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DNA nanomechanics allows direct digital detection of complementary DNA and microRNA targets

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Abstract

Techniques to detect and quantify DNA and RNA molecules in biological samples have played a central role in genomics research[1–3]. Over the past decade, several techniques have been developed to improve detection performance and reduce the cost of genetic analysis[4–10]. In particular, dramatic advances in label-free methods have been reported[11–17]. Yet, detection of DNA molecules at concentrations below femtomolar level requires amplified detection schemes[1,8]. Here we report a unique nanomechanical response of hybridized DNA and RNA molecules that serves as an intrinsic molecular label. Nanomechanical measurements on a microarray surface exhibit excellent background signal rejection that allows direct detection and counting of hybridized molecules. The digital response of the sensor provides a large dynamic range that is critical for gene expression profiling. We have measured differential expressions of miRNAs in tumor samples, which has been shown to help discriminate tissue origins of metastatic tumors[18]. 200 picograms of total RNA is found to be sufficient for this analysis. In addition, the limit of detection in pure samples is found to be 1 attomolar. These results suggest that nanomechanical readout of microarrays promises attomolar level sensitivity and large dynamic range for the analysis of gene expression, while eliminating biochemical manipulations, amplification, and labeling.

In a microarray experiment, single stranded probe DNA molecules with known sequences are immobilized on a surface at predefined locations. Hybridization of probe and target molecules is routinely measured by fluorescence, but it can also be detected by exploiting various nanoscale phenomena[19] including nanomechanics. For example; changes in surface stress[12] and added mass of target molecules[13], electrical forces[20], topographical changes in self assembled bar-coded nucleic acid probe tiles[21], and hydration induced surface tension[15] have been used for hybridization detection.

Our nanomechanical detection scheme relies on the changes in stiffness of DNA molecules immobilized on a surface before and after hybridization. We have used a recently developed atomic force microscope (AFM) technique[22,23] to investigate the stiffness of single and double stranded DNA molecules, which is illustrated in Fig. 1a. The resulting stiffness maps, such as in Fig. 1b, show hybridized DNA molecules as dark brown spots. This is verified by a series

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Author Information Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare competing financial interests. A patent application has been filed by Stanford University. Correspondence and requests for materials should be addressed to O. S. (sahin@rowland.harvard.edu)

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of experiments with non-complementary targets and complementary targets with varying concentrations.

When the sharp tip presses against the molecules on the surface, interaction forces increase at a rate proportional to local stiffness. The two force curves plotted in Fig. 1c show the surface is stiffer on the single stranded region. The responses of molecules against external forces are not only determined by their intrinsic mechanical properties, but also on their conformations on the surface. The higher stiffness observed on single stranded DNA is largely due to the influence of the gold substrate. Single stranded molecules either lay down on gold, or get squeezed under the influence of the tip without much resistance so that the tip is essentially feeling the stiffness of the substrate. This hypothesis is supported by the height measurements that show hybridized molecules to appear taller than single stranded regions (See supplementary information). Increased height of hybridized molecules also result in spot sizes larger than the size of DNA molecules due to tip convolution. The changes in the height of hybridized molecules have also been used to detect hybridization[25,26]; however these demonstrations required nano- to micromolar level target concentrations. In addition, height measurements provide limited discrimination against the roughness of the substrate surface; therefore atomically flat substrates had to be used. Measurement of a local material property like stiffness, however, provides the specificity necessary to discriminate single hybridized molecules on the surface.

Figure 1d shows calculated stiffness values over the dashed line in Fig. 1b. The stiffness values over single and double stranded molecules are well separated from each other. In addition, the values measured on different spots are sufficiently uniform to provide a clear signature for hybridized molecules. A computer can analyze the stiffness map with this signature and generate a binary image to count hybridized target molecules as seen Fig. 1e. RNA/DNA hybrids also exhibit the same mechanical signature as hybridized DNA (see supplementary information).

We have carried out hybridization experiments with varying target DNA concentrations and with two different areas of probe immobilization. The results plotted in Fig. 1f shows the dependency of surface density of hybridized molecules on target concentration and spot size; lower target concentrations and larger immobilization areas give less hybridized molecules at a given scan area. Concentration levels from 1 nM down to 1 aM are detectible. In addition, no hybridized molecules are found with the non-matching sequence. These numbers represent 3 to 8 orders of magnitude enhancements in the detection limit and detection range over the previously reported direct detection methods[11–16,20,21].

We have further investigated the performance of the presented technique under the complex background of biological samples by analyzing total RNAs extracted from tumor tissues. Detection and quantification of RNA requires the use of large amounts of starting material or additional enzymatic steps involving reverse transcription and amplification. Furthermore, small regulatory RNA molecules like microRNAs[27] are not directly compatible with conventional amplification schemes[28]. Therefore, the presented approach can simplify the analysis of microRNA content in biological samples to a great extent.

We have measured differential expressions of two microRNAs, hsa-mir-205 and hsa-mir-194, in colon and bladder tumors by analyzing total RNAs extracted from tissue samples. The extraction process includes disruption of cells and purification of RNAs. Material isolated this way is routinely used for gene expression profiling. Rosenfeld et al., have shown that microRNAs exhibit expression patterns that can be used to predict tissue origins of metastatic tumors and the particular microRNAs we investigate here discriminate tumors of gastrointestinal (colon) and nongastrointestinal-epithelial (bladder) origin[18]. The numbers of

RNA/DNA hybrids are plotted in Fig. 2a for hsa-mir-205 and in Fig. 2b for hsa-mir-194. (Numerical values from each measurement are provided in the supplementary section). The chart in Fig. 2c compares average expression levels normalized to 10 picograms. The data shows that hsa-mir-205 is expressed more in bladder tumor sample and hsa-mir-194 is expressed more in colon tumor sample. The observed inversion in differential expression in these microRNAs is in agreement with the results of Rosenfeld et al. and we further verified this result by conventional microarray analysis of the tumor samples (NCode™ Human miRNA Microarray V3). The average numbers of microRNAs are also in broad agreement with the numbers derived from quantitative PCR experiments[28]. Note that the inversion in the differential expressions of the two microRNAs was detectible down to 0.2 ng of total RNA. This represents a dramatic reduction in the amount of total RNA compared to conventional microarray experiments, which typically require amounts in excess of 1 μg. These arrays are generally printed on glass slides and use spot sizes around 100 × 100 μm. Their unprinted areas are blocked to eliminate unspecific adsorption.

An important advantage of the presented nanomechanical detection technique is its compatibility with multiplexed analysis in microarray platforms. Multiplexed analysis demands generation of stiffness maps and counting hybridized molecules for each array spot. Our instrument is not optimized for scan speed, yet it provides the throughput, reliability, and robustness for multiplexed analysis. As a demonstration, figure 3 shows a 25 μm wide stiffness map of hybridized molecules at 6 nm pixel size that is generated in 2 hours. In principle, a scan area of this size is sufficient to read out an array with a thousand spots by sampling 800 nm wide regions in each array spot. This level of multiplexing is sufficient for whole genome microRNA expression profiling and for many clinical applications. Throughput can be further enhanced by combining our high spatial resolution readout scheme with “DNA nanoarrays” formed by dip-pen lithography[29] and Supramolecular NanoStamping[30]. Multiplexed analysis can also create more complex situations due to probes with secondary structures, mismatches, potential unusual base pairing, and free termini. We have analyzed several probe sequences that are representative of these cases and validated the utility of our stiffness based hybridization detection principle under these situations (See supplementary information).

In summary, we observed that the nanomechanical signatures of hybridized DNA and RNA molecules provide an excellent background rejection that leads to digital readout with attomolar level detection limit in micro and nanoarray platforms, while eliminating external labeling and enzymatic manipulations of the target molecules. Furthermore, the analysis of microRNA contents in two tumor tissues illustrates the potential of this technique in medical applications. This opens the prospects of working with extremely small amounts of genetic material in combination with substantial simplifications and cost reduction in genetic analysis.

Methods Summary

Sample Preparation

The probe chips are prepared by immobilizing single-stranded and thiolated DNA molecules on a gold coated silicon substrate. Immobilization areas are defined lithographically using a standard lift-off procedure. After passivating the surface with mercaptohexanol[24], the chips are immersed in a solution containing target DNA or total RNA (no labels or any other chemical modifications) for overnight hybridization, washed with a buffer solution, and dried under a stream of nitrogen. Each sample is visually inspected and the samples that appear contaminated or scratched are not included in the AFM analysis.

AFM analysis

Samples are imaged in tapping-mode AFM under ambient conditions. T-shaped cantilevers are used to generate time-varying tip-sample forces. Effective elastic modulus is derived from these waveforms using our previously described mathematical procedure[22]. This procedure provides quantitative values on samples with flat surfaces. In the case of the tip interacting with DNA molecules, the calculated values provide a measure of stiffness, but they do not correspond to true elastic modulus values.

Methods

Substrate preparation

5 × 5 mm gold coated silicon chips were prepared by sputter coating (Au = 20 nm and Ti = 10 nm, 'AJA sputtering system', CNS, Harvard University) on Si wafer (3 inch, silicon quest international). Diamond scribe was used for cutting wafers into 5 × 5 mm chips. Substrates with 50 × 50 and 200 × 200 μm gold spots were defined by photolithography followed by standard lift-off procedure.

Immobilization of ssDNA on gold surface

Gold chips were pretreated with UV-ozone procleaner (Bioforce Nanosciences) for ~ 30 min and washed with nanopure water (deionized and filtered with inorganic membrane filter with 20 nm pores). Thiolated ssDNA (single stranded DNA, concentration 1μM) were immobilized on the freshly prepared gold surface through thiol-gold covalent bonding. High salt buffer was used for immobilization of ssDNA (1M NaCl, 50 mM phosphate buffer pH 7.2). Immobilization was done at room temperature under constant mixing, incubation time ~ 15 hrs. The immobilized ssDNA chip surfaces were passivated by washing with water and subsequent incubation with mercaptohexanol (MCH, 1mM in nanopure water) for ~1 hr.

Synthetic DNA samples

All oligos were purchased from MWG Biotech Inc. (Huntsville, AL 35805 USA) or from Integrated DNA Technologies (Coralville, IO 52241 USA) used within a month. Thiolated probes were reduced according to manufacturers' suggested protocols.

DNA Hybridization

Prior to hybridization, ssDNA immobilized chips were thoroughly washed with nanopure water. DNA/DNA hybridization reactions were carried out at room temperature under constant mixing in 1x SSC buffer (150 mM NaCl, 15 mM sodium citrate, pH 7.0). Volume of the hybridizing solution was 200 uL for 5 × 5 mm gold chips and 1 mL for 50 μm gold spots. Finally, hybridized chips were washed with low salt buffer (25 mM NaCl, 10 mM Tris HCl pH 7.4) and gently dried by weak stream of nitrogen.

Total RNA Hybridization

Total RNAs extracted from human bladder and colon tumors were purchased from Stratagene and kept at −70 °C until used. Samples were diluted with hybridization buffer (3x SSPE with Tween 20, pH 7.4 (Teknova)) to their final total RNA concentrations in Fig. 2. Hybridizations were carried out on a shaker for ~ 16 hrs at 42 °C. All microcentrifuge tubes, tweezers and gold coated silicon chips were cleaned with RNaseZap (Ambion) before immobilization of DNA molecules, and washed extensively with DNase, RNase free water (MP Biomedicals).

AFM measurement conditions

Tapping-mode AFM was used for the measurements under ambient conditions. Torsional harmonic cantilevers (HarmoniX Probes, Veeco Instruments) were used (resonance frequency f ~ 60 KHz) to generate time-varying tip-sample forces. Driving amplitude was adjusted so that peak tapping forces during the scan are approximately 30–50 nN (peak tapping forces are different on single and double stranded molecules). Set point amplitude is set to ~ 60 nm.

Calculation of elastic modulus

The numerical procedure to reconstruct tip-sample force waveform from the raw deflection signals involve averaging vibration waveforms over several consecutive oscillation cycles to reduce noise, correcting the effect of the torsional frequency response, and eliminating the cross talk from large vertical signals, and fitting the tip-sample force distance curves to a contact mechanics model. Details of these steps and data acquisition hardware are described in Ref. [22]. Calculations are carried out in Labview. Calibration of the time varying force waveform (Volts to Newtons conversion) is performed according to Sahin, O. (*Rev. Sci. Instrum.* **78**, 103707 (2007)). Effective elastic moduli of single and double stranded DNA in Fig. 1 are calculated for a tip radius of 7 nm. Subsequent images are calibrated by using the single stranded background in Fig.1 as a reference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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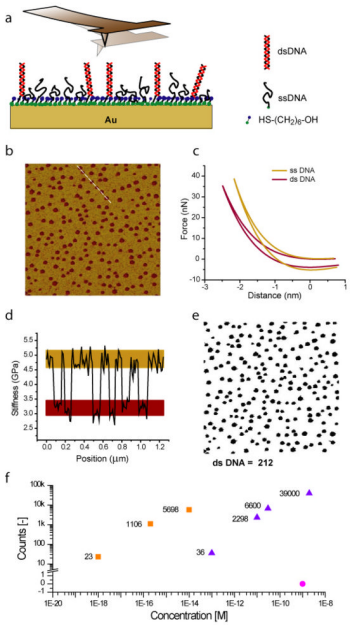

Figure 1.

Nanomechanical detection of DNA hybridization. (a) Schematic of the experiment. After hybridization, the surface of the array spot is scanned with the AFM to generate the stiffness map shown in (b). Scan size is 3 μm. Hybridized molecules are measured to be less stiff and appear as dark brown spots. (c) Stiffness at each pixel in (b) is calculated from force-distance curves. Effective stiffness values across the dashed line in (b) are plotted in (d). A computer generates a binary image (e) to count the hybridized molecules from stiffness values. The numbers of hybridized molecules found in 10 μm wide scan areas are plotted in (f) against varying target concentrations and for two probe immobilization areas (squares: 50 μm, triangles: 5 mm). Hybridization against a non-matching sequence is given by the pink circle.

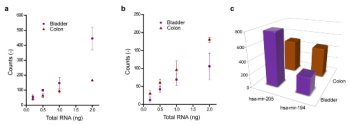

Figure 2.

Direct detection and quantification of miRNA expression in cancer tissues. Total RNAs isolated from bladder and colon tumor samples are analyzed for hsa-mir-205 and hsa-mir-194 content. The average numbers of target molecules found at varying amounts of total RNA are plotted for (a) hsa-mir-205 and (b) hsa-mir-194 (10 μm scan, 200 μm immobilization). Standard deviations are calculated whenever two or more measurements are available from experiments carried out in triplicates and they are given as error bars. (c) Comparison of expression levels in each sample estimated by normalizing the data to 10 pg, averaging, and scaling to entire area of immobilization.

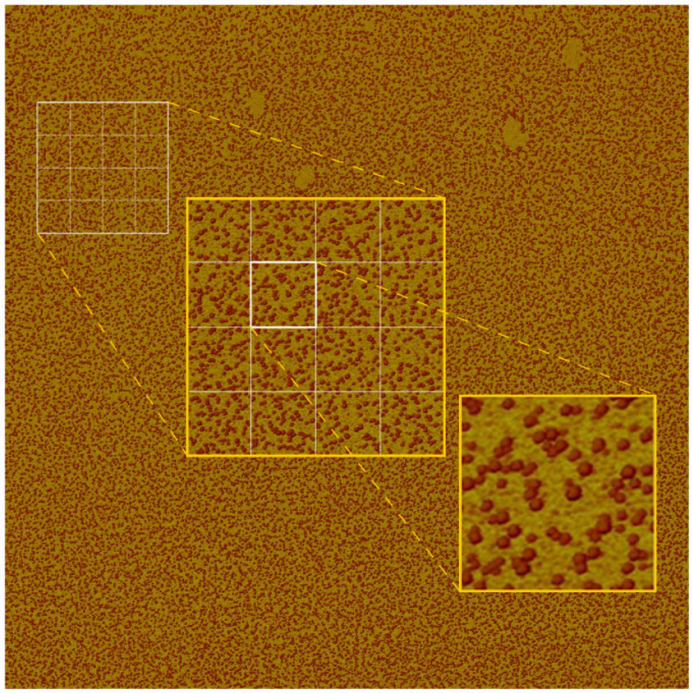

Figure 3.
High throughput and robust nanomechanical readout for multiplexed detection. 25 × 25 μm stiffness map at 6 nm pixel resolution shows individual hybridized molecules. Scan time is 2hrs. Target DNA concentration is 32 pM and immobilization area is 5 × 5 mm. The number of dsDNA is ~ 41250. The rectangular grid illustrates the possibility to share the scan area among different microarray spots. Insets are showing two levels of zoom into the stiffness data (not a rescan). The original image is given separately in supplementary information.