# Supplementary Information

## Deciphering Breast Cancer Cell Heterogeneity: A Quantum Dot-Conjugate Approach in MCF-7 and THP-1 Co-Cultures

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## S.1. Methodology

### S.1.1. Synthesis of Quantum dots

To synthesize the $QD^{\lambda}$ with color coded for blue, green and red respectively through Solvothermal chemical synthesis using chemicals include Cadmium Oxide (CdO) Cadmium acetate (CdAc), Zinc Oxide (ZnO), Zinc Acetate (Zn $(Act)_2$), Oleic Acid (OA), 1-Octadecene (1-ODE), Sulfur powder (S), Selenium powder (Se), Chloroform, Acetone, n-Hexanes, Trioctylphosphine Oxide (TOPO), Diphenyl phosphine (DPP) and Methanol are purchased with GC grade from Sigma-Aldrich, Merck-Millipore and SRL chem Ltd. All chemicals are kept safely under dry chemical cabinet hood to avoid oxidation. Previous reports include Peng et al., 2001[1] and Bae et al., 2008[2] protocols are used with substantial modification such as the temperature, and the rate of reactions to achieve respective fluorescence emissions for QDs. The individual protocols for the respective $QD^{\lambda}$ s are discussed below and which are reported previously [3-7].

#### *S.1.1.1. $QD^{450}$ (Blue emission Quantum dots)*

$QD^{450}$ was made by combining two precursor solutions, Sol A and Sol B, into a single solution. Then, in three necked flasks, add 10 mM Zn $(Act)_2$ and 1 mM CdO, add 7 mL OA, and allow the reactants to gradually increase in temperature from 90$^{o}$C to 150$^{o}$C for 1hrs under vacuum with a 10$^{o}$C interval, Sol B: 2. mM S powder was dissolved in 10 mL 1-ODE in the three-necked flask, followed by heating the reactants at Set the temperature of Sol A to

320$^{o}$C for nitrogen purging, then use a Syringe pump to add 3.0mL of Sol B to the Sol A. Allow 30 min for the reaction to complete, then add another 3.0mL of Sol B to the formed ZnS shells to allow them to overgrow. Allow one hour for the reaction to complete, with incremental assessments of fluorescence intensities every 10 min. Remove the heating mantle from the process, cool the reactants to ambient temperature, then add extra ethanol to precipitate the $QD^{450}$ out. For future use, keep the powder $QD^{450}$ at 4°C. In n-hexane or chloroform, these QDs can dissolve.

### *S.1.1.2. $QD^{525}$ (Green emission Quantum dots)*

$QD^{525}$ was made by combining three precursor solutions, Sol A, Sol B, and Sol C, into a single solution. Sol A: An 80:20 ratio of DPPSe and S was added to a three-necked flask, along with 10 mL 1-ODE, and the reaction was allowed to continue at 140$^{o}$C until a clear solution was obtained. Sol B: In a three necked flask, combine 128 mg of CdO, 2.5 mL of OA, and 8 mL of 1-ODE; allow to react at 260$^{o}$C for 30 min to obtain a clear pale-yellow solution; then add 3.0 mL of Sol A in Sol B; allow to react at 280$^{o}$C for 30 min; stop the reaction by removing the heating mantle; then purify the $QD^{\lambda}$ with excess Acetone and overcoat with shell. For an optimum utilisation, Zn/S was done in the same way utilising Sol C (Zinc and Sulphur were pre-prepared). The $QD^{\lambda}$ are coated with 2 to 3 monolayers of Zn/S shells and stored in n-hexane for later use.

### *S.1.1.3. $QD^{615}$ (Red emission Quantum dots)*

$QD^{615}$ was made by combining three precursor solutions, Sol A, Sol B, and Sol C, into a single solution. Sol A: In a three-necked flask, 138 mg CdAc, 356 mg ZnO, 20 mL 1-ODE, and 5mL TOPO were introduced and allowed to react, followed by a steady increase in temperature from 60$^{o}$C to 120$^{o}$C under vacuum, then to 310$^{o}$C under nitrogen purging until a clear solution was visible. Sol B was made afterwards with 1.60 g Se powder dissolved in 20

mL TBP. Sol C: 0.2 mL $(CH_3)_3Si)_2S$ in a three-necked flask with nitrogen in a 5 mL TOP Add 1.0 mL Sol B to Sol A at 310°C, then lower the temperature to 280°C for 10 min, then add 1.0 mL Sol C, allow the reaction to cool to 80°C for an hour, then add butanol and n-hexane in equimolar mixes of roughly 40 mL, then add excess methanol to precipitate off the $QD^{\lambda}$. $QD^{\lambda}$ can be stored as powder or dissolved in n-hexane or chloroform. Shells are prepared as mentioned in $QD^{525}$.

**S.1.2. Preparation of Water-soluble Quantum dots**

To make ligand exchange on the surface of the individual $QD^{\lambda}$, chemical-based ligand surface exchange was done using the 3-Mercaptopropionic acid (MPA), Chloroform, Methanol, Ethanol, Acetone and Millipore water were used. Previous reports with application changes include Larson et al., 2003[8], Liu et al., 2007[9]and Xing at al., 2007[10] for ligand exchange methods which were further mentioned in earlier research[5, 6]. To do water soluble $QD^{\lambda}$, 1.0 mL of MPA was dissolved in 10.0 mL of methanol, and the solution was then adjusted to pH 12 with the addition of a 11.0mL amount of TMAH (tri methyl ammonium hydroxide). A series of MPA−methanol solution (1.0. and 2.0 mL) was then added into a 1.0 mL $QD^{\lambda}$ (dissolved in Chloroform) were added to 10 mL Chloroform solution (100 mg/5mL QD solution, Conc 0.34 mM) and stirred for 30 min to get the precipitation of the $QD^{\lambda}$. Then, 10.0 mL of Millipore water was added into the mixture and kept stirring for another 20 min. The solution was separated into two phases finally, and the QDs were transferred into the supernatant aqueous phase from the underlying chloroform. The underlying organic phase was discarded, and the aqueous phase containing the $QD^{\lambda}$ was collected. The free MPA ligand in the $QD^{\lambda}$ aqueous solution was isolated by precipitating the $QD^{\lambda}$ with the addition of acetone. The supernatant was discarded, and the pellet was then redissolved in Millipore grade water and stored at 4$^{o}$C for further usage.

### S.1.3. Conjugation of Antibodies on the Quantum dots

In order to conjugate the antibodies (Abs) on the surface of $QD^{\lambda}$, antibodies Anti-EpCAM, Anti-CD45 and AntiCD44 are purchased from Thermofisher scientific inc. and Sigma Aldrich with purified form which having a terminal biotin group. All the Abs are stored at -20$^{o}$C to extend their shelf life. 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfo-succinimide (NHS) and 2-(N-morpholino) ethane sulfonic acid buffer (MES) is purchased form SRL. ltd and Sigma-Aldrich. Pathak et al., 2007[11, 12] and Chen et al., 2010[13] reports were mentioned for the conjugation process to be implemented in this current work with minor modifications.

$QD^{\lambda}$ primarily modified with MPA ligand ($QD^{\lambda/MPA}$) exchange for the active –COO- group as mentioned in the above step, the $QD^{\lambda/MPA}$ are then activated with EDC/NHS reaction as carboxy amine polyethylene glycol (PEG) and Streptavidin (SA) conjugation. To achieve successful conjugation of the Abs on the surface of QDs, an equimolar mixture of 1 mM solution of EDC and NHS were prepared in the MES buffer at 4$^{o}$C (all the reaction are to be worked under chilling condition to maintain the integrity of the Abs). 1mg of the individual $QD^{\lambda/MPA}$ (Quantum dots with MPA ligand) are measured and dissolved in Millipore water, followed by addition of the 200 µL of EDC and the reaction was kept at 37$^{o}$C for 15 min followed by addition of 200 µL of NHS for 15 minutes to form an intermediate unstable compound to form an active carboxylic group facilitate to form a peptide bond with first PEG and followed by SA. 1 µg/lit of respective molecule was added to the above solution and allowed to from a strong $QD^{\lambda/MPA/PEG}$ and $QD^{\lambda/MPA/PEG/SA}$ (Quantum dot with PEG and streptavidin).

### S.1.4. Cytotoxicity of the Quantum dots

For cytotoxicity experiments, the MTT and SDS stock solutions were produced per the manufacturer's instructions and stored at 4°C in a light-protected environment. Before applying the $QD^{\lambda/MPA}$, and $QD^{\lambda/MPA/PEG}$ individual cells were sown in the 96 well flat bottom microtiter plates overnight. Dissolve the necessary amount of stock $QD^{\lambda/MPA}$, and $QD^{\lambda/MPA/PEG}$ in the DMEM media with concentrations from 0.1, 1, 10, 20, 40, 60, 80 and 100 µg/mL (Without phenol and FBS). Microtiter plates with MCF-7, and THP-1 cells had 10 µL of each stock solution added to each well. For 24 hrs, these cells were grown in the favourable conditions. MTT-PBS (MTT dissolved in Phosphate buffer saline) solution was added to the cells and incubated for four hours to measure cell viability. To dissolve the formazan crystals generated by live cells, 100 µL SDS-HCl solution was added to each well. The MTT that interacted with the viable cells was measured at 570 nm using a multi-plate reader (Multiskan Go, Thermo Scientific).

### S.1.5. RGB extraction and surface plotting using Fiji-ImageJ

To obtain the surface plots for the respective RAW images of confocal microscopy, we followed the established protocols include the calibration of the scale[14], Conversion from RGB to grey scale and the extraction of the surface plot using the 3D plot-based plug-ins[14-16].

## SUPPLEMENTARY FIGURES

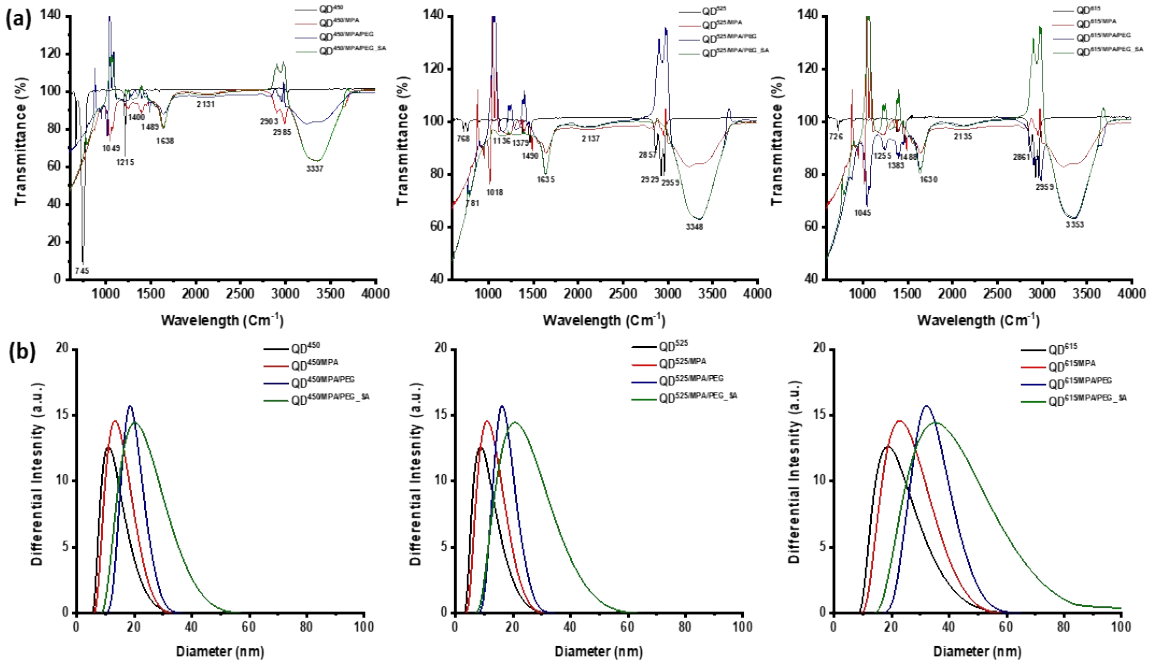



**Fig S1. Fourier Transform Infrared Spectroscopy and Dynamic Light Scattering** of the $QD^{\lambda}$ for surface modifications include naked $QD^{\lambda}$ with original surface ligands (Oleic acid, Diphenyl Phosphine and Trioctyl phosphine) in n-hexanes, 3-Mercaptopropoinc acid in Millipore grade water, PEG in Millipore grade water and Streptavidin modification in 1xPBS buffer at pH 7 for **(a)** $QD^{450}$ **(b)** $QD^{525}$ and **(c)** $QD^{615}$

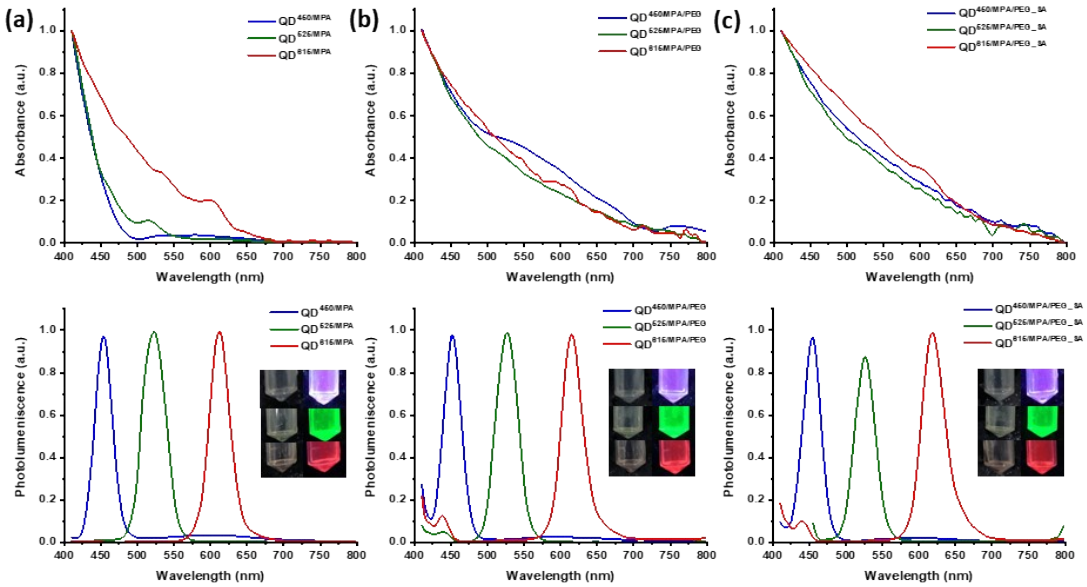



**Fig S2. UV-Visible absorption and Fluorescence spectrum** of the $QD^{\lambda}$ for surface modifications include 3-Mercaptopropoinc acid in Millipore grade water, PEG in Millipore grade water and Streptavidin modification in 1xPBS buffer at pH 7 for **(a)** $QD^{450}$ **(b)** $QD^{525}$ and **(c)** $QD^{615}$, the in-sights showed the bright fluorescence of $QD^{\lambda}$ in the hexane solution include the blue, green, and red fluorescence

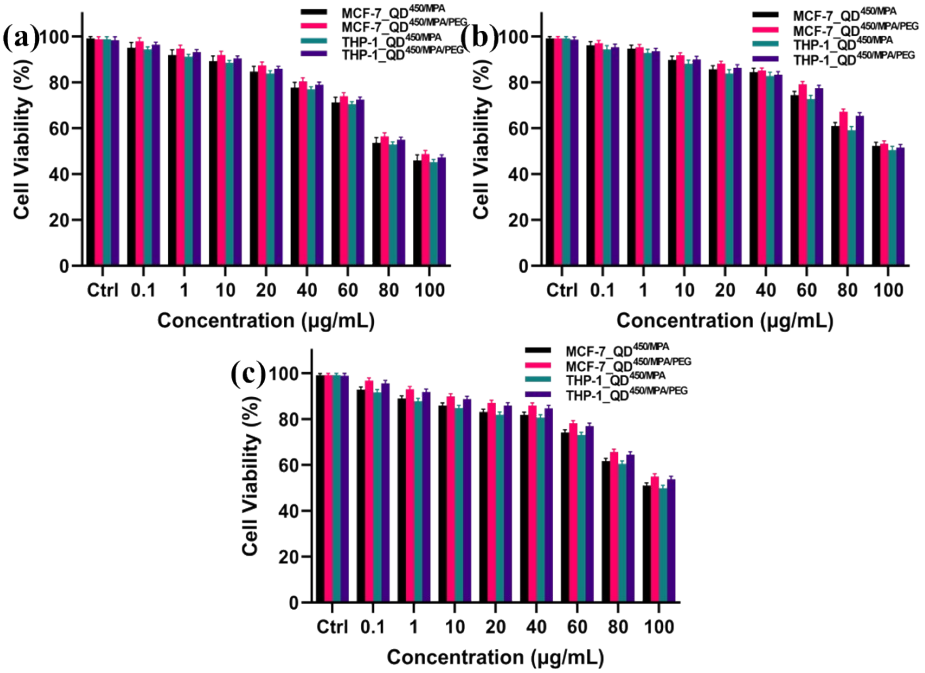
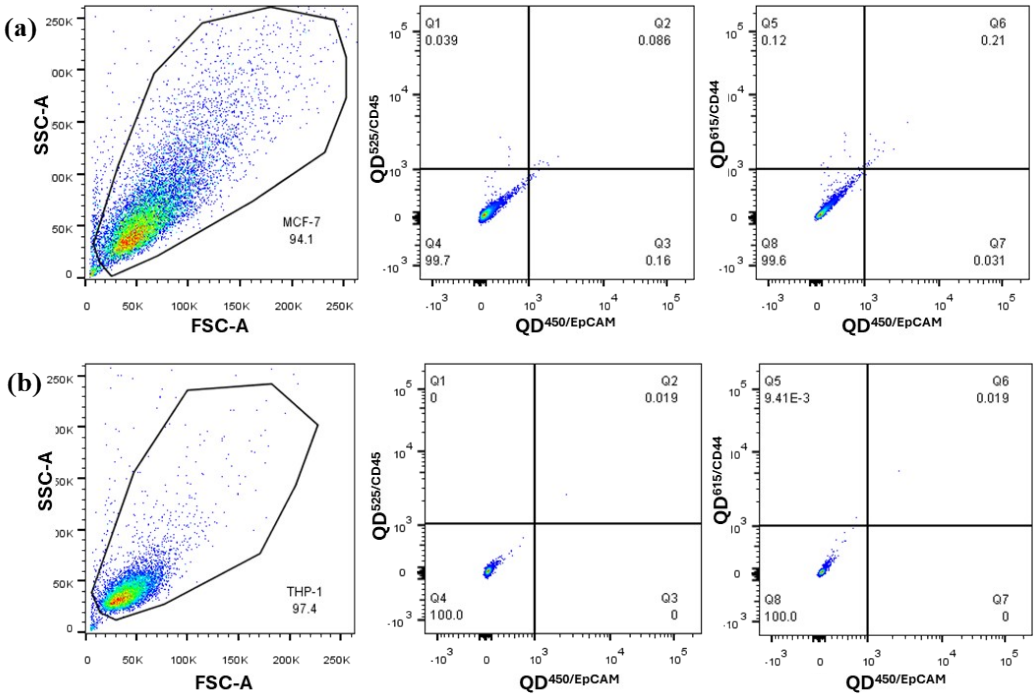



**Fig S3. Cytotoxicity of QD$^{\lambda}$ (QD$^{\lambda/MPA}$ and QD$^{\lambda/MPA/PEG}$)** suspended in the individual cultures of MCF-7 and THP-1 cell lines **(a)** QD$^{450/MPA/PEG}$ **(b)** QD$^{525/\ MPA/PEG}$ and **(c)** QD$^{615/}$ MPA/PEG




**S4 Fig. Flowcytometry dot plot images** for controls, where the cells lines are in co-culture incubated in 1x PBS were analysed through flowcytometry (a) MCF-7 and (b) THP-1 cancer cell lines with devoid of fluorescence emissions due to absence of QD$^{\lambda/ab}$

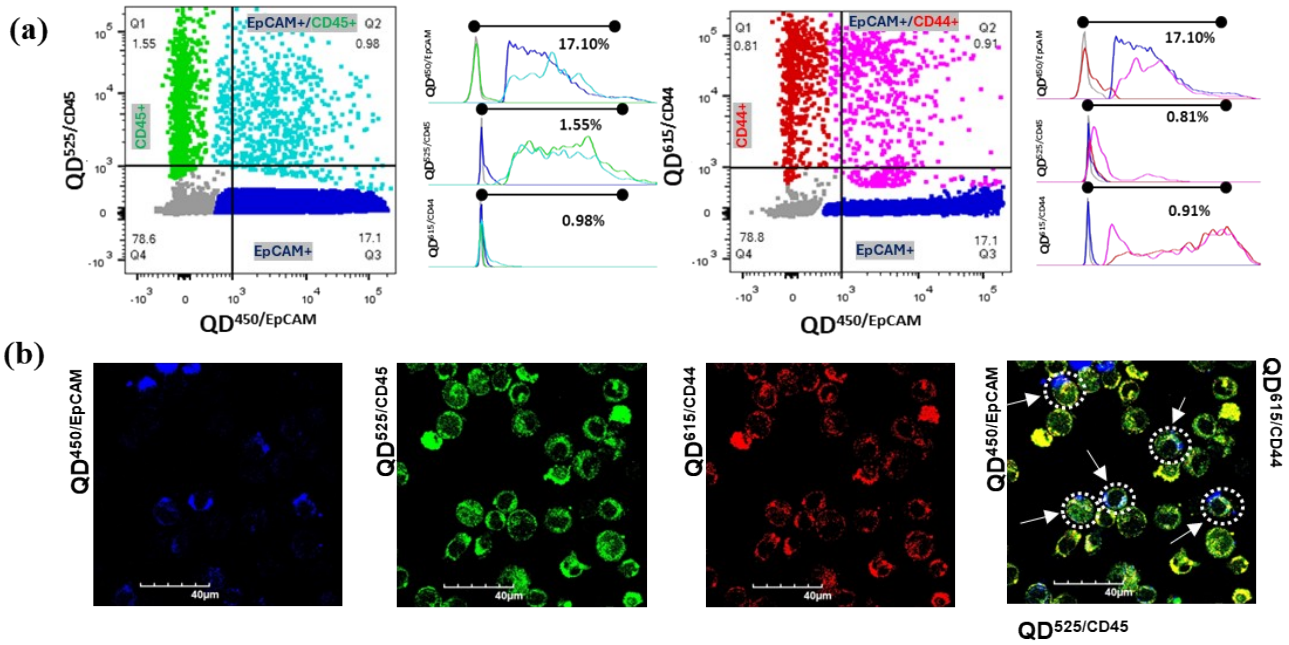



**Fig S5. Flowcytometry dot plot images** for capture the EpCAM+ and EpCAM- cells by using the $QD^{\lambda/Ab}$ conjugated with anti-EpCAM, anti-CD45 and anti-CD44 cells in co-culture cells in *in-vitro* incubated in 1x PBS media were analysed through flowcytometry (a) Dot plots of the MCF-7 and THP-1 with reference to $QD^{450/EPCAM}$ to $QD^{525/CD45}$ and $QD^{450/EPCAM}$ to $QD^{615/CD44}$ co-cultures with respective fluorescence emission (b) Sorted cells observed using the confocal laser microscopy

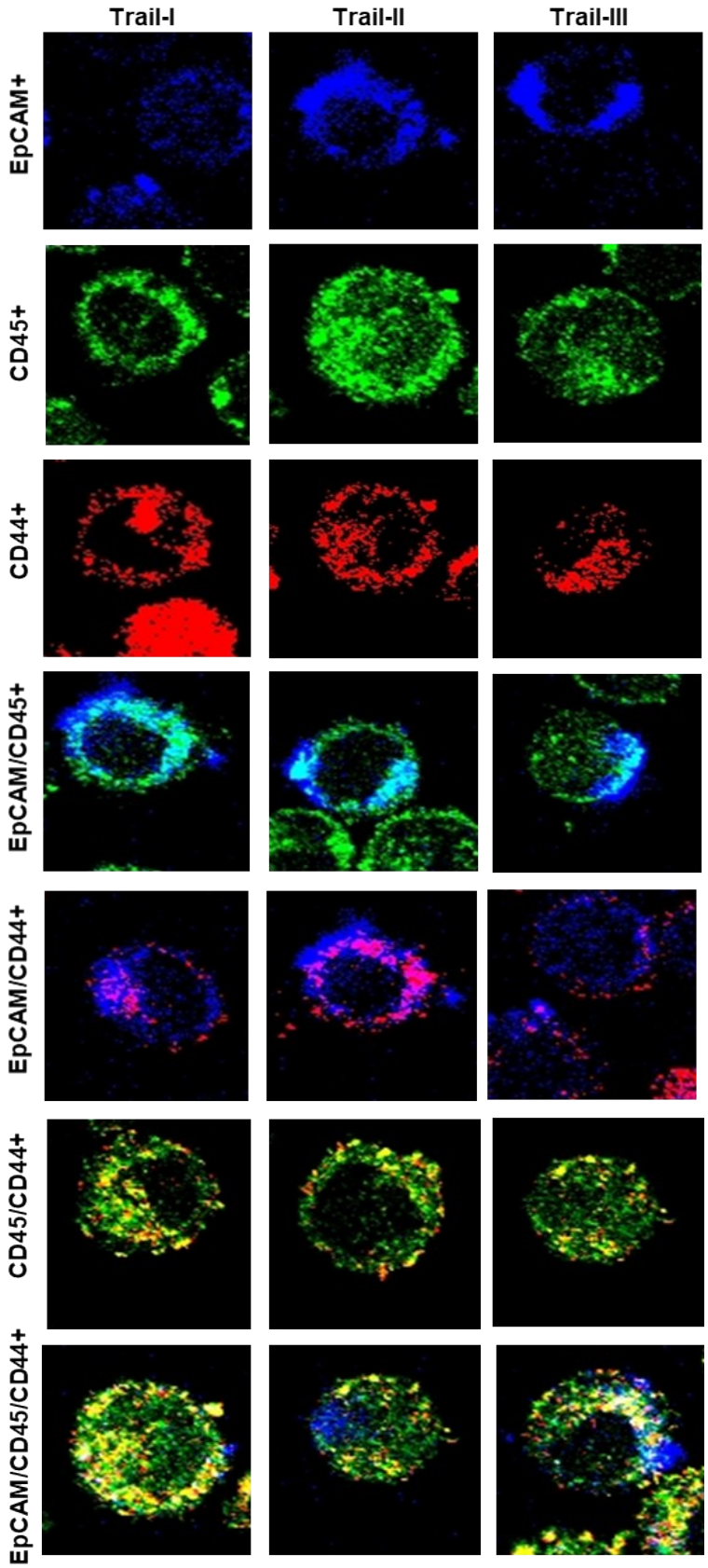



**Fig S6. Confocal microscopic images** of the FACS based collected cells with reference to respective $QD^{\lambda/Ab}$ conjugated bound on the surface of the cells for different trails for heterogeneity

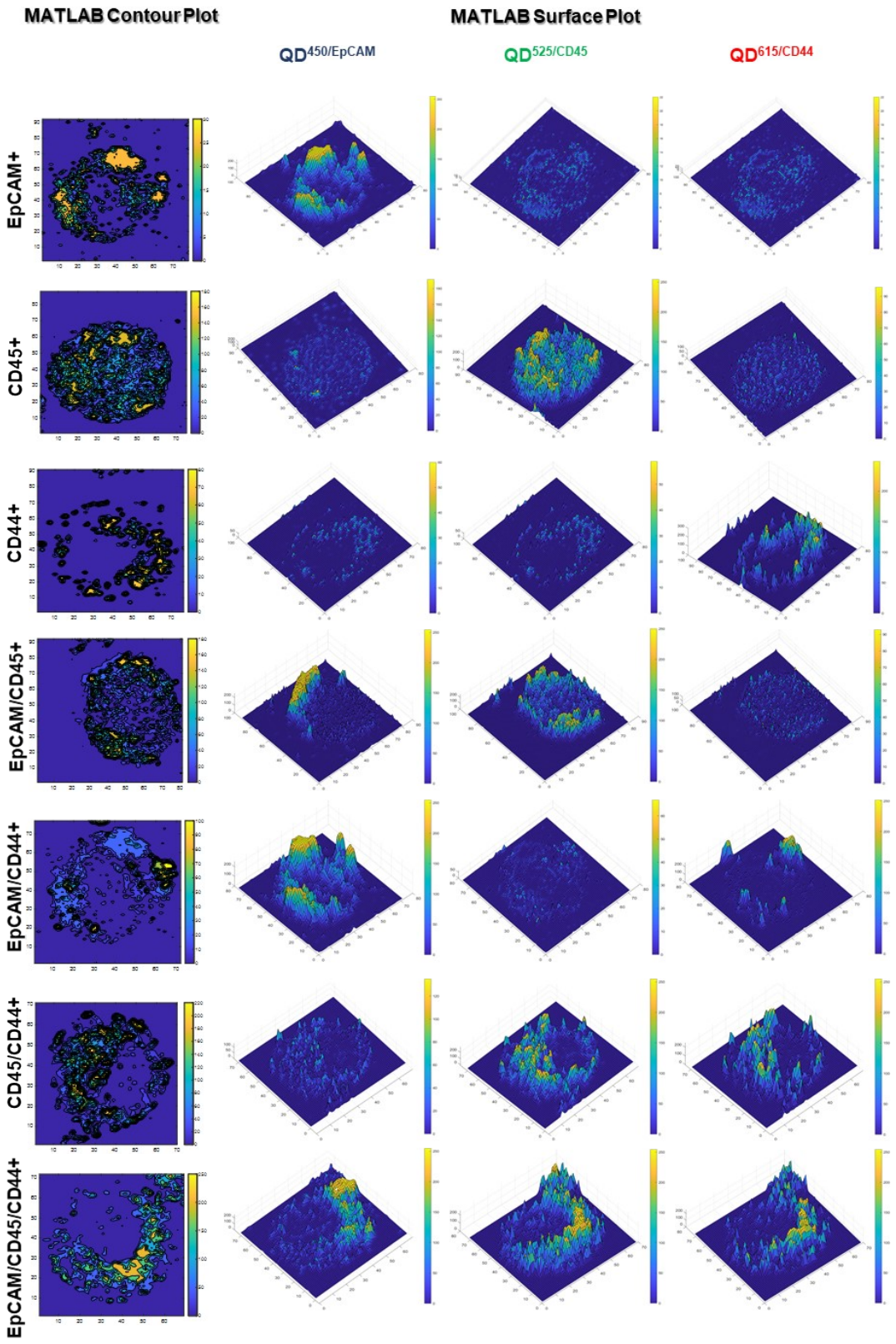



**Fig S7. MATLAB image analyses** for the Confocal microscopic images of the FACS based collected cells with reference to respective $QD^{\lambda/Ab}$ conjugated bound on the surface of the cells for different trails for heterogeneity with respective Counter and surface plots for $QD^{\lambda/Ab}$

## SUPPLEMENTARY TABLES

**Table S1.** Characteristics of the Quantum dots

| Quantum dot | Lattice Composition | ε ($L\ mol^{-1}\ cm^{-1}$) | Emission $\lambda_{max}$ (nm) | Φ (%) | FWHM (nm) |
|---|---|---|---|---|---|
| $QD^{450}$ | CdZnS/ZnS | $9.35X10^{-5}$ | 450 | 79.15 | 28.23 |
| $QD^{525}$ | CdSe/ZnS | $1.58X10^{-5}$ | 525 | 57.14 | 37.63 |
| $QD^{615}$ | CdSe/ZnS | $3.03X10^{-5}$ | 615 | 41.34 | 44.71 |

**ε -**Molar extension co-efficient**; Φ –** Quantum Yield**; FWHM-** Full width Half Maxima

**Table S2.** Characteristics of the surface ligands exchange Quantum dots

| Quantum dot | Emission $\lambda_{max}$ (nm) | Φ (%) | FWHM (nm) |
|---|---|---|---|
| $QD^{450/MPA}$ | 450.34 | 28.60 | 25.49 |
| $QD^{525/MPA}$ | 535.13 | 18.40 | 44.12 |
| $QD^{615/MPA}$ | 620.16 | 11.60 | 31.62 |

**Φ –** Quantum Yield**; FWHM-** Full width Half Maxima

**Table S3.** Quantum dots doses to be added to the co-cultures

| Co-Culture | Quantum dots | Concentration of QDs (µg/mL) |
|---|---|---|
| (MCF-7+THP-1) | $QD^{450}$/MPA | 40.0 |
| | $QD^{525}$/MPA | 40.0 |
| | $QD^{615}$/MPA | 40.0 |

**Table S4.** Quantum dots specific for cell lines

| Quantum dots | Chemical Composition¶ | Surface Modification§ | Cell Type | Cell line |
|---|---|---|---|---|
| **$QD^{450/EpCAM}$** | CdS/ZnS-OA capping | MPA-PEG-SA-EpCAM | Epithelial cells | MCF-7 |
| **$QD^{525/CD45}$** | CdSe/ZnS-DPP capping | MPA-PEG-SA-CD45 | Mesenchymal cells | THP-1 |
| **$QD^{615/CD44}$** | CdSe/ZnS-TOP capping | MPA-PEG-SA-CD44 | Epithelial cells | MCF-7 |

¶ **OA-** Oleic acid; **DPP-** Diphenyl Phosphine; **TOP-** Trioctyl phosphine
§ **MPA-** 3- mercaptopropoic acid; **PEG-** $NH_2$-$(PEG)_8$-COOH; **SA-** Streptavidin

**Table S5.** MATLAB general code for the image extraction and analysis

| Image extraction program | RGB extraction program |
|---|---|
| a = imread('image. file extension');<br>subplot(2,3,1);<br>imshow(a);<br>b = rgb2gray(a);<br>subplot(2,3,2);<br>imshow(b);<br>c = im2bw(a);<br>subplot(2,3,3);<br>imshow(c);<br>d = imadjust(b);<br>subplot(2,3,4);<br>imshow(d);<br>e = a;<br>e=rgb2gray(e);<br>subplot(2,3,5);<br>imhist(e);<br>imfinfo('EpCAM.png')<br>[height, width, colour_planes] = size(a)<br>%colormap('spring') | I = imread('image. file extension');<br>Ihsv = rgb2hsv(I);<br>Ih = histeq(Ihsv(:,:,3));<br>Ihsv(:,:,3) = Ih;<br>IO = hsv2rgb(Ihsv);<br>subplot(1,2,1),imshow(I),title('original image');<br>subplot(1,2,2),imshow(Ihsv),title('Color Histogram'); |

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