Fluorescent Cell Based Assays for High Throughput Analysis

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Abstract

Purpose. To characterize cell based conventional and time-resolved fluorescent (TRF) assays for high-throughput analysis. **Methods.** All cells were grown on 96-well filter plates and compared to solid polystyrene tissue culture plates. Calcein AM uptake was measured in cyclosporin or verapamil inhibited P-glycoprotein (Pgp) expressing MDCK and MES-SA/MX2 cells. Erythroid precursor cells derived from umbilical cord blood were used in a TRF-based immunoassay (europium-labeled specific antibodies) to measure cell differentiation and melphalan-induced cytotoxicity. **Results.** Intracellular fluorescence increased after drug treatment as measured by calcein AM uptake indicating inhibited Pgp activity. MDCK and MES-SA/MX2 cells showed a 1.4 and 4.5 fold increase in fluorescence, respectively, after treatment. Melphalan-induced erythroid cytotoxicity IC50 values of approximately 2µM were measured with both the TRF-based immunoassay and the traditional colony assay. **Conclusions.** A greater increase in calcein retention was observed in verapamil inhibited MES-SA/MX2 cells indicating a higher level of Pgp expression than that measured in MDCK cells. The TRF-based immunoassay provides a high-throughput alternative to the traditional colony assay for measuring melphalan-induced cytotoxicity. Not only can cells be cultured directly in the 96-well filter plate, but all the subsequent assay steps (such as media exchanges, drug treatment, washing, and reading) can also be performed in the same plate with a significant reduction in process time. These rapid and accurate results obtained in multiple assay systems meet the cell based fluorescent screening requirements for automated high-throughput.

Introduction

MultiScreen™ FL and PCF plates were developed to address the need for a low-fluorescent background, high signal to noise 96-well filter plate for cell-based assays. These plates provide a high throughput alternative to traditional polystyrene cell culture plates by allowing the user to grow the cells and do all steps associated with the assay (incubation, washing, reading) in a single plate. In addition, any adherent cell type which benefits from growth on a filter (epithelial, endothelial, etc.), can now be assayed under ideal growth conditions using the MultiScreen PCF.

Fluorescent assays were performed to demonstrate cell proliferation and/or the expression of the P-glycoprotein in a variety of suspension and adherent cell lines. The measurement of P-glycoprotein expression is valuable in the investigation of cell lines which exhibit a multi-drug resistance phenotype. The fluorescent assays demonstrated are examples of the advantages provided by growing the cells and performing all of the assay steps in a single filter plate. In addition, time-resolved fluorescence assays provide an appealing alternative to conventional fluorescence with higher signal to noise and ultimately, the improved sensitivity of the assay.

We also describe a new rapid-throughput cell-based assay of myelopoiesis that utilizes several novel technologies. The assay utilizes the MultiScreen FL in which the cells can be both cultured and assayed. Vacuum filtration for the wash steps prevents the loss of cells. To further decrease the high fluorescent background characteristic of cell-based assays and reduce the assay processing time, lineage-specific, lanthanide-conjugated antibodies are used to label the cells and binding is measured with time-resolved fluorescence. The assay can examine the differentiation of three major hematopoietic lineages, the myeloid, erythroid and megakaryocytic. Data generated by the CELISA™ (CEll-based Lanthanide-conjugated ImmunoSorbent Assay) assay correlate well with those generated by the low-throughput colony assay.

Materials and Methods

Materials: Millipore (Danvers, MA) MultiScreen plates used included the MultiScreen FL (order #S2EJ008L00) and the MultiScreen PCF (S2EJ016L00). Calcein AM, CyQuant and FluoReporter Blue were purchased from Molecular Probes (Eugene, OR). Cyclosporin A, verapamil, sodium fluorescein, melphalan and PBS were purchased from Sigma-Aldrich (St. Louis, MO). Hank's Balanced Salt Solution (HBSS) was purchased from Gibco/BRL (Gaithersburg, MD). DELFIA® enhancement solution, wash concentrate, Europium, Samarium and Terbium standard solutions, were purchased from EG&GWallac (Turku, Finland). K562 (chronic myelogenous leukemia cell line), MES-SA/MX2 (uterine sarcoma) and Madin Darby Canine Kidney (MDCK) cells were all cultured as recommended by the ATCC. MDCK cells were typically plated for 4 to 7 days prior to use in calcein AM uptake experiments. $CD36^+$ erythroid precursors were obtained from Poietics/BioWhittaker. Europium labeled antibodies to human glycophorin A and an isotype control were obtained by europium labeling immunoglobulins with reagents from EG&G Wallac/Akron (Akron, OH). Interleukin 3, stem cell factor, erythropoietin were purchased from R&D Systems (Minneapolis, MN). Methylcellulose was obtained from Stem Cell Technologies (Vancouver, BC).

Methods: Conventional and TRF fluorescence measurements were performed on the Wallac Victor2™ multilabel fluorescence plate reader in the top read mode. Calcein and CyQuant fluorescence were measured with l_{EX}:485nm, l_{EM}:535nm, CW-lamp energy at 10,000, normal emission aperture, with a 1 second measurement. FluoReporter Blue was measured at l_{EX}:355, l_{EM}:460nm and CW-lamp energy at 1,000. Europium TRF fluorescence was measured using l_{EX}:340nm, l_{EM}:615nm, with a 400 µsecond measurement after a 400 µsecond delay. Samarium TRF was performed using 340/640nm, a 50 µsecond delay and 100 µsecond measurement. Terbium TRF used 340/545nm and a 500 µsecond delay, 1400 µsecond reading.

Cell Proliferation and Calcein AM

CyQUANT and FluoReporter Blue Cell Proliferation Assays were performed as described by the manufacturer. Calcein AM uptake and fluorescence was determined as follows: cells were washed in sterile HBSS (by filtration for the MultiScreen FL plates, aspiration for the MultiScreen PCF or centrifugation for the plastic plate control) and 1µM calcein AM was added for 15 or 30 minutes at 37°C. The cells were then washed three times with cold, sterile HBSS and 100µl HBSS were added to each well before fluorescence determination.

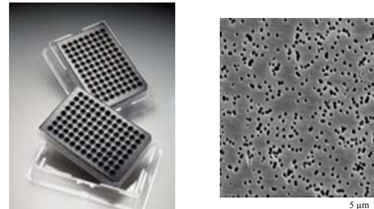
CELISA Assay

Cells (1,000/well $CD36^+$ precursors) were placed into wells of Millipore MultiScreen FL filter plates and cultured with lineage-specific growth factors for 5 days +/- experimental drug. After culture, culture supernatant was removed by vacuum filtration and each well was incubated with 200 µl europium-labeled antibody to glycophorin A (1 µg/ml) or an isotype control (1 µg/ml). After 60 minutes at room temperature, the wells were rinsed 3 times with Wash Buffer. All washes were done in a Millipore MultiScreen vacuum manifold with a vacuum pressure of 5" Hg. Delfia enhancement solution was added (100 µl/well) and, after 5 minutes, europium fluorescence was measured as described.

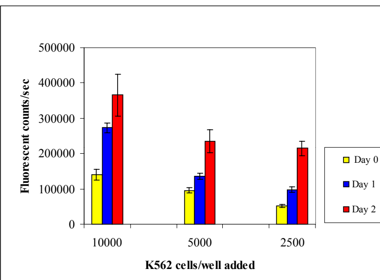
Colony Assay

Umbilical cord blood-derived $CD36^+$ precursors were seeded at 500/ml in methylcellulose with SCF, erythropoietin and IL-3 and assayed after 8 days culture at 37°C in 5% CO_2. Melphalan was added at day 0.

MultiScreen-FL Plates and 0.4µm FL Membrane

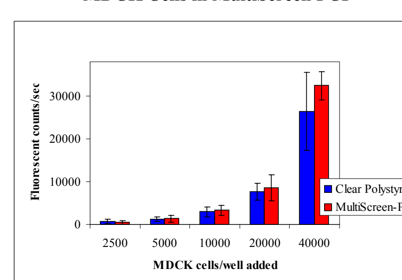


CyQUANT Cell Proliferation Assay on K562 Cells Grown in MultiScreen-FL



K562 cells were grown in MultiScreen FL plates for the incubation times indicated. Cell density was measured using the CyQUANT Cell Proliferation Assay as detailed in Methods. The results are an average of 6 wells per condition +/- the S.E.M.

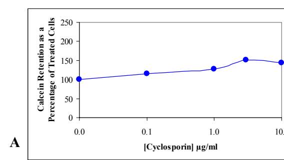
FluoReporter Blue Cell Proliferation Assay on MDCK Cells in MultiScreen PCF



MDCK cells were added to a MultiScreen PCF plate or clear polystyrene control plate at the concentration indicated and allowed to attach and proliferate for 3 days. The cell density was determined using the FluoReporter Blue assay as described in the Methods.

Calcein Fluorescence in Drug Treated MDCK and MES-SA/MX2 Cells

A



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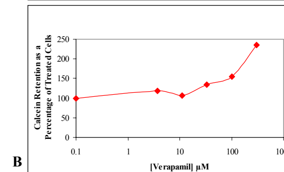
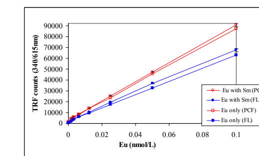
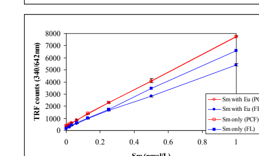


(A) MDCK cells were grown on MultiScreen PCF before addition of cyclosporin A at the indicated concentrations. (B) MES-SA/MX2 cells were added to MultiScreen FL then treated with verapamil at the indicated concentrations. Fluorescence was determined after the addition of calcein AM (1 µM). The increase in intracellular fluorescence shown is indicative of P-glycoprotein inhibition by cyclosporin A or verapamil. Tiberghien and Loor (1996) Anti Cancer Drugs 7:568-578.

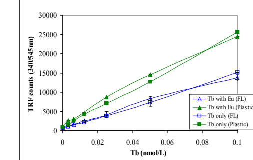
Europium and Samarium Standard Curves in MultiScreen FL and MultiScreen PCF





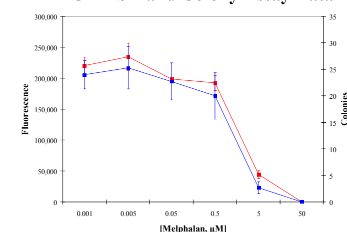
Europium and Samarium standard solutions were diluted to the concentrations indicated using Delfia Enhancement solution and added to either the MultiScreen FL or the MultiScreen PCF plates. The time-resolved fluorescence was determined at the appropriate wavelengths. As can be seen from the figure it is possible to read two different lanthanides in the same well with results similar to the lanthanide alone.

Terbium Standard Curves in MultiScreen FL and Polystyrene



Terbium standard solution was diluted to the concentrations indicated using Delfia Tb enhancer and added to either MultiScreen FL or clear polystyrene plates. The time-resolved fluorescence was determined at the appropriate wavelength. As can be seen from the figure it is possible to read Terbium in the presence of Europium with results similar to the lanthanide alone.

Melphalan Toxicity: Comparison of CELISA and Colony Assay Data



A comparison of the high-throughput CELISA assay and the colony assay utilizing $CD36^+$ precursors to screen for the toxicity of the chemotherapeutic Melphalan. Erythroid differentiation was followed by the expression of cell surface glycophorin A (the CELISA assay - red line) or the methylcellulose-based colony assay (blue line).

Summary and Conclusions

•Calcein AM concentrations were optimized (1-2 µM) and found to accurately measure > 5,000 cells/well. The measurement of calcein uptake after treatment with verapamil or cyclosporin A may be used to evaluate the presence of P-glycoprotein. Vacuum filtration allows these assays to be faster, more convenient and automatable.

•The new CELISA assay incorporates the features of europium-conjugated primary antibodies, time-resolved fluorescence and filter plate technology to dramatically increase the throughput of hematopoietic assays. The CELISA assay provides the ability to screen large libraries of compounds and/or recombinant proteins for either toxicity or drug discovery. Like the colony assay, the CELISA can determine drug effects on the myeloid, erythroid and megakaryocytic lineages.

•The MultiScreen-FL plate is compatible with Europium, Samarium and Terbium TRF detection with sensitivity comparable or better than polystyrene (1.5 pmol/L, 60 pmol/L and 12.5 pmol/L, respectively).

•The MultiScreen-PCF is the plate of choice for fluorescent applications which require cell adhesion and or monolayer formation. In addition, this plate is compatible with Europium and Samarium TRF detection with sensitivity of 3 pmol/L and 60 pmol/L, respectively.

•The MultiScreen-FL and PCF plates are compatible with both cell based and fluorescent assays which provide analytical tools specifically optimized for high-throughput screening and automation.

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