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SUPPORTING INFORMATION FOR

Selecting the appropriate indirect viability assay for 3D paper-based cultures: A data-driven study

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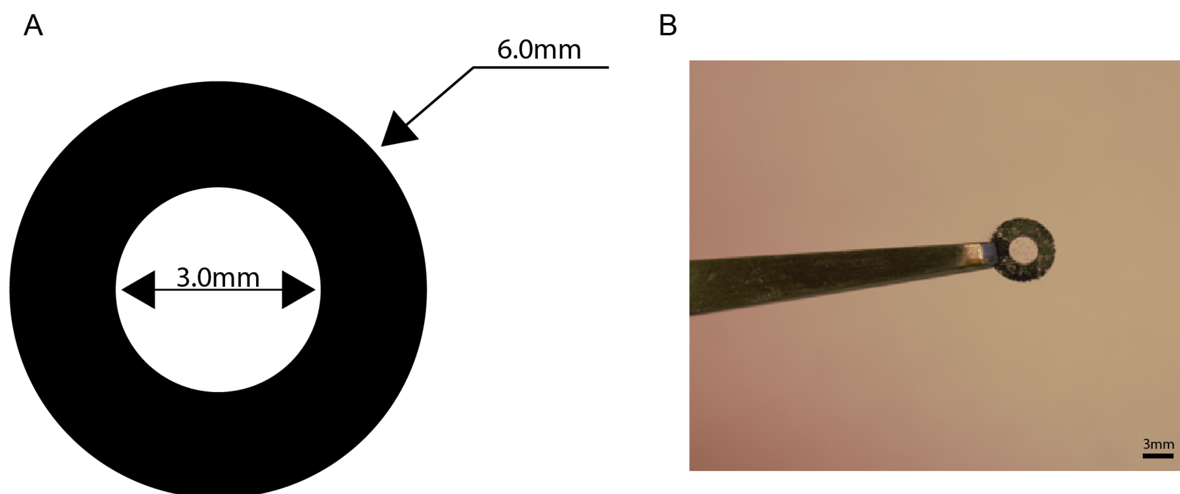

Figure S1. (A) Schematic and (B) photograph of the paper scaffolds used in this work. Each scaffold was printed as 6.0 mm in diameter and had a 3 mm diameter culture area surrounded by a 1.5 mm wax boundary. These dimensions are for the as-printed wax design, which can spread upon baking the scaffolds to give slightly different values. The scaffolds readily fit into the well of a standard 96-well plate.

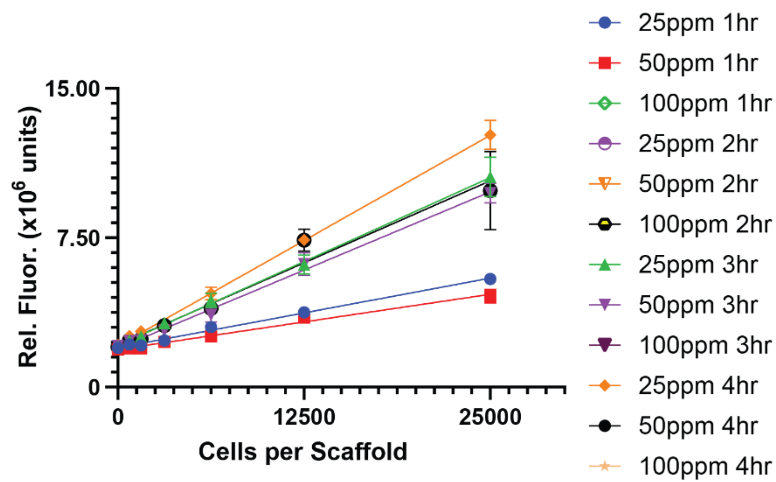

Figure S2. Optimization of resazurin data. Fluorescence datasets were obtained by incubating paper scaffolds containing 0-25,000 HCT116 cells in freshly prepared resazurin solutions. Each dataset was fit with a least-squares linear regression. Each point corresponds to the average and standard deviation of at least 4 separate cultures, prepared from at least 1 passage of cells.

Table S1. Summary of resazurin datasets obtained in Figure S2. [a]

Time (h)	Resazurin concentration (ppm)	Sensitivity (RFU/cell)	LoD [b] (Cells)	LoQ [b] (Cells)
1	25	165.0	644	1615
	50	109.1	1686	5061
3	25	373.5	849	2814
	50	249.3	826	2622
4	25	391.3	496	1671
	50	310.8	500	1864

[a] Values based on at least 4 separate cell-containing scaffolds, prepared from at least 1 passage of HCT116 cells.

[b] Limit of detection (LOD) and limit of quantification (LOQ) values were calculated using Eqns. 1 and 2 in the main text.

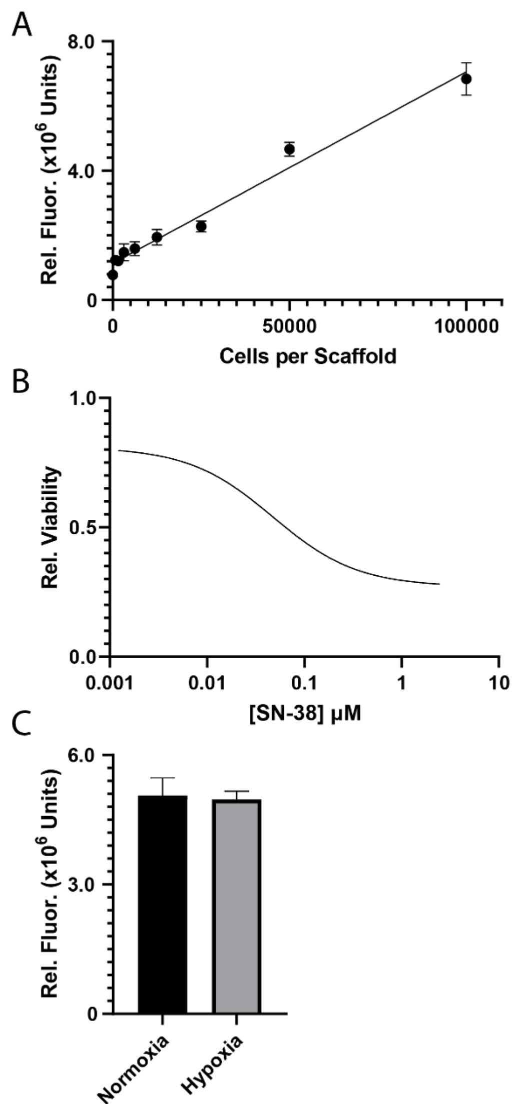

Figure S3. Evaluation of the propidium iodide-staining of cell-containing paper scaffolds using a flatbed scanner. A) Calibration curve prepared with HCT116 cells suspended in Matrigel for a final density ranging from 0–100,000 cells/scaffold. Values represent at least 4 separate cultures, prepared from at least 1 passage of cells. B) Dose-response relationship of 25,000 HCT116 cells treated with increasing concentrations of SN-38 for 48 h. Values represent at least 8 separate cultures, prepared from at least 2 passages of cells. C) Fluorescence intensity of 25,000 HCT116 cells in paper scaffolds after an overnight incubation under normoxic or hypoxic culture conditions. Values represent at least 8 separate cultures, prepared from at least 2 passage of cells.

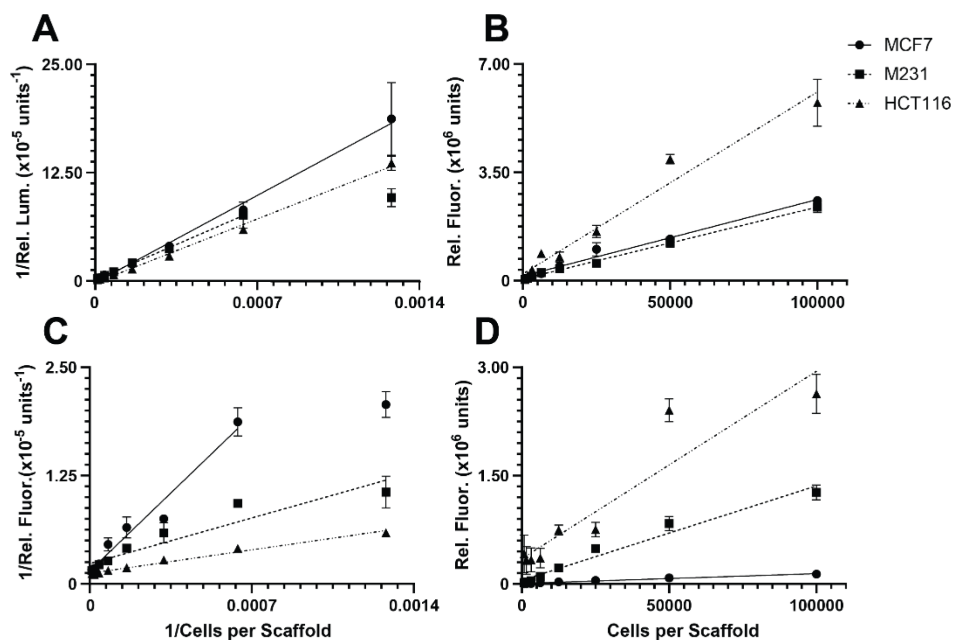

Figure S4. Calibration curves prepared from a second pass of each cell line. Each scaffold was deposited with either MCF7, M231, or HCT116 cells suspended in Matrigel for a final density ranging from 0–100,000 cells/scaffold. Background signals were determined from cell-free scaffolds. Datasets were fit with a least-square linear regression the A) CTG assay, B) resazurin assay, C) calcein-AM staining, and D) imaging of fluorescent proteins. All values are from at least 3 separate cultures, prepared from at least 1 passage of cells.

Table S2. Analytical figures of merit determined from the second replicate of the best-fit least square regression. [a]

	CellTiter-Glo 2.0	Resazurin	Calcein-AM	Fluorescent Protein
HCT116 Cells				
LoD (cells) [b]	7	N/A	1	N/A
LoQ (cells) [b]	23	3,457	4	N/A
Linear Range (cells)	781-100,000	781-100,000	781-100,000	781-100,000
Slope [c]	9.53	58.83	4649.00	25.96
Noise [d]	22.33	41,954.15	112.95	60,610.70
CV [b,e]	0.18 (0.08)	0.28 (0.08)	0.15 (0.16)	0.85 (0.13)
R^2	0.995	0.969	0.981	0.879
M231 Cells				
LoD (cells) [b]	87	9,023	7	N/A
LoQ (cells) [b]	290	35,512	22	N/A
Linear Range (cells)	1,562-100,000	781-100,000	781-100,000	781-100,000
Slope [c]	8.64	23.24	132.84	13.08
Noise [d]	249.74	87,940.00	294.75	1,304.84
CV [b,e]	0.19 (0.09)	0.76 (0.26)	0.40 (0.13)	0.49 0.23
R^2	0.999	0.997	0.887	0.970
MCF7 Cells				
LoD (cells) [b]	30	N/A	29	2,071
LoQ (cells) [b]	99	3,530	97	19,077
Linear Range (cells)	781-100,000	3125-100,000	1562-100,000	781-100,000
Slope [c]	6.96	24.76	38.88	1.36
Noise [d]	69.16	23,335.89	373.35	3,301.44
CV [b,e]	0.16 (0.15)	0.51 (0.08)	0.10 (0.19)	0.96 (0.42)
R^2	0.995	0.981	0.966	0.981

[a] Values are based on at least 3 separate cell-containing scaffolds obtained from at least 1 cell passage.

[b] Values are based on the IUPAC definition: LOD is the background signal plus 3 times the standard deviation of the blank and LOQ is the background signal plus 10 times the standard deviation of the blank.

[c] Slope values for the CTG and calcein-AM assays are the inverse of the transformed dataset to maintain a unit of readout unit per cell.

[d] Noise is the standard deviation of the signal from the blank.

[e] Coefficient of variation is the standard deviation at the given value divided by the average signal at the given value. Values are based on a low cell count of 3,125 cells per scaffold and a high cell count of 50,000 cells per scaffold.

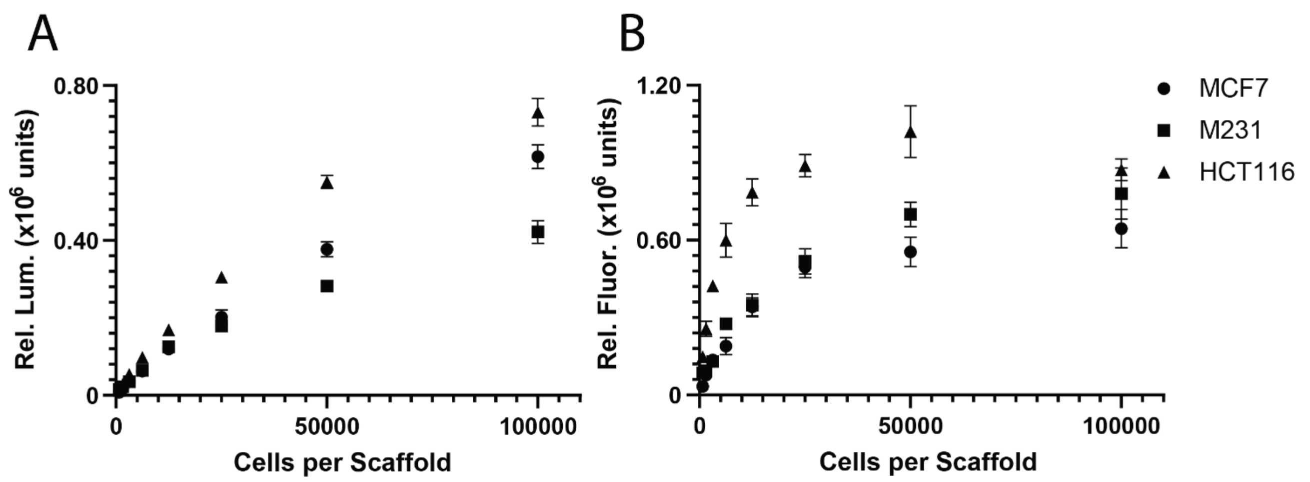

Figure S5. Raw signal vs. cell number datasets for the A) CTG assay and B) calcein-AM staining. These datasets were linearized in the main text with a Lineweaver-Burke transformation (Figure 3).

Table S3. Analytical figures of merit determined from the analysis with propidium iodide using HCT116 cells of the least-square linear regression. [a]

	PI
LOD (cells) [b]	6,641
LOQ (cells) [b]	24,465
Linear Range (cells)	781-100,000
Slope	2.28
Noise [c]	5,803
Coefficient of variation [b,d]	0.09 0.16
R^2	0.986

[a] Values based on at least 3 separate cell-containing scaffolds obtained from at least 1 cell passage.

[b] Values are based on the IUPAC definition: LOD is the background signal plus 3 times the standard deviation of the blank and LOQ is the background signal plus 10 times the standard deviation of the blank.

[c] Noise is defined as the standard deviation of the signal from the blank.

[d] Coefficient of variation is the standard deviation at the given value divided by the average signal at the given value. Values are based on a low cell count of 3,125 cells per scaffold and a high cell count of 50,000 cells per scaffold.

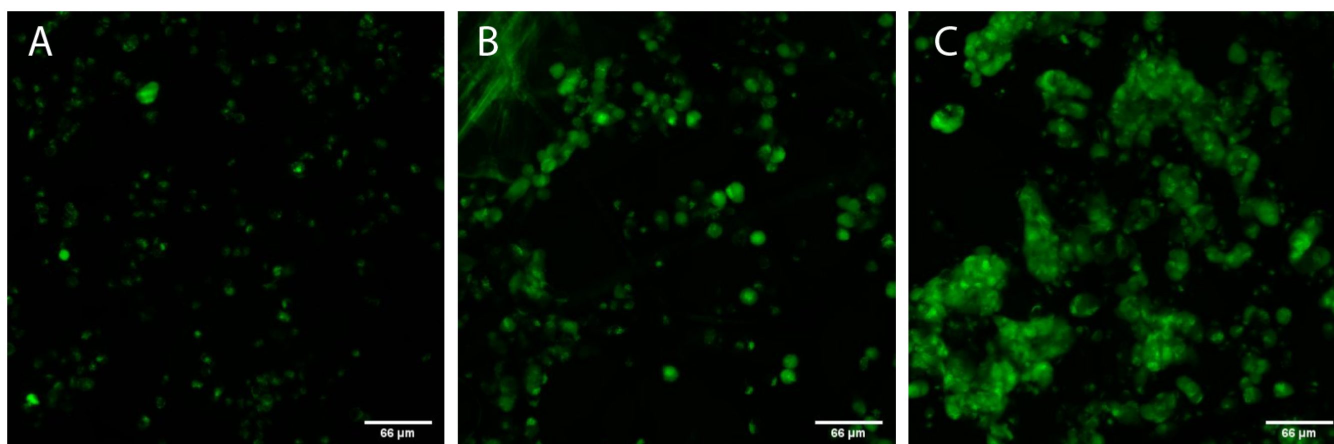

Figure S6. Representative confocal micrographs of the direct viability measurement in paper scaffolds used to calculate the IC_{50} of SN-38 after a 48 h dose at A) 2.5μM, B) 0.156μM, or C) 0.001μM. Images represent 1 scaffold, prepared from 1 passage of cells. The above micrographs were collected on a Zeiss LSM 710 spectral confocal laser scanning microscope with a 20X/0.80 Plan Apo objective, with a Z-resolution of 2 μm.

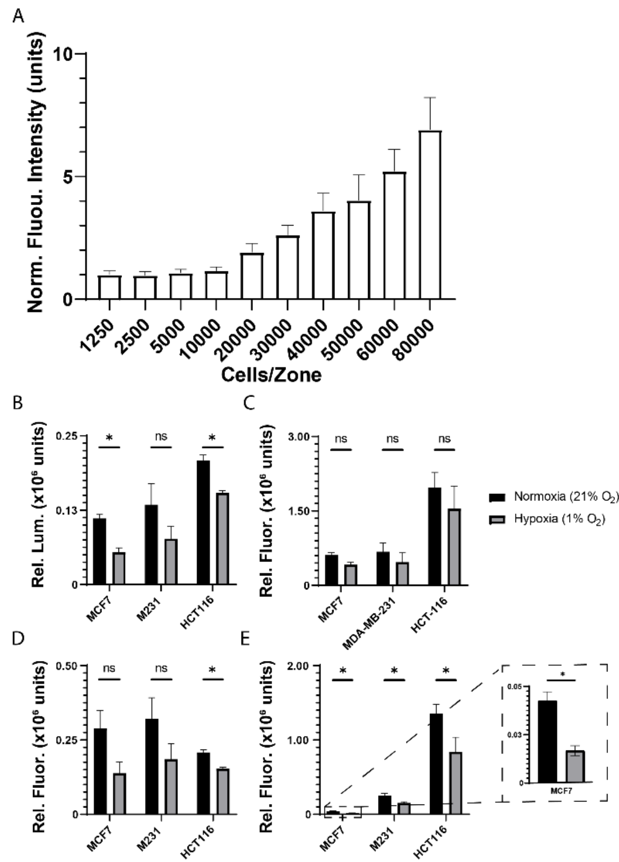

Figure S7. Evaluation of cellular and assay responses under hypoxic conditions. A) Fluorescence intensity of paper scaffolds with increasing densities of M231-HRE cells, corrected for cell number and normalized to scaffolds containing 1,250 cells/scaffold. These cells were engineered to express eGFP upon stabilization of hypoxia-inducible factor alpha, a canonical transcription factor indicative of cellular hypoxia. Fluorescence intensity is directly proportional to the extent and duration of hypoxia. B-E) Background corrected signal intensities of 10,000 or 40,000 cells per scaffold after a 24 h incubation in either normoxic (20% pO_2) or hypoxic (1% pO_2) conditions, determined with the B) CTG, C) resazurin (25 ppm resazurin incubated for 1 h), D) calcein-AM, and E) fluorescent protein assay. The datasets in A) represent ≥ 12 cultures prepared from ≥ 2 cell passages. The datasets in B-E) represent ≥ 7 cultures, prepared from ≥ 2 cell passages.

Table S4. 95% confidence interval values from fitted dose-response relationships of HCT116 cells with SN-38 in paper scaffolds. [a]

	CTG	**Resazurin**	**Calcein-AM**	**Fluorescent protein**	**Direct**
IC_{50} (nM)	42.6-276.3	6.4-596.6	47.5-678.3	18.4-9959	7.8-291.3
Span	0.03-1.01	0.43-1.01	0.02-1.18	-1.86-1.01	-1.15-15.99

[a] Values are based on at least 7 separate cell-containing scaffolds obtained from at least 2 cell passages for CTG, Resazurin, calcein-AM, and fluorescent protein. For the direct viability measure, values are based on at least 2 separate cell-containing scaffolds obtained from at least 1 cell passage.

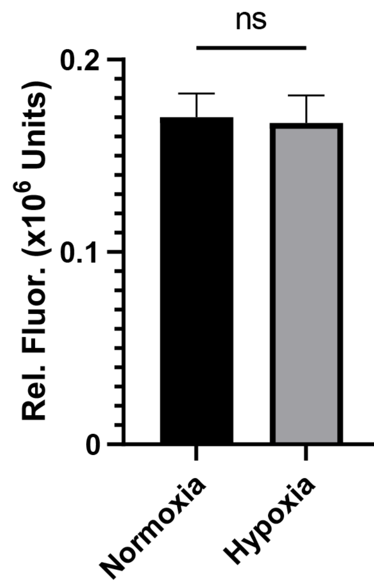

Figure S8. Fluorescence intensity comparison of 20,000 HCT116 cells deposited into paper scaffolds and incubated overnight under hypoxic (1% O_2) or normoxic (~20% O_2) conditions. All scaffolds were incubated for 4 h at normoxia before staining with propidium iodide. All scaffolds were stained immediately with propidium iodide following incubation at designated oxygen tensions. Values represent the average of at least 8 separate cultures prepared from at least 2 passages of cells.