High-throughput microtiter assay for Hoechst 33342 dye uptake

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Abstract

Exclusion of Hoechst 33342 dye is a characteristic common to stem cells, as well as chemotherapy-resistant cancer cells. Normally, these dye-excluding cells can be sorted from enzymatically dissociated tissues with a UV cell sorter/flow cytometer. UV-flow cytometry can be expensive, time-consuming and not readily available to all laboratories. We have developed a simple, high-throughput 96-well microtiter plate assay by which cell populations can be quickly screened for Hoechst dye uptake and exclusion. The method is compatible with green-fluorescent EGFP expressing cells, often used in stem cell biology. Useful applications for this assay will be the rapid screening of clonal stem cell populations and tumor cells for Hoechst dye uptake.

Introduction

Identification of stem cells is a challenge common to the many tissues in which they are sought. Recently, expression of ABCG2 (or BCRP), a cell surface, drug-resistance marker has been utilized as a means to identify presumptive stem cells. Specifically, ABCG2 expression confers upon cells the ability to exclude Hoechst dye 33342 (Kim et al. 2002; Scharenberg et al. 2002; Zhou et al. 2001), as well as resistance to at least 20 different chemotherapeutic agents, including methotrexate, doxorubicin, indolcarbazole, and others (review Doyle et al. 2003). This dye-excluding phenotype is abolished with 50 μ M verapamil treatment (Goodell et al. 1996). Identification of verapamil-sensitive subpopulations by flow cytometry and cell sorting has been used in a variety of tissues, including hematopoietic populations (Scharenberg et al. 2002), bone marrow, skeletal muscle

(Zhou et al. 2001) mammary gland (Welm et al. 2002), lung (Summer et al. 2003), as well as developing retina (Bhattacharya et al. 2003).

Hoechst dyes have been used previously for apoptosis assays, as well as cell cycle profiling by flow cytometry (Frey 1995). Hoechst-dye exclusion via active transporter (Lahmy et al. 1995) is associated with a multi-drug resistance phenotype. In adriamycin-resistant P388 cells, cocubation of these refractory cells in the presence of efflux blockers such as phenothiazines (trifluoperazine) or Ca^{2+} channel blockers (verapamil) promoted dye uptake/retention and allowed for flow cytometric DNA distribution histograms for cell cycle analysis. The multi-drug resistance phenotype has been evaluated using several dyes, including danorubicin, rhodamine 123, as well as Hoechst (Maynadie et al. 1994). Again, flow cytometry has been the method of choice for these assays. However, UV flow cytometry is a luxury not readily available to all laboratories.

In this report, we describe a simple, high-throughput assay in which Hoechst dye uptake can be assessed in a 96-well microtiter plate assay. We specifically included retinoblastoma cell lines that we hypothesize to contain subpopulations of either stem cells and/or chemoresistant cells that would exclude Hoechst dye. The assay we have developed is sensitive, linear, and parallels the protocol that can be used for flow cytometric analysis.

Materials

Equipment

NAPCO¹ 6000 incubator
 NUAire² biological safety cabinet (NU-425-400)
 PerSpective Biosystems³ Cytofluor II fluorescence multiwell plate reader

Cells

NIH 3T3, American Type Culture Collection (ATCC)⁴ (CRL-1658) is an adherent mouse fibroblast cell line that was used as a negative control for verapamil treatments.

Y79 (ATCC⁴ HTB-18) is a human retinoblastoma cell line that grows in suspension culture. Both Y79 cells and WERI-RB-27 cells (below) are thought to contain subpopulations of cells that exclude Hoechst dye as an indication of chemoresistance and/or stem cell-like properties.

WERI-RB27 (Sery et al. 1990) is a human retinoblastoma cell line that grows in suspension culture. These cells were a generous gift of Dr. John Ludlow (University of Rochester).

R28 is a non-tumorigenic, rat E1A-immortalized adherent retinal precursor cell line previously established in our laboratory (Seigel 1996; Seigel et al. 2004). R28 cells are not commercially

available, but can be obtained directly from our laboratory upon request.

Psi2 cells are an adherent mouse E1A retroviral packaging cell line and were kindly provided by Dr Roger Cone (Cone et al. 1988), while he was affiliated with Cold Spring Harbor Laboratories. Psi2 cells are now commercially available from ATCC4 (CRL-1808).

Solutions/Supplies

Culture medium: 4.98 g of DMEM powder (Sigma⁵ D5523-10L) is dissolved in 480 ml of water. This solution is sterilized either by autoclaving or filter-sterilization. The following sterile solutions are added to complete the DMEM: 5 ml of 200 mM L-glutamine (GIBCO⁶ 25030-081), 5 ml of MEM non-essential amino acids (GIBCO⁶ 11140), 50 ml of calf serum (Hyclone⁷ #SH30073.03), 15 ml of 7.5% NaHCO₃ (Sigma⁵ S-5761) and 0.125 ml of 50 mg/ml gentamicin (GIBCO⁶ #1570-060).

0.25% Trypsin-EDTA (GIBCO⁶ #25200-056) is diluted in CMF-PBS (see below) to 0.0625% for cell dissociation.

CMF-PBS (1 l): 0.015M NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, 26 mM NaHCO₃. To this solution is added 1 ml of 2% EDTA (in sterile water), 1 ml of 10% glucose (in sterile water) and 0.125 ml of 50 mg/ml gentamicin (GIBCO⁶ #1570-060).

Hoechst 33342 dye (Molecular Probes⁸ #H21492) is dissolved in sterile water to make a working solution of 10 mg/ml. Final concentration used is 5 µg/ml.

Verapamil Hydrochloride (MP Biomedicals⁹ #195545) is dissolved in sterile water to a 25 mM stock solution. Final concentration in the wells is 50 µM.

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PBS solution: 150mM NaCl (Baker¹⁰ #3624-05), 8 mM Na₂HPO₄ (Sigma⁵ #S-0751), 2.7 mM KCl (Sigma⁵ #P-9541), 15 mM KH₂PO₄ (Sigma⁵ #P-0662) in one liter dH₂O, pH 7.4

Corning¹¹ 96-well flat-bottom dishes (#25860), with 6.4 mm well diameter, for tissue culture and assay.

15 ml conical polystyrene tubes, Laboratory Products Sales¹² # L232151

Hemocytometer, Hausser Scientific¹³

Software: Prism 4 by Graphpad, Inc.¹⁴

Procedures

Microtiter plate assay (step-by-step)

1. Adherent cells are rinsed with 2 ml of CMF-PBS and trypsinized from stock cultures using 0.0625% trypsin for 5 min at 37 °C in a 5% CO₂ incubator. Trypsinized cell suspensions are counted by hemacytometer and plated in triplicate wells at increasing cell number (2000–10,000 cells per well) in 96 well microtiter dishes. Adherent cells are allowed to attach for 4 h prior to the assay to recover from trypsinization. (Suspension cultures are processed in 15 ml tubes, counted and dispensed in wells after incubation).

2. Cells (adherent or in suspension) are incubated with 5 ug/ml of Hoechst 33342 dye in a total of 100 μ l DMEM for 90 min at 37 °C in a 5% CO₂ incubator. Half of the Hoechst-containing wells receive 50 μ M Verapamil during the incubation, while half of the wells receive Hoechst alone. Tip: Hoechst dye is light-sensitive. Microtiter plates are covered with foil to protect them from light after Hoechst dye is added. A typical assay set-up is shown as a template in Figure 1.

3. After the 90 min incubation, supernatant is removed and the wells are rinsed with 100 μ l of cold DMEM culture medium at room tempera-

ture. The culture medium is removed and replaced with 100 μ l PBS for measurement in a PerSpective Biosystems Cytofluor II fluorescence multiwell plate reader (excitation 360 nm; emission 450 nm). Tip: For suspension cultures, be sure to set the plate reader for a brief shake to disperse the cells before reading the plate. Results are analyzed and graphed using Prism 4 software.

Results

We began by testing the sensitivity and linearity of the assay to measure Hoechst 33342 dye uptake in adherent and suspension-grown cells. Figure 2, 3 and 4 are graphs of individual experiments that represent multiple assays. Each cell line has been assayed multiple times as follows: R28 cells (8 times), Y79 (6 times), 3T3 cells (6 times), WERI-RB27 (4 times), Psi2 cells (3 times). In each graph, the standard deviation is calculated using triplicate wells for each condition. As seen in Figure 2, the 2000–10,000 cell range was linear with respect to cell number. This was true for adherent cells (R28 and NIH 3T3) as well as for cells in suspension (Y79). Verapamil treatment alone, without Hoechst treatment, had no effect on fluorescence measurements (not shown). Fluorescence intensity per well did vary by cell type, but remained linear within each cell type.

Many studies utilize EGFP-labelled, green fluorescent cells. Therefore, it was important to see if EGFP expression would interfere with the assay. In Figure 3, EGFP-labelled R28 cells were plated and compared with non-green R28 cells. The psi2 viral packaging cell line was included, as it expresses the immortalizing retroviral vector initially used to create the R28 cell line. As seen in Figure 3, the assay was again linear with respect to cell number. EGFP effects on Hoechst fluorescence detection were not statistically significant.

Since verapamil-sensitive Hoechst dye uptake is an important feature of stem cell and cancer cell biology, we tested whether the assay would be sensitive enough to detect verapamil-enhancing effects on Hoechst dye uptake. As seen in Figure 4, verapamil caused statistically significant enhancement of dye uptake for the retinoblastoma (RB) cell lines Y79 and WERI-RB27, but not NIH 3T3 cells (used as a negative control). The effect of

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	← Untreated controls →			← Hoechst 33342 →			← Verapamil →			← Hoechst + Verapamil →		
	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	0	0	0	0	0	0	0	0	0	0
B	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000	2000
C	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000	4000
D	6000	6000	6000	6000	6000	6000	6000	6000	6000	6000	6000	6000
E	8000	8000	8000	8000	8000	8000	8000	8000	8000	8000	8000	8000
F	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000	10000
G												
H												

Figure 1. Typical template for Hoechst dye uptake assay. Wells are plated in triplicate. Numbers indicate cell number ranging from 0 to 10,000 cells per well. Wells receive Hoechst, Verapamil, Hoechst + Verapamil, or remain as controls as indicated. Empty wells at the bottom can be used to test Hoechst dye intensity alone (without cells), at 0.625, 1.25, 2.5, and 5 $\mu\text{g}/\text{well}$.

Figure 2. Uptake of Hoechst 33342 dye is linear and dependent upon cell type. Adherent cells (3T3 fibroblasts, R28 retinal precursor cells), as well as cells grown in suspension (Y79 retinoblastoma cells) were analyzed. Hoechst dye uptake was linear with increasing cell number and varied according to cell type. This graph, as well as Figures 3 and 4, are individual experiments representative of multiple assays. Standard deviations are calculated from triplicate wells for each condition. Y-axis numbers are arbitrary fluorescence units generated by the plate reader.

verapamil was seen at a cell concentration as low as 2000 cells per well (Y79 cells).

Discussion

We have developed a simple, high-throughput 96 well microtiter assay that is (1) linear with respect to cell number for adherent and suspension cultures, (2) can accommodate EGFP-labelled, green fluorescent cells, and (3) can detect the dye-uptake enhancing effects of verapamil. The assay provides

Figure 3. Green fluorescent protein expression does not interfere with Hoechst 33342 dye exclusion assay. EGFP expression is often used to label putative stem cell populations. Therefore, we tested whether EGFP expression would interfere with the Hoechst dye exclusion assay. R28 cells with and without EGFP green fluorescence show significantly lower fluorescence intensities as compared with the psi2 packaging cell line (3T3 cells containing E1A retroviral vector) ($p < 0.05$ for 6000 and 8000 cells per well). Green fluorescence of the EGFP-R28 cells did not cause significant interference in the assay. Y-axis numbers are arbitrary fluorescence units generated by the plate reader.

excellent reproducibility, as one-way ANOVA comparisons of means from multiple experiments (within the same cell type/cell number) were not statistically different.

The limitation of this assay is that it does not replace the flow cytometer/cell sorter. This assay can only measure dye uptake of an entire population of cells and cannot account for differences within heterogeneous cell populations. Thus, this assay alone cannot isolate Hoechst-excluding subpopulations in a heterogeneous cell population.

Figure 4. Verapamil enhancement of Hoechst dye uptake in retinoblastoma cells is detectable in the microtiter assay. Retinoblastoma cell line Y79 and WERI-Rb27 cells demonstrate significantly higher levels of Hoechst fluorescence upon treatment with verapamil. In contrast, NIH 3T3 cells, used as a negative control, did not show a significant verapamil effect. Y-axis numbers are arbitrary fluorescence units generated by the plate reader.

One useful application for this assay in stem cell biology will be the analysis of multiple clonal cell populations derived from tissues of interest for rapid screening of Hoechst dye uptake. There are also applications in cancer cell biology, in which ABCG2 expression/Hoechst dye exclusion corre-

lates directly with exclusion of at least 20 chemotherapeutic agents, including methotrexate, doxorubicin, indolcarbazole, and others (Doyle et al. 2003).

This microtiter assay could easily be adapted as screening method for Hoechst dye-excluding

tumor cells. Importantly, EGFP labeled green fluorescent cells can be measured for Hoechst dye uptake without significant interference. Therefore, levels of green fluorescence could be measured (at an alternative wavelength) in lieu of actual cell number in circumstances where EGFP-labeled cells cannot be conveniently counted in wells. Further analysis of microtiter assay results, once the wells of interest were identified, can be accomplished by flow cytometry.

Our results show differences in dye uptake among different cell types. Dye uptake measurements on a per-well and per-cell basis may depend upon a number of factors, including cell size, adherence vs. suspension culture, the phase of the cell cycle and metabolic activity. Uptake of Hoechst 33342 is increased during S phase in HeLa cells (Frankfurt 1983), as well as in cells exposed to hyperthermia (Rice et al. 1985). These factors need to be taken into account for well-by-well comparisons of different cell types.

The presence of Hoechst dye-excluding subpopulations in our retinoblastoma cell cultures is tantalizing evidence for the existence of RB cells with either stem cell-like or chemoresistant properties. Additional experiments are underway for complete characterization of these RB subpopulations.

References

- Bhattacharya S., Jackson J.D., Das A.V., Thoreson W.B., Kuszinski C., James J., Joshi S. and Ahmad I. 2003. Direct identification and enrichment of retinal stem cells/progenitors by Hoechst dye efflux assay. *Invest. Ophthalmol. Vis. Sci* 44(6): 2764–2773.
- Cone R.D., Grodzicker T. and Jaramillo M. 1988. A retrovirus expressing the 12S adenoviral E1A gene product can immortalize epithelial cells from a broad range of rat tissues. *Mol. Cell. Biol.* 8: 1036–1044.
- Doyle L.A. and Ross D.D. 2003. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene* 22(47): 7340–7358.
- Frankfurt O.S. 1983. Increased uptake of vital dye Hoechst 33342 during S phase in synchronized HeLa S3 cells. *Cytometry* 4(3): 216–221.
- Frey T. 1995. Nucleic acid dyes for detection of apoptosis in live cells. *Cytometry* 21(3): 265–274.
- Goodell M., Brose K., Paradis G., Conner A.S. and Mulligan R.C. 1996. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* 183: 1797–1806.
- Kim M.H., Turnquist J. and Jackson M. et al. 2002. The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. *Clin. Cancer Res.* 8: 22–28.
- Lahmy S., Viallet P. and Salmon J.M. 1995. Is reduced accumulation of Hoechst 33342 in multidrug resistant cells related to P-glycoprotein activity? *Cytometry*. 19(2): 126–133.
- Maynadie M., Lizard G., Solary E., Roignot P. and Carli P.M. 1994. Multidrug resistance phenotype evaluation by immunofluorescence and functional tests: comparison of two monoclonal antibodies and three fluorescent dyes in three cells lines. *Anticancer Res.* 14(6B): 2605–2609.
- Rice G.C., Gray J.W. and Dewey W.C. 1985. FACS analysis of a hyperthermia-induced alteration in Hoechst 33342 permeability and direct measurement of its relationship to cell survival. *J. Cell Physiol.* 122(3): 387–396.
- Scharenberg C.W., Harkey M.A. and Torok-Storb B. 2002. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99: 507–512.
- Seigel G.M. 1996. Establishment of an E1A-immortalized rat retinal cell culture. *In Vitro Cell Dev. Biol.* 32: 66–68.
- Seigel G.M., Sun W., Wang J., Hershberger D., Campbell L. and Salvi R. 2004. Neuronal gene expression and function in the growth-stimulated R28 retinal precursor cell line. *Curr. Eye Res.* 28: 257–269.
- Sery T.W., Lee E.Y., Lee W.H., Bookstein R., Wong V., Shields J.A., Augsburger J.J. and Donoso L.A.J. 1990. Characteristics of two new retinoblastoma cell lines: WERI-Rb24 and WERI-Rb27. *Pediatr. Ophthalmol. Strabismus.* 27(4): 212–217.
- Summer R., Kotton D.N., Sun X., Ma B., Fitzsimmons K. and Fine A. 2003. Side population cells and Bcrp1 expression in lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 285: L97–104.
- Welm B.E., Tepera S.B., Venezia T., Graubert T.A., Rosen J.M. and Goodell M.A. 2002. Sca1 positive cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev. Biol.* 245: 42–56.
- Zhou S., Schuetz J.D., Bunting K.D., Colapietro A.M., Sampath J., Morris J.J., Lagutina J., Grosveld G.C., Osawa M., Nakauchi H. and Sorrentino S.P. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat. Med.* 7: 1028–1034.