Cell Viability Fluorescent Assays in 96-Well Filter Plates

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Abstract

Ideally suited for high throughput applications, cell based fluorescent assays are typically less expensive and hazardous than radioactive assays and are versatile, sensitive, and quantitative. Common uses include detection of cell-cell adhesion, cell viability, proliferation, cell cycle determination, and apoptosis as well as detection of specific proteins/receptors. We have developed several 96-well filtration devices to our popular MultiScreen® line that are both compatible with fluorescence determinations and cell growth. The MultiScreen-PCF is used for adherent or suspension cell culture and conventional fluorescence applications. In contrast, the MultiScreen-FL is a superior plate for time-resolved fluorescence (TRF) applications.

In our present study, we determined the incorporation of a fluorescent probe precursor (calcein AM) by measuring intracellular fluorescence both in suspension and in adherent cell lines. Calcein AM is cleaved by intracellular esterases to form calcein, a pH-independent, cytosolic fluorescent marker. We have demonstrated rapid, sensitive, and quantitative measurement of cell associated fluorescence using this probe with a variety of cell types (e.g.: leukemia, hybridoma, sarcoma, and epithelial cell lines). We have also examined cell growth using the CyQuant Cell Proliferation Assay Kit (Molecular Probes). The MultiScreen-PCF and FL plates possess some highly desirable features for automated high-throughput cell-based fluorescent screening procedures. In addition to culturing the cells directly in the plate, all the subsequent assay steps (such as media exchanges, drug treatment, washing, and reading) can also be performed in the same MultiScreen plate with a significant reduction in process time.

Introduction

•Fluorescence detection techniques, conventional and especially time-resolved fluorescence (TRF), are frequently used as part of the Drug Discovery high throughput screening (HTS) process because of their sensitivity and automation compatibility. Most of these techniques have used isolated and purified cell components as reagents for the assay protocol. The use of intact living cells will provide more valuable and physiologically relevant information in both HTS and secondary lead analysis assays.

•The purpose of this report is to display some applications results on 96-well filter plates that are both cell growth and fluorescent assay compatible. A comparison of filter and plate type performance including background, cross-talk, and overall signal:noise (S:N) ratios are also shown. By examining the results on a variety of filter types and pore sizes, each specific assay can be optimally configured for the target cells, reporter, and detection technology selected. Conventional fluorescence was shown on a calcein AM incorporation test and with the CyQuant Cell Proliferation Assay in suspension and attachment dependent cell lines.

Materials and Methods

Materials: Millipore MultiScreen plates used included the MultiScreen-FL (Fluorescence Plate, MAFLS4010), 0.4μm PCF (S2ER061M7) or 3.0μm PCF (S2ER061M8) on clear polystyrene plates, and 0.4μm or 3.0μm PCF membrane on black polystyrene plates (available upon request). Calcein AM and CyQuant Cell Proliferation Assay Kit were purchased from Molecular Probes (Eugene, OR). Cyclosporin A, sodium fluorescein, 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 and Europium standard solution for time-resolved fluorescence (TRF) determinations were purchased from Wallac OY (Turku, Finland). K562 (chronic myelogenous leukemia cell line), HB124 (hybridoma), 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.

Methods: Calcein uptake and fluorescence were determined as follows: cells were washed in sterile HBSS (by filtration for the MultiScreen plates or by centrifugation for the plastic plates) and calcein AM was added at the indicated concentrations 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 using the EG&G Wallac Victor² 1420 multilabel fluorescence plate reader in the top read mode (λ_{EX}:485nm, λ_{EM}:535nm). All measurements for conventional fluorescence were performed on the Wallac Victor² plate reader with a 1 second measurement. TRF was performed on the same instrument using λ_{EX}:340nm, λ_{EM}:615nm with a 400 μsecond measurement after a 400 μsecond delay. Cross-talk values for conventional fluorescence were assigned based on the readings obtained in wells containing PBS adjacent to wells containing 200μl sodium fluorescein (typically, 50ng/ml). For TRF, measurement of fluorescence in wells containing only DELFIA® enhancement solution adjacent to wells containing 0.1 nmol/L Europium standard in DELFIA® enhancement solution were used to evaluate cross-talk. Signal-to-noise ratio values were calculated based on the readings obtained in wells containing 200μl signal (sodium fluorescein or Europium) divided by those obtained in wells containing the same volume of blank (PBS or DELFIA® enhancement solution, respectively). CyQuant Cell Proliferation Assays were performed as described by the manufacturer. Briefly, the cells were seeded on the FL plates and allowed to proliferate up to 2 days. At each time point, the plate was processed on the same day and fluorescence was measured using the EG&G Wallac Victor² 1420 as described above for conventional fluorescence.

Scanning Electron Micrographs (SEM) of 0.4μm FL Membranes
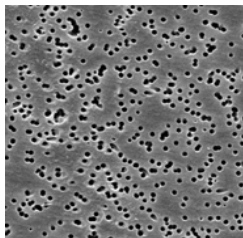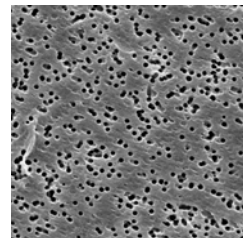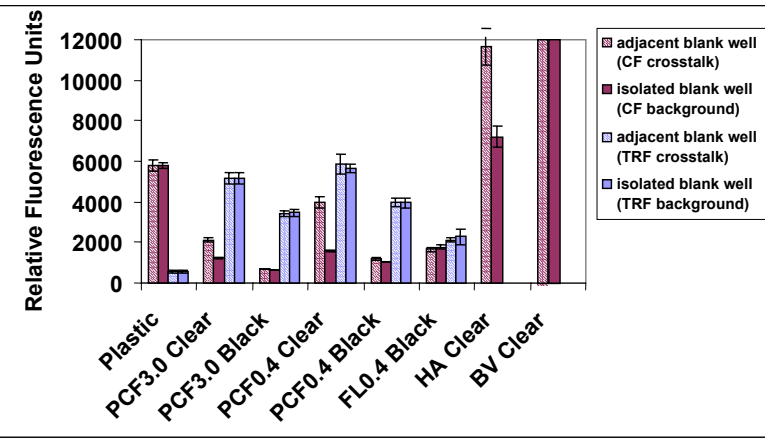

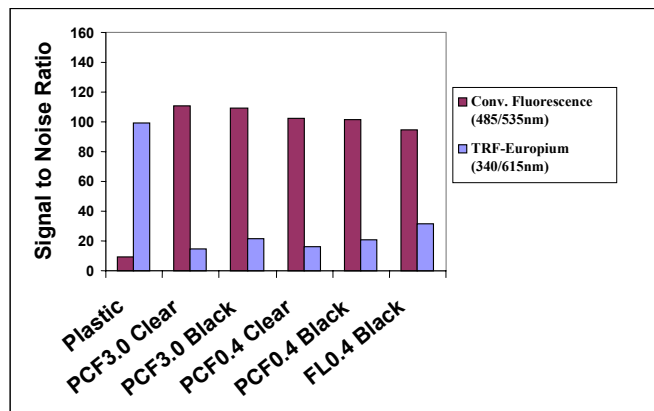Top   Bottom

MultiScreen-FL Plates

Background and Crosstalk Values for Conventional and Time-Resolved Fluorescence

Conventional fluorescence background and cross-talk values were determined using PBS as a blank solution and 5μg/ml sodium fluorescein for the signal. The average +/- SD for 8 wells is shown. Time-Resolved Fluorescence background and cross-talk values were determined using DELFIA enhancement solution and 0.1nmol/L Europium standard. The average +/- SD for 8 (crosstalk) or 16 (background) wells is shown

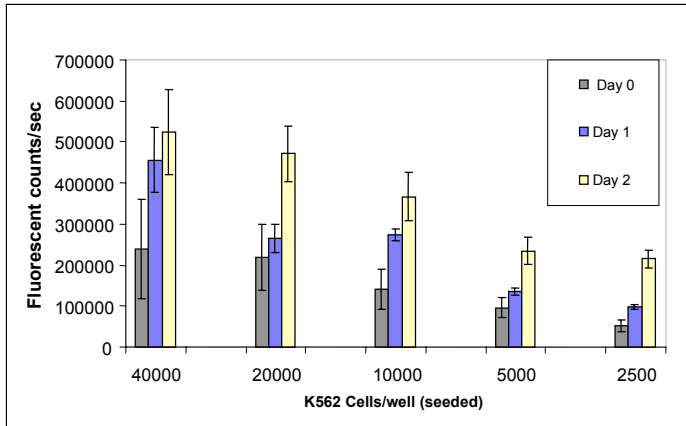## Signal to Noise Ratio for Conventional and Time-Resolved Fluorescence

The signal-to-noise ratio for several different 96-well plates was examined. The signal-to-noise ratio for each plate was calculated as described in the Materials and Methods.

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


K562 cells were grown in FL0.4 Black MultiScreen 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.

Optimal Calcein Concentration Determination
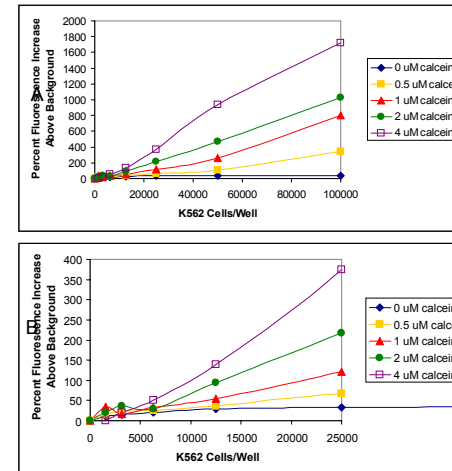
K562 cells were incubated in 0.4μm PCF black polystyrene 96-well plates at the indicated cell number. Calcein AM was added for 15 minutes at 37°C before extensive washing and the measurement of incorporated calcein fluorescence. The data in B is the same as A except the scale on the X-axis is expanded to demonstrate the sensitivity at each concentration. The results of two experiments with 6 replicates/experiment are shown.

## Time Course for Calcein Fluorescence in K562 Cells
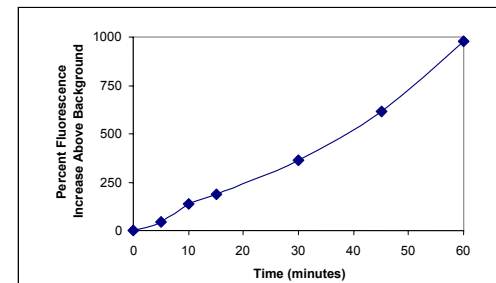
K562 cells (50,000 cells/well) were placed in a 3.0μm PCF clear polystyrene 96-well plate. The cells were incubated with 1μM calcein AM before washing extensively and the fluorescence measured. A representative experiment with 6 replicates per condition is shown.

Calcein Fluorescence in HB124 and MES-SA/MX2 Cells
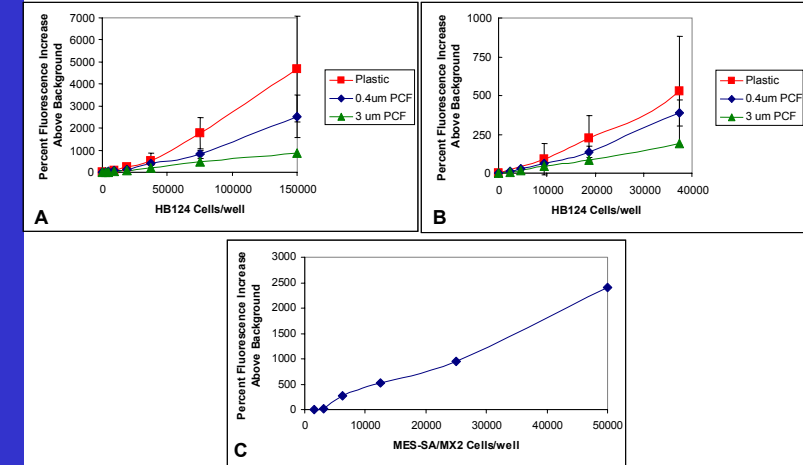
HB124 cells were placed in either plastic, 0.4μm PCF or 3.0μm PCF clear polystyrene 96-well plates at the indicated concentrations. MES-SA/MX2 cells were placed in 3.0μm PCF clear plates. The cells were incubated with 1μM calcein AM before washing extensively and the fluorescence measured. The results in A are identical to B except that the scale has been expanded to demonstrate the sensitivity at each concentration. The combined data from three separate HB124 experiments with 6 replicates per condition is shown. The combined data from two separate MES-SA/MX2 experiments with 6 replicates is shown.

Calcein Fluorescence in Cyclosporin Treated MDCK and Verapamil treated MES-SA/MX2 Cells
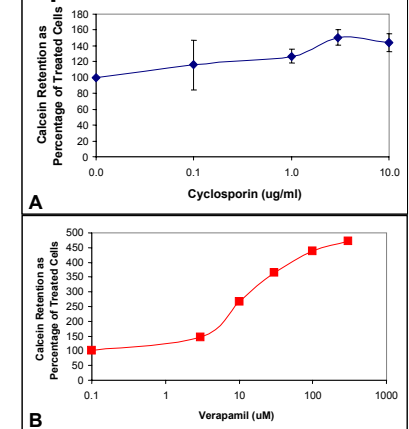
(A) MDCK cells were grown on 0.4μm PCF black polystyrene before addition of cyclosporin A at the indicated concentrations. (B) MES-SA/MX2 cells were added to 3.0 μm PCF clear plates then treated with verapamil at the indicated concentrations. Fluorescence was determined after the addition of calcein AM (1μM). The increase in intracellular fluorescence shown is indicative of P-glycoprotein inhibition by cyclosporin or verapamil. Tiberghien and Loor (1996) Anti-Cancer Drugs 7:568-578.

Summary and Conclusions

•Microporous filter & plate combinations were evaluated for performance in both Conventional Fluorescent (CF) and TRF detection:

– The MultiScreen-FL plates were found to have the best overall performance with respect to low background, no cross-talk, and S:N ratio.

– CF measurements demonstrated well-to-well cross-talk in most clear polystyrene plates and many filters showed unacceptably high backgrounds. The polycarbonate membrane (PCF) in the black polystyrene plates was found to be the best for this application.

– The MultiScreen-FL plate showed the best performance for Europium based TRF.

•Calcein AM concentrations were optimized (1-2 μM) and found to accurately measure > 5,000 cells/well. Time and concentration optimization is necessary for each cell type used. 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.

•Cell growth on the MultiScreen FL plate was measured with the CyQuant Cell Proliferation Assay.

•Since no single filter plate is optimal for all applications, a variety of 96-well filter plates are now available. The MultiScreen-FL and PCF plates are compatible with both cell based and fluorescent assays which provide analytical tools specifically optimized for HTS and automation.

•Thanks to Nancy Spicer and Brett Janosky for expert technical assistance.