A Simple Technique for Reducing Edge Effect in Cell-Based Assays

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Several factors are known to increase the noise and variability of cell-based assays used for high-throughput screening. In particular, edge effects can result in an unacceptably high plate rejection rate in screening runs. In an effort to minimize these variations, the authors analyzed a number of factors that could contribute to edge effects in cell-based assays. They found that preincubation of newly seeded plates in ambient conditions (air at room temperature) resulted in even distribution of the cells in each well. In contrast, when newly seeded plates were placed directly in the CO_2 incubator, an uneven distribution of cells occurred in wells around the plate periphery, resulting in increased edge effect. Here, the authors show that the simple, inexpensive approach of incubating newly seeded plates at room temperature before placing them in a 37° C CO_2 incubator yields a significant reduction in edge effect. (*Journal of Biomolecular Screening* 2003:566-570)

Key words: edge effect, cell-based assays, high-throughput screening

INTRODUCTION

CELL-BASED ASSAYS ARE WELL ESTABLISHED as powerful and widely used tools for drug discovery.[1] They combine the biological complexity of live cell responses with the scalability and process adaptation of high-throughput screening (HTS). However, the very biological complexity that imparts added value to cell-based assays also opens a Pandora's Box of sorts—it introduces a large number of potential sources of noise and variability that can deteriorate assay performance to levels unacceptable for HTS.[2] These factors include variations in cell plating, differential cell growth, differential cell response, uneven response across plates, plate-to-plate variability, run-to-run variability, and edge effect.

Edge effects are a class of phenomena that are widely recognized in fields as diverse as population biology[3] and dermatology.[4] They are also recognized as widespread factors that contribute to the deterioration of assay performance in HTS, but they are generally underaddressed in descriptions of assay procedures and in the scientific literature. There were several early reports on edge effects in microplate-based adsorption assays, due to thermal gradients[5,6] and differential adsorption characteristics across the plates.[7] A subsequent report suggested that well-to-well variations are of greater statistical significance in plate-based assays,[8] but this work was also based on binding, rather than cell-based, assays.

The causes of edge effect are complex, and undoubtedly a number of the factors cited above are involved in any given example of edge effect. It is common for screening departments to avoid use of the peripheral wells on plates to avoid edge effects, but this approach avoids, rather than solves, the problem, and it does so at the expense of capacity and throughput. Although that may be an acceptable work-around in some cases, it is clearly preferable to minimize the source of artifacts such as edge effect. Pre-incubation of plates with newly seeded cells at room temperature (RT) has been suggested as a technique to control edge effect in cell-based assays.[9] Here we evaluated this technique and show that it is a simple, inexpensive, and practical method for reducing edge effect in cell-based assays to be used for HTS.

MATERIALS AND METHODS

Cell lines and culture conditions

Chinese Hamster Ovary (CHO) cells stably transfected with the human insulin receptor (CHO-hIR)[10] and an insulin receptor subtrate-1 (IRS1)-GFP fusion protein, CHO-hIR IRS1-GFP, were propagated as a monolayer culture in Ham's F-12 Nutrient Mixture containing L-glutamine (Gibco™, Invitrogen Corporation,

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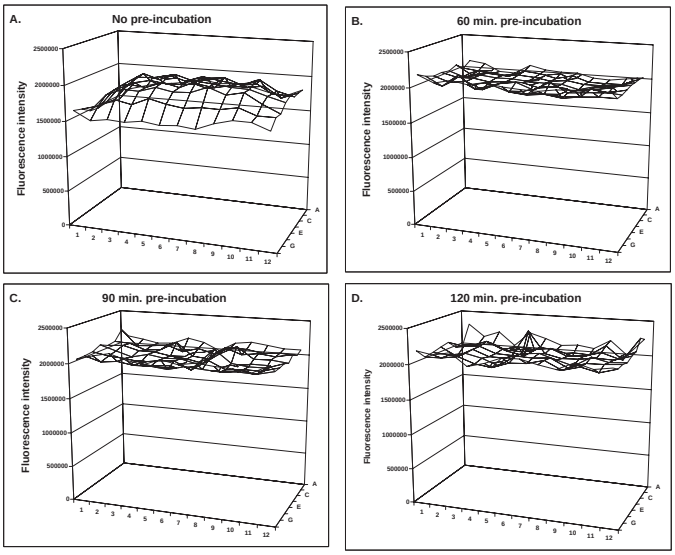

FIG. 1. Time course of CHO-hIR IRS1-GFP cells pre-incubated at different time intervals. CHO-hIR IRS1-GFP cells were seeded in 96-well plates at 10^5 cells/well and treated as follows: no pre-incubation (A), 60 min (B), 90 min (C), or 120 min (D) RT pre-incubation before a 24 h 37° C, 5% CO_2 incubation. Cells were then fixed in the presence of 10 μM Hoechst 33258 before fluorescence was measured.

Carlsbad, CA, USA), 10% fetal calf serum (FCS, Gibco™) supplemented with 0.5 mg/ml G418 (Gibco™). The cells were grown in a humidified incubator at 37° C and 5% CO_2, and passaged twice weekly by resuspension with 0.25% trypsin/0.53 mM EDTA solution (Gibco™) for 3 min at room temperature (RT). CHOhIR IRS1-GFP cells tested negative for *Mycoplasma* contamination. For all experiments, cells were seeded at 10^5 cells/well using a Multidrop dispenser (Thermo Labsystems, Vantaa, Finland) in black 96-well Packard Viewplates (Packard Bioscience, Company, Meriden, CT, USA) and incubated for 24 h at 37° C and 5% CO_2.

Hoechst 33258 measurements

To quantify number of cells per well, the cell nuclei were stained with 10 μM Hoechst 33258 (Sigma-Aldrich Co., St. Louis, MO, USA) in 0.1% formaldehyde (Bie & Berntsen Lab, Rødovre, Denmark) and 5.0% Triton X-100 (Sigma) in PBS (Gibco™) for 10 min at RT. The plates were then measured for Hoechst intensity (excitation 355 nm, emission 460 nm) using a fluorescence plate reader (SpectraFluor, Tecan Group AG, Männedorf, Switzerland).

Crystal violet staining and imaging

The cell growth pattern was evaluated by crystal violet staining. Cells were fixed and stained with a crystal violet solution, containing 0.5% (w/v) crystal violet (Sigma) in 25% (v/v) methanol (Sigma), for 15 min, followed by several washes with tap water. Plates of cells stained with crystal violet were imaged for visualization purposes by placing them upside down over an electroluminescent sheet (RS Components Ltd, Northants, UK) chosen to give extremely even illumination, and imaged from above using a CoolPix 950 digital camera (Nikon, Tokyo, Japan). For quantitative analysis, the plates were placed upside down over the same electroluminescent sheet and imaged using 100-msec exposures and 2 × 2 binning with an Orca-ER 12-bit CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IPLab Spectrum for Windows software (Signal Analytics, Fairfax, VA). A 50-mm still camera lens (Pentax, Slough, UK) with 3 screw-on close-up lenses (Tayon, Japan) was used to obtain a magnified image in which 4 adjacent wells filled the image field.

Well-by-well analysis of cell monolayer evenness

Selected wells were analyzed in 2 ways. First, the mean and standard deviation of the pixel intensities in the well were determined using the public-domain image analysis package ImageJ (U.S. National Institutes of Health, available at http://rsb.info.nih.gov/ij). The standard deviation was squared to give the variance for the pixel intensities in the well. Second, line plots were made from the pixel intensities along a vertical line drawn through the center of selected wells. The line plots were overlaid for comparison of the "flatness" of the pixel intensities across the well in the vertical dimension.

RESULTS and DISCUSSION

Room temperature pre-incubation reduces edge effect

Pre-incubation of plates with newly seeded cells at room temperature (RT) has been suggested as a technique to control edge effect in cell-based assays.[9] However, the technique has not previously been documented in the literature. Here we have evaluated the effect of pre-incubation at RT further using a cell line derived from the CHO-hIR parent cell line. This cell line, CHO-hIR IRS1-GFP, is stably transfected with an (IRS1)-GFP fusion protein, and it had been excluded from a cell-based HTS due to severe edge effects and instability of the cell line.

Edge effect in multiwell plates were quantified by measuring cell plating consistency (number of cells per well) by using the DNA-binding nuclear stain, Hoechst 33258.[11]

Figure 1 shows the effect of RT pre-incubations of 60, 90, and 120 min, respectively, on edge effect compared to control cells not pre-incubated at RT before placed in the CO_2 incubator. The data are depicted as a surface graph with each square corresponding to the fluorescence intensity value of 1 well. Based on these results, we decided to use 1 h of RT pre-incubation before placing plates in the CO_2 incubator.

Several additional experiments (data not shown) were performed in an attempt to further reduce or minimize the edge effect, including different plate stacking patterns (one-by-one, normal

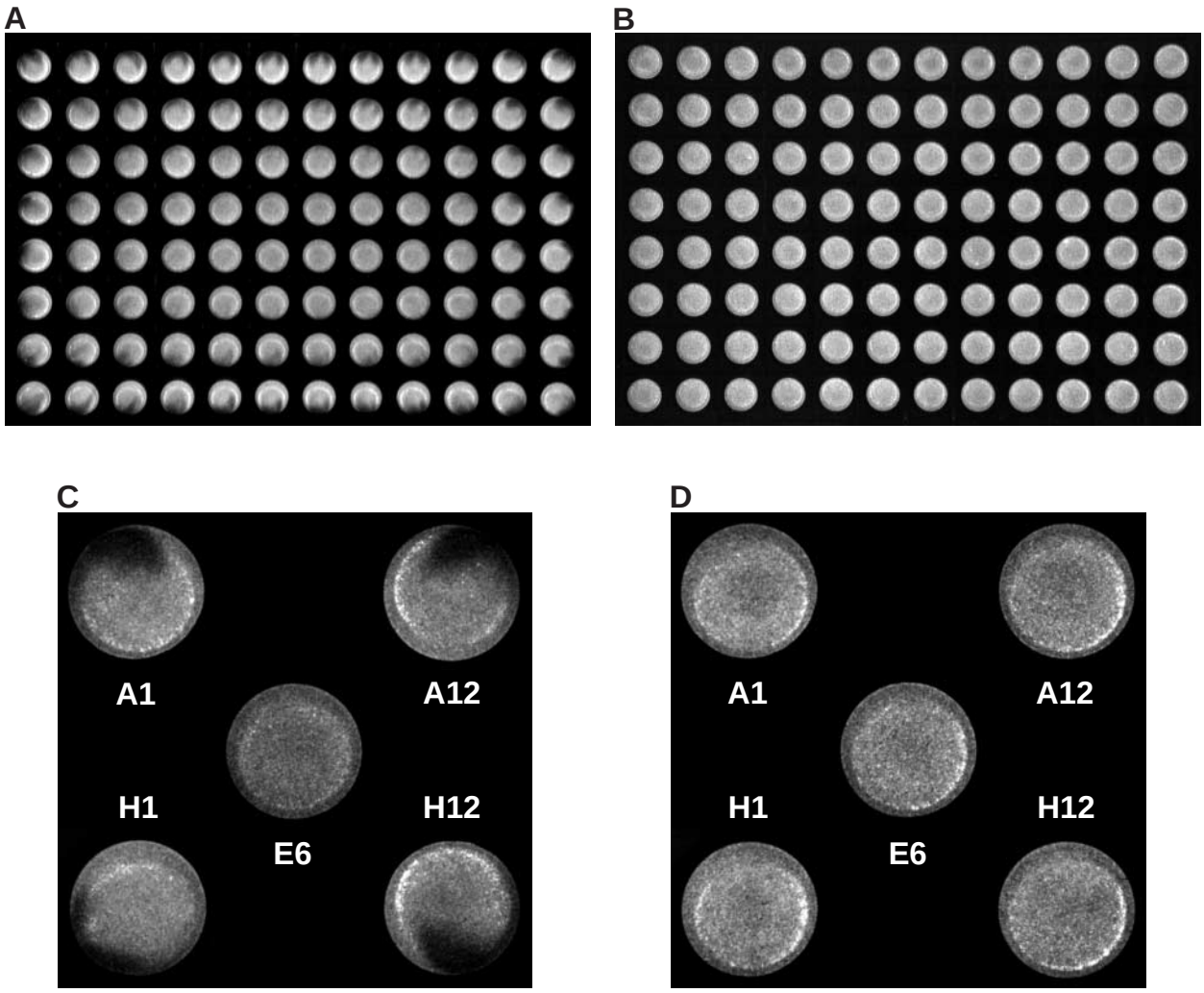

FIG. 2. Crystal violet stained 96-well plates seeded with 10^5 cells/well and either placed directly in CO_2 incubator (A), or pre-incubated for 1 h at room temperature (B). Composite images of selected wells of the same 2 plates are shown in panels (C) and (D). Each composite consists of wells A1, A12, E6, H1, and H12 of the control and pre-incubated plates, respectively. A more uniform distribution of cells is evident in the corner wells of the pre-incubated plates.

stacking vs. cross-stacking), reducing the volume/well (100 µl vs. 200 µl), changing trypsin concentrations upon trypsinization of cells prior to seeding (0.25% vs. 0.05%), wrapping the plates in Saran Wrap, which is CO_2 permeable, versus no wrapping, and seeding of cells with a Multidrop dispenser versus manual multiwell pipetting. None of these parameters significantly affected the edge effect observed in our system further.

Cells in edge wells distribute more evenly in RT pre-incubated plates

We used crystal violet staining to visualize cell localization in the wells of plates that were either pre-incubated at RT or placed directly in the 37° C incubator. Crystal violet binds to DNA and yields intense purple staining of cell nuclei.[12] Figure 2A shows that cells in the outer wells of plates placed directly in the incubator are localized toward the periphery of the well nearest the edge of the plate. If the plates were pre-incubated for 1 h at RT (Fig. 2B), this pattern disappeared and the cells plated evenly in all wells. The composite images of the corner wells of control (Fig. 2C) and pre-incubated (Fig. 2D) plates show this difference in more detail. A representative central well plated uniformly in both cases. These differences in cell uniformity are quantified in Figure 3.

In support of the above observations, plates incubated in a CO_2 incubator without a lid had the same reduced edge effect as plates pre-incubated at RT when measured with Hoechst 33258 staining. Furthermore, an even cell pattern was observed when the cells were crystal violet stained (data not shown).

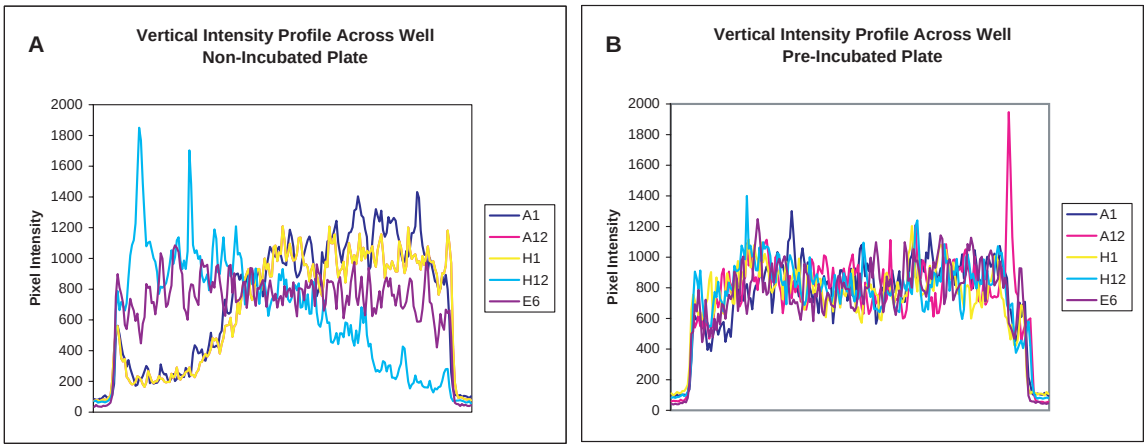

FIG. 3. Overlaid intensity profiles across the wells shown in Figure 2C (A) and 2D (B). The profiles for the pre-incubated wells (B) are indistinguishable, whereas the profiles for the control wells (A) can be clearly distinguished based on position on the plate.

We have also investigated whether edge effect was due to differences in cell motility between control and pre-incubated plates, by incubating plates at 37° C with a panel of cell motility inhibitors. Neither the myosin motility inhibitor wortmannin, the tubulin inhibitor nocodazole, the actin cytoskeleton inhibitor cytochalasin B, nor the dynein motility inhibitor EHNA (erythro-9-[3-(2-hydroxy-nonyl)]adenine) had any effect on the cell pattern observed when placing the cell plates directly in the CO_2 incubator (data not shown).

CONCLUSIONS

Our data indicate that room temperature pre-incubation reduces edge effect by minimizing thermal gradients in the edge wells of plates during the period between dispensing of the cell suspension into the wells and subsequent settling of cells to the bottom of the well and adhesion to the surface. Thermal gradients are greatest in the edge wells of plates, and they result in uneven distribution of the cells on the well bottoms. The uneven cell distribution in turn affects cell adhesion and morphology, and response to test compounds.

The data presented here show that pre-incubation of freshly seeded multiwell plates for 1 h at RT before placing them in the CO_2 incubator significantly reduces the edge effect for this class of cell-based assay. Room temperature pre-incubation of assay plates is a practical, inexpensive procedure that can significantly improve screening results for cell-based assays. The degree of effectiveness of this approach is likely to vary from one cell line to another, and it should be evaluated on a case-by-case basis before adoption.

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