Force Sensing for the Identification of Single-Cell Microorganisms*

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***Abstract* – Identification of cells of a specific type is a fundamental problem both for laboratory analysis and for future nanorobots operating in physiological environments for health care applications. This paper shows that force sensing with Atomic Force Microscopes by using tips functionalized with antibodies is an effective technique for detecting microorganisms that cannot be identified by optical microscopy. Experimental results are presented for the identification of Brown Tide Algae, which are single-cell marine microorganisms with dimensions on the order of 1-2 micrometers, and for several control experiments.**

***Index Terms* – Atomic Force Microscopes (AFMs); cantilever sensors; cell recognition; single-cell sensing; nanorobotics.**

I. Introduction

A nanorobot is an integrated system that can sense, "think" and act, and is built from nanoscale components, i.e., devices with dimensions in the range 1-100 nm in at least two of the principal directions. The dimensions of the nanorobot itself are on the order of a few micrometers, and therefore comparable to those of small biological cells. Artificial nanorobots do not yet exist, but many biological systems (e.g., cells) are essentially nanorobots, although they have limited computational power and perform only very specific tasks. Nanorobots of the future are expected to have revolutionary applications in health care and the environment.

Sensing is a fundamental capability for a robot, and the development of nanoscale sensors is currently an area of great research interest. Nanorobots operating in a physiological environment will need to recognize the different types of cells they encounter. Recognition of biomolecules with nanowire sensors has been demonstrated experimentally [1]. The attachment of molecules to a nanowire causes a change in conductivity of the wire, which can be detected by standard methods. The same principle may be applied for cell identification, and research in that direction is on-going at USC. However, cells tend to be larger than a nanowire sensor or the robot itself, and it is not clear how such sensors could be used in a nanorobot for cell identification. Therefore the quest for devices capable of single-cell recognition continues.

This paper reports results of research on a different approach for identifying cells, which is based on measuring the force required to break the bonds between a cell and antibodies to it. This work was conducted within a project that seeks to understand and control the development of Brown Tide Algae (BTA). These are single-celled, globular marine microorganisms with dimensions on the order of 1-2 μm. The BTA, whose scientific name is *Aureococcus anophagefferens*, is responsible for harmful blooms, known as brown tides, which cause major economic losses and can be life-threatening. Today there are no sensors capable of detecting BTA or other marine microorganisms *in situ*, i.e., in the marine environment. Water samples must be collected in the field and analyzed in the laboratory by using such methods as DNA sequencing or flow cytometry. Flow cytometry is an effective technique for high cell concentrations, but has difficulties for low concentrations, and does not have single cell resolution. The DNA sequencing method can reach very low concentrations or even detect single cells if amplification via PCR (polymerase chain reaction) is used, but it is elaborate and time consuming.

Cell identification is important not only for nanorobotics, but also for laboratory analysis applications, e.g., diagnostics. Here the size constraints are not as critical. However, the sensitivity of a sensor usually is proportional to its surface-to-volume ratio, and therefore micro and nanosensors are expected to perform much better than their macroscopic counterparts.

The remainder of this paper is organized as follows. First we describe in Section II the principles of force sensing with Atomic Force Microscopes (AFMs). Next we discuss our implementation and present experimental results (Section III), and finally draw conclusions in Section IV.

II. Force Sensing with an AFM

Atomic Force Microscopy is a major enabling technology for nanoscience and nanoengineering. AFMs are normally used for imaging at the nanoscale, but they also have found applications in nanomanipulation—see e.g. [2]—and as force sensors—see e.g. [3] for

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measurements of forces between DNA strands, and [4] for forces between antibodies and antigens; many other recent examples can be found in the literature.

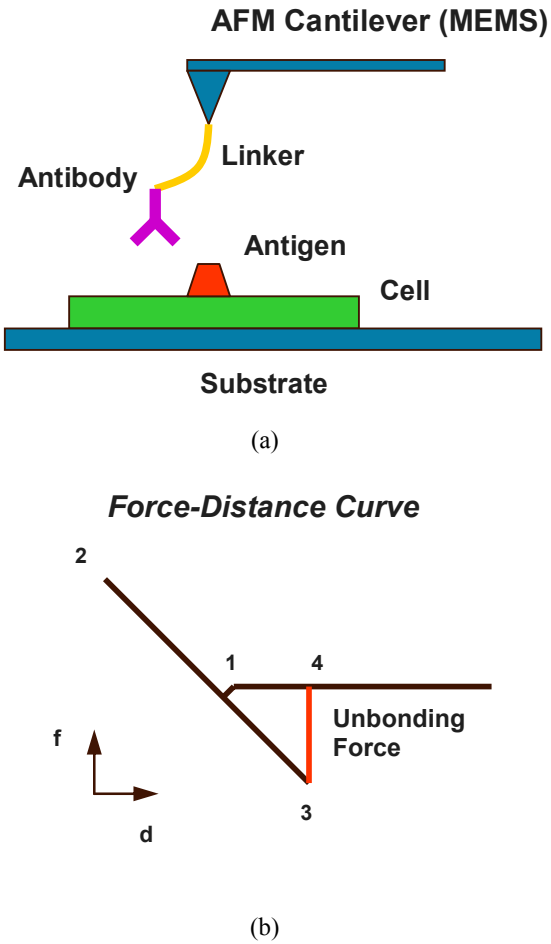

Fig. 1 Cartoon showing the principles of force sensing with the AFM. (a) A cell lying on a substrate and presenting an Ag that will be recognized by the Ab attached to the cantilever by a linker. (b) A force-distance curve showing an abrupt jump between points 3 and 4 during the tip retraction. The deflection that corresponds to this jump measures the force required to break the Ab-Ag bond.

Fig. 1 illustrates the principles of force sensing with the AFM. Fig. 1-a depicts a cell laying on a substrate surface and having certain antigens (Ags) on its surface. Above the cell is an AFM cantilever. An antibody (Ab) is attached to the cantilever's tip by means of a linker. The sensing procedure consists of performing cyclic motions in which the tip first approaches the surface along a vertical line and then retracts. The deflection of the cantilever is measured (and converted to force, assuming that the spring constant of the cantilever is known).

A typical force-distance (f-d) curve obtained in this manner is shown diagramatically in Fig. 1-b. Initially the distance between tip and sample is large and no force is sensed. As the tip approaches the surface there is a point 1 at which the tip "jumps to contact". (This effect is negligible for experiments in a liquid; in air it is due largely to capillary forces.) Continuing motion in the direction of the surface causes the cantilever to deflect by the same amount as the vertical motion, and the curve becomes a straight line until point 2 is reached and the tip begins to retract. If a bond was established between the Ab and the Ag, the cantilever will continue to deflect until point 3, when the bond is broken. The deflection returns to zero at point 4 and then follows the horizontal axis. The jump between points 3 and 4 corresponds to the force required to break the bond. If no bond is formed between tip and sample, there is no such jump.

Suppose now that we place several cells of different types on a surface, some of them being the cells we wish to identify (BTA in our case), and we load the tip with a monoclonal antibody (MAb) that is highly specific to the BTA. Then we probe the surfaces of the cells at several points by using the procedure outlined just above. There are Ab-MAb bonds formed only between the tip and the BTA, not the other cells. Therefore, the jumps in the f-d curves occur only for BTA cells and we can discriminate between them and the others.

III. Implementation and Experimental Results

The sensing procedure described in the previous section is deceptively simple, but its implementation raises a variety of complicated issues:

1) Choice of AFM, tip and substrate. We used throughout the experiments an MFP-3D (Asylum Research), which is the most modern AFM in our lab and is well suited for operation in liquids. Tips were very soft, with nominal spring constants on the order of 0.06 N/m (NP-S, Veeco, Santa Barbara, CA). For substrates we first tried mica and silicon, and then polycarbonate, for reasons explained below.

2) Tip functionalization. The specific chemistries used to attach antibodies to the tip will be discussed elsewhere. Here we point out that the linker length is an important issue. Hinterdorfer's group [4, 5] pioneered the use of flexible linkers, with lengths on the order of a few nanometers. A flexible linker allows the Ab to move and change its orientation relative to the Ag, thus increasing the probability of bonding. (The orientations of the Abs and Ags are not controllable.) In our experiments we found much more consistent data when we used very short, rigid linkers based on ethanolamine. We speculate that flexible linkers may be needed when studying the interaction of a single Ab-Ag pair, but rigid linkers may be better when multiple bonds are acceptable or desirable. The structure of the MAb we are using is not known, but we expect the MAb to attach covalently to the linker through amine bonds.

3) Cell immobilization. We spent a large amount of time and effort studying chemistries for attaching BTA to surfaces, and found none that were truly successful. Note that the cells must be sufficiently well anchored on the surface to withstand the effects of the AFM tip scanning

over them in a liquid environment. While many types of cells adhere readily to a variety of surfaces, these algae do not. We succeded in attaching BTA to mica or silicon surfaces functionalized with Ab's. This served to conduct certain experiments described below, but it is a time consuming procedure, which we found not generally useful. Finally, we discovered that a porous polycarbonate membrane with pores of sizes comparable to the cells is a suitable surface. No attachment chemistry is required. Instead, a swift and straightforward procedure is used, which involves depositing the cells on one side of the membrane while the other is being kept at a lower pressure by a pump [6, 7]. In essence, we use the membrane as a filter.

4) Operating environment. For Ab-Ag bonds to form, a liquid environment is required, and hence one must operate in an AFM liquid cell, or within a droplet of a liquid. The liquid itself is important. In our case, a PBS buffer was required (phosphate buffered saline).

5) Operating parameters. For example, it has been shown [8] that the rate at which the tip is moved has a strong influence on the results. Therefore, this and other parameters must be determined experimentally to maximize the desired effects.

The list of issues above is daunting, and they are not trivially resolved. However, once solutions have been found, the actual sensing procedure is straightforward and easy to implement.

In our first experiments, we functionalized both the tip and the substrate with Abs, and attached BTA to the Ab-coated substrate. Then we acquired f-d curves over the cell's surface, with the results shown at the bottom of Fig. 2. Note a well-defined peak in the histogram for forces around 250 pN. This value is within the range expected for Ab-Ag interactions.

As a control experiment, we flooded the sample with Abs. This fills all the available Ag sites on the cell's surface with Abs, and prevents any binding between tip and cell from occurring. Repeating the f-d curves yielded the results shown at the top of Fig. 2. The peak disappeared and the measured forces were below 100 pN. This can be attributed to nonspecific binding and noise. This experiment shows clearly that the forces being measured without blocking are due to specific Ab-Ag binding and therefore can be used for cell identification.

We compared f-d curves obtained on BTA, on the polycarbonate surface itself, and on two other types of microscopic algae, both immobilized on polycarbonate membranes as explained earlier. The results are shown in Fig. 3. The BTA data is similar to that in Fig. 2, although it shows less of a peak and different values of the mean force. We believe that this is due to a different pull rate used in the two experiments, and to a variable number of Ab-Ag bonds being formed because different cantilevers have different numbers of antibodies that can bind with the cell surface. In contrast, the forces measured for the other algae are all below 100 pN, and are similar to those obtained for the naked membrane. If we filtered out the force values below, say 100 pN, and integrated the result, we would have a BTA detector with high signal to noise ratio.

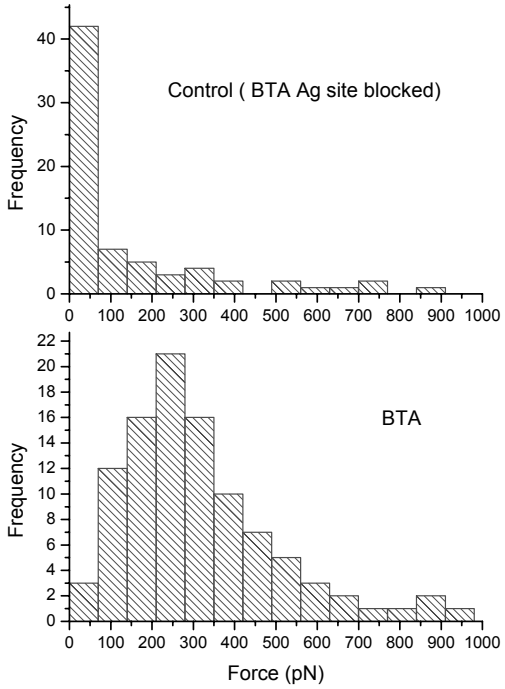

Fig. 2 Histograms of unbonding forces taken when the Ag sites are blocked (top), and in the normal, unblocked situation (bottom).

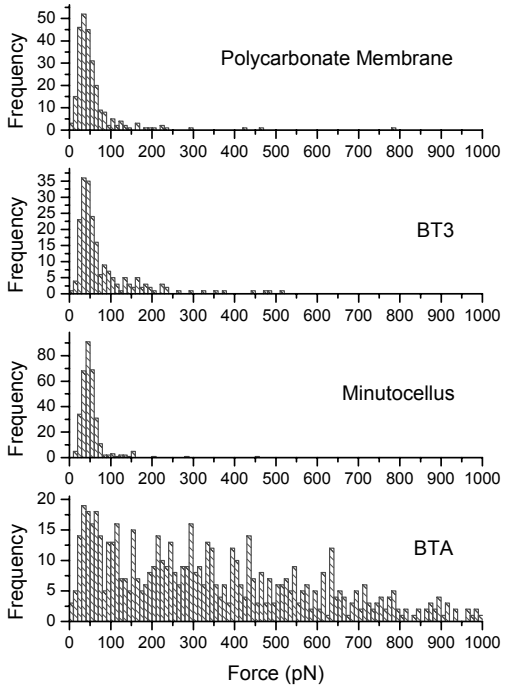

Fig. 3 Force histograms. The three top histograms correspond to control experiments for the naked polycarbonate membrane, and for BT3 and Minutocellus algae. The bottom histogram is for the BTA.

IV. Conclusion

We have shown in this paper that it is possible to detect specific single-celled marine microorganisms (brown tide algae, or BTA) at the single-cell level, amidst other similar microbes. This is done by using as a force sensor an AFM with a tip functionalized with monoclonal antibodies (MAbs) to the BTA. The MAb-Ag interaction is highly specific, as expected and demonstrated by our experimental results. For the first time, BTA cells were immobilized on a surface and studied by force microscopy.

Our approach is not restricted to BTA or other microorganisms, and can be used, in principle, to identify arbitrary cells, provided that Abs for such cells are available.

Tip functionalization is a relatively complicated procedure that takes about two days, but can be done off line and in batches. Functionalized tips have a shelf life of several weeks. Sample preparation on polycarbonate membranes is very simple and takes a few minutes. In addition, a membrane can easily be cleared of all cells and re-used. Therefore, it is possible to build a force-based sensor that operates continuously, on successive batches of cells. In our experiments functionalized tips were used for hundreds of f-d curves without losing the Abs. AFMs with multiple tips are beginning to appear. Functionalizing these tips separately with different Abs would result in simultaneous detection of cells of several types.

Each force-distance (f-d) curve is typically acquired at 0.5 Hz, and therefore takes 2 sec. If we use 60 f-d curves per cell, each cell is processed in 2 min. Therefore, analysis of a sample with some 30 cells can be done in about one hour. These are very conservative estimates. One can easily gain a factor of two in speed by reducing the tip travel length by half in the f-d curves. In addition, the number of f-d curves neded to make a decision can be reduced substantially through maximum likelihood detection or similar statistical techniques, because the approximate probability density functions for the BTA versus other cells are known from the histograms, which need to be found experimentally only once.

The force sensing technique discussed in this paper performs the cell identification task with single-cell resolution for cells that cannot be recognized by optical microscopy. In addition, its speed and complexity compare favorably to other methods which have coarser resolutions. Determining the number of cells of a specific type in a given volume would require further research, to establish a relationship between concentration and the number of cells trapped on the sample substrate. This seems quite doable, but we have not attempted it yet.

Force sensing with and AFM is an effective *laboratory* technique for identifying BTA and similar cells, as we have shown above. Whether it can be used by autonomous nanorobots is still an open question. An AFM is a macroscopic instrument. However, the force sensing in an AFM is performed by a microscopic cantilever, and cantilever sizes are shrinking –for example, Roukes' group at Caltech is building sub-micron cantilevers [9]. Cantilever deflection in an AFM is usually measured by an optical system involving a laser and a photodetector, which are hard to incorporate in a nanorobot. However, piezoresistive cantilevers are also available. These convert deflection directly into an electrical signal, and are more promising at the nanoscale.

We believe that an *in situ* detector could be built on a chip by using extant microfluidics technology in conjunction with piezoresistive cantilevers with tips functionalized with antibodies.

In summary, the work reported here shows that force information suffices to identify cells of a specific type in an environment that may contain a variety of other cells. The methods discussed in this paper are very promising for laboratory tests and may lead to chip-sized *in situ* detectors, but their application to the autonomous nanorobots of the future remains an open issue that requires further research.

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