Electronic Supplementary Material (ESI) for Biomaterials Science.

Supplementary information

Materials and Methods

Expression, Synthesis, and Purification of Affibody-Streptavidin Fusion Proteins

The coding sequence as described by Brasino *et al* [1] for the EGFR binding affibody designated as ZEGFR:1907 or the nonbinding parent Z domain was synthesized by Integrated DNA Technologies (IDT) and ligated into the pET21b+ vector with a N-terminal T7 epitope tag and a C terminal 6 x His tag (19).The ZEGFR:1907 construct was again mutated via the Q5 site directed mutagenesis kit (New England Biolabs) to substitute a single codon at express Cysteine at site Asparagine 23 (N23) of the (designated as N23C). pTSA-13 (Stv-13) vector (Addgene) that encodes a 119 residues streptavidin sequence was bought in the form of bacterial stab. The DNA extracted from the vector was used to amplify streptavidin sequence using a standard PCR reaction (Applied Biosystems, Gene Amp, PCR systems 9700). The following forward and reverse primers (IDT) 5'-aaaaaa**aagctt**GGCATCACCGGCACCTGGTACAACCAGCTCG-3' (GC content: 51%) and 3'-aaaaa**ctcgag**CACCTTGGTGAAGGTGTCGTGGCCGACC-5' (GC content: 56%) were used to incorporate a HindIII and an XhoI site on the amplified sequence. The underlined part of the primer sequences are the annealing nucleotides. The Pet21b+ vector (with N23C sequence) was digested with HindIII and XhoI restriction enzymes for 1 h at 37^{o}C. The amplified sequences were ligated into the digested Pet21b+ vector with T4 DNA ligase reaction (NEB) to produce N23C-STV.The ligated plasmid was transformed into BL21(DE3) *E. Coli* using heat shock and an ampicillin (Sigma Aldrich) resistant agar plate was streaked with the transformed bacteria to grow on overnight.

A single colony of the transformants were pricked and grown overnight in 5 mL of Luria Broth (LB) supplemented by 5 µl of ampicillin. This culture was diluted 1:100 the following day into 50 mL of ampicillin-containing (Sigma-Aldrich) LB and grown in a 250 mL Erlenmeyer flask until the OD600 was approximately 0.8, at which point affibody expression was induced by adding 1M IPTG (Isopropyl β-D-1-thiogalactopyranoside). After inducing for 3 h, the cells were pelleted by centrifuging at 10,000g for 5 min, the supernatant was discarded, and the pellet was stored at −20°C until the following day. Cells were lysed by resuspending in 25 mL of equilibration buffer (PBS, pH 7.4 with 5 mM imidazole) followed by probe sonication on ice (1 min and 1min off, total on 4mins, 25%

power) to lyse the pellets. Lysates were centrifuged at 12,000*g* for 20 min at 4°C after which 200 µl of HisPur Ni-NTA suspension (Thermo Fischer) was added to the lysates and incubated for 1h at RT on a rotisserie shaker. The Ni-NTA beads were centrifuged at 700*g* for 2 min and washed 5 times with 25 mM imidazole wash buffer. The adsorbed affibody-STV (N23C-STV) were eluted by adding elution buffer (250 mM imidazole in water) with the beads for 15 min on the shaker.

Synthesis of Affibodies Conjugated to Maleimide-Benzophenone.

Newly purified affibody-STV (N23C-STV) were immediately quantified using a NanoDrop Spectrophotometer (NanoDrop Lite, Thermo Fisher), diluted to 50 µM in PBS pH 7.4 and reacted with approximately a 20:1 molar excess of 4N-maleimido-benzophenone (Sigma-Aldrich). This was done by first dissolving the maleimide benzophenone in DMSO to prepare 10 mM stock solution and then mixing this solution at 1:9 ratio with the affibody-STV solution. This mixture was then reacted in the dark overnight at RT, after which excess maleimide benzophenone was removed through a centrifugal desalt column (Zeba, Themo Fisher) with MW cut off 7 kDa. The BP reacted fusion protein was designated as N23BP-STV.

10 µl of N23BP-STV was mixed with sample loading buffer, denatured by heating for 10 min at 70°C in thermocycler (Applied Biosystems, Gene Amp, PCR systems 9700) and loaded in SDS polyacrylamide gels (Thermo Fischer) and electrophoresis and run for 25 min at 200V in MES running buffer (50mM MES,50mM Tris base,1% SDS,1mM EDTA). The gel was washed three times, stained with Coomassie (Simply Blue Stain, Thermo Fischer) and imaged with Typhoon FLA 9500 scanner to observe the purity of the samples.

MDA-MB-468 Breast Cancer Cell Culturing

MDA-MB- 468 breast carcinoma cells were procured from the American Type Culture Collection (ATCC) and grown in 75 cm^2 culture flasks at 37°C under 5% CO_2 in the presence of Dulbecco's Modified Eagle Media (DMEM, with glucose and glutamine, phenol red but no sodium pyruvate, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Thermo Fisher).

Culture of Immortalized Human Keratinocyte Cells (HaCaT)

An aneuploid immortal keratinocyte cell line from adult human skin HaCaT was procured from ATCC. The HaCaT cells used in this study were transfected with a FRET-based sensor of ERK activity, the extracellular signal-regulated kinase activity reporter or EKAR[2]. EKAR was developed with a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) on either side of it, flanking a phospho binding domain that upon phosphorylation induces a conformational change which again reduces the distance between the CFP (donor) and YFP (acceptor), thus leading to FRET which can then be represented as ERK activity[3,4]. Following a standard protocol,[2,4,5] the HaCaT cells were transfected with EKAR by using lipofectamine reagent (Thermo).After EKAR transfection, HaCaT cells were cultured at 37°C with 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% heat inactivated Fetal Bovine (FBS,Gibco) and1% penicillin-streptomycin (Thermo Fisher).

Culture of Caco-2 Cells

Caco-2 human adenocarcinoma cell line was obtained from the American Type Culture Collection (ATCC) and grown in 75 cm^2 culture flasks at 37°C under 5% CO_2, 95% humidified incubator in the presence of Dulbecco's Modified Eagle Media (DMEM, with glucose and glutamine, phenol red but no sodium pyruvate, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Thermo Fisher). Cells were passaged at 70-90% confluence.

Confocal Microscopy Analysis Studies

MDA-MB- 468 human breast carcinoma cells were grown to confluence at 37°C under 5% CO_2. The cells were trypsinized with 0.025 % Trypsin-EDTA (Gibco). 12 mm poly-L-lysine coated glass coverslips (Corning BioCoat) were placed into 4 wells of a 24 well plate. Then 500 µl of the cell suspension was seeded in the wells of cell culture plates on to the coverslips at a seeding density of 25000 cells/well and grown for 24 h. Cells were then incubated with 500 µl of 1,0.5,0.25 and 0 µM of N23BP-STV with UV irradiation (890

μW/cm2 365 nm light) after 3 h for 30 mins after which the cells were kept back in incubator overnight. The cells then were washed thrice to remove excess fusion proteins with 500 μl PBS buffer and reacted with 500 μl of 0.1 μM biotinylated quantum dots (Qdot™ 655 Biotin Conjugate, Invitrogen). After reacting with biotinylated QDs for 3 h, cells were washed three times with PBS to remove unbound QDs. Next, 500 μl of 5 μM 2'-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole, trihydrochloride (Hoechst 33342, Thermo Scientific, Germany) was reacted to each of the well for 20 minutes to stain the nucleus of the live cells. After washing the cells again with PBS, the coverslips were taken out from the well and mounted on to a glass slide using Prolong™ Glass Antifade Mountant (Invitrogen, USA). Mounted coverslips on the glass slides were kept at room temperature overnight and imaged in Nikon A1R laser scanning confocal microscopy. The biotinylated QD fluorescence and Hoechst-stained cells were imaged using Cy5 and DAPI channels respectively.

Flow Cytometry Measurements

MDA-MB- 468 human breast carcinoma cells were grown to confluence at 37°C under 5% CO_2. The cells were trypsinized with 0.025% Trypsin-EDTA and 500 μl of the cell suspension was seeded in two wells of a 24-well cell culture plates @ 25000 cells/well and grown for 24 h. Cells were then incubated with 500 μl of 1 μM N23BP-STV and after 3 h, UV irradiated for 30 min and incubated further overnight. The cells then were washed to remove excess fusion proteins with PBS and reacted with 50 0μl of 0.1 μM biotinylated QDs (Qdot™ 655 Biotin Conjugate, Invitrogen). As a control, no QDs were added to N23BP-STV conjugated cells. After reacting with biotinylated QDs for 3 h, cells were washed three times with PBS to remove excess of QDs, dislodged using 250 μl of trypsin-EDTA, collected and centrifuged for 5 min at 150*g*. The supernatant was discarded and resuspended in 50μl of DMEM. To this, 500 μl of paraformaldehyde (4% PBS solution) was added to fix the cells and resuspended in PBS solution and stored at ~4^{o}C. Using BD FACSCelesta instrument (BD Biosciences) the fluorescence of the cells modified with N23BP-STV and biotin-QDs was measured using an excitation wavelength of 408 nm against control cells where no biotin-QDs were added. The raw data was then analyzed using Flowing Software (OmicX). In order to further measure the presence of QDs on the

cell surface at different time points, after photo-crosslinking to N23BP-STV to MDA-MB-468 cells followed by incubating further for 24,48 and 72h, cells were treated with the biotinylated QDs. As controls, cells were not reacted with the N23BP-STV fusion protein but treated with the biotinylated QDs. After reacting the cells with QDs, the cells were washed and trypsinized and fixed with paraformaldehyde solution. Flow cytometry analysis of 468 cells-N23BPSTV-QD against 468 cells-no protein-QD was done at 408nm excitation wavelength.

Cell proliferation Studies as Function of N23BP-STV Concentration

MDA-MB- 468 human breast carcinoma cells were grown to confluence at 37°C under 5% CO2. The cells were trypsinized with 0.025% Trypsin-EDTA (Gibco), seeded in 20 wells of a 96 well cell culture plate @5000 cells/well and grown overnight. Next, cells were washed with 100 µl of PBS twice and 100 µl of 1, 0.50, and 0.25 µM of N23BP-STV were added to each set of five wells, reacted for 3 h, UV treated for 30 min and incubated overnight. Excess fusion protein was removed by washing the cells with PBS. For the control variant, no protein was added to the cells. Next the cells were treated with different protein concentrations, trypsinized and resuspended in 500 µl of fresh media. The cell concentrations were determined by taking 10 µl aliquots from each of the stocks and counted manually using a hemocytometer. The cells were next reseeded at 2000 cells/well in triplicates to grow the cells up to three different time points of 24, 48, and 72 h. At the varying time points, the cells were trypsinized and counted.

Viability staining of N23BP-STV conjugated cells

To carry out the study, MDA-MB- 468 human breast carcinoma cells were grown to confluence at 37°C under 5% CO2. The cells were trypsinized with 0.025% Trypsin-EDTA (Gibco), seeded in 18 wells of a 24 well cell culture plate @50000 cells/well and grown for 36h. Next, cells were washed with 500 µl of PBS twice and 500 µl of 1, 0.50, µM of N23BP-STV were added to each set of three wells. Control cells were not treated with protein. One set of protein conjugated cells in triplicates were reacted for 3 h, UV treated for 30 min and incubated overnight.Other set of protein conjugated variant in triplicates were not exposed to UV. Excess fusion protein was removed by washing the cells with

PBS. The cells were next treated with 5μM Calcein and PI solution 36h post protein addition and reacted for 30minutes. Then the cells were washed and fixed with 4% paraformaldehyde solution. Then the cells were analyzed in BD FACSCelesta instrument (BD Biosciences) with a 488 and 561 laser to measure mean fluorescence intensity of Calcein and PI.

Using DNA Interactions to Drive Cell-Cell Interactions

For these studies, MDA-MB-468 human breast carcinoma cells were grown to confluence at 37°C, trypsinized and seeded in 8 wells of a 96 well cell culture plate at 5000 cells/well and grown overnight. Next, cells were washed with 100 µl of PBS twice and 100 µl of 1 µM N23BP-STV was added to each well, reacted for 3 h, then UV treated for 30 min and kept back in the incubator overnight. Excess fusion protein was removed by washing the cells with PBS multiple times. Next 100 µl of 0.01 mg/ml DiO (Benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate, Thermo Fischer Scientific, USA) was added to the cells in 4 wells and 100 µl of 5 µM DiI (1,1'-Dioctadecyl-3,3,3',3'Tetramethylindocarbocyanine Perchlorate, Thermo Fischer Scientific, USA) was added to rest of the 4 wells and reacted for 20 minutes. After the two sets of cells were stained DiO or DiI, the cells were trypsinized and spun down separately. The DiO stained cells were incubated with 400 µl of 1 µM DNA sequence-**1** (5'bio-CCC TAG AGT G-3'; IDT, USA) and the DiI stained cells were treated with 400 µl of 1 µM DNA sequence **2** (5'-CCC TAG AGT G-bio3'; IDT, USA) for 3 h at 37^{o}C. Next, 100 µl of DiO stained DNA sequence**1** treated cells were mixed with 100 µl of DiI stained sequence **2** treated cells and 200 µl of 1 µM DNA linker (5'-CAC TCT AGG GCA CTC TAG GG -3') for 1 h at 37 ^{o}C. In the control stock, both DiO and DiI stained and sequence 1 and 2 treated cell suspensions were mixed but no DNA linker was added. After 24 h incubation, the cells were washed and fixed with 4% paraformaldehyde and imaged in Nikon A1R laser scanning confocal microscopy. GFP and Cy5 channels were used to image the DiO and DiI stained cells respectively.

Spheroid Formation Methods

Preparing poly hydroxyethyl methacrylate (polyHEMA) Coated Wells

A stock solution of 30 mg/mL poly hydroxyethyl methacrylate (polyHEMA, Sigma Aldrich, USA) was prepared in 95% ethanol. The solution was heated at ~60^{o}C with continuous stirring for 2-3h to dissolve the polyHEMA. Next, 100 µl of the polyHEMA solution was dispensed to individual wells of a 96 well plate, kept open overnight for removal of ethanol by air drying until the polyHEMA solidified.

Spheroid Forming Tests with DNA and MDA-MB-468 Cells

Cell monolayers were detached from their culture flask using a standard trypsinization protocol and seeded in 96 well plates at 5000 cells/well. After overnight growth, 100 µl of 1 µM N23BP-STV was added to the cells. After initial reaction with the fusion protein for 3h, cells were exposed to UV radiation for 30 min for photoconjugation followed by another incubation overnight. Next, cells were treated with 100 µl of 5 µM Hoechst in DMEM for 20 min to stain the nucleus. After washing the cells with PBS three times, cells were trypsinized, spun down and resuspended in 1 µM of DNA Sequence **1** (5'bio-CCC TAG AGT G-3') or DNA Sequence **2** (5'- CCC TAG AGT G-bio3') for 3 h. Then 100 µl from each cell stock were mixed together and 200 µl of 1 µM complementary DNA linker (5'-CAC TCT AGG GCA CTC TAG GG -3') was added and reacted in the incubator at 37^{o}C for 1 h. As controls, cells were reacted with 1 µM of N23BP-STV but no DNA linker (control 1) and native cells without any protein or DNA treatment (control 2) were used. Next 200 µl of the DNA treated cells containing ~ 15000 cells were added to the polyHEMA coated wells. The medium was replaced every 48h with standard culture medium. Brightfield imaging for analysis of size distribution and shape of spheroids was captured at the desired time points with Nikon Widefield microscope. To test for mechanical stability, the formed spheroids were pipetted out and transferred to another non poly HEMA coated standard cell culture treated well of a 96 well plate. The spheroids were washed with PBS thrice and then fresh media was added to the well. The spheroids were again imaged to study the retention of spheroid integrity.

Spheroid Forming Tests with DNA and Dye-Stained MDA-MB-468 Cells

MDA-MB-468 human breast carcinoma cells were grown to confluence at 37°C, trypsinized and detached from the culture flask. Then the cells were seeded in 12 wells

of a 96 well cell culture plate at 5000 cells/well and grown overnight. Next, cells were washed with 100 µl of PBS twice and 100 µl of 1 µM N23BP-STV was added to each well, reacted for 3 h, UV treated for 30 min and incubated overnight. Excess fusion protein was removed by washing the cells with PBS multiple times. Next 100 µl of 0.01 mg/ml DiO (Benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate, Thermo Fischer Scientific) was added to the cells in 6 wells and 100 µl of 5 µM DiI (1,1'-Dioctadecyl-3,3,3',3'Tetramethylindocarbocyanine Perchlorate, Thermo Fischer Scientific) was added to rest of the 6 wells and reacted for 20 min. After the two sets of cells were stained DiO or DiI, the cells were washed with PBS, trypsinized and spun down separately. The DiO stained cells were incubated with 400 µl of 1 µM DNA sequence-**1** (5'bio-CCC TAG AGT G-3') and the DiI stained cells were treated with 400 µl of 1 µM DNA sequence **2** (5'-CCC TAG AGT G-bio3') for 3 h at 37°C. Next, 100 µl of DiO stained DNA sequence**1** treated cells were mixed with 100 µl of DiI stained sequence **2** treated cells and 200 µl of 1 µM DNA linker (5'-CAC TCT AGG GCA CTC TAG GG -3',IDT,USA) for 1 h at 37 °C. Next, 200 µl of the suspension containing ~15000 DNA treated, DiO and DiI stained cells with DNA linker were added to the polyHEMA coated wells. The medium was replaced every 48 h with standard culture medium. Images were captured with FITC and Cy5 channels in Nikon Widefield microscope. After 96h of spheroid growth, the stable cell spheroids were transferred to another standard cell culture well (no polyHEMA coated). The spheroids were washed with PBS multiple times and fresh media was added.

Spheroid Forming Tests Using Different Concentrations of DNA Linker

To study the spheroid formation in presence of different amount of DNA linker strands, spheroids were assembled using DNA hybridization as described above but repeated using 0.5 and 0.25 µM linker DNA strands. MDA-MB-468 cells were conjugated to 1 µM N23BP-STV and stained with Hoechst. The cells were reacted to 1 µM of DNA Sequence **1** (5'bio-CCC TAG AGT G-3') or DNA Sequence **2** (5'- CCC TAG AGT G-bio3') for 3 h. Then cells from two stocks were mixed in 1:1 ratio. Next, 200 µl of 0.5 and 0.25 µM complementary DNA linker (5'-CAC TCT AGG GCA CTC TAG GG -3') was added to each mixture and reacted in the incubator at 37°C for 1 h. Next, 200 µl of the cell mixture

containing ~ 15000 cells and different amounts of DNA linker were added to the polyHEMA coated wells. The medium was replaced every 48h with standard culture medium. Brightfield and DAPI imaging were done at the desired time points with Nikon Widefield microscope. After 96h the spheroids were pipetted out and transferred to another non polyHEMA coated well. The spheroids were washed with PBS and then fresh media was added to the well. The spheroids were imaged again.

Spheroid Tests conjugating each set of cells with complementary DNA strands

MDA-MB-468 human breast carcinoma cells were seeded in 12 wells of a 96 well cell culture plate at 5000 cells/well and grown overnight. Next, cells were photo-crosslinked to 1 μM N23BP-STV and incubated overnight. Next 100 μl of 0.01 mg/ml DiO (Benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl]-, perchlorate, Thermo Fischer Scientific) was added to the cells in 6 wells and 100 μl of 5 μM DiI (1,1'-Dioctadecyl-3,3,3',3'Tetramethylindocarbocyanine Perchlorate, Thermo Fischer Scientific) was added to rest of the 6 wells and reacted for 20 min. After the two sets of cells were stained DiO or DiI, the cells were washed with PBS, trypsinized and spun down separately. The DiO stained cells were incubated with 400 μl of 1 μM DNA sequence **1** (5'bio-CCC TAG AGT GCA CTC TAG GG-3', IDT,USA) or (5'bio CCCTAGAGTGCACTCTAGGG-3'; IDT,USA) and the DiI stained cells were treated with 400 μl of 1 μM DNA sequence **2** (5'-CCC TAG AGT GCA CTC TAG GG-bio3', IDT,USA) or (5'bio CCCTAGAGTGCACTCTAGGG-3'; IDT,USA) for 3 h at 37^{o}C. Next, 100 μl of DiO stained DNA sequence**1** treated cells were mixed with 100 μl of DiI stained sequence **2** treated cells and 200 μl of fresh media added to the mix. Next, 200 μl of the suspension containing ~15000 DNA treated, DiO and DiI stained cells were added to the polyHEMA coated wells. The medium was replaced every 48 h with standard culture medium. Images were captured with FITC and Cy5 channels in Nikon Widefield microscope.

Spheroid Forming Tests with DNA and Using Cell-Adhesive Surfaces

Cell monolayers were detached from their culture flask using a standard trypsinization protocol and seeded in 96 well plates at 5000 cells/well. After conjugation with the 1 μM

fusion protein, cells were treated with 100 µl of 5 µM Hoechst in DMEM for 20 min to stain the nucleus. After washing the cells with PBS three times, cells were trypsinized, spun down and resuspended in 1 µM of DNA Sequence **1** (5'bio-CCC TAG AGT G-3'; Stock 1) or DNA Sequence **2** (5'- CCC TAG AGT G-bio3'; Stock 2) for 3 h. Then 100 µl from each cell stock were mixed together and 200 µl of 1 µM complementary DNA linker (5'-CAC TCT AGG GCA CTC TAG GG -3') was added and reacted in the incubator at 37°C for 1 h. Then 200 µl of ~ 15000 DNA treated cells with DNA linker were added to non polyHEMA coated standard cell culture treated wells which are cell adhesive in contrast to poly HEMA coated wells. As control, cells were reacted with 1µM of N23BP-STV, DNA Sequence **1** and **2** and incubated on no poly HEMA coated cell adhesive well without linker DNA. The medium was replaced every 48h with standard culture medium.

Calcein/PI Staining

After 96 h of growth, the spheroids were transferred to a nonpoly HEMA coated well and washed with PBS. Next, 100 µl of DMEM containing 50 µl of 0.1 µM Calcein and 50 µl of 2 µM PI was added to the spheroids in each well and reacted at 37°C for 30 minutes. Stained spheroids were washed with PBS and imaged in FITC and TxRED channels in a Nikon widefield microscope.

Mechanical stability of spheroids

The matured spheroids were washed with PBS and then consecutively transferred from one non poly HEMA coated well to another and imaged at desired points to observe if any visible changes in spheroid diameter has occurred. Study up to seventh consecutive spheroid transfers were done and imaged.

Immunostaining

Matured MDA-MB-468 spheroids were transferred to a non poly HEMA coated well and fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed spheroids were permeabilized with 0.5% Triton X-100 (Sigma Aldrich) in PBS for 15 min at room temperature and washed 3 times with PBS. For blocking, spheroids were incubated with 3% BSA in 4 °C for 1 h. Next, spheroids were incubated with Alexa Fluor 488 conjugated

anti E cadherin antibody (CD324 (E-Cadherin) Monoclonal Antibody (DECMA-1), Alexa Fluor 488, eBioscience™; Invitrogen) in 4 °C overnight with the concentration of 1:100 in 3% BSA solution. Next day, the spheroids were repeatedly washed with 3% BSA solution and then imaged in confocal microscope.

Spheroid Tests with Different Amounts of N23BP-STV Conjugated to Cells

Cells were counted and seeded in a 96 well plate with 5000 cells/well density. After overnight growth, 100 µl of 1µM, 0.5µM,0.25 µM N23BP-STV solution was added to the wells. After initial reaction with the fusion protein for 3h, cells were exposed to UV radiation for 30 min. Then the cells were kept back to the incubator again overnight. Next, the cells were stained with 100 µl of 5µM Hoechst for 20 minutes. After washing the cells with PBS thrice, cells were trypsinized, spun down and resuspended in 1µM of Sequence 1 (5'bio-CCC TAG AGT G-3'; Stock 1) / Sequence 2 (5'- CCC TAG AGT G-bio3'; Stock 2) DNAs and reacted for 4 h. Then 100 µl from each cell stock were mixed together and 200 µl of 1µM complementary linker DNA strands (5'-CAC TCT AGG GCA CTC TAG GG -3') were added to the mixture. The mixture was reacted in the incubator at 37^{o}C for 1h. Then 200µl of cell suspension with linker DNA containing ~ 15000 cells was added in each of the poly HEMA coated well for all the three protein concentration variants. The medium was replaced every 48h with standard culture medium.

Statistical Analysis of Spheroids grown with 0.5µM and 0.25µM N23BP-STV Conjugated Cells

In order to carry out statistics, 8 MDA-MB-468 spheroids were grown for each of the 0.5µM and 0.25µM N23BP-STV conjugated cells for 96 h employing the above-mentioned method. Bright field images were captured at 24/48/72/96h time points. Spheroid diameter and circularity were measured using ImageJ software. The polygon tool was used to outline spheroids and projected area to obtain circularity measurements. Circularity is measured as $(4\Pi\times[\text{Area}])/[\text{Perimeter}]^2$, and ranges from 0 for infinitely elongated polygon to 1 for perfect circle. Levels of statistical significance are indicated on the respective graphs where calculation of p values has been done using t-test. A two

factor ANOVA was performed to analyze statistical significance of the effect of growth time and protein concentration on the spheroid sizes.

Spheroid Tests with Different Amounts of Cell Seeding Density to Poly HEMA Coated Surface

Cells were counted and seeded in a 96 well plate with 5000 cells/well density. After overnight growth, 100 µl of 1µM N23BP-STV solution was added to the wells. After initial reaction with the fusion protein for 3h, cells were exposed to UV radiation for 30 min. Then the cells were kept back to the incubator again overnight. Cells were trypsinized, spun down and resuspended in 1µM of Sequence 1 (5'bio-CCC TAG AGT G-3'; Stock 1) / Sequence 2 (5'- CCC TAG AGT G-bio3'; Stock 2) DNAs and reacted for 4 h. Then 100 µl from each cell stock were mixed together and 200 µl of 1µM complementary linker DNA strands (5'-CAC TCT AGG GCA CTC TAG GG -3') were added to the mixture. The mixture was reacted in the incubator at 37^{o}C for 1h. Then 15000/10000/5000 cells were added in the poly HEMA coated well, each variant in triplicates in presence of 1µM linker. The spheroids were grown for 96h and the medium was replaced every 48h with standard culture medium.Bright field images were captured and spheroid diameters were measured using ImageJ software.

Spheroid Tests with HaCaT Cells

HaCaT cell monolayers were detached from their culture flask via a standard trypsinization protocol. Cells were counted and seeded in a 96 well plate with 5000 cells/well density. After overnight growth, 100 µl of 1µM N23BP-STV was added to the cells and photoconjugated to the cells as described above for the MDA-MB-468 cells. Two control variants were used in this study. Cells in control 1 were conjugated with 1µM N23BP-STV, DNA Sequence **1** or **2** but were not treated with DNA linker whereas control 2were only native cells without any protein or DNA conjugation. The HaCaT cells used in this study had a green fluorescent protein as Extracellular Kinase Activity Reporter (EKAR) sensor in it and the spheroid images can be captured in FITC channel[2–6]. After washing the cells with PBS three times to remove excess protein, cells were trypsinized,

spun down and reacted with biotin DNA sequence 1 and DNA sequence 2 as described above for 4 h. For control 1, no linker DNA was added. Then 100 µl from each cell stock were mixed together and 200 µl of 1 µM complementary DNA linker strands (5'-CAC TCT AGG GCA CTC TAG GG -3') were added to the mixture. The mixture was then reacted in the incubator at 37^{o}C for 1h. Then ~ 15000 treated cells were added to the polyHEMA coated well to obtain a single spheroid of uniform size. The medium was replaced in every 24h with standard culture medium. Imaging in Nikon widefield microscopy was done to capture images in Brightfield/FITC channel at 24,48, 72 and 96 h. After 96 h of growth, the spheroids were found to be stable enough to transfer to another plate.

Statistical Analysis of HaCaT Spheroids grown with 0.5µM N23BP-STV Conjugated Cells

In order to carry out statistics, 8 HaCaT spheroids were grown with 0.5µM N23BP-STV conjugated cells for 72h. Bright field images were captured at desired time points. Spheroid diameter was measured using ImageJ software and plotted against time.

Spheroid Tests with CaCo-2 Cells

CaCo-2 cell monolayers were detached from their culture flask and seeded in a 96 well plate with 5000 cells/well density. After overnight growth, CaCo-2 cells were photoconjugated with1µM N23BP-STV. Control cells were native CaCo-2 cells without any protein or DNA treatment. After washing the cells with PBS to remove excess protein, cells were treated with 100 µl of 5 µM Hoechst in DMEM for 20 min for staining. Next, the cells were washed, trypsinized, spun down and reacted with DNA sequence **1** and DNA sequence **2** as described above for 3 h.. Then 100 µl from each cell stock were mixed and 200 µl of 1 µM complementary DNA linker strands were added to the mixture. The mixture was then incubated at 37^{o}C for 1h. Then ~ 15000 protein and DNA conjugated CaCo-2 cells along with DNA linker and control CaCo-2 cells were added to the polyHEMA coated well. The medium was replaced in every 48 h with standard culture medium. Imaging in Nikon widefield microscopy was done to capture images in Brightfield/DAPI channel at 24,48, 72 h and 96 h. After 96 h of growth, the spheroids were transferred to another non poly HEMA coated standard cell culture well.

Doxorubicin (DOX) Tests with DNA Assembled Spheroids

The spheroids grown for 96 h in above mentioned method were transferred to a new non poly HEMA coated standard cell culture treated well and washed thrice with PBS. To compare the drug resistance property of 3D spheroids with 2D cultured cells, cells from monolayer were detached from surface by trypsinization and seeded at 5000 cells/well density in a 96 well plate. The cells, grown overnight, were then conjugated with affibody-STV, DNA Sequence **1** and **2**. The 1:1 mixture of the cells treated with DNA Sequence **1** and **2** were then added to a non poly HEMA coated standard cell culture treated well with a seeding density of 15000 cells/well without DNA linker strands.1000 ng/ml of DOX (Doxorubicin hydrochloride, Fisher Scientific, USA) in DMEM were prepared. 200µl of each DOX solution was added to the 3D spheroids and 2D cultured cells and 200µl of fresh media was added to the control 3D spheroids and 2D cultured cells. Then the treatments were kept incubated with the spheroids and 2D cells for 72h. Next, spheroids and 2D cells were washed and cell viability assays were run using Alamarblue (Invitrogen). For this, 10 µl of Alamarblue was added to 90 µl of DMEM and incubated with DOX treated spheroids, 2D DNA conjugated cells and controls for 4 h at 37^{o}C. Figure S7 showed the UV absorbance curve of 2D cultured DOX treated cells in comparison to the control cells where no DOX treatment was done and also UV absorbance curve of DOX treated spheroids against the control spheroids without any DOX treatment. The absorbance value at 570 nm for each variant was corrected from background absorbance at 600 nm and tabulated in Figure 10. The cell viability of 2D cultured cells and 3D spheroids were calculated by considering the control sample viability as 100 % as no DOX treatment was done on those variants. It was evident from the cell viability study that the spheroids were not susceptible to DOX treatment revealing higher drug resistance in comparison to 2D cells.

FIGURES

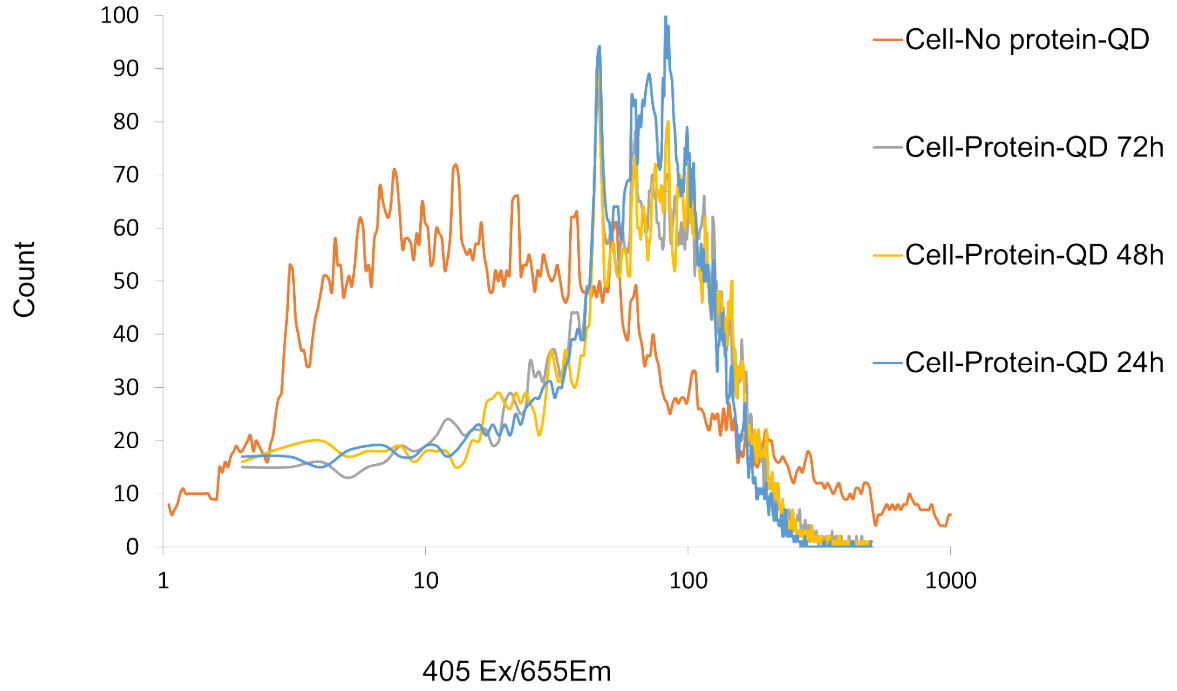

Figure S1. A: Flow cytometry study of cells-N23BPSTV treated with Biotin QD and grownup to 24/48/72h of timepoints against control cells with no protein but treated with QDs.

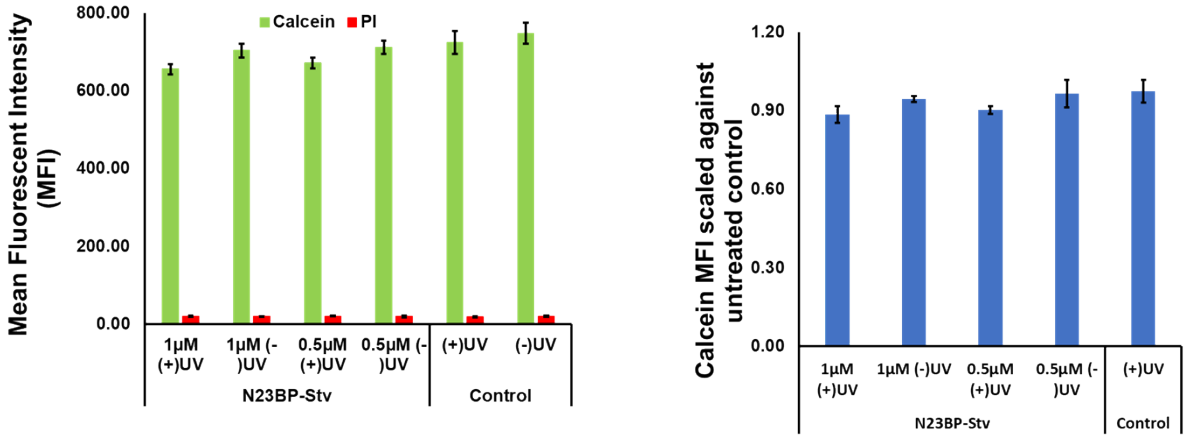

Figure S2. Flow cytometry data from calcein and PI staining of MDA-MB-468 cells treated with either 1 or 0.5 μM N23BP-STV and +/- UV light.

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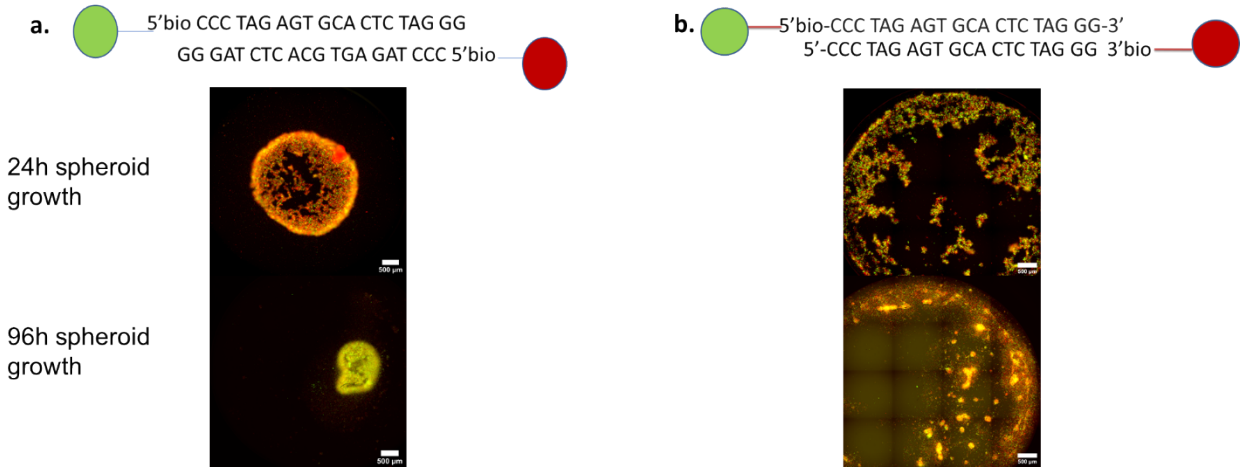


Figure S3: a. 24h and 96h timepoint images of DiO and Dil stained MDA-MB-468 cells conjugated with N23BP-STV, DNA sequences (5'bio CCCTAGAGTGCACTCTAGGG-3' and 5'bio CCCTAGAGTGCACTCTAGGG-3' where DNA complementarity caused cell-cell interactions in x-y directions) and then incubated in poly HEMA well; b. DiO and Dil stained MDA-MB-468 cells conjugated with N23BP-STV, DNA sequences (5'bio-CCC TAGAGTGCACTCTAGGG-3' and 5'-CCCTAGAGTGCACTCTAGGG-bio3' where complementarity caused cell-cell interactions in z direction) and then incubated in poly HEMA coated well. Scale bar corresponds to 500 µm.

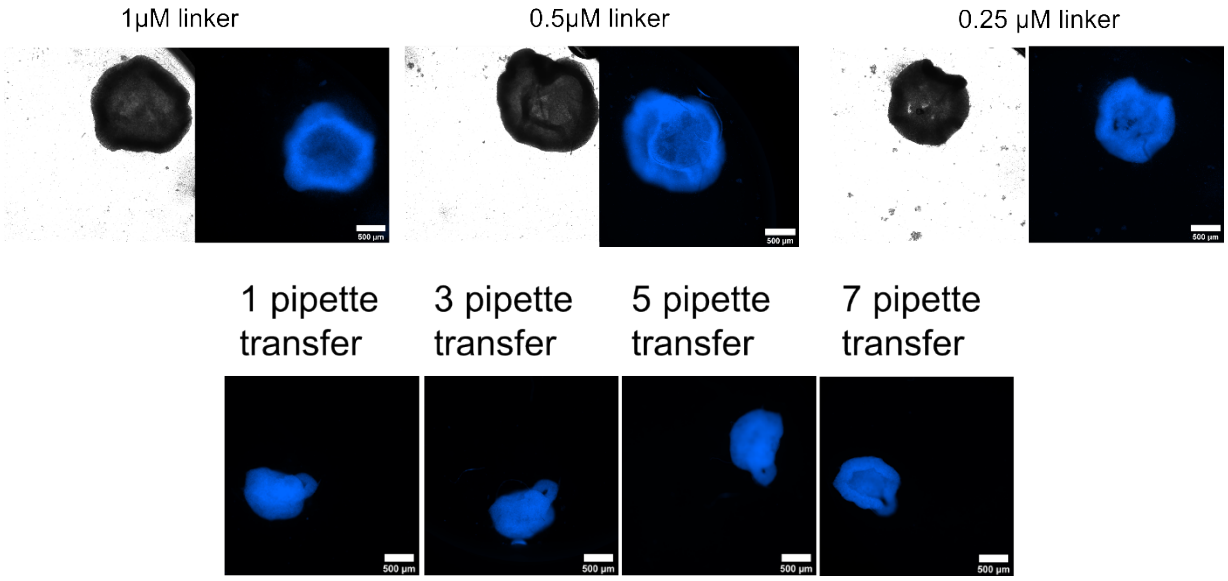


Figure S4. BF and DAPI images of matured spheroid grown with 1/0.5/0.25 µM of linker DNA strands after transferring to a non poly HEMA coated standard cell culture treated well and washing thrice with PBS. Mechanical stability of spheroids were measured by 7 consecutive pipette transfers of spheroids to different wells.

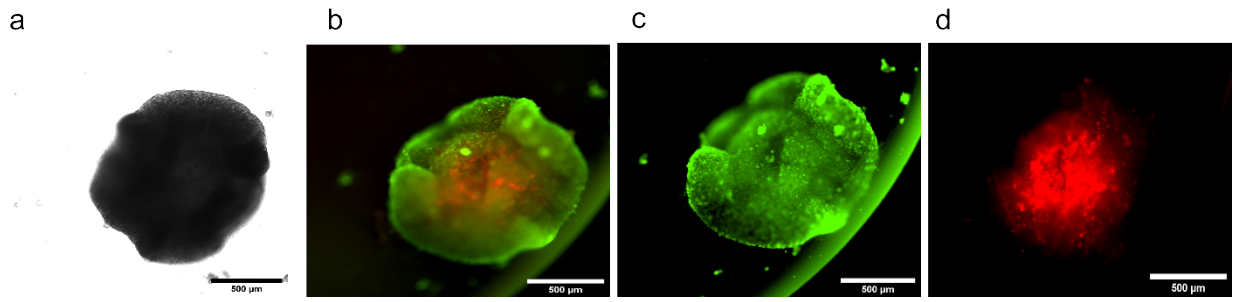


Figure S5. a. Bright field image of 4 days old MDA-MB-468 spheroid; b. Same spheroid after staining with Calcein/PI in dual channel; c. FITC channel showing live cells; d. Spheroid in TxRed channel showing PI stained dead core of spheroid.

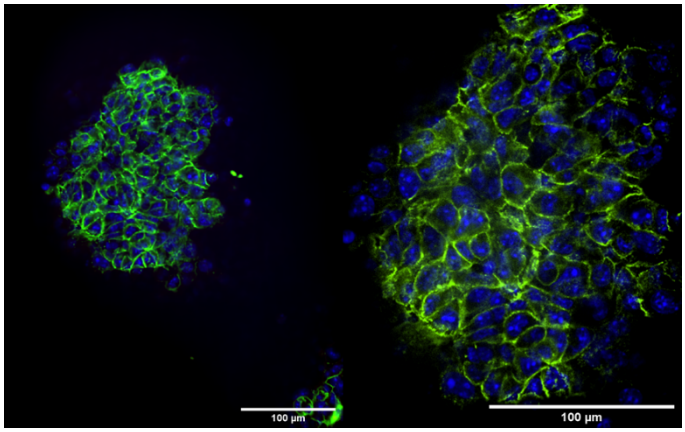
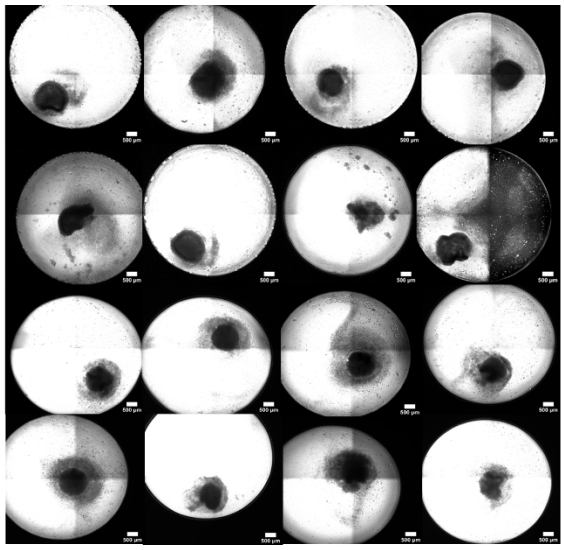


Figure S6. Confocal microscopy image of a 4 days old MDA-MB-468 spheroid stained with Alexa fluor 488 conjugated anti e-cadherin antibody where the cell nucleus were counterstained with DAPI.

a.

b.



c.

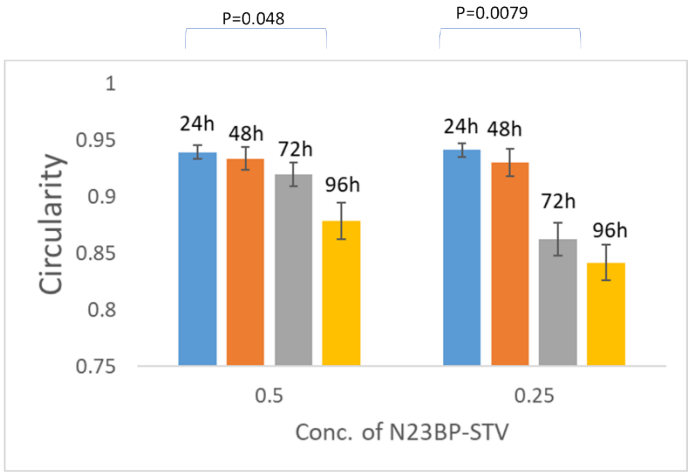


Figure S7: Bright field image of 96 h old MDA-MB-468 spheroids grown for statistical analysis; a. 0.5 µM N23BP-STV; b. 0.25 µM N23BP-STV; c. Changes of circularity of spheroids with time and concentration of N23BP-STV. Error bars represent standard error. $p < 0.05$; calculated by t test (n=8).

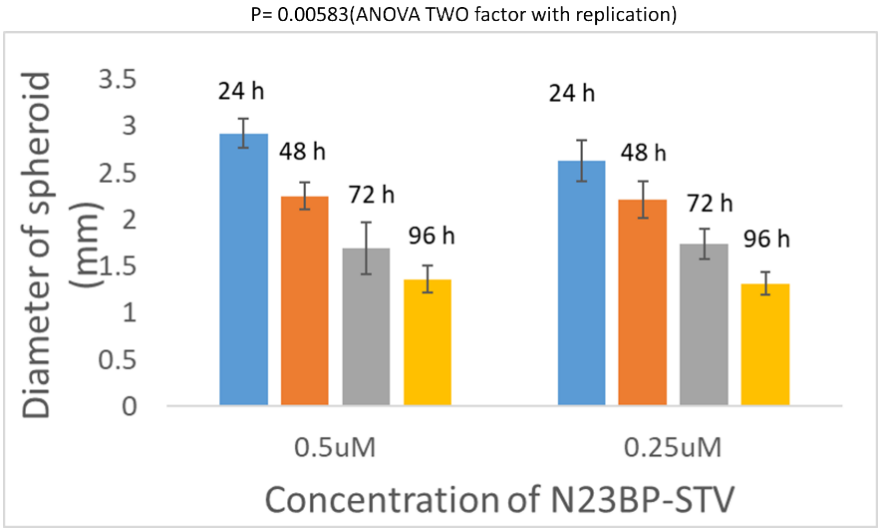


Figure S8: Changes of spheroid diameter with time and concentration of N23BP-STV. Error bars represent standard error. p value was calculated by a two factor ANOVA analysis.

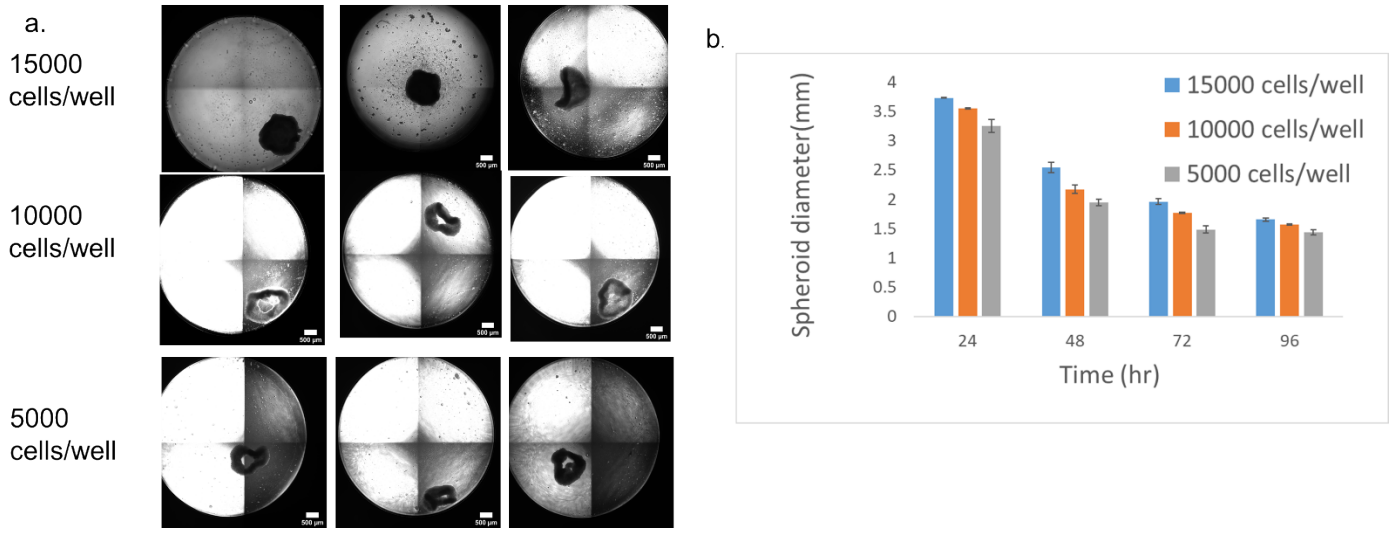


Figure S9: a. Bright field images of MDA-MB-468 spheroids after 96 h where cells were conjugated with 1 μM N23BP-STV and reacted with 1 μM DNA (Sequence 1 and Sequence 2) and incubated in polyHEMA coated wells using different seeding densities of 15000, 10000 and 5000 cells/well in presence of 1 μM complementary linker strands. b. Changes of spheroid diameter with time for different seeding density.

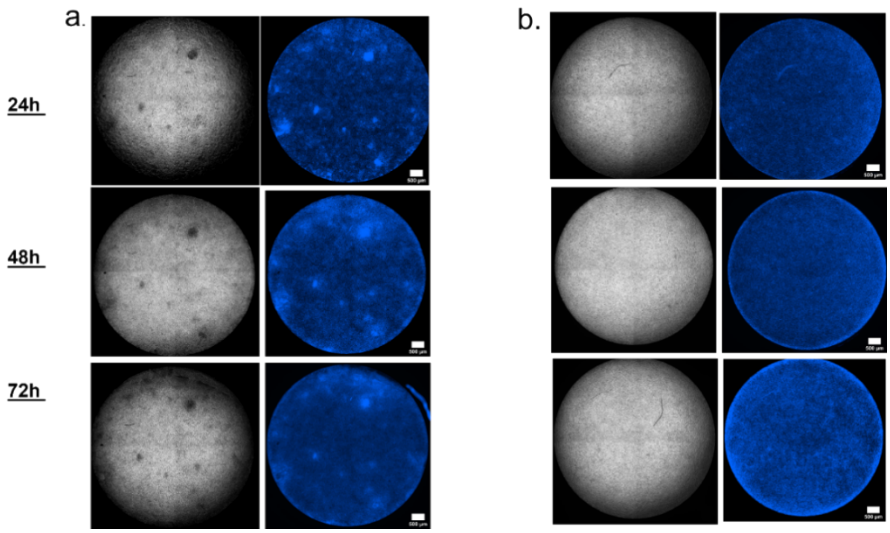
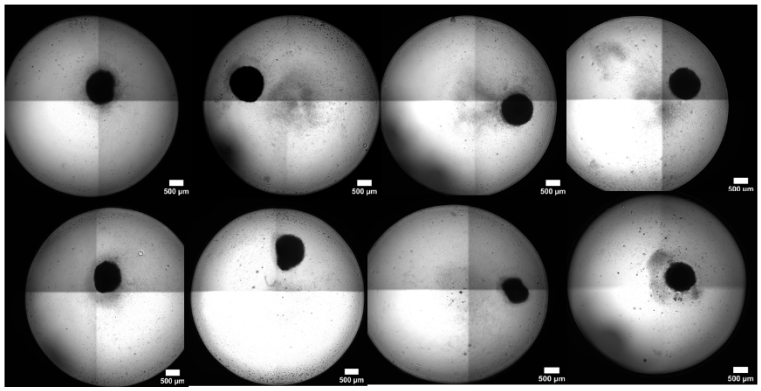


Figure S10. a. Mix of (1μM N23BP-STV+ 1μM Sequence 1+ MDA-MB-468 cells) and (1μM N23BP-STV+ 1μM Sequence 2+ MDA-MB-468 cells) in presence of 1μM linker strands were incubated in a non polyHEMA well plate. b. Only cells incubated in a non polyHEMA well plate. Scale bar corresponds to 500μm.

a.



b.

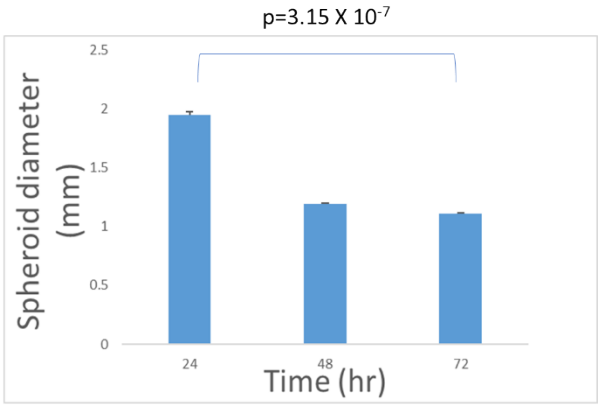


Figure S11: Bright field image of 72 h old HaCaT spheroids grown for statistical analysis with 0.5uM N23BP-STV; b. Change of spheroid diameter with time; c. Change of circularity of spheroids with time. Error bars represent standard error. p values were calculated by t test (n=8)

Caco-2 aggregates: After transfer

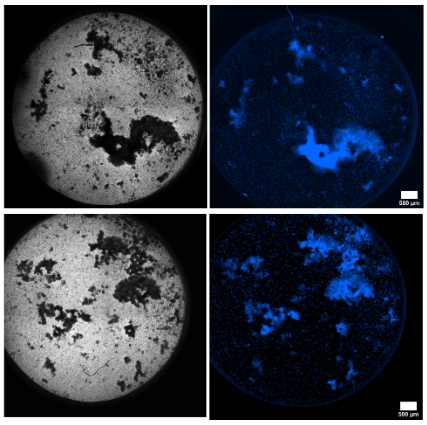


Figure S12. Caco-2 cell aggregates after transfer to a non-coated well.

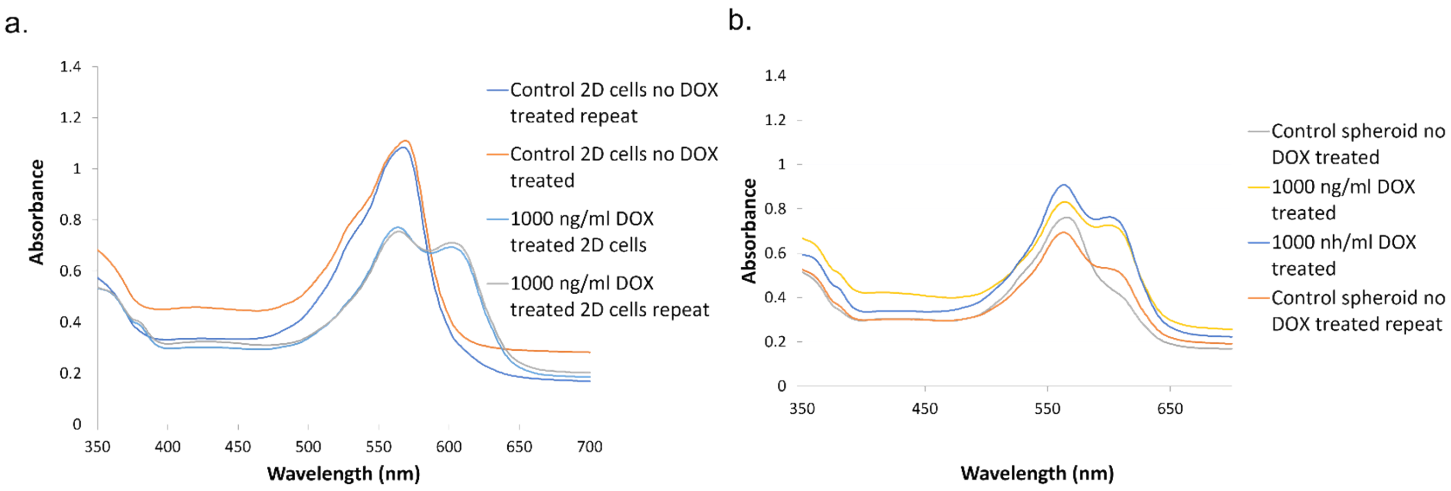


Figure S13. UV absorbance plot of a. 3D spheroids and b. 2D cultured cells.

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