Supplementary Information

Supplementary information consists of the following sections.

1. RNA/DNA sequences used in this study
2. Height and stiffness measurements on hybridized molecules
3. Stiffness maps at varying concentrations of target DNA
4. Stiffness measurements on RNA/DNA hybrids.
5. Averages and standard deviations of hybridized miRNAs measured at different concentrations of total RNA
6. Original high pixel density version of the stiffness map in Fig. 3
7. Hybridization detection under sub-optimal conditions
    - 7. a- Probes with secondary structures and G-quads
    - 7. b- Mismatches, potential unusual base pairing, and free termini
    - 7. c- Compromised hybridization conditions

1. RNA/DNA sequences used in this study

Following are the sequences (5’ to 3’) used in this study.

Data in Fig. 1 (b-e)

1. GTG CCG AAT CTG TGA TGA TGA GGA T

2. ATC CTC ATC ATC ACA GAT TCG GCA C (Modification (5'): Thiol)

Data in Figures 1(f), 3, Supplementary Figures 1, 3 and 5

3 ATC CTC ATC ATC ACA G (Modification (5'): Thiol)

4 CTG TGA TGA TGA GGA T

Data in Fig. 2

5. TCC ACA TGG AGT TGC TGT TAC A (Modification (5'): Thiol). Used as the probe for hsa-mir-194.

6. CAG ACT CCG GTG GAA TGA AGG A (Modification (5'): Thiol). Used as the probe for hsa-mir- 205.

Data in Supplementary Fig. 4

7. UCU UUG GUU AUC UAG CUG UAU GA (microRNA hsa-mir-192)

8. TCA TAC AGC TAG ATA ACC AAA GA (Modification (5'): Thiol)

9. TCT TTG GTT ATC TAG CTG TAT GA. (used for control experiments)

Data in Supplementary Fig. 6, (probes with secondary structures)

10. AGA TCA GTG CGT CTG TAC TAG CAC A (Modification (5'): Thiol)

11. TGT GCT AGT ACA GAC GCA CTG ATC T

12. AGA TCA GTG CGT CTG TAC TAG CAG T (Modification (5'): Thiol)

13. ACT GCT AGT ACA GAC GCA CTG ATC T

14. TTA GGG TTA GGG TTA GGG TTA GGG (Modification (5'): Thiol)

15. CCC TAA CCC TAA CCC TAA CCC TAA

Data in Supplementary Fig. 7, (probes testing mitmatches, unusual base pairing, and free termini)

16. CAG ACT CCG GAG GAA TGA AGG A (Modification (5'): Thiol)

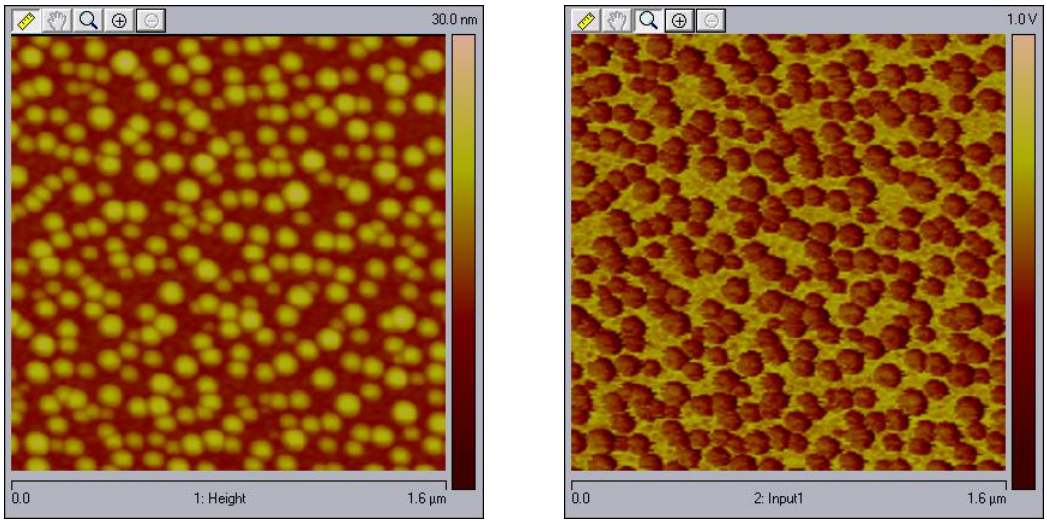
17. UCC UUC AUU CCA CCG GAG UCU G (miRNA hsa-mir-205)

18. TCC AAA TGG AGT TGC TGT TAC A (Modification (5'): Thiol)
19. UGU AAC AGC AAC UCC AUG UGG A (miRNA hsa-mir-194)
20. CAG ACT CCG GTG GGA TGA AGG A (Modification (5'): Thiol)
21. UCC UUC AUU CCA CCG GAG UCU G (miRNA hsa-mir-205)
22. TCC ACA TGG GGT TGC TGT TAC A (Modification (5'): Thiol)
23. UGU AAC AGC AAC UCC AUG UGG A (miRNA hsa-mir-194)
24. GGT GGA ATG AA (Modification (5'): Thiol)
25. {UUC AUU CCA CC }G GAG UCU G (miRNA hsa-mir-205)
26. ACA TGG AGT TG (Modification (5'): Thiol)
27. AAC AG {C AAC UCC AUG U} GG A (miRNA hsa-mir-194)

The hybridization against non-matching sequence in Fig. 1(f) was tested by using sequences 3 & 9. Data in supplementary Fig. 2 is obtained by using only sequence 10, no targets. Data in supplementary Fig. 8 is obtained with sequences 6 (probe) and 17 (target).

2. Height and stiffness measurements on hybridized molecules

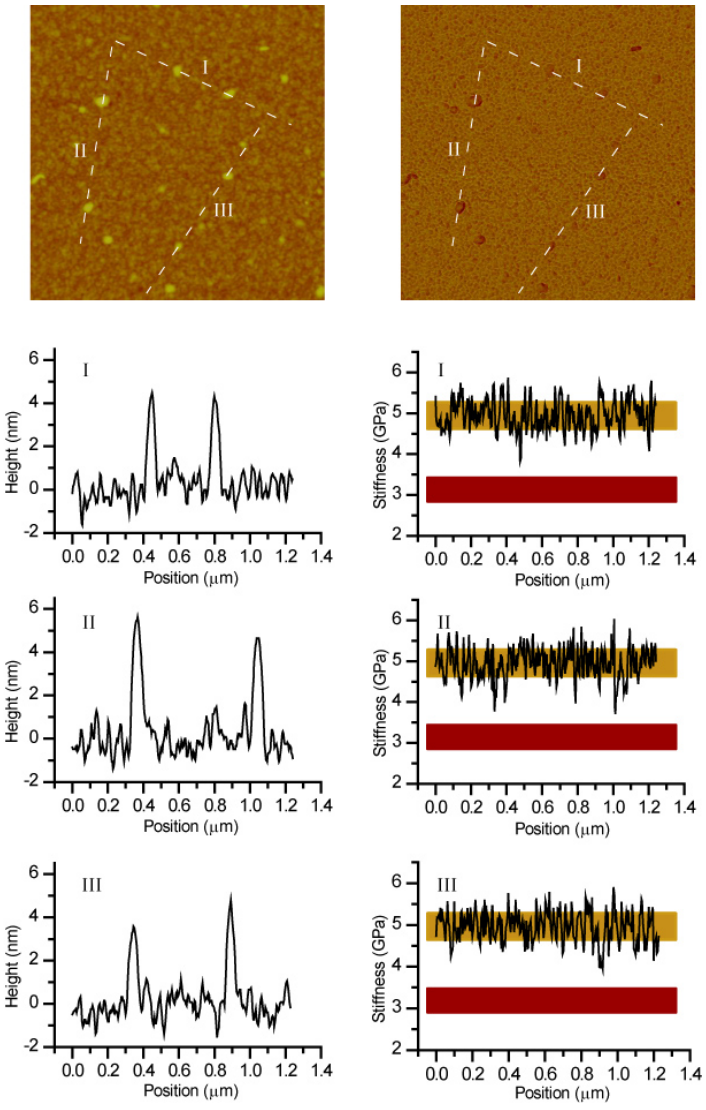
In our tapping-mode based imaging system, topography and stiffness maps are generated simultaneously. Figure S1 shows an example of topography and stiffness maps over the immobilized molecules on the microarray surface hybridized to 2 nM 16 base long target DNA. We see that height of the surface increases around the hybridized molecules approximately 2 to 8 nanometers. This suggests that hybridized molecules are not necessarily lying down on the surface.



Supplementary Figure 1: topography (left) and stiffness (right) maps of a hybridized microarray. Color bar represents 30 nm in the topography map and 1 decade of elastic modulus (1 GPa and 10 GPa) in the stiffness map.

Increased height of hybridized molecules provides a number of advantages in our measurements. AFM tip convolves with tall features on the surface. As a result hybridized molecules appear larger than their physical dimensions. Also note that non uniformity in the heights of hybridized molecules is reflected as a variation in spot sizes in the stiffness map. Height measurements provide auxiliary information that may be incorporated in the detection process. Although heights of hybridized molecules are not uniform, they cannot extend beyond the finite contour length of hybridized molecules. If a spot in the stiffness map has a corresponding height which is beyond a threshold level, that spot may not be counted as a hybridized DNA molecule. On the other hand, height

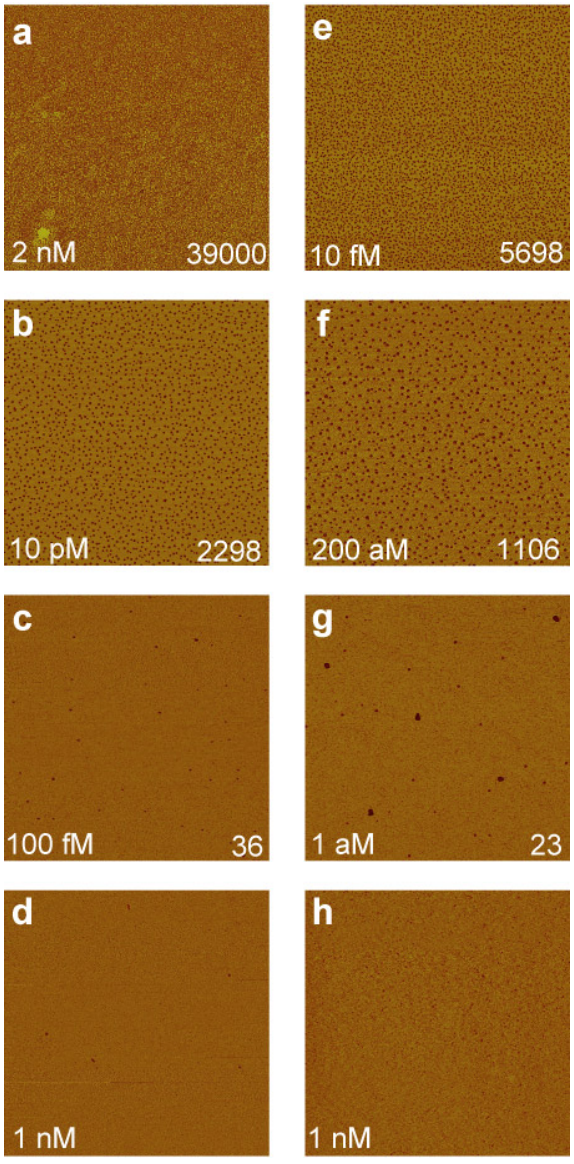
information alone does not provide specificity to judge whether a topographical feature is a hybridized DNA or not. In supplementary figure 2 below, we show height and stiffness maps of the surface of an unhybridized array surface (sequence 10). As seen in the line profiles, there are features with heights around 3 to 6 nanometers. The stiffness map shows that those features don't have the stiffness level of hybridized DNA reported in figure 1, which is marked with the red rectangle. In general, roughness of the substrates, non-specific adsorption, or secondary structures of probes can create height variations. Specific material information, which acts as intrinsic labels, is therefore needed to reliably identify hybridized molecules.



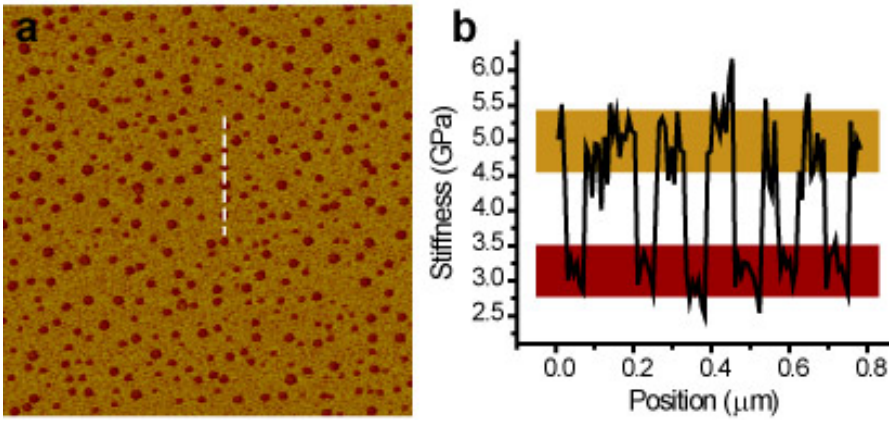
Supplementary Figure 2: topography (left) and stiffness (right) maps of an unhybridized microarray surface. Numerical values of height and stiffness across the dashed lines are given below the images. The red rectangles correspond to the stiffness levels of hybridized DNA. Note that 3 to 6 nm tall features seen in the topography map do not exhibit stiffness levels of hybridized DNA.

3. Stiffness maps at varying concentrations of target DNA

Supplementary Figure 3.
Stiffness maps with varying target concentrations and non-matching sequences. 5 x 5 mm (a-d) and 50 x 50 μm (e-h) immobilization areas are used to detect. Scan size is 10 μm. Target concentrations and the numbers of hybridized molecules are given on the bottom left and right corners of the corresponding images. Hybridizations against non-matching sequences are given in (d) and (h). Features larger than a threshold value (see section S2) are eliminated in the counts. For example 5 large features in figure (g) are not included in the reported counts. These excluded spots have heights larger than 40 nm.



4. Stiffness measurements on RNA/DNA hybrids.



Supplementary Figure 4. Nanomechanical detection of RNA/DNA hybridization. Stiffness map (a) and stiffness values across a section (b) show that RNA/DNA hybrids exhibit similar mechanical characteristics as hybridized DNA molecules. Scan size is 3 microns.

5. Averages and standard deviations of hybridized miRNAs measured at different amounts of total RNA

The measurements of miRNA content in tumor tissues for different amounts of total RNAs presented in Fig. 2 are carried out in triplicates. The numbers of hybridized spots counted on each sample are listed in the following table. Samples are visually inspected under optical microscope and those that appear contaminated or scratched are not analyzed. Standard deviations are estimated if two or more data points are available.

hsa-mir-205

Conc.	**Bladder**				**Colon**			
[ng/mL]	miR-205	Average	Std.dev	CV[%]	miR-205	Average	Std.dev	CV[%]
0.2	68				41			
0.2	59				48			
0.2	41	56.00	13.75	24.55	33	40.67	7.51	18.46
0.5	103				57			
0.5	92				78			
0.5	106	100.33	7.37	7.35	53	62.67	13.43	21.43
1	117				92			
1	191				81			
1	132	146.67	39.12	26.67	102	91.67	10.50	11.46
2	498				165			
2	392							
2		445.00	74.95	16.84		165.00		

hsa-mir-194

Conc.	**Bladder**				**Colon**			
[ng/mL]	miR-194	Average	Std.dev	CV[%]	miR-194	Average	Std.dev	CV[%]
0.2	5				24			
0.2	14				37			
0.2	17	12.00	6.24	52.04		30.50	9.19	30.14
0.5	46				68			
0.5	33				65			
0.5	48	42.33	8.14	19.24	48	60.33	10.79	17.88
1	61				112			
1	87				109			
1	58	68.67	15.95	23.22	67	96.00	25.16	26.21
2	132				185			
2	80				175			
2		106.00	36.77	34.69		180.00	7.07	3.93

6. Original high pixel density version of the stiffness map given in Fig. 3

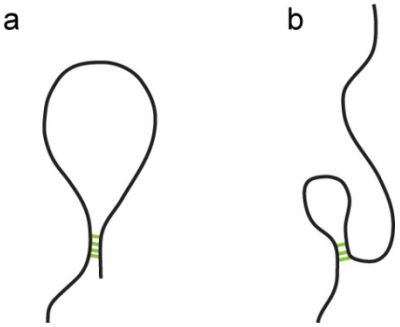
Supplementary Figure 5. The picture is given in a separate file due to high pixel density. It is linked to the online version of the paper.

7. Hybridization detection under sub-optimal conditions

Large sequence variety of DNA or RNA targets in genomic analysis and different laboratory practices in array hybridization creates a range of sub-optimal experimental conditions that may hinder the possibility of discriminating hybridized DNA molecules based on stiffness measurements. We have investigated several cases related to probes with secondary structures or quadruplex-DNA, probes with base pair mismatch, unusual base pairing or free termini, and also different hybridization temperatures. These sub-optimal cases also affect hybridization kinetics that may lead to reduced sensitivity; however, we were able to detect distinct and consistent stiffness values of the hybridized molecules. In the following sections we show stiffness data recorded under a variety of sub-optimal conditions.

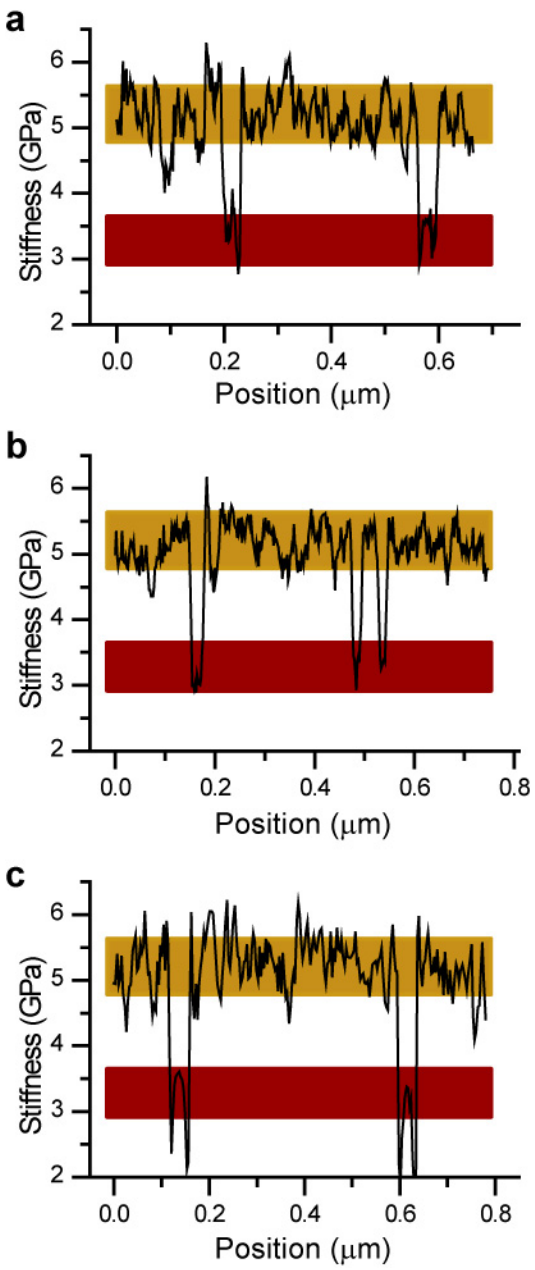
7. a- Probes with secondary structures and G-quadruplex DNA

Probe sequences that can form secondary structures are tested using the sequences investigated by Gao *et al*. [*Nucleic Acids Research* **34**, 3370-3377 (2006)]. The probe target pairs with sequences 10/11 and 12/13 have secondary structures sketched below



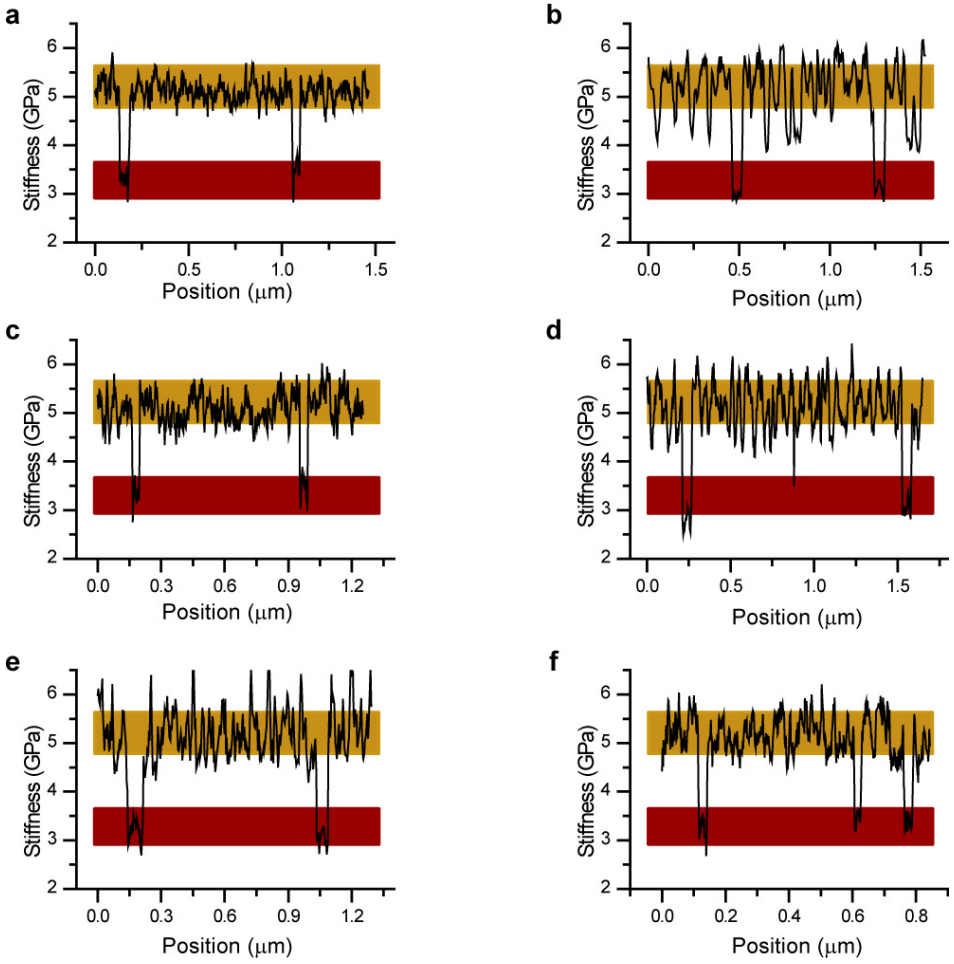
Here **a** represents the secondary structure formed by the complementary sequences 10/11, and **b** represents the secondary structures formed by the complementary sequences 12/13. Solution phase melting temperature of these structures under the hybridization conditions used are 60.5 °C and 46.8 °C. In addition, we have investigated a probe sequence forming G-quadruplex structures. We used the telomeric repeat $(TTAGGG)_4$, sequences 14/15. Zhao *et al.* [*J. Am. Chem. Soc.* **126**, 13255-13264 (2004)] showed that hybridization kinetics of this particular sequence, when immobilized on the surface, exhibits the characteristics of two surface states for the probes (i.e. folded or unfolded). Therefore, this sequence should be representative of probes with G-quadruplex structures.

The data in supplementary figure 6 show that hybridized molecules with these sequences are still detectible with stiffness values at ~ 3.2 GPa.



Supplementary Figure 6. Stiffness values across sections of hybridized array surfaces using probes with secondary structures (a, b), or G-quadruplex DNA (c).

7. b- Probes with mismatches, potential unusual base pairing, and free termini

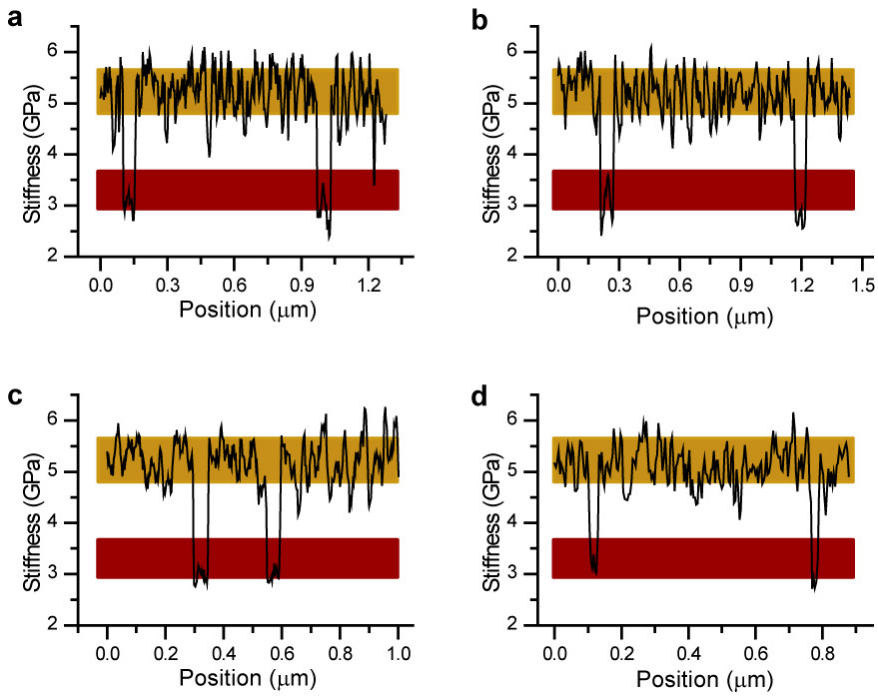


Supplementary figure 7. Stiffness values across sections of hybridized array surfaces using probes with single base pair mismatch (a, b), potentially unusual base pairing (c, d), or probes shorter than targets resulting in free termini (e, f).

We have tested the effectiveness of stiffness based detection of hybridized molecules in the presence of mismatches (sequences 16/17 and 18/19), potentially unusual base pairing, such as with edited RNAs (sequences 20/21 and 22/23), and also for the case of probes that are shorter than targets (sequences 24/25 and 26/27). The mismatches are placed either close to the center or near the 5' end of the probe. The positions of sequence mismatches are underlined in materials and methods section. Shorter probes testing free termini are selected such that the longer overhang is either extending from the 5' end or 3' end of the probe. The data in supplementary figure 7 show that hybridized molecules

with these sequences are detectible with stiffness values at ~ 3.2 GPa, similar to the other sequences tested in this work.

7. c- Compromised hybridization conditions



Supplementary Figure 8. Stiffness values across sections of array surfaces hybridized at different temperatures: (a) 25°C, (b) 36 °C, (c) 42 °C, and (d) 48 °C.

We have investigated the effect of hybridization temperature on the stiffness of hybridized duplexes. For this purpose we used probe sequences complementary to hsa-mir-205 and carried out hybridization with synthetic target miRNAs at four different temperatures between 25 and 48 °C. The data in the figure above shows that the stiffness values observed on the hybridized molecules are detectible in all four cases with stiffness around ~ 3.2 GPa. We note that temperature can affect hybridization efficiency and stability of secondary structures. Careful design of microarray hybridization experiments is essential to eliminate or minimize these effects.