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Whole-Cell Sensing for a Harmful Bloom-Forming Microscopic Alga by Measuring Antibody–Antigen Forces

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***Abstract*—*Aureococcus anophagefferens*, a harmful bloom-forming alga responsible for brown tides in estuaries of the Middle Atlantic U.S., has been investigated by atomic force microscopy for the first time, using probes functionalized with a monoclonal antibody specific for the alga. The rupture force between a single monoclonal antibody and the surface of *A. anophagefferens* was experimentally found to be 246 ± 11 pN at the load rate of 12 nN/s. Force histograms for *A. anophagefferens* and other similarly-sized algae are presented and analyzed. The results illustrate the effects of load rates, and demonstrate that force-distance measurements can be used to build biosensors with high signal-to-noise ratios for *A. anophagefferens*. The methods described in this paper can be used, in principle, to construct sensors with single-cell resolution for arbitrary cells for which monoclonal antibodies are available.**

***Index Terms*—Atomic force microscopy, *Aureococcus anophagefferens*, biosensors, force-distance measurements, single-cell identification.**

I. Introduction

SINGLE-CELLED microalgae play an essential ecological role in aquatic environments as the producers of organic material that constitutes much of the base of the food webs in these ecosystems [1]. Despite this usually beneficial role, some microalgal taxa produce toxic or noxious substances that can disrupt plankton community structure or function, and result in significant ecological damage, economic loss, and human health effects [2]. Detecting, predicting and ultimately preventing these harmful algal blooms (HABs) are presently major foci in environmental plankton biology [3].

Microalgal taxa have traditionally been identified by light and electron microscopy based on morphological features of the cell and its organelles. Unfortunately, many small species of algae (cells 2–10 μm in size) lack sufficient morphological detail to enable their differentiation and identification. Therefore, our knowledge of the diversity and ecological importance of these species has only recently begun to advance [4]–[9]. For these minute species, genetic and immunological markers remain the only viable means of obtaining sufficient taxonomic characters for identification and enumeration in natural water samples.

Among these latter approaches, genetic techniques have received the most attention during the last decade. These methods are powerful, but they generally require lengthy procedures and fairly rigorous or stringent conditions (e.g., exact temperature control). They also require the extraction of nucleic acids or the introduction of compounds into the cells. Although less extensively exploited in ecological research, immunological approaches offer advantages over genetic techniques in that antibody-based approaches typically are more forgiving with respect to reaction conditions, and often target cell surface antigens, obviating the need for extraction of cellular constituents or permeabilization of cell walls. Thus, antibody-based approaches hold great promise for the development of *in situ* techniques for assaying for the presence and abundance of specific microalgae [10]–[13].

Early application of antibodies for the identification of algae involved the use of polyclonal antibodies and epifluorescence microscopy to identify several minute eukaryotic and prokaryotic microalgae [3], [14]–[17]. These approaches have improved in recent years to include the use of monoclonal antibodies, and indirect detection techniques that provide the potential to dramatically increase the speed of analysis [18], [19]. Often, however, these techniques require abundances of the target alga that are greater than typical abundances under nonbloom conditions.

Atomic force microscopy (AFM) is a powerful technique used to image the morphology of surfaces at molecular, even atomic resolution, as well as to study the physical characteristics of the surfaces [20], [21]. Probing of interaction

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forces at the nanoscale has become possible, through experiments such as stretching of proteins [22] and unzipping of DNA [23], [24]. Biomolecular recognition studies have been performed on various receptor–ligand complexes, such as biotin–avidin [25]–[27], and antibody–antigen [28]–[36], as well as molecules involved in cellular recognition [37]. At the cellular level, the morphology of cells, their biocompatibility, and preferential binding between cells and various surfaces have been studied [38]. Estimates for turgor pressure as well as other mechanical properties of cells have been obtained [39], [40]. Limited probe-based cell recognition studies have been done, such as imaging human red blood cells using an AFM tip functionalized with Helix pomatia lectin, which is specific for N-acetylgalactosamine-terminated glycolipids present on the membrane surface of red blood cells [41], and studying the morphology and height profile differences for an *E. coli* cell imaged with a bare tip and a tip functionalized with antibody [42].

Aureococcus anophagefferens is a minute (2–4 μm) pelagophyte alga that is the cause of recurrent harmful algal blooms known as "brown tides" in coastal lagoons of the middle Atlantic states [43], [44]. This noxious alga lacks flagella and other distinctive morphological features. It is difficult to distinguish from co-occurring species of algae, complicating its detection in natural water samples and thwarting research to understand the factors leading to these harmful blooming events.

In this paper we report the development of a highly sensitive AFM-based method for identifying this alga. For the first time, *A. anophagefferens* cells were immobilized onto a surface and studied by AFM. We present proof-of-concept for a biosensor device capable of identifying single, whole algal cells by using force spectroscopy with AFM tips functionalized with monoclonal antibodies to surface antigens of the target alga.

Our work was conducted in the context of a large program on networked, embedded sensing, with the specific goal of monitoring marine microorganisms. However, our results have much broader applicability in the detection of single cells, algal or not, for which monoclonal antibodies are available.

II. Methods

A. Tip Functionalization With MAb

Silicon nitride AFM tips (NP-S, Veeco) were rinsed with chloroform, and then cleaned by treatment with UV-ozone for 30 min. (T10X10, UVOCS). The tips were rinsed with deionized water, ethanol, and then dried with Ar or N_2. Then they were placed on a glass coverslip and the coverslip was placed in a solution of ethanolamine-HCl in DMSO for 12 h. Molecular sieves were used to remove any residual water from the DMSO solution. The tips were removed from the solution and rinsed with DMSO, ethanol, and deionized water. They were then placed in a solution of glutaraldehyde (5%–10%, v/v) in water for 1 h, and then rinsed with water [31]. Finally, the tips were treated in an MAb [18] solution that was specific for surface antigens of *A. anophagefferens* (8.5 μg/mL of MAb in 100 mM phosphate buffer, pH 7.2) for 1 h, rinsed with phosphate buffer, and then stored in phosphate buffer at 4 °C until use. Tips could typically be stored for several weeks after they had been functionalized [28].

B. Algal Immobilization

Two immobilization schemes for fixing the brown tide alga (BTA) (see Appendix for cell information) onto a surface have been used in this study.

1) Surface MAb Method: A small piece of silicon wafer ($\sim$ 1 cm^2) was treated with a 10% v/v solution of polyethyleneimine (P3143-100 mL, Sigma) in deionized water for 3 h, followed by the addition of 100 μL of MAb (66 μg/mL) specific for *A. anophagefferens* cells. Approximately 1 mL of a culture in late exponential phase (10^6 cell/mL) was added onto the MAb-treated surface and left for 4 hours to allow cells to bind to the MAb on the surface. The sample was then washed with DI water to remove unbound cells from the surface.

2) Polycarbonate Method: Mechanical trapping of the brown tide alga, *Minutocellus polymorphus*, and an unidentified 3 μm alga (BT3) that was not *A. anophagefferens* but co-occurred with it, was accomplished by trapping cells within the 2-μm-diameter pores of track-etched polycarbonate isopore membrane filters (Cat. No. TTTP04700, Millipore). This approach was first demonstrated by Kasas and Ikai [45] and subsequently used by others [38], [46]. A membrane filter was placed inside a 13-mm syringe filter holder (Cat. No. 09-753-10A, Fisherbrand). Two milliliters of stock BTA solution (10^6 cell/mL) followed by 5 mL of DI water was gently injected through the filter using a syringe. The membrane was removed from the holder and washed with DI water and dried using a Kimwipe tissue. Finally, the membrane was fixed onto a glass microscope slide using epoxy (6-Minute Epoxy, Tower Hobbies) which had been precured 8 min. The last step is important because using uncured epoxy will result in a membrane surface where epoxy will wick through the pores and harden, which can look very similar to algal cells when imaged by AFM.

C. AFM Imaging and Force Spectroscopy

The spring constants of the cantilevers were determined using a standard software feature of the AFM (MFP-3D, Asylum Research). AC mode imaging in PBS buffer solution was done to locate cells bound on the MAb surface or trapped within pores of the membrane filter as shown in Fig. 1. After the image was acquired, force–distance (f–d) experiments were carried out by switching the AFM into dc mode, relocating the tip over the cell of interest, disabling the x- and y-direction scans, and then tracking the deflection signal as the tip approached and retracted in the z direction (normal to the surface), repeatedly, in a controlled fashion. The f–d experiments were performed using a z-direction travel distance of 500 nm and varying load rates from 15 to 60 nN/s. The maximum load was set at 5 nN without specifying additional residence time

Interpretation of f–d curves can be a complicated matter, particularly when multiple interactions between tip and surface are involved. Much of the research in force spectroscopy until now has been focused on resolving the binding force of single-molecule, ligand-receptor interactions [47], [48]. In order to achieve this, one needs to screen for individual f–d curves that correspond to single binding events according to some criteria [29],

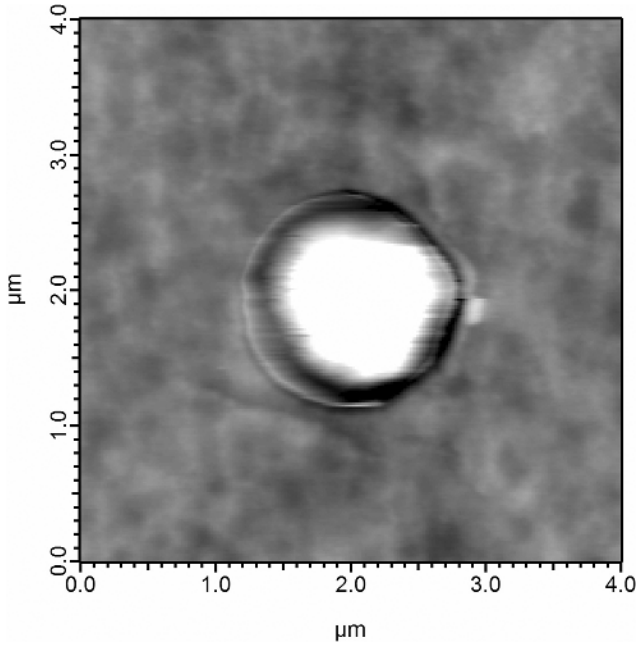

Fig. 1. AFM topography image acquired in ac mode in PBS buffer solution, showing a cell of *A. anophagefferens* protruding out of a 2-μm pore of a polycarbonate membrane filter. The topography image is used to find the location of the cell of interest for force spectroscopy experiments.

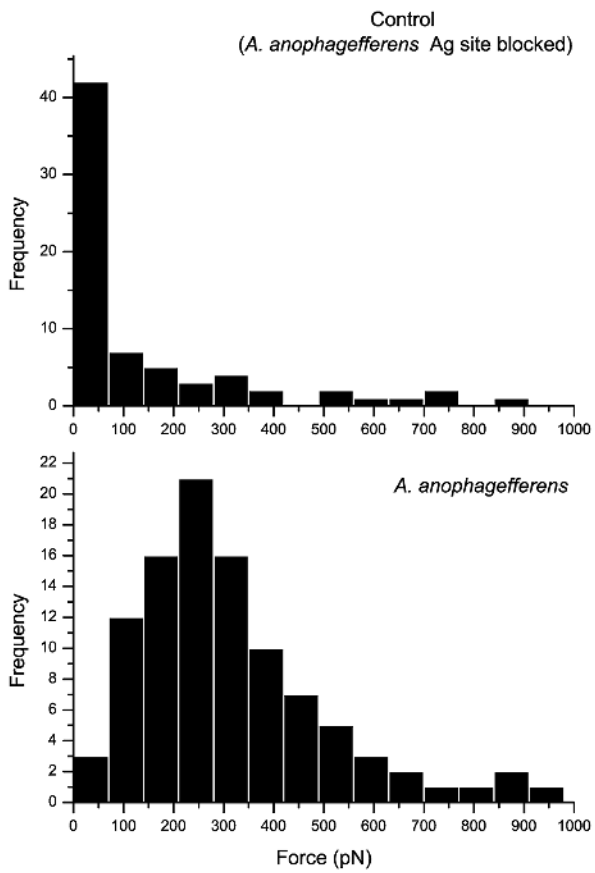

Fig. 2. Force histogram showing interaction between functionalized tip and the surface of *A. anophagefferens*, and the control experiment where the *A. anophagefferens* antigen sites have been blocked with MAb (Control). Histogram bin size $= 70$ pN; load rate $= 12$ nN/s.

[49], [50]. Our focus is on building sensors with minimal data processing requirements, and therefore the discrimination of the force involved in the unbinding of a single antibody–antigen complex is of secondary concern. We simply measured the final rupture force in the retracting curves and ignored single antibody–antigen issues.

III. Results

A. Algal Immobilization

AFM-based experiments require that the specimen to be studied is fixed in position since it is being probed mechanically during the imaging process. It is common to use surfaces having specific properties or to modify the surfaces chemically to immobilize biomolecules and whole cells [51], [52]. *A. anophagefferens* proved to be a difficult cell to study because it has no affinity towards most surfaces, whether these surfaces are chemically modified or not. Even the use of a MAb-coated silicon surface had limited success, since the algae were easily detached from the surface in the AFM imaging process. Mechanical trapping was therefore the method of choice for immobilizing these cells.

B. Individual Binding Force for Single MAb–Ag Complex

Fig. 2, bottom (labeled *A. anophagefferens*), is a histogram of rupture forces between a probe functionalized with the MAb to the target alga immobilized by using the Ab method described above. The histogram for the f–d rupture force taken at 12 nN/s load rate exhibits a well-defined peak corresponding to a force of 246 ± 11 pN, which is within the expected range for single Ab–Ag bonds [28]. For these reasons, we believe that the data in Fig. 2 corresponds to a single Ab–Ag interaction. To obtain such a cleanly delineated peak, we had to prepare and test a number of AFM probes; most of them produced less distinctive histograms, as shown in Figs. 3 and 4. Fig. 2, top (labeled "Control"), shows the histogram obtained after flooding the immobilized alga with excess MAb, thereby blocking the antigen sites on the surface of the alga. One can clearly see a reduction in the average binding force. This confirms that the measured rupture forces are due to MAb–algal interaction.

The linking system used to bind the MAb to the tip affects the quality and performance of the probe in measuring specific Ab–Ag binding events. Hinterdorfer *et al.* have used a flexible linker based on polyethylene glycol (PEG) with length between 6 and 8 nm to give the Ab the flexibility needed to seek and bind to the recognition site [28]. In our case, ethanolamine was used as the linker. It has a relatively short length of approximately 2 nm and is less flexible than PEG. We expect that the MAb, whose structure is unknown, was covalently attached to the linker at varying locations on the Ab, where amine functional groups are exposed. The specific attachment points may influence how the Ab binds the Ag, and the measured rupture forces. Because our ultimate goal is to detect whole algal cells, we do not aim for force signatures having the resolution of single molecule binding events. Rather, we take the opposite approach and seek to attach many Abs to the probe-linker system, thereby

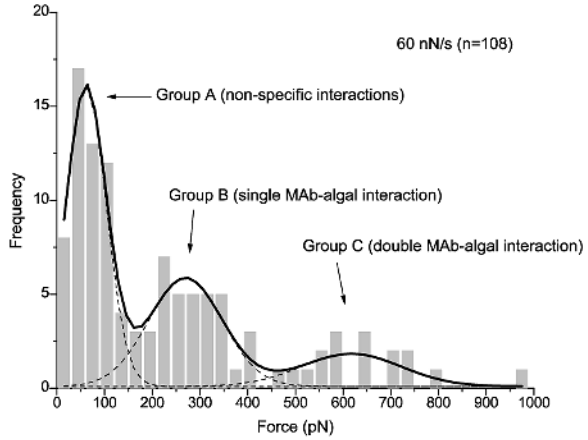

Fig. 3. Histogram of rupture forces measured for MAb-functionalized probe with a cell of *A. anophagefferens* measured at 60 nN/s. The histogram was fitted using Gaussian curves to group the rupture forces. Group A, Group B, and Group C can be attributed to nonspecific interactions, single MAb–algal interaction, and double MAb–algal interaction, respectively. Bin size = 30 pN.

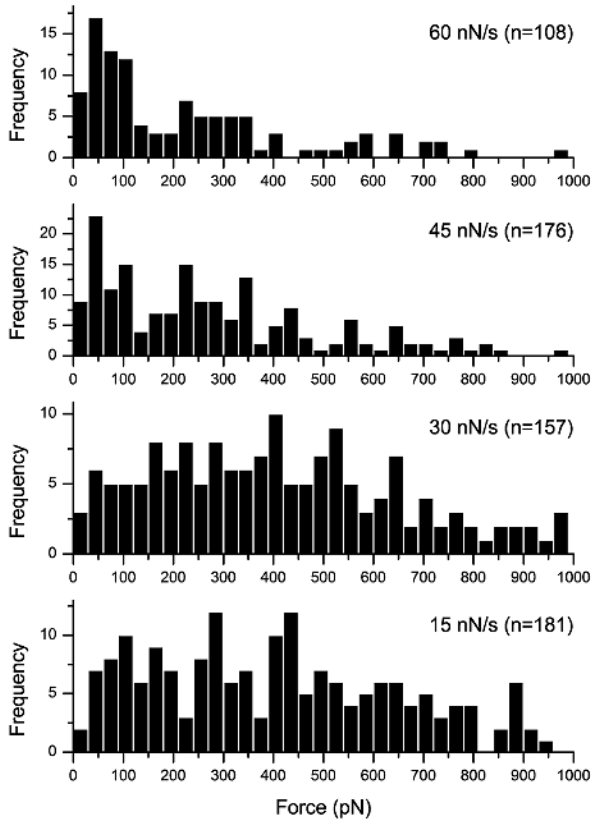

Fig. 4. Histogram showing the effect of load rate on the rupture force (bin size = 30 pN). Maximum load was set to 5 nN, z-direction travel distance of the tip was 500 nm, with zero residence time at the point of maximum load.

increasing the chance that the Ab will interact with the surface in a desired manner. The idea is to distinguish between specific recognition events versus unspecific ones, regardless of the number of Ab–Ag complexes affecting the final rupture force. Therefore, the rest of the force spectroscopy experiments described below were performed using probes so prepared, and without any probe selection tests.

TABLE I
LOAD RATE EFFECT ON RUPTURE FORCE GREATER THAN 100 pN

Loadrate (nN/s)	Contact Time (s)	Binding Frequency > 100 pN (%)
15	0.57	90.04
30	0.28	91.76
45	0.19	69.52
60	0.14	61.82

C. Parameters Affecting Rupture Force

We studied the effect of load rate on rupture forces between MAb and the alga's surface. Fig. 3 shows a rupture force histogram performed at 60 nN/s. One can distinguish three groupings of unbinding peaks: a sharp distribution at less than 100 pN ("Group A"), a slight broader distribution at about 300 pN ("Group B"), and a very broad distribution at 600 pN ("Group C"). We think that the grouping at less than 100 pN is due to nonspecific interactions, the grouping at about 300 pN corresponds to a single MAb–algal interaction, and the last grouping at near 600 pN is associated with two such interactions. Fig. 4 shows that a slower rate of pulling generally leads to an increased number of unbinding events above 100 pN. At the slowest load rate in Fig. 4 (15 nN/s), one can no longer resolve the three groupings discussed previously. Slower load rate corresponds to longer contact time between the probe and the surface. Contact time is the duration of time that the probe remains in physical contact with the surface during the course of a force-distance experiment. One can calculate this by doubling (to account for the approach and withdraw processes) the time it takes the probe to reach the specified force load at a given load rate. Table I shows clearly that longer contact times result in more rupture events greater than 100 pN. We believe that longer contact times allow for a greater number of specific MAb–algal bonds to form between the MAb functionalized probe and the alga's surface.

Hinterdorfer's 6-nm linker required approximately 1 ms to properly bind to the receptor [28]. Because we used a relatively short and rigid linker system, we believe that the time needed for the formation of a MAb–algal bond will be affected. For estimation purposes, if we use the formula presented in [28] and only correct for the length of our linker, our antibody would need approximately 10 ms to find and bind the receptor (assuming that the dissociation constant kD is roughly the same for both systems). The contact times reported in Table I are at least an order of magnitude greater than the required time for an individual MAb to bind a receptor. Therefore, we did not feel the need to incorporate an additional residence time for the probe to be in contact with the algal surface. The maximum number of rupture forces greater than 100 pN occurs at load rates lower than 30 nN/s.

Load rate experiments are important because they establish the acceptable load rate parameters to use for discriminating between algal cells and other cells of similar shape and size. We used load rates of 15 and 30 nN/s for the brown tide algal recognition experiments, to maximize the specific binding interactions. Fig. 5 shows histograms of rupture forces for tips functionalized with *Aureococcus*-specific MAbs and (from

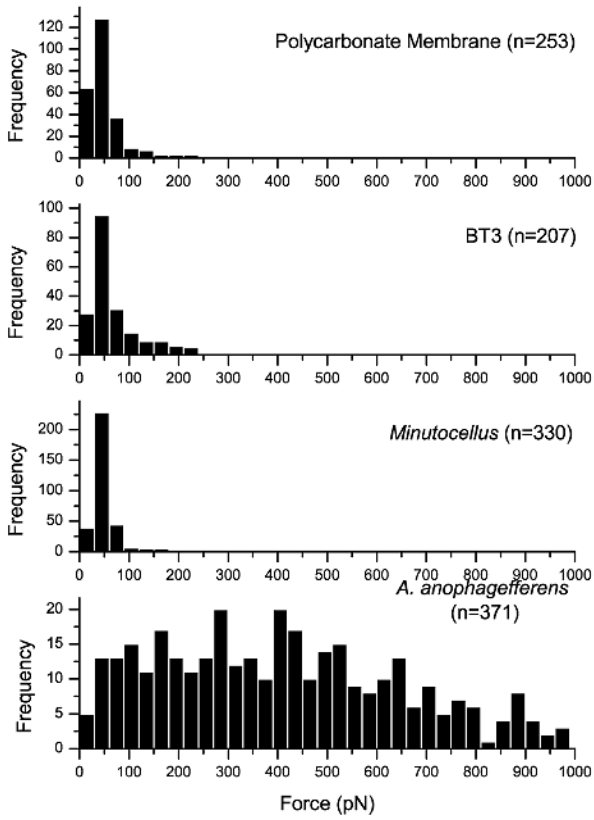

Fig. 5. Histogram of rupture forces showing interaction between the AFM tip functionalized with monoclonal antibody specific for BTA with various surfaces (bin size = 30 pN). Significant binding forces greater than 100 pN are only seen for the BTA cell surface.

TABLE II
BINDING FREQUENCIES FOR *A. ANOPHAGEFFERENS* AND OTHER SURFACES

Cell or Surface	Total Binding Frequency	Binding Frequency > 100 pN	Binding Frequency > 100 pN (%)
Aureococcus Anophagefferens	**371**	**337**	**90.84**
Minutocellus polymorphus	330	17	5.15
BT3	207	47	22.71
Polycarbonate	253	28	11.07

top to bottom) a naked polycarbonate membrane, BT3, and *Minutocellus polymorphus* and *A. anophagefferens* cells, all immobilized on the polycarbonate membrane. BT3 and *Minotocellus* are algae of similar size as the brown tide alga, that co-occur with it, and are optically indistinguishable from it. Rupture forces below 100 pN can be attributed to nonspecific interactions between the MAb and the surface being probed. It is readily apparent that a significant portion of the binding events greater than ∼100 pN were unique to the surface of the brown tide alga and not observed for other cells, as shown in Table II.

It is known that the rupture force for MAb–algal interaction can take on a spectrum of values depending on the load rate, as first discussed by Merkel *et al.* [53]. They measured biotin–streptavidin and biotin–avidin interactions ranging from 5 to 170 pN for load rates which were varied by six orders of magnitude. Although we did not study the effects of load rate for such a broad range, we did see indications that the measured rupture forces taken at different load rates were consistent with their finding. We measured the single interaction force to be 246 ± 11 pN, 252 ± 24 pN, and 277 ± 17 pN for load rates of 12, 45, and 60 nN/s, respectively.

IV. DISCUSSION AND CONCLUSION

Our experimental results show that the rupture force signature attributed to specific and nonspecific interactions between a MAb-functionalized AFM tip and a cell surface can be exploited for cell identification. For example, simply integrating the force histogram binding events above a threshold set by the nonspecific rupture forces (∼100 pN in our case) will suffice to detect the brown tide alga *Aureococcus anophagefferens* with a high signal-to-noise ratio in the presence of other cells optically indistinguishable from the target algal species.

The force signature is very dependent on the quality of the functionalized probe, and it is difficult to control the number and the orientation of the Abs. Research efforts in this field have been traditionally focused on determining the binding force between individual complementary molecular complexes [50]. Using dilute concentration of linkers as well as the antibody to be covalently attached are some of the strategies for minimizing the number of bound molecules. In our case, the AFM tip was immersed in a 8.5-μg/mL MAb solution and allowed to react for an hour. The radius of the AFM probe tip (5–40 nm for the probe we used) is another factor that will determine the number of bound biomolecules interacting with another surface in the area of contact. Finding the tip radius is not a straightforward process [40]. These issues make it difficult to estimate the number of Abs that are attached to the AFM probe, but we do know that it is greater than three, as shown in Fig. 5 (labeled *A. anophagefferens*).

Contrary to efforts in minimizing the number of Abs functionalized on the AFM probe, it may be more desirable to have a large number of Abs to ensure that the results are not significantly affected by variations in individual Abs or their orientation. Our work establishes that a linker based on ethanolamine, which is readily available and inexpensive, is well suited for biosensing applications of the AFM. There are advantages to using PEG as linkers [28], but PEG also has disadvantages such as variation in polymer length, a time-consuming synthesis process, and purchase cost.

Detection and identification of microorganisms by force spectroscopy is inherently a method with single-cell resolution and has several other features which could make it an attractive alternative to other techniques used today. The MAb functionalized probe is robust and has a fairly long shelf life (∼2 weeks). Our experience shows that the functionalized probe, once prepared, can be used to perform thousands of f–d experiments. All of the three cell types we studied neither ruptured nor showed any signs of damage after the force spectroscopy experiments were performed. This suggests that the load of 5 nN is suitable for work with cells of this type. With our approach, the amount of MAb used is conserved because a single probe can be used

repeatedly for many samples. This is in contrast to existing immunological methods in which the antibody is not recycled after detection, as is the case for techniques such as ELISA and flow cytometry. Detection time is comparable to other laboratory techniques. About 100 f–d curves should be enough to determine with a high degree of confidence whether the cell is specific for the antibody bound to the probe. It takes approximately 2 s to obtain an f–d curve, or about 3 min for 100 f–d curves. One can easily minimize this time by decreasing the load and the travel length parameters while maintaining the same load rate. We used 500-nm travel for our experiments, but one can reduce that to 250 nm without affecting the rupture force measurements. This will cut the detection time by half. The number of f–d curves can also be reduced, for example by using maximum-likelihood detection, since the probability density functions for *A. anophagefferens* versus nontarget algae hypotheses are approximately known from the experimental histograms.

We envision a future *in situ* detection technology that leverages the force spectroscopy methods being developed today. MEMS technology can be applied to miniaturize and simplify the *in situ* detection process. Note that AFM imaging of the surface is not required if the cell positions are known in advance. Micrometer-sized orifices at predefined positions on solid support can be microfabricated and pumps can be used to draw a volume of the water sample into a detection apparatus. The support will essentially act as a filter, trapping cells of interest based on size. Multiple functionalized probes can be positioned over orifices at known positions, and their deflection tracked using cantilevers having piezoresistive sensors. This will speed up the process and simplify the deflection measurements. After the cells are tested, they can be removed from the orifices by reversing the pressure to dislodge them. The detector would then be ready to make further measurements.

Finally, it is important to recognize that the sensing methods described in this paper are not restricted to algae or other marine microorganisms. They can be applied to any cell type for which monoclonal antibodies are available. This may have a strong impact on the detection of cells and microorganisms of importance not only for environmental monitoring but also for health care applications. Both laboratory and *in situ*, or implanted (embedded) sensing applications, can be envisaged.

APPENDIX
CELL PREPARATION

A. anophagefferens and other algae were grown and stored with the following conditions:

Strain CCMP1794 of *A. anophagefferens* from Barnegat Bay, NJ, was cultured in modified f/2 medium at 20 °C under a 12 : 12h light : dark cycle. Cells were harvested in the late exponential phase, preserved with a final concentration of 1% glutaraldehyde, and stored at 4 °C in dark glass.

Minutocellus polymorphus is a Bacillariophyte that was isolated from Great South Bay, NY, in 1986. It was grown in f/2 $(+\mathrm{Si})$ under the same temperature and light conditions as *A. anophagefferens*.

BT3 is an unidentified alga with spherical, nonflagellated cells similar in size to *A. anophagefferens*. It was originally isolated from Great South Bay, NY, in 1987 during a brown tide. It was grown in f/2 $(-\mathrm{Si})$ under the same temperature and light conditions as *A. anophagefferens*.

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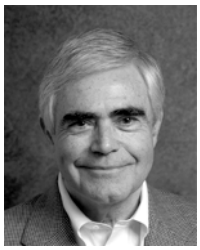

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