# Quantitative and multiplex microRNA assays from unprocessed cells in isolated nanoliter well arrays

# SUPPLEMENTARY INFORMATION

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### **Reagents and materials**

Polyethylene glycol diacrylate (PEGDA) 700, polyethylene glycol (PEG) 200, PEG 400, 2-Hydroxy-2methylpropiophenone (Darocur® 1173), 100x TE (Tris-ethylenediaminetetraacetic acid, EDTA) buffer, Tween® 20, 3-(trimethoxysilyl)propyl methacrylate, acetic acid, sodium dodecyl sulfate (SDS), and sodium chloride (NaCl) were purchased from Sigma. Nuclease free water was purchased from Millipore. Methanol was acquired from Macron. 1x phosphate-buffered saline (PBS, 10 mM PO<sub>4</sub><sup>3-</sup>, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) was bought from Corning. Tris-HCl (pH 8.8) buffer was obtained from Teknova. NEB2 buffer, T4 DNA ligase, adenosine triphosphate (ATP), and Proteinase K were ordered from New England Biolabs. DNA ligase was stored in 10 mM Tris-HCl, 50 mM KCl, 1 mM 1,4-dithiothreitol, 0.1 mM EDTA, 50% (v/v) glycerol (pH 7.4 @ 25°C). Proteinase K was stored in 20 mM Tris-HCl, 50% (v/v) glycerol 1 mM CaCl<sub>2</sub> (pH 7.4 @ 25°C). Nucleic acids including DNA probes (Table S1), DNA linker, and miRNA targets (Table S2), were acquired from Integrated DNA Technologies. Nucleic acids were stored at -20°C in 1x TE. The biotinylated linker had a 5' phosphorylation modification, a 3' biotin modification, and a 5' to 3' sequence of TAA AAT ATA TAA AAA AAA AAA A. Buffer compositions are listed in Table S3.

probe name	5' modification	sequence 5' to 3'	3'	concentration
			modification	
miR-21-5p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAT CAA CAT CAG TCT GAT AAG CTA	inverted dT	247 μM
let-7a-5p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAA ACT ATA CAA CCT ACT ACC TCA	inverted dT	50 µM
miR-210-3p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAT CAG CCG CTG TCA CAC GCA CAG	inverted dT	100 µM
miR-155-5p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAA CCC CTA TCA CGA TTA GCA TTA A	inverted dT	100 µM
cel-miR-54-3p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAC TCG GAT TAT GAA GAT TAC GGG TA	inverted dT	100 µM
cel-miR-238-3p probe	Acrydite <sup>TM</sup>	GAT ATA TTT TAT CTG AAT GGC ATC GGA GTA CAA A	inverted dT	100 µM
biotinylated probe	Acrydite <sup>TM</sup>	ATA GCA GAT CAG CAG CCA GA	biotin	10 µM

Table S1. DNA probe sequences, modifications, and concentration used in prepolymer solution.

Table S2. Synthetic miRNA synthetic targets.

target name	sequence 5' to 3'
miR-21-5p target	UAG CUU AUC AGA CUG AUG UUG A
let-7a-5p target	UGA GGU AGU AGG UUG UAU AGU U
miR-210-3p target	CUG UGC GUG UGA CAG CGG CUG A
miR-155-5p target	UUA AUG CUA AUC GUG AUA GGG GU
cel-miR-54-3p target	UA CCC GUA AUC UUC AUA AUC CGA G

Table 55. Burler Compositions.					
name	composition				
1x TE	10 mM Tris-HCL, 1 mM EDTA (pH 8.0 @ 25 °C)				
1x TET	1x TE, 0.05% (v/v) Tween® 20				
1x PTET	50 mM Tris-HCL, 5 mM EDTA, 25% (v/v) PEG400, 0.05% (v/v) Tween® 20 (pH 8.0 @ 25°C)				
1x R50 rinse buffer	1x TET, 50 mM NaCl				
NEB2 buffer	10 mM Tris-HCl, 50 mM NaCl, 10mM MgCl <sub>2</sub> , 1 mM DTT (pH 7.9 @ 25 °C)				
settling buffer	1x TE, 137 mM NaCl				
lysis buffer	1x TE, 0.2% (v/v) Tween® 20, 989 mM NaCl, 8% (w/v) SDS, 64 U/mL proteinase K				

Table S3. Buffer Compositions.

#### Well array fabrication

Glass slides were acrylated using standard methods<sup>1,2</sup>. Clean slides were dipped in a one part 3-(trimethoxysilyl)propyl methacrylate, three parts acetic acid, and five parts deionized (DI) water solution for 30 min at room temperature. Slides were then rinsed with methanol, then DI water, and finally dried with argon gas. Device schematics were designed in AutoCAD 2017 (Autodesk). The well arrays with 30 µm wells had the wells arranged in tight-packed configuration with 30 µm as the minimum spacing between wells. The well arrays with  $300 \,\mu\text{m}$  wells had the wells arranged in a square grid with  $300 \,\mu\text{m}$  or  $50 \,\mu\text{m}$  as the minimum spacing between wells or in a tight-packed configuration with 30 µm as the minimum spacing between wells (Figure S1). SU-8 wafer molds were fabricated using standard methods and feature heights were measured with a profiler (Veeco, Dektak 150). Devices with wells in tight-packed configurations had heights of 38.6 µm and devices with wells in square grid configurations had heights of 35.0 µm. The SU-8 molds were used to make polydimethylsiloxane (PDMS) molds using standard soft lithography protocols<sup>3</sup>. The PDMS molds were used to fabricate the well arrays on acrylated glass slides using degas-driven flow<sup>4,5</sup> (Figure S2). Clean PDMS molds were placed on the acrylated glass slides and then the assembly was degassed under vacuum for 1 hour. Immediately following removal from vacuum, 10 µL of Norland Optical Adhesive 81 (NOA81) well arrays was applied over the inlet of the PDMS mold to load NOA81 via degas-driven flow. NOA81 was allowed to fill the mold for 15 min (shielded from light), excess NOA81 was removed from on top of the inlet, and then devices were cured for 6 min using UV light (Black-Ray ® UV Bench Lap, UVP). After curing, molds were peeled off from the well arrays and any excess NOA81 cured in the well inlet was removed using a scalpel.



Figure S1. Schematics of well array geometries. Tight packed configuration of (A) 30  $\mu$ m wells with 30  $\mu$ m minimum well spacing and (B) 300  $\mu$ m wells with 30  $\mu$ m minimum well spacing. Square grid configuration of (C) 300  $\mu$ m wells with 50  $\mu$ m minimum well spacing and (D) 300  $\mu$ m wells with 300  $\mu$ m minimum well spacing. The well arrays contained index marks that substituted wells (not shown in schematics). Scale bar is 300  $\mu$ m.



Figure S2. Well array fabrication protocol.

# Hydrogel post fabrication

Prepolymer solutions containing 20% (v/v) PEGDA 700, 40% (v/v) PEG 200, 5 % (v/v) Darocur<sup>TM</sup> photoinitiator (2-hydroxy-2-methylpropiophenone), and 35% (v/v) 3x TE buffer were mixed with solutions containing DNA probes (9 parts prepolymer solution, 1 part DNA probe solution) to obtain the final desired concentrations. NOA81 300 µm well arrays were placed in DI water and degassed for 1 min in a bath sonicator (Branson 2800) to remove any air trapped in the wells. The devices were then placed with the well array facing down over 508 µm spacers formed using 3 layers of polyvinyl chloride electrical tape (Del City) attached to a glass plate. 1x TET buffer was introduced between the well array and the glass plate. The 1x TET in the wells was then exchanged for the prepolvmer solution containing DNA probes. The exchange was done by first aspirating excess 1x TET using vacuum without drying the wells. Then, 5  $\mu$ L of prepolymer was applied directly onto the well array and spread back and forth with pipette tip for 1 min. Excess solution was then aspirated without drying the wells. The loading, spreading, and aspiration process was repeated 3 times. Finally, 5 uL of prepolymer solution was applied onto the wells and a flat PDMS film was applied on top of the array. The flat PDMS films were formed by curing PDMS a 1-2 mm layer of PDMS on a clean polystyrene dish (Falcon). The cured PDMS was then cut into 1.5 x 1.5 mm squares. The flexibility of the PDMS film helped prevent bubble trapping when applied to well array. Hydrogel posts were photopolymerized within the wells as described in the main text. Post location alignment was done using printed acetate transparencies attached to the monitor of the imaging scope computer. Posts  $\leq 40 \,\mu m$  were photopolymerized using exposure times of 200 ms and posts > 40 µm were made using exposure times of 100 ms. Following post photopolvmerization, the PDMS film was removed and devices were rinsed by flowing 1x TET over the well array, then placing the device with the wells facing down on the spacers and rinsing 3 times with 1x TET by iterative loading and purging of the solution between the glass plate and the well array without drying the wells. For devices with multiple posts made with different compositions, the prepolymer solution loading, post photopolymerization, and rinsing steps were sequentially repeated for each composition. For hydrogel post treatment with potassium permanganate, the well arrays on the spacers were continuously rinsed by loading and purging the potassium permanganate in 0.1 M Tris-HCl (pH 8.8) solution for 3 min. Devices were then rinsed with 1x TET.

# miRNA assays in isolated well arrays

For the assays with synthetic microRNA, the 30  $\mu$ m well array top layer was rinsed with DI water and sonicated for 1 min. The array was then placed on spacers and 250  $\mu$ L 1x TET was loaded. The 1x TET was then exchanged for hybridization buffer containing the desired concentration of target miRNA. The 300  $\mu$ m well array bottom layer with the hydrogel posts was kept in 1x TET in the spacer chamber. Similarly, the 1x TET was exchanged for hybridization buffer. Both layers were removed from the spacers and 20  $\mu$ L of their respective solutions was placed on top of each array to prevent the wells from drying. The devices were then sandwiched together and immediately sealed using magnets (Figure S3). To load targets into the post containing layer before sandwich assembly, 20  $\mu$ L of the solution containing target was applied directly to the array and spread back and forth with pipette tip for 5 seconds before assembling the sandwich and sealing with magnets. For experiments in which the same miRNA mass was delivered to wells with different number or sizes of posts, miRNA target was only included in the top layer. Sealed devices were placed in a polystyrene dish containing 500  $\mu$ L of DI water to reduce evaporation and heated to 55 °C for 90 min.

Following hybridization, the magnets were removed and the device was rinsed by flowing 1x R50 buffer over the well array, then placing the device face down on the spacers and rinsing 3 times with 1x R50 by iterative loading and purging of the solution between the glass plate and the well array without drying the wells. The ligation buffer was prepared fresh and contained 100  $\mu$ L of NEB2 buffer, 25  $\mu$ L of 10 mM ATP, 869  $\mu$ L of 1x TET, 4  $\mu$ L of 10  $\mu$ M A10 universal adapter with biotinylated A12 spacer (biotinylated linker) in 1x TE, and 2  $\mu$ L of 400 units/mL T4 DNA ligase solution. Following ligation, the well array was washed by rinsing 3 times with 1x R50 by iterative loading and purging of the solution between the glass plate and the well array without drying the wells. Then, the labeling step was done by loading 1x R50 buffer containing 10  $\mu$ g/mL streptavidin-R-phycoerythrin (SA-PE, Invitrogen) and incubating for 1 hour at room temperature. Labeling time and SA-PE concentration were experimentally

determined (Figure S4). Labeling time and SA-PE concentration were increased from the particle-based assay<sup>6</sup> because labeling in the well array platform was done using just diffusion without any convective mixing. Following labeling, the well array was washed 3 times with 1x R50 as described earlier and then incubated 1x R50 at room temperature for  $\geq 1$  hour prior to imaging. For imaging, the solution in the well arrays was exchanged to 1x PTET by removing the array from the spacers, applying 20 µL of 1x PTET on the array, spreading back and forth with a pipette tip for 5 seconds and then covering with a micro cover glass. Imaging was done in 1x PTET to match conditions used in prior work<sup>6–8</sup>. After completion of the assay, the devices can be stored in 1x R50 buffer for a few days at 4°C before imaging. Longer term stability of the SA-PE fluorophores and bound molecules has not been characterized. All surfaces were kept nuclease free by cleaning them with RNAseZap<sup>TM</sup> and RNAse AWAY<sup>TM</sup> (ThermoFisher) and then rinsing with DI water prior to the assay. Devices can be reused by incubating devices in 1x TET at 70°C for 1 hour in order to remove bound miRNA.



Figure S3. miRNA assay in isolated well arrays protocol. The device consists of two well arrays made from NOA81 on acrylated glass slides. The top array has wells with 30  $\mu$ m diameters. The bottom array has wells with 300  $\mu$ m diameters and contains PEGDA posts functionalized with DNA probes complimentary to miRNA targets. Step 1. The two arrays are sandwiched together and sealed using magnets to isolate each 300  $\mu$ m well as a separate reactor. Hybridization proceeds for 90 min at 55 °C and free miRNA in each reactor is captured by the DNA-probes in the PEGDA posts. Step 2. The magnets are removed and the sandwich is opened. The bottom array is washed with 1x R50 buffer. Step 3. 1x R50 buffer is exchanged for ligation solution. Ligation proceeds for 60 min at room temperature and biotinylated linkers are ligated to the captured miRNA targets. Step 4. The bottom array is washed with 1x R50 buffer. Step 5. 1x R50 buffer is exchanged for labeling solution which contains SA-PE. Labeling proceeds for 60 min at room temperature and SA-PE binds to the biotin in the biotinylated linkers. Step 6. The bottom array is washed with 1x R50 buffer prior to imaging.



Figure S4. SA-PE concentration optimization. (A) Composite brightfield and fluorescence images of 80  $\mu$ m posts with biotinylated probes in 300  $\mu$ m wells after incubating in 1x R50 buffer with 2  $\mu$ g/mL for 45 min (left) and (right) 10  $\mu$ g/mL for 60 min. Arrays were rinsed with 1x R50 buffer prior to imaging, as previously described. Images were captured through a 10x objective. (C) Profile plots were obtained using a 30 pixel high, 100 pixel wide window from the top center post in each image.. Scale bar is 100  $\mu$ m.

#### **Reactor volume estimation**

The volume of each reactor in the well array sandwiches was statistically estimated. Because the well array sandwiches were assembled manually without any alignment, different numbers of 30 µm wells overlapped with each 300 µm well, which resulted in some variability in the expected reactor volumes. We estimated the number of 30 µm wells that overlapped with each 300 µm well using AutoCAD. We randomly placed an array of 30 µm wells over a single 300 µm and counted the number of wells that were overlapping. The average number of 30 µm wells in contact with a 300 µm well was  $27.5 \pm 1.6$  (standard error of 0.29 for n = 30 trials). Therefore, the average total volume of the wells top layer that overlap with a single 300 µm well in the bottom layer was ~0.8 nL and the total volume of a sealed reactor in the array sandwich was ~3.2 nL. Thus, 5.7% and 1.3% of the observed well to well in fluorescence signal variability after a binding assay can be attributed to differences in the analyte mass delivered because of alignment when analytes are included in the top layer only before assembly and when analytes are included in both layers before assembling, respectively. For simplicity, we ignored the indexing marks in the well arrays and also ignored the volume occupied by PEGDA posts.

### Assay performance

In our well based system we did not utilize convective mixing to enhance target delivery to the PEGDA hydrogels, therefore we assumed mass transport occurs mainly via diffusion. As an approximation, we modeled our reactors as a cylinder with a cylindrical concentric post at its center. The diameter of each reactor was  $L_W = 300 \,\mu\text{m}$ , determined by larger wells in the bottom layer. The diameter of the hydrogel posts was  $L_P = 40 \,\mu\text{m}$ . The height of the cylindrical reactor was set by the height of the combined layers ( $H_W = 35.0 \,\mu\text{m} + 38.6 \,\mu\text{m} = 71.8 \,\mu\text{m}$ ). The height of the post was approximated as the height of the bottom layer ( $H_P = 35.0 \,\mu\text{m}$ ). The diffusivity of miRNA outside of the post was assumed to be the diffusivity of miRNA in free solution,<sup>9,10</sup> with diffusion coefficient  $D_W \sim 10^{-10} \,\text{m}^2 \text{s}^{-1}$ . The diffusivity of miRNA within the hydrogel posts<sup>11</sup> was set as  $D_P \sim 10^{-11} \,\text{m}^2 \text{s}^{-1}$ . For simplicity, using axial symmetry we estimated the total mass transport time as the addition of radial and axial diffusion times inside and outside of the posts. The total diffusion time is thus given by  $t_D \sim \left(\frac{1}{2}L_W - \frac{1}{2}L_P\right)^2 / D_W + (H_W - H_P)^2 / D_W + \left(\frac{1}{2}L_P\right)^2 / D_P + (H_P)^2 / D_P \sim 350 \,\text{s}$ . Because of the high probe incorporation in the PEGDA hydrogels, the assay is mass transport limited with Damköhler numbers Da >> 1.<sup>11</sup> From equation (1) in the main text,  $\tau$  is the time constant describing the timescale of capturing all of the targets in the reaction volume. Given that assay times are dominated by mass transport times,  $\tau \sim t_D$ .

In equation (1) in the main text, note that because we measure the fluorescence intensity *I* using wide-field imaging,  $A_p$  is the 2D area of the probe-containing region of particles or posts measured during imaging. Because our particles and posts are short (<40 µm), we assume that we measure the fluorescence across the entire height of the posts or particles in the 2D image captured, and thus the measured fluorescence intensity *I* does not depend on particle or post height. Additionally, because we report mean fluorescence intensity averaged across the 2D area, we did not need to assume that fluorescence was uniformly distributed across the particle. Thus, while the total particle and post area exposed to solution will affect the binding kinetics, the measured equilibrium fluorescence  $I_{max}$ , depends on the 2D area imaged.

# Multiplex miRNA assays with synthetic targets

condition	1	2	3	4
number of wells per condition (n)	4	12	11	9
post	mass/well (amol)			
cel-miR-238	0	0	0	0
cel-miR-54	0.5	0.5	0.5	0.5
miR-21	0	5	0.5	0.05
let-7a	0.05	0	5	0.5
miR-210	0.5	0.05	0	5
miR-155	5	0.5	0.05	0

Table S4. miRNA target concentrations used in multiplex assay with synthetic targets (Figure 6).

The synthetic miRNA target masses loaded to each well for the multiplex assays are listed in Table S4. Net mean signal was calculated by subtracting the mean signal of the *cel*-miR-238 (negative control) post from the mean signal of each other post in each well and then normalizing by the average net mean signal from the *cel*-miR-54 post (positive control). Note that treating PEGDA posts with potassium permanganate resulted in lower signals for negative controls, meaning less non-specific binding of SA-PE.

# Imaging and image analysis

Device readout can be done with any adequately-equipped standard fluorescence microscope or slide-scanner. The X-Cite 120LED light source from Lumen is remarkably stable and did not require routine calibration. Fluorescence micrographs for quantitative analysis were captured using the 20x objective, unless otherwise specified. Devices with settled cells were imaged using the 5x objective and cells per well were counted manually in ImageJ. Images were analyzed using custom ImageJ and MATLAB scripts. Using ImageJ, the mean fluorescence signal was calculated using by calculating the mean intensity using a window placed over the regions of interest. Background subtracted intensities were calculated by subtracting the mean intensity calculated from a window placed outside of the region of interest. Using MATLAB, 100-200 pixel windows were placed around the regions of interest and the background signal was calculated from the edge region of these windows. The background subtracted mean fluorescence signal was calculated by adding pixel intensities above a threshold of mean background signal plus 3 times the standard deviation (SD) of the background, then subtracting the mean background intensity from each of those pixels, and then dividing the total background subtracted signal by the measured pixel area of each post. Pixel to µm conversion factors were calculated using a Graticules stage micrometer. The MATLAB script was employed to analyze images of posts with non-uniform fluorescence and of varying shapes and sizes. Net mean signals were calculated by subtracting the background subtracted mean signal from negative controls from the background subtracted mean signal of the data points of interest. The lower limit of detection (LLOD) of the assay was determined using previously established methods<sup>7</sup>. The noise of the assay was defined as the SD of the negative controls. The LLOD was determined from the extrapolated value obtained from a linear fit of the net signal vs. miRNA target amount plotted in log-log axes that would be equal to a net signal to noise ratio (SNR) of 3.

# **Enzymatic amplification assays**

Streptavidin- $\beta$ -galactosidase conjugates (SAB, Invitrogen) were bound to 200 µm circular posts containing biotinylated DNA probes in a (see SI). The 300 µm well well arrays were then sealed against a 30 µm well array containing fluorescein di- $\beta$ -D-galactopyranoside (FDG, Thermo Fisher) substrate dissolved in 1x PBST buffer containing 0.2% (v/v) dimethylsulfoxide (DMSO). High viscosity ethyl cyanoacrylate adhesive (World Precision Instruments) was applied around the glass slides of the array sandwich to keep the device sealed after the removal of the magnets for imaging.

The 200  $\mu$ m circular posts were photopolymerized in 300  $\mu$ m wells in the bottom layer using 100 ms exposure times. Devices were rinsed with PEG200, then 3 times with 1x TET, and then stored in 1x TET overnight at 4 °C. Devices were then rinsed with 1x PBS with 0.05% (v/v) Tween 20 and incubated with 1  $\mu$ g/mL<sup>1,12</sup> streptavidin- $\beta$ -galactosidase conjugates (SAB) in 1x PBS with 0.05% (v/v) Tween 20 for 1 hour at room temperature. Devices were then rinsed 3 times with 1x PBS with 0.05% (v/v) Tween 20 and incubated in 1x PBS with 0.05% (v/v) Tween 20 for 1 hour at room temperature. Devices were then rinsed 3 times with 1x PBS with 0.05% (v/v) Tween 20 and incubated in 1x PBS with 0.05% (v/v) Tween 20 for 1 hour. 200  $\mu$ M of fluorescein di- $\beta$ -D-galactopyranoside (FDG) dissolved in 1x PBST buffer containing 0.2% (v/v) dimethylsulfoxide (DMSO) was loaded into the 30  $\mu$ m wells in the top layer array. Before sandwich assembly, 5  $\mu$ L of the FDG solution was added to the top layer and excess solution was removed from the bottom layer.

The images in Figure 1B and Figure 1C were captured using 10x and 5x objectives, respectively. Note that in our results shown in the main text the signal in the wells without functionalized posts was higher than the background signal of the substrate if the arrays were not incubated with SAB (Figure 2C). We determined that there is some non-specific sticking of SAB to the NOA arrays that leads to this higher signal (data not shown).

#### **Fluorescence** calibration

We constructed a calibration curve of SA-PE fluorescence by loading free SA-PE in well arrays and then imaging using the same conditions as used in quantitative assay measurements (Figure S5). SA-PE was loaded in 1x PTET buffer in well arrays covered with a microcover class (Figure S5A). The fluorescence intensity in each well  $(I_{well})$ was measured using 150 pixel (90  $\mu$ m) circular windows placed over each well. The background signal ( $I_{ba}$ ) was measured by placing the window outside of the wells. The net mean fluorescence for a given SA-PE concentration [X] was calculated by  $I_{net} = (I_{well}[X] - I_{bg}[X]) - (I_{well}[0] - I_{bg}[0])$ , which accounted for background fluorescence arising from excess fluid above the 35 µm deep wells and the NOA array itself. The net mean fluorescence was measured for 0, 0.0001, 0.001, and 0.01 mg/mL concentrations of SA-PE (n = 4, 7, 7, and 6 wells, respectively). Then, the net mean fluorescence for each condition was converted to net mean fluorescence\*area. The area of the 150 pixel window was converted to µm using the pixel density of images captured through a 20x objective in our imaging setup (0.6 µm/pixel). The total SA-PE mass in this 150 pixel window was calculated using the concentration of SA-PE, the volume of the window, and the molecular weight of SA-PE. The molecular weight for SA-PE was estimated as 292.8 kDA using the molecular weight values for streptavidin and Rhodophyta Phycoerythrin listed in the vendor website. By plotting net mean fluorescence\*area vs. mass and calculating the slope of a linear fit we estimated the fluorescence efficiency constant ( $F_e$ ) of our system as  $F_e \sim 1*10^6$  AFU  $\mu m^2$ amol<sup>-1</sup> (Figure S5B). We then estimated the expected net mean fluorescence vs. mass for SA-PE binding to 40  $\mu$ m posts (66 pixels) by dividing net mean fluorescence\*area by the 2D projected area of the 40 µm posts (Figure S5C, Figure 2F). We assumed complete binding of the loaded SA-PE. The linear fit was obtained by finding the fit of log-transformed data.



Figure S5. SA-PE fluorescence calibration curve. (A) Fluorescence micrographs (at different contrasts to aide viewing across multiple orders of magnitude) of free SA-PE loaded into wells. From left to right: 0, 0.0001, 0.001, and 0.01 mg/mL SA-PE. (B) Plot of net mean fluorescence\*area vs. mass. (C) Plot of expected net mean fluorescence vs. mass for SA-PE binding in devices with wells containing 40  $\mu$ m square posts. Dashed lines indicate linear fit. Error bars represent ± SD (n ≥ 4 wells for each condition). Scale bar is 100  $\mu$ m.

#### Probe incorporation in posts of different sizes

In order to determine that posts of different sizes had similar probe incorporation efficiencies, we photopolymerized square posts of different area ( $A_P$ ) functionalized with biotinylated DNA probes within 300 µm wells and incubated the array with a solution containing SA-PE, as described previously. The resulting mean fluorescence of the posts was measured in ImageJ using square windows from images captured through a 10x objective (Figure S6). We performed ANOVA to determine if there was a statistically significant difference in mean signal between the posts of different area. We obtained an F value = 1.08, which is lower than 3.86, which is the critical value for a critical

significance level ( $\alpha$ : alpha) = 0.05. Thus, we cannot reject the null hypothesis and conclude that there was no statistically significant difference in fluorescence signal between posts of different area.



Figure S6. SA-PE Binding in posts of different area ( $A_P$ ). (A) Brightfield and fluorescence (at same contrast) composites following SA-PE binding assay of well arrays with (from left to right) 5300, 1700, 900, and 300  $\mu$ m<sup>2</sup> posts. (B) Mean fluorescence for posts of each area. Error bars represent ± SD (n = 6 wells for each condition). Scale bar is 100  $\mu$ m.

### Cell handling and miRNA assays from cells in well arrays

Frozen Calu-6 cells were stored in 100  $\mu$ L aliquots with 100k cells and were reconstituted by first adding 900  $\mu$ L of media and warming to 37 °C in a water bath (myBath, Benchmark). Cells were then centrifuged for 2 min at 6 g. Supernatant was then aspirated leaving the cell pellet which was then resuspended in media. The centrifugation and resuspension in media step was then repeated one more time and then cells were kept at room temperature in media and used in experiments within 30 min.

Cells were settled into devices with 300 µm wells spaced 50 µm apart containing settling buffer. Settling was performed at room temperature by placing a 5  $\mu$ L drop of the cell suspension at the desired densities over the device region with wells containing PEGDA posts. After 10 min, devices were imaged through a 5x objective for cell counting. Cells per well were counted manually in ImageJ. As cell density per well increased, accurately counting the number of cells became more challenging due to the overlap of cell boundaries. The layer containing cells was the bottom layer and the top layer had 30 µm wells loaded with lysis buffer. Note that both lysis layer solution and the top layer were kept at 55 °C prior to assembly for proper solubility. Before assembly, 20 µL of excess lysis buffer was applied to the top layer and excess settling buffer was gently aspirated from the bottom layer. After assembly, the reactor had final concentrations of 1x TET, 335 mM NaCl, 1.9% (w/v) SDS, and 14.9 U/mL proteinase K. For the miRNA assays, cells were counted prior to assembly as cells are lysed upon SDS delivery. Cells were passively settled into the wells and so we determined that after assembly  $92 \pm 30\%$  (n = 14 wells) of cells are retained (Figure S7). Calu-6 cells suspended in media were settled into 300 µm wells as described previously and top layers with 30 µm wells containing media were applied onto the bottom layers as done for miRNA assays, but without using magnets for sealing in order to enable imaging. Wells from 3 different devices were imaged through 10x objectives before and after sandwich assembly in order to count cells per well before and after assembly and determine the percentage of cells retained after assembly.

For assays measuring miRNA from cells, the same method described above in the Imaging and image analysis section was employed. Wells with no cells were used as the negative controls. Because the net mean signal of some

wells with cells was <0, those wells were not included in linear fit calculation with log-log axes, resulting in a LLOD of ~16 cells. If we estimate the LLOD with linear axes and include the wells with net mean signal <0 in the calculation of the linear fit, we estimate an LLOD of ~6 cells. Note that performing the linear fit with linear axes results in a lower LLOD despite the fact that the wells with net mean signal <0 are considered. We report the higher, more conservative LLOD of ~16 cells obtained with the linear fit calculation using log-log axes because a more even weighting is given across the entire range of cells/well and it is more consistent with our previously reported methods for calculating LLOD.

For multiplex assays from cells (Figure 7D), the posts functionalized with probes complementary to *cel*-miR-238 were used as negative controls and had a net signal of  $1.1 \pm 0.7$  AFU (n = 16 wells). 0.12 amol of *cel*-miR-54 was delivered to each well by including synthetic *cel*-miR-54 target in the lysis solution in the top layer. The positive control posts functionalized with probes complementary to *cel*-miR-54 had a net signal of  $180 \pm 31$  AFU (n = 16 wells). The same of *cel*-miR-54 concentration was used in multiplex assays with synthetic targets (Figure 6), but *cel*-miR-54 was included in both the top and bottom layers before the sealing of the reactors resulting in a mass delivered of 0.5 amol. Given the differences in mass delivered, the resulting fluorescence should have a ratio of  $I_{top+bottom}/I_{top} \sim 3.5$  (Figure 2) and we observe a ratio of  $I_{top+bottom}/I_{top} \sim 2.9$ . The signal was ~16% higher than expected when cells and lysis reagents were present in the assay indicating that the assay performance was not detrimentally affected. Copy numbers for each miRNA target (#/cell) were calculated using experimentally measured values for net mean fluorescence signal per amol (AFU/amol) and net mean fluorescence signal per cell (AFU/cell). AFU/amol was estimated from assays performed with synthetic targets in configurations matching the multiplex cell assays (Figure 6). The average of the ratio of net mean fluorescence to the loaded miRNA mass for each data point was used as an estimate for AFU/amol.



Figure S7. Cells retained after well array sandwich assembly. Representative brightfield images of Calu-6 cells in media settled into 300 µm wells before and after application of a top layer with 30 µm wells.

#### miRNA assays from cells using hydrogel particles arrays

Hydrogel particles functionalized with DNA probes complimentary to miRNA targets were fabricated using stopflow lithography, as detailed in prior work<sup>7,13,14</sup>. For assays from cells, Calu-6 cells were reconstituted as previously described. In order to make cell handling similar to the cell handling done in cell assays in the well arrays, reconstituted cells were first rinsed with 1x PBS and then resuspended in settling buffer. After 10 min, cells were resuspended in settling buffer and particles for miR-21, let-7a, miR-210, miR-155, *cel*-miR-54, and *cel*-miR-238 miRNA targets were added. Then, 11.6  $\mu$ L of lysis buffer was added to the tube containing cells and particles resulting in a final reactor volume of 50  $\mu$ L. The miRNA assay hybridization, ligation, and labeling steps then proceeded as detailed in prior work<sup>6,7</sup>. For the multiplex assay, particles with probes complementary to *cel*-miR-238 were used as the negative control and particles with probes complimentary to *cel*-miR-54 were used as a positive control by including 100 amol of synthetic *cel*-miR-54 target in the reaction (Figure S8A). Assays with varying amounts of synthetic amount of miR-21 were performed to construct a calibration curve for miR-21 (Figure S8B). ~40 particles for each target were included in each reactor and after completion of each assay 4-6 particles of each type were imaged from each reactor for analysis. miRNA copy numbers per cell were estimated using experimentally determined values for AFU/amol and AFU/cell, as described previously. Following assay completion, particles with probes complementary to *cel*-miR-238 had a mean signal of 2.2 ± 0.5 AFU and particles with probes complementary to *cel*-miR-238 had a net mean signal of  $87 \pm 8$  AFU. Signal for miR-210 was below the LLOD.



Figure S8. miRNA assays using hydrogel particles. (A). Multiplex assay from Calu-6 cells. Plot shows the net mean fluorescence signal measured from particles with probes complementary to miR-21, let-7a, miR-210, and miR-155 following completion of the assay. (B) Calibration curve for miR-21. Assays were performed with synthetic miR-21 target masses of 0, 10, 30, 60, 100, and 500 amol were done in different tubes. Error bars represent  $\pm$  SD (n  $\geq$  4 particles for each condition). The dashed line indicates the linear fit.

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