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SUPPORTING INFORMATION FOR

Supported gel slab scaffolds as a three-dimensional cell-based assay platform

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Materials and Methods.

Paper-scaffold preparation. Paper scaffolds were prepared as detailed previously.[1, 2] Briefly, sheets of 47.5-μm thick Lensx90 paper (Berkshire Corporation) were wax patterned to contain a series of 3 mm circles surrounded by a 1.5 mm wax border. The wax-patterned sheets were baked for 10 minutes, cooled to room temperature, and the individual scaffolds removed from the sheet with a hole punch (Office Max). Before use, the scaffolds were sterilized in an Anprolene AN74i ethylene oxide sterilizer (Andersen Sterilizers Inc). The cell-containing regions of the scaffolds were deposited with 0.5 μL of either cell-laden or cell-free ECM.

Optimized CTG and the resazurin assay conditions to measure cell viability in the SGS scaffolds. For the CTG assay, each scaffold was placed in a 96-well plate containing 150 μL of a 1:1 solution of 1X DPBS and CTG reagent. The scaffolds were agitated on an XY shaker for 20 min at room temperature before 100 μL of the solution was transferred to an opaque 96-well plate. Luminescence intensity (350-850 nm) was recorded on a SpectraMax i3x Multi-Mode microplate reader (Molecular Devices). For the resazurin assay, each scaffold was placed in a 96-well plate containing 20 μL of a resazurin stock (0.66 mM in 1X DPBS) and 100 μL of fresh culture medium. The scaffolds were incubated for 5 h at room on an XYZ shaker before 100 μL of solution was transferred to a clear-bottom 96-well plate. Fluorescence intensity (λex = 560 nm and λem = 590 nm) was recorded on a SpectraMax i3x Multi-Mode microplate reader.

Calibration curves. Relationships between cell number and the signal generated from optimized CTG or resazurin assay conditions were measured as follows. Monolayer cultures were prepared by depositing 781-50,000 cells suspended in 100 μL of culture medium directly into 96-well plates. Background signals associated with the monolayer culture format were obtained from 100 μL of culture medium only. The SGS scaffolds were deposited with 781-100,00 cells suspended in ECM and placed in a 96-well plate containing 100 μL of culture medium. Background signals associated with this format were determined from SGS scaffolds deposited with ECM only. The cells were incubated overnight before analysis.

Dose-response relationships. SGS scaffolds deposited with 1.0×10^4 HCT116 or M231 cells suspended in ECM were incubated overnight in culture medium and then transferred to a 96-well plate containing 200 μL of culture medium containing 0.04 - 1 μM SN-38 or 0.02 - 6.25 μM doxorubicin. Cell viability was measured with the CTG assay after a 72-h dose. Drug stock solutions were prepared at 1000x the dosing concentration in DMSO and stored at -20 °C until needed. No-drug controls and SGS scaffolds deposited with only ECM were maintained in culture medium containing 0.1% (v/v) DMSO for 72 h before analysis. The datasets were fit with a three-parameter logistic regression curve; IC50 values obtained for each dataset were compared with an extra sum-of-squares F-test.

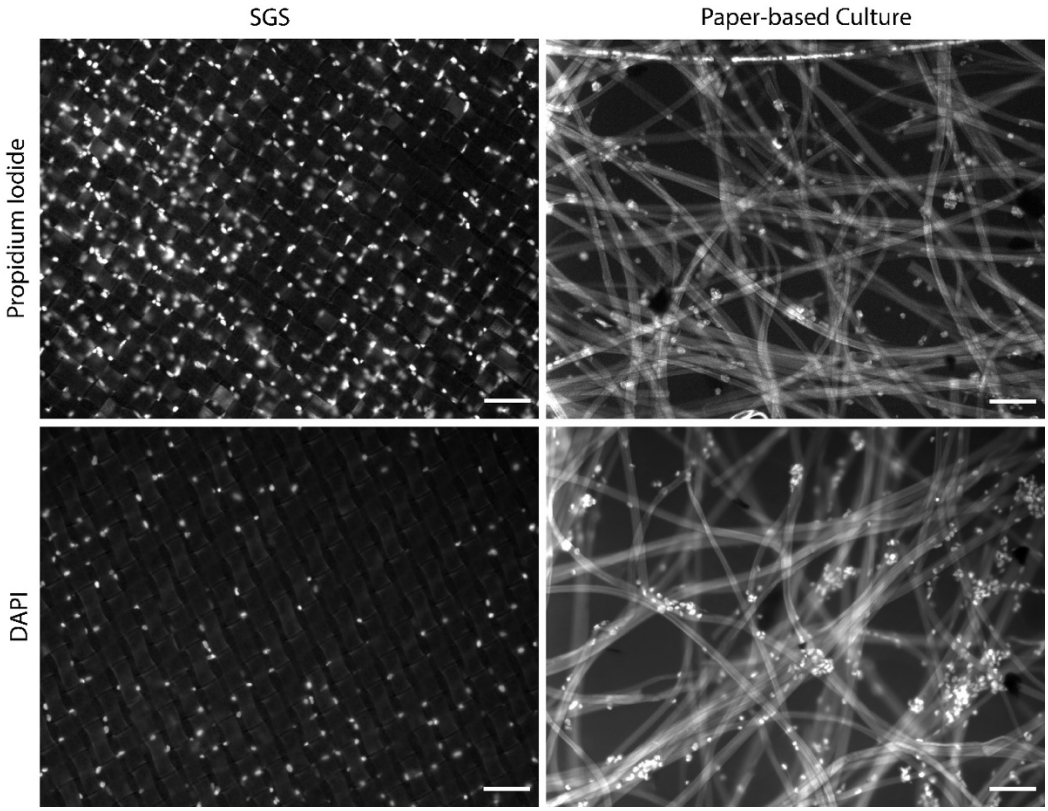

Figure S1. Widefield fluorescence micrographs of cell-containing (left column) SGS scaffolds with a nylon bottom piece and (right column) paper scaffolds. The nylon mesh in the SGS scaffolds had an average pore size of 7 μm. Each scaffold was deposited with 1.0×10^4 M231 cells suspended in ECM, incubated overnight, and stained before analysis. The cells were fixed and permeabilized in a paraformaldehyde solution (3.7% v/v in PBS) before staining with PI or DAPI. The micrographs were collected on a Nikon TE-2000 inverted microscope with a 10X/0.80 Plan Apo objective. The scale bar in each panel represents 100 μm.

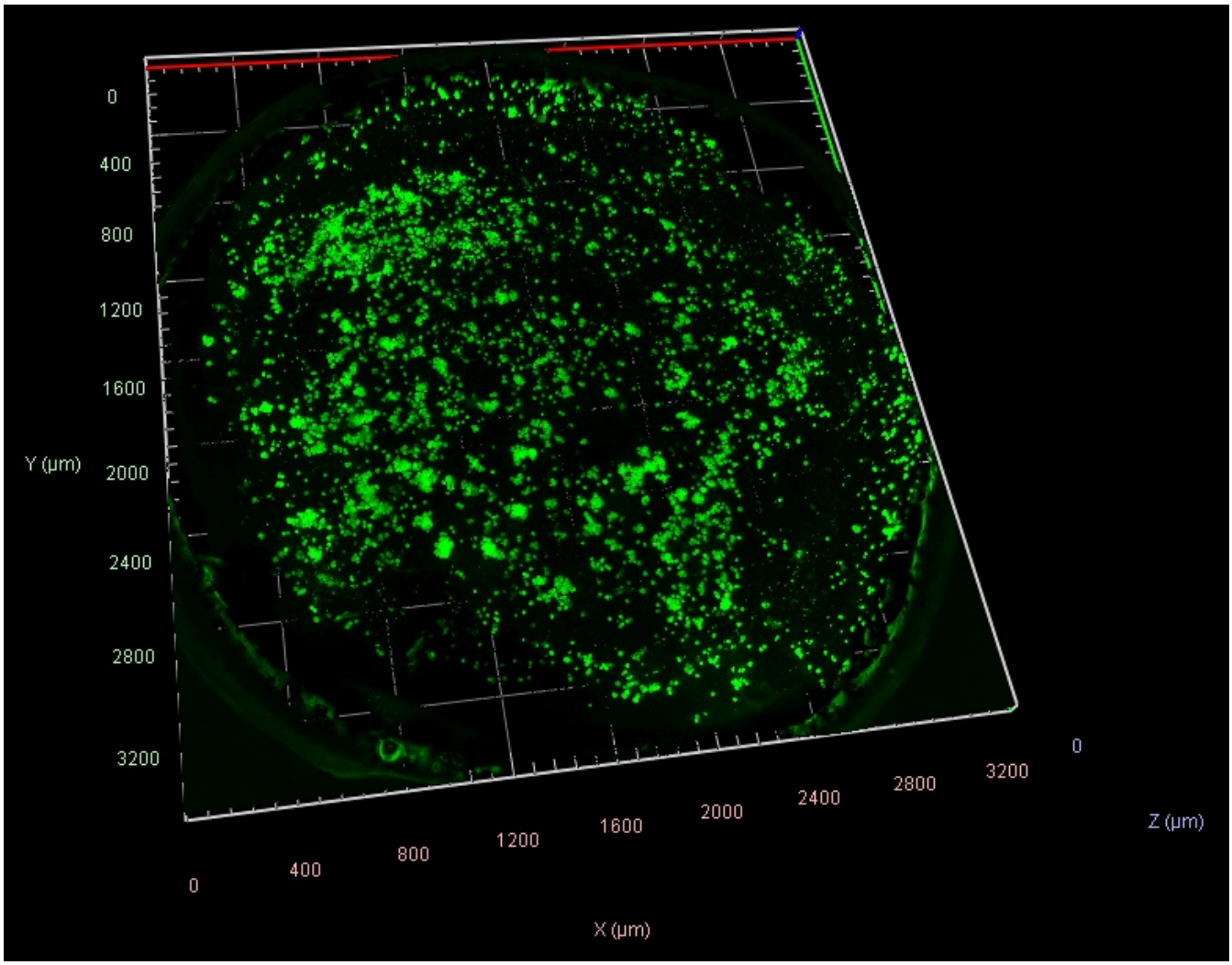

Figure S2. A larger version of Figure 2Ci from the main text of the manuscript. A composite confocal micrograph with a z-resolution of 4 µm of an SGS scaffold with a porous PET bottom piece deposited with 1.0×10^4 CellTracker green-labeled M231 cells suspended in ECM at a final density of 1.2×10^7 cells/cm^3. The composite image is an 11x12 tiled array, captured 24 h after cell deposition.

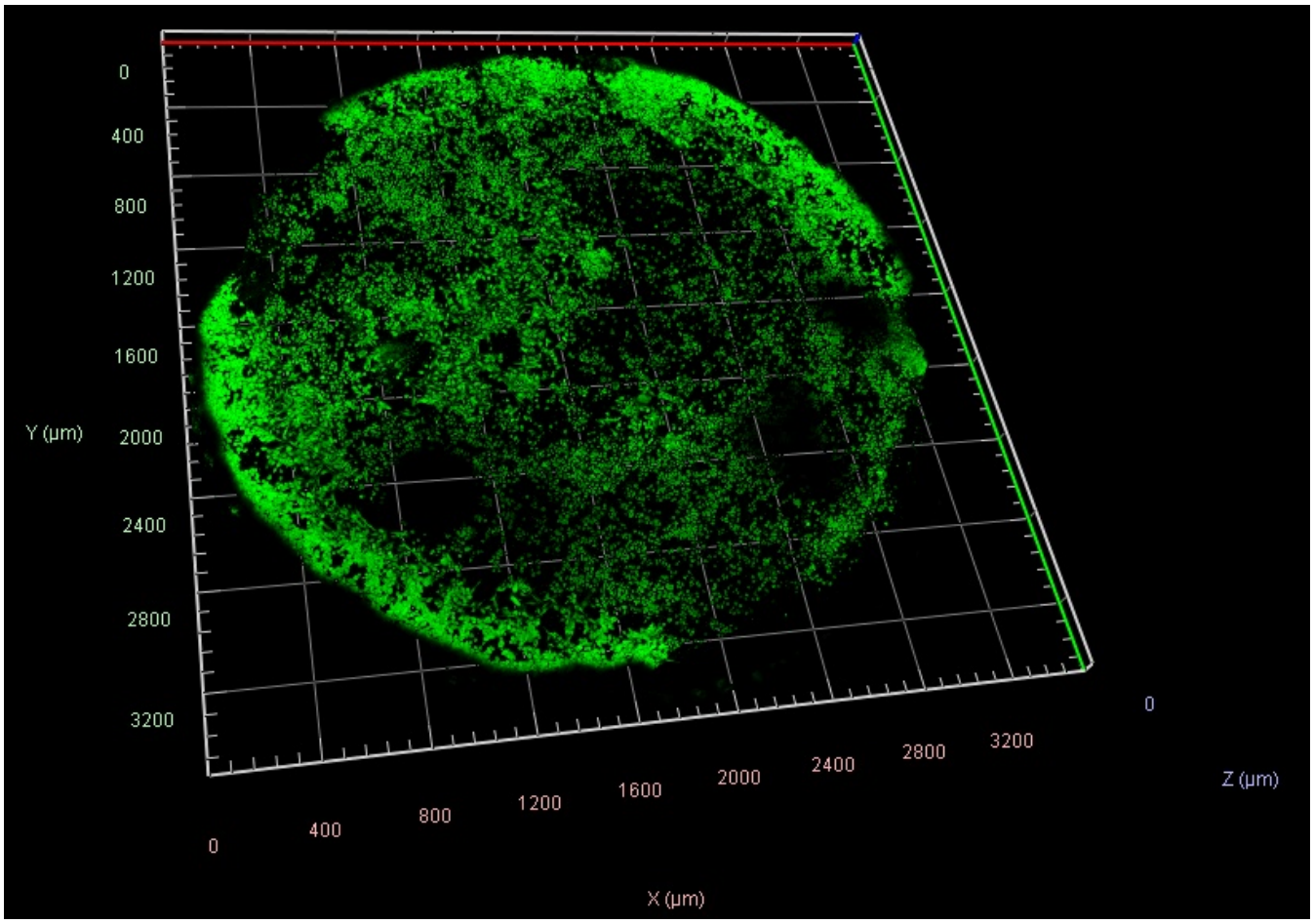

Figure S3. A larger version of Figure 2Cii from the main text of the manuscript. A composite confocal micrograph with a z-resolution of 4 µm of an SGS scaffold with a porous PET bottom piece deposited with 6.0×10^4 CellTracker green-labeled M231 cells suspended in ECM at a final density of 7.2×10^7 cells/cm^3. The composite image is an 11x12 tiled array, captured 24 h after cell deposition.

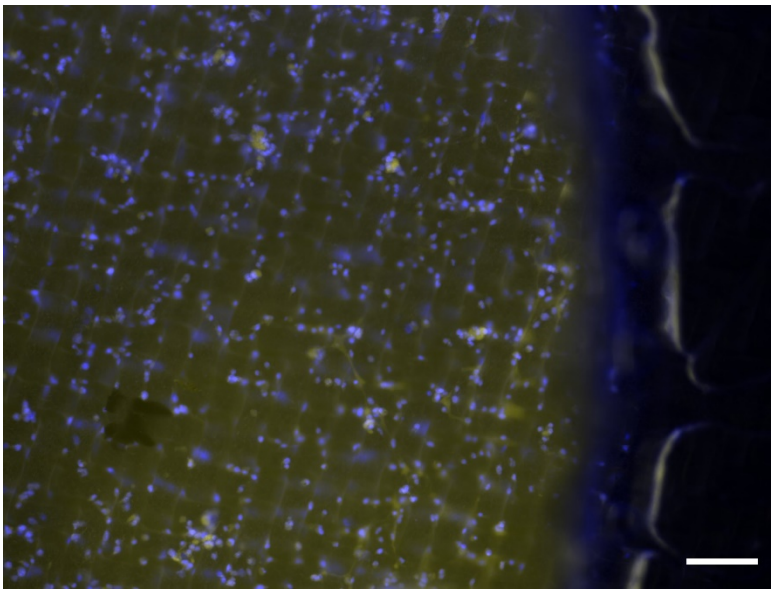

Figure S4. A widefield fluorescence micrograph of an SGS scaffold with a nylon bottom piece (7 μm pore size) deposited with $1.0x10^4$ M231 cells suspended in ECM at a final density of $1.2x10^7$ cells/cm^3. The ECM was pre-labeled with NHS-FITC (1 mg/mL, 10 min, on ice). The cells were fixed and permeabilized in paraformaldehyde (3.7% v/v in PBS) and then stained with PI (7.2 μM, 1% RNase-A, 1X DPBS, 45 min, 37°C). The ECM is yellow; the PI-stained nuclei are blue. The micrograph was collected on a Nikon TE-2000 inverted microscope with a 10X/0.80 Plan Apo objective. The scale bar in each panel represents 100 μm.

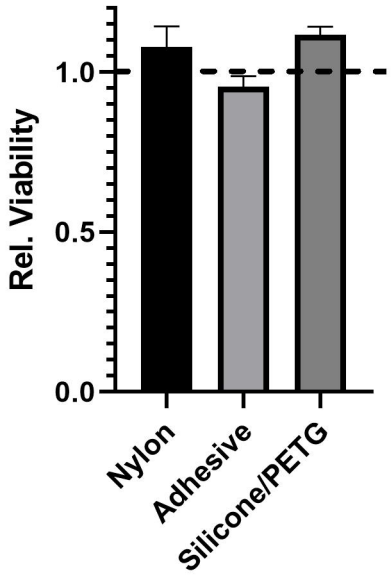

Figure S5. Viability of M231 cells in the presence of a component of the SGS scaffolds, relative to a negative control containing only cells. In each setup, $6.0x10^4$ M231 cells were suspended in ECM, deposited in a paper scaffold, and placed in a 6-well plate containing culture medium and 1) a 15 mm x 20 mm piece of nylon mesh, 2) a 15 mm x 20 mm piece of a silicone/PETG sheet, 3) a 15 mm x 20 mm piece of a silicone/PETG sheet covered in spray adhesive. Viability was measured with the CTG assay. An unpaired *t*-test with Welch's correction to account for unequal standard deviations found no difference between the negative control and any of the component-containing cultures. All values correspond to at least 8 scaffolds prepared from at least 2 cell passages.

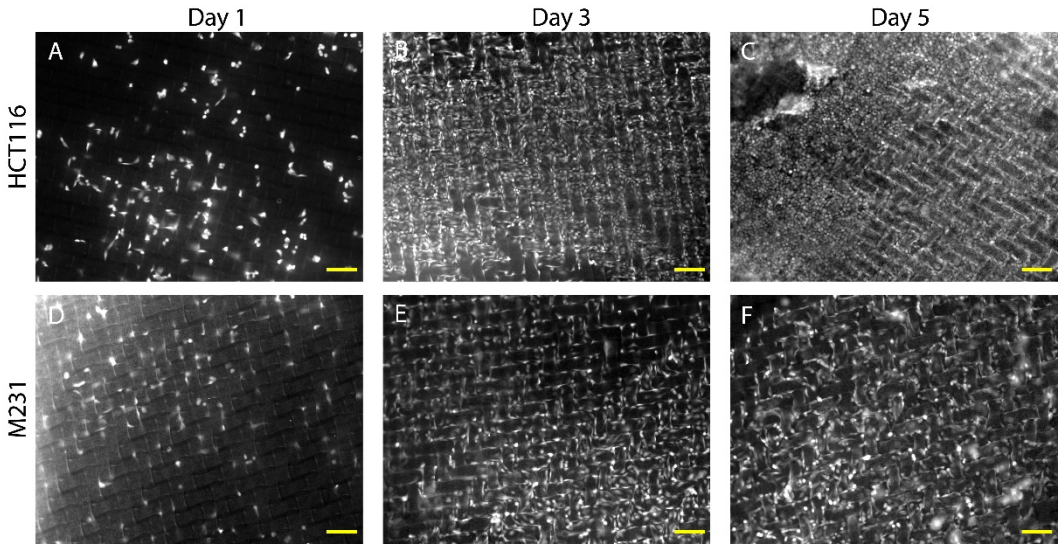

Figure S6. Representative widefield fluorescence micrographs of SGS scaffolds with a nylon mesh bottom piece, deposited with $1.0x10^4$ (A-C) HCT116 or (D-F) M231 cells suspended in ECM. The cells were stained with calcein-AM, and micrographs were collected (A, D) one, (B, E) three, and (C, F) five days after deposition. The micrographs were collected on a Nikon TE-2000 inverted microscope with a 10X/0.80 Plan Apo objective. The scale bar in each panel represents 100 μm.

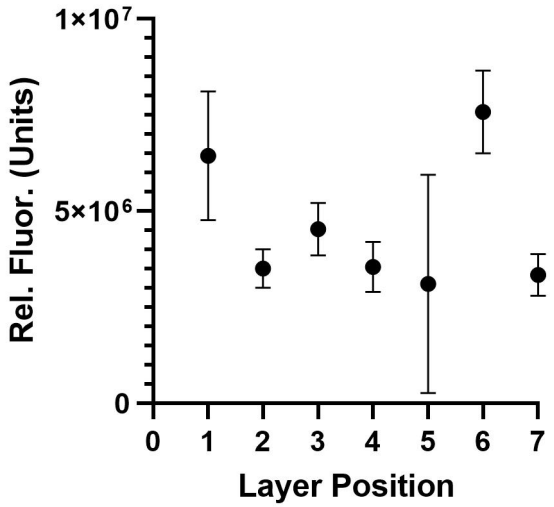

Figure S7. Relative fluorescence intensity of SGS scaffolds (nylon bottom piece) after staining with PI (7.2 μM, 1% RNase-A, 1X DPBS, 45 min, 37ºC). Each scaffold was deposited with $6.0x10^4$ M231 cells suspended in ECM, stacked, and placed in a holder that limited nutrient and oxygen exchange to the top of the stack. Increases in layer number correspond to increasing distances from the source of fresh culture medium. A schematic of this setup is shown in Figure 4 of the manuscript. After 120 h, the scaffolds were physically separated and stained with PI. Each scaffold was imaged on a Sapphire Biomolecular Flat Bed Scanner (Azure Biosciences, λ_{ex}=520 nm, λ_{em}=607 BP70 nm, 10 μm resolution). The average fluorescence intensity of the cell-containing regions was determined with the AzureSpot software package (Azure Biosciences). Each value corresponds to the average and standard error of the mean of at least 3 tumor stacks prepared from at least 1 cell passage.

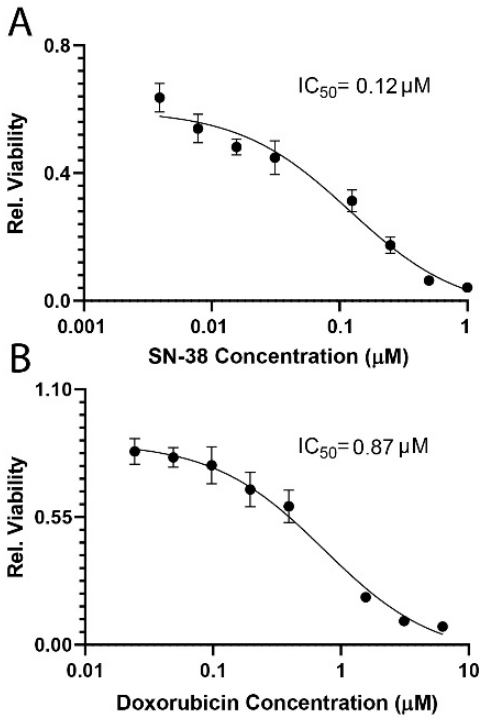

Figure S8. Dose-response relationships of A) HCT-116 cells against SN38 and B) M231 cells against doxorubicin. The viability values are plotted relative to a no-drug control. These plots determined the potency and efficacy of a drug-cell pair for a given set of dosing conditions. Potency was defined as the drug concentration needed to elicit 50% of the maximal inhibitory (IC_{50}) response. Efficacy was defined as the maximal response that can be expected. A) Viability of $1.0x10^4$ HCT116 cells suspended in ECM and deposited in an SGS scaffold, 48 h after dosing with the active metabolite of irinotecan (SN-38). B) Viability of $1.0x10^4$ M231 cells suspended in ECM and deposited in an SGS scaffold, 72 h after dosing with doxorubicin. Cellular viability was determined with the CTG assay. Each data point represents at least 8 replicate scaffolds prepared from 2 passages of cells. The lines represent three-parameter logistical fits, which were used to estimate the potency and efficacy of the drug-cell combination.

Table S1. Compounds from the Approved Oncology Drugs set provided by the National Cancer Institute screened against M231 cells deposited as monolayers in a 96-well plate or SGS scaffolds for 72 h. [a]

Compound Number	NSC [b]	CAS [c]	Toxicity hit in 2D [d,f]	Toxicity hit in 3D [e,f]
1	740	59-05-2		
2	6396	52-24-4	X	
3	26271	50-18-0		
4	49842	143-67-9	X	
5	82151	23541-50-6	X	X
6	122819	29767-20-2	X	X
7	180973	54965-24-1		
8	279836	65271-80-9	X	X
9	609699	119413-54-6	X	X
10	713563	107868-30-4		
11	750	55-98-1	X	
12	8806	3223-07-2		
13	26980	50-07-7	X	X
14	63878	69-74-9	X	X
15	85998	18883-66-4		
16	123127	25316-40-9	X	X
17	218321	53910-25-1		
18	296961	20537-88-6		
19	613327	95058-81-4	X	X
20	715055	184475-35-2	X	
21	752	154-42-7	X	X
22	9706	51-18-3	X	
23	27640	50-91-9	X	
24	66847	50-35-1		
25	92859	1327-53-3		
26	125066	11056-06-7	X	
27	226080	53123-88-9	X	
28	312887	75607-67-9	X	
29	616348	100286-90-6	X	
30	718781	183321-74-6	X	
31	755	50-44-2		
32	13875	645-05-6		
33	32065	127-07-1		
34	67574	2068-78-2	X	
35	102816	320-67-2	X	
36	125973	33069-62-4	X	X
37	241240	41575-94-4		

38	362856	85622-93-1		
39	628503	114977-28-5	X	
40	719276	129453-61-8		
41	762	55-86-7	X	
42	18509	5451-09-02		X
43	34462	66-75-1	X	
44	71423	595-33-5		
45	105014	4291-63-8	X	
46	127716	2353-33-5	X	
47	246131	56124-62-0	X	X
48	369100	99011-02-6		
49	683864	162635-04-3	X	
50	719344	120511-73-1		
51	1390	315-30-0		
52	19893	51-21-8	X	
53	38721	53-19-0		
54	75520	70-00-8		
55	109724	3778-73-2		
56	138783	3543-75-7		
57	256439	57852-57-0	X	X
58	409962	154-93-8	X	
59	701852	149647-78-9	X	X
60	719345	112809-51-5		
61	3053	50-76-0	X	X
62	24559	18378-89-7	X	X
63	45388	4342-03-4	X	
64	77213	366-70-1		
65	119875	15663-27-1		
66	141540	33419-42-0	X	
67	256942	56390-09-1	X	X
68	606869	123318-82-1	X	X
69	702294	52205-73-9		
70	719627	169590-42-5		
71	3088	305-03-3		
72	25154	54-91-1	X	
73	45923	298-81-7		
74	79037	13010-47-4	X	
75	122758	302-79-4		
76	169780	24584-09-6		
77	266046	61825-94-3	X	
78	608210	71486-22-1	X	
79	712807	154361-50-9		
80	721517	118072-93-8		

[a] Compound number in the table corresponds to the compound number plotted in **Figure 5** of the manuscript

[b] National Service Center (NSC) number

[c] Chemical Abstracts Service (CAS) number

[d] 2D culture format consisted of 500 M231 cells deposited into 96-well plates

[e] 3D culture format consisted of 1.0×10^4 M231 cells suspended in ECM and deposited into SGS scaffolds

[f] A toxicity hit was defined as a compound whose luminescence signal from the CTG assay was greater than three standard deviations lower than the average signal generated by the no-drug (negative) control. Viability was measured 72 h after dosing.

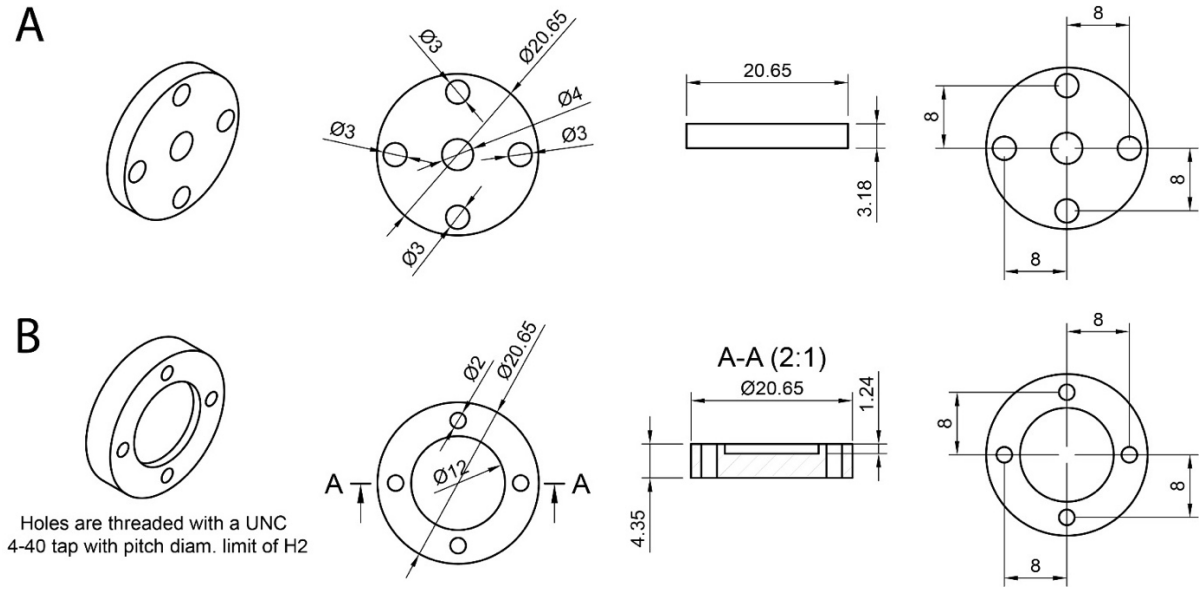

Figure S9. Engineering diagrams of the holder used to generate the stacked invasion assay and the spheroid-on-demand stacks are described in the manuscript. This holder ensured the SGS scaffolds were in conformal contact throughout the experiment and limited the exchange with fresh medium to the top of the stack only. A) Top acrylic plate, which was laser cut from a 3.125 mm-thick sheet of clear cast acrylic (McMaster-Carr) on an Omtech 55W CO_2 laser engraver and cutter. The laser settings to cut the acrylic were: 35% power, 8 mm/sec, 2 passes. The cut pieces were cleaned in a 70% (v/v) ethanol solution and dried overnight. B) Bottom plate, 3D printed on a Form 3B+ printer with Biomed Clear resin (FormLabs). The newly printed pieces were soaked in fresh isopropanol for 20 min, dried overnight at room temperature, and cured for 1 h at 60 ^{o}C in the Form Cure accessory (FormLabs). The four screw holes in the bottom plate were tapped with a UNC 4-40 tap with a pitch diameter limit of H2. Before use, the top and bottom plates were soaked overnight in 1X DPBS and sterilized in an ethylene oxide chamber (Andersen Sterilizer). The scale and reported values are in millimeters.

SGS compatibility with indirect viability assays.

Indirect viability assays assess the toxicity or proliferative effects of unknown compounds, comparing differences in cell number between two culture conditions or between a drug and no-drug control. Calibration curves relating signal and cell number. We recently evaluated the performance of four indirect viability assays for cells suspended in ECM and deposited in paper scaffolds,[2] measuring: i) residual ATP pools with the CellTiter Glo (CTG) assay, ii) redox potential of the cells with the resazurin assay, iii) esterase activity ability of viable cells to retain cleaved calcein-AM, and iv) fluorescence densitometry measures of cells engineered to express a fluorescent protein constitutively.

Figure S10 contains representative calibration curves of assay signal versus cell number for the CTG and resazurin assays. The cells were assessed as monolayers deposited directly into a well plate or after being suspended in an ECM and deposited into an SGS scaffold. The CTG datasets for the i) HCT116 and ii) M231 cell lines could be fit with a single linear regression when plotted as their reciprocal values. This transformation accounts for the enzymatic conversion of luciferin to oxyluciferin, which is responsible for producing the detected photons. The resazurin datasets were not transformed before analysis. Datasets with two linear regressions are attributed to the presence of pericellular hypoxia, which is supported by the dataset presented in **Figure S10**. These datasets measured the expression of eGFP, which was engineered into M231 cells downstream of three hypoxia recognition elements. We showed that eGFP expression is proportional to the extent and duration of hypoxia.[3] **Figure S11** shows hypoxia in both SGS and paper scaffolds at high cell densities.

The analytical figures of merit associated with the calibration curves in **Figure S10** are summarized in **Table S2**. **Figure S12** contains replicate datasets of each calibration curve.

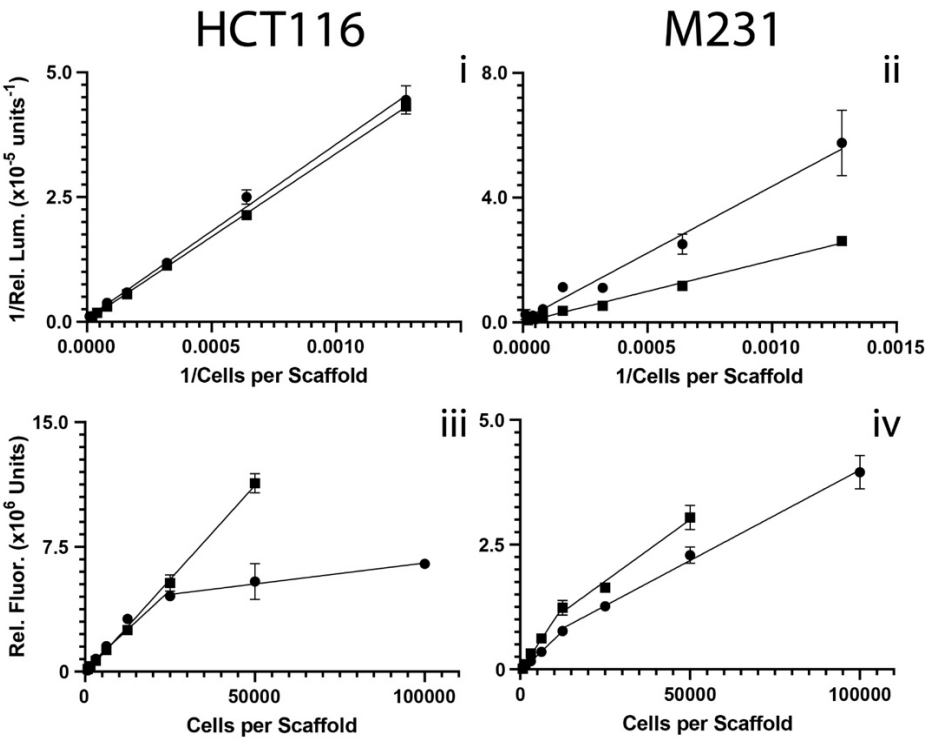

Figure S10. Representative calibration curves relating signal generated from the CTG or resazurin assay to the number of cells deposited as a monolayer (■) or in the SGS scaffolds (●). The monolayer cultures contained 781-50,000 cells per well, and the SGS scaffolds were deposited with 781-100,000 cells suspended in ECM. Each value corresponds to the average and standard error of the mean of values from at least 4 scaffolds prepared from at least 1 cell passage, collected 24 h after deposition and measured with the i-ii) CTG assay or iii-iv) resazurin assay.

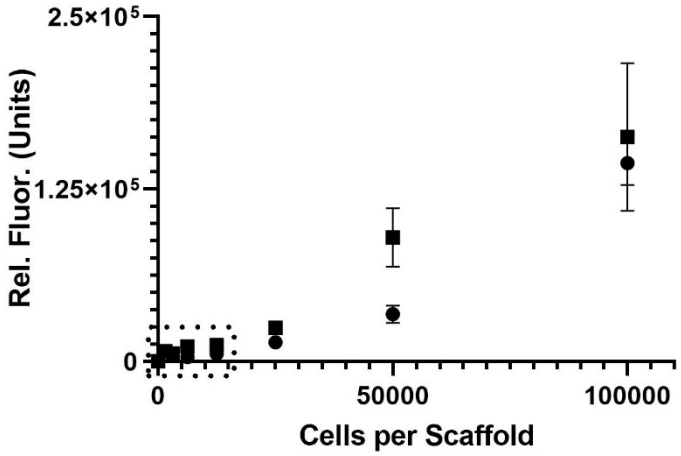

Figure S11. Measurement of pericellular hypoxia in SGS (■) and paper (●) scaffolds deposited with M231 cells engineered to express an enhanced green fluorescent protein (eGFP) upon stabilization of hypoxia-inducible factor-1 alpha (HIF1α). The eGFP gene was downstream of three hypoxia recognition elements, which the HIF1α transcription factor binds to under hypoxic conditions. Scaffolds containing cell free-ECM served as the background signal controls. The scaffolds were imaged on a SpectraMax i3x Multi-Mode microplate reader, 24 h after deposition. Each value represents the average and standard error of the mean for at least 4 replicate cultures prepared from at least 1 cell passage.

Table S2. The analytical figures of merit of indirect viability assays for cells deposited as monolayer cultures or in the SGS scaffolds [a,b]

	CTG Assay		Resazurin Assay	
	SGS	2D	SGS	2D
HCT116 cells				
Sensitivity (unit/cell) [c,d]	28.7	30.0	188.6 *25.3*	226.2
LOD (cell) [e,f]	9	3	N/A	1,017
LOQ (cell) [d]	30	11	482	2,146
M231 cells				
Sensitivity (unit/cell) [c,e]	23.4	50.4	65.4 *36.3*	103.3 *49.3*
LOD (cell) [d]	18	8	486	1,580
LOQ (cell) [cd]	36	26	3,386	5,275

[a] These values summarize the data plotted in Figure 3

[b] The cells were suspended in ATCC ECM before deposition in the SGS scaffolds

[c] The sensitivity value reported for the CTG assay was inverted to maintain a unit of RLU/cell.

[d] Italicized values correspond to the linear regressions of high cell density regions.

[e] Values are calculated based on 3 times (LOD) or 10 times (LOQ) the standard deviation of the blank divided by the slope of the trend line.

[f] N/A values for the LOD indicate that the values could not be calculated.

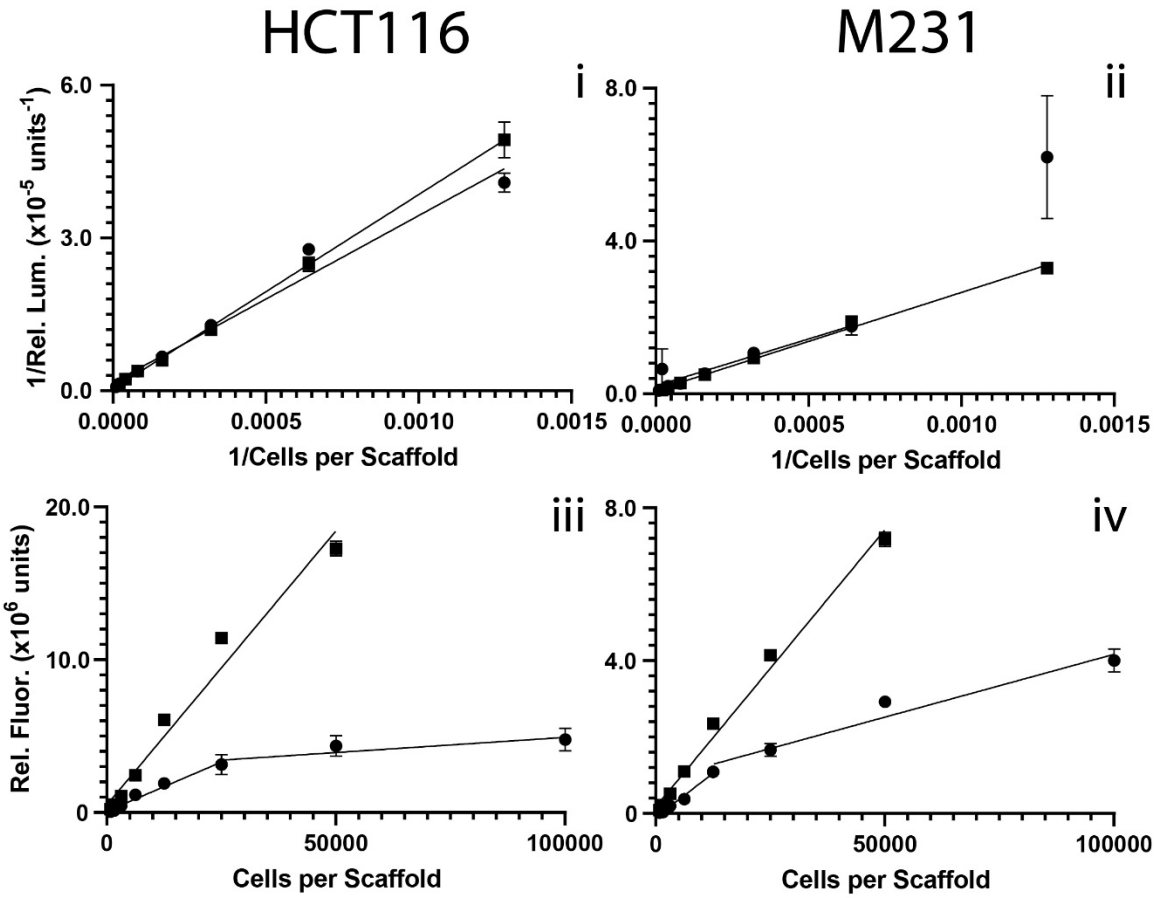

Figure S12. A second replicate of the calibration curves, relating the signal generated from the CTG or resazurin assay to the number of cells deposited as a monolayer (■) or in the SGS scaffolds (●). The monolayer cultures contained 781-50,000 cells per well, and the SGS scaffolds were deposited with 781-100,000 cells suspended in ECM. Each value corresponds to the average and standard error of the mean of values from at least 4 scaffolds prepared from at least 1 cell passage, collected 24 h after deposition and measured with the i-ii) CTG assay or iii-iv) resazurin assay.

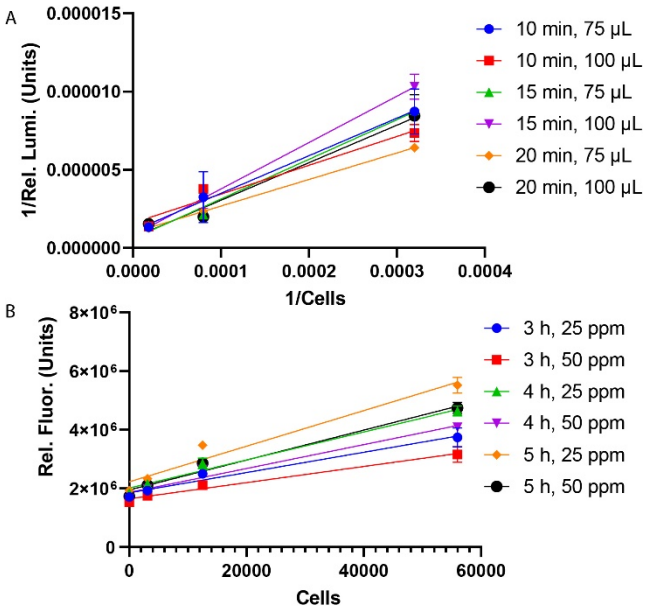

Figure S13. Calibration curves relating the number of M231 cells deposited in the SGS scaffolds to the signal generated by the A) CTG or B) resazurin assay. SGS scaffolds with nylon mesh bottom pieces were deposited with M231 cells suspended in ECM (3,125-50,000 cells per scaffold) and analyzed after a 24-h period. The scaffolds were agitated on an A) XY shake plate or B) an XYZ shake plate at room temperature for the indicated time. Each value corresponds to the average and standard error of the mean of at least 3 scaffolds prepared from at least 1 cell passage.

Table S3. The analytical figures of merit for the CTG assay conditions tested in **Figure S12.** [a]

SHAKE TIME	VOLUME	SENSITIVITY [b]	LOD [c]	LOQ [c]
10 min	75 µL	41.59	69	120
10 min	100 µL	54.22	56	95
15 min	75 µL	39.43	187	432
15 min	100 µL	33.72	55	66
20 min	75 µL	58.89	46	62
20 min	100 µL	41.66	62	99

[a] The optimized conditions used in this work are highlighted in red.

[b] The reported values are the inverse of slopes obtained from the linear regressions to maintain a unit of readout unit per cell.

[c] Values were calculated with the IUPAC definitions, where a limit of detection (LOD) is 3 times the standard deviation of the blank divided by the slope of the trend line. The limit of quantitation (LOQ) is 10 times the standard deviation of the blank divided by the slope of the trend line.

Table S4. The analytical figures of merit of the resazurin assay conditions tested in **Figure S12.** [a]

SHAKE TIME	CONC.	SENSITIVITY	LOD [b]	LOQ [b]
3 h	25 ppm	34.58	7,264	33,716
3 h	50 ppm	27.57	4,902	26,172
4 h	25 ppm	47.8	1,557	13,693
4 h	50 ppm	40.73	2,063	18,778
5 h	25 ppm	60.72	562	13,355
5 h	50 ppm	51.04	1,862	16,027

[a] The optimized conditions used in this work are highlighted in red.

[b] Values were calculated with the IUPAC definitions, where a limit of detection (LOD) is 3 times the standard deviation of the blank divided by the slope of the trend line. The limit of quantitation (LOQ) is 10 times the standard deviation of the blank divided by the slope of the trend line.

R Studio code for comparing viability profiles in the tumor-on-demand stacks.

```
library(readxl)
source("pairtest.R")
invasion<-read_excel("3.xlsx",col_names = FALSE) # the excel name can be changed here
#rowSums(invasion)
invasionper<-invasion/rowSums(invasion)
groupsize<-dim(invasionper)[1]/2
print(groupsize)

#Orthogonal transformation
invarionorthogonal1<-invasionper[,1]*(-3)+invasionper[,2]*(-1)+invasionper[,3]*1+invasionper[,4]*3
invarionorthogonal2<-invasionper[,1]*1+invasionper[,2]*(-1)+invasionper[,3]*(-1)+invasionper[,4]*1
invarionorthogonal3<-invasionper[,1]*(-1)+invasionper[,2]*3+invasionper[,3]*(-3)+invasionper[,4]*1
invarionorthogonal<-cbind(invarionorthogonal1,invarionorthogonal2,invarionorthogonal3)
tmpdata = invarionorthogonal[1:groupsize,]-invarionorthogonal[(groupsize+1):(2*groupsize),]

for (i in 1:3){
  print(shapiro.test(tmpdata[,i])$p)
}


#original
pairalltest(tmpdata) #1st row, t test p-value; 2nd row, step-down p-value; 3rd, one-sample Wilcoxon signed rank test; 4th, step-down p-value
#use 2nd row if normal holds for all variables

#power transform (if any of 3 orthogonal transformed variables is not normal)
addvalue=1.5 # make all data positive
indexlocal=c(rep(1,groupsize),rep(0,groupsize))
invasionorthogonallocal=cbind(as.numeric(unlist(invarionorthogonal[,1])),as.numeric(unlist(invarionorthogonal[,2])),
                      as.numeric(unlist(invarionorthogonal[,3])))
translambda=powerTransform((invasionorthogonallocal+1.5) ~ indexlocal)$lambda
transtmp = invasionorthogonallocal+1.5
transnew = transtmp
for (i in 1:dim(transtmp)[2]){
  transnew[,i]=BoxCox(transtmp[,i],translambda[i])
}
tmpdata = transnew[1:groupsize,]-transnew[(groupsize+1):(2*groupsize),]

for (i in 1:3){
  print(shapiro.test(tmpdata[,i])$p)
}

pairalltest(tmpdata)
```

R Studio source code for "pairtest.R".

```
stepdown<-function(orderedpvalues){
  pp1 = orderedpvalues[1]*3; pp2=max(pp1, (2*orderedpvalues[2])); pp3=max(pp2, orderedpvalues[3])
  return(c(pp1,pp2,pp3))
}

pairalltest<-function(tmpdata){ #the difference size = (L, 3)
  ttestresults=c(t.test(tmpdata[,1])$p.value, t.test(tmpdata[,2])$p.value, t.test(tmpdata[,3])$p.value)
  print(ttestresults)
  print(stepdown(ttestresults[order(ttestresults)]))
  wtestresults=c(wilcox.test(tmpdata[,1])$p.value, wilcox.test(tmpdata[,2])$p.value,
wilcox.test(tmpdata[,3])$p.value)
  print(wtestresults)
  print(stepdown(wtestresults[order(wtestresults)]))
}
```

References.

1. Lloyd C, Boyce M, Lockett M. Paper-based invasion assays for quantifying cellular movement in three-dimensional tissue-like structures. *Curr Prot Chem Biol* **9**, 1-20 (2017).

2. Sitte ZR, Larson TS, McIntosh JC, Sinanian M, Lockett MR. Selecting the appropriate indirect viability assay for 3D paper-based cultures: a data-driven study. *Analyst* **148**, 2245-2255 (2023).

3. Truong AS, Lockett MR. Oxygen as a chemoattractant: confirming cellular hypoxia in paper-based invasion assays. *Analyst* **141**, 3874-3882 (2016).