ADVANCED HEALTHCARE MATERIALS

Supporting Information

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New Strategy for Promoting Vascularization in Tumor Spheroids in a Microfluidic Assay

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# indicates equal contribution of these authors

Figure S1

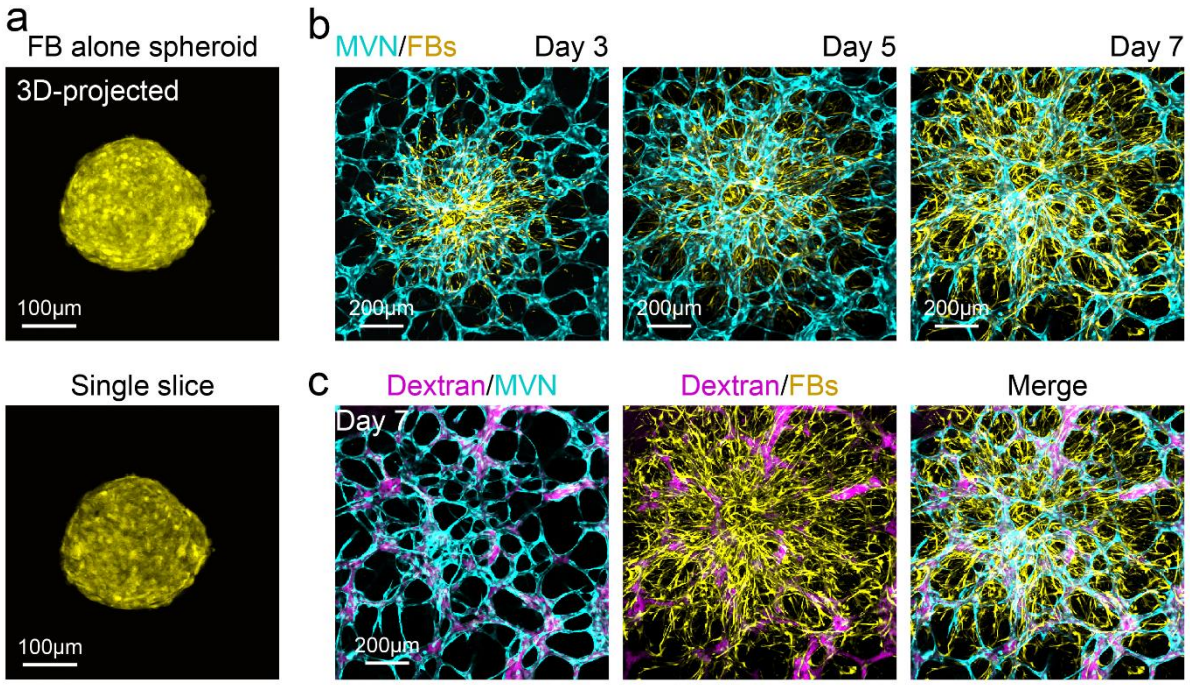


Figure S1. Vascularization of FB alone spheroid. a) Representative images of FB alone spheroid. b) Representative confocal images of microvessels in FB alone spheroid on day 3, 5, and 7. c) Images of MVNs perfused with Texas Red Dextran (10 kDa) on day 7.

Figure S2

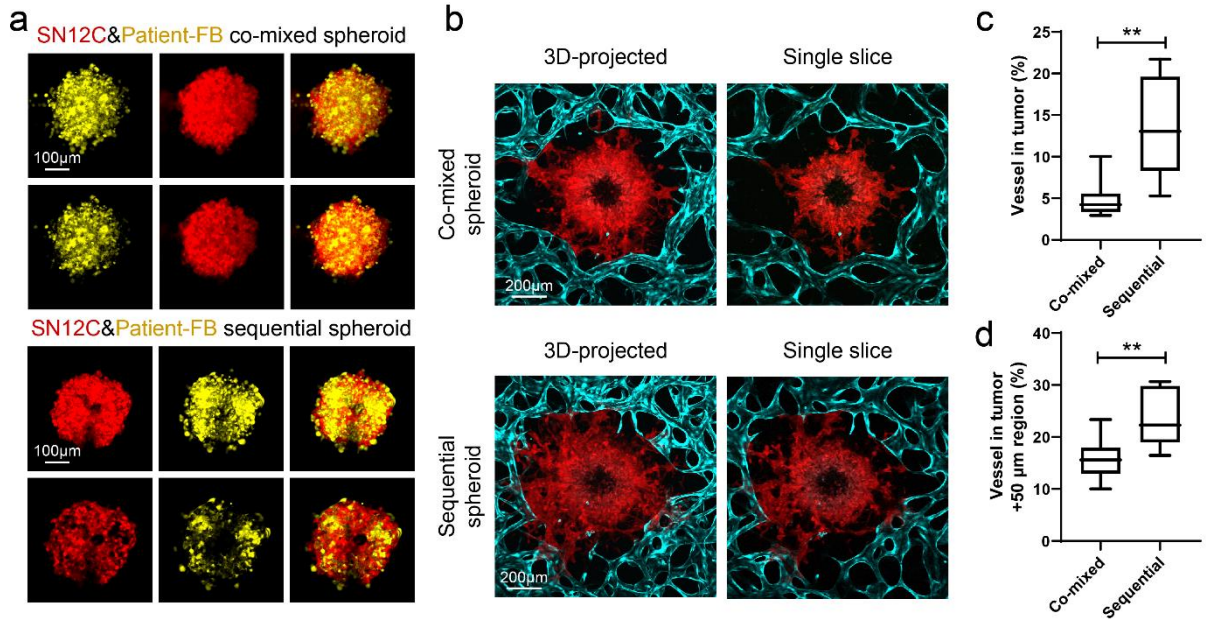


Figure S2. Sequential SN12C tumor spheroid made with cancer patient-derived thyroid FBs shows a higher vascularization level than the co-mixed spheroid. a) 3D-projected image and single slice image of SN12C tumor spheroids formed by co-mixed or sequential methods using cancer patient-derived thyroid FBs. b) 3D-projected images and single slice images of vascularized tumor spheroids formed by co-mixed or sequential methods. c) Statistical analysis of vessel percentage in the tumor region or d) tumor +50 μm region. Bars represent mean ± S.D. Two-tailed t tests were performed for the statistical comparisons. Data were collected from at least 9 tumor spheroids for each group. ** $p < 0.01$.

Figure S3

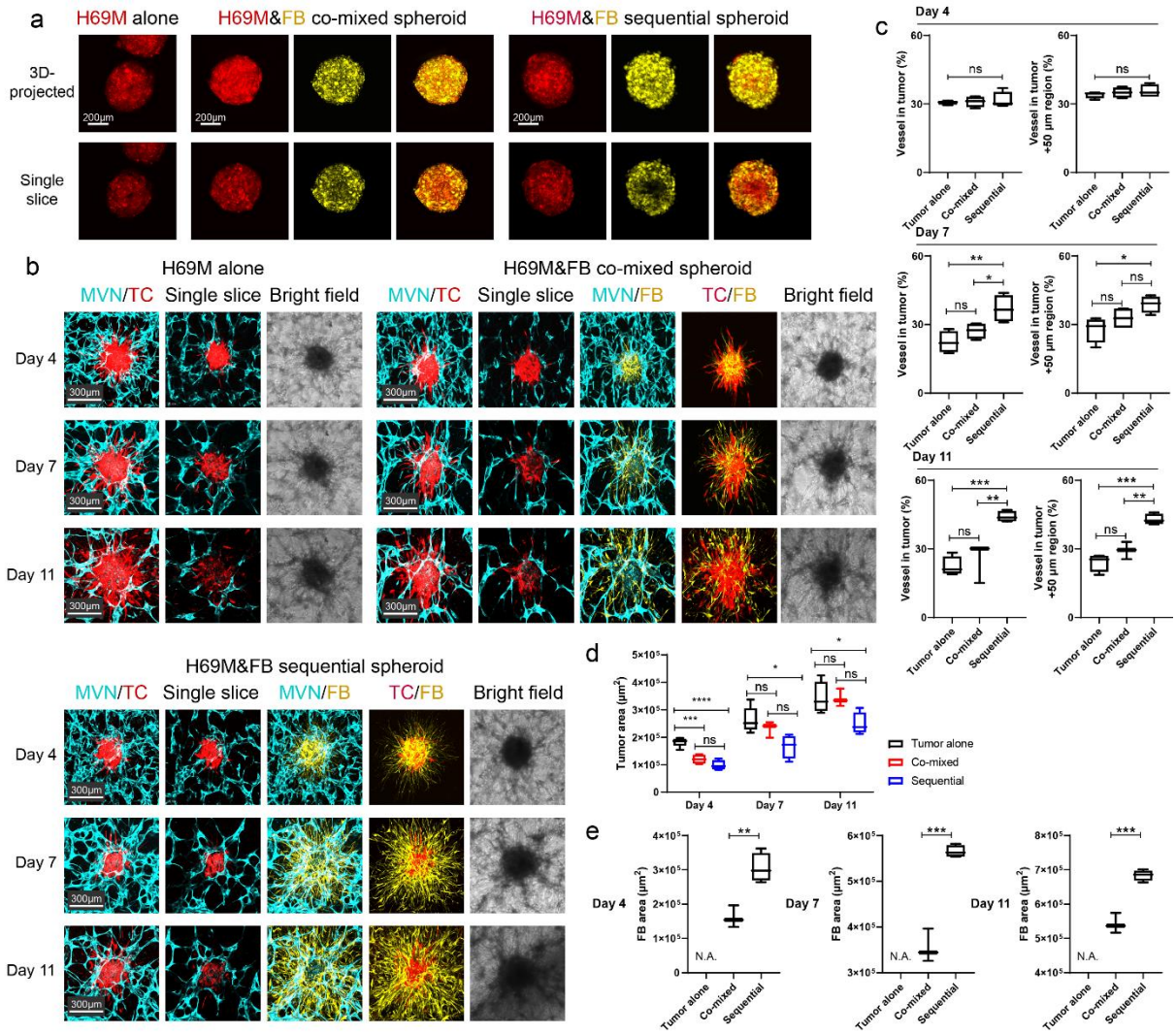


Figure S3. H69M tumor spheroids formed by the sequential method promote vascularization. a) Representative confocal images of H69M tumor spheroids made using the three different methods. b) Representative confocal images of vascularized H69M tumor spheroids formed by three different methods on days 4, 7, and 11. c) Statistical analysis of vessel percentage in the tumor region and tumor +50 μm region on days 4, 7, and 11. d) Statistical analysis of tumor area over 7 days. e) Tumor spheroid associated FB area analysis on day 4, 7, and 11. Bars represent mean ± S.D. One-way ANOVA was performed for the statistical comparisons in (c) and (d). In figure (c), day 4, no significant differences; day 7 left $p<0.01$, right $p<0.05$; day 11, left $p<0.001$, right $p<0.001$. In figure (d), day 4, $p<0.0001$; day 7, $p<0.05$; day 11, $p<0.05$. Significance determined by Tukey's multiple comparisons test of mean value between each group. Two-tailed t tests were performed for the statistical comparisons in (e). Data were collected from at least 6 tumor spheroids for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure S4

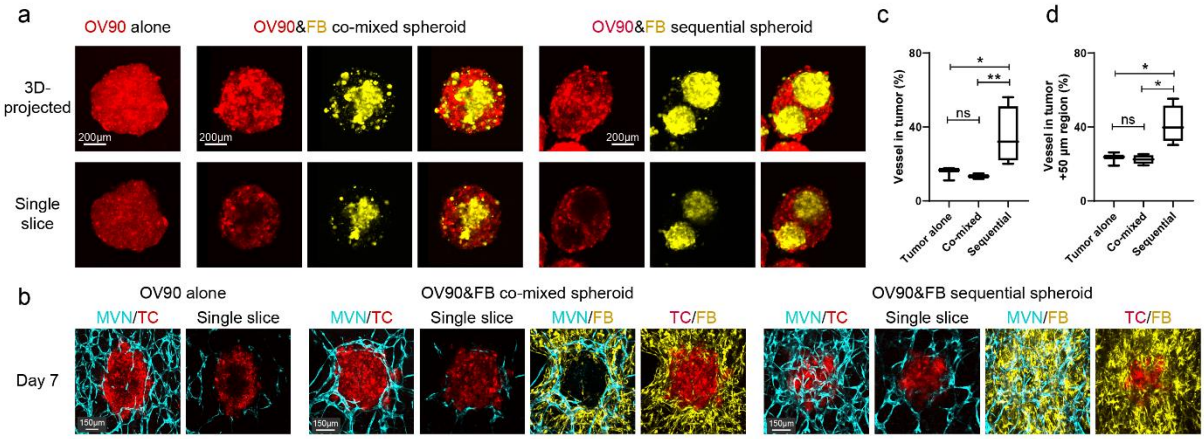


Figure S4. Vascularization of OV90 sequential tumor spheroids is superior to the other methods. a) Representative confocal images of OV90 tumor spheroids formed by three different methods. b) Representative confocal images of vascularized OV90 tumor spheroids formed by three different methods. c) Statistical analysis of vessel percentage in the tumor region and d) in tumor +50 μm region. Bars represent mean ± S.D. One-way ANOVA was performed for the statistical comparison. Figure (c), $p < 0.05$. Figure (d), $p < 0.05$. Significance determined using Tukey's multiple comparisons test of mean value between each group. Data were collected from at least 5 tumor spheroids for each group. * $p < 0.05$, ** $p < 0.01$.

Figure S5

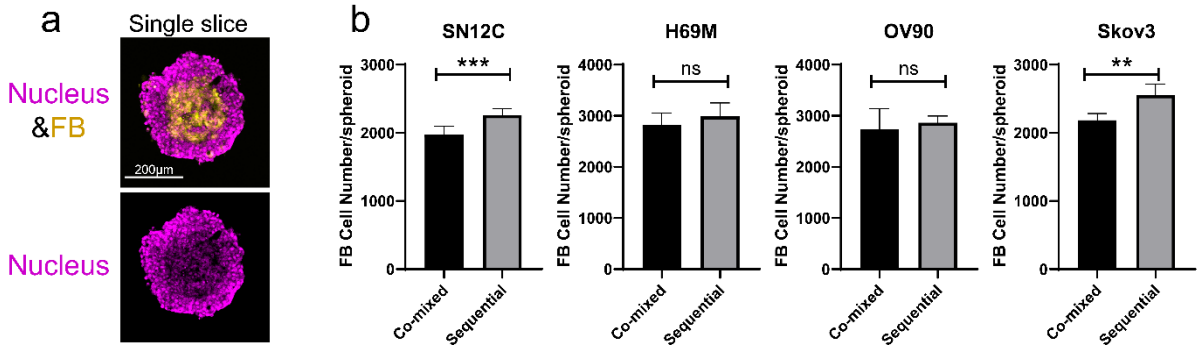


Figure S5. FB cell number in tumor spheroids. a) Fluorescent image of OV90 co-mixed tumor spheroid with nuclear staining. b) FB cell numbers in tumor spheroids made with SN12C, H69M, OV90, or Skov3 using co-mixed or sequential methods. Bars represent mean ± S.D. Two-tailed t tests were performed for the statistical comparisons. Data were collected from at least 6 tumor spheroids for each group. ** $p < 0.01$, *** $p < 0.001$.

Figure S6

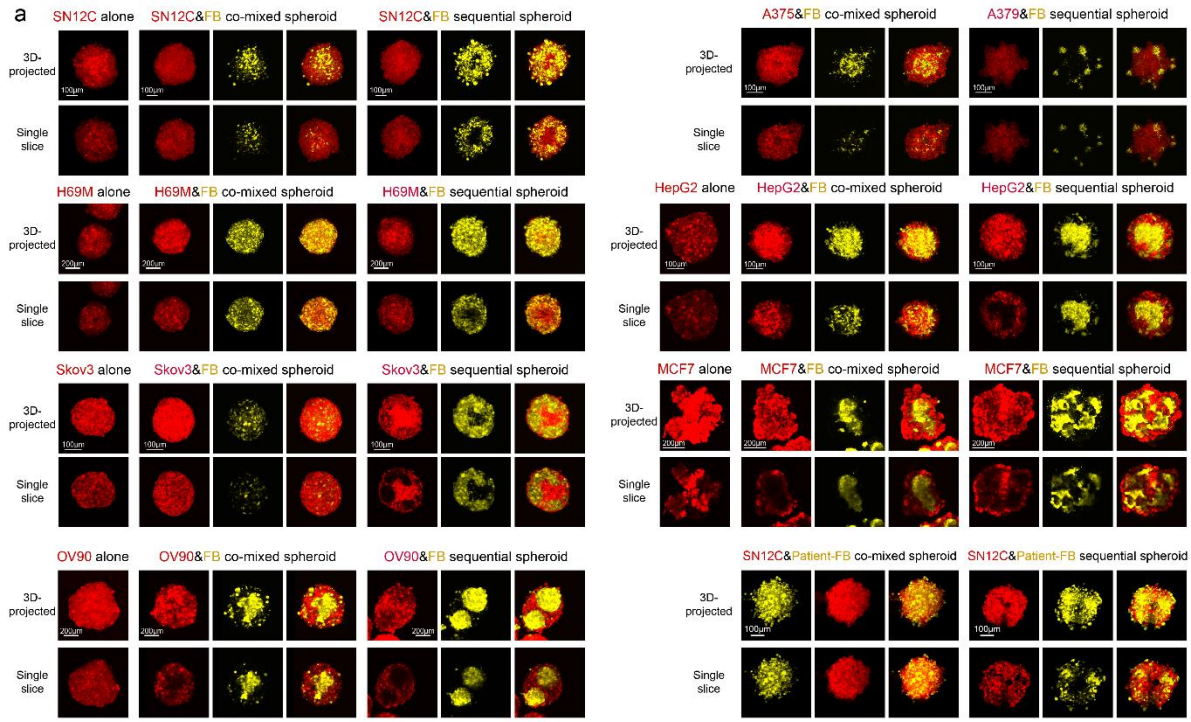


Figure S6. Different types of tumor spheroids formed by tumor cell alone, co-mix, and sequential methods. 3D projected and single slice images of tumor spheroids made of SN12C, H69M, Skov3, OV90, A375, HepG2, or MCF7 showed the different patterns of FBs and tumor cells in tumor spheroids.

Figure S7

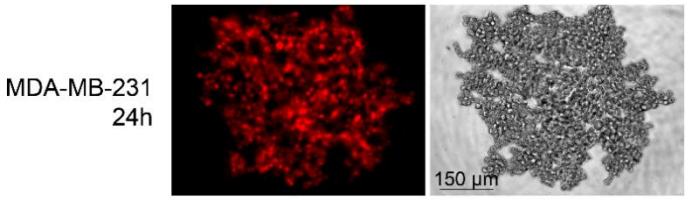


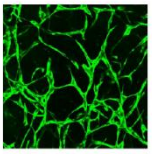
Figure S7. MDA-MB-231 cells alone do not form tumor spheroids. Epi-fluorescent and bright field images of MDA-MB-231 cultured in the ULA plate for 24 hours. Tumor cell clusters were weakly connected and easily dissociated by pipetting (not shown).

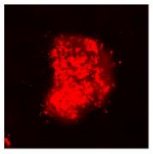
Figure S8

1.

Prepare images for analysis

Image › Stacks › Z Project...,
Chose Max Intensity





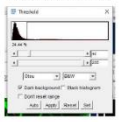
Change images to 8-bit

Image › Type › 8-bit

2.

Threshold tumor image

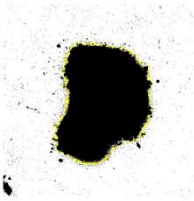
Image › Adjust › Threshold...



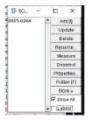
3.

Create ROIs of tumor spheroid

Analyze › Analyze Particles...



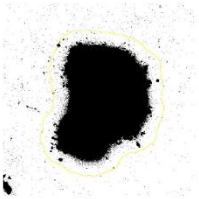
Tumor area ROI added



4.

Generate ROI with 50 µm larger

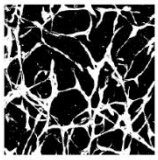
Edit › Selection › Enlarge...



Add to ROI manager

5.

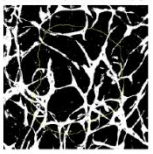
Threshold Vessel image



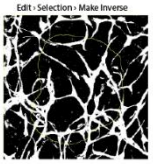
6.

Remove vessels out of tumor+50 µm region

Click on the +50 µm ROI in ROI manager



Edit › Selection › Make Inverse



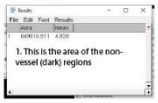
Edit › cut

7.

Measure the non-Vessel area

Edit › Selection › Create Selection

Analyze › Measure

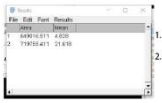


8.

Measure area of the entire image

Make sure no ROIs selected

Analyze › Measure



1. This is the area of the non-vessel (dark) regions
2. This is the area for entire image

The difference between area 2 and area 1 is the area of vessel in the tumor and nearby +50 µm region.

Vessel area in tumor and nearby 50µm region = Area 2 (719755.4) – Area 1 (649016.5) = 70738.9 µm²

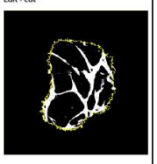
9.

Similarly remove vessels out of tumor ROI

Click on the tumor ROI in ROI manager

Edit › Selection › Make Inverse

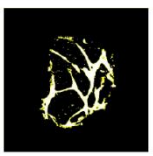
Edit › cut



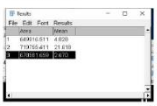
10.

Measure the non-Vessel area

Edit › Selection › Create Selection



Analyze › Measure



1. This is the area of the non-vessel (dark) regions
2. This is the area for entire image
3. This is the area of the non-vessel (dark) regions (exclude from tumor)

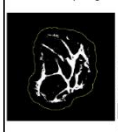
The difference between area 2 and area 3 is the area of vessel in the tumor region.

Vessel area in tumor = Area 2 (719755.4) – Area 3 (649016.5) = 41173.75 µm²

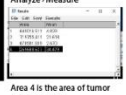
11.

Measure tumor region and tumor + 50µm region

Click on the +50 µm region ROI in ROI manager

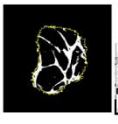


Analyze › Measure

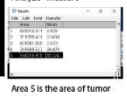


Area 4 is the area of tumor + 50µm region

Click on the tumor ROI in ROI manager



Analyze › Measure



Area 5 is the area of tumor region

12.

Use excel to calculate Vessel in tumor or/and nearby 50µm region percentage

Original data | Calculated

1	649015.5	4.828	Tumor area	168376.9
2	719755.4	21.619	Tumor area plus 50µm	269688.6
3	678681.7	2.69	Vessel area in tumor	41173.75
4	2090868.6	164.79	Vessel area in tumor and nearby 50µm regi	70738.9
5	1002176.9	55.224	Vessel in tumor area %	24.45232
			Vessel area in tumor and nearby 50µm regi	26.22984

Vessel in tumor area % = 100 x Vessel area in tumor (41173.75) / tumor area (168376.9) = 24.45332 %

Vessel in tumor area and nearby 50 µm region % = 100 x Vessel area in tumor and nearby 50 µm region (70738.9) / tumor area plus 50 µm (269688.6) = 26.22984 %

Figure S8. Vessel percentage in the tumor region and nearby 50 μm region analysis using ImageJ.