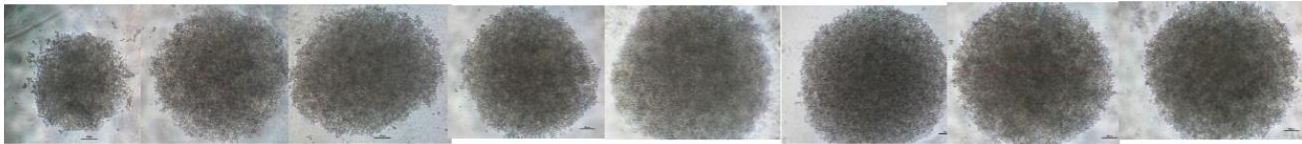


(a)



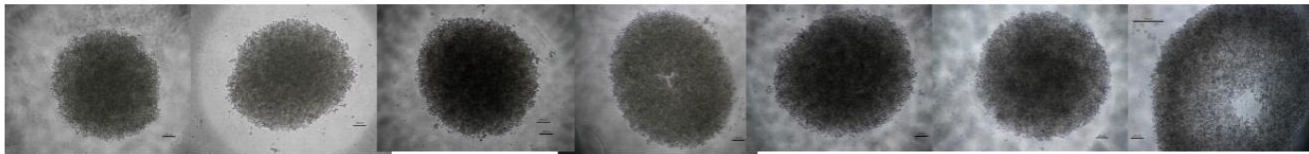
Day 4

3000 CELLS	4000 CELLS	5000 CELLS	6000 CELLS	7000 CELLS	8000 CELLS	9000 CELLS	10000 CELLS
1424.5	1433	1691	1696	2047	2024	1870.5	1856



Day 5

3000 CELLS	4000 CELLS	5000 CELLS	6000 CELLS	7000 CELLS	8000 CELLS	9000 CELLS	10000 CELLS
1262.5	1502	1898	1732.5	1933	1997.5	2019	1974



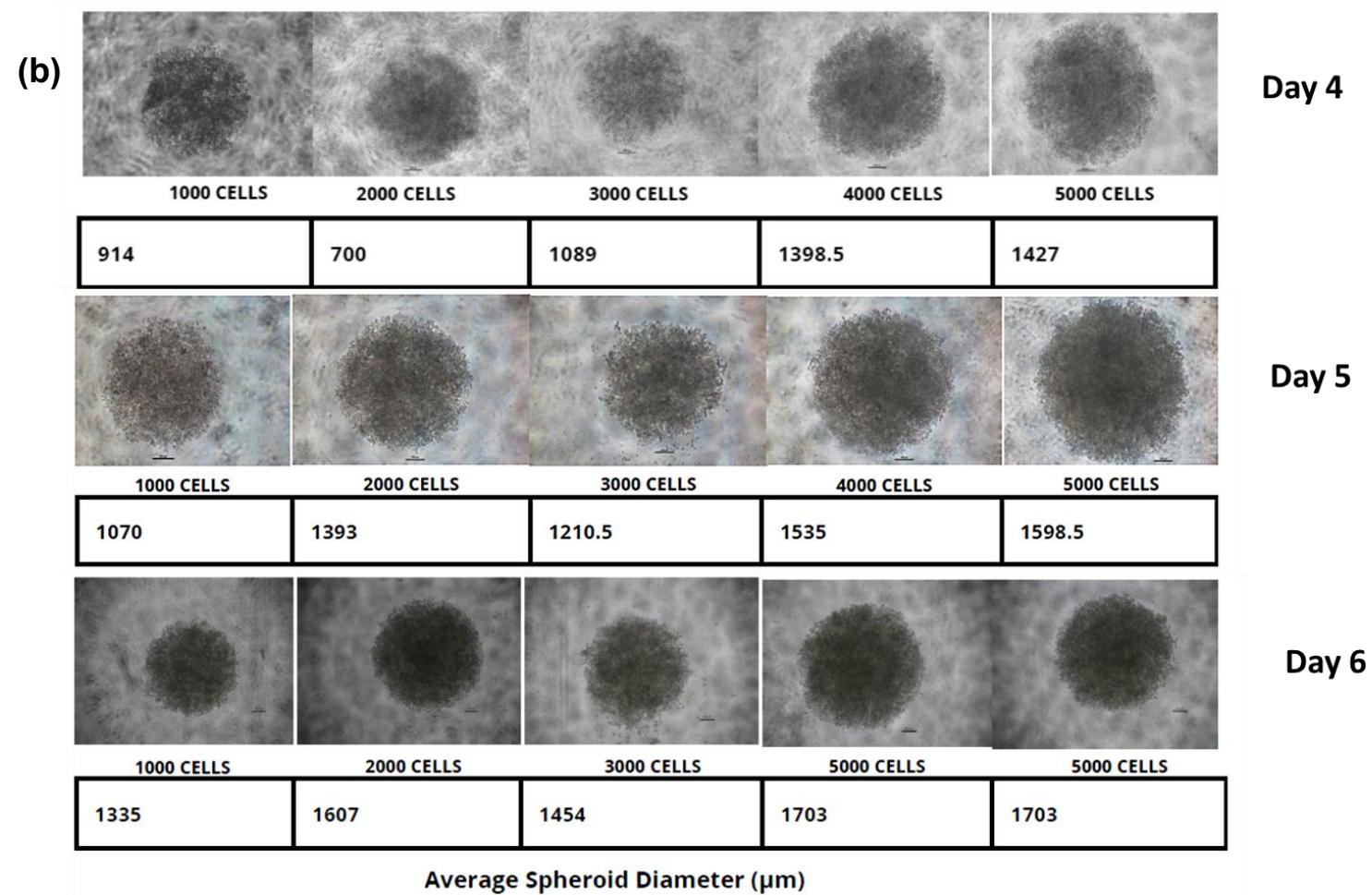
Day 6

4000 CELLS	5000 CELLS	6000 CELLS	7000 CELLS	8000 CELLS	9000 CELLS	10000 CELLS
1738	1992	1768	2040	2126.5	2110	2400

Average Spheroid Diameter (μm)

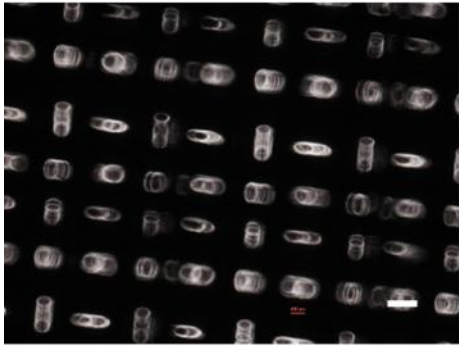
SI1. a: Three-dimensional (3D) culture developed from MDA-MB-468 TNBC cells $[(1-10) \times 10^3 \text{ cells}/20 \mu\text{L}]$ in complete medium. The average change in diameters (depicted in the boxes below the image in microns) of the 3D culture was recorded on days 4, 5, and 6 post-seeding under normal oxygen conditions (NOC). The best cellular aggregate was observed when $4-6 \times 10^3 / 20 \mu\text{L}$.

Magnifications=4x; scale bar=100 μm

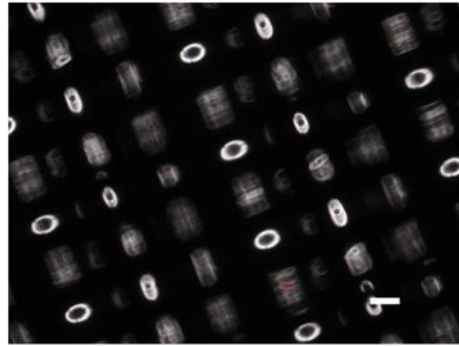


SI1. b: Three-dimensional (3D) culture developed from MDA-MB-468 TNBC cells $[(1-5) \times 10^3 \text{ cells}/20 \mu\text{L}]$ in complete medium. The average change in diameters (depicted in the boxes below the image in microns) of the 3D culture was recorded on days 4, 5, and 6 post-seeding under low oxygen conditions (LOC). The best cellular aggregate was observed when $3-5 \times 10^3 / 20 \mu\text{L}$. **Magnifications=4x; scale bar=100 μm**

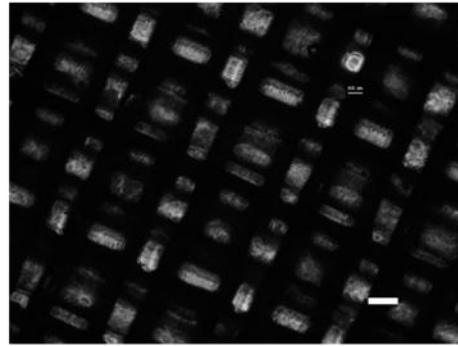
(a)



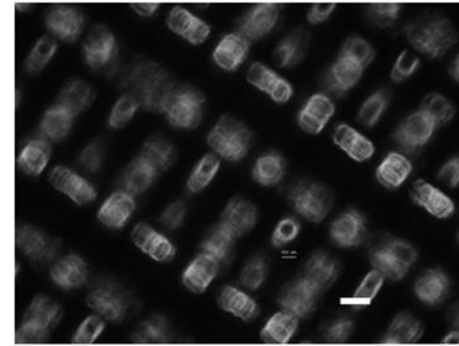
1 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



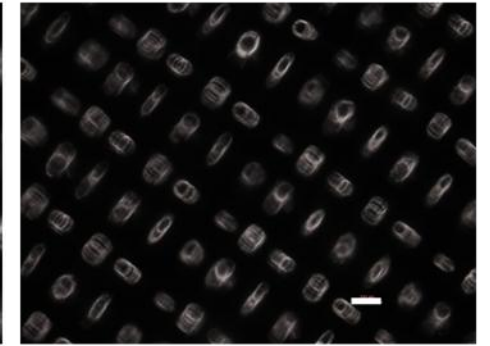
2 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



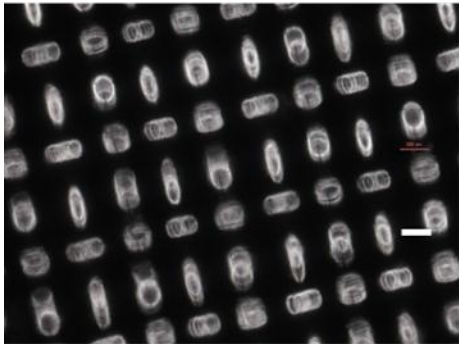
3 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



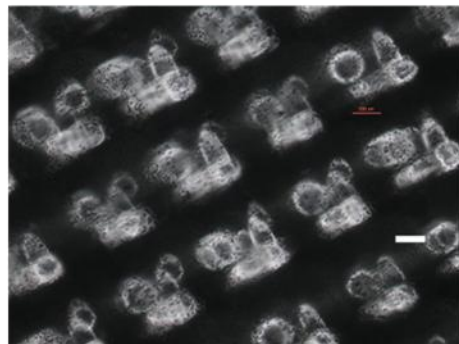
4 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



5 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



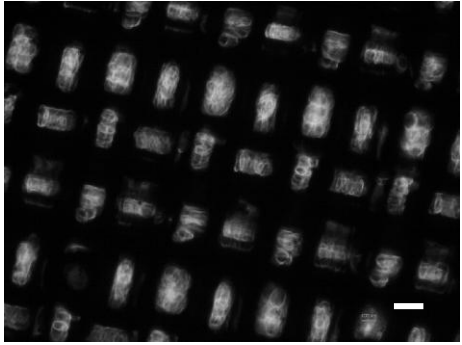
6 x 10⁴ 20µl/cells – day 2 post seeding (NOC)



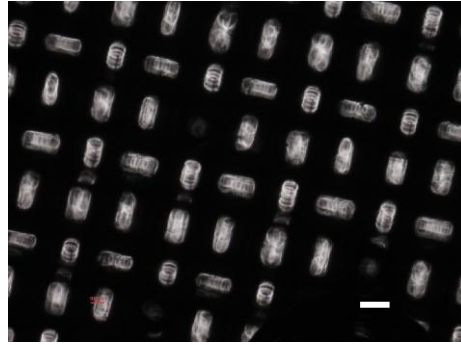
7 x 10⁴ 20µl/cells – day 2 post seeding (NOC)

SI2. a: The spheroids from the MDA-MB-468 formed using the scaffold approach were detected starting from day 2 post-seeding under normal oxygen conditions (NOC). Images were taken on day 2. The quantity and number of spheroids were observed to be related to the volume and the density of the cell suspension added to the scaffold culture dish. The cell density/volume used was $1-7 \times 10^3 / 20 \mu\text{L}$. Magnification=4x; scale bar in white = 200µm

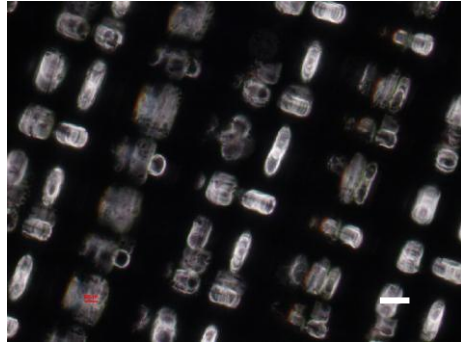
(b)



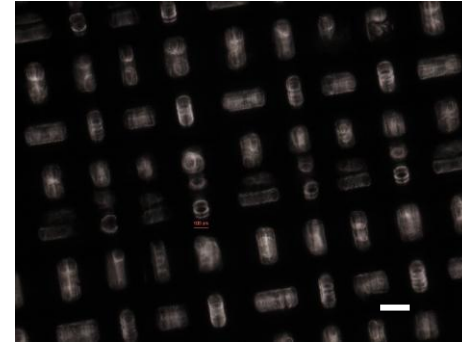
1 x 10⁴ 20μl/cells – day 2 post seeding (LOC)



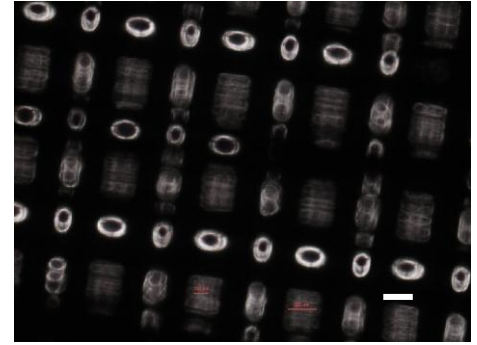
2 x 10⁴ 20μl/cells – day 2 post seeding (LOC)



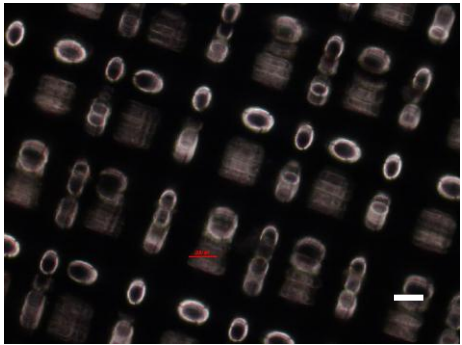
3 x 10⁴ 20μl/cells – day 2 post seeding (LOC)



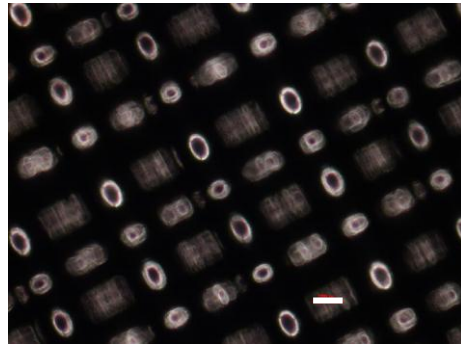
4 x 10⁴ 20μl/cells – day 2 post seeding (LOC)



5 x 10⁴ 20μl/cells – day 2 post seeding (LOC)

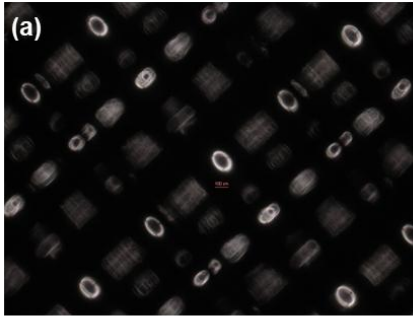


6 x 10⁴ 20μl/cells – day 2 post seeding (LOC)

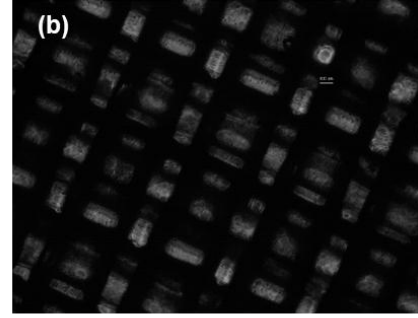


7 x 10⁴ 20μl/cells – day 2 post seeding (LOC)

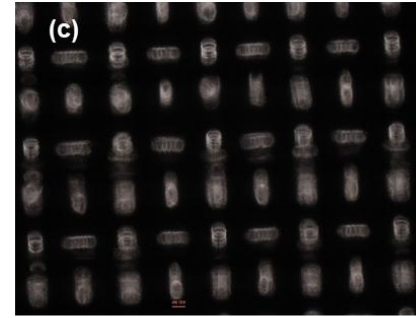
SI2. b: The spheroids from the MDA-MB-468 formed using the scaffold approach were detected starting from day 2 post-seeding under low oxygen conditions (LOC). Images were taken on day 2. The quantity and number of spheroids were observed to be related to the volume and the density of the cell suspension added to the scaffold culture dish. The cell density/volume used was $1-7 \times 10^3 / 20 \mu\text{L}$. Magnification=4x; scale bar in white = 200μm



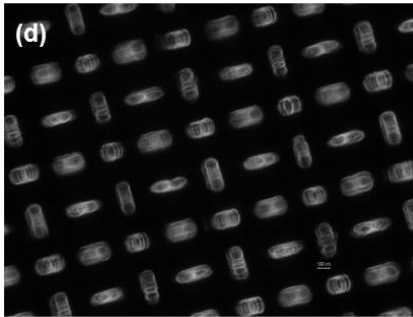
1 x10³ 20μl/cells – day 4 post seeding
(NOC)



4 x10³ 20μl/cells – day 4 post seeding
(NOC)



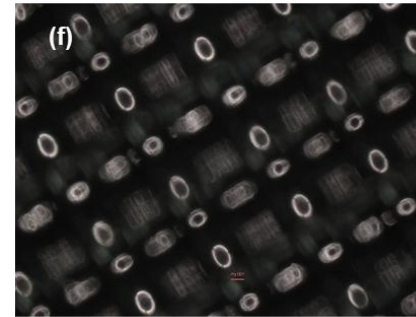
7 x10³ 20μl/cells – day 4 post seeding
(NOC)



1 x10³ 20μl/cells – day 4 post seeding
(LOC)

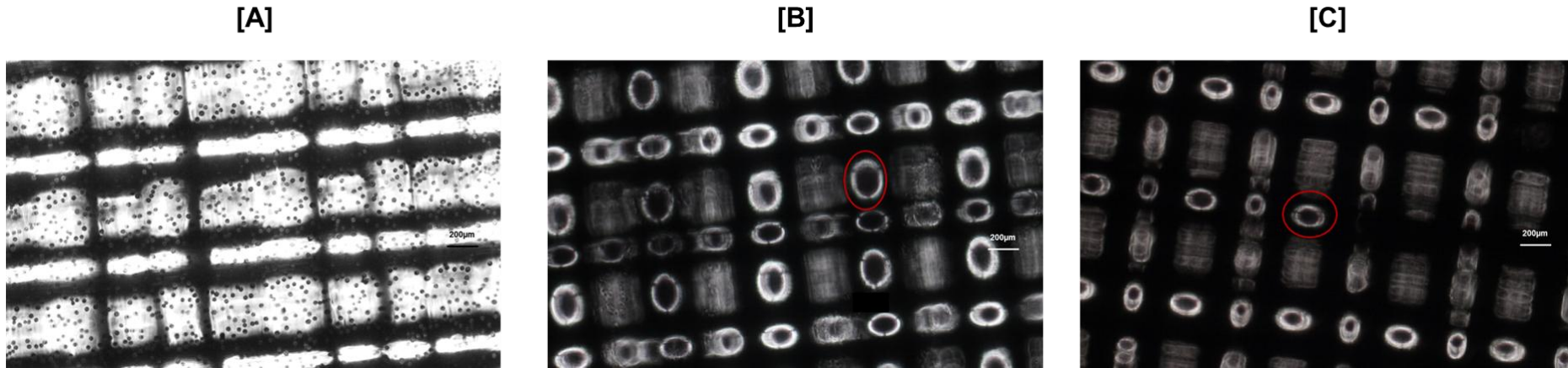


4 x10³ 20μl/cells – day 4 post seeding
(LOC)



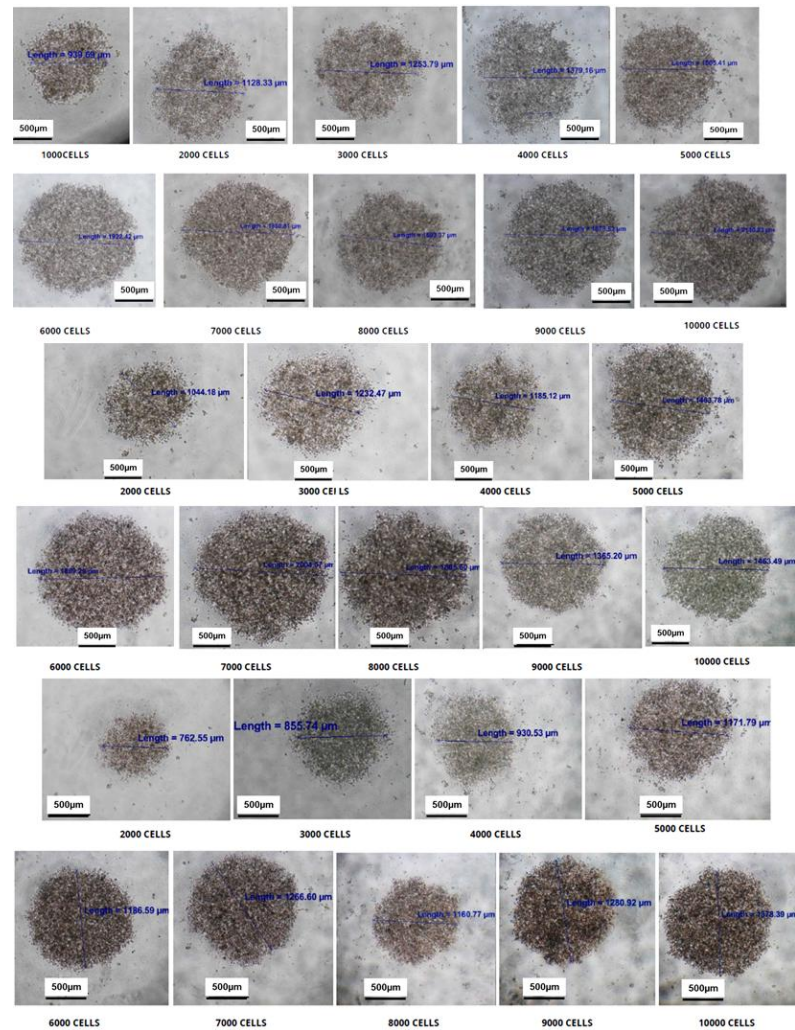
7 x10³ 20μl/cells – day 4 post seeding
(LOC)

SI3. : The spheroids from the MDA-MB-468 formed using the scaffold approach were detected after 4 days under (a), (b) and (c) normal oxygen (NOC) or (d), (e)and (f) low oxygen (LOC) conditions. The images were taken on day 4. The quantity and number of spheroids were observed to be related to the volume and the density of the cell suspension added to the scaffold culture dish. The cell density/volume used was 1,4, and 7× 10³ /20 μl. Magnification=4x; scale bar= 100μm



SI4: Formation of MDA-MB-468 spheroids using the 3D scaffold culture [A] Day 0 post-seeding image showing single MDA-MB-468 cells distributed on the scaffold surface. [B] normal (NOC) and [C] low oxygen conditions showing the appearance of early 3D compact aggregate formation (red circles). The cell density/volume used was 5×10^3 /20 µL / 1.5 mL. [B] and [C] captured on day 2 post-seeding.. Magnification 4x; Scale bar = 200µm

(a)



Day 4

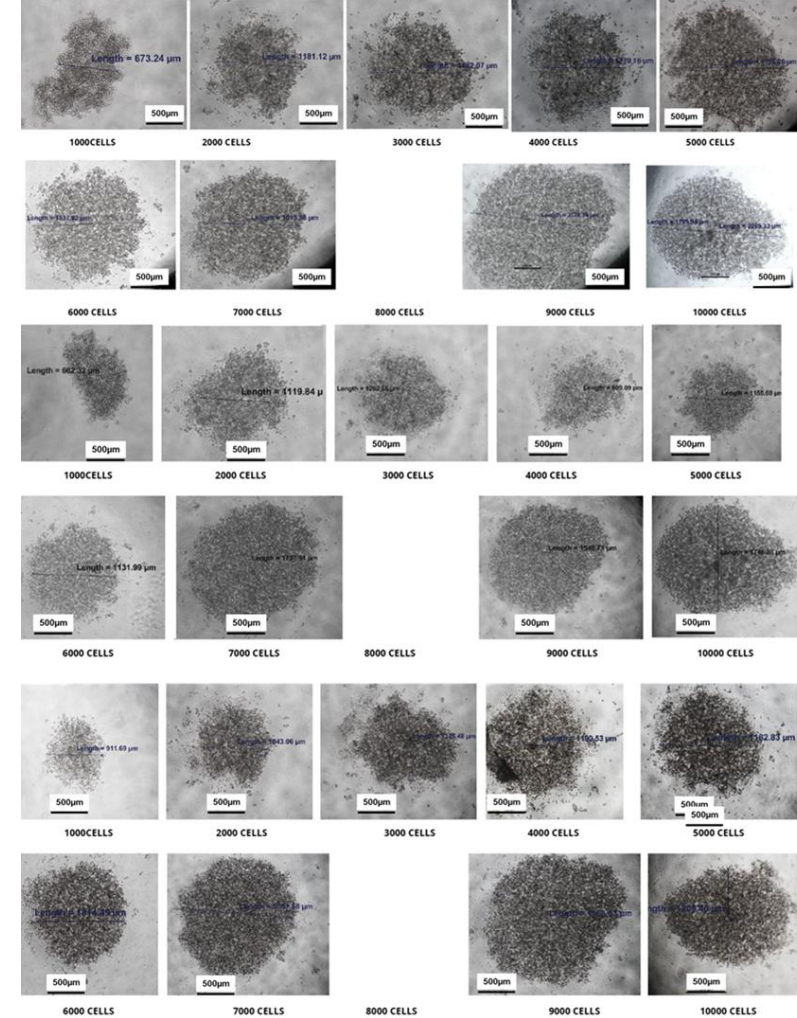
Day 5

Day 6

SI5 a: Three-dimensional (3D) culture developed from MDA-MB-231 TNBC cells [(1-10) × 10³ cells/20 μL] in complete medium. The average change in diameters (depicted on the images in microns) of the 3D culture was recorded on days 4, 5, and 6 post-seeding under normal oxygen conditions (NOC). The best cellular aggregate was observed when 4–7 × 10³ /20 μL.

Magnifications = 4x; scale bar = 500 μm

(b)

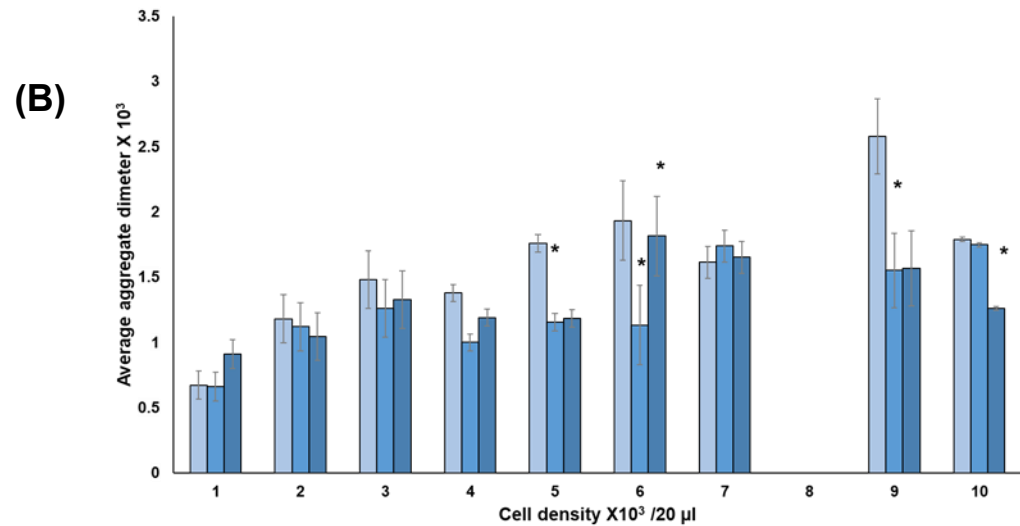
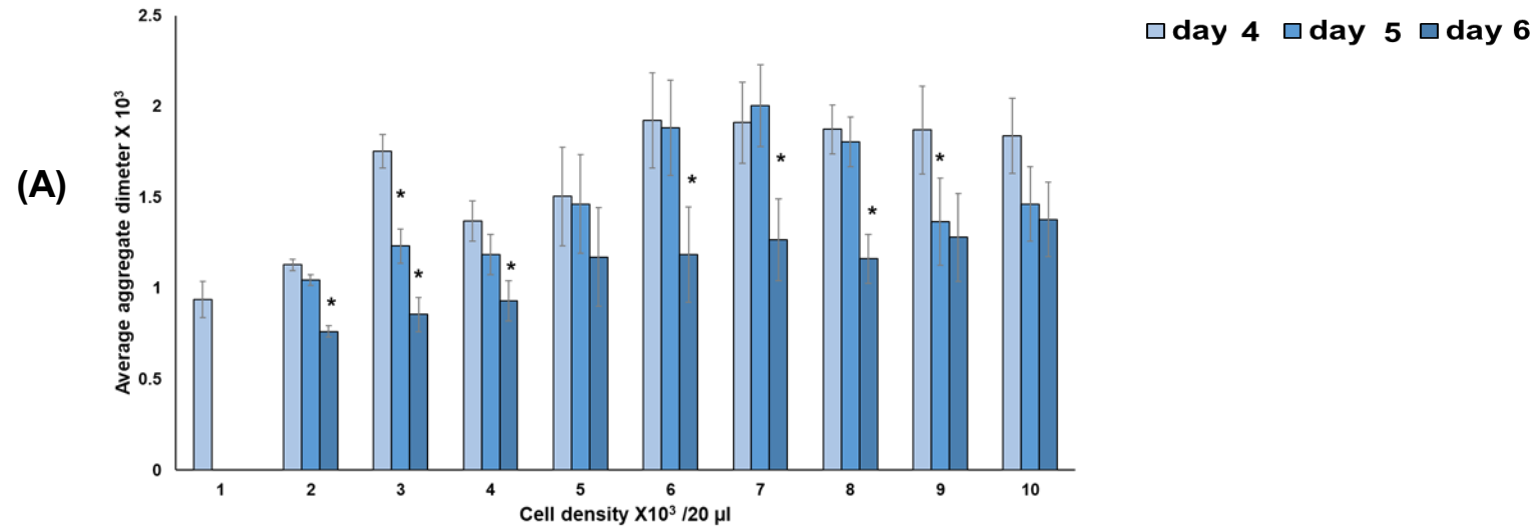


Day 4

Day 5

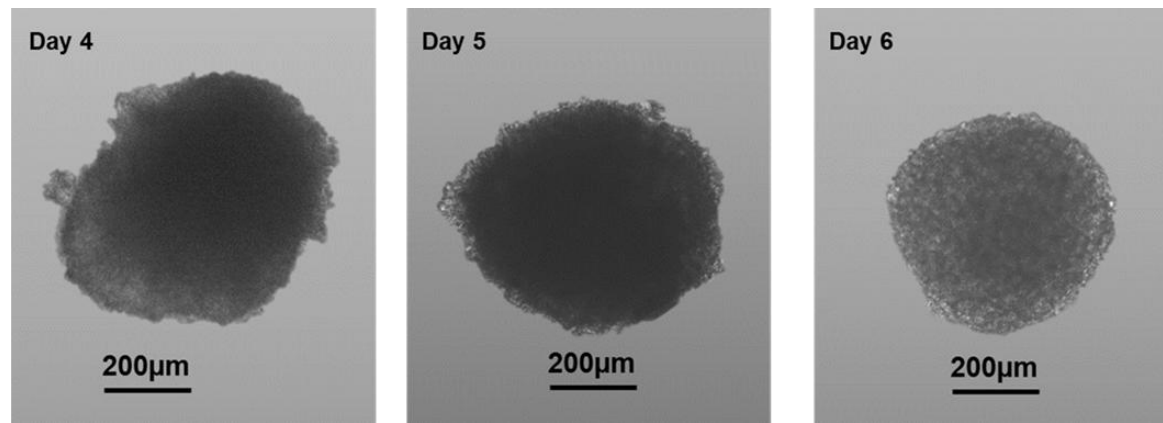
Day 6

SI5 b: Three-dimensional (3D) culture developed from MDA-MB-231 TNBC cells [(1-10) × 10³ cells/20 μL] in complete medium. The average change in diameters (depicted on the images in microns) of the 3D culture was recorded on days 4, 5, and 6 post-seeding under low oxygen conditions (LOC). HD yielded the most uniform, compact spheroids at 4–7 × 10³ cells/20 μL. No 3D aggregates were formed at 8 × 10³ cells/20 μL cell density. **Magnifications= 4x; scale bar = 500 μm**

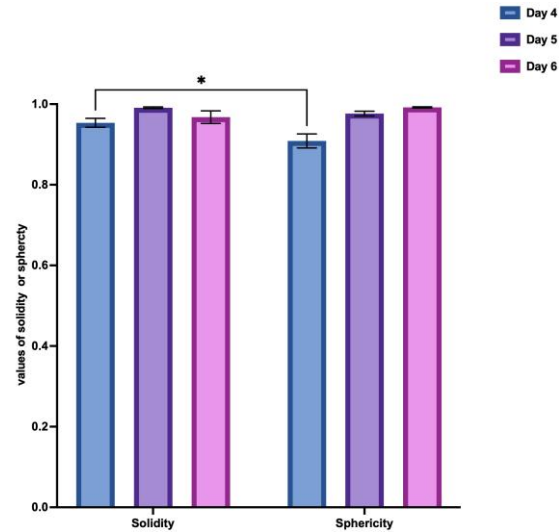


SI6:3D cultures formation by MDA-MB-231 TNBC cells across seeding densities and oxygen conditions. Cells were seeded at $(1-10) \times 10^3$ cells/ $20 \mu\text{L}$ in complete medium and cultured at 37°C , $5\% \text{CO}_2$ under **(A)** normal oxygen (NOC) or **(B)** low oxygen (LOC). Mean percentage change in spheroid diameter was recorded on days 4, 5, and 6. Under NOC, HD yielded the most uniform, compact spheroids at $4-7 \times 10^3$ cells/ $20 \mu\text{L}$. Under LOC no 3d aggregates were formed at 8×10^3 cells/ $20 \mu\text{L}$. Data are shown as mean $\pm$ SD; two-way ANOVA, $p < 0.05$

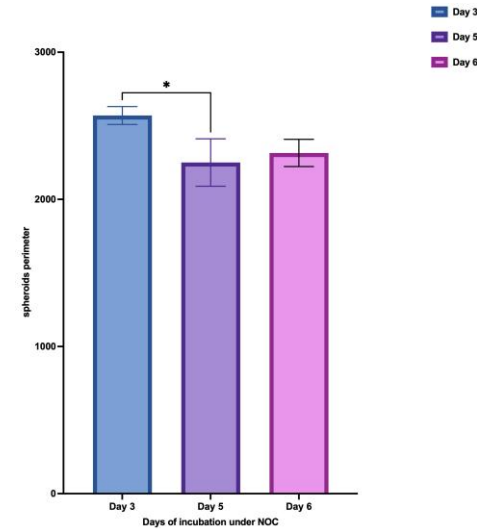
(A)



(B)



(C)



S17:3D culture formation of MDA-MB-231 TNBC cells was assessed using ultra-low attachment (ULA) plates under normal (NOC). **[B,C]**. Morphometric parameters, including solidity, sphericity, and perimeter, were quantified using AnaSP image analysis software. Images are representative of a single well that was followed longitudinally across the captured time period. Red arrows indicate cellular debris. Data are expressed as the mean $\pm$ standard error of the mean (SEM) from three independent biological replicates. Statistical significance was determined as $p < 0.05$ (*) using one-way ANOVA. Spheroid images were captured using an inverted light microscope (Nikon Eclipse TE2000-U) at 4 $\times$ magnification. **NOC: normal oxygen condition; scale bar=200 micron**

SI Table1:Primers used for the quantitative polymerase chain reaction

Gene Symbol	Gene Name	Primer Sequence (5' - 3')	
<i>HIF1A</i>	<i>Hypoxia-inducible factor-1-alpha</i>	F:	CCT CTG TGA TGA GGC TTA CCA TC
		R:	CAT CTG TGC TTT CAT GTC ATC TTC
<i>NES</i>	<i>Nestin</i>	F:	CTT CCC TCC GCA TCC CGT CA
		R:	AAA GCC AGC ATG TCA CCC TC
<i>SNAI1</i>	<i>Snail family transcriptional repressor-1</i>	F:	CGG TTC CGA TGC CCT GAG GCT C
		R:	CGT CAC ACT TCA TGA TGG AAT TG
<i>TWIST1</i>	<i>Twist basic helix-loop-helix transcription</i>	F:	ACC ATC CTC ACA CCT CTG CAT
		R:	TTC CTT TCA GTG GCT GAT TGG
<i>VEGFA</i>	<i>Vascular endothelial growth factor A</i>	F:	AGG GCA GAA TCA TCA CGA AG
		R:	CAC ACA GGA TGG CTT GAA G
<i>CD44</i>	<i>Cluster of differentiation 44</i>	F:	CGC CAA ACA CCC AAA GAA
		R:	GTG TTG TCC TTC CTT GCA TT
<i>GAPDH</i>	<i>Housekeeping gene</i>	F:	ACC ACA GTC CAT GCC ATC AC
		R:	TCC ACC ACC CTG TTG CTG TA

F: Forward; **R:** Reverse;

SI Table 2: Comparison of Spheroid Culture Techniques in MDA-MB-468 TNBC Cells under Varying Oxygen Conditions in this study

Parameter	Hanging Drop (HD)	Liquid Overlay (ULA/Agarose)*	Scaffold-Based
Spheroid Formation Time	Moderate (24–36 h)	Dependent on HD pre-formation	Fast (24–48 h)
Structural Integrity	Moderate under (NOC)	High (especially ULA under NOC)	Variable (less compact under LOC)
Morphometric Uniformity (Sphericity)	Variable	High under NOC, moderate under LOC	Moderate; perimeter fluctuation observed
Ease of Handling & Imaging	Moderate; prone to disruption	High (ULA) / Moderate (Agarose)	Low (scaffold material obscures visualisation)
Gene Expression Consistency	Moderate	High (notably <i>CD44</i> , <i>TWIST1</i> , <i>VEGFA</i> under NOC)	less responsive under LOC
Hypoxia Simulation (<i>HIF1A</i> , <i>VEGFA</i>)	Poor under LOC	NOC mimics pseudo-hypoxia response	Inconsistent, sometimes inverse expression
Reproducibility	Moderate	High	Moderate

LOC: low oxygen condition; **HD:** hanging drop; **NOC:** normal oxygen condition; **ULA:** ultra-low attachment; *, Rat tail collagen I added to the complete media