Supporting information for

Distinct Tubulin Dynamics in Cancer Cells Explored by a Highly Tubulin-Specific Fluorescent Probe

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1. Abbreviations

EMT, Epithelial-Mesenchymal Transition; GFP, Green fluorescent protein; EGFP, enhanced green fluorescent protein; FC, Fluorescein-labeled colchicine; DC, dansyl-labeled colchicine; NBC, NBD-labeled colcemid; DAPI, 4',6-Diamidino-2- phenylindole; RH, relative humidity; DTT, Dithiothreitol; GSH, Glutathione; NAC, N-acetyl cysteine; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel; PVDF, polyvinylidene difluoride; MT, microtubule; Gly, glycine; Met, methionine; Lys, lysine; Cys, cysteine.

2. Experiment section

2.1 Materials and Reagents

All commercial reagents and solvents were purchased from vendors and were used without further purification or distillation. Cell images were captured on LSM710 confocal microscope from Carl Zeiss. Absorption spectra were recorded on UV-2450 (Japan) spectrophotometer. Fluorescence was detected on FlexStation 3 multimode reader from Molecular Devices. Gel images were captured on fluorescent gel imager (PharosFX ™ Plus Molecular Imager, Bio-Rad). DMEM high glucose medium, RPMI1640 medium and fetal bovine serum were purchased from Invitrogen (Life Technologies Corporation, China). Ac-α-tubulin IgG (GST), α-tubulin IgG (GST), E-cadherin IgG (Santa), GAPDH IgG (Abcam), TGF-β, NativeMark™ Unstained Protein Standard (Life Technologies, USA) were purchased from brand agent (Biopike Corporation, China). PC3 (TCHu158) was purchased from National Center for Medical Culture Collection (CMCC). A2780/Taxol (BG028), A2780 (BG072), HCT8 (BG207) and MCF7 (BG336) were purchased from Bogoo Biotechnology Co., Ltd. (Shanghai, China). Microtubule polymerization assay kit (Cytoskeleton, catalog no. BK011P) was

purchased from Univ-Bio Corporation (Shanghai, China).

2.2 Cell Culture

Cells were grown in tissue culture flasks in complete growth medium (DMEM high glucose and/or RPMI1640 medium, pH 7.4, supplemented with 10 % fetal bovine serum, 100 mg/mL streptomycin, and 100 units/mL penicillin) in a carbon dioxide incubator (37 °C, 5 % CO_2, 90 % relative humidity (RH)).

2.3 Fluorescence spectroscopy.

General Fluorescence kinetics are recorded in 384 well microplate by using a FlexStation 3 multimode reader. Generally, tubulin and other interferants were dissolved in general buffer (80 mM PIPES, 2.0 mM $MgCl_2$ and 0.5 mM EGTA) or in aggregation buffer (80 mM PIPES, 2.0 mM $MgCl_2$, 0.5 mM EGTA, 20 % glycerol, 1.0 mM GTP and DAPI) with or without taxol; OC9 was dissolved in DMSO with concentration of 10 mM as stock solution and was diluted to 10X in working buffer before using; Microtubule polymerization level was indicated by DAPI following the enclosed experimental protocol of microtubule polymerization assay kit, or by turbidity assay (λ = 340 nm); in the detection of OC9 containing systems, 5.0 μL OC9 (10X) ,which prewarmed in multimode reader at 37 ^{0}C for 5.0 min, was diluted by related solution of tubulin or interferants to 50 μL final volume before test; the fluorescence kinetics was immediately monitored after mixing of analytes in the kinetic model of FlexStation 3 multimode reader at 37 ^{0}C.

Fluorescence responses of OC9 with tubulin and other putative interferants The fluorescence kinetics of OC9 (10 μM) was detected (Ex = 430 nm , Em = 510 nm) immediately in the presence of 4.0 μM tubulin, BSA, actin, trypsin, and collagen; and 10 mM

N-acetylcysteamine (NAC), amino acids (Gly, Met, Lys, Cys), dithiothreitol (DTT), glutathione (GSH) and metal ions ($CuCl_2$, $FeSO_4$, $CaCl_2$, $ZnSO_4$) respectively in general buffer. The OC9 only solution in general buffer was chosen as the negative control.

Quenching effects of OC9 on intrinsic tryptophan fluorescence The fluorescence kinetics of 4.0 μM tubulin were detected (Ex = 430 nm, Em = 510 nm) immediately in the presence of different concentrations of OC9 (0, 2.0, 4.0, 8.0, 20, 40, 80, and 100 μM) respectively in general buffer until the fluorescence intensity reached stable, and then intrinsic tryptophan fluorescence was monitored following (Ex = 295 nm, Em = 336 nm).

The Stern-Volmer plot The fluorescence quenching is analyzed by the Stern–Volmer equation: [22]

$$F_0 / F = 1 + K_{SV}[Q] = K_q \tau_0 [Q] + 1$$

where F_0 and F are the fluorescence intensities in the absence and presence of OC9, respectively. [Q] is the concentration of the quencher, and K_{SV} is the Stern–Volmer dynamic quenching constant, which is equal to the product of double molecule fluorescence quenched rate constant K_q and the fluorescence life expectancy τ_0 of fluorescent protein without the quencher.

According to the Stern–Volmer curves, the possible quenching mechanism can be interpreted. The largest diffusion constant and τ_0 of tubulin as biomacromolecule is respectively $2.0\times10^{10}\ M^{-1} \cdot s^{-1}$ and 1.0×10^{-8} s, when K_q calculated from the Stern–Volmer equation is higher than $2.0\times10^{10}\ M^{-1} \cdot s^{-1}$, the quenching mechanism can be interpreted as Static Quenching Style induced by the quencher combined into the fluorescent protein-quencher complex with the fluorescent protein; Otherwise the quenching

mechanism can be interpreted as Dynamic Quenching Style induced by the quencher crashing with the fluorescent protein.

In current work, the K_q of tubulin intrinsic tryptophan fluorescence quenched by OC9 was calculated as $1.87\times10^{13}\ M^{-1}\cdot s^{-1}$ from Stern–Volmer curves (Fig. S1), which is obviously higher than $2.0\times10^{10}\ M^{-1}\cdot s^{-1}$, indicating a static quenching mechanism instead of a crashing style during the OC9-tubulin interaction.

Affinity fluorescence kinetics of OC9 with tubulin or taxol pretreated tubulin (MT). The microtubule polymerization kinetics of 20 μM tubulin in general buffer, or in aggregation buffer with 20 μM taxol was monitored respectively by DAPI (Ex = 360 nm, Em = 450 nm) according to the enclosed experimental protocol of microtubule polymerization assay kit. After the fluorescence intensity reached stable, OC9 was added and the affinity fluorescence kinetics from OC9/tubulin were synchronously monitored (Ex = 430 nm, Em = 510 nm) immediately and respectively until the fluorescence intensity reached stable (Fig. 1C).

Fluorescent titration of tubulin or taxol pretreated tubulin (MT) by OC9. The microtubule polymerization kinetics of 20 μM tubulin in aggregation buffer with 20 μM taxol was recorded (Ex = 360 nm, Em = 450 nm) until the fluorescence intensity reached stable (Fig. S2A). Then, the affinity induced fluorescence of both the resulted solution and 3.0 μM tubulin in general buffer were detected (Ex = 430 nm, Em = 510 nm) immediately and respectively in the presence of various concentrations of OC9, until the fluorescence intensity reached stable (Figs. S2B-C).

Dissociation Constant (K_d) and binding sites (n) of OC9 to Tubulin and MT

The dissociation constant (K_d) was determined according the literature.[27] First the inner

filter effect was corrected for the observed fluorescence values using the equation:

$$F_{corrected} = F_{observed} \text{ antilog } [(A_{ex} + A_{em}) / 2]$$

where A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths, respectively;

and k_d can be expressed in the equation:

$$1 / X = 1 + K_d / L_f \qquad (1)$$

where L_f represents the free OC9 concentration which can be expressed as:

$$L_f = L – n[PL_n] \qquad (2)$$

where n is the number of binding sites of OC9 in tubulin, L is the original concentration of OC9, $[PL_n]$ is the concentration of OC9-bound tubulin; and X is the fractional occupancy which can also be expressed as:

$$X = \Delta F / \Delta F_{max} = [PL_n] / P_0 \qquad (3)$$

where ΔF is the enhancement of fluorescence intensity from tubulin-OC9 interaction in equilibrium, and ΔF_{max} is the maximum fluorescence change when tubulin is completely liganded by OC9 and can be calculated by plotting 1/ΔF versus 1/L; P_0 is the total concentration of tubulin.

Combining the formula (1), (2) with (3) gives the equation:

$$L = K_d / (1 - X) - nP_0(1 - X) + (nP_0 - K_d) \quad (x<=1)$$

which can be fitted by using GraphPad Prism 5 and affords K_d and n.

Time windows of microtubule stability with OC9 in an in-vitro model The fluorescence kinetics of 10 μM OC9 with 10 μM tubulin in general buffer, 20 μM OC9 with 20 μM tubulin in general buffer or aggregation buffer, and 10 μM OC9 with 10 μM tubulin in aggregation

buffer with 10 μM taxol was detected (Ex = 430 nm , Em = 510 nm) immediately and respectively until the fluorescence intensity reached stable.

2.4 Native-PAGE analysis of tubulin oligomers

The polymerized microtubule was obtained by treatment of 20 μM tubulin with 20 μM taxol in aggregation buffer, and the aggregative level was indicated by turbidity assay (λ = 340 nm) (Fig. S3). This resulted polymerized microtubule solution and 20 μM tubulin in general buffer were added non-reductive and native loading buffer (1X), subjected to 3.5 %-6.0 % polyacrylamide gel without SDS and electrophoresed in buffer without SDS. Apparent molecular mass of bands was determined by using the NativeMark™ Unstained Protein Standard (Life Technologies, USA). After native-PAGE, the gel was incubated with 10 μM OC9 for 3.0 h at room temperature and then acquired pictures by the fluorescent gel imager (PharosFX ™ Plus Molecular Imager, Bio-Rad), followed by commassie briliant blue staining.

2.5 Confocal microscope

General Cells were fixed with 37 °C prewarmed paraformaldehyde for 15 min, permeabilized with permeabilization buffer (Triton X-100) for 15 min and blocked with blocking buffer for 15 min. Mouse anti-α tubulin antibody (1:200) was added and incubated for 1.0 h at room temperature and then Dylight 549-Goat Anti-Mouse IgG (1:500) was added and incubated for 30 min in darkness at room temperature. Cells were washed three times with PBS and incubated with 10 μM OC9 for 3.0 h at room temperature. Fluorescently stained cells were analyzed with confocal microscope and/or in 3D mode scanning from the matirx side to the top side of the cell and taking pictures every 0.25 μm of cells.

Vincristine treatment. MCF7 cells were plated in confocal culture dishes at 6.0×10^4 cells/dish and grown for 24 h, 1.0 μM vincristine was added for 6.0 h. Immunofluorescence and OC9 staining is following the protocol described above.

Microtubule depolymerization with ice and recovery. MCF7 cells were plated in confocal culture dishes at 6.0×10^4 cells/dish and grown for 24 h, then cells were incubated in ice for 2.0 h and then reincubated in 37 ^oC incubator respectively for 0 min, 5.0 min, 20 min and 30 min. Immunofluorescence and OC9 staining is following the protocol described above.

Living cells imaging. Living cells (MCF7, HCT8) were plated in confocal culture dishes at 6.0×10^4 cells/dish and grown for 24 h, then 10 μM OC9 was added and in situ images were captured in confocal microscope with the time consumption.

Cells in EMT progress. PC3 cells were plated in confocal culture dishes at 3.0×10^4 cells/dish and grown for 24 h, then cells were added TGF-β (10 ng/ml) for 0 h, 24 h and 72 h. Immunofluorescence and OC9 staining is following the protocol described above.

Supplementary Figures

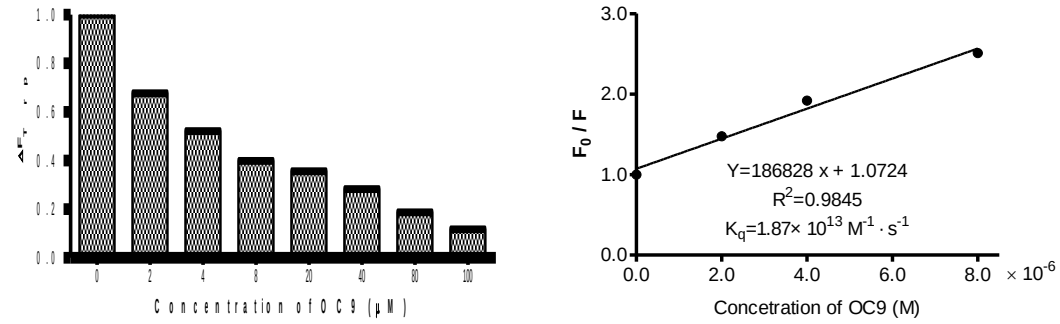
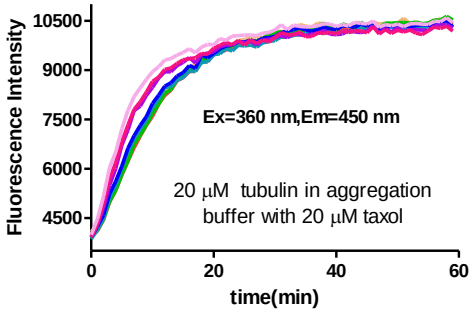


Figure S1. Quenching effects of OC9 on intrinsic tryptophan fluorescence (Ex: 295 nm, Em: 336 nm) of 4 μM tubulin; the histogram (left) represents the fluorescence intensity normalized of samples with different OC9 concentration to the tubulin only control. right: the Stern-Volmer plot.

A



B C

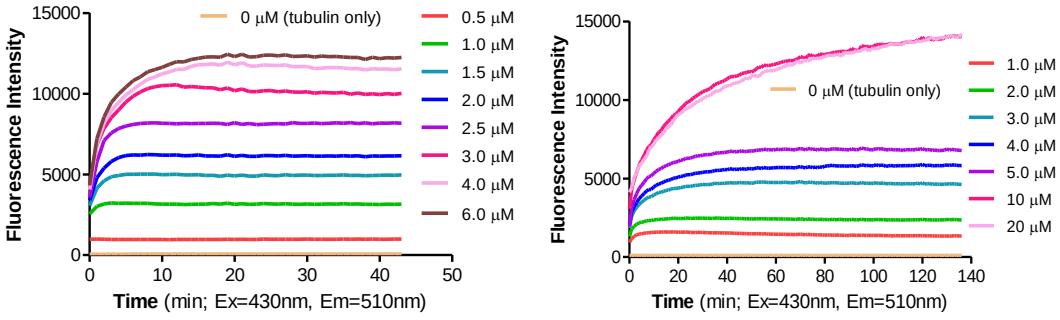


Figure S2 Fluorescent kinetics of microtubule polymerization by DAPI in aggregation buffer (A). 20 μM tubulin was dissolved in aggregation buffer with 20 μM taxol, the fluorescence by DAPI (Ex=360 nm, Em=450 nm) were detected immediately in 37 ^{0}C multimode reader until the fluorescence reached stable, this taxol pre-treated tubulin is used in the titration assay. Then fluorescent titration of various concentration of OC9 in 3 μM tubulin (B) or 20 μM taxol pre-treated tubulin (C) were performed. Fluorescence is detected at Ex = 430 nm and Em = 510 nm.

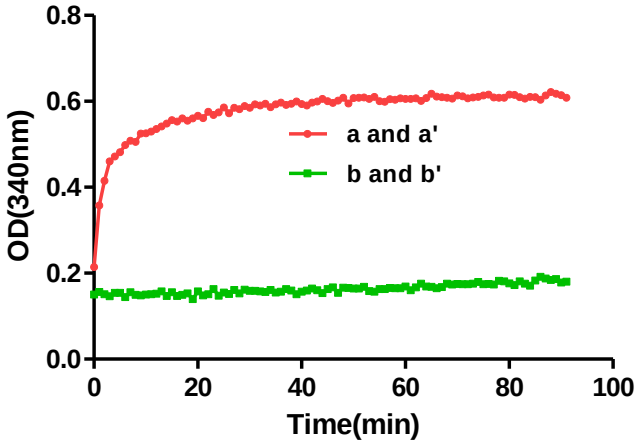


Figure S3: Absorbent kinetics of microtubule polymerization by turbidity. The microtubule polymerization kinetics of 20 μM tubulin in aggregation buffer with 20 μM taxol (a and a') and 20 μM tubulin in general buffer (b and b') were detected immediately in 37^{0}C multimode reader in kinetic mode of absorbance (λ = 340 nm) until the absorbance value reached stable.

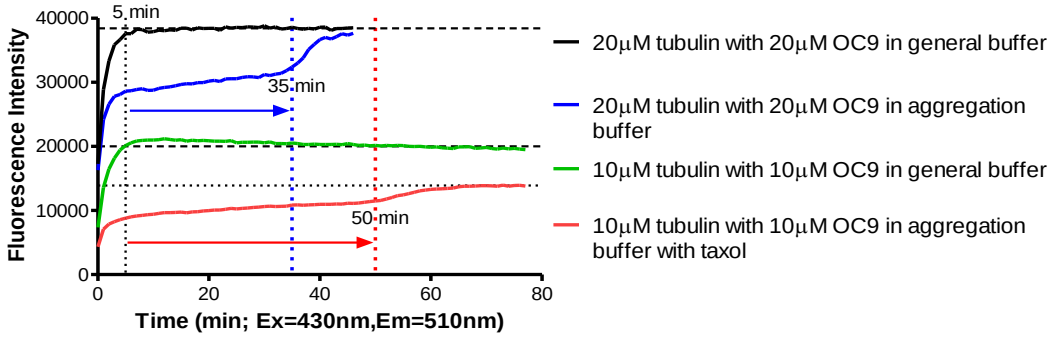


Figure S4: Time windows of microtubule stability with OC9 in an in-vitro model. Fluorescence of medium-promoted (blue) and taxol-promoted (red) microtubules in aggregation buffer with OC9 is shown; black and green indicate changes in the fluorescence of tubulin in the general buffer. Vertical dashed lines indicate time windows. These data shown that the fluorescence responses of OC9 to 10 μM tubulin (green) and 20 μM tubulin (black) were stabilized in 5 min after OC9 added in general buffer. In the aggregation medium, however, considerably more time is required for the fluorescence response to stabilize (blue); The fluorescence intensity remained relatively stable for approximately 30 min after the first sharp increase, then rapidly increased again before stabilizing. The presence of taxol further delayed the stabilization of the fluorescence response (red), which occurred approximately 45 min after the first increase. This lengthy period of relative microtubule stability, during which the in-situ state of tubulin experienced little interference, offered a useful time window for tubulin imaging in living cells. The maximum fluorescence intensity of the taxol-treated solution (red) is noticeably lower than that of the taxol-free mixture (green), indicating that OC9 has no obvious effect on taxol-promoted and stabilized microtubules. This finding is consistent with the previous observation (Fig. 1C, red).

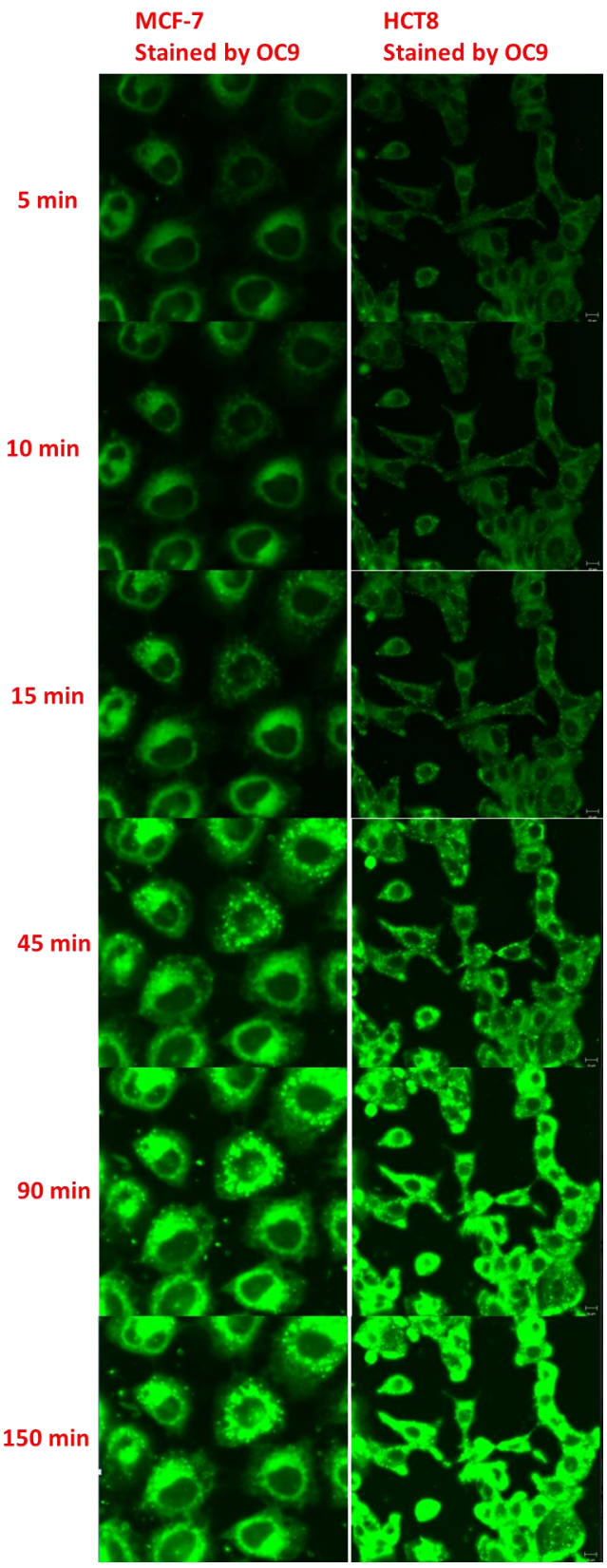
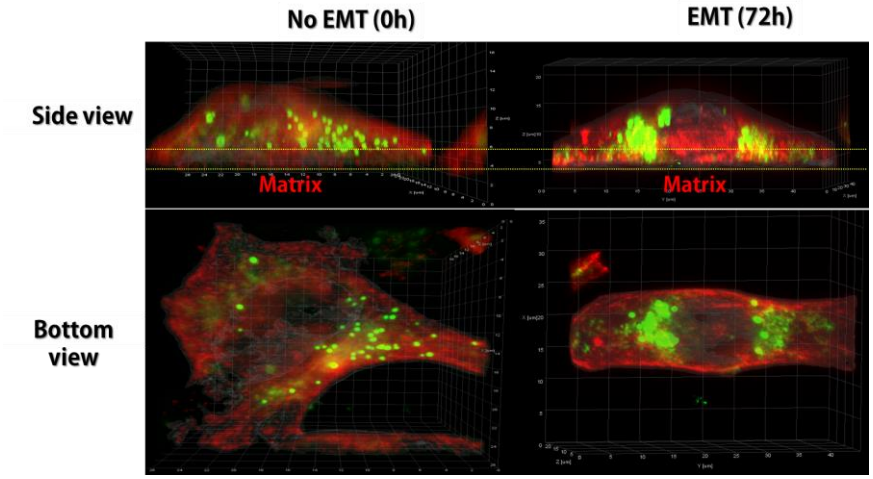


Figure S5: Living cells imaging by OC9 in confocal microscope. Cells were added 10 µM OC9 and captured images in real-time mode of confocal microscope with the time consumption. Bar: 10 µm

A



B

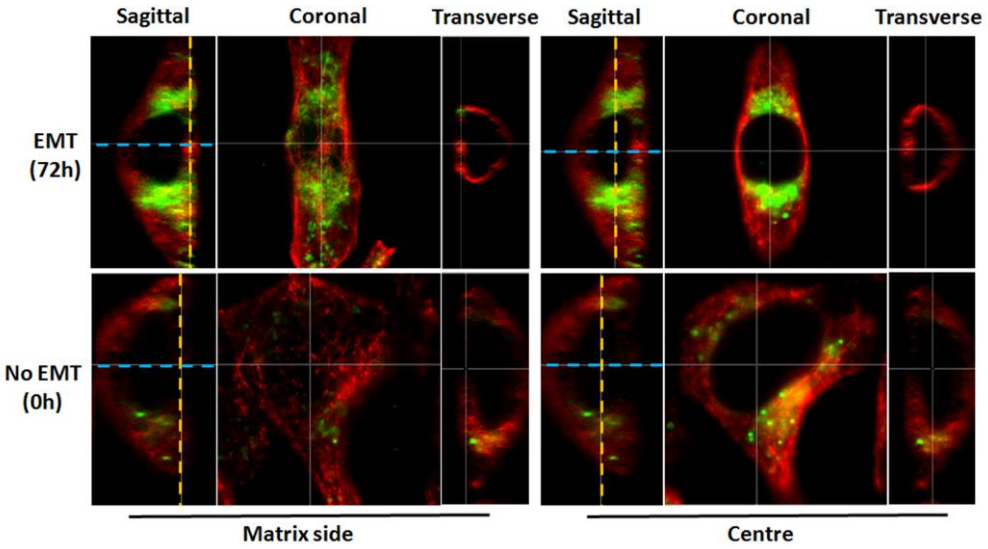


Figure S6: 3D visualization of cellular tubulin oligomers in single EMT or parental PC3 cell. EMT is induced by TGF-β (10 ng/mL) for 72 h. 3D shapes of single EMT or parental PC3 cell. ***A.*** The side and bottom view are presented. ***B***. The profile map of single PC3 cell with or without 72 h-treatment of TGF-β. The yellow and blue dashed line in sagittal plane refer to the orthogonal layer of coronal and transverse plane respectively. The coronal planes show the matrix-side and center view of the cells respectively. Pictures are taken from bottom (matrix) to top of the stained cells by every 0.25 μm intervals with confocal microscope in 3D scanning mode.