Supplementary Information

Controlling Cellular Packing and Hypoxia in 3D Tumor Spheroids via DNA Interactions

Sven A. Saemundsson,[a] Shane D. Curry,[a] Bryce M. Bower,[a] Ethan J. DeBoo,[a] Andrew P. Goodwin,[a,b] Jennifer N. Cha[a,b,c] *

a. Department of Chemical and Biological Engineering, University of Colorado, Boulder, 596 UCB, Boulder CO, 80303, USA.

b. Materials Science and Engineering Program, University of Colorado, Boulder, 596 UCB, Boulder CO, 80303, USA.

c. Biomedical Engineering Program, University of Colorado, Boulder, 596 UCB, Boulder CO, 80303, USA.

* To whom correspondence should be addressed: Jennifer.Cha@colorado.edu

MATLAB Code for the Analysis of AlexaFluor 488 Conjugated AntiHIF1a Antibody-Stained Spheroids

```
clear; clc;
groups = ['1'; '2'; '3'; '4'; '5'; '6'];
% 1 - no DNA, 2 - 20bp DNA, 3 - 100bp DNA
% 4 - 200bp DNA, 5 - 400bp DNA, 6 - not stained to get background
replicates = ['1'; '2'; '3']; % 3 samples from each group
imgmaster = zeros(2048,2048,18);
roid_intensity = zeros (size(replicates,1), size(groups,1));
k=0;
%set up loop to open each image
for i = 1:size(groups,1)
    for j = 1:size(replicates,1)
        k=k+1
        filename = [groups(i) '_' replicates(j) '.tif'];
        %assemble filename string


        img = imread(filename); %open file


        %start image processing to get spheroid binary mask
        mask = imbinarize (img,"adaptive",'ForegroundPolarity','dark'...
            ,"Sensitivity",0.58);
        mask2 = imfill (mask, "holes");
        SE = strel('disk',7);
        mask3 = imopen (mask2, SE);
        mask4 = bwareaopen (mask3, 300000);
        %stop image processing, spheroid binary mask ready


        celldata = regionprops (mask4,img,"MeanIntensity");
        %get mean pixel intensity of spheroid using original image and its
        %mask


        celldata_num = cat(1,celldata.MeanIntensity);
        %turn data into numbers


        roid_intensity (j,i) = max(celldata_num);
        %write value to matrix. If more than 2 particles in the mask,
        %spheroid is going to be the brightest one, so take max


        imgmaster(:,:,k) = mask4; %save each mask for visual inspection
        %if a spheroid part of the mask has holes or gaps, adjust
        %sensitivity and/or strel size


    end
```

```
end


%save masks as image stack
outputFileName = 'img_stack.tif'
for z = 1:k
    imwrite(imgmaster(:,:,z),outputFileName,
'writemode','append','Compression','none');
end
```

MATLAB Code for the Analysis of Hypoxia-Stained Spheroids

```
clear; clc;
groups = ['1'; '2'; '3'; '4'; '5'; '6'];
% 1 - no DNA, 2 - 20bp DNA, 3 - 100bp DNA
% 4 - 200bp DNA, 5 - 400bp DNA, 6 - not stained to get background
replicates = ['1'; '2'; '3']; % 3 samples from each group
imgmaster = zeros(2048,2048,18);
roid_intensity = zeros (size(replicates,1), size(groups,1));
k=0;
%set up loop to open each image
for i = 1:size(groups,1)
    for j = 1:size(replicates,1)
        k=k+1
        filename = [groups(i) '_' replicates(j) '.tif'];
        %assemble filename string


        img = imread(filename); %open file


        %start image processing to get spheroid binary mask
        mask = imbinarize (img,"adaptive",'ForegroundPolarity','dark'...
            ,"Sensitivity",0.61); %play w/ sensitivity to get a good mask
        mask2 = imfill (mask, "holes");
        SE = strel('disk',10);
        mask3 = imopen (mask2, SE);
        mask4 = imdilate (mask3, SE);
        mask5 = imclearborder (mask4);
        mask6 = bwareaopen (mask5, 200000);
        SE2 = strel ('disk',30);
        mask7 = imdilate (mask6, SE2);
        mask8 = imfill (mask7, 'holes');
        mask9 = imerode (mask8, SE2);
        %stop image processing, spheroid binary mask ready


        celldata = regionprops (mask7,img,"MeanIntensity");
        %get mean pixel intensity of spheroid using original image and its
        %mask
```

```
        celldata_num = cat(1,celldata.MeanIntensity);
        %turn data into numbers


        roid_intensity (j,i) = max(celldata_num);
        %write value to matrix. If more than 2 particles in the mask,
        %spheroid is going to be the brightest one, so take max


        imgmaster(:,:,k) = mask9; %save each mask for visual inspection
        %if a spheroid part of the mask has holes or gaps, adjust
        %sensitivity and/or strel size
    end
end


%save masks as image stack
outputFileName = 'img_stack.tif'
for z = 1:k
    imwrite(imgmaster(:,:,z),outputFileName,
'writemode','append','Compression','none');
end
```

MATLAB Code for the Analysis of Rhodamine 6G Stained Spheroids

```
clear; clc;


groups = ["no"; "20"; "100"; "200"; "400"; "nostain"];
% 1 - no DNA, 2 - 20bp DNA, 3 - 100bp DNA
% 4 - 200bp DNA, 5 - 400bp DNA, 6 - not stained to get background
replicates = ["1"; "2"; "3"]; % 3 samples from each group
imgmaster = zeros(2048,2048,18);
roid_intensity = zeros (size(replicates,1), 25, size(groups,1));
k=0;


%set up loop to open each image
for i = 1:size(groups,1)
    for j = 1:size(replicates,1)
        l=1;
        k=k+1
        filename = convertStringsToChars(strjoin(["D:\Users" + ...
            "\sasae\OneDrive" + ...
            " - UCB-O365\AXR Confocal\7_16_24 mono Rhodamine 6G" + ...
            " transport experiment 40min\" groups(i) "_" replicates(j) ...
            ".nd2"],''));
        %in line above, define folder with .nd2 imgs
        I = BioformatsImage (filename);
        % open .ndt (needs bioformats toolbox install)
```

```
        pxsize = I.pxSize(1); %get microns per pixel
        finalerode = round (10/pxsize);%10um erode, convert that to pixels
        img = getPlane(I, 1, 'R6G', 1);% get rhodamine plane as image

        %start image processing to get spheroid binary mask
        mask = imbinarize (img);
        mask2 = imfill (mask, "holes");
        SE = strel('disk',10);
        mask3 = imerode (mask2, strel('disk',15));
        mask3half = imclearborder (mask3);
        mask3plus = imdilate (mask3half,strel('disk',15));
        mask4 = imdilate (mask3plus, SE);
        mask5 = imclearborder (mask4);
        mask6 = bwareaopen (mask5, 200000);
        SE2 = strel ('disk',30);
        mask7 = imdilate (mask6, SE2);
        mask8 = imfill (mask7, 'holes');
        mask9 = imerode (mask8, SE2);
        masktop = mask9;
        %stop image processing, initial spheroid binary mask ready
        SEfinal = strel('disk',finalerode);
        for m = 1:25
            if sum(sum(masktop)>0)
                maskbottom = imerode (masktop,SEfinal); %make eroded mask
                mask_measure = masktop - maskbottom; %subtract to get a "ring"
                celldata = regionprops (mask_measure,img,"MeanIntensity");
                %get mean pixel intensity of the ring of spheroid using
                %original image and its mask (radial Pix intensity profile)
                celldata_num = cat(1,celldata.MeanIntensity);
                %turn data into numbers
                roid_intensity (j,m,i) = max(celldata_num);
                %write value to matrix. If more than 2 particles in
                %the mask,"ring" is going to be the brightest one, so take max
                masktop = maskbottom; %set small mask as big mask
else
            end
        end
            %imgmaster(:,:,k) = mask9; %save each mask for visual inspection
            %if a spheroid part of the mask has holes or gaps, adjust
            %sensitivity and/or strel size
    end
end
%write into separate matrices
roid_no = roid_intensity (:,:,1);
roid_20 = roid_intensity (:,:,2);
roid_100 = roid_intensity (:,:,3);
roid_200 = roid_intensity (:,:,4);
roid_400 = roid_intensity (:,:,5);
%stop write into separate matrices
```

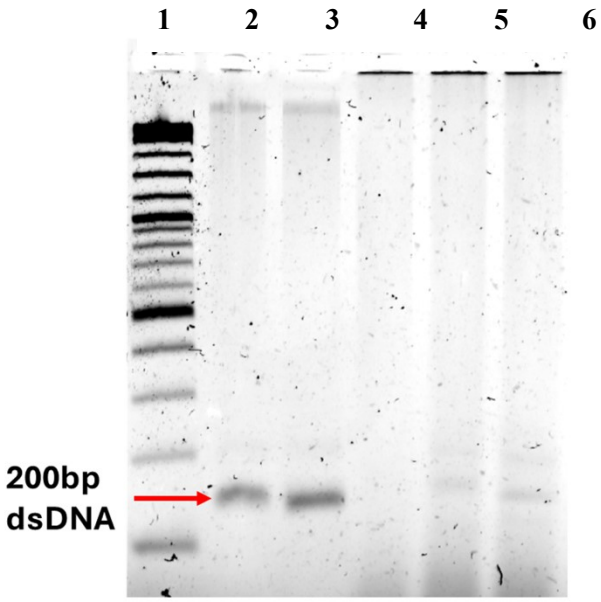

Figure S1. Agarose gel electrophoresis of biotinylated single stranded 200 base pair DNA (lanes 2 and 3) reacted with N23BP-STV (lanes 5 and 6).

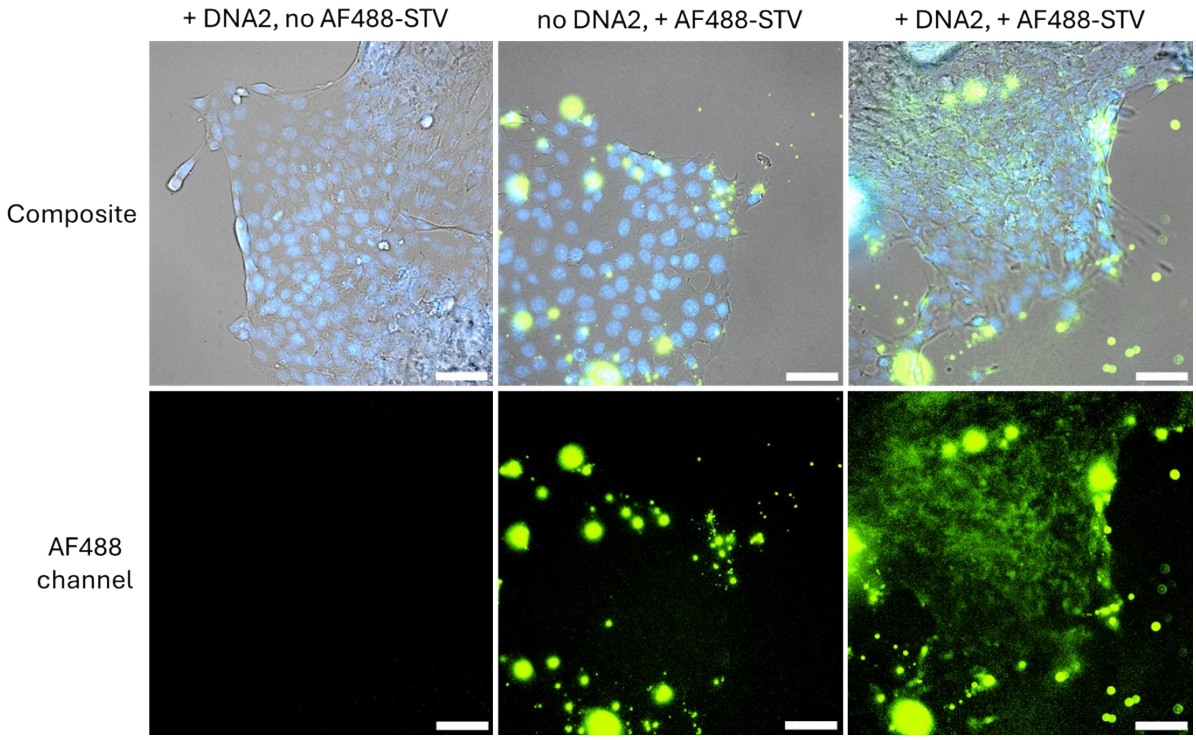

Figure S2. Wide-field images of 4T1 cells after reacting with photocrosslinkable affibody-streptavidin fusion proteins followed by reacting with 200 bp biotinylated DNA. Scale bar: 50 μm

DNA Length	Sequence
20 bp DNA 1	5’ – /Biosg/ CCC TAG AGT G – 3’
20 bp DNA 2	5’ – CCC TAG AGT G /3Bio/ – 3’
20 bp Linker	5’ – CAC TCT AGG GCA CTC TAG GG – 3’
100 bp DNA 1	5’ – /Biosg/ TGG GAA GAA AAA TCT ACG TTA ATA AAA CGA ACT AAC GGA ACA ACA TTA TT – 3’
100 bp DNA 2	5’ – ACA GGT AGA AAG ATT CAT CAG TTG AGA TTT AGG AAT ACC ACA TTC AAC TA /3Bio/ – 3’
100 bp Linker	5’ – TAG TTG AAT GTG GTA TTC CTA AAT CTC AAC TGA TGA ATC TTT CTA CCT GTA ATA ATG TTG TTC CGT TAG TTC GTT TTA TTA ACG TAG ATT TTT CTT CCC A – 3’

Table T1. 20 base and 100 base pair biotinylated DNA sequences ordered from IDT

DNA Product	Primer Sequence
200 bp DNA 1	5’ – /Biosg/ ATT GAT GCC ACC TTT – 3’
200 bp DNA 2	5’ – /Biosg/ TGA ATC TGG TGC TGT A – 3’
400 bp DNA 1	5’ – /Biosg/ GTT TAG CTC CCG CTC TGA TT – 3’
400 bp DNA 2	5’ – /Biosg/ GGG TTG AGT GTT GTT CCA GT – 3’

Table T2. Primers used in PCR to generate 200 base and 400 base pair biotinylated DNA sequences from an M13 phage DNA template

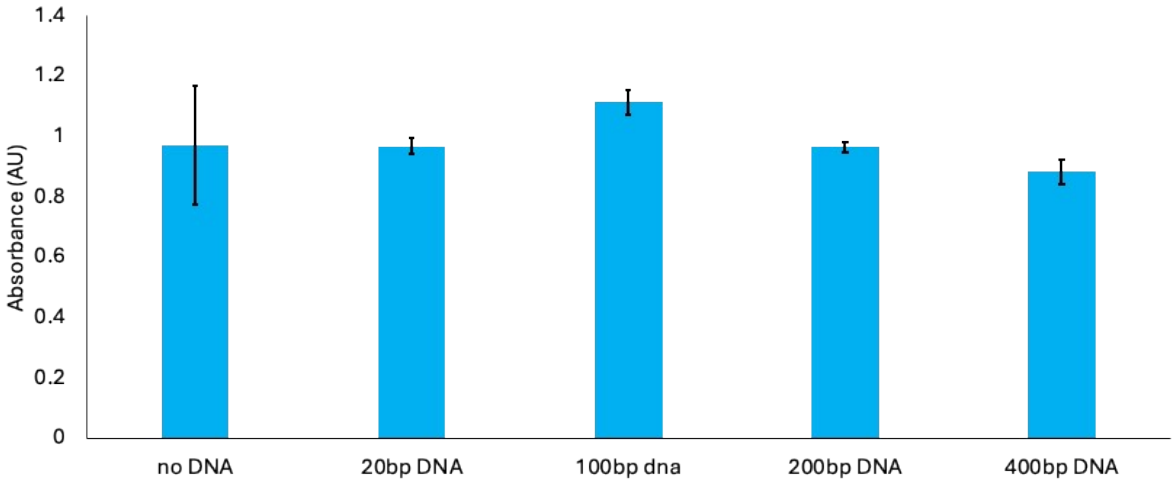

Figure S3. MTT results of 4T1 cells after conjugation with biotinylated DNA.

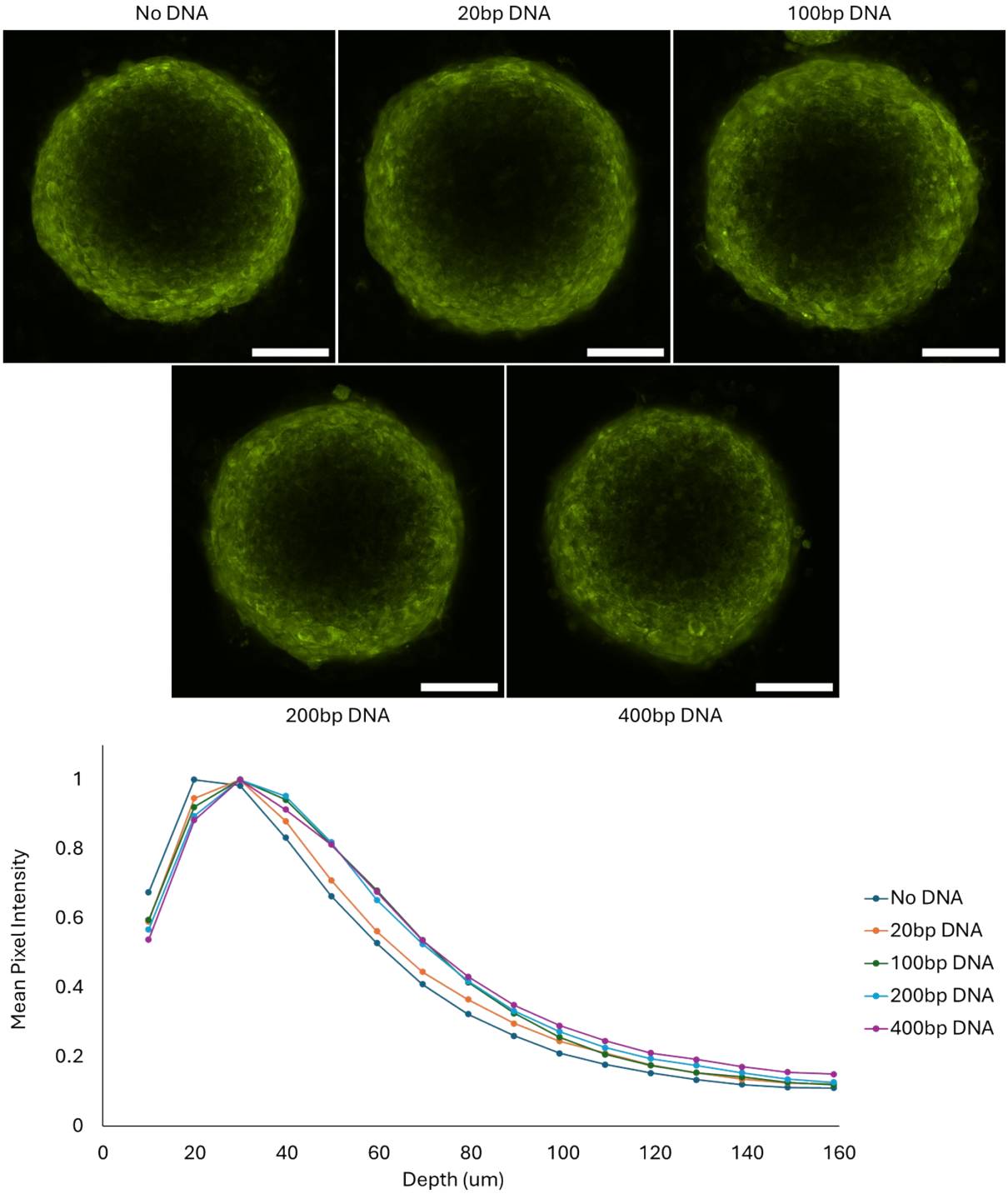

Figure S4. Radial distribution profile of 3D 4T1 spheroids assembled with varying lengths of DNA after treated with Rhodamine 6G for 20 min.