Supplementary Information

A Platform for Multiplexed Colorimetric microRNA Detection using Shape-encoded Hydrogel Particles

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Methods

Microfluidic device fabrication

Microfluidic channels for particle synthesis were fabricated in polydimethylsiloxane (PDMS) using previously published methods.[1] PDMS (Sylgard 184, Dow Corning) was mixed in a 10:1 base: crosslinker ratio and poured over a SU-8 mask, fabricated using standard photolithography techniques. After curing overnight at 65°C, the PDMS channels were cut and cleaned by sonicating in ethanol. Inlets and outlets were punched using a biopsy punch (1.5 mm inlet, 4 mm outlet). The PDMS channels were placed on a glass coverslip coated in half-cured PDMS (10:1 ratio, cured for 30 min at 65°C). The resulting device was baked overnight at 65°C to complete the bonding process. Channel heights and widths were 60 μm and 300 μm respectively. Channel lengths varied from 3 to 4 mm.

Particle Synthesis

All buffer solutions were filtered through a 0.2 μm syringe filter prior to use. Particles were synthesized using stop flow lithography (SFL) as shown in Figure 1A. A prepolymer solution consisting of 20% PEGDA-700, 40% PEG-600, 5% Darocur 1173 photoinitiator (all from Sigma-Aldrich, used without further modifications), and 35% 3x Tris-EDTA buffer (TE, EMD Millipore) is mixed in a 9:1 ratio with the acrydite-modified DNA probe of interest (either a biotin probe or miRNA-specific probe). The different probes used are listed in Table S1. All DNA oligo probes and synthetic RNA targets were purchased from Integrated DNA Technologies (IDT) with RNase-free HPLC purification. This solution is introduced into the PDMS channel using compressed air-driven flow. The flow is stopped and a particle is polymerized by exposing the channel with UV light (M365L2-C4, Thorlabs, 365 nm LED) for 100 ms through a photomask (mylar transparency mask, Fineline Imaging) inserted in the F-stop. The mask defines the 2D-extruded shape of the particles. Flow is then resumed to clear the particle and bring fresh prepolymer into the UV exposure zone. The flow-stop-expose process is run in a continuous cycle to achieve semi-continuous particle synthesis. Particles are collected from the outlet and washed twice with TET (TE buffer with 0.05% Tween-20). Each wash consists of adding 400 μL of buffer to the microfuge tube containing the particles, vortexing briefly, centrifuging for 20s to sediment the particles, and removing 400 μL of supernatant.

Unreacted groups in the particles are then oxidized by incubating particles for 5 min with 500 μM $KMnO_4$ in 0.1 M Tris-HCl. Biotin probe particles are washed 3x with TBST (Tris buffered saline + 0.1% Tween-20). miRNA particles were washed 1x with TET, 2x with Rinse buffer (TE buffer with 0.1% Tween-20 + 50 mM NaCl), and then stored in hybridization buffer (TET + 500 mM NaCl) at 4°C. The particles used in the current study have a fixed 2D perimeter of 1000 μm and same height, resulting in a consistent surface area to volume ratio (0.051 ± 0.0029 μm^{-1}) for comparable target and enzyme diffusion.

Table S1. Names and Sequences of different DNA probes used for particle fabrication.

Name	Sequence
Biotin probe	5'/Acryd/ATA GCA GAT CAG CAG CCA GA/Bio/3'
miR-21 probe	5'/Acryd/GAT ATA TTT TAT CAA CAT CAG TCT GAT AAG CTA/InvdT/3'
miR-221 probe	5'/Acryd/GAT ATA TTT TAG AAA CCC AGC AGA CAA TGT AGC T/InvdT/3'
miR-141 probe	5'/Acryd/GAT ATA TTT TAC CAT CTT TAC CAG ACA GTG TTA/InvdT/3'
miR-16 probe	5'/Acryd/GAT ATA TTT TAC GCC AAT ATT TAC GTG CTG CTA/InvdT/3'
let-7a probe	5'/Acryd/GAT ATA TTT TAA ACT ATA CAA CCT ACT ACC TCA/InvdT/3'
cel miR-54 probe	5'/Acryd/GAT ATA TTT TAC TCGGAT TAT GAA GAT TAC GGG TA/InvdT/3'
Universal biotinylated linker	5'/Phos/TAA AAT ATA TAA AAA AAA AAA A/Bio/3'

Precipitate Reaction Characterization

Characterization experiments were done with particles co-polymerized with different concentrations of biotin probe incorporated, with the shape encoding the concentration. The biotin concentrations were calculated based on the biotin probe concentration in the prepolymer solution and the incorporation rate (11%) for this formulation of hydrogel particles based on previous work.[2] The particles were suspended in TBST (~15 particles of each concentration) to which streptavidin alkaline phosphatase (S-AP, Sigma Aldrich) was added to a final concentration of 10 µg/ml in a 50 µL reaction volume. Particles were mixed with the enzyme on a tube revolver (speed 30 with shaking, ThermoFisher) for 30 minutes at room temperature (21.5°C). The tube holder was angled at 90° so that the tubes rotated in the same plane in a rolling motion. The particles were then washed three times with TBST. Then 50 µL of substrate solution was added to 50 µL of particle solution. The particles were placed back on the tube revolver for varying amounts of reaction time at room temperature. After the specified time, particles were washed once with 100 µL of TBST and then deposited onto an imaging well on a glass slide.

The substrate solution contained nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP), a precipitating substrate commonly used with S-AP in blotting and *in-situ* hybridization.[3] The solution was made by dissolving a tablet of NBT-BCIP (SigmaFAST BCIP/NBT, Sigma Aldrich) in nuclease-free (NF) water. For studies assessing reaction time, "1x substrate" was used, in which the tablet was dissolved in 10 ml of NF water (the recommended concentration for the product resulting in 0.15 mg/ml BCIP, 0.30 mg/ml NBT, 100 mM Tris, and 5 mM $MgCl_2$). For studies assessing substrate concentration, the tablet was dissolved in either 5 ml or 2 ml of NF water resulting in 2x and 5x substrate solutions respectively. In all cases, the solution was filtered through a 0.45 µm small volume syringe filter.

miRNA Detection Assay

Particles were co-polymerized with oligonucleotide DNA probes specific to different miRNA. The shape of the particle encoded for the miRNA target. The particles were suspended in hybridization buffer (~10 particles for each miRNA in each reaction) to which the synthetic miRNA targets were added at different concentrations. The final NaCl concentration in the 50 µL reaction volume was 350 mM, which previous studies have shown gives high signal while reducing cross-reactivity.[4] Target hybridization was conducted for 90 min at 55°C on a thermoshaker as shown in Figure 3A. Particles were washed three times in Rinse buffer (TE buffer with 0.1% Tween-20 + 50 mM NaCl) and 245 µL of ligation solution (40 nM biotinylated universal linker (IDT), 250 nM ATP, 800 U/ml T4 DNA ligase in NEBuffer 2 (New England Biolabs)) was added to the 50 uL of particles. Particles were placed on the tube revolver at room temperature for 30 min for the ligation reaction. Particles were washed 3 times with Rinse buffer again

and then S-AP enzyme solution was added to a final concentration of 10 µg/ml in a 50 µL reaction volume. The particles and enzyme were mixed on the tube revolver at room temperature for 30 min. Particles were washed again with 3x Rinse buffer and then 50 µL of 5x substrate solution was added to 50 µL of particles. The precipitation reaction was carried out for 1 hour on the tube revolver at room temperature. For imaging, particles were washed once with 100 µL of Rinse buffer and then deposited onto an imaging well on a glass slide.

For total RNA experiments 500 ng of total RNA extracted from various different human tissue sources (colon, breast, and prostate) were used. The colon and breast tissue total RNA were purchased from BioChain, while the prostate tissue total RNA was purchased from Ambion. The total RNA was first heated to 95°C on a thermoshaker for 5 min and then cooled down over a period of 8 min before being added to the particles in hybridization buffer. Particles with a probe for *cel*-miR-54, a miRNA only found in *C. elegans* were used a negative control.

Scaling Analysis for Substrate Depletion during Multiplexed miRNA Detection

For multiplexed miRNA detection (3-5 miRNA panel), we assume there is at most ~10 fmol of target miRNA. Based on previous work in the Doyle group, we expect ~50% of the miRNA hybridize within 90 minutes2, thus giving about 5 fmol of S-AP enzyme bound in the reaction. Given the enzymatic activity (517 units/mg) and molecular weight (195 kDa) of S-AP, this results in ~5 x 10-4 units in the reaction. One unit of S-AP hydrolyzes 1 µmol of p-nitrophenyl phosphate per minute at 23°C, pH 10.45. We assume a similar activity rate for NBT/BCIP in our reaction buffer. Therefore, over the course of a 60-minute reaction, the bound enzyme could convert at most 0.03 µmol of substrate. At 5x substrate concentration (~2 mM NBT/BCIP), there is ~0.1 µmol substrate present. Given these upper bounds on the enzymatic activity possible, we believe that in the time frame of the reaction, substrate depletion should be minimal.

Imaging and Analysis

Imaging wells were created by attaching clear circular reinforcement labels (Avery) to a cleaned glass slide. The particles to be imaged were deposited inside the imaging well. Each well could hold ~15 µL of volume and so the particles from each reaction typically filled two wells. The glass slide was inserted into the slide holder slot on the phone imaging stand as shown in the schematic in Figure 1B. A Samsung Galaxy S6 phone with a 4X macro lens attached (easymacro.com) was placed on top of the phone stand to photograph the particles. Images were taken on top of an orange rectangle (with RGB values 255,180,0) printed on white photo paper. In order to have a controlled lighting environment for imaging, adhesive LED lights (Chibitronics) were attached to the phone stand (powered by a cell battery and copper tape provided with the lights). To standardize ambient lighting conditions, the phone stand was used in a laminar flow cabinet with the overhead cabinet light on (in a dark room, so that no other ambient light was present). The default camera application and settings were used.

The images were then cropped to exclude the imaging well in ImageJ (NIH) and then analyzed using a custom MATLAB script (see sample provided below). In the script, each image was separated into its RGB components. Both the blue and red channels were used to isolate particles using a fixed threshold as shown in Figure 1C (the blue channel worked best for low precipitate particles, while the red channel worked best at high precipitate levels). Particles were identified manually based on the shape, however this could be automated with existing shape detection algorithms. The script calculated the average red channel values for each particle along with the average background red value. Red channel values were inverted (255-value) and then normalized by the area of the control shape to account for the slight variation in project areas. Normalized net signal was calculated as background subtracted signal of the

particle in question minus that of the control particle (0 nM biotin for reaction characterization, 0 fmol synthetic miRNA for calibration curves, or *cel*-miR-54 for total RNA). In cases where particles were touching each other and thresholding could not identify separate particles, average red channel values for particles were determined manually using ImageJ's polygon area selection tool.

The average net red channel signal was plotted against the biotin loading per particle on a semi-log scale and a line of best fit was fitted to the data using Origin Lab. The limit of detection (LOD) at each time point was determined as the biotin loading where the net signal is equal to 3 times the standard deviation of the control particles (3σ method). For total RNA data, a student's t-test was employed to determine whether the different between tumor and normal signal was significant.

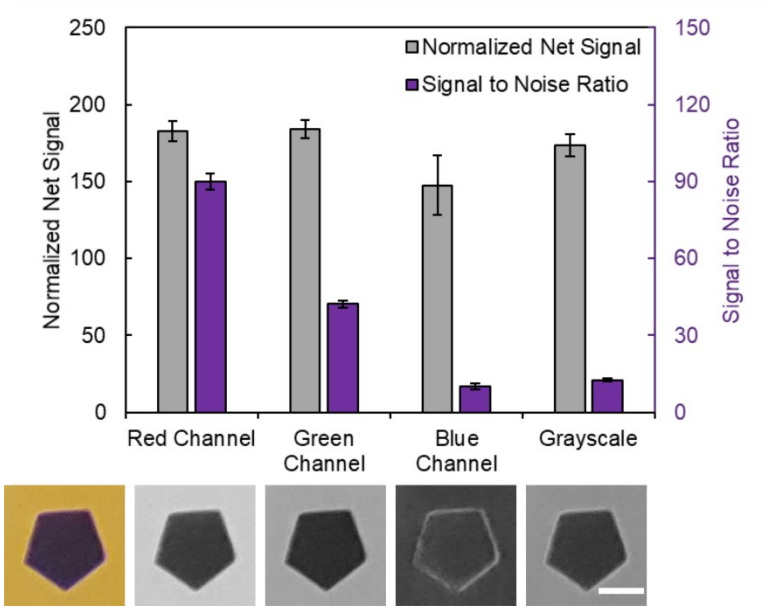

Figure S1. Comparison of RGB channel intensities for 55 nM biotin concentration. Normalized net signal from each individual color channel and grayscale transformation on the left axis. Corresponding signal-to-noise ratio on the right axis. Error bars represent 1 standard deviation. A representative particle image in each channel or after grayscale transformation is shown below each category. Scale bar is 200 µm.

A comparison of the signal from each of the RGB channels as well as grayscale is shown in Figure S1. Grayscale transformation from an RGB image in MATLAB obeys the following equation:

$$Grayscale = 0.2989 \times R + 0.5870 \times G + 0.1140 \times B$$

While net signal values are similar for red and green channels ($p>0.05$, student's t-test), the noise (standard deviation of the control particles) in the green channel results in a lower signal-to-noise ratio (SNR). The blue channel and grayscale net signals are lower than red or green channels ($p<0.05$) and the noise is greater, resulting in a very low SNR. Thus, red channel signals result in the largest SNR, giving higher sensitivity compared to the other channels or a combination of all three.

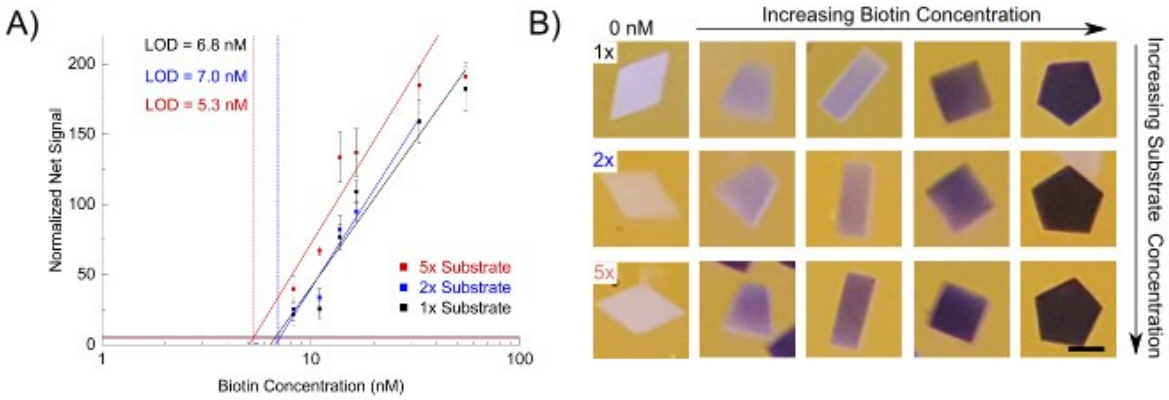

Figure S2. A) Normalized net signal as a function of biotin probe concentration using 1x, 2x, and 5x concentrated NBT-BCIP substrate solution. Reaction time was 60 minutes for all three. Vertical dashed lines show the limit of detection. Horizontal lines show the 3σ level. Error bars represent 1 standard deviation. Pearson's correlation coefficients for the semi-log fits are 0.968, 0.983, and 0.944 for 1x, 2x, and 5x respectively. B) Representative particle images at various biotin concentrations for each substrate concentration tested. Scale bar is 200 µm.

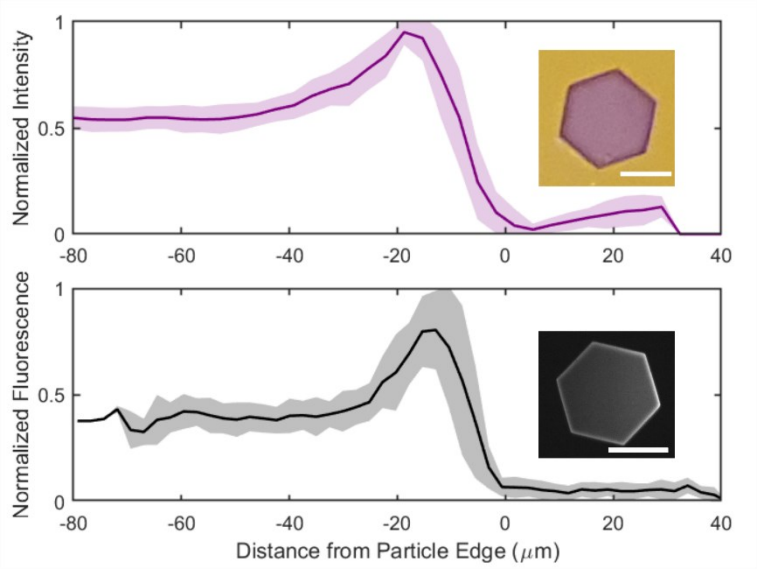

Figure S3. Comparison of boundary layer profile in colorimetric and fluorescence miRNA assay. Top: Normalized red channel intensity for the particle shown (3 fmol miR-21, 1 hr reaction with 5x NBT-BCIP substrate) as a function of distance from particle edge. Bottom: Normalized fluorescence values for the particle shown (500 amol miR-21, labelled with streptavidin-phycoerythrin) as a function of the distance from the particle edge. In both cases, 20 intensity line scans around the particle boundary were averaged. Shaded regions represent 1 standard deviation. Scale bars are 200 µm.

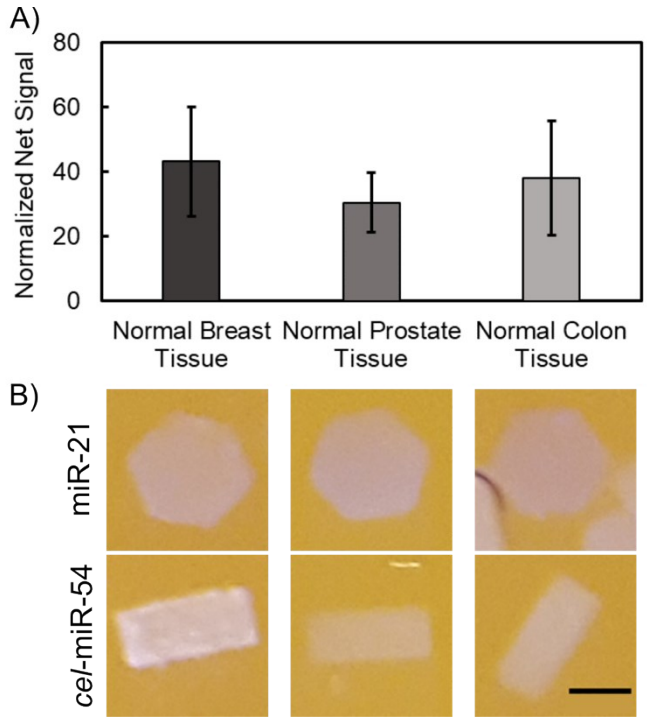

Figure S4. A) Net signal from miR-21 particles from 500 ng total RNA extracted from three different types of tissue. Negative control particles for cel-miR-54 were used for normalization. Error bars represent one standard deviation. No significant differences based on pair-wise comparisons using student's t-test ($p>0.05$). B) Representative image of particles after 1 hour of reaction. Scale bar is 200 µm.

Phone Imaging Setup

A sample CAD file of the phone imaging stand is available in the NIH 3D print repository: https://3dprint.nih.gov/discover/3dpx-012992

Sample MATLAB Script for Image Analysis

This script will produce a table ('measurements') for all the identified objects as labelled in the associated figure that is created.

```
%Importing the image--------------------------------
[filename, pathname] = uigetfile({'*.jpg;*.tif;*.png'},'Choose
Image'); %This creates an easy-to-use GUI to select the photos.
I=imread([pathname filesep filename]);

%--- Split image---------------------
[Ired,Igreen,Iblue]=imsplit(I); %split image into rgb channels
Iinverted=imcomplement(Ired); %invert red channel image

%Threshold the image-------------------------------
bluethresh = 120/255;
redthresh = 70/255; %adjust these thresholds as necessary for optimal
particle identification
```

```
bwIblue = imbinarize(Iblue,bluethresh);
bwIred = imbinarize(Iinverted,redthresh);

%%fill and remove all objects containing fewer than 1000 pixels:
bwIblue2 = imfill(bwIblue,4,'holes');
bwIred2 = imfill(bwIred,4,'holes');

bwIblue3 = bwareaopen(bwIblue2,1000);
bwIred3 = bwareaopen(bwIred2,1000);

bwI=bwIblue3+bwIred3; %Add the two binary images generated. Combines
the particles detected using the red and blue channels separately.

bwI(bwI==2)=1; %any overlap is set back to 1.

%find background average rgb values----------------------------
backgroundthresh=10/255;
background=imbinarize(Ired,backgroundthresh)-bwI;
bgred=regionprops(background,Ired,'MeanIntensity');

%print to command window
sprintf('background red intensity: %0.4f',bgred.MeanIntensity)

% trace boundaries of particles-------------------------------
[boundary,labels]=bwboundaries(bwI,'noholes');

figure, imshow(I)
hold on
for k = 1:length(boundary)
    b=boundary{k};
    plot(b(:,2),b(:,1),'w','LineWidth',0.5)
end

%measure properties using Ired for intensity values
measurements=regionprops('table',labels,Ired,'MeanIntensity','Centroid
','Perimeter','Area');

objects=max(max(labels));
measurements.labelnumber=[(1:objects)'];

% Label particles in image------------------------------
str={};
for i=1:objects
     str{i}=sprintf('%i',measurements.labelnumber(i));
end

text(measurements.Centroid(:,1)+10,measurements.Centroid(:,2),str,'Col
or','w','FontSize',10);
```

References

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3 J. A. Schumacher, E. J. Zhao, M. J. Kofron and S. Sumanas, *Biotechniques*, 2014, **57**, 254–6.

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5 Molecular. Probes, *Certificate of Analysis*, 2017.