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A Reagent-Free Screening Assay for Evaluation of the Effects of Chemicals on the Proliferation and Morphology of HeLa-GFP Cells

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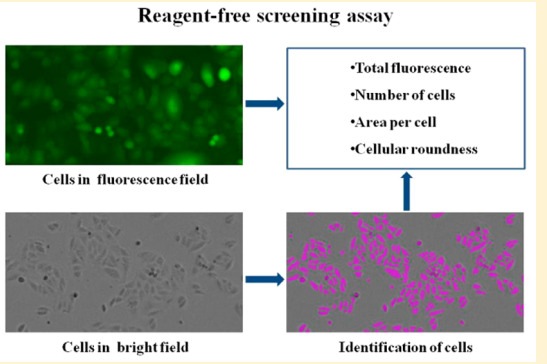
Supporting Information

ABSTRACT: A reagent-free screening assay using HeLa cells with green fluorescent protein expressed in the cytoplasm was developed to describe adherence and proliferation of cells after seeding and to evaluate the dose- and time-dependent effects of three classes of chemicals, including metals ($CdCl_2$, MeHg, $HgCl_2$, and $NiCl_2$), flame retardants [tris(1,3-dichloro-2-propyl) phosphate, heptadecafluoro-1-octanesulfonic acid, tetrabromobisphenol A, and tris(2-chloroethyl) phosphate], and phenolic fungicides (2,4,6-trichlorophenol, 4-*tert*-butylphenol, 3-bromophenol, and 2,4-dibromophenol), on the proliferation and morphology of cells. A seeding density of 5000 cells/well was determined to be optimal, and the most suitable duration of xenobiotic exposure was 120 h beginning 58 h postseeding. Dose- and time-dependent alterations in total fluorescence in the fluorescent field and number of cells, area per cell, and cellular roundness in the bright field were identified after exposure to each of the target chemicals. Results were comparable to those of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or other end points published in the literature for the same chemicals.



INTRODUCTION

A long and growing list of chemicals are released into the environment due to human activity and have potential to cause long-term, adverse effects on wildlife and humans.[1,2] In the 20th Century, thousands of chemicals have been registered in the European Union (EU) market, and during the production and use of these chemicals, some of them inevitably escape into the environment.[3−6] Recently, there have been increased efforts in toxicology to develop and validate novel approaches for rapid screening of chemicals.[7] For example, in the White Paper "Strategy for a Future Chemicals Policy" published in 2001, the European Commission (EC) proposed that the REACH (Registration, Evaluation and Authorization of Chemicals) system should deal with both existing and new chemicals.[8] In that system, considering the cost of performing traditional *in vivo* testing and issues of animal welfare, one alternative approach that was proposed is to structure the evaluation beginning with less expensive, high-throughput *in vitro* testing before progressing to more definitive trials. Such alternatives include cell-based test systems, including primary cultured cells or immortal cell lines.[9−13]

Currently, one of the most used *in vitro* testing systems is based on immortalized and/or genetically modified cell lines.[14,15] Xenobiotic-elicited proliferation of cells represents a sensitive end point in screening for toxicity to cells and thus represents an integrative, relevant model for assessing basal toxicity.[16] Furthermore, the inhibition of cellular proliferation is often used as an end point in *in vitro* toxicity tests. However, two major limitations have affected the use of cell lines when measuring cytotoxicity of chemicals. (1) Some cytotoxicity assays, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or LDH, are based on measurement of the activity of specific enzymes, where some chemicals might

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directly affect activity of these enzymes but do not cause cytotoxicity, thus resulting in biased measurements. (2) Usually, only one end point (e.g., MTT) has been determined in a cytotoxicity assay, and thus, information obtained might not be sufficient to support reliable assessments of the hazards of chemicals.

High-content screening (HCS) is a recent advance in integration and automation of quantitative microscopy and analyses of images that combines the efficiency of high-throughput techniques with imaging of cells to collect quantitative data from multiple parameters.[17−19] HCS has already been applied in the development of pharmaceuticals and biotechnology applications and is quickly growing in popularity for *in vitro* determination of the toxic potential of chemicals.[20−27] However, in previous studies of HCS *in vitro*, some reagents specific to the HCS were necessary.[20,21,23,24] Most reagents used were cytotoxic, and thus, the assay could not support a long-term or online monitoring of the effects of chemicals.

In this study, a modified and stable version of the HeLa cell line with constitutive expression of green fluorescent protein (GFP) in the cytoplasm was used (1) to establish a reagent-free HCS assay, (2) to develop cellular growth curves and determine the most appropriate seeding density and when to initiate exposures, (3) to evaluate the effects of three classes of environmental chemicals on the proliferation and morphology of living cells, and (4) to compare sensitivities between the newly developed HCS assay and the MTT assay.

MATERIALS AND METHODS

Cell Culture and Growth Curves. The HeLa-GFP cell line, which includes constitutive expression of green fluorescent protein (GFP) in the cytoplasm of the human cervical carcinoma cell line (HeLa), was created by transfection of pGFP-N1. Stable cells were obtained from the Cell Resource Center (Peking Union Medical College, Beijing, China) and cultured in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum (Gemini Bio-Products), 100 units/mL penicillin, and 100 μg/L streptomycin at 37 °C in a 5% CO_2 atmosphere. To determine the optimal density for seeding cells prior to exposure and determine when to initiate exposures, various numbers of cells (500, 1000, 2000, 5000, and 10000 cells/well) were seeded into 96-well plates (Corning Coster, Corning, NY). The proliferation and morphology of cells were monitored by use of a SpectraMax i3Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) every 24 h after seeding. Data obtained were then used to produce growth curves.

Exposure of Cells to Chemicals. On the basis of the growth curves produced, an initial density for the seeding of plates of 5000 cells/well was considered to be most suitable for subsequent exposure experiments, with exposure beginning and ending times after seeding of 58 and 178 h, respectively. Briefly, cells were seeded into 96-well plates. After incubation for 58 h, 100 μL of exposure medium was added to each well, and cells were treated with various concentrations of each chemical for 120 h. In this study, three classes of chemicals, including metals [cadmium chloride ($CdCl_2$), methylmercury chloride (MeHg), mercury(II) chloride ($HgCl_2$), and nickel chloride ($NiCl_2$)], flame retardants [tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), heptadecafluoro-1-octanesulfonic acid (PFOS), tetrabromobisphenol A (TBBPA), and tris(2-chloroethyl) phosphate (TCEP)], and phenolic fungicides [2,4,6-trichlorophenol (TCP), 4-*tert*-butylphenol (4-TBP), 3-bromophenol (3-BP), and 2,4-dibromophenol (2,4-DBP)], were selected because these chemicals have been shown to elicit cytotoxicity in cell lines.[28−32] Sources of each chemical are given in the Supporting Information. Metals were dissolved in phosphate-buffered saline (PBS), while organic compounds were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. For each of the chemicals tested, 9 or 10 concentrations (0.25−600 μM) were used. During exposure, four parameters related to cellular proliferation and morphology were measured using the SpectraMax i3Multi-Mode Microplate Reader every 12 h. Each treatment contained three replicate wells.

HCS Assay. During exposure, bright-field and fluorescent images were obtained (four times) using a SpectraMax i3Multi-Mode Microplate Reader. For each well, four images were collected. Images were analyzed using SoftMax Pro Microplate Data Acquisition and Analysis Software (version 6.4). The whole process, including imaging, image montage, and data analysis, was automated, and more details about setting information on the SpectraMax i3Multi-Mode Microplate Reader are presented in the Supporting Information. Four parameters were examined in this study, including total fluorescence in the fluorescent field and the number of cells, area per cell, and cellular roundness in the bright field. Of the four parameters tested, the total fluorescence and number of cells were related to cell proliferation, and the two other parameters measured, area per cell and cellular roundness, were used to describe morphologies of cells. Background correction was applied to all images.

MTT Assay. Cell viability was determined with the MTT assay according to a published protocol.[33] After a 116 h exposure period, 25 μL of the MTT stock solution (5 g/L) was added to each well, and the cells were incubated with MTT for 4 h prior to removal of the culture medium. After that, the culture medium was removed, and 100 μL of DMSO was added to each well. Plates were incubated for 20 min, and MTT activity was determined at 470 nm. In this study, the MTT assay was performed concurrent with the HCS assay. Six replicates were included for each treatment concentration for each chemical.

Statistical Analyses. Statistical analyses were performed using Origin 9.0. The data are presented as means ± the standard error. Normality of data sets was examined using the Kolmogorov−Smirnov test. Homogeneity of variances was checked by Levene's test. One-way analysis of variance (ANOVA) was used to determine the significance of differences between the control and chemical exposure groups. A level of significance for type I error was set at a P value of <0.05. Median effect concentrations (EC_{50}) for three parameters tested (e.g., total fluorescence, number of cells, and MTT assay) were calculated by nonlinear regression analysis.

RESULTS AND DISCUSSION

Growth Curves. Bright-field images were obtained and used for the development of the reagent-free HCS assay, and representative images are shown in Figure S1. Specifically, two parameters, including the total fluorescence and number of cells, were used to construct growth curves (Figure S2). The rates of cell division were high with seeding densities of 2000, 5000, or 10000 cells/well. A seeding density of 5000 cells/well was used for subsequent cell exposures. Furthermore, for the first time to the best of our knowledge, the most appropriate duration after seeding to begin exposure was determined using

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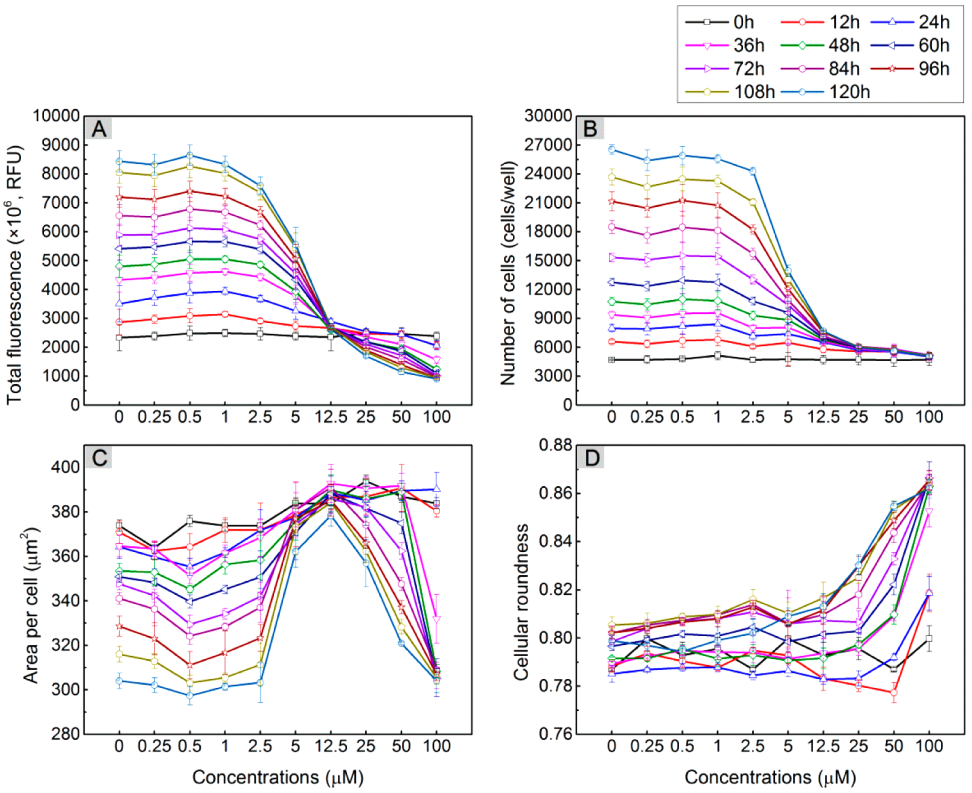

Figure 1. Dose- and time-dependent effects of $CdCl_2$ on (A) the total fluorescence, (B) the number of cells, (C) the area per cell, and (D) cellular roundness. Values represent means ± the standard error of the mean (n = 3). RFU indicates relative fluorescence units.

Table 1. Lowest Observed Effect Concentrations (LOECs) and Median Effect Concentrations (EC_{50}) for the Total Fluorescence, Number of Cells, and MTT Activity after Exposure to Different Concentrations of Each Chemical[a]

	total fluorescence		no. of cells		MTT activity	
chemical	LOEC (μM)	EC_{50} (μM)	LOEC (μM)	EC_{50} (μM)	LOEC (μM)	EC_{50} (μM)
$CdCl_2$	5	8.21 ± 0.71	5	5.59 ± 0.24	5	4.92 ± 0.38
MeHg	25	30.42 ± 1.42	25	27.64 ± 1.04	25	25.76 ± 1.66
$HgCl_2$	25	51.01 ± 2.32	25	59.18 ± 5.73	50	72.23 ± 5.17
$NiCl_2$	NA	NA	NA	NA	NA	NA
TDCIPP	50	64.19 ± 4.06	50	35.71 ± 12.43	50	43.18 ± 10.30
PFOS	150	128.06 ± 17.76	150	131.71 ± 4.79	150	126.24 ± 10.26
TBBPA	50	73.24 ± 2.76	50	72.03 ± 4.75	50	66.24 ± 10.08
TCEP	NA	NA	NA	NA	NA	NA
TCP	75	150.05 ± 10.93	75	155.46 ± 6.89	75	110.23 ± 11.16
4-TBP	200	359.36 ± 19.64	200	337.10 ± 8.15	400	387.91 ± 11.51
3-BP	150	206.97 ± 18.70	150	232.16 ± 7.34	150	238.84 ± 28.19
2,4-DBP	50	63.87 ± 3.13	50	72.54 ± 14.13	50	56.03 ± 1.71

[a] n = 3. NA indicates not available.

the newly developed HCS assay. In previous studies using cells for toxicity testing of chemicals, the time at which to initiate exposures was determined on the basis of microscopic observation, and no related data were used to support the determination.[34−36] In our study, the four HCS parameters were used to describe the process of cellular adherence and proliferation. These results suggested that for some parameters, especially for the number of cells, a rapid increase could be observed starting 58 h postseeding. Two parameters, area per cell and cellular roundness, were used to describe cellular morphology in this study. During cellular adherence, it is expected that the area per cell becomes larger and larger and cellular roundness becomes lower and lower. The results obtained in this study were consistent with these hypotheses. Additionally, the data suggested that, at least at 58 h postseeding, cellular adherence was complete and cellular proliferation was beginning. That is the reason why 58 h postseeding was selected as the most appropriate time to initiate exposure to chemicals.

Dose- and Time-Dependent Effects of Chemicals on the Proliferation of Cells. No significant effects on the total fluorescence or the number of cells were observed after exposure to $NiCl_2$ or TCEP, but treatment with each of the other 10 chemicals tested resulted in dose- and time-dependent decreases in the two parameters (Figure 1 and Figures S3−S13). The lowest observed effect concentrations (LOECs) and

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EC_{50} values for the two parameters tested after exposure for 120 h to each of the chemicals tested are presented (Table 1). Metals such as $CdCl_2$, $HgCl_2$, and MeHg are typical environmental pollutants and have a global distribution in various environmental media.[37−39] Results of previous studies have demonstrated that some metals significantly inhibit proliferation of cells *in vitro*,[40,41] and compared with MeHg and $HgCl_2$, $CdCl_2$ was more toxic.[40,41] Therefore, these results were consistent with the results obtained in this study. Flame retardants and phenolic fungicides are two groups of relatively emerging environmental pollutants, and their environmental risks have received a great deal of attention.[42,43] In this study, exposure to each of the phenol fungicides and each of the flame retardants, except for TCEP, caused a dose- and time-dependent decrease in the extent of cellular proliferation. Previously, it has been reported that TDCIPP is more toxic than TCEP *in vitro* and *in vivo*,[44,45] and thus, these results were consistent with the study presented here. Taken together, the results from this study suggest that this newly developed assay is a relatively reliable approach for screening of chemicals.

Dose- and Time-Dependent Effects of Chemicals Tested on Cellular Morphology. No significant effects on area per cell and cellular roundness were observed after exposure to $NiCl_2$ or TCEP, but treatment with each of the other 10 chemicals tested resulted in dose- and time-dependent changes in the two parameters (Figure 1 and Figures S3−S13). Cellular morphology is an important toxic end point for chemical exposure. However, such toxicity information is limited because quantitative measurement of such a parameter requires specific instruments and software. In this study, two parameters, including area per cell and cellular roundness, were integrated into the HCS assay. The results of this study demonstrated that treatment with most of chemicals changed the area per cell and cellular roundness. The parameters area per cell and cellular roundness are related to cellular adherence, and from the growth curves produced, we could observe that trends in changes of these two parameters were opposite. However, results of this study demonstrated that some chemicals simultaneously increased the area per cell and cellular roundness. A possible explanation for that observation is that exposure to chemicals inhibited proliferation of cells, which was evidenced by the decreased number of cells.

The Results of the Reagent-Free HCS Assay Developed Were Comparable to MTT Activity. Exposure to $NiCl_2$ or TCEP for 120 h did not change the MTT activity, but treatment with each of the other chemicals tested ($CdCl_2$, MeHg, $HgCl_2$, TDCIPP, PFOS, TBBPA, TCP, 4-TBP, 3-BP, and 2,4-DBP) decreased MTT activity in a dose-dependent manner (Figure S14). LOECs and EC_{50} values for the MTT assay after exposure for 120 h to each chemical tested are presented (Table 1). The results were comparable for two parameters in the reagent-free assay, the total fluorescence and number of cells.

In this study, the reagent-free assay based on the proliferation and morphology of cells was evaluated after multiple durations of exposure for a range of end points. Results suggested that this assay could support a long-term or online monitoring of the effects of chemicals and thus might be a novel approach for screening of chemicals. However, on the basis of the data from this study, this newly developed assay appears to lack sensitivity. It might be due to the use of immortalized HeLa cells or/and a low level of GFP expression. Therefore, further studies are needed to improve the sensitivity of this assay by using more sensitive cells. Additionally, another further objective is to improve the throughput of this assay by moving this assay from a 96-well plate to a 384-well plate and/or decreasing the total assay duration from nearly 7.5 to <2 days.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.6b00277.

Sources of chemicals; the HCS assay; representative images and identification of cells (Figure S1); growth curves (Figure S2); effects of MeHg (Figure S3), $HgCl_2$ (Figure S4), $NiCl_2$ (Figure S5), PFOS (Figure S6), TBBPA (Figure S7), TDCIPP (Figure S8), TCEP (Figure S9), 4-TBP (Figure S10), 3-BP (Figure S11), 2,4-DBP (Figure S12), and TCP (Figure S13) on the total fluorescence, number of cells, area per cell, and cellular roundness; and MTT activity after exposure to each of three classes of chemicals (Figure S14) (PDF)

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Notes

The authors declare no competing financial interest.

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