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High Content Imaging of the HepaRG cell line treated with aflatoxin B_1, benzo[a]pyrene, cyclosporin A, rotenone and trichostatin A over a range of chemical concentrations

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1. Overview

A fully automated high throughput workflow is applied to perform high content imaging analysis of the HepaRG cell line to characterise, with single-cell resolution, the phenotypic responses of this cell line after individual exposure to five chemicals. We have simultaneously measured 9 endpoints (**detailed endpoint information are reported in the Dataset file**), able to provide information on two important cell-health parameters: mitochondrial toxicity and cytotoxicity. The dataset can be used to guide the experimental design of *in vitro* toxicity testing.

2. Materials and Methods

a. Cell exposure

The HepaRG cell line was provided by Biopredic International (Rennes, France) in cryopreserved vials. For the exposure of chemicals, the cells were plated on 96-well plates according to the standard protocol (Joossens et al., 2019).

The effect of exposure to aflatoxin B_1 (CAS No. 1162-65-8, purity ≥ 99.2%, Sigma), benzo[a]pyrene (CAS No. 50-32-8, purity ≥ 99.0%, Sigma), cyclosporine A (CAS No. 59865-13-3, purity > 99.9%, Sigma), trichostatin A (CAS No. 58880-19-6, purity > 98%, Sigma), rotenone (CAS No. 83-79-4, purity > 95.9%, Sigma), after 24 h of treatment with each chemical, was evaluated on individual basis.

The chemicals, diluted in cell media with the final concentration of DMSO corresponding to 0.1%, were tested at 8 decreasing concentrations with a dilution factor of 2.5 (**Table 1**). "C1" corresponded to the highest concentration of the chemical, whilst the "C8" corresponded to the lowest concentration of that chemical. The vehicle control was used as negative control, whereas valinomycin (CAS No. 2001-95-8, purity > 98% Sigma) and cadmium chloride (CAS No. 233-296-7, purity > 99.9%, Sigma) were tested as positive controls.

Three technical replicates were analysed for each condition, as reported in **Fig. 1**. Moreover, the analyses were carried out in three biological replicates (i.e., batches).

	C1	C2	C3	C4	C5	C6	C7	C8
Aflatoxin B_1	50 µM	20 µM	8 µM	3.2 µM	1.28 µM	0.512 µM	0.205 µM	0.082 µM
Benzo(a)pyrene	50 µM	20 µM	8 µM	3.2 µM	1.28 µM	0.512 µM	0.205 µM	0.082 µM
Cyclosporine A	50 µM	20 µM	8 µM	3.2 µM	1.28 µM	0.512 µM	0.205 µM	0.082 µM
Rotenone	10 µM	4 µM	1.6 µM	0.64 µM	0.256 µM	0,102 µM	0.041 µM	0.016 µM
Trichostatin A	50 µM	20 µM	8 µM	3.2 µM	1.28 µM	0.512 µM	0.205 µM	0.082 µM
Valinomycin	100 µM	40 µM	16 µM	6.4 µM	2.56 µM	1.024 µM	0.41 µM	0.164 µM
Cadmium chloride	165 nM	66 nM	26.4 nM	10.6 nM	4.22 nM	1.69 nM	0.67 nM	0.27 nM

Table 1. Concentrations of chemicals tested in high content imaging.

Batch 1

TP_509

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C1			Sol CTL			CdCl2 @ C2			Sol CTL		
B	CdCl2 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	chem5 @ C1	CdCl2 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2	chem5 @ C2
C	Val @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	chem5 @ C1	Val @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2	chem5 @ C2
D	Val @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	chem5 @ C1	Val @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2	chem5 @ C2
E	CdCl2 @ C3		Sol CTL		Sol CTL		CdCl2 @ C4		Sol CTL		Sol CTL	
F	CdCl2 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	chem5 @ C3	CdCl2 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4	chem5 @ C4
G	Val @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	chem5 @ C3	Val @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4	chem5 @ C4
H	Val @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	chem5 @ C3	Val @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4	chem5 @ C4

TP_510

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C5			Sol CTL			CdCl2 @ C6			Sol CTL		
B	CdCl2 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	chem5 @ C5	CdCl2 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6	chem5 @ C6
C	Val @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	chem5 @ C5	Val @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6	chem5 @ C6
D	Val @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	chem5 @ C5	Val @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6	chem5 @ C6
E	CdCl2 @ C7		Sol CTL		Sol CTL		CdCl2 @ C8		Sol CTL		Sol CTL	
F	CdCl2 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	chem5 @ C7	CdCl2 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8	chem5 @ C8
G	Val @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	Chem5 @ C7	Val @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8	chem5 @ C8
H	Val @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	chem5 @ C7	Val @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8	chem5 @ C8

Batch 2

TP_679

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C1			Sol CTL			CdCl2 @ C2			Sol CTL		
B	CdCl2 @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	CdCl2 @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2
C	Val @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	Val @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2
D	Val @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	chem4 @ C1	Val @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2	chem4 @ C2
E	CdCl2 @ C3		Sol CTL		Sol CTL		CdCl2 @ C4		Sol CTL		Sol CTL	
F	CdCl2 @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	CdCl2 @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4
G	Val @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	Val @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4
H	Val @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	chem4 @ C3	Val @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4	chem4 @ C4

TP_680

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C5			Sol CTL			CdCl2 @ C6			Sol CTL		
B	CdCl2 @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	CdCl2 @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6
C	Val @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	Val @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6
D	Val @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	chem4 @ C5	Val @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6	chem4 @ C6
E	CdCl2 @ C7		Sol CTL		Sol CTL		CdCl2 @ C8		Sol CTL		Sol CTL	
F	CdCl2 @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	CdCl2 @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8
G	Val @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	Val @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8
H	Val @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	chem4 @ C7	Val @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8	chem4 @ C8

Batch 3

TP_719

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C1			Sol CTL			CdCl2 @ C2			Sol CTL		
B	CdCl2 @ C1	chem4 @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	CdCl2 @ C2	chem4 @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2
C	Val @ C1	chem4 @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	Val @ C2	Chem4 @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2
D	Val @ C1	chem4 @ C1	chem5 @ C1	chem1 @ C1	chem2 @ C1	chem3 @ C1	Val @ C2	chem4 @ C2	chem5 @ C2	chem1 @ C2	chem2 @ C2	chem3 @ C2
E	CdCl2 @ C3		Sol CTL		Sol CTL		CdCl2 @ C4		Sol CTL		Sol CTL	
F	CdCl2 @ C3	chem4 @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	CdCl2 @ C4	chem4 @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4
G	Val @ C3	chem4 @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	Val @ C4	Chem4 @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4
H	Val @ C3	chem4 @ C3	chem5 @ C3	chem1 @ C3	chem2 @ C3	chem3 @ C3	Val @ C4	chem4 @ C4	chem5 @ C4	chem1 @ C4	chem2 @ C4	chem3 @ C4

TP_720

	1	2	3	4	5	6	7	8	9	10	11	12
A	CdCl2 @ C5			Sol CTL			CdCl2 @ C6			Sol CTL		
B	CdCl2 @ C5	chem4 @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	CdCl2 @ C6	chem4 @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6
C	Val @ C5	chem4 @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	Val @ C6	chem4 @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6
D	Val @ C5	chem4 @ C5	chem5 @ C5	chem1 @ C5	chem2 @ C5	chem3 @ C5	Val @ C6	Chem4 @ C6	chem5 @ C6	chem1 @ C6	chem2 @ C6	chem3 @ C6
E	CdCl2 @ C7		Sol CTL		Sol CTL		CdCl2 @ C8		Sol CTL		Sol CTL	
F	CdCl2 @ C7	chem4 @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	CdCl2 @ C8	chem4 @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8
G	Val @ C7	chem4 @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	Val @ C8	chem4 @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8
H	Val @ C7	chem4 @ C7	chem5 @ C7	chem1 @ C7	chem2 @ C7	chem3 @ C7	Val @ C8	chem4 @ C8	chem5 @ C8	chem1 @ C8	chem2 @ C8	chem3 @ C8

Fig. 1 The layout of microplates in the study: TP_509 and TP_510 correspond to panel A, Batch 1, TP679 and TP680 correspond to panel B, Batch 2, TP_719 and TP_720 correspond to panel C, Batch 3. Chem 1 corresponds to aflatoxin B_1, chem 2 corresponds to benzo[a]pyrene, chem 3 corresponds to cyclosporine A, chem 4 corresponds to rotenone, and chem 5 corresponds to trichostatin A.

b. Cell staining and image acquisition

Mitochondrial toxicity and cytotoxicity were tested using the HCS Mitochondrial Health Kit, (Invitrogen). The staining combined three different fluorescent dyes to analyse the nucleus (Hoechst 33342), the state of the cell membrane (cell membrane damage, Image-IT™ DEAD Green™ viability stain), and the mitochondrial membrane potential (MitoHealth stain). To acquire and analyse the fluorescence images of stained cells in the 96-well plates, a Cellomics ArrayScan® VTI high content imaging instrument (Thermo Scientific) was used (the acquisition protocol is reported in the Dataset file). The image acquisition, analysis, visualisation, and storage were performed with the HCS Studio™ Cell Analysis software (Thermo Scientific HCS Studio™ 2.0 Cell Analysis Software, Thermo Scientific, catalogue no.: SX000041A).

3. Data analysis

a. Quality control

The variability between the technical replicates was evaluated by calculating the coefficient of variation (values greater than 20% were not considered for the subsequent analysis).

b. Cell population analysis

Cell population analysis allows to extrapolate the composition of the cellular population and distinguish healthy and suffering cells at the level of a single cell. This is possible thanks to a combination of the features measured by the MitoHealth kit and the Compartmental Analysis BioApplication software (Thermo Fisher), a Cellomics tool which quantifies intracellular intensity changes in specific sub-cellular regions across multiple fluorescent channels. Five different cellular conditions are recognised: healthy cells, detached cells, cells with mitochondrial damage, cells with cellular damage, cells with both mitochondrial and cellular damage. Detached cells are calculated comparing Valid Object Count at each condition and Valid Object Count of the negative control. Healthy cells, Mitochondrial Damage, and Mitochondrial and Cell Damage correspond to events set up in the acquisition parameters, whilst cell Damage is calculated by subtraction. The cell viability corresponds to the sum of healthy cells and cells with mitochondrial damage.

c. Identification of endpoints affected by the treatment

The aim of this analysis was to identify, for each chemical, the endpoints exhibiting significant effects (increased or decreased fluorescence intensity) without using a parameterized model such as the Hill function to obtain an EC50. Signal to Noise Ratio (SNR) normalization was applied. SNR was defined as the measured value minus the mean of the negative controls and then divided by the standard deviation of negative controls. Values between -3 and 3 were considered as corresponding to “no effect” as the result of exposure in the treated cells relative to the negative control cells (Hansjosten et al., 2018).

4. Data analysis

a. Quality Control and Cell Viability

For representative purposes, the distribution of the number of attached cells in negative controls (**Fig. 2**) and the percentage of live cells (**Fig. 3**) are reported.

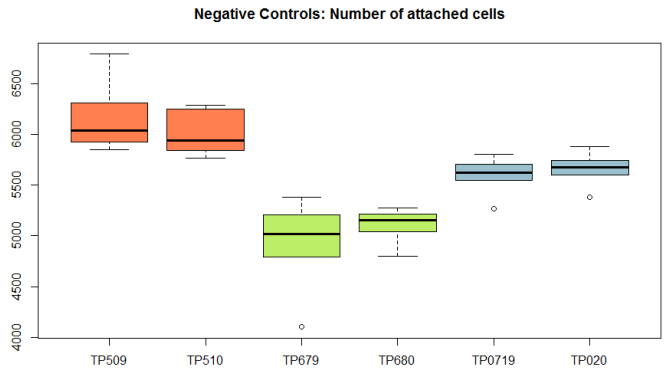

Fig. 2 Box plots reporting the distribution of the number of attached cells for negative controls in the six analysed microplates. Box plots show the five-number summary: the minimum value, first (lower) quartile, median, third (upper) quartile, and maximum value.

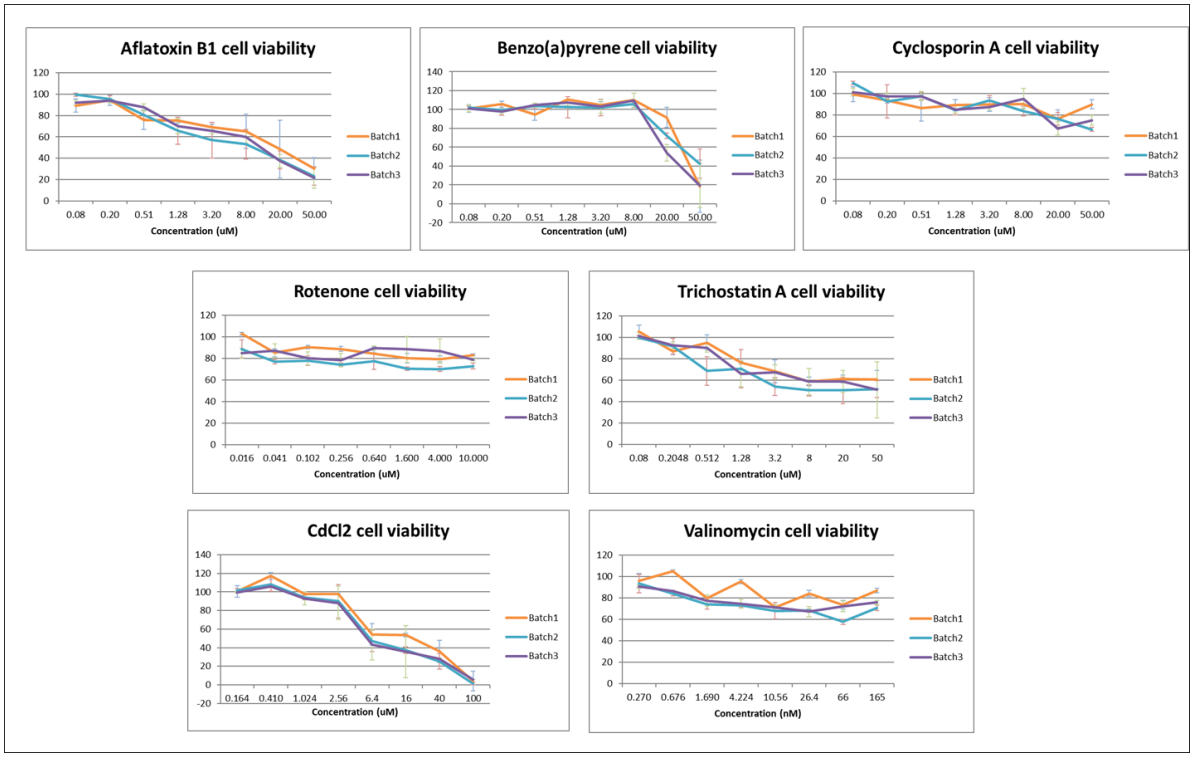

Fig. 3 Cell viability at different concentrations of chemicals, expressed as a percentage of live cells.

b. Population analysis

Population analysis is reported as the percentage of damaged and healthy cells at each condition tested in three biological replicates.

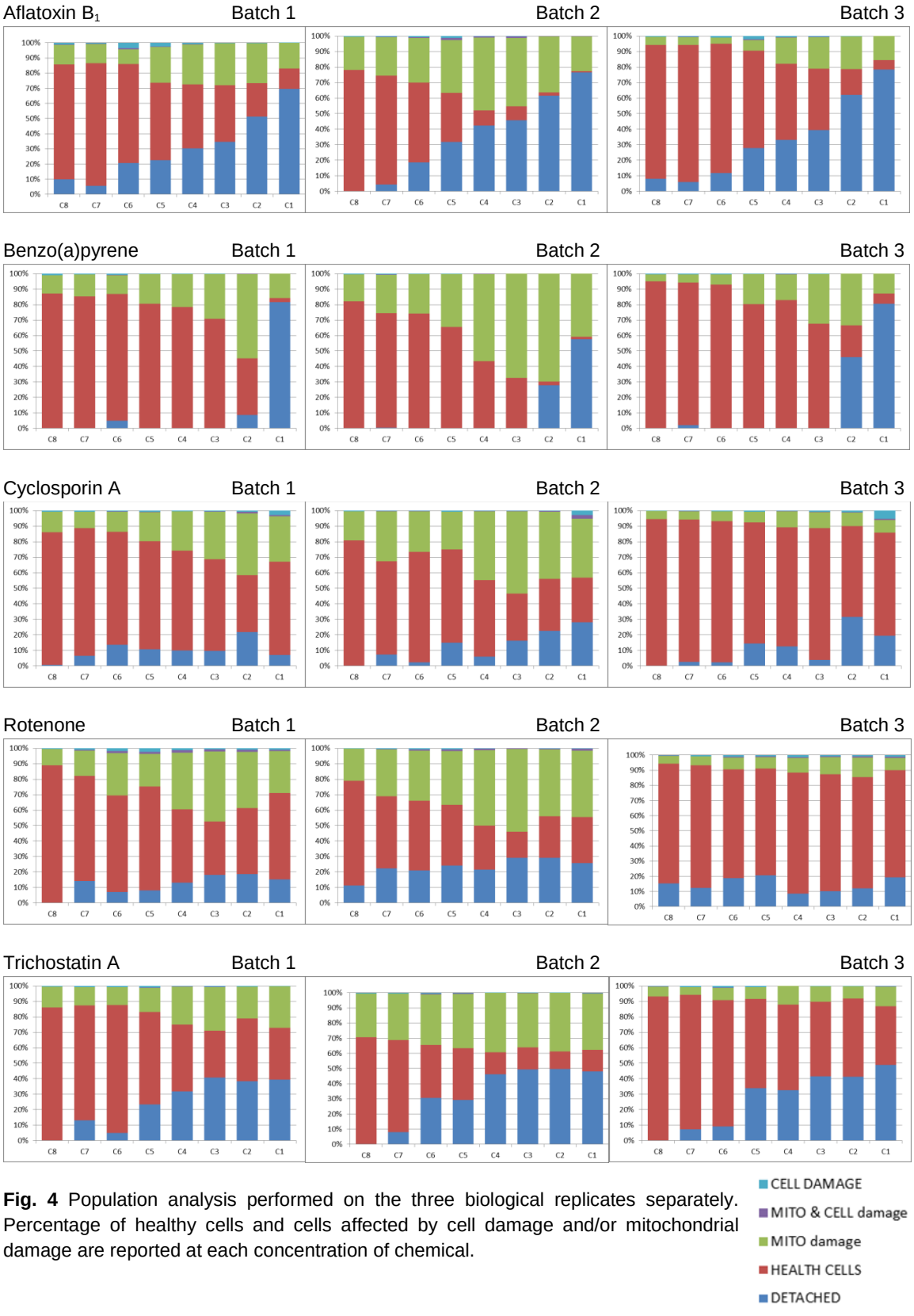

Fig. 4 Population analysis performed on the three biological replicates separately. Percentage of healthy cells and cells affected by cell damage and/or mitochondrial damage are reported at each concentration of chemical.

c. Affected endpoints

Raw data and normalised data are reported in the Dataset file. Fig. 5, 6, and 7 show how the chemical treatment affects the different endpoints after SNR normalization.

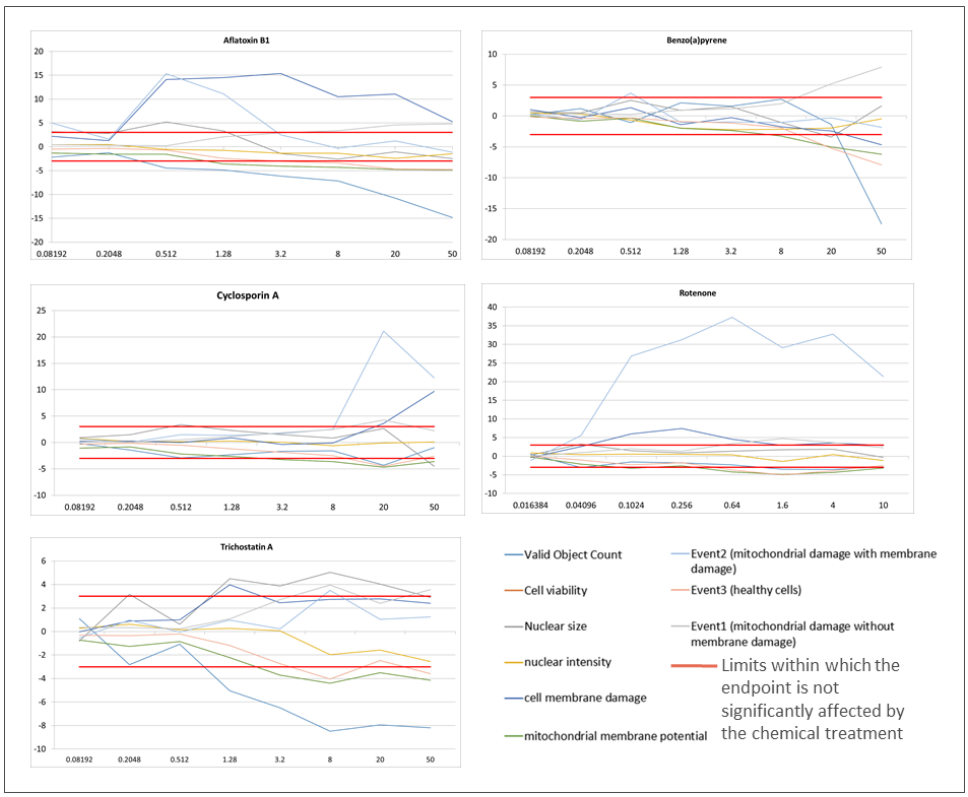

Fig. 5 – Affected endpoints in the first biological replicate (Batch 1).

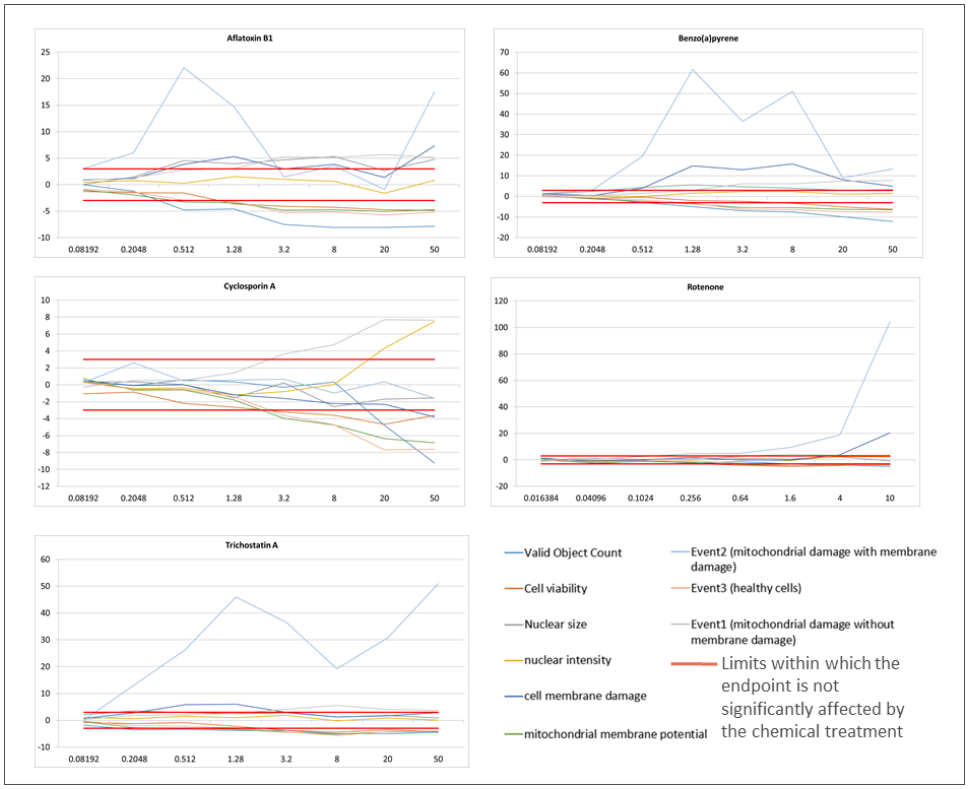

Fig. 6 Affected endpoints in the second biological replicate (Batch 2).

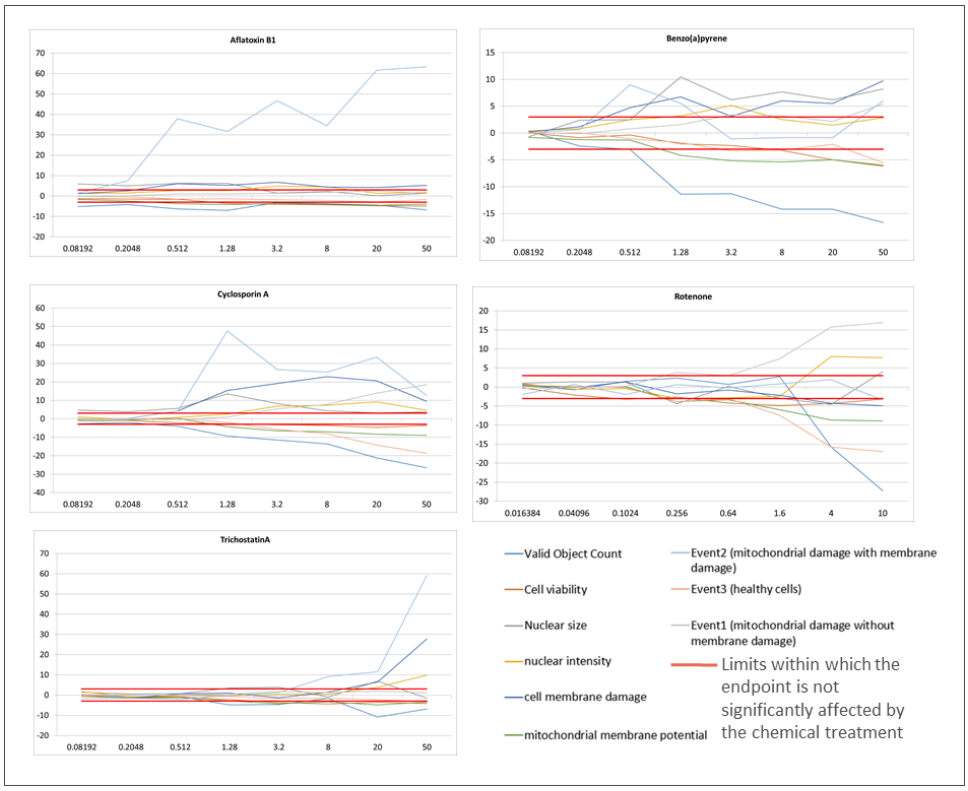

Fig. 7 Affected endpoints in the third biological replicate (Batch 3).

5. Preliminary conclusions

The method applied here provided a useful measure to study the effects of chemicals on the selected cellular endpoints. The three batches demonstrated satisfactory biological reproducibility. This dataset may be used to support the subsequent experimental design of future toxicological studies.

6. References

Hansjosten, I., Rapp, J., Reiner, L. *et al.* Microscopy-based high-throughput assays enable multi-parametric analysis to assess adverse effects of nanomaterials in various cell lines. *Arch Toxicol* **92**, 633–649 (2018). https://doi.org/10.1007/s00204-017-2106-7

Joossens, E., Macko, P., Palosaari, T. *et al.* A high throughput imaging database of toxicological effects of nanomaterials tested on HepaRG cells. *Sci Data* **6**, 46 (2019). https://doi.org/10.1038/s41597-019-0053-2