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## Method

## Paired image- and FACS-based toxicity assays for high content screening of spheroid-type tumor cell cultures

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## ARTICLE INFO

## Article history:

Received 20 October 2014

Revised 17 December 2014

Accepted 15 January 2015

## Keywords:

Toxicity assay

High-throughput

Spheroid-type tumor cell culture

## ABSTRACT

**Novel spheroid-type tumor cell cultures directly isolated from patients' tumors preserve tumor characteristics better than traditionally grown cell lines. However, such cultures are not generally used for high-throughput toxicity drug screens. In addition, the assays that are commonly used to assess drug-induced toxicity in such screens usually measure a proxy for cell viability such as mitochondrial activity or ATP-content per culture well, rather than actual cell death. This generates considerable assay-dependent differences in the measured toxicity values. To address this problem we developed a robust method that documents drug-induced toxicity on a per-cell, rather than on a per-well basis. The method involves automated drug dispensing followed by paired image- and FACS-based analysis of cell death and cell cycle changes. We show that the two methods generate toxicity data in 96-well format which are highly concordant. By contrast, the concordance of these methods with frequently used well-based assays was generally poor. The reported method can be implemented on standard automated microscopes and provides a low-cost approach for accurate and reproducible high-throughput toxicity screens in spheroid type cell cultures. Furthermore, the high versatility of both the imaging and FACS platforms allows straightforward adaptation of the high-throughput experimental setup to include fluorescence-based measurement of additional cell biological parameters.**

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## 1. Introduction

Recently developed tissue culture protocols allow the generation of patient-derived cell lines from several tumor types in a manner that preserves the genetic and phenotypic aspects of the original tumor [1,2]. These culture protocols are therefore superior over 'traditional' adherent culturing protocols [3,4]. It may be expected that spheroid-type culture protocols will rapidly become the standard in establishing and culturing human tumors from diverse tissues. One obvious application of such cultures is to use them in drug screens aimed at identifying effective drugs or drug combinations for targeting specific tumor subtypes, or even individual tumors, thus contributing to the personalization of cancer care. However, this imposes an experimental challenge as traditional cytotoxicity assays have been developed and optimized for traditional adherent cell culture models. High throughput toxicity screens are usually based on plate-based proliferation or viability assays [5]. The most commonly used approach is to measure the per-well amount of some aspect of cellular metabolism or biomass

as a proxy for the number of viable cells [6]. The principal methods are (i) determination of ATP in cell lysates by luciferin/luciferase-generated bioluminescence, (ii) reduction of tetrazolium salts such as MTS and MTT to formazan by cellular dehydrogenases, and (iii) determination of the total amount of nucleic acid per well by fluorescent DNA-binding cyanine dyes [7–10].

Importantly, these widely used methods can give highly variable results for the same drug-cell line combination. Indeed, in two large pharmacogenomic datasets (TCGA [11] and the Cancer Cell line Encyclopedia [12]) the concordance of drug response data for the same cell line was very poor. This was due to the different platforms that were used to measure drug response, rather than to differences in cell line identity or culture conditions [13,14].

We reasoned that toxicity assays in spheroid-type cultures should ideally assess cell viability on a per-cell basis, rather than an indirect measurement of 'cellular activity' per-well. Therefore, we chose two highly versatile platforms based on imaging and FACS, allowing well- and cell-based analysis of drug response. We demonstrate that both methods produce highly concordant toxicity values in drug-treated human spheroid cultures. Both methods use widely available equipment and low-cost chemicals, yet produce highly accurate and reproducible results in high-throughput

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format. The methods are ideally suited for high-throughput drug discovery screens in spheroid-type tumor cultures.

## 2. Materials and methods

### 2.1. Cell culture

Patient-derived colonosphere lines were established as described before [15]. The colonospheres are cultured in non-adherent 10 cm dishes in Stem Cell Medium with 10 ng/mL b-FGF (Abcam), which is refreshed twice a week. All cell culture was carried out at 37 °C in a 5% CO<sub>2</sub> humidified incubator.

#### 2.1.1. Generation of single cells

Colonospheres are spun down at 491g for 5 min and washed twice with PBS, and are then incubated with 3 mL Accumax at 37 °C for 3 min or until a suspension is formed. The suspension is filtered through a 40 µm cell strainer and the strainer is flushed with PBS. Take the single cells up in Stem Cell Medium in a 10 cm dish and add 10 ng/mL b-FGF. The cells are maintained in a humidified 37 °C incubator with 5% CO<sub>2</sub> for 3–5 days to form colonospheres.

### 2.2. Compound treatment

PSC-833 and irinotecan were obtained from commercial vendors (Novartis, Basel, Switzerland and Campo; Pfizer, Capelle a/d IJssel, The Netherlands). Three to five day old colonospheres were filtered with a 70 µm cell strainer and seeded in appropriate density for each cell line in 150 µL Stem Cell Medium with 10 ng/mL b-FGF in 96-well plates. PSC-833 and irinotecan were digitally dispensed with the HPD-300 (Hewlett Packard and Tecan). Compounds were diluted in at least 75% DMSO before digital titration. Concentration series were 0–50 µg/mL for Irinotecan and 0–4.5 µM for PSC-833 with a constant ratio of 2.5. In each well normalization to 1% DMSO was performed by back filling with 100% DMSO again using the HPD-300. Colonospheres were incubated in a humidified 37 °C incubator with 5% CO<sub>2</sub> for 1–4 days with no further changes of media or re-addition of compounds.

### 2.3. Toxicity/proliferation assays

#### 2.3.1. Image-based toxicity assay

To prevent the fluorescent dyes from being pumped out of the colonospheres by ATP binding cassette transporters [16,17], Verapamil 50 µM was added to each well. Plates were placed in a humidified 37 °C incubator with 5% CO<sub>2</sub> for 30 min before adding the fluorescent dyes. Calcein Green AM 4 µM and Draq5™ 2 µM were added to each well. Plates were placed in the incubator for 10 min prior to reading. The plate was spun down for 30 sec at 49 g and images were acquired on an ArrayScan VTi (Thermo Scientific) at 5× magnification using a custom protocol (Supplementary Document 2). 12 images per well were made to visualize whole wells. All DNA content was identified and visualized with DRAQ5™. Live cells were identified with Calcein Green AM. The intensity of the Calcein Green signal, per DRAQ5™ positive area is calculated. The levels of intensity were normalized to and expressed as a relative percentage of the plate-averaged vehicle treated control.

#### 2.3.2. Flow cytometry Nicoletti assay

Medium with the compounds was aspirated off and replaced with 150 µL buffer, (PBS + 0,1% Triton-X + 0,1% sodium citrate, pH 7.4, supplemented with 50 µg/mL propidium iodide (PI)). Cells were incubated overnight at 4 °C [8]. Cell analysis was performed

directly from the plate using the high-throughput sampler on a 3-laser Canto II flow cytometer (Becton Dickinson, Mountain View, CA). Fluorescent labeled beads (CS & T beads, Becton Dickinson, Mountain View, CA) were used to standardize the flow cytometer and verify optical path and stream flow. This procedure enables controlled standardized results and allows the determination of long-term drifts and incidental changes within the flow cytometer. No changes were observed which could affect the results. The PI fluorescence signal at FL2-A versus counts was used to determine sub-G1 and cell cycle distribution. Levels of apoptosis were normalized to and expressed as a relative percentage of the plate averaged vehicle treated control.

#### 2.3.3. CellTiter-Glo assay (Promega)

Measurements were made according to the manufacturer's protocol. Briefly, plates were allowed to equilibrate at room temperature for 30 min. Equal volumes of CellTiter-Glo reagents were added directly to the wells. Plates were incubated at room temperature for 10 min on a shaker and fluorescence was measured on a luminometer [7]. Luminescence reading was normalized to and expressed as a relative percentage of the plate-averaged vehicle treated control.

#### 2.3.4. CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega)

Measurements were made according to the manufacturer's protocol. Briefly, 20 µL of MTS reagent was added directly to the wells and plates were incubated for 2 h in a humidified 37 °C incubator with 5% CO<sub>2</sub>. The plate was spun down and the supernatant transferred to a new plate. Absorbance was measured at 490 nm on a 96-well plate reader [18]. Absorbance was normalized to and expressed as a relative percentage of the plate-averaged vehicle treated control.

#### 2.3.5. MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega)

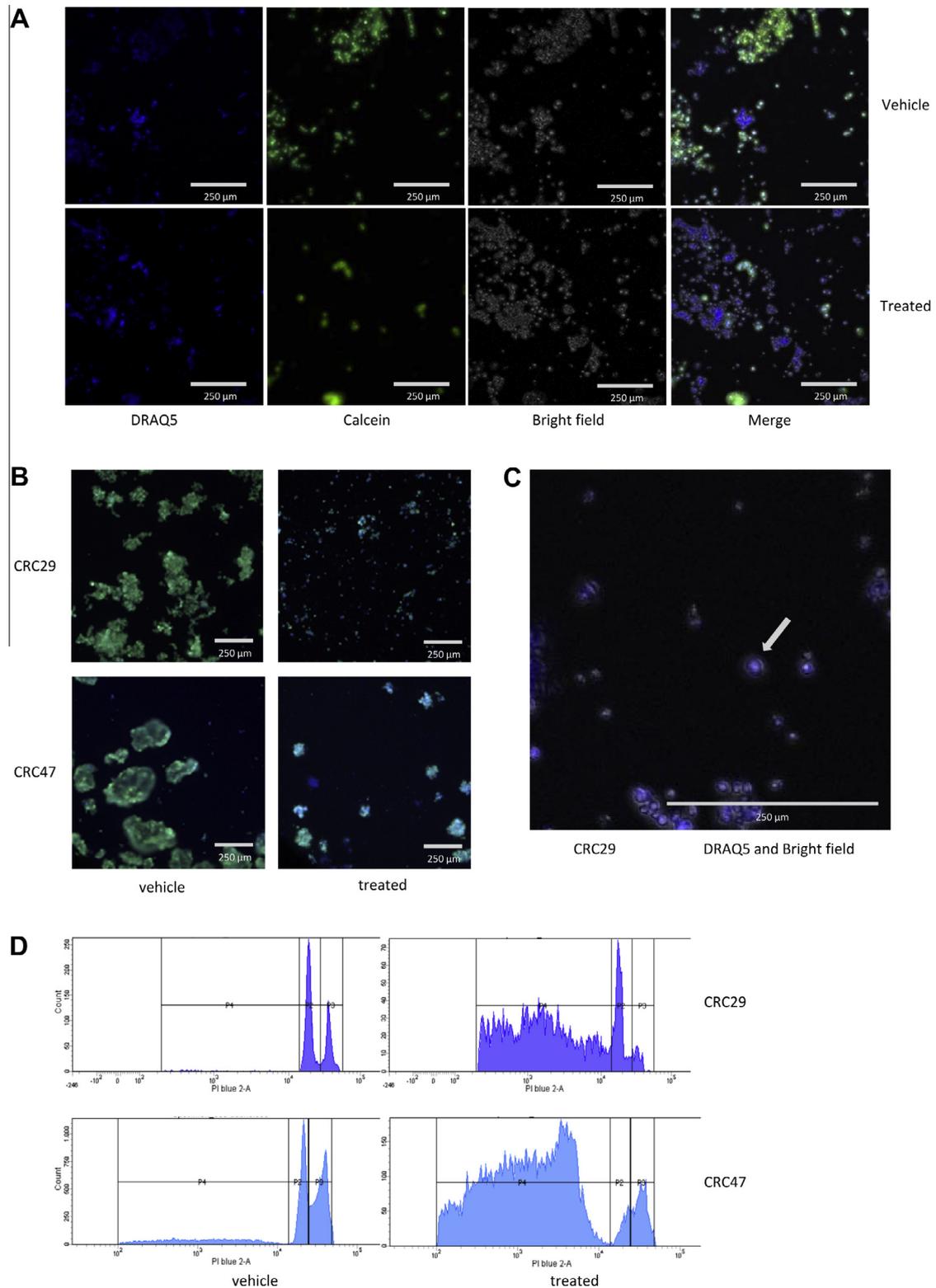
Measurements were made according to the manufacturer's protocol. Briefly, 20 µL of reagent was added directly to the wells and plates were incubated for 1 h in a humidified 37 °C incubator with 5% CO<sub>2</sub>. Fluorescence was measured on a 96-well plate reader at excitation/emission 400/505 and 485/520 nm. Fluorescence reading was normalized to and expressed as a relative percentage of the plate-averaged vehicle treated control.

### 2.4. Statistical analysis

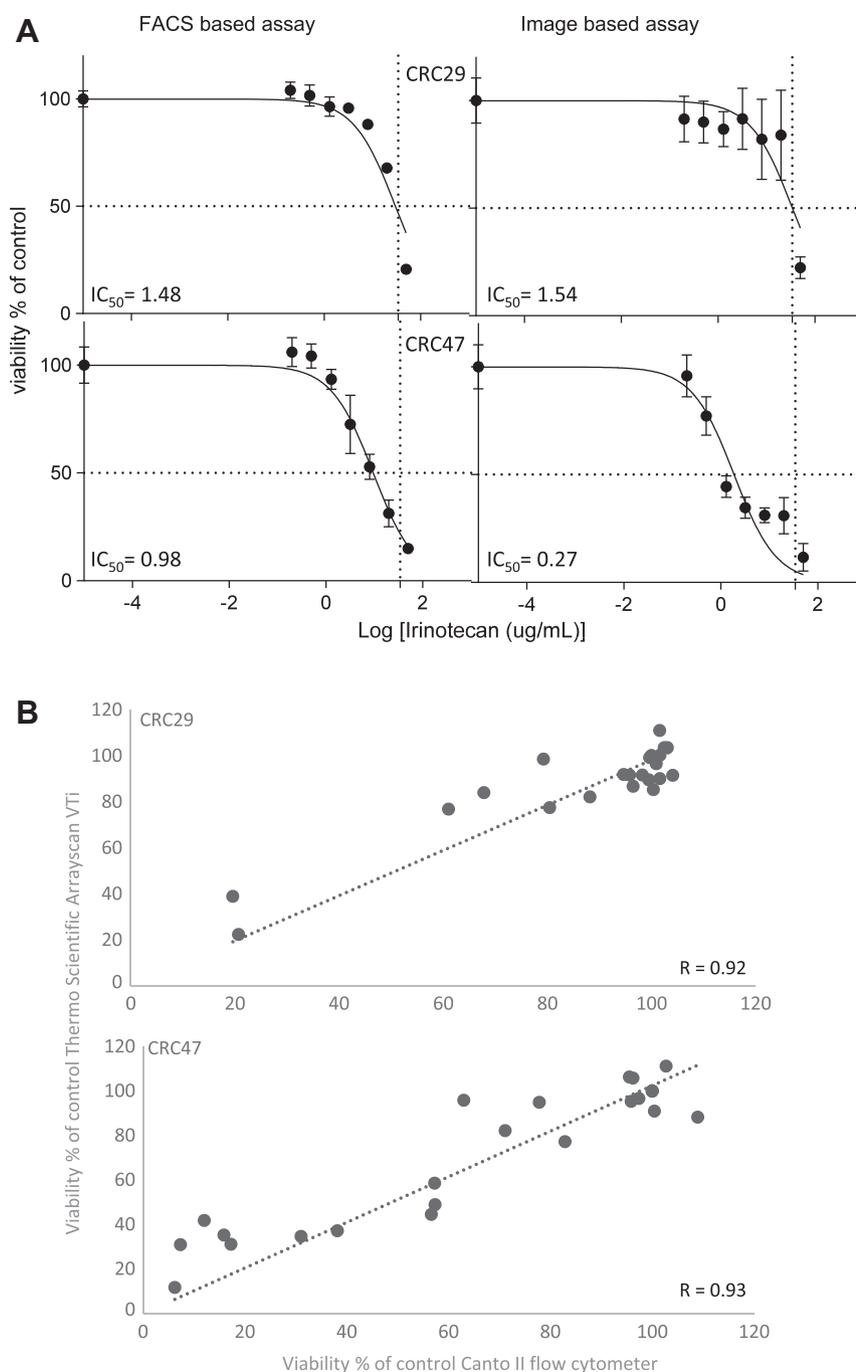
Differences per treatment group were calculated with an unpaired *t*-test or an ANOVA, when appropriate. Correlations between the assays were calculated via the Pearson's test. Z-factors were calculated to quantify the suitability of this assay as high-throughput screen. Differences of repeated measurements with different assays were calculated with the Friedman test. All statistical tests were performed with GraphPad Prism software (GraphPad, San Diego, CA). Differences with a *P* value of less than .05 were considered statistically significant.

## 3. Results

We have previously reported on the isolation and characterization of a series of cancer stem cell (CSC)-enriched non-adherent spheroid cultures from human colorectal tumors [15]. While such cultures are relatively resistant to oxaliplatin and 5-FU [19,20], they are sensitive to irinotecan, provided that drug efflux is inhibited [15]. Here, we used irinotecan treatment of two phenotypically distinct colonosphere cultures to develop a new toxicity assay for spheroid-type tumor cell cultures. CRC29 is relatively undifferentiated,



**Fig. 1.** Paired image- and FACS based toxicity assays. (A) Representative pictures, generated by the Thermo Scientific ArrayScan, of vehicle treated (upper panel) and irinotecan-treated (50  $\mu$ g/mL; lower panel) CRC29 colonospheres. DRAQ5 identifies all live cells and cell fragments that are present in the wells. (B) Representative pictures of vehicle-treated and irinotecan-treated (50  $\mu$ g/mL; lower panel) CRC29 and CRC47 colonospheres stained with Calcein Green and DRAQ5. CRC47 colonospheres also received PSC-833 (4  $\mu$ M). CRC29 spheroids rapidly disintegrate following drug addition. CRC47 spheroids stay intact but gradually become smaller as the outer cells die and lose Calcein fluorescence. (C) The resolution of the ArrayScan camera allows analysis of single cells in high-throughput format. The DRAQ5 image shows chromatin condensation of single drug-treated cells, characteristic of apoptosis. (D) Representative pictures of the FACS-based Nicoletti assay are shown for vehicle-treated (left panels) and irinotecan-treated (right panels) CRC29 and CRC47 colonosphere lines. Drug treatment induces cell death which is measured as a peak of cell fragments with sub-G1 DNA content. In addition, irinotecan induces a G1 arrest in CRC29 and a G2 arrest in CRC47 cells.

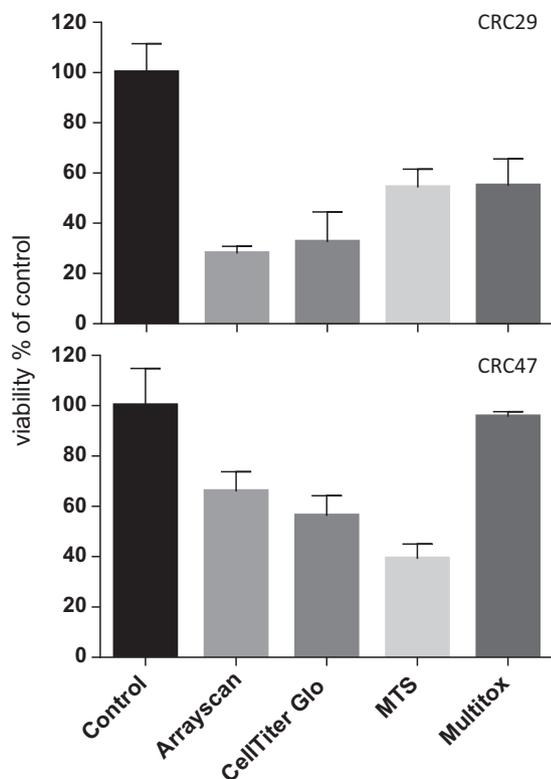


**Fig. 2.** Correlation of image- and FACS-based toxicity assays. (A) IC<sub>50</sub> curves generated on the imaging platform (left) on the FACS platform (right). Small uniform colonospheres were treated for 72 or 48 h with concentration series of irinotecan (CRC29) or irinotecan and PSC-833 (CRC47). (B) Image based and FACS-generated data points of the same sample series were plotted to calculate the concordance of the two assays. The correlation (*r*) values of the data points were calculated with the Pearson's test for each colonosphere line, *p* < 0.01.

does not express drug efflux pumps, and is sensitive to irinotecan monotherapy (Supplemental Fig. 1A and 1B). CRC47 is well-differentiated and expresses high levels of CK20 and ABCB1 (Supplemental Fig. 1A and 1B). Therefore, CRC47 killing by irinotecan requires ABCB1-mediated drug efflux inhibition with PSC-833 (Supplemental Fig. 1C).

Equal numbers of small uniform colonospheres (CRC29, CRC47) were plated in 96-well plates and were treated with a concentration series of irinotecan (0–50 µg/mL) in the absence or presence of PSC833 respectively for 48–72 h. The drugs were added using a robotic liquid handler (HPD300; Tecan). To identify all cells (live

and dead) the DNA was stained with DRAQ5™, a far-red emitting fluorescent DNA dye [21]. We chose this dye as its spectral properties do not interfere with those of commonly used anti-cancer drugs or with Calcein-type live cell dyes. Furthermore, this DNA binding dye is extremely photo stable compared to older generation DNA dyes and it can be used in live cells [22]. Live cells were identified by subsequent addition of Calcein Green AM [23,24]. The intensity of Calcein fluorescence per DRAQ5™ positive area was then measured. Analysis of the generated images showed that in both cell lines the drug response was homogenous throughout the wells. The DNA of single cells showed the condensed chromatin which



**Fig. 3.** Concordance between toxicity assays. Tumor cells (CRC29, upper panel; CRC47, lower panel) were treated with irinotecan (50  $\mu\text{g}/\text{mL}$ ). Drug-induced toxicity was then simultaneously measured with 4 distinct assays (ArrayScan, Multitox, MTS, Cell Titer Glo). The percentage of viable cells was then plotted as % of control values.

is characteristic of apoptotic cells. Furthermore, we noted that drug-induced cell death in spheroids starts in the outer cell layer. These cells detach from the spheroid structure, ultimately leaving a small proportion of live (Calcein-positive) cell clusters and single cells (Fig. 1A and B). The percentage of drug-induced cell death, based on the combined DRAQ5 and Calcein fluorescence data was then calculated and plotted relative to vehicle-treated control.

In parallel with the image based analyses an additional series of similarly treated samples were analyzed for sub-G1 DNA content (apoptosis) and cell cycle distribution [8]. To this end, the cells were stained with propidium iodide and were analyzed on a 3-laser Canto II flow cytometer with a high-throughput sampler. The generated DNA profiles clearly show irinotecan-induced cell death. Notably, this was accompanied by a G1 arrest in one cell line (CRC29) and by a G2 arrest in the other (CRC47; Fig. 1D).

Dose–response curves were generated for both assays (Fig. 2A). Cross-comparison of the toxicity data revealed a very high and significant concordance of the results obtained on the two platforms (Fig. 2B; Supplementary Table 1). Furthermore, the calculated Z-factors of both cells lines, CRC29 0.78 and CRC47 0.68, show its suitability as a method for high content screening.

Finally, we compared image based-generated toxicity values with those generated by frequently used additional toxicity assays, such as Cell Titer-Glo, Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay and MultiTox-Fluor Multiplex Cytotoxicity Assay. To this end, both cell lines were treated with irinotecan (50  $\mu\text{g}/\text{mL}$ ; 48/72 h) and the percentage of viable cells was analyzed in parallel with all 4 assays. In both cell lines, the results from all toxicity assays were significantly different calculated via Friedman test, CRC29  $p < 0.001$  and CRC47  $p = 0.003$ . When comparing

each assay to the image based toxicity assay via *t*-test, no significant differences were found between the Cell Titer Glo assay and our novel image based assay. (Fig. 3). The toxicity values obtained with the MTS and the multitox assay were significantly different and poor ( $p < 0.02$ ).

#### 4. Discussion

We present a robust method for high content screening of drug-induced cell death in spheroid-type cultures, involving paired imaging- and FACS-based analysis of cell viability and cell cycle profiles. It provides a low-cost approach for accurate and reproducible high-throughput analysis of cell death and cell cycle arrest in spheroid-type cell cultures. However, the method can also easily be adapted for the study of many other aspects of spheroid biology, by making use of the rapidly expanding toolbox of fluorescent reporters for cell biological parameters. Furthermore, the new generation of ArrayScan equipment allows three-dimensional imaging, and this will greatly help in fully exploiting the benefits of spheroid-type cell cultures.

We propose that cell-based assays such as the one presented in this report can serve as an ideal screening method when performing toxicity screens. The only well-based viability assay that generated data that were significantly concordant with that obtained with the image based assay was the Cell-Titer-Glo assay which measures ATP content per well (Promega). Cell-Titer-Glo may therefore be considered as a good alternative for cell-based assays in those experimental settings where only well-based viability information is needed.

DRAQ5 is a relatively new DNA-binding dye in the far red spectrum [22] comparable to Hoechst 33342 and with the same efficacy for staining live cells. Although Hoechst 33342 would have been a more conservative choice, DRAQ5 is extremely photo stable, is cell-permeant, and does not interfere with simultaneous imaging of many widely used fluorescent proteins (GFP, YFP, CFP, dsRed, mCherry, Dendra2) and accurately reflects the spatial concentration of DNA in living cells.[25]. Furthermore, unlike Hoechst 33342 and Rhodamine123, it is not a substrate for ABC-transporter-mediated efflux [26,27].

All materials used for the described methods are widely available and relatively cheap. Furthermore, the assays are fast and highly reproducible due to automated drug dispensing and a protocol that does not involve washing steps. Therefore, the described assay provides an ideal platform for testing large numbers of drugs in concentration series on patient-derived spheroid-type cell cultures. These features are extremely relevant and valuable for the development of diagnostic protocols that are aimed at personalizing cancer treatment.

#### Acknowledgements

The authors thank all members of the Surgical Oncology research group for the input and constructive feedback during work discussions, the employees of the Cell Screening Core for the excellent technical assistance. The employees and in particular Jeroen van Velzen for the technical assistance when working at the flow facility. This study was supported by the PON foundation and “Vrienden UMC Utrecht” (K.T.) and the Dutch Cancer Society (KWF) - The Netherlands project number: UJ2011-5226 (T.T.V.).

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fob.2015.01.003>.

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