Supporting Information For

Label-Free Nanoplasmonnic-Based Short Noncoding RNA Sensing at Attomolar Concentrations Allows for Quantitative and Highly Specific Assay of MicroRNA-10b in Biological Fluids and Circulating Exosomes

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S1. Nucleic Acid Sequences.

Table S1. Nucleic acid sequences used in this study

| strand | name        | sequence                  | MW (kDa) | modification |
|--------|-------------|---------------------------|----------|--------------|
| A      | ssDNA-10b   | 5'-CACAAATTCGGTTCTACAGGGA-3' | 7.1      | 5'thiol-C6   |
| B      | target miR-10b | 5'-UACCCUGUAGAACCAGAAUUGUG-3' | 7.0      | none         |
| C      | miR-16      | 5'-UAGCAGACGCUAUAUUGGCG-3' | 7.1      | none         |
| D      | miR-126     | 5'-CAUUAUUACUUUGUACGCG-3'  | 6.3      | none         |
| E      | miR-141     | 5'-UAACACUGUCUGUAAAGAUGG-3' | 6.7      | none         |
| F      | miR-122     | 5'-UGGAGUGUGACAAUGGUGUUUG-3' | 6.8      | none         |
| G      | miR-10a     | 5'-UACCCUGUAGAUCGAAUUGUG-3' | 6.9      | none         |

S2. Spectroscopy and Microscopy Characterization, and qRT-PCR Assay.

Absorption and extinction spectra in the range of 300-1100 nm were collected with a Varian Cary 50 Scan UV-visible spectrophotometer using 1 cm quartz cuvette. All the absorbance spectra were collected using 0.3 mL of reaction solution diluted in 2.0 mL of acetonitrile. Acetonitrile was used as a background for these measurements, and the background was run before collecting the absorbance spectra. All extinction spectra were measured in PBS buffer (pH 7.2) at room temperature unless otherwise specified. Here, the blank silanized glass coverslips immersed in PBS buffer were used as background, which was determined before collecting the extinction spectra. All AFM measurements were conducted in air utilizing tapping mode on a Bruker BioScope Catalyst with SSS-NCHR probes (Nanosensors) (tip radius ~2 nm). Images were collected using a tip velocity of 42 N/m over 1-2 uM scan sizes of three to five regions of each samples. All microscopy files were plain fitted and 2D fitted using Gwyddion. RNA was quantitated using the NanoDrop 2000 Spectrophotometer (Thermo) and samples were diluted to 3.0 ng/µL. Samples were converted to cDNA for miRNA-10b and RNU6B using RT primers (Life Technologies) and the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) according to manufacturer’s protocol. Analysis by qRT-PCR was performed using the ViiA 7 Real-Time PCR System (Life Technologies) and fold changes were obtained by normalizing to control, normoxia conditions for each cell line, using the model presented in Pfaffl, 2001. Scanning electron microscopy (SEM) micrograms were acquired using a JEOL-FESEM at 15 kV. The average edge lengths of the nanoprisms were determined from the SEM images using ImageJ software. Approximately 500 nanoprisms were counted to determine the average values. Transmission electron microscopy (TEM) images of the exosomes were collected using Tecnai G212 Bio Twin TEM microscope at 80 kV operating voltage. The TEM images were captured using AMT CCD camera.
S3. Silanization of Glass Coverslips and Attachment of Nanoprisms. The glass coverslips were silanized by MPTES for the chemical attachment of gold nanoprisms on the glass surface.\(^2\)\(^,\)\(^3\) The glass coverslips were incubated for 30 minutes in an aqueous solution of 20% (v/v) of RBS 35 detergent solution at 90° C followed by 5 minutes of sonication. The coverslips were completely rinsed with nanopure water and again immersed in a solution of concentrated hydrochloric acid and methanol (1:1 v/v) for another 30 minutes. After 30 minutes, the coverslips were thoroughly cleaned with nanopure water and placed overnight in a vacuum oven at 60° C. The next day, the coverslips were allowed to cool to room temperature and then incubated in 10% MPTES in anhydrous ethanol solution. After 30 minutes, the solution-covered coverslips were sonicated for 5 minutes, rinsed with anhydrous ethanol, and sonicated in anhydrous ethanol solution for 10 minutes. The rinsing step was repeated at least 5 times and then the silanized glass coverslips were baked in a vacuum oven for 3 hours. The MPTES-functionalized coverslips (substrate) were then incubated in a freshly prepared nanoprisms reaction solution for 30 minutes. The gold nanoprisms attached to the silanized glass coverslips were rinsed with ethanol, and dried under nitrogen flow. Finally, substrate-bound gold nanoprisms were further utilized for the development of LSPR-based miR sensor.

S4. Data Processing and Statistical Analysis. Calibration curves using commercially-obtained miR-10b were performed five times independently and all measurements were reported as mean ± standard deviation (σ). The maxima of UV-visible extinction spectra were used to determine the \(\lambda_{\text{LSPR}}\) and the \(\Delta\lambda_{\text{LSPR}}\) has been derived by taking the difference between the LSPR-based sensor’s responses before and after hybridization (\(\Delta\lambda_{\text{LSPR}}\)). The LODs were calculated by measuring the \(\Delta\lambda_{\text{LSPR}}\) for the blank (mixed -S-PEG6:-SC6-ssDNA-10b functionalized gold nanoprisms) and then obtained the Z (mean + 3σ) value. The Z value was converted into the relative concentration using the calibration curve. Briefly, the blank measurement was obtained as the \(\Delta\lambda_{\text{LSPR}}\) response for LSPR-based sensors after incubation in the respective media without any target miR-10b. Calibration curves constructed using commercially-obtained miR-10b in PBS buffer was used to determine the concentration of miR-10b in total RNA extracted from cell lines and exosomes. Culture media, sup-1, and sup-2 containing miR-10b was obtained using RPMI calibration (AsPC-1 and BxPC-3 cells) curves under hypoxia and normoxia conditions. Similar calibration curves were established using DMEM (PANC-1 cells). For patient plasma samples (PDAC, CP, and NC), and Sup-1 and Sup-2 samples, miR-10b concentration was obtained using the miR-10b calibration curve in human plasma. The miR-10b concentrations in total extracted RNA from exosomes isolated from patient plasma were calculated using the PBS buffer calibration curve.
**Table S2.** Calibration curve and the limit of detection (LOD) derived for human plasma supplemented with commercially-obtained miR-10b through LSPR-based sensor designed by using various edge lengths of gold nanoprisms.

| $\lambda_{\text{LSPR}}$ (nm)$^a$ | Edge length (S.D.)$^{b,c}$ | Equation from calibration curve | $R^2$ value | $Z$ value (nm) | LOD (fM) |
|-------------------------------|-----------------------------|-------------------------------|-------------|---------------|---------|
| 750                           | 34 (2.6)                    | $Y = 0.6271\ln(X)+8.6786$   | 0.97        | 2.44          | 47.5    |
| 800                           | 42 (3.5)                    | $Y = 0.5442\ln(X)+10.866$   | 0.97        | 2.04          | 0.091   |
| 820                           | 47 (4.9)                    | $Y = 0.5333\ln(X)+10.494$   | 0.94        | 1.80          | 0.083   |

$^a$The LSPR dipole peak position ($\lambda_{\text{LSPR}}$) of gold nanoprisms in acetonitrile. $^b$S.D. represents standard deviation. $^c$At least 500 nanoprisms from two different batches of synthesis were counted to determine the average edge-length.

**Table S3.** Calibration curve and the LODs derived with the commercially-obtained miR-10b through LSPR based sensor developed with ~42 nm edge length nanoprisms in different physiological media under various conditions.

| Physical media | Media condition | Equation from calibration curve | $R^2$ value | $Z$ value (nm) | LOD (aM) |
|----------------|-----------------|--------------------------------|-------------|---------------|---------|
| RPMI           | Hypoxia         | $Y = 0.4035\ln(X)+8.9509$     | 0.97        | 1.80          | 20.1    |
|                | Normoxia        | $Y = 0.4509\ln(X)+9.8236$     | 0.94        | 1.50          | 9.61    |
| DMEM           | Hypoxia         | $Y = 0.4086\ln(X)+8.9136$     | 0.96        | 1.80          | 27.5    |
|                | Normoxia        | $Y = 0.4169\ln(X)+9.2527$     | 0.94        | 1.80          | 17.2    |
| PBS buffer     |                 | $Y = 0.5105\ln(X)+10.599$    | 0.96        | 1.80          | 32.6    |
Table S4. \( \Delta \lambda \text{LSPR} \) responses from LSPR-based sensors, which were constructed with three different edge length of miR-10b in human plasma.

| Nanoprisms’s edge-length (nm) | miR-10b concentration (nM) | Average \( \Delta \lambda \text{LSPR} \) (nm) | Standard deviation (nm) |
|------------------------------|-----------------------------|--------------------------------|------------------------|
| 34                           | 10                          | 10.6                           | 1.3                    |
|                              | 1.0                         | 7.8                            | 1.1                    |
|                              | 0.1                         | 6.6                            | 0.5                    |
|                              | 0.01                        | 5.4                            | 0.5                    |
|                              | 0.001                       | 3.9                            | 0.5                    |
|                              | 0.0001                      | 3.2                            | 0.4                    |
|                              | 0.00001                     | 2.2                            | 0.5                    |
| 42                           | 10                          | 12.8                           | 1.5                    |
|                              | 1.0                         | 10.3                           | 1.4                    |
|                              | 0.1                         | 8.4                            | 0.8                    |
|                              | 0.01                        | 8.0                            | 1.1                    |
|                              | 0.001                       | 6.4                            | 1.0                    |
|                              | 0.0001                      | 5.8                            | 1.2                    |
|                              | 0.00001                     | 4.8                            | 0.8                    |
|                              | 0.000001                    | 3.9                            | 0.4                    |
|                              | 0.0000005                   | 3.5                            | 1.1                    |
|                              | 0.0000001                   | 2.0                            | 0.5                    |
| 47                           | 10                          | 12.0                           | 0.8                    |
|                              | 1.0                         | 9.8                            | 1.5                    |
|                              | 0.1                         | 7.8                            | 1.0                    |
|                              | 0.01                        | 7.3                            | 1.3                    |
|                              | 0.001                       | 6.0                            | 0.5                    |
|                              | 0.0001                      | 5.2                            | 0.8                    |
|                              | 0.00001                     | 4.4                            | 0.5                    |
|                              | 0.000001                    | 3.8                            | 0.5                    |
|                              | 0.0000005                   | 3.2                            | 0.5                    |
|                              | 0.0000001                   | 2.5                            | 0.6                    |
Table S5. ∆λ_{LSPR} responses from LSPR-based sensors, which were constructed with 42 nm edge-length gold nanoprisms in different physiological media.

| Type of media        | miR-10b concentration (nM) | Average ∆λ_{LSPR} (nm) | Standard deviation (nm) |
|----------------------|----------------------------|-------------------------|-------------------------|
|                      | 10                         | 11.5                    | 0.6                     |
| PBS buffer           | 1.0                        | 9.8                     | 0.3                     |
|                      | 0.1                        | 8.6                     | 0.5                     |
|                      | 0.01                       | 7.8                     | 0.4                     |
|                      | 0.001                      | 7.0                     | 0.5                     |
|                      | 0.0001                     | 5.9                     | 0.3                     |
|                      | 0.00001                    | 4.4                     | 0.5                     |
|                      | 0.000001                   | 3.5                     | 0.5                     |
|                      | 0.0000005                  | 2.6                     | 0.6                     |
|                      | 0.0000001                  | 1.9                     | 0.7                     |
| DMEM (hypoxia)       | 10                         | 9.6                     | 0.6                     |
|                      | 1.0                        | 8.1                     | 0.2                     |
|                      | 0.1                        | 7.8                     | 0.8                     |
|                      | 0.01                       | 6.8                     | 0.4                     |
|                      | 0.001                      | 5.6                     | 0.5                     |
|                      | 0.0001                     | 4.9                     | 0.4                     |
|                      | 0.00001                    | 4.0                     | 0.4                     |
|                      | 0.000001                   | 3.4                     | 0.3                     |
|                      | 0.0000005                  | 2.5                     | 0.3                     |
|                      | 0.0000001                  | 2.0                     | 0.2                     |
| RPMI (hypoxia)       | 10                         | 10.5                    | 1.3                     |
|                      | 1.0                        | 9.3                     | 1.0                     |
|                      | 0.1                        | 7.8                     | 0.5                     |
|                      | 0.01                       | 7.2                     | 0.9                     |
|                      | 0.001                      | 6.5                     | 0.6                     |
|                      | 0.0001                     | 5.2                     | 0.4                     |
|                      | 0.00001                    | 4.3                     | 0.4                     |
|                      | 0.000001                   | 3.7                     | 0.4                     |
|                      | 0.0000005                  | 2.9                     | 0.2                     |
|                      | 0.0000001                  | 2.4                     | 0.3                     |
Table S6. Determination of LSPR-based miR-10b concentration in different biological compartments of Sham transfected (to generate control cells) pancreatic cancer cells under hypoxic and normoxic conditions.

| Cell lines | Growth condition | Biological compartment | Concentration (fM) | Standard deviation (fM) |
|------------|------------------|------------------------|--------------------|-------------------------|
| ASPC-1     | Normoxia         | Media                  | 0.22               | 0.10                    |
|            |                   | Cells                  | 0.13               | 0.05                    |
|            |                   | Exosomes               | 0.91               | 0.40                    |
|            |                   | Sup-1                  | 0.12               | 0.08                    |
|            |                   | Sup-2                  | 0.02               | 0.01                    |
|            | Hypoxia           | Media                  | 0.62               | 0.20                    |
|            |                   | Cells                  | 10.35              | 0.80                    |
|            |                   | Exosomes               | 45.70              | 1.00                    |
|            |                   | Sup-1                  | 0.26               | 0.10                    |
|            |                   | Sup-2                  | 0.38               | 0.07                    |
| BxPC-3     | Normoxia         | Media                  | 0.61               | 0.20                    |
|            |                   | Cells                  | 0.05               | 0.02                    |
|            |                   | Exosomes               | 1.10               | 0.80                    |
|            |                   | Sup-1                  | 0.11               | 0.08                    |
|            |                   | Sup-2                  | 0.08               | 0.03                    |
|            | Hypoxia           | Media                  | 3.58               | 1.00                    |
|            |                   | Cells                  | 1.32               | 0.60                    |
|            |                   | Exosomes               | 1.94               | 0.50                    |
|            |                   | Sup-1                  | 0.20               | 0.07                    |
|            |                   | Sup-2                  | 0.10               | 0.04                    |
| PANC-1     | Normoxia         | Media                  | 0.29               | 0.02                    |
|            |                   | Cells                  | 0.05               | 0.03                    |
|                  | Exosomes |       | Sup-1 | 0.15 |       | Sup-2 | 0.03 | 0.05 |
|------------------|----------|-------|-------|------|-------|-------|------|------|
| Hypoxia          | Media    | 1.23  |       | 0.7  |       |       |      |      |
|                  | Cells    | 0.50  |       | 0.2  |       |       |      |      |
|                  | Exosomes | 3.25  |       | 0.7  |       |       |      |      |
|                  | Sup-1    | 0.33  |       | 0.1  |       |       |      |      |
|                  | Sup-2    | 0.1   |       | 0.06 |       |       |      |      |
Figure S1. UV-visible absorption spectra of gold nanoprismss dissolved in acetonitrile (solid lines) and after attachment onto silanized glass substrate and submersed in PBS buffer (dotted lines) for three different edge lengths: (34 nm: blue lines), (42 nm: red lines) and (47 nm: black lines).
Figure S2. (A) UV-visible extinction spectra of gold nanoprisms functionalized with 1.0 µM/1.0 µM ratio of -C6-ssDNA-10b/-S-PEG6 (blue solid) attached to silanized glass, after incubation with 10 nM of miR-10b (solid red), after treatment with 15 units of RNase H enzyme for 2h (blue dotted), and after rehybridization with 10 nM of miR-10b (dotted red). All extinction spectra were collected in PBS buffer. (B) Change in LSPR dipole peak position of gold nanoprisms functionalized with 1.0 µM/1.0 µM ratio of -C6-ssDNA-10b/-S-PEG6 upon hybridization and dehybridization for several cycles. After each hybridization and dehybridization steps the LSPR-based sensors were thoroughly rinsed with PBS buffer.

Discussion Related to Figure S2: The working principle of our LSPR-based sensor is that the attachment of miR-10b to nanoprism-bound –ssDNA-10b increases the local dielectric environment and modulates $\Delta \lambda_{\text{LSPR}}$. Therefore, we expect that the breaking of the –ssDNA:RNA duplex would allow us to recover the original sensor. To prove our hypothesis, the LSPR-based sensors containing –ssDNA:RNA duplexes were incubated with DNA:RNA cleaving enzyme RNase H solution for 2h, which resulted in blue-shift of the $\lambda_{\text{LSPR}}$ back to the position where it was observed before miR-10b incubation. A control experiment was conducted by incubating the LSPR-based sensor alone (without hybridized miR-10b) and enzyme RNase H solution and this combination, as expected, did not display any noticeable $\Delta \lambda_{\text{LSPR}}$. Incubation of the same LSPR-based sensor into 10 nM of miR-10b solution resulted in ~12 nm red-shifts of $\lambda_{\text{LSPR}}$. These experimental data demonstrate two important aspects of this sensing platform. First, the observed $\Delta \lambda_{\text{LSPR}}$ values mentioned above that occurred upon incubation of the LSPR-based sensor in miR-10b solution were due to DNA:RNA duplex formation. Second, the sensor can be readily regenerated. We further investigated the regeneration process, the miR-10b hybridization and enzymatic cleaving of the DNA:RNA duplex by repeating the sensing/cleaning procedures at least 5 times on the same LSPR-based sensor while monitoring the $\lambda_{\text{LSPR}}$ shift, which was nearly identical each time before and after hybridization and dehybridization of miR-10b. This experimental result is extremely important in the context of long-term stability of the sensors. The inert character of gold nanostructures towards actual biological constituents present in the human plasma as
well as strong gold-sulfur bond, which holds the –ssDNA-10b from detachment, likely provide the long-term stability of the sensors. These features will enhance the potential for developing these sensors into cost-effective point of care diagnostic tools. A controlled experiment was carried out parallel to this experiment where the LSPR-based sensors were incubated in an enzyme RNase H solution for 2 hours, but $\Delta \lambda_{\text{LSPR}}$ was negligible.

**Figure S3.** Extinction spectra of sensing platform constructed with mixed -SC6-ssDNA-10b/-S-PEG6 (red) and after incubation in human plasma solution containing 10 nM of each of miR-16, miR-122, miR-126, and miR-141, washed with PBS buffer (blue). All extinction spectra were measured in PBS buffer to determine the $\lambda_{\text{LSPR}}$.

**Discussion Related to Figure S3:** Because of the presence of hundreds of miRs in real biological samples, it is extremely important to analyze the specificity of the sensors. The LSPR-based miR-10b sensors were incubated for 12 h in normal human plasma solution containing 10 nM each of miR-16, miR-122, miR-126, and miR-141 because these miRs are normally present in human plasma. A very small (1.7 ± 0.2 nm) $\lambda_{\text{LSPR}}$ red-shift was observed upon incubation in mixed miRs, whereas 10 nM pure miR-10b incubation in human plasma resulted in ~12 nm $\lambda_{\text{LSPR}}$ red-shifts (data not shown). These experimental data unequivocally demonstrate that the LSPR-based sensors,
which contain the antisense -ss-DNA-10b attached to the gold nanoprism are highly specific towards their target miRs, which in this instance miR-10b.

**Figure S4.** Average $\lambda_{\text{LSPR}}$ shift ($\Delta\lambda_{\text{LSPR}}$) of the sensing platforms, which were prepared with 42 nm edge-length gold nanoprisms as a function of miR-10b concentration in PBS buffer (A), DMEM (B) under normoxia (blue diamonds) and hypoxia (red squares), and RPMI (C) under normoxia (blue dots) and hypoxia (red triangles).
Figure S5. Extinction spectra of the sensing platform constructed with mixed -SC6-ssDNA-10a/-S-PEG6 (blue) and after incubation in human plasma solution containing leftover miR-10a (red) resulted in ~6 nm $\lambda_{\text{LSPR}}$ red-shifts. Insert shows the expanded version near to the LSPR dipole peak of the gold nanoprisms.
### Table S7. Statistical analysis for analysis performed on patient samples.

| Fraction | Measurement                | Comparison     | p-value\(^a\) |
|----------|----------------------------|----------------|---------------|
| Plasma   | Concentration by LSPR      | CP v. PDAC     | <0.02         |
| Plasma   | Concentration by LSPR      | CP v. NC       | <0.001        |
| Plasma   | Concentration by LSPR      | PDAC v. NC     | <0.005        |
| Exosomes | Concentration by LSPR      | CP v. PDAC     | <0.01         |
| Exosomes | Concentration by LSPR      | CP v. NC       | <0.003        |
| Exosomes | Concentration by LSPR      | PDAC v. NC     | <0.005        |
| Exosomes | qRT-PCR                    | CP v. PDAC     | <0.005        |
| Exosomes | qRT-PCR                    | PDAC v. NC     | <0.005        |
| Sup-1    | qRT-PCR                    | CP v. PDAC     | <0.02         |
| Sup-1    | qRT-PCR                    | PDAC v. NC     | <0.008        |

\(^a\)One-way ANOVA followed by Bonferroni adjustment was performed for each fraction shown in Figure 8, based on diagnosis and by comparison with normal values where indicated (Figure 8). The above table provides the p values for all statistically significant differences. There were significant differences between patients with CP, PDAC, and normal control (NC) for Sup-1 and Sup-2.

**References:**

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