

Supporting Information:

A Platform for High-Throughput Testing of the Effect of Soluble Compounds on 3D Cell Cultures

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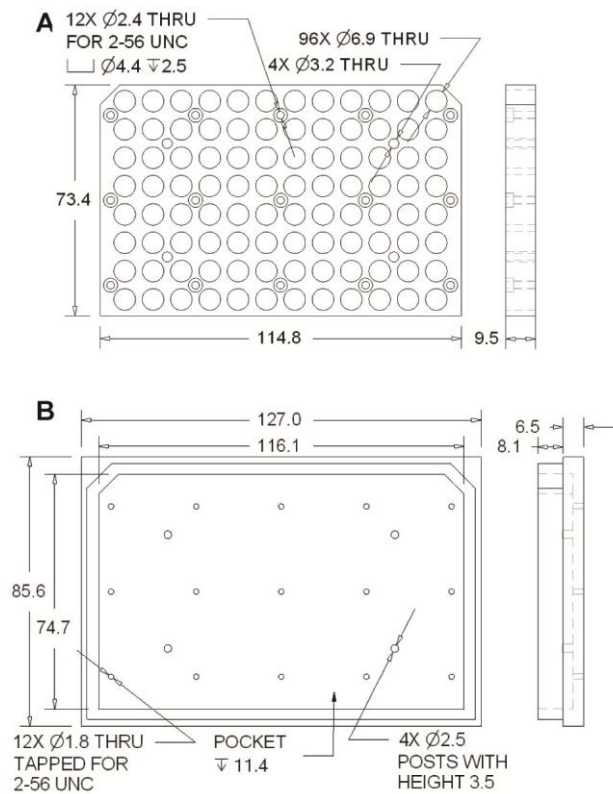
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Figure S1: Dimensional scheme of the 96-well holder

(A) Drawing of the 96-hole insert (top part of the 96-well holder) with key dimensions in millimeters. The 96 holes with a diameter of 6.9 mm were spaced according to the specifications of a standard 96-well plate; the fifteen holes with a diameter of 2.4 mm were for 2-56 UNC screws, and the holes with a diameter of 3.2 mm were for alignment with four posts emanating from the base. (B) Drawing of the base plate, a single-well dish, with key dimensions in millimeters.



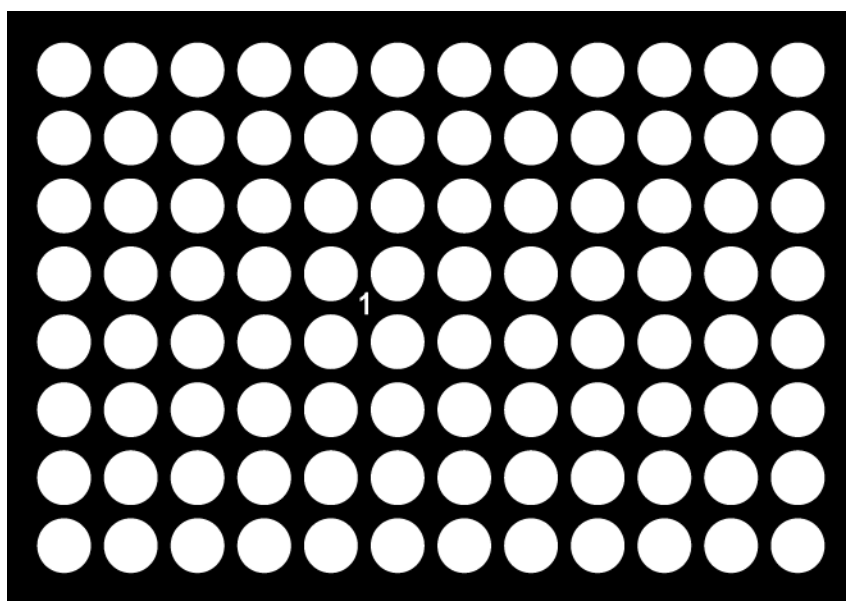
Additional comments on the design:

Sealing the plate from the bottom: A piece of an adhesive PET sheet was added under the single-well dish to avoid any leakage through the screw holes and also to minimize possibilities of contamination during the assembly of the plate.

Choice of screws: The screws had socket head caps (type 316 stainless steel) with a 2-56 thread. Variation of the screws length demonstrated that 1/2 inch screws were optimal length for the acrylic plates we used for the experiments presented here: shorter screw did not provide sufficient compression while longer screws interfered with equipment that processes 96-well plates (e.g. plate reader). By using an automatic screwdriver sensitive to torque, we were able to evaluate that the necessary torque is around 0.25 Nm.

Figure S2: Pattern for wax-printing of the 96-zone paper

After printing this pattern on chromatography paper (Whatman filter paper CHR 114) with a solid ink office printer (Xerox Phaser 6150), we heated it for two minutes in an oven at 150°C. Holes were then die cut into it for the four alignment posts and the fifteen screws using a custom made die cut (same dimension of the 96-hole insert, figure S1) and a die press with a pressure of 50 Psi. For cell cultures purposes, the 96-zone paper sheets were also autoclaved, dried and re-baked for 1 min on a hotplate to insure, that the hydrophobicity of the patterned wax in the paper was restored after the autoclaving cycle.



Preparation of PDMS-patterned paper-based layer:

The layers of paper (Whatman CHR 114) were pre-cut to dimensions for the 96-well holder using a laser cutter (VersaLASER VLS3.50, Universal Laser Systems Inc.). We prepared PDMS as directed by the manufacturer (44g allowed to prepare around 20 layers). We poured it in a stamp pad and placed it in an oven for 10 minutes at 60°C. The stamp itself was a two-part assembly, a support and a stamp holder, printed with a 3D printer (Dimension Elite from Stratasys), in acrylonitrile butadiene styrene (ABS). A piece of rubber was die cut with the same dimension of the 96-hole insert presented figure S1, and then glued to the stamp holder with double-sided adhesive foam. We aligned a layer in the support thanks to posts created in the support according to the 15 bolts and then stamped it with the stamp 'pre-inked' in the pad filled with PDMS. We, then cured the PDMS on a hotplate for 30 seconds at 220°C and repeat the whole process on the other side of the layer. To remove any residue from laser cutting, we rinsed the PDMS-paper layers in milliQ water on an orbital shaker for a few hours before being autoclaved.

Cell culture:

Reagents for cell culture and analysis were from Invitrogen unless otherwise noted. We cultured MDA-MB-231 cells (ATCC) as recommended by ATCC in Eagle's Minimal Essential Medium (EMEM, ATCC) with 10% Fetal Bovine Serum (FBS), 1% Glutamax, and 1% penicillin-streptomycin . For the experiments with the designed platform, we put 200 uL of media per well. The cells (MDA-MB231) were suspended at a concentration of 5×10^6 cells/mL in half Matrigel, half growth media in volume. The cells were plated by adding 4 uL/hydrophilic zone. The sheets of paper were then put in a dish containing growth media and let equilibrate (overnight). The

next steps were the stacking of the layers, the assembly and fastening of the 96-well holder, and the addition of the media in the different wells. For the experiments presented in this manuscript, the 96-well holders were placed on an orbital shaker in an incubator at 36°C, 5% CO₂.

The cells were transfected to express fluorescence (GFP or mTomato) as described elsewhere.²⁴ It allowed us to also visualize them by imaging the fluorescence of the cell with the gel scanner.

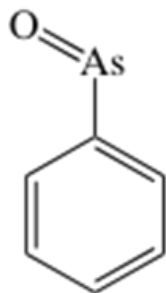
Alamar Blue assay:

Alamar Blue (AB) was diluted at 10% in media and then added to the well (100 µL/well for the 96-well holder and 0.5 mL/well for the 12-well plate in the experiments referred as ‘Testing the toxicity of the materials for the 96-well holder’). After incubation (36°C, 5% CO₂) under agitation during 4 hours (5 hours for the 12-well plate), the intensity of fluorescence of the AB was recorded with a plate reader (Spectramax Gemini XS; Excitation: 544 nm, Emission: 590 nm). For the 12-well plate, twice 50 µL from each well were transferred to a 96-well plate before reading.

Calcein-AM staining:

To visualize live cells, after destacking the layers were incubated in Hank’s Balanced Salt Solution (HBSS) containing 4 µg/mL Calcein-AM for 20 min. After incubation, the paper layers were rinsed three times with cold HBSS and imaged using the gel scanner. Fluorescent intensity from Calcein-AM, in principle, can also be calibrated to calculate the absolute number of live cells. A custom-made image analysis software processed the resulting images, and calculated the intensity of fluorescence in each zone and each layer.²⁴

Toxicity of Phenylarsine oxide (PAO)



We prepared a 25-mM solution of PAO (Sigma Aldrich) in DMSO (Sigma Aldrich). All the subsequent dilutions were realized in growth media. After assembling the 96-well holder, we added 180 μ L of growth media to each well. We prepared six different solutions of media containing phenyl arsine oxide (PAO) at concentration ten times greater than the desired ones.

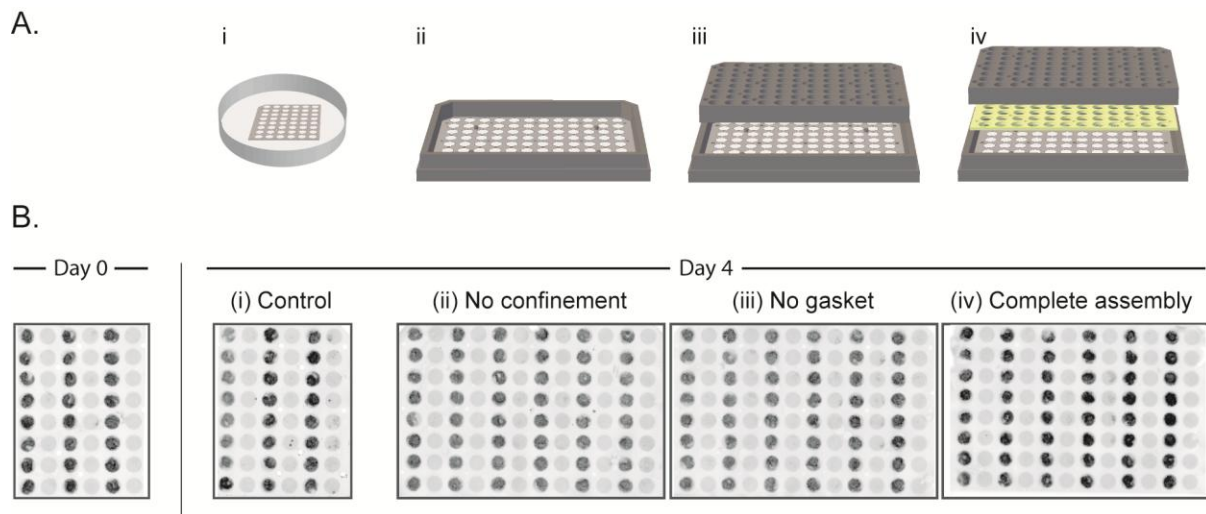
Confocal imaging of the cells

The imaging was performed on Zeiss LSM700 laser-scanning confocal microscope with solid-state laser excitation (405, 488 and 555 nm). Cellulose fibers were imaged via reflection mode. 3D images were reconstructed using Zeiss Zen software. For imaging experiments, we impregnated wax-patterned paper with a suspension of MDA-MB-231 cells in FBS-MEM media and Matrigel (v/v 1:1), (6 μ L, 24,000 cells/zone). We stained the cells after 2 hours (“day 0”) of 4-day culture in the presence of 0, 0.38, 1 or 3.8 mM concentration of CPA. Staining protocol: the samples were incubated with solution of Calcein (4 μ g/mL in serum-free MEM media, 20 min shaking at 36°C). We rinsed the samples with cold serum-free media (3X) and imaged mounted in growth medium on microscope cover slide. Images are described in Figure S5 (day 4) and supporting information movies S1 (day 0).

Figure S3: Testing the Effect of Confinement of 3D Substrates to Wells of the 96-Well Holder.

To test the effect of confinement induced by small wells on cells, we monitored the growth of cells in 96-zone layers inside the 96-well plate, and the growth of cells in analogous 96-zone layers inside the single-well dish (no 96-hole insert, no confinement) or inside a standard cell culture dish.

(A) Scheme of the experiment that evaluated the possible effect of confinement on cells inside a 96-well holder. We plated MDA-MB-231 GFP cells in multi-zone sheets of paper (every other column) and tested them in four geometries: (i) Multi-zone paper in a Petri dish (control) (ii) 96-zone sheet + bottom part of the 96-well holder (no confinement) (iii) 96-zone sheet + 96-well holder (assembly without gasket) (iv) 96-zone sheet + 96-well holder +PDMS gasket (complete assembly). (B) After four days of culture, we stained the multi-zone sheets with calcein and scanned with a gel scanner. The confinement induced by the 96-hole insert did not affect the growth of the cells (e.g. compare (i) and (iv)).



Matlab script to plot figures 5C and 5D

```
clear all; close all

dir='';
file1 = '1L CPA.xls.xls';

% read the data from xls file (output of image analysis script)
A1=xlsread(fullfile(dir, file1));

% define CP concentrations
CPA = [0 10 38 100 380 1000 3800 7600];

% plot the data from single layer
figure(2);
subplot(4,1,1);

%convert data to rectangular matrix; 8 concentrations, 6 replica
X=reshape(A1,6,8);

% plot the data using box plot
h=boxplot(X,CPA);
ylim([0 40000 ] );

% calculate the mean of drug-free zones; plot the normalized data
X0= mean(X(:,1));
figure(3);
subplot(4,1,1);
h=boxplot(X/X0,CPA);
ylim([0 1.3]);
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% read multilayer data from xls file
file = 'L1-L3 CPA.xls.xls';
A =xlsread(fullfile(dir, file));

% sort the data to find the most intense zone (technical outlier)
temp=sort(reshape(A,1,numel(A)));

for ii=2:4
figure(2);
subplot(4,1,ii);

%convert data to rectangular matrix; 8 concentrations, 6 replica
X=reshape(A(ii-1,:),6,8);

% calculate the mean of drug-free zones
X0= mean(X(:,1));

% each image contained one visible technical outlier (significantly
% stronger intensity) these 2 lines identify and remove this outlier
MAX=max(max(X));
X(X>0.99*MAX)=NaN;

%plot the data
boxplot(X,CPA);
ylim([0 14000 ] );

% plot the normalized data
figure(3);
subplot(4,1,ii);
boxplot(X/X0,CPA);
ylim([0 1.3]);
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
```


Figure S4: Confocal microscopy images of 3D construct of cells exposed to cyclophosphamide (CPA). (A) Confocal images of calcein-stained cells in Matrigel in 200- μ m-thick paper after culture for four days; (B-D) the same cells in the presence of sub-lethal (B), near-IC50 (C) and lethal concentration of CPA (D). Images were acquired with 10x objective and provide an overview of ca. 60% of the area of the culture zone. (E-H) Morphology of cells from images from (A-D) under 20x magnification. Animated 3D reconstruction of the cells before culture (day 0) are described in movie S1. The distributions of cells in paper observed by confocal microscopy were in agreement with the intensity of fluorescence measured by the gel scanner and presented figure 5C.

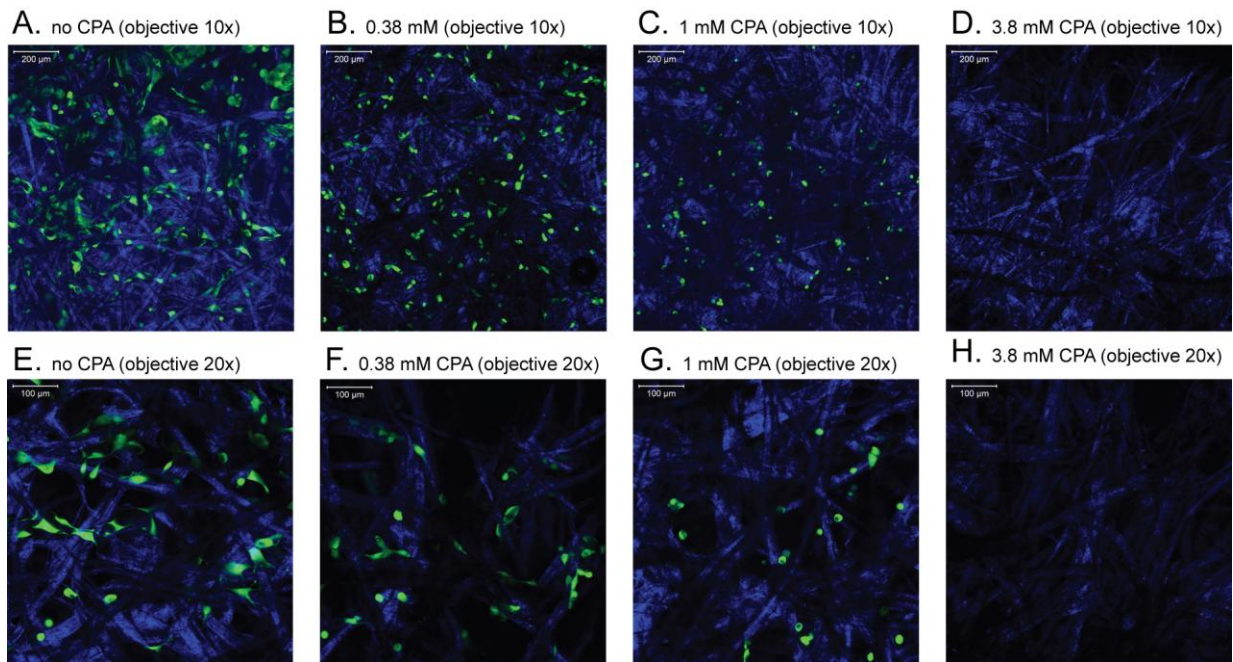


Figure S5: Long-term Exposure of 3D Cell Cultures to Paclitaxel (Tax).

We exposed cells to Paclitaxel (Taxol) using the procedure identical to the long-term exposure of cells to cyclophosphamide (Figure 5). Briefly, we exposed the MDA-MB-231 cells in 2D, 200 μm -thick and 600- μm -thick 3D constructs to paclitaxel for three days. (A) In 2D culture, the IC50 was similar to the inhibitory constant reported in the literature (3 nM). We estimated the number of live cells using Alamar Blue; the intensities were normalized with respect to the wells containing no drug. Blue circles represent an average of six experiments; red bars are $2 \times$ (standard deviation). (B) In 3D-200- μm constructs, the apparent IC50 was 5 nM. (C) In 3D-200- μm , the IC50 varied from 10 nM in the top to 100 nM in the bottom layers. In (B) and (C), the box plots describe normalized intensity of fluorescence of calcein stain in cell-containing zones (3-6 replica per concentration). See Figure 5 for the description of the statistical values represented by the box plot.

