APPLICATION NOTE

Varioskan LUX reader and EVOS FL system

Evaluation of the toxic effects of PFOS in hESCs differentiating into cardiomyocytes

Measurement of cell viability and mitochondrial membrane potential using the Varioskan LUX reader and EVOS FL system

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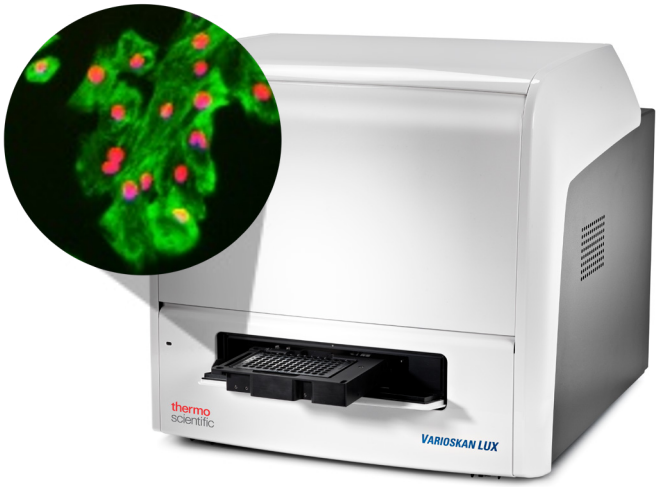

Introduction

Exposure to environmental pollutants during pregnancy may cause birth defects, such as congenital heart defects (CHDs), a leading cause of infant mortality resulting from malformation of the heart structure during fetal development [1,2]. *In vitro* differentiation systems using human embryonic stem cells (hESCs), which simulate early cardiogenesis as it would occur *in vivo* [3], can be effectively employed as models to evaluate the effects of drugs or environmental pollutants on human cardiac development, especially at very early stages [4,5]. The environmental pollutant perfluorooctanesulfonic acid (PFOS) has been shown to disrupt ESC-dependent cardiogenesis in mice [6]. In this study, we evaluated the potential toxicity of PFOS in the induced cardiac differentiation of hESCs in a model system. We demonstrate the benefits of using the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader and the Invitrogen™ EVOS™ FL Imaging System to detect changes in cell viability and mitochondrial membrane potential (MMP) of differentiating hESCs upon treatment with PFOS.

Cell viability on differentiation days 2, 4, and 8 after chemical treatment was measured with Invitrogen™ PrestoBlue™ HS Cell Viability Reagent, a one-step resazurin-based cell viability assay [7], which is a cost-effective, sensitive, and simple method to detect cellular viability. The PrestoBlue HS reagent is based on resazurin, an indigo-colored, nontoxic redox dye that detects the metabolic activity of living cells. Once the dye has entered the cells, resazurin is reduced to resorufin, which can be excited at 560 nm and emits light at around 590 nm. Cellular viability can be measured by simply adding premixed PrestoBlue HS Cell Viability Reagent to culture medium containing cells, incubating for 1–2 hr, and then measuring the fluorescence with a microplate reader such as the Varioskan LUX Multimode Microplate Reader.

PFOS has been shown to cause mitochondrial toxicity and depolarization of the mitochondrial membrane [8]. Thus, we measured MMP by staining the differentiating cardiac cells with JC-1 dye (part of the Invitrogen™ MitoProbe™ JC-1 Assay Kit) after PFOS treatment. JC-1 is a cationic monomer dye that emits green fluorescence. When live cells are stained with JC-1, some JC-1 monomers enter the

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mitochondria and, under normal metabolic conditions (high membrane potential), self-organize into aggregates, which emit red fluorescence. The membrane potential–dependent accumulation of J-aggregates in mitochondria can be detected by measuring the emission of green (~525 nm) and red (~590 nm) fluorescence—the decrease or increase in the red-to-green fluorescence intensity ratio can indicate the depolarization or hyperpolarization of mitochondria, respectively [9,10].

Materials and methods

Materials

See Table 1 for detailed information on the sources of the key reagents and materials.

Preparation of reagents

The preparation of all reagents that were used in this study is presented below.

EDTA (0.5 mM): To 50 mL of DPBS, 50 µL of Invitrogen™ UltraPure™ 0.5 M EDTA, pH 8.0, was added under sterile conditions.

Y-27632 (10 mM): Under sterile conditions, 100 mg of Y-27632 powder was dissolved in DMSO to obtain a final concentration of 10 mM. Aliquots of 100 µL were prepared and stored at –20°C.

30X Matrigel™ matrix–DMEM/F-12 solution: Matrigel matrix (Corning Life Sciences, Tewksbury, MA, USA) was resuspended and diluted 1:1 by adding 10 mL of cold Gibco™ DMEM/F-12 medium to one vial of Matrigel matrix stock (10 mL) under sterile conditions. The Matrigel matrix was aliquoted (400 µL) and kept at –20°C. **Note:** Matrigel matrix can easily solidify at room temperature, so it is important to keep everything cold when diluting and aliquoting.

10% (w/v) BSA-DPBS: 5 g of BSA was dissolved in 50 mL of DPBS, sterile-filtered, divided into 1 mL aliquots, and stored at 4°C (these aliquots can be stored for up to 1 year).

Table 1. List of key materials and reagents employed.

Material or reagent	Source	Cat. No.
Penicillin-Streptomycin, Liquid	Thermo Fisher Scientific	A15140122
PrestoBlue HS Cell Viability Reagent		P50201
JC-1 (Mitochondrial Membrane Potential Probe)		T3168
Essential 8 Medium		A1517001
Essential 8 Supplement (50X)		A1517101
96-Well Microplate, Nunclon Delta surface		161093
60 mm Cell Culture Dish, Nunclon Delta surface		150288
Vitronectin (VTN-N) Recombinant Human Protein, Truncated		A14700
DPBS, no calcium, no magnesium		14190144
UltraPure 0.5 M EDTA, pH 8.0		15575020
RPMI-1640 Medium		A11875093
TrypLE Express Enzyme		A12604021
DMEM/F-12		11330032
Bovine Serum Albumin (BSA)	Sigma	B2064-100G
L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate		A8960-5G
Y-27632 dihydrochloride, 100 mg	MedChemExpress	HY-10583
CHIR-99021, 10 mM in 1 mL DMSO	Selleck	S7037
WNT-C59, 5 mg		S2924
PFOS	Sigma	77282
Dimethyl sulfoxide (DMSO)	Amresco	0231-500mL

L-Ascorbic acid 2-phosphate sesquimagnesium salt hydrate (64 mg/mL): 0.64 g of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate was dissolved in 10 mL of double-distilled water (ddH_2O) under sterile conditions. The resulting solution was divided into 1 mL aliquots and stored at –20°C.

WNT-C59 (20 mM): 5 mg of WNT-C59 powder was dissolved in DMSO to a final 20 mM solution under sterile conditions; the resulting solution was divided into 10 µL aliquots and stored at –20°C.

CHIR-99021 (10 mM): 1 mL of 10 mM CHIR-99021 stock solution was divided into 30 µL aliquots and stored at –20°C.

PFOS solutions: PFOS powder was dissolved in the required amount of DMSO to get a 300 mM stock solution under sterile conditions. This stock solution was further diluted to final concentrations of 240, 180, 120, 60, 10, and 1 mM with DMSO and stored at 4°C or –20°C.

JC-1 stock solution (2 mg/mL): 5 mg of JC-1 powder was aseptically dissolved in 2.5 mL of DMSO to obtain a 2 mg/mL stock solution. The resulting solution was divided into 50 µL aliquots and stored at –20°C.

Preparation of culture media

Culture media that were used in this study are described below.

Essential 8 Medium: Under sterile conditions, 490 mL of Gibco™ Essential 8™ Medium was mixed with 10 mL of Gibco™ Essential 8™ Supplement (50X) to prepare 500 mL of complete Essential 8 Medium, and stored at 4°C for up to two weeks.

Essential 8 Medium with 10 µM Y-27632: 50 µL of 10 mM Y-27632 was added to 50 mL of complete Essential 8 Medium under sterile conditions, and this resulting medium was always used fresh.

CDM3 medium: 250 µL of 10% BSA, 166 µL of L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, and 500 µL of penicillin-streptomycin were mixed with RPMI-1640 medium under sterile conditions; the final volume was adjusted to 50 mL with RPMI-1640 medium.

CDM3 medium with 6 µM CHIR-99021: 50 mL of CDM3 medium was supplemented with 30 µL of CHIR-99021 10 mM stock solution, under sterile conditions.

CDM3 medium with 2 µM WNT-C59: 5 µL of 20 mM WNT-C59 was added to 50 mL CDM3 medium under sterile conditions.

CDM3 medium with PFOS: Media containing 1, 10, 60, 120, 180, 240, and 300 µM PFOS were prepared by adding from each PFOS stock solution to CDM3 medium at a 1:1,000 ratio. 0.1% DMSO in CDM3 medium was used as a control.

CDM3/PrestoBlue HS Cell Viability Reagent premixed medium: CDM3 medium was mixed with PrestoBlue HS Cell Viability Reagent (Cat. No. P50200) at a ratio of 9:1 just before use.

CDM3 medium with JC-1 (2 µg/mL): The 2 mg/mL JC-1 solution was added to CDM3 medium at a 1:1,000 ratio, and mixed in completely; this medium was prepared just before use, as storage is not recommended.

Preparation of culture dishes

For hESC maintenance and expansion: In a sterile hood, Gibco™ Vitronectin (VTN-N) Recombinant Human Protein was diluted with DPBS to a final concentration of 5 µg/mL. Then, 60 mm cell culture dishes were coated by incubating with 2 mL of diluted VTN-N at 37°C for at least 1 hr.

For cardiac differentiation: In a sterile hood, 400 µL of 30X Matrigel matrix–DMEM/F-12 solution was diluted with 11.6 mL of cold DMEM/F-12 medium, and the diluted Matrigel matrix was added to 96-well plates (100 µL/well). The Matrigel matrix–coated plates were kept at 4°C overnight before use. The solution can alternatively be stored at 4°C for up to two weeks.

Maintenance and expansion of hESCs

Maintenance and expansion of hESCs were carried out as previously described [11]. Briefly, H9 hESCs were cultured as colonies in Essential 8 Medium on VTN-N–coated 60 mm cell culture dishes in a humidified 37°C incubator supplemented with 5% CO_2. The medium was replenished every day. When the cells became ~70–90% confluent, they were washed with DPBS, detached with 0.5 mM EDTA, gently resuspended as aggregates in Essential 8 Medium by pipetting, and reseeded in new VTN-N–coated 60 mm cell culture dishes supplemented with complete Essential 8 Medium.

Cardiac differentiation and exposure to toxicant

Cardiac differentiation was conducted as previously described [3], and the experimental setup and plate layout is shown in Figure 1. Briefly, when hESCs became ~70–90% confluent, the colonies were washed with DPBS and digested with Invitrogen™ TrypLE™ Express Enzyme until the colonies could easily detach from the plate. The digestion time depended on the colony status and generally varied from 6 to 8 min. After digestion, the cells were washed with DMEM/F-12 to remove the TrypLE enzyme and resuspended by gentle pipetting as single cells in complete Essential 8 Medium containing the ROCK inhibitor Y-27632 (ROCKi, STEMCELL Technologies). Then the cells were seeded in 96-well plates precoated with Matrigel matrix according to the workflow steps described in Figure 1. The blue wells were filled with DPBS, and the remaining wells were filled with cells at a density of 1–1.8 x 10^4/cm^2. The next day, the cells were incubated with Essential 8 Medium without ROCKi, which was replenished daily afterwards. When the cells reached about 90% confluence (2–3 days later, which was then designated day 0), cardiac differentiation was induced by replacing the Essential 8 Medium with CDM3 medium containing CHIR-99021. At the same time, cells were treated with 300, 240, 180, 120, 60, 10, and 1 µM PFOS (Figure 1, wells in shades of orange), or 0.1% DMSO (Figure 1, white wells in column 9), in 6 replicates each. On day 2, the medium was replaced with CDM3 medium containing WNT-C59 and PFOS/DMSO, and the medium was replenished every other day. Beating cardiomyocytes could be detected between days 6 and 8. Cell viability and JC-1 staining assays were conducted on days 2, 4, and 8.

Cell viability assay using the PrestoBlue reagent

Prior to cell viability assays, cells on differentiation days 2, 4, and 8 were incubated for 1–2 hr at 37°C with 100 µL CDM3 medium premixed with the PrestoBlue HS Cell Viability Reagent, to allow the reduction of resazurin to resorufin. Then, using bottom reading, fluorescence was measured with the Varioskan LUX Multimode Microplate Reader with excitation at 560 nm (with bandwidth of 5 nm) and emission at 590 nm. Background fluorescence was measured for 6 wells containing CDM3 medium only, averaged, and subtracted from the fluorescence values of wells containing cells treated with either PFOS or DMSO vehicle control. Viability values were calculated by normalizing the average fluorescence values of PFOS-treated groups to the ones in DMSO-treated groups (set as 100%) and expressed as percentage of control.

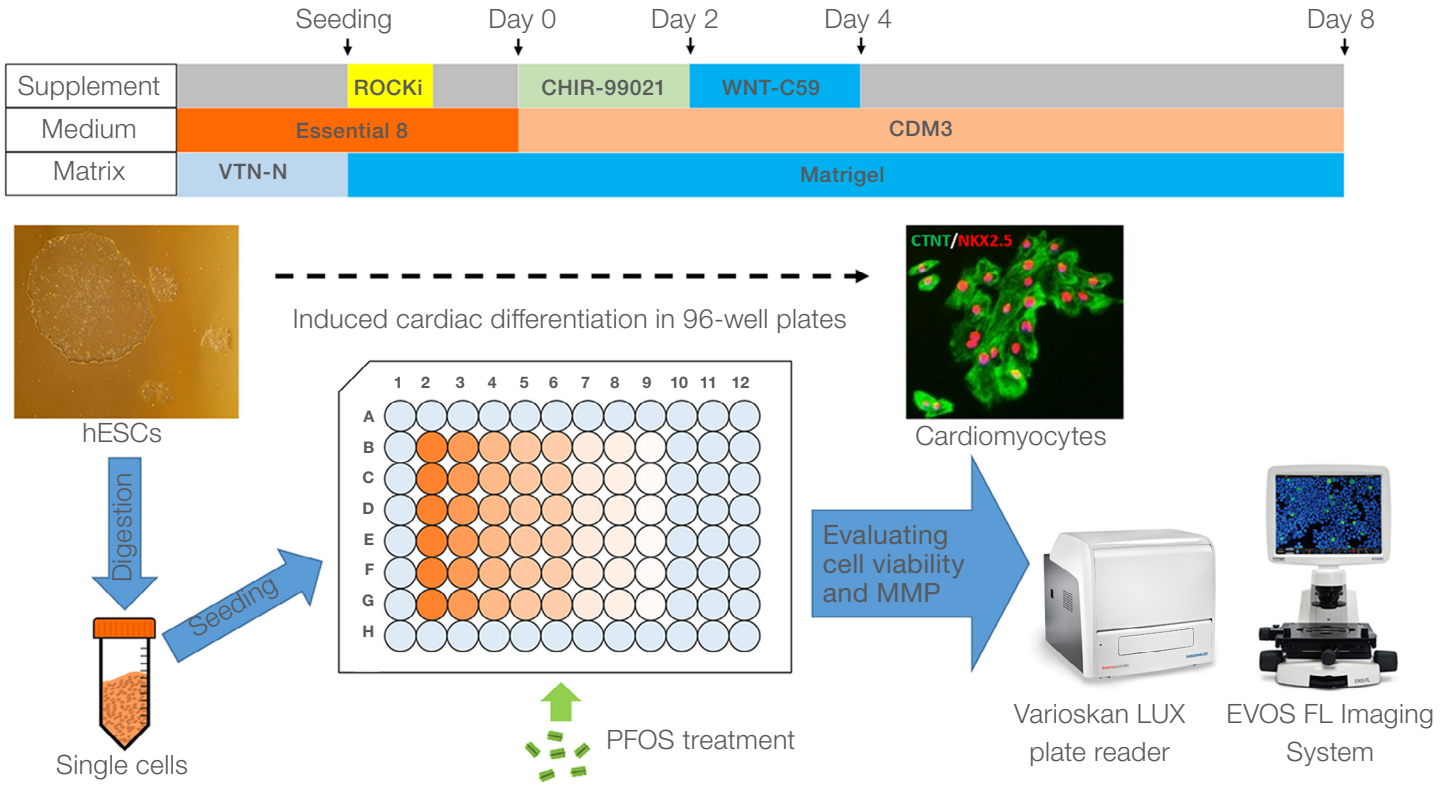

Figure 1. Experimental layout for evaluation of potential developmental cardiac toxicity of PFOS. hESCs were seeded as single cells in 96-well plates and differentiated into beating cardiomyocytes for 8 days, as indicated. Cell viability and MMP were detected with the Varioskan LUX Multimode Microplate Reader and EVOS FL Imaging System. In the schematic of the 96-well plate, blue wells are filled with DPBS (the wells from B10 to G10 were filled with CDM3 medium only prior to cell viability measurements); white wells are filled with cells treated with DMSO; orange wells are filled with cells treated with different concentrations of PFOS.

MMP detection

The MMP was measured using the JC-1 dye. Briefly, on differentiation days 2, 4, and 8, cells were incubated for 20–30 min at 37°C with 100 µL CDM3 medium containing JC-1, to allow JC-1 to enter into the cells and form J-aggregates in the mitochondria. After incubation, the cells were washed twice with DPBS to remove unbound JC-1 and incubated with 100 µL CDM3 medium just before measuring. Using bottom reading, fluorescence was measured with the Varioskan LUX Multimode Microplate Reader at excitation/emission wavelengths of 490/530 nm to detect the green fluorescence emitted by JC-1 monomer, and 525/590 nm to measure the red fluorescence emitted by J-aggregates. The average ratio of red fluorescence to green fluorescence (FL590/FL530) of 6 technical repeats was then calculated. The fluorescence data of the PFOS-treated groups were normalized to the data of the DMSO-treated group to get the relative membrane potential.

Results and discussion

The potential developmental cardiac toxicity of PFOS was evaluated with an *in vitro* hESCs-based cardiac differentiation system. Cell viability and MMP changes after PFOS treatment were used as toxicity indicators.

The results in Figure 2 show that PFOS concentrations of 1 µM or less did not significantly affect cell viability (Figure 2A) or MMP (Figure 2B) at any time point during the cardiac differentiation process. However, PFOS levels of 60 µM or higher induced noticeable cell death, as determined by the PrestoBlue HS cell viability assay, even after only 2 days of treatment and differentiation (Figure 2A). These data were consistent with visualization of the cells using the EVOS FL imaging system. Images show a decreasing number of cells, as well as red and green fluorescence, with increasing PFOS concentrations and days after induction of differentiation (Figure 3).

Interestingly, treatment with 10 µM and 60 µM of PFOS resulted in a concentration-dependent change of the MMP. The extent of differentiation was also dependent on PFOS concentration (Figure 2B). These data indicated that although 10 µM and 60 µM PFOS did not cause the expected cell death, these treatments might have already disrupted the differentiation process. Similar effects caused by PFOS and its analogs have been previously reported [12,13]. It was also observed that when the cells were exposed to PFOS at concentrations above 120 µM, the MMP decreased, indicating damage to mitochondrial membrane integrity, which was accompanied by a decrease in cell viability as differentiation continued (Figures 2 and 3). As illustrated in this study, utilizing orthogonal approaches—where a number of parameters are simultaneously studied—in combination with imaging enables better understanding of the cytotoxicity of compounds such as PFOS.

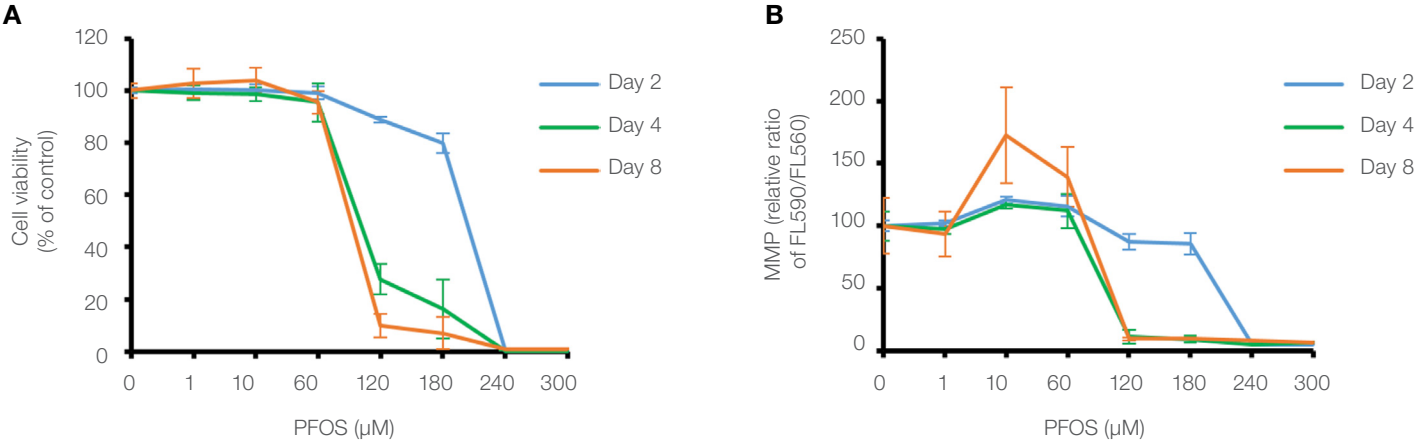

Figure 2. Effects of PFOS on cell viability and MMP. hESCs were differentiated into beating cardiomyocytes and treated with increasing concentrations of PFOS along the differentiation process. Cells were collected at days 2, 4, and 8, and **(A)** assayed with PrestoBlue HS Cell Viability Reagent or **(B)** stained with JC-1 for MMP changes.

Conclusions

We employed the Varioskan LUX Multimode Microplate Reader and EVOS FL Imaging System to elucidate the effects of the known toxicant PFOS on differentiation of hESCs into cardiomyocytes. We measured cell viability with the one-step PrestoBlue HS Cell Viability Reagent and tracked changes in MMP with the JC-1 assay. The combination of the Varioskan LUX Multimode Microplate Reader with the EVOS FL Imaging System allowed us to follow the experiments both visually and quantitatively and provided evidence for the potential developmental toxicity of PFOS during cardiac differentiation.

References

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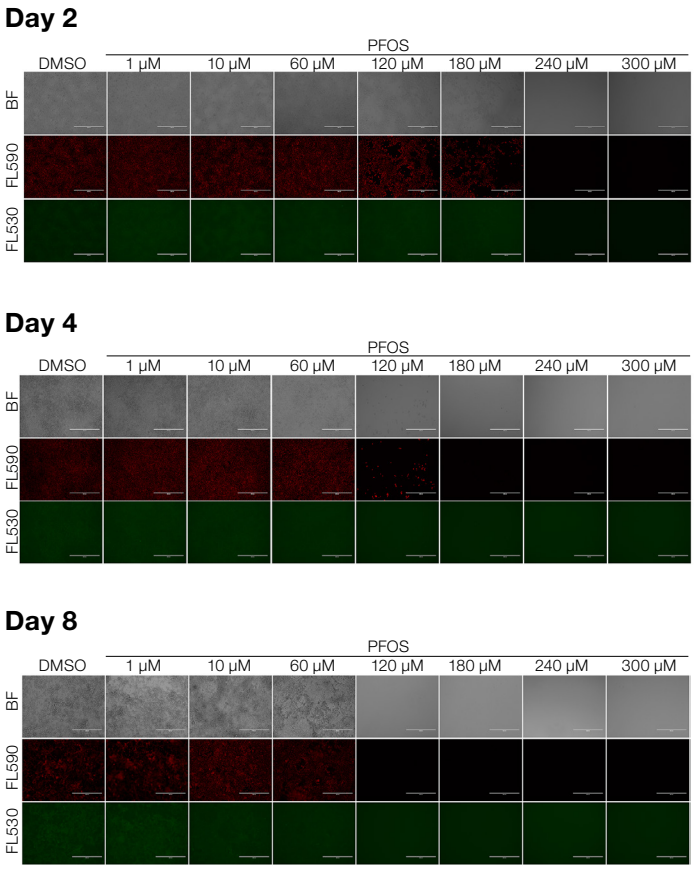

Figure 3. Effects of PFOS treatment on cell number. hESCs were differentiated into cardiomyocytes for 8 days and treated with the indicated PFOS concentrations during the differentiation process. Images were acquired on the EVOS FL Imaging System on days 2, 4, and 8. Representative images from brightfield imaging (BF, top panels), 590 nm fluorescence of the PrestoBlue cell viability assay (FL590, middle panels), and 530 nm fluorescence of JC-1 for MMP detection (FL530, bottom panels) are shown.

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