells as if they were going to be imaged, and stop at the step previous to gelatin immobilization.
2. Prepare the dye mixture so that there is 3 μL of the mixture for every mL of the bacterial suspension.
    a. If not placed on the gelatin slide, incubate for 15 minutes in a dark location inside the centrifuge tube.
    b. If placed on the gelatin slide, incubate for 30 minutes in a dark location.
3. Trap 4 μL of the stained bacterial suspension between the slide and a cover slide.
4. Visualize, document and save the images under a fluorescent microscope.
    a. Advised to verify the presence of green color – SYTO9 (all cells stained)
    b. Followed by the presence of propidium iodide (membrane integrity compromised stained)
5. As control, the cells must be incubated in 70% isopropanol during 5 minutes after the first centrifuge, followed by the exact same procedure previously described.

Appendix C: Competence test under the imaging conditions

1. Prepare cells as though you are going to image them under the previously tested conditions. At the step where you would normally apply them to gelatin, simply store the 750 µL of cells at room temperature for 30 minutes.
2. After half an hour, set up five tubes containing 100 µL each of the concentrated cells. Number them 1-5.
3. Add 5 µL of chromosomal DNA conferring antibiotic resistance.
4. Incubate at room temperature for 30 minutes.
5. Add 55 µL of 10X DNase digestion buffer.
6. Add 5 µL of DNase I (at 5 mg/mL in 0.15 M NaCl).
7. Incubate 15 minutes at 37C.
8. Dilute and plate on LB and on selective LB-strep agar to determine transformation efficiency. Plate 10^1-10^7 on LB and 10^0-10^5 on LB-strep
9. Repeat steps 4-10 using tubes 2-5, noting the time of DNA addition in order to keep track of how long the cells are competent. The goal is to collect data for 3 hours.
10. Make a plot of competence over time to decide if they are losing competence over time.