Antibody recognition force microscopy (Ig-RFM) to identify and map the nanoscale distribution of protein molecules on the surface of live microorganisms

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Antibody-recognition force microscopy (Ig-RFM) is a relatively new technique that uses atomic force microscopy (AFM) to study antibody-antigen interactions, identify proteins, and map the nanoscale spatial location of single molecules in complex biological structures. It is a type of AFM that is designed to detect minute binding events (e.g., picoNewton forces) that occur between antibodies that are covalently coupled to an AFM tip and antigens that are, for example, exposed on a cell surface. This is a powerful technique because it permits the study of live cells or isolated biomolecules (e.g., protein or DNA) under physiological conditions. Here we describe the use Ig-RFM to probe the cell surface of live bacterial cells using AFM tips that were functionalized with protein-specific polyclonal antibodies. In doing so we were able to identify specific protein molecules that were targeted to the external cell surface. We were also able to map the nanoscale distribution of individual protein molecules on the cell surface and within an extracellular polymeric substance.

Keywords AFM; antibody; atomic force microscopy; bacteria; microorganism

1. Single-molecule imaging in biogeochemistry

In the field of geomicrobiology there is often a desire to understand the molecular-scale biogeochemical reactions that occur in the nanoscale space between a microorganism and a mineral surface. Here microbial biomolecules catalyze both the formation of new minerals (i.e., through precipitation or crystallization of new mineral phases) or the dissolution of existing mineral phases. This in turn controls the bioavailability of beneficial and toxic elements for the surrounding ecosystem. As such, the nanoscale biogeochemical reactions that occur at a mineral-microbe interface can have profound affects (both positive and negative) on water quality, mineral distribution, migration of subsurface contaminants, and soil productivity.

Analysis of the biogeochemical reactions that occur in the environment as a result of the actions of individual microbial biomolecules on particular mineral phases is a difficult and often time-consuming task that requires the use of highly sophisticated equipment. These tools must permit the study of both organic and inorganic components, operate on spatial scales of nanometers to micrometers, enable three-dimensional measurements, and allow for the examination of microbial activities under “physiological” or “environmental” conditions. Many of the biogeochemical processes that are of interest to geomicrobiologists occur between specific protein molecules that a bacterium expresses on its exterior surface (or within an extracellular polymeric substance, EPS) and a particular mineral phase that exists in the environment. Over the past ten years scanning probe microscopy (SPM) has proved to be very useful at providing detailed biological and geological information in these systems.

Two forms of SPM, atomic force microscopy (AFM) and scanning tunneling microscopy (STM), are particularly useful for studying mineral-microbe interactions on the molecular scale. AFM has been used to examine the nanoNewton-scale molecular unbinding events that occur between single microbial protein molecules and the Fe(III)-mineral hematite (Fe₂O₃) [1] and the molecular adsorption density of microbial protein molecules on hematite [2]. These studies have also provided a more detailed understanding of the intermolecular forces involved in protein folding and polymer elongation [1]. In addition, AFM has been used to identify specific protein molecules that are expressed on the exterior surface of a bacterium and measure the nanoNewton binding forces that occur between a protein and a mineral or artificial surface [3-7]. STM has been successfully used to measure the nanoAmpere tunneling current between single electrically-conductive microbial protein molecules and the (111) surface of gold [8].

![Fig. 1 Examples of retraction force curves collected between an antibody-functionalized AFM tip and bacterial cell surface. The three traces show no binding (top), specific binding (middle), or nonspecific binding (bottom) between the tip and cell. Figure modified from Lower et al., 2009 [15].](image-url)
In the study described here, we used AFM to probe the cell surface of live *Shewanella oneidensis* MR-1 cells using AFM tips that were functionalized with protein-specific antibodies (i.e., AntiOmcA or AntiMtrC) to determine if OmcA or MtrC were targeted to the external cell surface [9]. This technique, termed antibody-recognition force microscopy (Ig-RFM), detects minute binding events (e.g., picoNewton forces) that occur between antibodies (e.g., AntiOmcA) covalently coupled to an AFM tip and antigens (e.g., OmcA) that are exposed on a cell surface. These attractive forces manifest themselves as one or more discrete sawtooth signatures in the force-separation profile (Fig. 1). Ig-RFM has been used extensively to study antibody-antigen interactions, identify proteins, and map the nanoscale spatial location of single molecules in complex biological structures and under physiological conditions [10-13].

*S. oneidensis* MR-1 is a ubiquitous dissimilatory metal reducing bacterium that is well known for its ability to respire a variety of anaerobic terminal electron acceptors including solid-phase Fe(III)-minerals. This presents a unique physiological challenge to the bacteria because unlike oxygen, which can freely diffuse across the bacterial membrane during aerobic respiration, Fe(III)-minerals (e.g., goethite or hematite) are highly insoluble terminal electron acceptors (TEAs) that reside outside the cell. Therefore, *S. oneidensis* MR-1 must shuttle electrons from the internal cytoplasmic membrane (where they are generated as a result of the oxidation of carbon compounds) to Fe(III)-minerals located outside the bacterium’s cell wall. Previous studies suggest that *S. oneidensis* MR-1 displays outer membrane proteins OmcA and MtrC on the exterior cell surface to catalyze the terminal reduction of Fe(III)-minerals through direct contact with the mineral [1, 3, 5, 14-17]. Therefore, the objective of the study described here was to use Ig-RFM to determine whether OmcA or MtrC are actually targeted to the external surface of live *S. oneidensis* MR-1 cells when a Fe(III)-mineral serves as the TEA [15]. And if so, how were the proteins distributed on the exterior cell surface and relative to the Fe(III)-mineral [15].

### 2. Covalently linking antibody molecules to AFM tips

The method that was used for covalently linking antibody molecules to AFM cantilevers was provided by Ebner et al., 2007 and Ebner, Hinterdorfer, and Gruber, 2007 [18-19]. This method consists of a three-step process in which single antibody molecules are covalently coupled to silicon nitride (Si$_3$N$_4$) cantilevers via a flexible, heterofunctional polyethylene glycol (PEG)-linker molecule (Fig. 2). The PEG-linker molecule consists of a NHS (N-hydroxysuccinimide) group at one end and an aldehyde group at the other end (i.e., NHS-PEG-aldehyde). The NHS group is designed to form a covalent bond with an AFM tip and the aldehyde group is designed to form a covalent bond with an antibody. In doing so, antibody molecules become covalently attached to a Si$_3$N$_4$ AFM tip creating an antibody-functionalized AFM tip, which can then be used for Ig-RFM (Fig. 2). Prior to conducting the Ig-RFM experiments, the specificity of each polyclonal antibody (i.e., AntiOmcA and AntiMtrC) for OmcA or MtrC was verified by Western blot analysis of proteins resolved by nondenaturing (and denaturing) polyacrylamide gel electrophoresis (data not shown).

All glassware was first washed with mild detergent, rinsed twice with distilled H$_2$O, rinsed with MilliQ-H$_2$O (Millipore Corp.), and then air-dried. Next, the glassware was rinsed with 100% ethanol and then air-dried with N$_2$. The clean glassware was stored in a large glass desiccator under N$_2$ until needed. Si$_3$N$_4$ cantilevers were gently cleaned with chloroform, dried with N$_2$ and then place in an Ozone Cleaner for 20 minutes. The cantilevers were then rinsed with 100% ethanol and air-dried with N$_2$.

First, the cleaned Si$_3$N$_4$ cantilevers were aminated overnight using ethanolamine (Fig. 2). In a small glass beaker, 3.3 g ethanolamine-HCl (Sigma-Aldrich) was dissolved in 6.0 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich) by heating the solution to 60°C until all the ethanolamine dissolved. Next, 3 Å pore molecular sieve pellets (Sigma-Aldrich) were added to the solution such that no monolayer of pellets had formed on the bottom of the beaker. The cantilevers were carefully arranged on a cleaned glass cover slide and this slide was carefully lowered into the ethanolamine solution so that the cover slide rested on top of the molecular sieve pellets. Care was taken so that no air bubbles formed between the glass cover slip and molecular sieve pellets. The beaker was covered with an

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**Fig. 2** Coupling of antibody molecules to Si$_3$N$_4$ AFM tips.
upside-down glass Petri dish so that the DMSO did not evaporate and the amination reaction was allowed to proceed overnight at room temperature. The following day the cantilevers were carefully removed from the ethanolamine solution, gently washed 3-times with DMSO, 2-times with 100% ethanol, air dried with N₂ and then stored in a glass desiccator under N₂ until needed.

Second, the active NHS ester of the NHS-PEG-aldehyde linker molecule was used to form a covalent linkage between the linker molecule and the amine groups on the AFM tips (Fig. 2) [18]. To do this, 3.5 mg NHS-PEG-aldehyde was dissolved in 0.5 mL chloroform (CHCl₃) and the solution was poured into the small glass reaction chamber such that the entire bottom of the chamber was covered up to a depth of approximately 2-4 mm (i.e., enough solution to cover the Si3N₄ cantilevers). Next, 10 µL triethylamine (Sigma-Aldrich) was added, and the ethanolamine coated AFM tips were carefully immersed in this solution for 2 hours at room temperature. The reaction chamber was covered with an upside down glass Petri dish to prevent the CHCl₃ from evaporating. After 2 hours, the tips were washed 3-times with CHCl₃, dried with N₂ and then stored in a glass desiccator under N₂ until needed.

Finally, the antibody molecules (i.e., AntiMtrC or AntiOmcA) were covalently tethered to these tips via the linker molecule’s aldehyde group (Figs. 2 and 3) [19]. The inside of a cleaned glass Petri dish was coated with clean sheet of parafilm (Sigma-Aldrich) and the PEG-linker-functionalized tips were carefully pressed into the parafilm in such a way that the cantilevers were fixed in place in the parafilm and the tips pointed inwards in a circular manner. Into the center of the circle, 200 µL of antibody (0.2 mg/mL) dissolved in phosphate buffered saline (PBS; Sigma-Aldrich) was added. Care was taken to ensure that the solution coated each of the AFM tips. Then, 2 µL 1M NaCNBH₃ (32 mg NaCNBH₃, 50 µL 100mM NaOH and 450 µL H₂O; Sigma-Aldrich) was added to the antibody-PBS solution and carefully mixed with a pipette. The solution was allowed to react with the AFM cantilevers for 1 hr at room temperature. An upside down glass Petri dish was used to cover the reaction chamber to prevent the PBS from evaporating. After 1 hour, 5 µL of 1M ethanolamine (dissolved in 20% NaOH) was added to the reaction solution in order to passivate any remaining unreacted aldehyde groups. The tips were washed several times in PBS and stored in PBS buffer at 4°C. We have been able to successfully store functionally active (i.e., as determined by force spectroscopy) antibody-functionalized AFM tips for up to 4 weeks under these conditions.

3. AFM with antibody-functionalized AFM tips

Our AFM (Veeco/Digital Instruments; Bioscope AFM and NanoScope IV controller) rests on an inverted optical microscope (Axiovert 200M, Zeiss), which allows accurate positioning of the tip over the sample. For the experiments described here, an antibody-functionalized tip was positioned such that the scan would frame a single bacterium (Fig. 3). To mitigate the possible effects of cell senescence, data was collected in as tight a window as the instrumental and experimental constraints would allow. In general, all data were collected within 30 minutes from the time the cells were harvested for imaging. Cell viability was confirmed after force measurements by placing the AFM cantilever on an LB agar growth plate and observing cell growth.

Force curves were collected by monitoring the deflection of the cantilever as a piezoelectric scanner vertically translated the antibody-coated tip towards the sample (i.e., bacterium growing on a hematite substrate; Fig. 3). To generate force-volume images, the AFM cantilever deflected 10-20 nm, which corresponded to a “loading force” of approximately 1-2 nN. The tip was then pulled away from the sample, completing one approach-retraction cycle, which took approximately 1 second (scan rate = 1.0 Hz). This vertical approach-retraction cycle was repeated laterally across a 32 x 32 matrix grid on the sample, thereby generating force-volume images. Each force...
volume image took approximately 20-30 minutes to collect (image capture approximately 2 x 32^2/scan rate). In “relative” trigger mode, force volume imaging also yields a measure of sample topography, based on the scanner height at which a specific, constant cantilever deflection (e.g. 10 nm) was achieved.

4. Analysis of the force-distance and force-volume data

For analytical purposes, force-volume data were either used in the original 32 x 32 matrix form or in interpolated forms as large as 993 x 993. The interpolation was performed by simple averaging within the boundaries of the original 32 x 32 matrix, to avoid processing artifacts. The number of retraction profiles containing sawtooth signatures was counted to obtain the number of specific binding events for a given substrate. We counted only those events with high specificity or stringency, where the stringency threshold was defined as 10 times the instrumental noise (resulting in threshold values between 0.1 nN and 0.3 nN). The raw pixilated force-volume data were deconvoluted using a regularized filter algorithm.

In this study we defined affinity as the work required to separate the tip from the sample surface, calculated by integrating force over separation distance. The specific binding force between the tip and sample is directly proportional to the number of proteins undergoing simultaneous extension. In a system with uniform distribution of both binding proteins on the cell surface and antibody on the tip surface, the number of binding events is in turn proportional to the contact area between the two interacting surfaces. We thus expect large affinity where there is large contact area. Although the increased contact area between the cell and the pyramidal AFM tips does enhance the apparent affinity at the cells’ perimeter, the increased contact area alone should not account for the highly localized affinity observed.

As noted in Figure 1, attractive interactions between an antibody-tip and cell, resulted in relatively short-range, non-specific and longer-range specific adhesive forces. To distinguish between these two interactions, we integrated each force curve beginning at >20 nm and ending at the full retraction of the piezoelectric motor [15]. This integration procedure quantifies the work of binding, measured in Joules, between the antibody-tip and a particular position on the sample. While this integration procedure does not totally exclude non-specific binding, it does select for those events associated primarily with specific antibody-antigen binding [15].

5. Bacterial preparation and Ig-RFM imaging of live cells

Wild-type *S. oneidensis* MR-1 and ΔomcAΔmtrC double mutant cells were cultivated anaerobically with Fe(III), in the form of Fe(III) chelated to nitrilotriacetic acid (NTA), serving as the TEA [15]. The ΔomcAΔmtrC double mutant is deficient in both OmcA and MtrC but produces other proteins native to the outer surface of *S. oneidensis* MR-1 [15]. Growth conditions have been described elsewhere [20, 21] and were selected based on previous studies [1, 15, 20-22] that suggest *S. oneidensis* MR-1 targets OmcA and MtrC to the cell surface during anaerobic growth on Fe(III).

We performed Ig-RFM in fluid (i.e., defined growth medium) on living *S. oneidensis* MR-1 cells using a Digital Instruments Bioscope AFM [1, 15, 20, 23]. The AFM cantilever was scanned across the surface of a bacterium and the antibody-functionalized tip was repeatedly brought into and out of contact with different regions of a bacterium, “fishing” for a binding reaction with cytochrome molecules exposed on the cell surface.

Binding events were observed upon separating AntiOmcA- or AntiMtrC-functionalized tips from wild-type *S. oneidensis* MR-1 cells (Fig. 4). For control, 0.1 µM free AntiOmcA or AntiMtrC was added to the imaging fluid to prevent recognition of surface-exposed cytochromes [1, 13, 15]. This effectively eliminated binding between the antibody-functionalized tip and cell surface [15]. Binding events occurred with approximately the same frequency for AntiMtrC compared to AntiOmcA [15]. Since these measurements involve one bacterial strain, grown, harvested, and imaged under identical conditions, the differences in the force spectra can be attributed to the presence or absence of surface-exposed cytochromes [15]. These results (Fig. 4) show that both OmcA and MtrC are expressed on the external surface of wild-type cells when Fe(III) serves as the TEA.

Several retraction force-distance profiles are shown in Fig. 4, demonstrating the forces between the probe and cell surface after AFM tip contact. These retraction profiles exhibit attractive forces resisting probe-cell separation (Figs. 1 and 4). Such attractive forces were only observed after a tip came into contact with a cell or adjacent regions. This supports the observation that specific binding only occurs near the bacterium-hematite interface, where antibody-binding proteins (i.e., OmcA and MtrC) are localized.

To validate the stringency in our detection of specific binding events, force volume imaging was also performed with bare- or un-functionalized-AFM tips (i.e., Si$_3$N$_4$ tips or Si$_3$N$_4$ with only the NHS-PEG-aldehyde linker molecule covalently attached to it but no antibody; Fig. 3A). The resulting force-separation profiles rarely exhibited sawtooth signatures with consistent contour lengths [15]. However, some attractive forces, consistent with general adhesion forces (van der Waals interactions occurring near the region of tip-sample contact), were sometimes observed at the cell edges. The presence of this attractive, non-specific adhesion force suggests that the biomolecules localized to the bacterium-hematite interface are adhesive in nature (Fig. 1). The absence of well-defined sawtooth signatures (Fig. 1)
observed using these unbaited control tips supports the stringent detection of antibody-specific binding events observed when using tips baited with antibody [15].

We confirmed the detection of OmcA and MtrC on the surface of wild-type S. oneidensis MR-1 by conducting Ig-RFM on ΔomcAΔmtrC double mutant S. oneidensis MR-1 cells that were deficient in both OmcA and MtrC [15]. We found that the force spectra showed a dramatic reduction of adhesion frequency (Fig. 4) supporting the notion that OmcA and MtrC ligands were probed on the wild-type S. oneidensis MR-1 cells. The binding events that were observed for the double mutant were only nonspecific in nature (Figs. 1 and 4). This indicated that the antibodies on the tip do not participate in specific interactions with other proteins on the surface of S. oneidensis MR-1 [15].

As an additional control we also conducted measurements on wild-type S. oneidensis MR-1 cells using Si$_3$N$_4$ tips conjugated with the PEG-linker but not functionalized with polyclonal antibody (Fig. 3). Similar to the results with the double mutant, the unbaited tips were largely unreactive with the surface of the bacteria [15]. Those binding events that were observed were non-specific in nature [15]. Taken together, these results demonstrated that the antibody-coated tips have a specific reactivity with OmcA and MtrC molecules. Further, these force measurements show that MtrC and OmcA are present on the external cell surface when Fe(III) serves as the TEA [15].

To map the distribution of cytochromes on living cells, Ig-RFM was conducted on living S. oneidensis MR-1 cells that were growing on a hematite (Fe$_2$O$_3$) thin film (Fig. 3) [15]. The cells were grown anaerobically to mid-log phase with Fe(III)-NTA serving as the TEA and then the cells were deposited onto the hematite thin film along with anaerobic growth medium that lacked Fe(III)-NTA [15]. The cells were allowed to attach to and grow on the hematite surface (without drying) overnight in an anaerobic chamber. The following day the liquid was carefully removed and immediately replaced with fresh anaerobic imaging solution (i.e., PBS). Ig-RFM was preformed on the cells by raster scanning an antibody-functionalized AFM tip across the sample surface thereby creating an affinity map [15, 24].

Figure 5 is the antibody-cytochrome recognition images for MtrC and OmcA. The corresponding height (or topography) images of the bacteria cells are also shown in Figure 5 [15]. OmcA molecules were found to be concentrated at the boundary between the bacterial cell and hematite surface (Figs. 5E-F). MtrC molecules were also detected at the edge of a cell (Figs. 5C-D). Some MtrC, unlike OmcA, was observed on the cell surface distal from the point of contact with the mineral (Figs. 5C-D). Both OmcA and MtrC were also present in an extracellular polymeric substance (EPS) on the hematite surface (Figs. 5D and 5F), which is consistent with previous results showing MtrC and OmcA in an EPS produced by cells under anaerobic conditions [25, 26]. This discovery is interesting in light of the research by Rosso et al., 2003 [27] and Bose et al., 2009 [28] that found Shewanella could implement a nonlocal electron transfer strategy to reduce the surface of hematite at locations distant from the point of cell attachment. The Ig-AFM results (Fig. 5) suggest the possibility that MtrC and/or OmcA are synthesized by S. oneidensis to reduce...
crystalline Fe(III) oxides at points distal from the cell. However, additional experiments showing reductive dissolution features coinciding with the extracellular location of MtrC and/or OmcA need to be performed to test this hypothesis. It is also important to note that the affinity maps shown here were collected on only a few cells because it so challenging to produce large numbers of high-quality images. Future work should be conducted on a population of cells. Until this time, these affinity maps can be used to provide an unrefined, lowest order estimate of the number of cytochromes on the outer surface of living *S. oneidensis* MR-1.

Perhaps the strongest indication for true proteins (i.e., OmcA and MtrC) localization is the sharp localization of high affinity at the cell edges and on specific regions of the cell surface (Fig. 5). Based on the observation that the cells are deformed into slightly pyramidal shapes, the contact area between the pyramidal tip and compliant cell should roughly vary as the overlapping area between two inverted triangles sliding apart, the intersection of which varies gently as \((h-x)^2\). If proteins were uniformly distributed and contact area were the only cause of the apparent edge affinity, there should be a gentle decrease in affinity as the tip moves from the cell edge to the cell top surface. This effect, however, is not observed even though the instrumental resolution (signal:noise) would certainly allow detection of smaller separation forces. The abrupt decrease in cell-edge affinity and the appearance of “affinity-islands” on the tops of some

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**Fig. 5** Ig-RFM of live *S. oneidensis* MR-1 cells growing on hematite. Height (A) and corresponding Ig-RFM (B) image for a bare tip. Height (C) and corresponding Ig-RFM (D) image collected with an AntiMtrC-functionalized tip. Height (E) and corresponding Ig-RFM (F) image collected with an AntiOmcA-functionalized tip. Each panel also contains a thin white oval that shows the location of the bacterium on the hematite. A color-coded scale bar is given to the right. Height is given in micrometers (\(\mu m\)) and the work required to separate the tip from the surface is given in attojoules (aJ). Figure modified from Lower et al., 2009 [15].
cells (i.e., the cells that were probed with AntiMtrC-tips; Fig. 5) thus negate the possibility of uniform distribution of binding proteins over the cell surface.

As supplemental evidence of true protein localization, two experimental checks were performed. In typical operation, the cantilever is scanned at 0° in a direction such that the facets on the AFM tip (i.e., the flat sides of the pyramidal tip) approach the cell, allowing for large contact area. Changing the scan angle by 45° allows the edge (between facets) of the pyramidal tip to approach the cell and significantly decreases the contact area along this orientation. Although the magnitude of the sawtooth events does decrease (suggesting fewer bonds in parallel), the specific binding events still occurred near the bacterium-hematite interface (data not shown), thus supporting true localization of binding proteins.

Here we show that Ig-RFM, a new imaging technique, can be used to understand the molecular-scale biogeochemical reactions that occur in the nanoscale space between a living microorganism and a mineral surface. These results reveal that S. oneidensis MR-1 localizes two proteins molecules, OmcA and MtrC, to the exterior cell surface and at points distal to the bacterium, when hematite serves as the TEA [15]. This observation supports the hypothesis that OmcA and MtrC catalyze the reduction of an iron oxide mineral (Fe³⁺ → Fe²⁺) via a direct protein-mineral electron transfer reaction [15, 29].

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References


