Supporting Information

Synthesis and Characterization of Fluorescent Polymeric Nano-thermometer: Dynamic Monitoring of 3D Temperature Distribution in Co-Cultured Tumor Spheroids

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1. Materials and Methods

1.1 Materials:

The Pluronic F-127 (F127), Rhodamine-B (Rh-B), Urea, Paraformaldehyde, and Hydrochloric acid solution (~3 $mol \cdot L^{-1}$) were purchased from Sigma Aldrich (St. Louis, USA). All reagents were of analytical grade and used without further purification. Double-distilled (DD) water (Merck, Millipore Corp. Germany) was used for all experimental purposes, and it was also used for FPNTs syntheses and analyses. The PDMS prepolymer was purchased from Sylgaurd 184 (Dow Corning Co., Midland, MI).

1.2 Preparation of Fluorescent Polymeric Nano-thermometer (FPNTs):

To prepare FPNTs, the first step was mixing of Pluronic F-127 and Rh-B dye (10000:1) in 100 mL of water and stirring for 2.5-3 hours at room temperature (27 ^{0}C) to produce the dye-micelle complex, which serves as the structural core of the compound. Following the completion of the first step, the urea and paraformaldehyde were added with vigorous stirring for 15 minutes before increasing the temperature to initiate the polymerization process. To prepare the dye-micelle polymer complex (DMPC), the mixture was heated at 60 ^{0}C with magnetic stirring for 150 minutes at 60 ^{0}C. To eliminate the insoluble contaminants from the obtained DMPC solution, three layers of filter paper were used to filter out the bigger particles in the solution. Afterward, the filtered solution was mixed with hydrochloric acid to obtain an acidic pH (pH ~3), and the mixture was heated at 100 ^{0}C in a boiling water bath for 120 minutes to allow the formation and cross-linking of the polymer. Which resulted in the formation of Rh-B-embedded polymeric nanoparticles or fluorescent polymeric Nano-thermometers (FPNTs). Further, the prepared FPNTs were washed three times by centrifugation (at 11000 rpm, and 20 ^{0}C) for 45 minutes each time with sterilized DD water, and then FPNTs were air dried. Finally, 0.01mg/ml solution was used in all the characterization and bio experiments. Besides, to investigate the stability and optical properties of the FPNTs, FPNTs were stored at -20 ^{0}C for almost 6 months. Later particles were defrosted at 4 ^{0}C and allowed to obtain room temperature before measuring the intensity.

1.3 Characterization of FPNTs:

To conduct SEM measurement, diluted FPNTs solution was drop cast on a silicon wafer (0.5 x 0.5cm^2) and allowed to evaporate the water of the sample. Prior to the measurements, the dried sample was sputtered with a gold (Au) and imaged. Further, the JSM-7610F, a high-resolution thermal field emission scanning electron microscopy (JEOL Ltd. Tokyo, Japan), was used to image the samples. The DLS of FPNTs were measured by a Malvern Zetasizer Nano ZS90 DLS system (Worcestershire, UK). Briefly, FPNTs were loaded in a DLS cuvette (Malvern Zetasizer, Worcestershire, UK) and samples were loaded in the chamber. Further, at 25 ^{0}C, a laser beam with a wavelength of

633 nm was directed at the diluted sample, and the strength of the scattered signal was measured at a 173^0 angle. The same machine was used to measure the zeta potential of the FPNTs too at the same time.

The Fourier-transform infrared (FTIR) spectroscopy [JASCO FT/IR-4200 spectrometer (JASCO Inc. TX. USA)] was used to measure the bonding characteristics of the FPNTs. The liquid sample was dropped on the sensor (crystal) and followed the steps to measure the spectrum as mentioned by the manufacturers. The absorbance mode was used to acquire the whole spectrum (4000 cm^{-1} to 400 cm^{-1}). For 1H Nuclear Magnetic Resonance (1H NMR) analysis, about 20 mg of FPNTs was directly mixed with 1 mL d-DMSO and the mixture was placed in an NMR tube. The spectrum was obtained on a Digital Nuclear Magnetic Resonance Spectrometer Bruker NMR instrument 700 MHz.

The fluorescence spectroscopy [JASCO FP-6500 fluorescence spectrophotometer (JASCO Inc. TX. USA)] was used to measure the intensity variations of the FPNTs with different temperatures. As aforementioned, 0.01mg FPNTs were diluted in 1 ml of sterilized DD water. On the other hand, during this measurement permanent heating and temperature measurement setup was built. Briefly, a thin heating wire (coiling shape) was fixed inside the quartz cuvette and applied voltage and current through an external direct current (DC) power supplier (GPD-3303, GwINSTEK, Taiwan) A K-type thermocouple was attached to a digital meter (NoEnName_Nuill, China) was inserted into the same quartz cuvette to measure the exact temperature. Further, the whole setup was mounted in fluorescence spectroscopy and the cuvette was filled with 3.5ml of FPNTs for the intensity measurements. The temperature was increased from 25 ^{0}C to 100 ^{0}C with 10 ^{0}C intervals and subsequent intensity spectra were acquired and plotted by using Origin 8.0 software. Normal DD water was used as the background and the whole experiment was done in a dark environment.

1.4 Fabrication of PDMS Temperature Calibration (PTC) Chip:

For the temperature calibration, PTC comprises three layers: - Glass-PDMS-Glass substrate, as shown in Figure S1. The top and bottom glasses were cleaned with standard acetone and IPA cleaning. In parallel, the 10:1 (v/v) ratio of Silicone elastomer 184-A (Sil-more industrial Ltd. Taipei, Taiwan) and curing agent Sylgard 184-B (Sil-more industrial Ltd. Taipei, Taiwan) was mixed thoroughly and removed bubbles by vacuum suction. Further, PDMS was poured (7-8 mm thick) on a pre-cleaned silicon wafer (3") and cured at 80 ^{0}C for 2 h. Later PDMS was slowly peeled off see Figure S1(a) and macro-well arrays (radius 5mm) were created with the help of a PDMS biopsy puncher see Figure S1(b). After the punching process, PDMS was cleaned thoroughly, corona plasma cleaned (Model BD-20,

Electro-Technic products, INC. USA) [Figure S1(c)] and bonded on the bottom glass substrate, and the top glass slide was kept detached [Figure S1(d)]. The top glass layer was used as a lid to control the liquid evaporation.

1.5 Temperature-dependent fluorescence intensity calibration of FPNTs on PTC chip:

Firstly, the wells of the PTC chip were loaded with the diluted FPNTs and air-dried [Figure S2(a)]. Further, wells with FPNTs were filled with glycerol to maintain a uniform temperature [Figure S2(b)], and later PDMS was covered with a glass slide (top layer) [Figure S2(c)]. Later, a K-type thermocouple was pierced into the well through the side wall of the PDMS to measure the local/solution temperature and this whole setup was placed on a heating pad (SP1848-27145 Thermoelectric Peltier Cooler). The temperature of the heating pad was controlled through a temperature controller (GX66S, Yeong Shin Co. Ltd. Taiwan), and the K-type thermocouple was also plugged into the same controller to read the solution/local temperature back [Figure 3(a)]. However, the K-type thermocouple has a temperature reading accuracy of 1.1 ^{0}C. The whole setup with a heating pad was placed on a fluorescent upright microscope (70X Olympus, Japan) and images were acquired by a CCD camera (M-shot, Micro-shot Technology Limited, China). When the FPNTs were exposed to light, temperature-sensitive Rh-B dye was excited with a green light at the wavelength of ~553nm and emitted with red color at the wavelength of 627nm as shown in Figure 3(a). The temperature calibration was done from 25 ^{0}C to 100 ^{0}C with a 5 ^{0}C interval. Finally, the captured images were processed with open-source software Image J (NIH, USA) and quantified the fluorescence intensities by the same software by using its in-built functions to extract the mean pixel intensity and standard deviation for each fluorescent spot in the picture. Precisely, the average value of 15 intensity points was plotted (Figure 3(c)) at each temperature point by using Origin software.

1.6 Open µ-Well 3D Cell Culture Chip Design and Fabrication:

The open µ-well 3D cell-culture platform was created to co-culture the HCT-8 (colon cancer) and NIH3T3 (fibroblast) cell lines. AutoCAD software (Autodesk Inc. USA) designed the micro-wells dimension and mask. The well-established soft lithography technology was used to create the SU-8 pillars [Figure S3(a)] and then the PDMS molding technique was used to create open micro-wells (500µm diameter and ~500µm depth) for 3D cell culture as shown in Figure S3(b)[1].

1.7 Determination of Half-Maximal Inhibitory Concentration (IC50) on 2D Cultures:

HCT-8 (Colon Cancer cell line) and NIH3T3 (fibroblast cells) were initially seeded with 3000 cells/well with a complete growth medium. Further, the prepared FPNT was mixed uniformly in PBS and added individually to each well of the 96-well plates at a final concentration of 100, 50, 25, 10, 5, 1, 0.1, 0.01, and 0.001 µM in the cell culture

medium for 72 h. After 72 h of incubation, the medium containing FPNT was slowly removed, cleaned with PBS three times, and replaced with 100 µl of CCK-8 mixed medium. In all 2D experiments, cell viability was quantified by using CCK-8 (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) colorimetric assay at an absorbance of 450 nm according to the manufacturer's instructions. Plates were incubated for 30 min and absorbance was measured by using a benchtop plate reader (GloMax® Explorer Multimode Microplate Reader, Promega, Taiwan). Background absorbance was subtracted from each sample before calculations of the absorbance ratio. Cell viability [56.23±7.43 for HCT-8 and 38.02±10.84 for NIH3T3] values were calculated at different log concentrations [Figure S4] and sigmoidal dose-response curves were calculated through origin software.

1.8 *In Vitro* 3D Co-culture on an open µ-Well 3D Cell Culture Chip:

The HCT-8 and NIH3T3 cells were used in this study and obtained from the Bioresource Collection and Research Center (BCRC) in Hsinchu, Taiwan. As a growth media, a 1:1 mixture of RPMI-1640 (Dow Corning Co., Midland, MI USA) and DMEM (Dow Corning Co., Midland, MI USA) was used to co-culture the 3D tumor spheroids. To produce the 3D tumor spheroids in all of the tests, a 1:1 mixture of HCT-8 and fibroblast cells were used in each experiment as shown in Figure S5(a)[1]. Figure S5(b) shows the Cellular arrangement (HCT-8 (Red), Fibroblast (Green)) of the tumor microenvironment after 9 days.

1.9 Dynamic Temperature Measurement inside the 3D Co-Cultured Tumor Spheroids:

The FPNTs were used to test the temperature inside the 3D Co-Cultured Tumor Spheroids. Prior to seeding cells on the µ-well 3D cell culture platform, the prepared FPNTs (5µl) were mixed with a 1:1 ratio of cells. As previously stated[1], after the introduction of cells and FPNTs into the µ-well 3D cell culture platform, the gravitational force will pull the cells into the micro-wells within 4-5 minutes. The cells outside the wells were slowly sucked out without disturbing the cells inside the wells. Further, a 500 µl medium was added to the chip and kept for incubation in a humidified incubator (MCO-18AIC CO_2 Incubator, Sanyo, US) with 5 % CO_2, at 37 ^{0}C. Spheroids were observed regularly with the help of an inverted microscope (70X Olympus, Japan). Pipettes were used to collect co-cultured tumors at 2, 5, and 9 days after the start of the experiment. The tumors were aspirated into a pre-pasted reinforcing ring on the coverslip slide and carefully cleaned the spheroids. Further, those spheroids were incubated with Hoechst for 30 mins in a humidified incubator. After the incubation, spheroids were cleaned 3 times with PBS and added to the mounting medium, and sealed the spheroids for imaging. The slicing and images of the 3D spheroids were done by using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). The FPNT will be providing the red

fluorescence emission (627 nm wavelength), and the strength of the fluorescence emission is inversely proportional to the temperature gradient[2]. The z-scanning intensity profile was measured along the prescribed marking line (yellow color) (n = 3) as shown in Figure S6. Similar to the previous study[1] the z-stacks were separated into 10 μm of each slice, and the average of these 10 slices and 15 intensity points within a 100 μm region was calculated. We analyzed the temperature gradient inside the co-cultured tumor spheroids with a fluorescence-based nano-temperature sensor (FPNTs). The measured intensities on confocal microscopy were approximated based on normalized intensities of different regions [center (80-100μm), exterior, and interior (80-100μm)] as shown in Figure S6. The measured values represent the temperature difference (~2.9 ^{0}C) from the core region (34.9 ^{0}C) to the peripheral region (37.8 ^{0}C) in desmoplastic 3D tumor spheroids and these results were consistent with previous studies[3].

2. Reaction Mechanism for the FPNTs Preparation:

Temperature-sensitive FPNTs are spherical probes, composed of Urea (NH_2CONH_2) and Paraformaldehyde [$HO(CH_2O)_nH$] resins and the Rh-B ($C_{28}H_{31}ClN_2O_3$) conjugate with Pluronic F127 ($C_3H_6O{\cdot}C_2H_4O$)x. The Rh-B and Pluronic F127 conjugate through a hydrophobic and hydrophobic interaction where the carboxylic part of Rh-B interacts with the methyl group of Pluronic F127 [S7(a)] and formed a dye-micelle complex (DMC). Urea and paraformaldehyde (UF) resins are primarily made up of urea and paraformaldehyde with paraformaldehyde acting as the cross-linker. The UF resins are formed in water at a pH above 7 at the start of the reaction, because the methylol derivatives that form in the first steps condense rapidly under acidic conditions, as illustrated in S7(b)[4]. Condensation of the amine group and hydroxyl group forms the backbone of the UF resin. As shown in figure S7(c) covalent linkage of DMC with UF resin prevents the leaching of the fluorophore. Rh-B molecules were uniformly integrated into the polymer composite nanoparticles and covalently encased in the 3D cross-linked UF resin with preserved fluorescence characteristics S7(d).

3. The quenching ability of FPNTS:

We analyzed the fluorescent intensity of FPNTs for repeated 50 cycles and observed there were significant changes in the intensity as shown in Figure S8. Each cycle duration was for 60 seconds. The first 30 seconds of illumination were done, and the next 30 seconds were kept unexposed. As shown in Figure S8, the intensity profile of FPNTs is nearly linear for the entire 50 cycles, and it was observed that the fluorescence intensity changed slowly for the first 5 cycles and rapidly for the subsequent 5 cycles (a total of 10 cycles) [inset S8].

Table 1: *Comparison of performance characteristics of the temperature sensors*

Type of Sensor	**Temperature Range (^{0}C)**	**Sensitivity**	**Reversibility**	**Calibration Environment**	**Cell types**	**Cellular level**	**Refs**
CdSeS/ZnS@polymer	25-45	-1.55%/°C	Positive	In water	HepG2	Dotted inside cell	[84]
CdSe QDs	30-60	0.015%/°C	Positive	In PBS	Mouse tissue	Local tissue	[74]
Nano needle	24-60	N/A	Positive	In water	N/A	The local region in the cell	[75]
Au nanoclusters	15-45	N/A	Positive	In cell	HeLa	Dotted in cell	[27]
Nanodiamonds (N-vacancy centers)	54	N/A	Positive	In air	WS1	Dotted in cell	[76]
Au/Pd Thermocouple	17-37	N/A	N/A	In cell	N/A	Subcellular	[85]
Si Resonator	30-59	-1900 ppm/K	N/A	In cell	N/A	Single-cell	[86]
gTEMP fluorescent protein	5-50	N/A	Positive	In cell	HeLa	Subcellular	[77]
FPNTs	25-100	1.1^{0}C	Positive	In Glycerol	3D Co-Culture	HCT-8 and NIH3T3	Present work

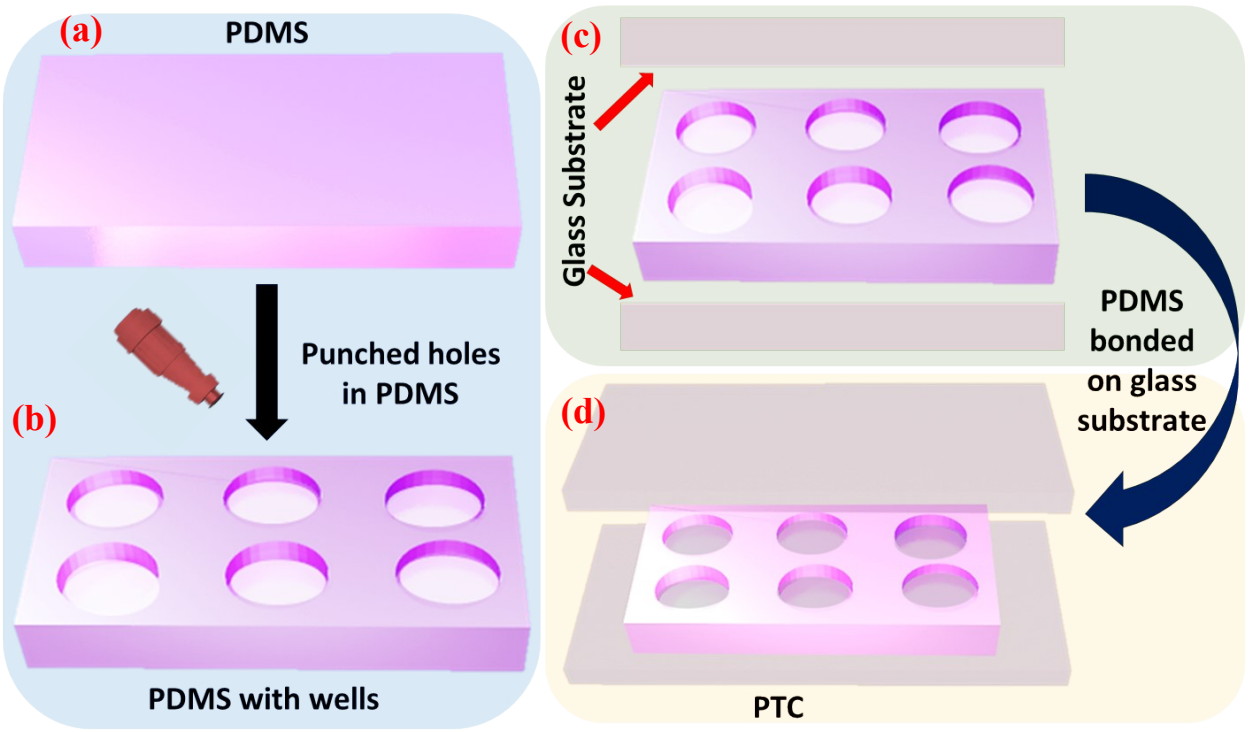

Figure S1 Schematic Illustration of the Preparation of the PTC (a) After the curing process PDMS was progressively peeled away from the silicon substrate (b) The wells were created using a PDMS biopsy puncher with a radius of ~5 mm and a depth of 7-8 mm (c) After cleaning of the glass slide and PDMS, corona plasma treatment has been done to make good adhesion between both the surface (glass slide and PDMS). (d) PTC with an attached bottom glass slide and top glass slide (dissociate).

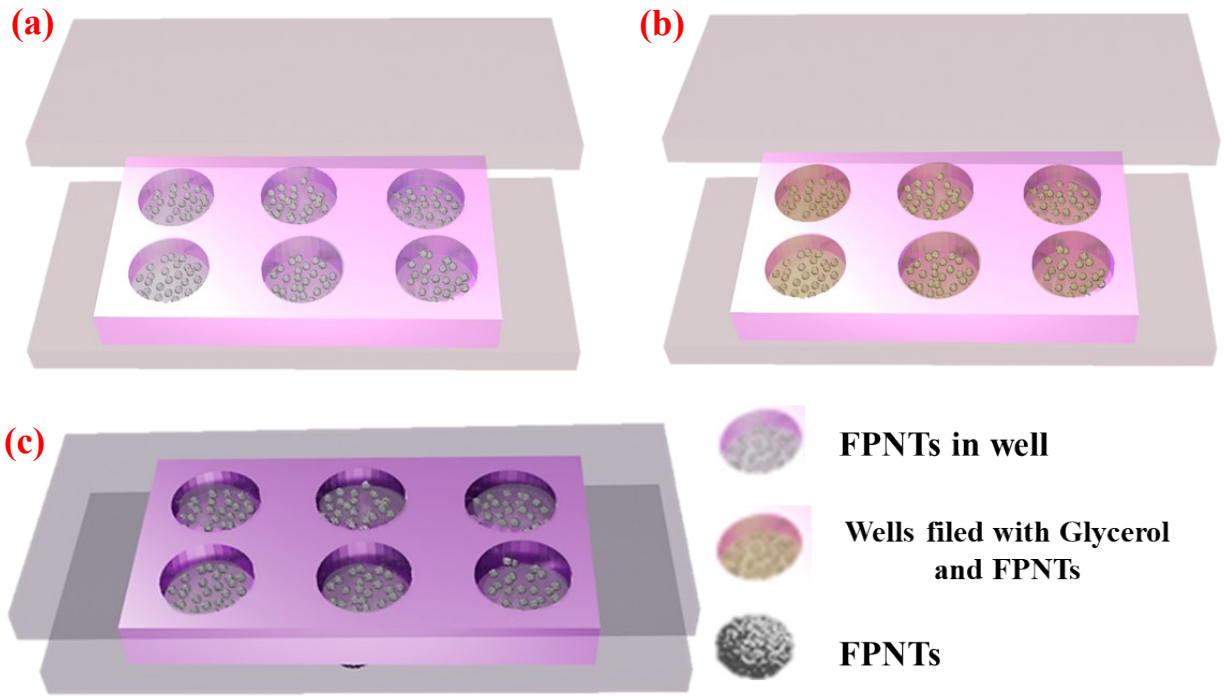

Figure S2 FPNTs and glycerol loading in PTC wells for temperature calibration (a) PTC chip was loaded with the diluted FPNTs and air dried (b) Wells were filled with glycerol to maintain a uniform temperature (c) PDMS was covered with a glass slide (top layer).

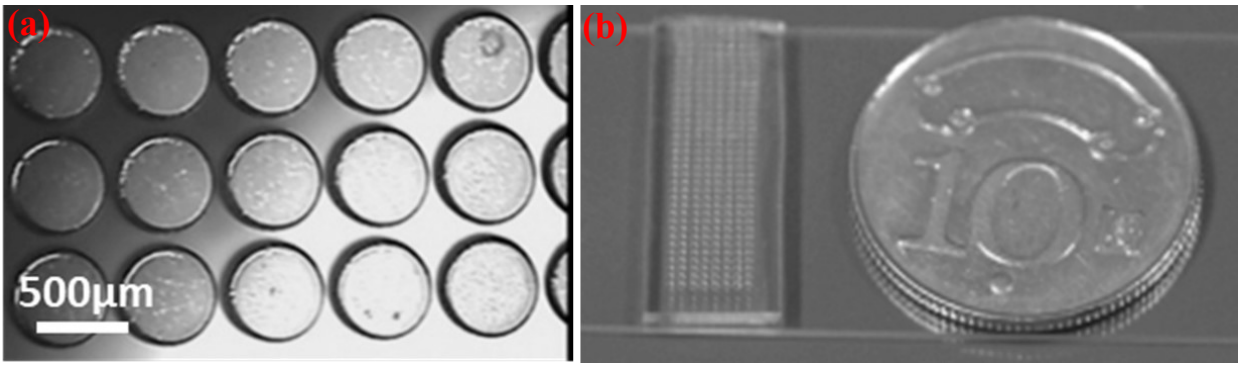

Figure S3 Schematic Illustration of the fabrication of the Open µ-well 3D cell-culture chip (a) Soft lithography technology was used to create the SU-8 pillars (b) PDMS molding technique to create the open µ-Well 3D Cell Culture Chip.

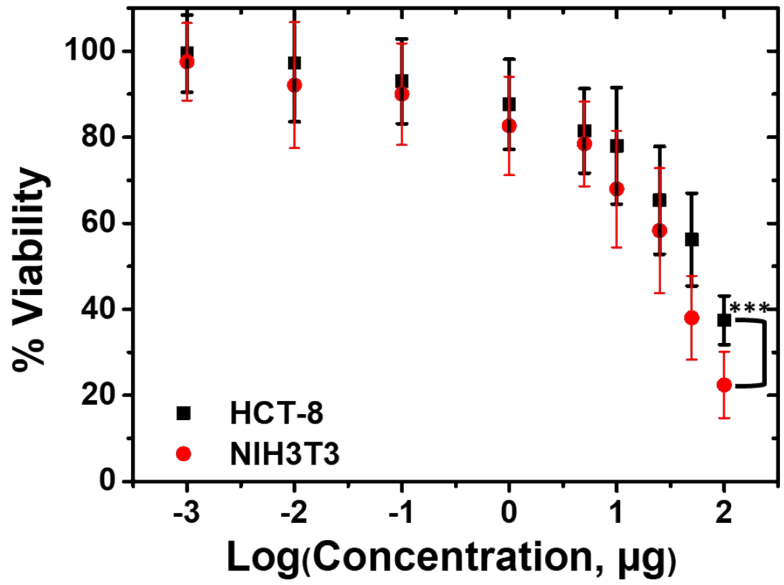

Figure S4 IC-50 value determination. The FPNTs were used to treat HCT-8, and NIH3T3 cell lines with different concentrations (100−0.001 µg). IC-50 curve of the FPNTs on 2D HCT-8 [56.23±7.43 µgmL^{-1}], and NIH3T3 [38.02±10.84 µgmL^{-1}] are means ± SD of three independent biological repeats. Data are represented as mean ± s.e.m., n = 3, and p-values are calculated using two-way ANOVA with Bonferroni correction, ***$p < 0.001$.

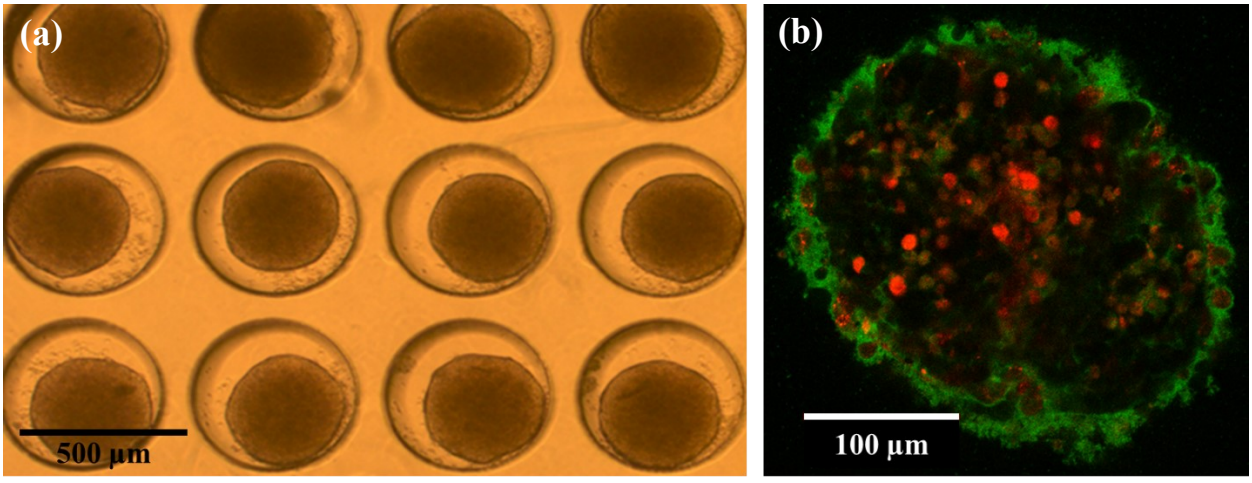

Figure S5 The spheroid formation and its cellular arrangement. (a) co-cultured, even-sized 3D spheroids formation in the micro-well chip (b) Cellular arrangement (HCT-8 (Red), Fibroblast (Green)) of tumor microenvironment after 9 days.

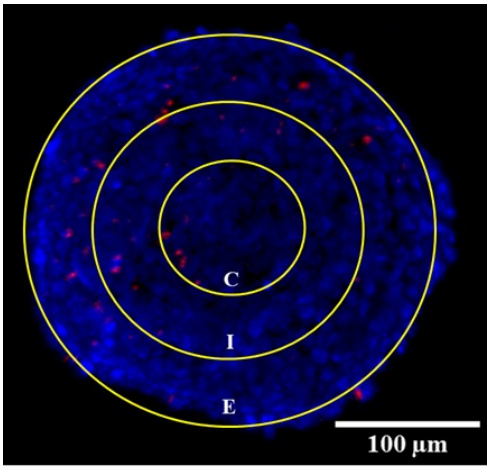

Figure S6 Spheroids were treated with FPNTs, and the fluorescence intensity density in the center region (C), Interior region (I), and Exterior region (E) were measured. The center region showed a significantly higher fluorescence intensity compared to the exterior region.

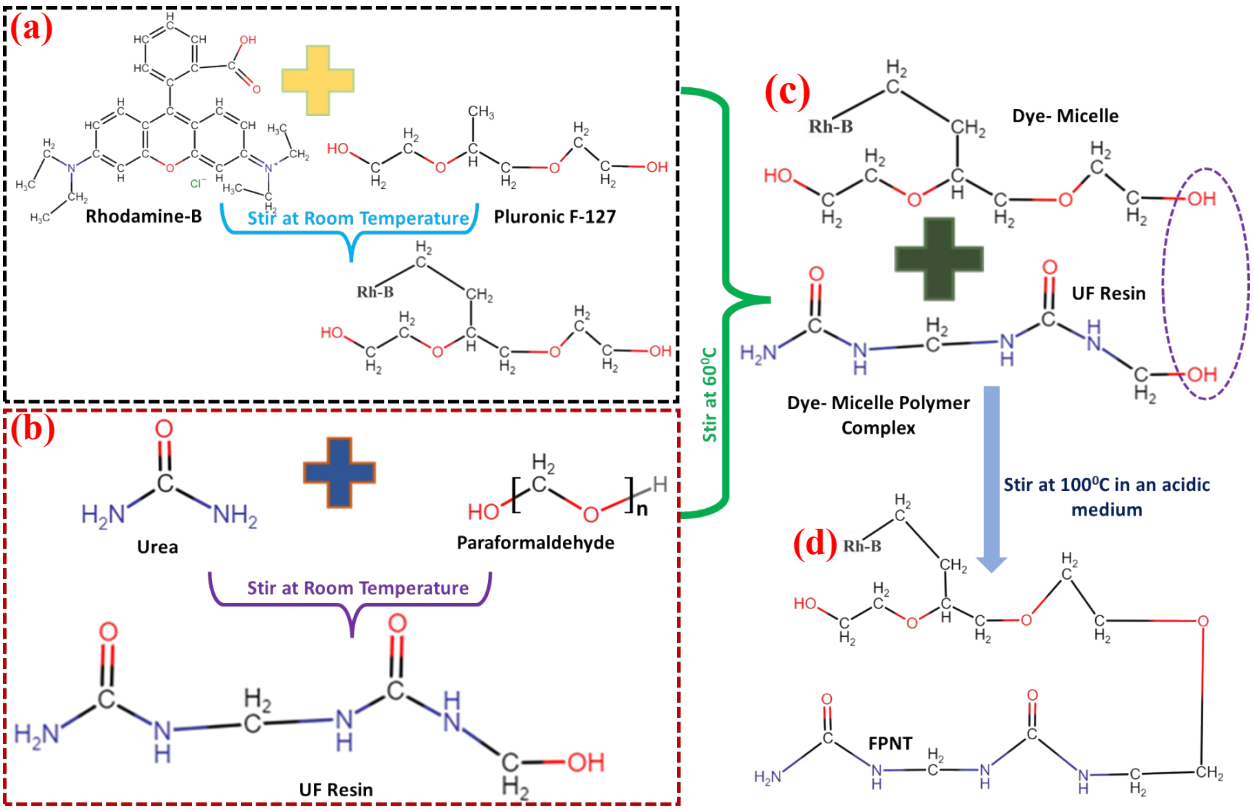

Figure S7 Reaction mechanism for the FPNTs preparation (a) Rh-B dye was mixed with Pluronic F-127 to make dye-micelle complex through hydrophilic-hydrophobic interactions (b) Under neutral pH (pH 7), UF resin forms methyl derivatives (c) The high temperature-based acid catalysis (at ~pH 3) and condensation (hydroxyl methyl terminal groups) reaction resulted in highly cross-linked three-dimensional networked particles (d) Rh-B molecules were homogeneously incorporated into the polymer

composite nanoparticles and covalently enclosed in the three-dimensionally cross-linked UF resin with unchanged fluorescent properties.

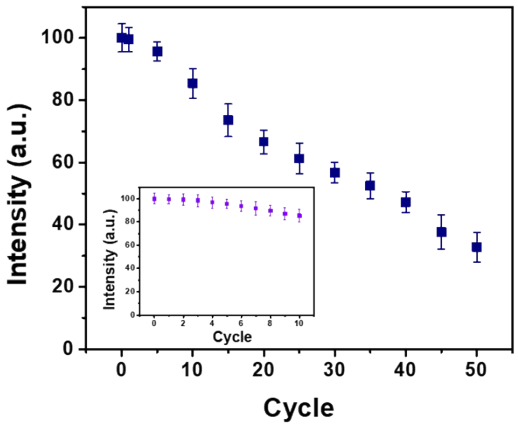

Figure S8 Fluorescence quenching of FPNTs with repeated cycles. FPNTs were exposed for 50 cycles and each cycle duration was for 60 seconds. The first 30 seconds of illumination were done, and the next 30 seconds were kept unexposed. The intensity profile of FPNTs changed slowly for the first 5 cycles and later for the next 5 cycles (a total of 10 cycles), the intensity change was fast [inset] and finally, the intensity profile was changed linearly.

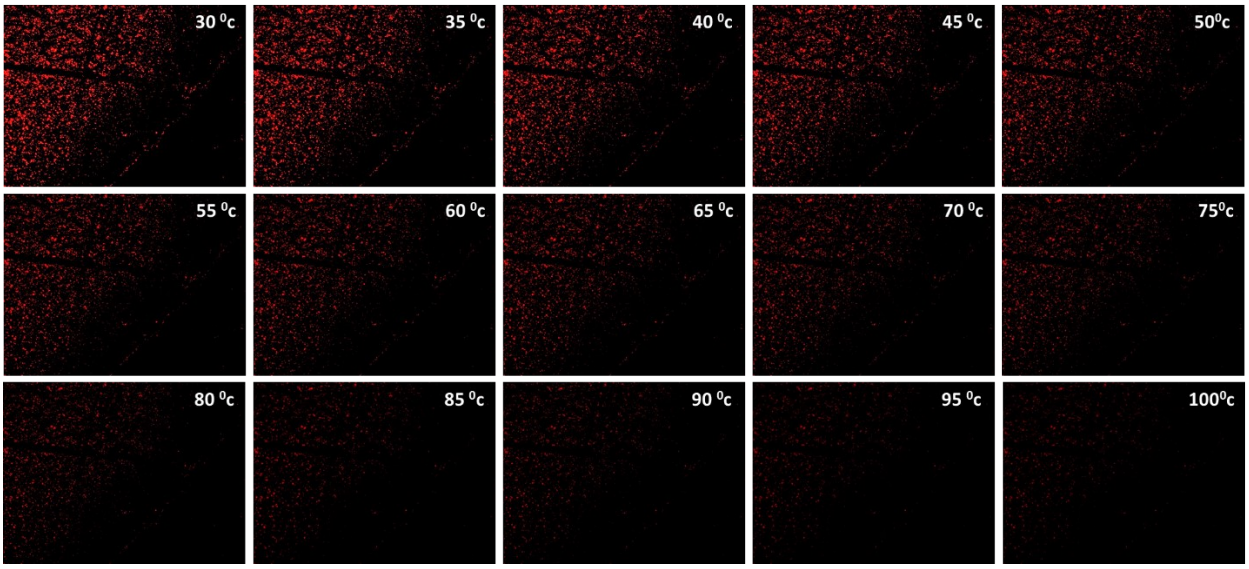

Figure S9 Fluorescence images of FPNTs at different temperatures illustrate the correlation between fluorescence intensity and temperature. FPNTs were placed inside the PTC well and heated (25 ^{0}C and 100 ^{0}C). Due to the heating of FPNTs fluorescence intensity varies. (Scale bar = 100 µm).

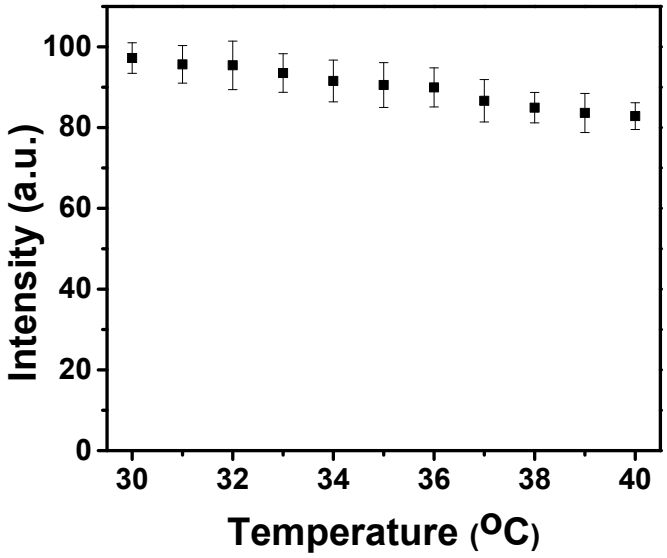

Figure S10 Fluorescence response of FPNTs when temperature varies from 30 to 40 ^{0}C.

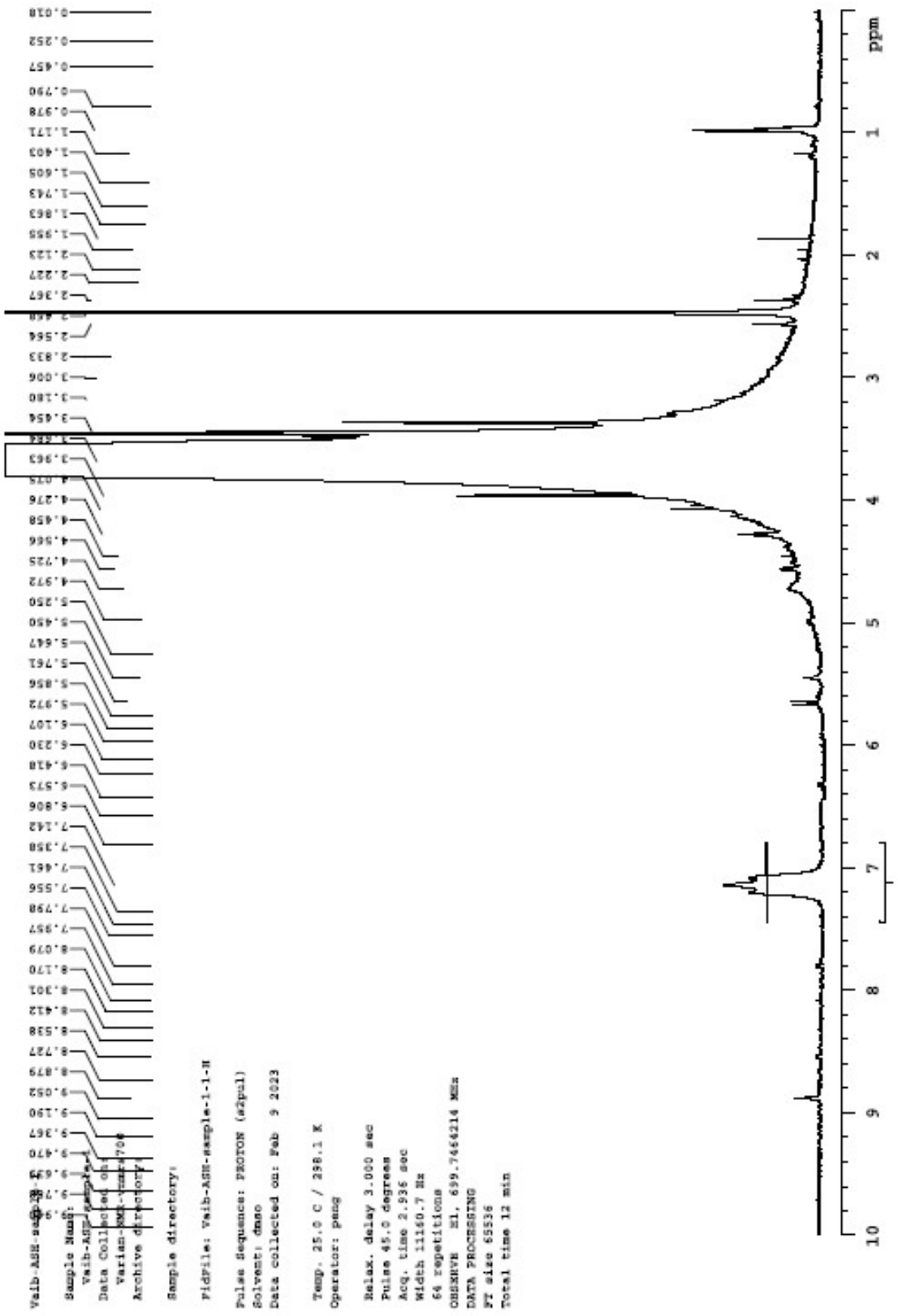

Figure S11 ***Proton NMR spectra of FPNTs at RT***

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