SUPPLEMENTARY MATERIALS FOR:

Physiologically relevant oxygen tensions differentially regulate hepatotoxic responses, gene induction and CYP1A activity in HepG2 cells

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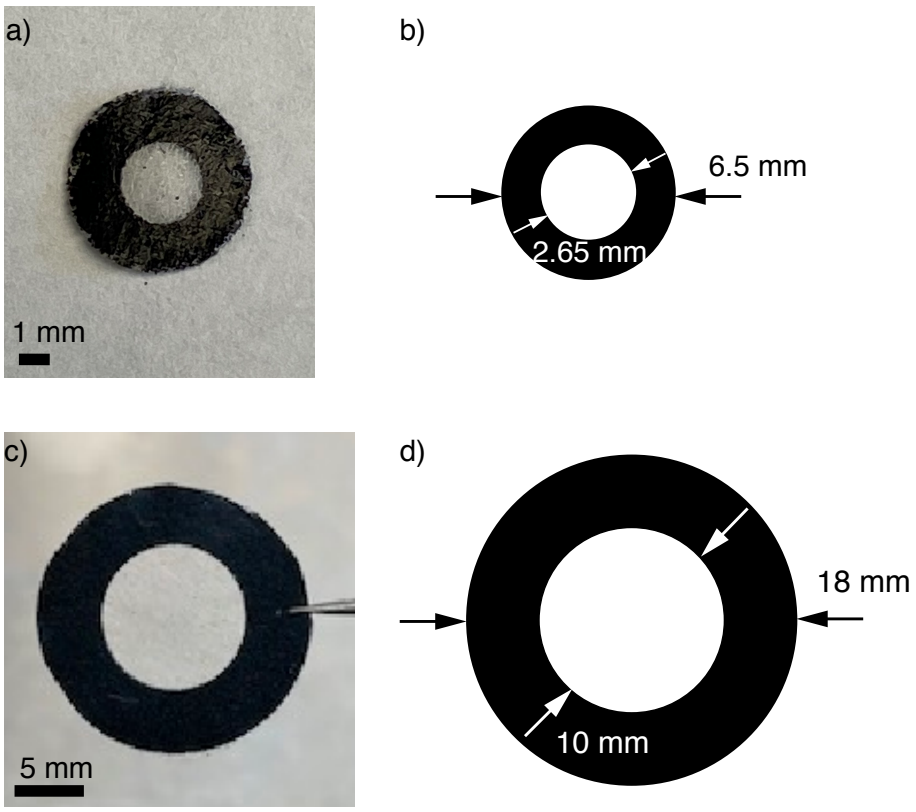
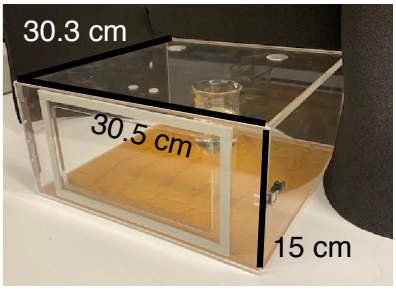


Figure S1. Photographs and schematics of the small and large zone paper scaffolds used in this work. Both scaffolds were drawn with Adobe Illustrator. The designs were patterned onto Whatman 105 lens paper with a Xerox ColorQube 8650 wax printer. (a) Photograph and (b) schematic of a small single zone scaffold, which contained a 2.65 mm seeding region surrounded by a 1.925 mm thick border. (c) Photograph and (d) schematic of a large single zone scaffold, which contained a 10 mm seeding region surrounded by a 4 mm thick border.

a.



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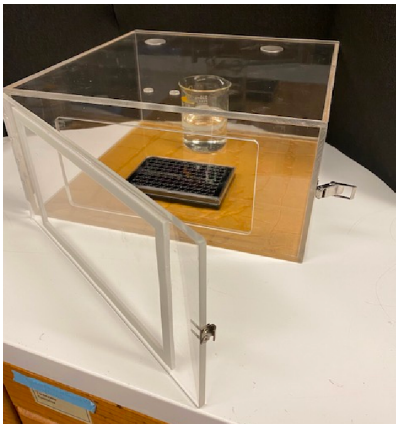

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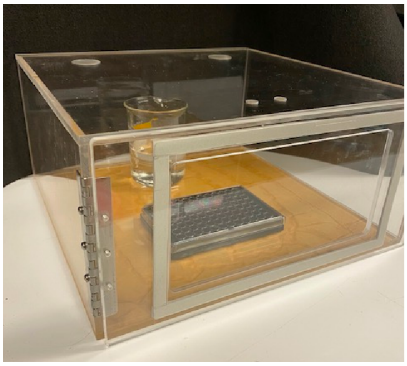

Figure S2. Photographs of a home-built hypoxia chamber assembled from 0.635 cm-thick cast acrylic sheets. (a) The 30.5 x 30.3 x 15 cm chamber contains a (b) 20.3 x 10.3 cm A latched door on the front face. The door was fitted with a 0.32 x 0.95 cm (W x H) thick foam gasket to form an airtight seal. (c) The chamber's top face contained two 2.9 cm-diameter holes, where CO_2 and O_2 gas sensor were mounted.

Table S1. Table of primer sequences for qPCR

Gene	**Forward Primer (5' – 3')**	Tm (°C)	**Reverse Primer (5' – 3')**	Tm (°C)	Conc. (nM)	Efficiency (%)
β-Actin	CTGGCACCCAGCACAATG	57.1	GCCGATCCACACGGAGTACT	59.1	800	99.28
GAPDH	GAGTCCACTGGCGTCTTCAC	62.1	GGTGCTAAGCAGTTGGTGGT	65.5	800	115.36
CYP1A1	GCACAGAGGTAGTCTCACTGCTTG	59.3	AAGGGCAGAGGAATGTGATGTT	56.7	600	102.06
CYP1A2	CTTCGGACAGCACTTCCCTG	62.2	AGGGTTAGGCAGGTAGCGAA	65.7	400	119.44
CYP2E1	TTGAAGCCTCTCGTTGACCC	61.6	CGTGGTGGGATACAGCCAA	65.0	600	98.61
CYP3A4	CTTCATCCAATGGACTGCATAAAT	53.6	TCCCAAGTATAACACTCTACACAGACAA	57.1	100	97.37
UGT1A1	TGACGCCTCGTTGTACATCAG	61.0	CCTCCCTTTGGAATGGCAC	63.8	400	98.57
UGT1A6	AGCCCAGACCCTGTGTCCTA	64.4	CCACTCGTTGGGAAAAAGTCA	60.2	400	107.17
SULT1A1	GTCACCGAGCTCCCATCTTC	61.7	GTCTCCATCCCTGAGGGAATC	61.1	200	97.66
SULT1E1	TGGTGGCTGGTCATCCAAA	61.3	GAACCTGTCCTTGCATGAATTTC	59.7	200	105.26
SULT2A1	TCCAGTTATTCCCCAAGTCTTTCT	61.1	AAACATCTCTGGGATTTCTCATGAG	60.2	600	95.41
AhR	ACATCACCTACGCCAGTCGC	64.1	TCTATGCCGCTTGGAAGGAT	64.3	600	86.27

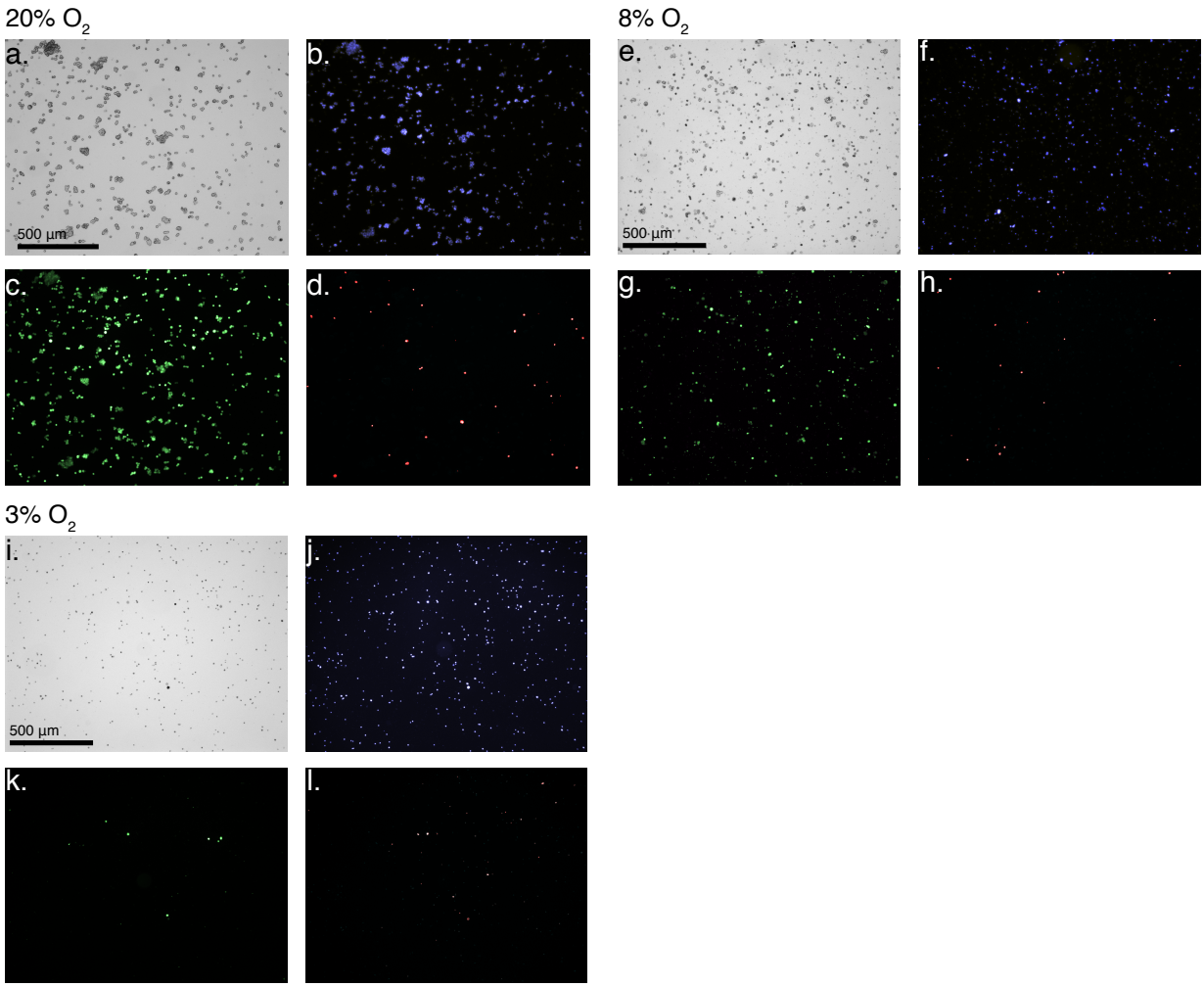

Figure S3. Representative widefield images of HepG2 cells stained with calcein-AM, propidium iodide (PI), and Hoechst 33342 after a 48 h exposure to 20% (a-d), 8% (e-h), or 3% (i-l) oxygen.

Image acquisition: The cells were imaged with a Nikon TE-2000i microscope equipped with a QICAM Fast 1394 digital camera (QImaging). Images were collected with a 10x objective. Calcein-labeled cells were imaged with a filter cube containing a 470 ± 20 nm excitation filter, a 525 ± 25 nm emission filter, and a 495 nm dichroic mirror. PI-labeled cells were imaged with a filter cube containing a 560 ± 20 nm excitation filter, a 630 ± 35 nm emission filter, and a 585 nm dichroic mirror. Hoechst-labeled cells were imaged with a filter cube containing a 350 ± 22 nm excitation filter, a 460 ± 25 nm emission filter, and a 400 nm dichroic mirror. All fluorescence images were collected with a 1000 ms integration time.

Image analysis: Each image was analyzed with ImageJ, using a previously described process. First, each fluorescence image was thresholded using the Otso method. Next, particles in close

proximity were separated using a watershed process. Lastly, the cells were analyzed using the ‘analyze particles’ function with a size setting of 10-inifinte pixel range and 0-1.00 circularity range. **Table S2.** Percentage of viable cells at each oxygen tension.[a]

Table S2. Percentage of viable cells at each oxygen tension. [a]

	20% O_2	**8% O_2**	**3% O_2**[b]
Percentage live FDA/(FDA+PI)	89.9 +/- 3.5	90.9 +/- 2.0	42.4 +/- 13.9
Percentage not dead 1-(PI/Hoechst)	89.7 +/- 3.9	94.0 +/- 1.4	97.2 +/- 0.7

[a] Cells were stained with a combination of calcein-AM, PI, and Hoechst 33342.

[b] At 20 and 8% O_2 both viability calculations, percent live and percent not dead, were statistically equivalent. However, the percent live calculation for 3% O_2 isn't equivalent to the percentage not dead; this observation has been observed by Kang et al. who stained hepatocytes with Calcein-AM along an oxygen gradient (6.9% and 0.3% O_2). At low oxygen tensions the 40-50% of the cells did not stain with Calcein-AM.

Table S3. Statistical comparison of EC_{50} between culture conditions and oxygen tensions. [a]

EC_{50} Acetaminophen F-Test

2D 20% O_2	2D 8% O_2	2D 3% O_2	3D 20% O_2	3D 8% O_2	3D 3% O_2	
	*	*	*			2D 20% O_2
		NS		*		2D 8% O_2
					NS	2D 3% O_2
				*	NS	3D 20% O_2
					NS	3D 8% O_2
						3D 3% O_2

EC_{50} Cyclophosphamide F-Test

2D 20% O_2	2D 8% O_2	2D 3% O_2	3D 20% O_2	3D 8% O_2	3D 3% O_2	
	*	*	*			2D 20% O_2
		NS		NS		2D 8% O_2
					*	2D 3% O_2
				*	NS	3D 20% O_2
					*	3D 8% O_2
						3D 3% O_2

EC_{50} Aflatoxin B1 F-Test

2D 20% O_2	2D 8% O_2	2D 3% O_2	3D 20% O_2	3D 8% O_2	3D 3% O_2	
	NS	NS	NS			2D 20% O_2
		*		NS		2D 8% O_2
					*	2D 3% O_2
				*	*	3D 20% O_2
					NS	3D 8% O_2
						3D 3% O_2

[a] * indicates a P-value <0.05. NS indicates not significant.

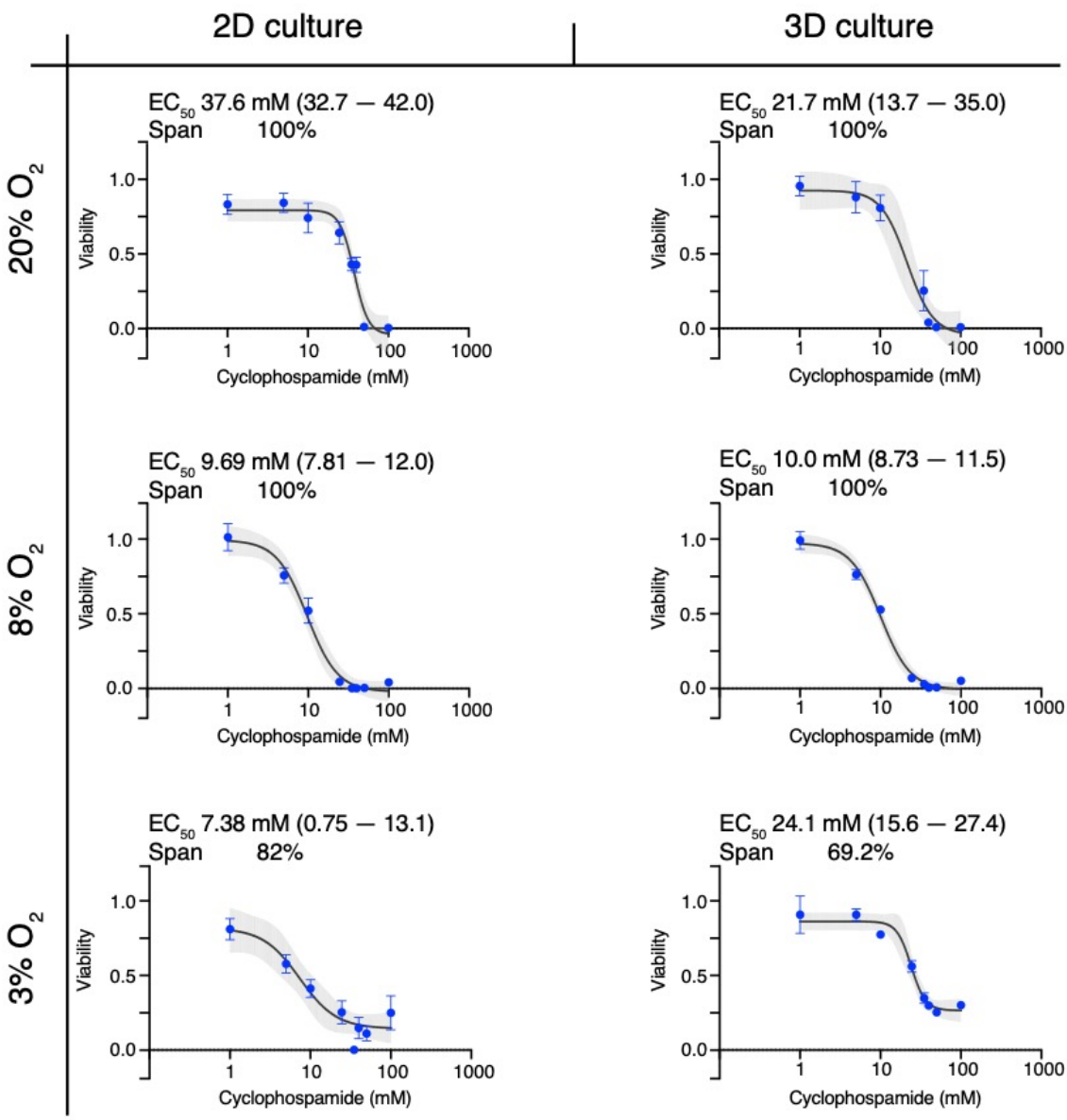

Figure S4. Dose-response relationships of 40,000 HepG2 cells in monolayer or 3D cultures after a 48 h exposure to cyclophosphamide at atmospheric (20%), periportal (8%), or perivenous (3%) oxygen tensions. Plotted points are the average and SEM of at least six data points collected from different cell passages (N=2-3) with each pass containing at least three technical replicates (n=3). The black lines connecting the points represent the best-fit 4PL model and the gray shaded areas represent the 95% confidence intervals of those fits.

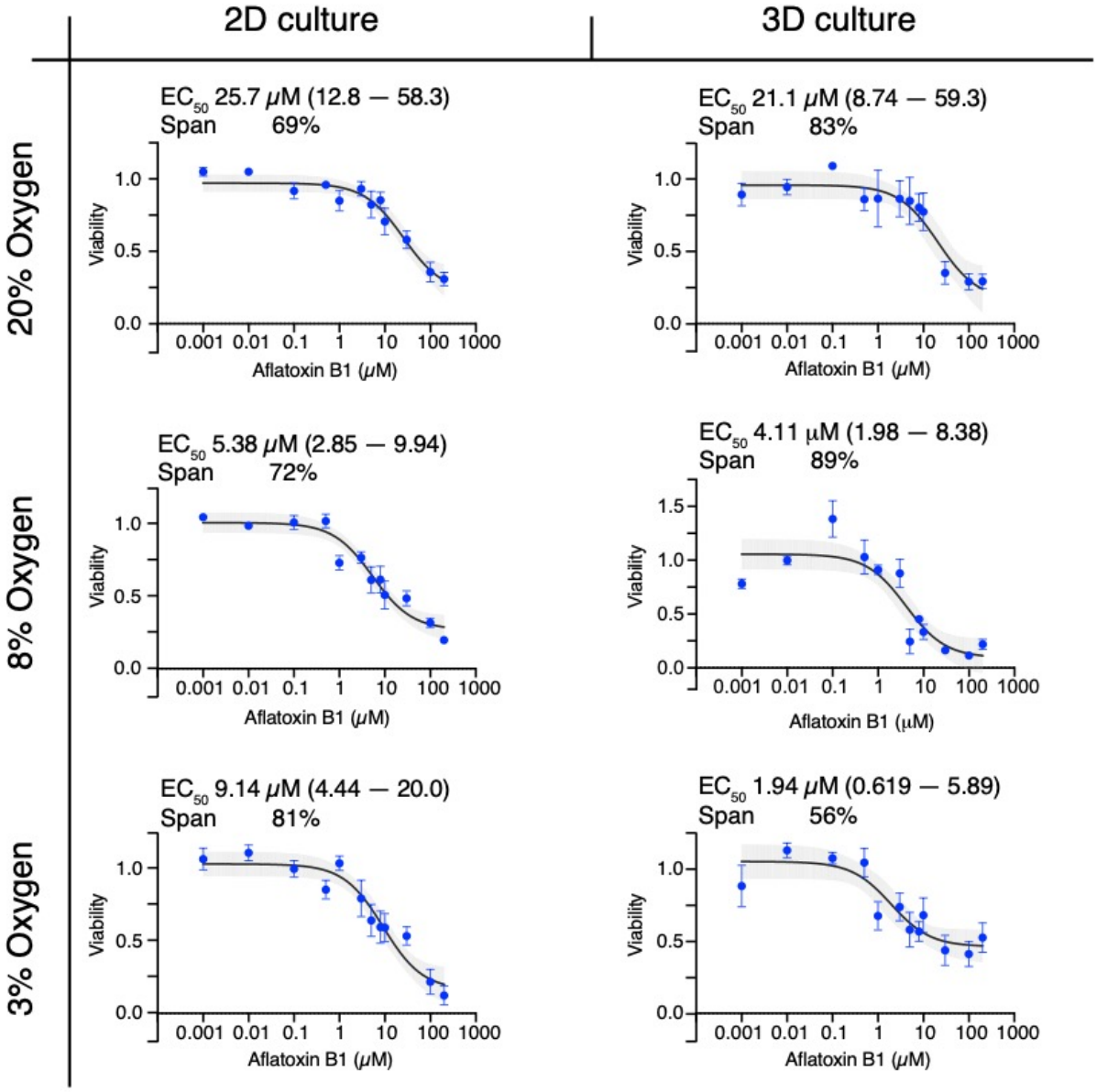

Figure S5. Dose-response relationships of 40,000 HepG2 cells in monolayer or 3D cultures after a 48 h exposure to aflatoxin B1 at atmospheric (20%), periportal (8%), or perivenous (3%) oxygen tensions. Plotted points are the average and SEM of at least six data points collected from different cell passages (N=2-3) with each pass containing at least three technical replicates (n=3). The black lines connecting the points represent the best-fit 3 PL model and the gray shaded areas represent the 95% confidence intervals of those fits.

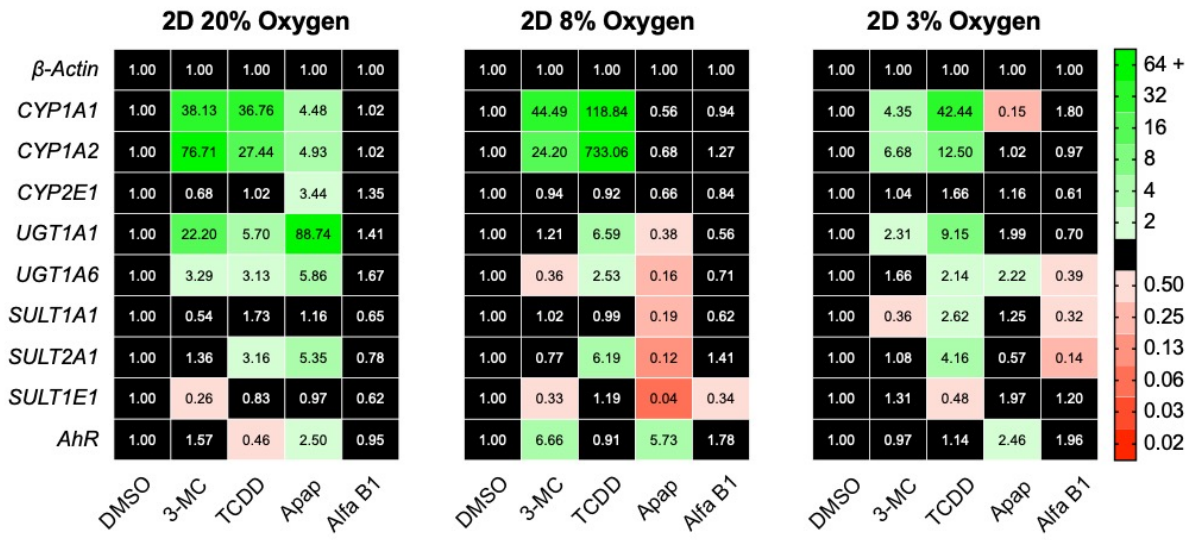

Figure S6. Transcript-level regulation of HepG2 cells cultured in a 2D culture format, plated on traditional plasticware after a 48 h incubation at 20%, 8%, or 3% O_2. The cells were treated with 5 μM 3-MC, 1 nM TCDD, 10 mM acetaminophen (apap), or 10 nM aflatoxin B1 (afla B1). Each value is the fold change of the average ΔΔCt value of at least 4 datapoints collected from different cell passages (N=2-3), each pass contained at least three technical replicates (n=3). A fold-change >2 indicates a significant increase in expression; <-2 indicates a significant decrease. The numerical values are the average ΔΔCt values.

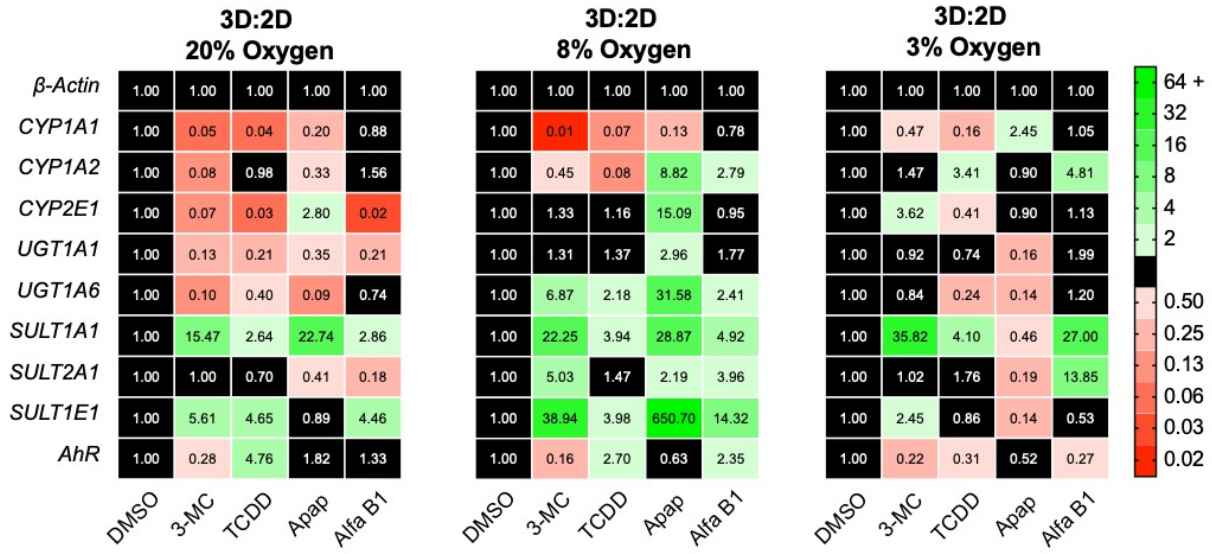

Figure S7. Transcript-level regulation ratio of 3D to 2D HepG2 cells cultured for a 48 h incubation at atmospheric (20%), periportal (8%), or perivenous (3%) oxygen tension. The cells were treated with 5 μM 3-MC, 1 nM TCDD, 10 mM acetaminophen (apap), or 10 nM aflatoxin B1 (afla B1). Each value is the fold change of the average 3D ΔΔCt divided by the 2D ΔΔCt value of at least 4 datapoints collected from different cell passages (N=2 – 3), each pass contained at least three technical replicates (n=3). A fold-change >2 indicates a significant increase in expression; <-2 indicates a significant decrease. The numerical values labels represent the average ΔΔCt value.

Table S4. Numerical values of CYP1A fold induction corresponding Figure 6b.

	2D 20% O_2	3D 20% O_2	2D 8% O_2	3D 8% O_2	2D 3% O_2	3D 3% O_2
Basal	0.96	1.18	0.79	0.95	1.03	0.74
0.1% DMSO	0.98	0.96	1.00	1.00	1.00	1.01
0.1 nM TCDD	12.64	1.74	1.08	0.94	2.58	0.74
1 nM TCDD	12.89	2.05	2.63	0.80	2.99	0.93
10 nM TCDD	17.57	2.22	1.95	0.81	3.55	0.58
1 µM 3-MC	16.16	1.32	1.43	1.02	3.72	0.78
3 µM 3-MC	13.55	1.84	2.19	0.80	2.52	0.78
5 µM 3-MC	11.08	3.56	11.33	1.22	2.19	0.88

Calculation 1. The following equations calculate the oxygen at the surface of cells in the monolayer formats, as described previously by Al-Ani et al., (2018) Oxygenation in cell culture: Critical parameters are routinely not reported, *PLoS One*, 13 (10), e0204269.

Eqn 1. Oxygen delivered to the surface of the cell per second ($mol/s * cm^2$)

$$\text{Maximum deliverable oxygen} = \frac{\text{Diffusion coefficient of } O_2 \text{ in medium}}{(\text{Medium depth}) * 1000} * \text{Surface oxygen tension}$$

Eqn 2. Cellular oxygen requirement ($mol/s * cm^2$)

$$\text{Cellular oxygen requirements} = (O_2 \text{ consumption} * \text{Cell density}) * 10^{-18}$$

Eqn 3. Oxygen concentration at liquid surface (mol/L)

$$\text{Oxygen at liquid suface} = (1 - \%CO_2 - 0.06) * \text{Atmospheric pressure} * \%O_2 * \left(\frac{O_2 \text{ Solubility in medium}}{0.209}\right)$$

Eqn 4. Oxygen concentration at cell surface (mol/L)

$$\text{Oxygen at cell surface} = (O_2 \text{ at liquid surface}) * \left(1 - \frac{\text{Cellular } O_2 \text{ requirement}}{\text{Deliverable } O_2}\right)$$

Constants used in each equation

Molar percentage of CO_2	5.0 %
Atmospheric pressure	1.00 atm
Medium depth	0.361 cm
Corresponds to 100 µL of medium in a standard 96 well plate	
Oxygen consumption rate	34 amol/cell∗s
Cell density	125,000 cells/cm^2
Diffusion constant of O_2	2.86x10^{-5} cm^2/s
Oxygen solubility	2.00x10^{-4} mol/L

O_2 at cell surface when incubator at 20% O_2.

$$\text{Oxygen at liquid suface} = 1.78 \times 10^{-4}\ mol/L$$

$$\text{Cellular oxygen requirements} = 4.25 \times 10^{-12}\ mol/cm^2 * s$$

$$\text{Maximum deliverable oxygen} = 1.41 \times 10^{-11}\ mol/cm^2 * s$$

$$\text{Oxygen at cell surface} = 1.24 \times 10^{-4}\ mol/L$$

O_2 at cell surface when incubator at 8% O_2.

$$\text{Oxygen at liquid suface} = 6.81 \times 10^{-5}\ mol/L$$

$$\text{Maximum deliverable oxygen} = 5.40 \times 10^{-12}\ mol/cm^2 * s$$

$$\text{Oxygen at cell surface} = 1.45 \times 10^{-5}\ mol/L$$

O_2 at cell surface when incubator at 3% O_2.

Oxygen at liquid suface $= 2.56 \times 10^{-5}\ mol/L$

Maximum deliverable oxygen $= 2.02 \times 10^{-12}\ mol/cm^2 * s$

Oxygen at cell surface $= -2.81 \times 10^{-5}\ mol/L$ †

† A negative value indicates that there is ≤ 50% oxygen deficiency for the cells.