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A rapid microwell fluorescence immunoassay for cellular protein detection

Carole Lavigne[1,#], Armèle de Guignée[2] and Alain R. Thierry[2,3]

[1]Beausejour Medical Research Institute, Moncton, New Brunswick, Canada

[2]Laboratoires des Défenses Antivirales et Antitumorales, UMR 5124, Université de Montpellier II, Montpellier, France

[3]Modélisation et Ingénierie des systèmes complexes biologiques pour le diagnostic, Sysdiag CNRS FRE3009, Montpellier, France.

[#]Corresponding Author: Carole Lavigne, Ph.D., National HIV and Retrovirology Laboratories, Public Health Agency of Canada, 100 Eglantine Drwy, Tunney's Pasture, Ottawa, Ontario, Canada, K1A 0K9. Phone: (613) 941 8857; Fax: (613) 952 6588; Email: carole_lavigne@phac-aspc.gc.ca

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ABSTRACT

In this paper, we describe a simple, rapid, specific, sensitive, and reliable method, the FICP method (Fluorescence Immunoassay for Cellular Protein detection) which is readily applicable to the detection of proteins directly on cells cultured in 96-well plates. In order to illustrate this method, we report on the detection of two different proteins, the cell cycle proteins cyclin D1 and $p21^{CIP1/WAF1}$, in untreated and 2-cyclopenten-1-one treated breast cancer cells. When the FICP method was compared with Western blot procedure, FICP was found to be superior for many characteristics. By using this method, we were able to quantify biological effects of a specific compound on protein levels in non-lysed cells and perform statistical analysis. Therefore, we believe this screening assay could be very useful for detecting poorly expressed proteins and for drug development.

INTRODUCTION

One of the main limitations to the development of drugs is the availability of a rapid, quantitative and sensitive assay for cellular protein detection (1). The presence of specific proteins in tissues or cells has been traditionally detected by biochemical methods such as Western immunoblotting or immunological methods such as ELISA and immunocytochemistry. Recently, proteomic technologies have created new possibilities in the discovery of novel drug targets and have been applied to study numerous protein interactions, to measure binding affinities, specificities, and levels of protein expression in various preparations, and in the investigation of various disorders (2-6). However, the use of protein microarrays to study protein abundances or protein-protein interactions in the complex cellular environment is still under development (7).

There are few high-throughput methods for the analysis of gene expression and function directly in mammalian cells and high-throughput technologies for quantitative cell biology are still at the developmental stage (8). Recently, some cell (9) and tissue microarrays (10) have emerged for the study of gene activity, protein expression, and cell-surface exploration and offer the potential to accelerate high-throughput functional genetic studies. A new focus in the cell microarray field has been the adaptation of cell arrays for RNAi in a high-throughput format (11, 12) and the development of a new protein microarray platform, Dissociable Antibody MicroArray (DAMA) staining allowing global analysis of protein expression and subcellular localization (13). Although cell microarray technology has many possible experimental uses, several technical limitations need to be addressed before its full potential can be fulfilled (8, 12, 14). The main obstacles are the creation of the DNA constructs and the production of diverse libraries of RNAi types from which slides are printed, and the efficiency of nucleic acid delivery in different cell lines and primary cells that are difficult to transfect. Also, the most pressing need in cell microarrays is continued improvement in the flexibility,

accuracy and speed of image analysis and in the tools for analyzing the resulting quantitative data.

In this study, we describe a fluorescence immunoassay for cellular protein detection (FICP) in microwell plates that can be used to perform drug screening rapidly, at a low cost, and with specificity for the targeted proteins. We have adapted standard techniques for immunofluorescence microscopy (15) to the use of a 96-well microplate for the screening of drug effects on a specific protein. The assay procedure allows rapid screening of proteins variation in many cell samples by measuring protein abundance/well.

In order to demonstrate the full potential of this method, we have used an experimental model based on cyclopentenone prostaglandins. Cyclopentenone prostaglandins (PGA1, PGA2, PGJ2 and their metabolites) are potent inhibitors of tumor cell proliferation *in vitro* and are able to suppress tumorigenicity *in vivo* by regulating the expression of a variety of stress-induced and cell cycle related genes (16). 2-Cyclopenten-1-one (cyclopentenone) causes cell cycle arrest in G1 by down-regulating cyclin D1 gene expression and cyclin dependent kinase 4 (CDK4) and inducing the CDK inhibitor $p21^{CIP1/WAF1}$ (17-19). Recently, MCF-7 cells treated with cyclopentenone were found to arrest not only in G1 but also at the G2 -M phase via inhibition of cyclin B1 expression and aurora-B kinase activity (20). We have found that cellular distribution of lipoplex DLS-siRNAs targeting cyclin D1 mRNA in MCF-7 cells varied with cyclopentenone or 17β-estradiol treatments (21). The amount of rhodamine-labeled siRNAs present in cells treated with cyclopentenone was lower when compared to untreated cells. In contrast, in cells treated with 17β-estradiol rhodamine fluorescence was more intense indicating that more siRNAs were present when compared to untreated MCF-7 cells. We believe that siRNAs following delivery by DLS might localize specifically to their mRNA targets in the cells and that the variations in the amount of siRNAs present in cells might reflect the effects of cyclopentenone or 17β-estradiol on cyclin D1 gene expression. In the present study, we used the microwell cell-based fluorescence immunoassay FICP to demonstrate at the protein level that cyclopentenone represses the expression of cyclin D1 and induces the expression of the CDK inhibitor $p21^{CIP1/WAF1}$ in MCF-7 cells.

MATERIALS AND METHODS

Cell culture

The human breast carcinoma cancer cell line MCF-7 was purchased from the American Type Culture Collection and plated in 25 cm^2 flasks. Cells were cultivated in Dulbecco's Modified Eagle Medium (Gibco-Invitrogen, Grand Island, N.Y.) supplemented with 2 mM L-glutamine (Sigma-Aldrich, St-Louis, MO) and gentamicin 50 ug/mL (Gibco- Invitrogen, Grand Island, NY) in the presence of 10% fetal bovine serum (BioMedia, Drummondville, QC, Canada). Cells at exponential growth were rinsed with PBS before being treated with trypsin-EDTA (Gibco-Invitrogen, Grand Island, N.Y.) and centrifuged for 10 minutes at 1100 r.p.m. The pellet was resuspended in complete culture medium and plated in black 96-well microplates with clear bottoms (Corning Costar, Fisher Scientific, USA) at a density of 10,000 cells per well. Cells were incubated 24 hours at 37°C in a 5% CO_2 atmosphere to allow them to adhere to the bottom of the well. Then, cells were treated with different doses of 2-cyclopenten-1-one diluted in sterile water (Sigma-Aldrich, St-Louis MO) for 4 hours.

Microwell fluorescence immunoassay

Cells were fixed directly in the 96-well microplate by using cold methanol. Culture medium was removed by pipetting and cells were washed gently twice with 100 µL Dulbecco's Phosphate Buffered Saline (PBS) with $MgCl_2$ and $CaCl_2$ pH 7.4 (Gibco-Invitrogen, Grand Island, NY) by pipetting carefully to do not disturb or damage the layer of cells. To fix the cells, a volume of 100 µL of -20°C methanol was added and the microplate was put on ice for 10 minutes. Cells were then washed twice with 100 µL of PBS at 22°C.

To permeabilize the fixed cells, a solution (100 µL/well) of 0.5% Triton X-100 (Sigma-Aldrich, St-Louis, MO) in PBS was added, and cells were incubated for 12 minutes on ice. After rinsing twice with PBS at 22°C, cells were incubated at 22°C for 5 minutes in the presence of 100 µL of a 22°C solution of 5% serum in PBS, and rinsed with 100 µL of a 22°C solution of 0.2% gelatin (Sigma-Aldrich, St-Louis, MO) in PBS for 5 minutes. These steps make possible prevention of random non-specific binding of antibodies and avoid false positive results.

A volume of 40 µL (4 µg/mL) of mouse monoclonal anti-

cyclin D1 antibody (1:50 in the blocking buffer PBS/Gelatin; clone A-12, Santa Cruz, CA) was added, and cells were incubated for 1 hour at 37°C. As negative controls, primary antibody was replaced with PBS. After incubation, cells were washed once with PBS, and incubated with PBS/Serum and PBS/Gelatin for 5 minutes each at 22°C. After incubation, a volume of 40 μL (8 μg/mL) of Goat FITC-conjugated anti-mouse IgG (1:50 in the blocking buffer PBS/Gelatin; sc-2010, Santa Cruz, CA) was added, and cells were incubated in the dark for 1 hour at 37°C. Cells were washed twice at room temperature with PBS (5 minutes each), and the fluorescence was measured at 485 nm excitation 520 nm emission wavelengths, using a FLUOstar OPTIMA microplate reader (BMG Labtechnologies, USA) according to the manufacturer's instructions.

Western blotting

MCF-7 cells were plated in culture flasks and allowed to grow until 80% of confluence. Cells were treated with different doses of 2-cyclopenten-1-one for 4 hours. Whole cell extracts were prepared by lysing cells in ice-cold RIPA (Radio-Immunoprecipitation Assay) buffer (Santa Cruz Biotechnology, CA) containing freshly added protease inhibitors provided with the RIPA lysis buffer kit for 30 minutes on ice. Cells were disrupted and homogenized by passing through 21 gauge needle. After centrifugation at 10,000g for 10 minutes at 4°C, the supernatant was collected and protein content determined using a Bradford assay (Bio-Rad, USA). Supernatants were kept frozen at -80°C until analysis. Extracted proteins (100 μg) were heated at 100°C for 4-5 minutes in 2X SDS gel loading buffer (100 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 200 mM dithiothreitol, and 0,2% bromophenol blue). Proteins were separated by SDS polyacrylamide gel electrophoresis on 10% separating gel and 5% stacking gel, and transferred on Immobilon-P PVDF membranes and immunoblotting of the cyclin D1 protein was carried out using the BM Chemiluminescence Blotting substrate (POD) kit. After overnight incubation in 50 mL solution 1%, membranes were incubated with diluted anti-cyclin D1 antibody (1:250 with blocking solution 0.5%) for 1 hour at 22°C. After incubation with antibody, membranes were washed three times for 15 minutes each time with 50 mL of Tris buffered saline (TBS: 50 mM Tris base, 150 mM NaCl, pH 7,6) containing 0.05% Tween (TBS-Tween), and incubated for 1 hour at 22°C with POD-conjugated anti-mouse antibody diluted 1:5000 in blocking solution 0.5%. After incubation, membranes were washed 4 times for 20 minutes each time in 50 mL of PBS-Tween 0.05%, and then incubated for 60 seconds with the pre-mixed detection reagent (20 μL/cm^2 of membrane). Autoradiography was performed using Kodak X-Omat 5000RA film. This experiment was repeated three times.

Cell proliferation

Cells were seeded in clear 96-well plates (Corning Costar, Fisher Scientific, USA) at a density of 10,000 cells/well. After 24 hours, the culture medium was replaced by fresh medium. 2-Cyclopenten-1-one was added to the cells in the presence of 10% fetal bovine serum at a final concentration varying from 150 to 1200 μg/mL and cells were incubated at 37°C for 4 hours in a 5% CO_2 atmosphere. In untreated control cells, cyclopentenone was replaced by an equal amount of sterile water. After incubation, cell number was evaluated using the MTT Cell Proliferation Assay (22). Briefly, cells were rinsed twice with PBS and 50 μL of fresh culture medium was added to each well, followed by the addition of 8 μL of a solution of 4 mg/mL MTT (Sigma-Aldrich, St-Louis, MO). After 2 h incubation, the purple formazan crystal was solubilized by adding 120 μL of lysis buffer (1 % HCl 12 N and 5% Triton X-100 in isopropanol), and the absorbance was measured at 570 nm on a SpectroMax Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). This experiment was repeated twice.

Statistics

Statistical analysis of differences between treatment groups was evaluated by Student's *t* test for unpaired observations using the Analysis Toolpak of Microsoft Excel. $P< 0.05$ was considered significant.

RESULTS

Amount of cyclin D1 varies upon cell density

The biological model used to test the FICP method was based on the detection of the cell cycle protein cyclin D1 in MCF-7 cells. Cells were cultured and plated as described in Materials and Methods by varying the cell density from 5000 to 20,000 cells per well. As shown in Fig. 1, the fluorescence signal significantly increased with the amount of cells ($p<0.01$ and 0.001). These results showed that the immunoassay is able to detect a variation in the amount of a given protein based only on the number of cells.

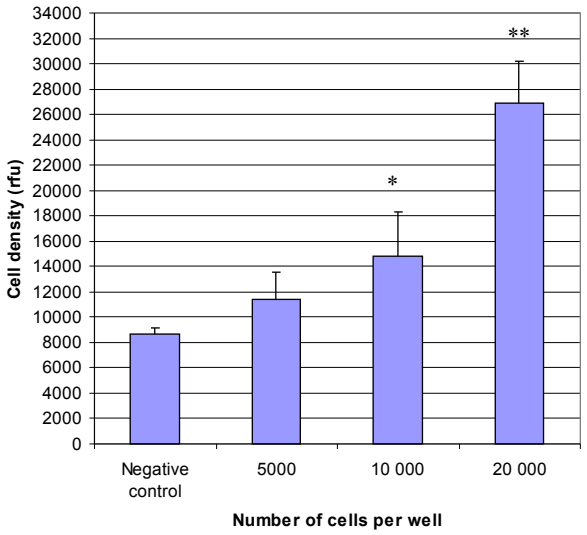

Fig. 1: Effect of cell density on the signal in immunofluorescence detection of cyclin D1 protein. MCF-7 cells were plated in a 96-well microplate at different cell densities as described in Materials and Methods. As negative control, the primary antibody was replaced by an equal amount of PBS. Results are representative of two separate experiments, each done in 12 replicates. Data shown represent the mean of 12 replicates ± SD from one experiment. * p< 0.01, ** p< 0.001.

Inhibition of cyclin D1 protein expression by cyclopentenone

2-Cyclopenten-1-one is known to cause cell cycle arrest in G1 and down-regulate cyclin D1 protein expression in MCF-7 cells (19, 23). To determine whether the fluorescence immunoassay FICP was able to specifically detect a variation in the amount of cyclin D1 protein we treated the cells with different concentrations of cyclopentenone for 4 hours. In these experiments, MCF-7 cells were plated in 96-well microplates at a density of 10,000 cells/well or in 75 cm^2 flasks at a concentration of 10 x 10^6 cells/flask. After 24 hours plating, the culture medium was replaced by fresh medium supplemented with cyclopentenone at a final concentration varying from 150 to 1200 µg/mL. After 4 hours incubation, cyclin D1 expression was measured by either the fluorescence immunoassay or Western Blot as described in Materials and Methods. The results are summarized in Fig. 2. The amount of cyclin D1 protein detected by the fluorescence signal inversely decreased with the concentration of cyclopentenone. At cyclopentenone concentrations higher than 150 µg/mL, the amount of cyclin D1 protein was significantly decreased compared to control cultures, with maximum inhibition at 600 µg/mL ($p<0.05$ for 300 µg/mL and $p<0.001$ for 600 and 1200 µg/mL, Fig. 2A). Results

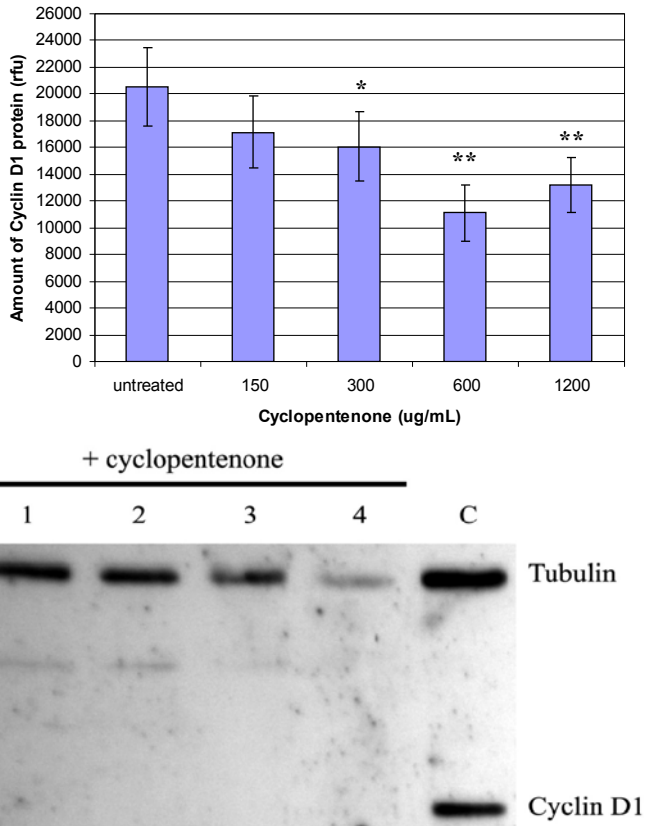

Fig. 2: Inhibition of cyclin D1 protein expression by cyclopentenone. MCF-7 cells exposed to cyclopentenone and untreated cells were processed after 4 hours of treatment. (A) Amount of cyclin D1 protein in each microwell was detected by immunofluorescence. Results are representative of four separate experiments, each done in 6 replicates. Data shown represent the mean of at least 5 replicates ± SD from one experiment. (B) CyclinD1 protein detection by Western blotting. Wells 1, 2, 3, and 4 represent cells treated with 150 µg/mL, 300 µg/mL, 600 µg/mL, or 1200µg/mL of cyclopentenone, respectively. Well C represents untreated control cultures. Results are representative of the amount of protein obtained in three separate experiments, and represent a single immunoblot. * p< 0.05, ** p< 0.001.

obtained by immunoblot analysis showed a diminution in expression of cyclin D1 protein in cells treated with cyclopentenone when compared to untreated control cells (Fig. 2B). Detection of cyclin D1 protein was clearly visible in untreated control cultures however no cyclin D1 bands were detected in cells treated with 150 µg/mL and higher concentrations of cyclopentenone. The intensity of the tubulin signal decreased at increasing concentrations of cyclopentenone probably due to the increasing cytotoxicity at doses higher than 300 µg/mL of cyclopentenone.

Cell proliferation inhibition

Since cyclopentenone causes an arrest in cell cycle and inhibits tumor cells proliferation (17-19) it was important

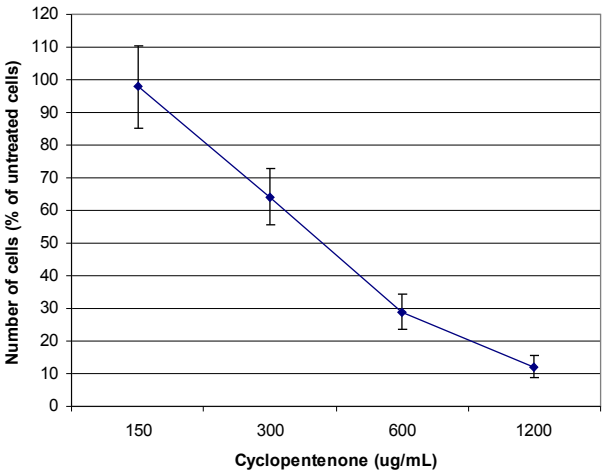

Fig. 3: Effect of cyclopentenone on MCF-7 cell proliferation. Cells were plated in 96- well plates at a density of 10,000 cells per well. Viable cells were measured using the MTT Cell Proliferation Assay 4 hours after treatment with increasing doses of cyclopentenone. Data shown represent mean of 12 replicates ± SD from two separate experiments, each done in 6 replicates.

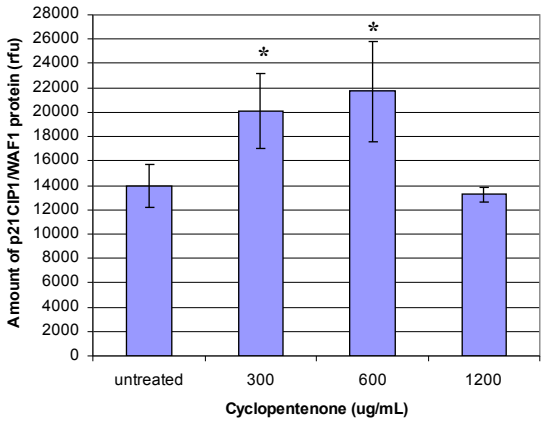

Fig. 4: Activation of $p21^{CIP1/WAF1}$ protein expression in MCF-7 cells by cyclopentenone. Untreated and treated cells were processed after 4 hours of treatment and amount of $p21^{CIP1/WAF1}$ protein in each microwell was detected by immunofluorescence. Results are representative of three separate experiments done in triplicate. Each bar represents the mean of at least 3 replicates ± SD from one experiment. * $p < 0.01$.

to assess whether the diminution in the production of cyclin D1 observed in the fluorescence immunoassay and the Western Blot method was correlated with an inhibition in the proliferation of the cells. As shown in Fig. 3, the number of cells decreased in a dose-response manner with increasing concentration of cyclopentenone in the culture medium.

Specificity of the fluorescence immunoassay

To evaluate the specificity of the FICP assay, we next evaluated the effect of cyclopentenone on the expression of the cyclin dependent kinase (CDK) $p21^{CIP1/WAF1}$ protein. This protein was chosen, as the treatment with cyclopentenone has resulted in an induction of its protein expression in tumor (17-19). Therefore, we repeated the experiment as described above, and investigated the amount of $p21^{CIP1/WAF1}$ protein in parallel with those of cyclin D1 protein. The induction of $p21^{CIP1/WAF1}$ and cyclin D1 proteins were evaluated in separate wells of the same microplate. Results showed that a significant increase ($p<0.01$) in the amount of $p21^{CIP1/WAF1}$ protein was observed in cells treated with 300 and 600 µg/mL of cyclopentenone compared to untreated control cells (Fig. 4). At higher doses, such as 1200 µg/mL, amount of p21 decreases apparently due to toxicity of the compound.

DISCUSSION

In this paper, we describe a method for detection of protein in cells plated in a 96-microwell that we called FICP method (Fluorescence Immunoassay for Cellular Protein detection), which has potential applications for drug screening. FICP is an improvement in drug screening techniques because it allows quantitative determination of biological effects of test compounds directly on targeted proteins in the cell environment. In addition, biological effects of a specific compound on protein levels can be calculated (% of inhibition) and statistics can be performed by using this method.

The FICP method was adapted from standard protocols for immunofluorescence microscopy which are used for detection of proteins from cells fixed on microscopic slides. The number of rinses was diminished from 3 to 2 at each step to decrease the risk of cell loss and shorten the duration of the assay. The major advantage provided by the FICP is the possibility of rapidly detect proteins in non-lysed cells plated in a 96-well microplate. The FICP method can be used for testing different compounds at different doses in a same plate by using small amounts of drug, leading to the cost effective investigation of very expensive drugs such as antisense oligonucleotides or small interfering RNAs.

Table 1: Comparison of FICP method with immunoblotting in detecting proteins.

	WESTERN BLOT	FICP
Time	2 days/12 samples	3 hours/90 samples
Nature of biological material	Extract from lysed cells	Fixed and permeabilized cells
Quantity of biological material	1 x 10^7 cells/sample	1 x 10^4 cells/sample
Sensitivity	25-100 µg	5-10 µg
Specificity	High	High
Reproducibility	Low	High
Variations between experiments	> 15%	< 15%
Statistics	Not available	Available

The immunofluorescence assay described in this study can be optimized to detect more than one protein at a time which allows studies of expression levels of two or more proteins in the same experimental conditions, saving time, precious test compounds and cells. Using FICP, we have clearly delineated cyclopentenone dual biological effect on cell cycle by quantifying inhibition of cyclin D1 expression and induction of p21 on a same microplate. Variation and amount of protein values obtained here are expected as previously described (19, 23). These results show that the fluorescence immunoassay FICP is able to specifically detect variations in expression of different proteins into cells treated with the same compound.

In Table 1, the FICP method is compared with Western blot procedure respective to sensitivity, reproducibility and specificity. It is obvious that FICP presents several advantages and is superior for many characteristics over the immunoblotting procedure. FICP is as specific as immunoblotting but is 5 to 10 times more sensitive and more reproducible than Western blot procedure, based on the amount of protein (Table 1). In this study, the FICP was able to detect a dose-response diminution of protein expression where immunoblotting failed to do it probably because of the limitation on the amount of total proteins that can be loaded on the electrophoretic gel or the loss of cyclin D1 protein during the cell washes in the Western blot procedure, due in part to cytotoxicity-induced membrane permeability. In FICP, cells are directly fixed after incubation with cyclopentenone allowing a more precise detection of protein levels. These results suggest that the FICP is more suited to detect variations in protein expression. In addition, by using FICP, protein detection can be achieved by using 1000 times less cells which may represent an important advantage when using, for instance, primary cells.

One limitation of the FICP method described here could be the difficulty to detect proteins expressed at very low levels with low-affinity antibodies due to the sensitivity of the multiwell fluorescence plate reader. Nevertheless, this limitation can be overcome by measuring signal by a fluorescence-labelled primary antibody, thus avoiding the use of a secondary antibody and cutting down as well cost and needed time period for this procedure. The use of fluorochromes such as Alexa Fluor series or cy3 CYBR gold that have been recently made available, may also improve the immunofluorescence assay method FICP described in this study and should be tested.

In conclusion, we have developed a microwell cell-based immunoassay that is a sensitive and accurate alternative to immunoblot analysis for rapid and inexpensive screening of large numbers of cell samples. FICP constitutes a screening assay with relative measurement of protein abundance/well. FICP has been developed in 96-well plates but could theoretically be adapted to 384-well plates and other formats, as well as robots use. The FICP method described here provides a rapid, simple, specific, sensitive, and reliable method for cellular protein detection and we believe this method could be very useful for detecting variations in protein expression and for drug development. More has to be done to determine reproducibility, variance, and linearity of the dynamics of the assay, to perform similar evaluation on other human cell lines and evaluate detection of more than two proteins per assay. Finally, this method also allows visualization of the targeted protein into cells, as classical immunofluorescence methods, by microscopic examinations of cells plated in the microwell plates.

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