MICROCANTILEVER ARRAYS FOR MULTIPLEXED BIOMOLECULAR ANALYSIS

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ABSTRACT
An accurate, efficient, and quantitative method for detection of multiple biomolecules, such as DNA and proteins, would benefit many bio-medical applications. These applications include diagnostics of complex diseases such as cancer, drug discovery, and development of fundamental scientific knowledge regarding signaling pathways. We have developed a chip-level microcantilever array designed for high-throughput biomolecular analysis. In particular, biological reactions on one surface of a microcantilever beam change its surface tension due to intermolecular energetic and entropic interactions. These interactions generate sufficient torque to deflect the cantilever beam. Integration of microfluid cells on the chip allows for individual functionalization of each cantilever. Each cantilever is designed to respond uniquely to a specific target analyte allowing for simultaneous and quantitative analysis of multiple bio-molecules. Experiments testing the physical response of the microarray describe the repeatability of the cantilevers while providing information regarding the limits on the detection time for reaction-induced deflections to dominate over random drift of the cantilevers. Statistical analysis shows that the cantilevers exhibit thermomechanical sensitivity within ±7% variation. The maximum observed trend of the long-term drift is about 2.1nm/min, which suggests reactions should be completed within 10 minutes, for a reliable bioassay.

INTRODUCTION
An accurate, efficient, and quantitative method for detection of multiple biomolecules, such as DNA and proteins, would benefit many bio-medical applications. These applications include diagnostics of complex diseases such as cancer, drug discovery, and development of fundamental scientific knowledge regarding signaling pathways. Current methods of biomolecular analysis, such as gel electrophoresis and mass spectrometry (MS) are limited by low throughput and efficiency and/or high cost per assay. Commercially available DNA microarrays provide sensitive assay for thousands of DNA sequences (useful for genetic analysis) but require the use of molecular labels (Duggan et al., 1999). These labels can affect the behavior and functionality of the specific biomolecules due to the physical attachment of the labels. High-throughput diagnostics of proteins remains a greater challenge due to their low concentrations and their fragility (compared to DNA). With these considerations, an analysis tool is needed that can quantitatively detect both DNA and low concentrations of proteins in a high-throughput, cost-effective, and label-free manner.

A new type of chemo-mechanical sensor that relies on reaction-induced changes in surface free energy to generate mechanical motion has been recently developed and studied (Berger et al., 1997; Fritz et al., 2000; Wu et al., 2001a, b; Hansen et al., 2001). In particular, chemical or biological reactions on one surface of a microcantilever beam change its surface tension due to intermolecular energetic and entropic interactions. These interactions generate sufficient torque to deflect the cantilever beam (Figure 1). Current reported biomolecular interaction induced surface tension ranges from 1 to 50 mJ/m² (Fritz et al., 2000; Wu et al., 2001a, b, c).

![Figure 1. Specific biomolecular interactions between target and probe molecules alter intermolecular interactions within a self-assembled monolayer on one side of a cantilever beam. This can produce a sufficient torque to bend the cantilever beam.](image-url)
of these cantilevers on a microchip can provide a cost-effective method of bio-molecular detection. From experiments using single cantilevers contained in a fluid cell filled with a stationary solution, Wu et al. (2001a) and Hansen et al. (2001) reported that not only can DNA hybridization be detected using cantilever deflections, the technique has sufficient resolution to detect single base pair mismatches. Subsequently, it was shown that prostate specific antigen (PSA), a serum marker important for detecting prostate cancer, can be detected at clinically relevant concentrations and conditions (Wu et al., 2001b,c). While these past experiments have demonstrated the effectiveness of cantilever-based nanomechanical biomolecular detection, the underlying physics of the intermolecular interactions that result in cantilever motion have yet to be fully understood and require a great amount of further experimental investigation.

The challenges faced in the development of cantilever-based high-throughput multiplexed bioassays include individual cantilever functionalization on the chip, and parallel detection of cantilever deflections. We have developed a microcantilever array on a chip for multiplexed biomolecular analysis. This device utilizes adsorption (i.e. specific binding) of biomolecules (DNA or proteins) onto the surface of the cantilever to induce detectable nanomechanical movement. Integration of microfluid cells on the chip allows for individual functionalization of each cantilever. Each cantilever will respond uniquely to its target analyte allowing for simultaneous and quantitative detection of multiple bio-molecules. The characterization of the nanomechanical movement of each cantilever is accomplished by utilization of a ray-optics based experimental apparatus. Experiments that test the physical response of the microarray provide useful information about noise, drift and the limits of measurement. These are now described in detail.

**CHIP DESIGN AND FABRICATION**

The microcantilever array is fabricated using a silicon substrate and a glass or PDMS cover. To allow for individual cantilever functionalization specific to the target analyte, the microarray integrates a reaction chamber with each cantilever. The reaction chambers have one inlet and one outlet port to promote chamber filling. Currently, a micropipette is used to inject fluid into the reaction chamber (Figure 2). At the free end of the cantilever, a rigid paddle acts as a mirror surface for the optical readout method described in the next section.

The fabrication process is shown in Figure 3. Deep reactive ion etching (DRIE) is used to etch trenches on the Si wafer, which will later provide rigidity to the paddle. Low pressure chemical vapor deposition (LPCVD) silicon nitride is deposited and patterned to form the cantilever structures. A low temperature oxide (LTO) layer is deposited and patterned and acts as the mask for bulk silicon etching. Gold is thermally evaporated on the wafer and is patterned on the cantilevers. An eight-hour TMAH bulk silicon etching is used to release the cantilever structures. Hydrofluoric acid (HF) removes the LTO mask. When released, the cantilevers bend towards the gold surface 60 μm or 140 μm due to residual stress, depending on the length of the cantilevers.

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** (a) A schematic of the cantilever and the reaction chamber. A micro reaction chamber is integrated on the chip for each cantilever to allow separate functionalization. Each chamber has one inlet and one outlet port to promote bubble-free filling. (b) SEM pictures of a cantilever and its paddle. The cantilever has a 200 μm x 40 μm leg and a 100 μm x 100 μm paddle for optical readout. The ridge on the paddle provides rigidity to the paddle.

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** Fabrication Process. The trenches for the ridge on the Si chip are etched in step 1. LPCVD nitride is deposited on both sides (step 2) and cantilevers are patterned on one side (step 3). Oxide is deposited (step 4) and patterned as the mask for bulk Si etching (step 5). Gold is evaporated and patterned on the cantilevers (step 6). TMAH etches the bulk silicon to release the cantilevers (step 7) and the oxide is stripped (step 8). A Glass or PDMS cover bonds to the silicon chip (step 9).
Pyrex® glass was chosen as the cover material because it bonds well with Si to form a robust reaction chamber for the cantilever. A PDMS cover can be used also in place of the glass. The glass wafer has a 0.1 µm thin film of amorphous Si, bonds well with Si to form a robust reaction chamber. The Si wafer is bonded to the glass wafer through a 350 °C anodic bonding process. It was later determined that this high-temperature bonding process caused the paddle to excessively deform so that it was no longer effective as a reflection mirror. Thus, the room-temperature application of a PDMS cover was preferred. The PDMS cover is made of Sylgard® 184 Silicone Elastomer. It is fabricated using a silicon wafer mold processed with DRIE. The cover is then aligned with the silicon chip to form individual reaction chambers. The microcantilever array is finally subjected to oxygen plasma to make the PDMS surface hydrophilic and then immersed and stored in clean DI water.

EXPERIMENTAL RESULTS AND DISCUSSION

A schematic of the optical readout system is shown in Figure 4. A ray-optics based method is used for parallel detection of multiple cantilever beam deflections. A low power He-Ne laser beam (λ = 632.8 nm) is expanded and collimated to illuminate an area of the cantilever array. The initial deflections of the cantilevers allow the rigid paddles to reflect the cantilever deflection signal away from the direction of the chip's specular reflection. The light that is reflected from the microarray passes through an optical lens and is captured by a CCD camera off the image plane. If the cantilever bends, the angle of the paddle and reflected light changes, causing the spot to move on the CCD screen. Two reflection spots are shown. A centroid algorithm is used to calculate the displacement of the spots.

The cantilevers were tested for drift and thermomechanical response. To determine the drift behavior, the microarray temperature is controlled to a value near room-temperature with the reaction chambers containing deionized water. Cantilever drift as a function of time under these conditions can be determined by monitoring displacement of reflected spots for no external stimuli. The silicon nitride and gold films form a thermomechanical bimorph, which can be actuated by a temperature change. Hence, the thermomechanical response of each cantilever can be compared to determine the statistical variations in thermomechanical properties between each cantilever. Because the thermal bimorph is mechanically analogous to the reaction-induced surface stress, thermomechanical characterization provides some indication of the expected response to biomolecular reactions. To determine the temperature response of each of the cantilevers, the temperature controller is cycled several times, each time returning to the baseline temperature. Figures 5a and 5b show the same three cantilevers’ behavior for two different experiments on different days. In Figure 5a, the cantilevers are held at 23 °C first for 30 minutes. Next, six temperature cycles to 24 °C are completed in 48 minutes, followed by holding the microarray at 23 °C again for 30 minutes. Results of similar experiments are shown in Figure 5b except that two six-step temperature cycles are performed.

The thermomechanical response of the cantilevers is investigated with more experiments similar to the examples in Figure 5. Fitting a 4th order polynomial to the baseline temperature data points and then normalizing all the data to this line removes the drift signal. The complete temperature response data for these three cantilevers is shown in Figure 6. The theoretical thermomechanical sensitivity of the cantilevers, \( S_T = \delta / \Delta T \) where \( \delta \) is cantilever deflection and \( \Delta T \) is temperature change, is given by (Miyatani and Fujihira, 1997)

\[
\delta = \frac{3\sigma (1-\nu) L_p^2}{E_{SN} I_p} \left( 1 + \frac{L_p}{L_s} \right)
\]

(1)

Here, \( t \) is the cantilever thickness, \( \sigma \) is the surface stress, \( \nu \) is Poisson’s ratio, \( E \) is the elastic modulus, \( L_p \) is the length of the sensitive leg of the cantilever, and \( L_s \) is the length of the rigid paddle. The surface stress due to the mismatch of thermal expansion between silicon nitride and gold is

\[
\sigma_{st} = (\alpha_{SN} - \alpha_{Al}) L_p E_{Al} \alpha_{Al}
\]

(2)

where \( \alpha \) is the thermal expansion coefficient. The cantilevers used are 200 µm long, 40 µm wide and 0.6 µm thick, with a rigid paddle of 100 µm by 100 µm at the end. These cantilevers have the thermomechanical sensitivity of 58 nm/K according to equations (1) and (2). The temperature response data in Figure 6...
shows that the observed average thermomechanical sensitivity of the cantilevers is 18 pixel/K and varies by ±7%, which corresponds about ±4 nm/K. This current sensitivity is sufficient for qualitative detection of clinically relevant surface stress changes; however, the random drift signal could dominate for long reaction times.

Figure 5. Experimental results of drift and temperature response tests. (a) The cantilevers are held at 23 °C for 30 minutes, followed by 6 temperature cycles to 24 °C, and are held again at 23 °C. (b) The experiment is similar to (a) except the cantilevers experience 2 six-step temperature cycles. The unit of displacement is CCD pixels, where 1 pixel corresponds ~3 nm of cantilever deflection.

The long-term drift signal can be observed in Figure 5. The drift is random in nature and is not caused by temperature fluctuations in the liquid since this variation can only cause deflections much smaller than those observed (Yue, 2001; Dunphy, 2001). In an attempt to isolate the source of drift, a fixed spot was imaged to determine if the readout apparatus was a significant source of drift. No significant drift was observed from this fixed reflected spot suggesting that the optical readout system does not appreciably contribute to the drift behavior. The long-term drift of the cantilevers can dominate the signal compared to the deflection that will be induced by bio-reactions on the cantilever surface.

Figure 6. Thermomechanical sensitivity of the cantilevers. Three cantilevers are investigated in 5 separate experiments. In each experiment, the cantilevers experience several temperature cycles from 23 °C to 24 °C. The thermomechanical sensitivity is extracted by normalizing all the data to the base temperature line. The variation of the temperature response is less than 7%, which corresponds to a calculated sensitivity variation of ±4 nm/K.

According to equation (1), a surface stress change by 1 mJ/m² can deflect the 200 μm long cantilever by 2 nm. The worst-case long-term drift signal can dominate the bio-reaction signal by one order of magnitude. However, multiple experiments demonstrate the existence of a statistical maximum drift trend for 3 different cantilevers and 213 data points (Figure 7). The worst-case change in cantilever bending due to drift was found

Figure 7. The long-term drift and its maximum trend as a function of time. Multiple experiments show the drift distribution over time for 3
different cantilevers and 213 data points. There is a maximum trend, 0.7 pixel/minute, which corresponds to ~2.1 nm/min.

to be about 21 nm in 10 minutes. Although the source of the drift is still not fully understood, this cantilever-array technique remains viable to analyze biomolecular reactions as long as the time required for reactions fall within a timescale where the bio-reaction signal dominates the drift signal. For example, a biomolecular binding with 10 mJ/m² surface stress change (corresponding to 20 nm deflection) could still be qualitatively investigated if the reaction is completed in 5 minutes. Wu et al. (2001a) reported that DNA hybridization on a cantilever was accomplished within about 30 minutes in a 1 ml fluid cell. It is suggested that the experimental times were limited by the diffusion rate of the target molecules (Wu et al., 2001c). In contrast, the microarray chip contains 1 μl reaction chambers. Because diffusion times are related to the volume by the relation \( t_d = \left( \frac{\mu V}{D} \right)^{1/2} \), a 1000 fold reduction in volume should reduce the diffusion time by a factor of 100, which should allow the same DNA molecules to reach the cantilever in 18 seconds. The drift within this time scale is negligible. In addition, for short times (0 - 10 minutes) the drift of the cantilevers can be monitored and approximated as a predictable linear trend thus allowing for drift correction.

SUMMARY
We have developed a chip-level microcantilever array designed for high-throughput biomolecular analysis. In particular, biological reactions on one surface of a microcantilever beam change its surface tension due to intermolecular energetic and entropic interactions. These interactions generate sufficient torque to deflect the cantilever beam. Integration of micro fluid cells on the chip allows for individual functionalization of each cantilever. Each cantilever will respond uniquely to its target analyte allowing for simultaneous and quantitative detection of multiple biomolecules. The cantilevers demonstrate repeatable thermo-mechanical sensitivity, which fluctuates only 7% (about ±4 nm/K). This current sensitivity is sufficient for qualitative detection of clinically relevant surface stress changes; however, the random drift signal dominates at this concentration for long reaction times. The drift demonstrates a repeatable maximum trend that will allow for the drift signal to be minimized for short assay/reaction times making the microcantilever array a viable method of high-throughput biomolecular analysis.

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