

Preliminary examination of time-resolved fluorometry for protein array applications

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ABSTRACT: The advantages of time-resolved fluorometry over conventional fluorometric analysis are well known. However, time-resolved fluorescence has not as yet found wide applications in protein microarray or other multiparametric methods of analysis. Here we describe a general method which is suitable for multiparametric and microarray analysis, based on time-resolved fluorometry. A polystyrene surface is coated with different monoclonal antibodies, specific for certain analytes. The analyte mixtures are then universally biotinylated, using an active biotin ester. After removing excess biotin, the biotinylated samples are applied on the polystyrene surface, incubated and the excess is washed away. The bound moieties are then quantified by adding a universal detection reagent containing streptavidin, labelled with a fluorescent europium chelate. After washing and drying of the solid surface, the immobilized moieties are detected by using solid-phase, laser-excited time-resolved fluorometric analysis. In a preliminary examination of this principle, we have demonstrated that we can correctly identify upregulation of three secreted proteins, following stimulation of a breast carcinoma cell line with various steroids. Our method should be suitable for high-density microarray analysis of proteins, captured by specific monoclonal antibodies or other binding reagents. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: protein microarrays; time-resolved fluorescence; europium chelates; tumour markers; biotinylation

INTRODUCTION

Microarrays are miniature devices that offer the capability of performing thousands of assays simultaneously, in relatively small areas, and with relatively minimal reagent consumption (1–10). Microarrays have already found important applications in DNA sequencing and gene expression studies (4, 7, 11, 12). Commercial products and instrumentation are now available. On these microdevices, it is possible to immobilize either short oligonucleotides or longer DNA sequences. After target-probe hybridization, the signal is detected using either radioactivity or fluorescence. It is usually possible to immobilize many thousands of DNA molecules in relatively small areas, and, with newer techniques, the density can reach the level of whole genomes.

Despite the rapid improvements and progress on DNA microarrays, protein microarrays are much less widely used (13). Many years ago, Ekins proposed the use of multianalyte immunoassay, which is a form of a protein microarray technology (14, 15). Examples of multianalyte immunoassays have been widely published and

are based on either specific detection of multiple emission signals from fluors (e.g. various lanthanide chelates) (16–19) or fluorescence emitted from spatially distributed microspots (20). The procedure of screening expression cDNA libraries with antibodies is also a form of a protein microarray technology. Other possible protein microarray formats include solid-phase immobilization of large number of monospecific antibodies, which could capture specific proteins from complex mixtures in an addressable format. With the development of efficient methods for detection of such microspots with high sensitivity and specificity, protein microarrays could find important new applications in future studies.

The advantages of time-resolved fluorometry over conventional fluorescence have been widely discussed in the literature (16, 17, 21–23). In short, time-resolved fluorometry affords 100–1000 times more sensitivity due to background signal rejection from the application of pulsed excitation and time-gated detection. Reagents that can detect spatially separated fluorescent microspots have recently been developed (20, 24–28). In this paper, we describe a model application of time-resolved fluorescence for the multiparametric analysis of tissue culture supernatants which were universally labelled with biotin. On a solid phase polystyrene surface, we have immobilized four specific monoclonal antibodies, either directly or through a secondary antibody. We have then exposed these antibodies to tissue culture supernatants obtained from breast carcinoma cell lines, which have been

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stimulated with either alcohol (control) or with steroid hormones, which are known to induce specific gene expression, with protein secretion into the supernatants. The selected proteins were chosen because their mode of regulation by steroid hormones is known and monoclonal antibodies are available for their specific capture. Using this system, we were able to detect the expected stimulatory effect of various hormones on certain genes. These preliminary data suggest that the general principles used in this study may be applicable to high-density measurements in protein microarray formats.

MATERIALS AND METHODS

Cell line

The breast carcinoma cell line BT-474 was obtained from the American Type Culture Collection (Rockville, MD).

Steroid compounds

All steroids used were obtained from Sigma Chemical Co., St. Louis, MO. Stock solution (10^{-2} mol/L) were prepared in absolute ethanol. More dilute solutions were prepared in the same solvent.

Stimulation experiments

The BT-474 breast carcinoma cell line was cultured in phenol red-free RPMI medium (Gibco BRL, Gaithersburg, MD) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L) and fetal calf serum (10%), at 37°C, 5% CO₂, in plastic culture flasks. Once confluent, the cells were transferred into fresh plastic culture flasks, using the same medium but without any addition of fetal calf serum. Stimulation was carried out with 70% confluent cells and was initiated by adding various steroids dissolved in 100% ethanol. All steroids were used at 10^{-8} mol/L final concentration. A negative control (only alcohol added) was included in each experiment. Tissue culture supernatants were collected after 7 days (typically 50 mL per flask) and concentrated about 20-fold with Centricon[®] concentrators (cut-off 10 kDa; Amicon Corp.). This step would efficiently eliminate low molecular weight, amino-containing moieties, including amino acids.

The concentrated supernatants were biotinylated by mixing with an equal volume of 0.5 mol/L sodium bicarbonate solution (pH 9.1), adding into this solution 5 mg NHS-LC-LC-Biotin, then mixing and incubating at room temperature for 3 h (29) (Pierce Chemical Co., Rockford, IL). The biotinylated supernatants were then extensively dialysed in 5 L 0.1 mol/L sodium bicarbonate solution, with change of the solution at least three times to ensure complete removal of unreacted and hydrolyzed biotin.

Coating of microtitre plates

White, 96-well polystyrene microtitre plates were coated directly or indirectly with four monoclonal antibodies. Since the direct and indirect coating of these monoclonal antibodies gave similar results (data not shown), we will describe here only the direct coating of the microtitre wells with the monoclonal antibodies. For indirect coating, we have used a sheep anti-mouse immunoglobulin from Jackson ImmunoResearch (West Grove, PN).

The microtitre plates were coated with either a mouse monoclonal prostate-specific antigen antibody (coded 8301; Diagnostic Systems Laboratories, Webster, TX) or with a mouse monoclonal hK2 antibody (coded G586; supplied by Hybritech Inc., San Diego, CA), or with a pS2 mouse monoclonal antibody (catalog No. MS-111-PABX; obtained from Neomarkers, Union City, CA). A ferritin mouse monoclonal antibody (used as a negative control), was also obtained from Diagnostic Systems Laboratories.

All antibodies were coated at a concentration of 500 ng/100 µL per well in 0.1 mol/L Tris buffer (pH 7.8) overnight at room temperature.

Detection reagent

We have recently described the preparation of a poly-vinylamine-streptavidin complex multiply labelled with the europium chelate of 4,7-bis(chlorosulphophenyl)-1,10-phenanthroline-2,9-dicarboxylic-acid (BCPDA) (25, 30). This is a universal detection reagent which can be used in conjunction with biotinylated moieties. We have previously shown that this reagent is suitable for solid-phase time-resolved fluorometric analysis and demonstrated its suitability for immunoassay, immunohistochemistry and in microarray applications (31). The stock solution of this reagent is diluted 10-fold in a 60 g/L bovine serum albumin solution.

Assay procedure

The microtitre plate strips were washed six times with a 0.05% Tween-20 solution in 0.1 mol/L Tris buffer (pH 7.8). We then added to each well the biotinylated tissue culture supernatants from cells treated with either alcohol, oestradiol (oestrogen), dihydrotestosterone (DHT, androgen) or norgestrel (progestin). The supernatants were prediluted 100-fold in a 60 g/L bovine serum albumin solution and incubated with the antibodies for 30 min. The wells were then washed and reacted with the streptavidin detection reagent (diluted 10-fold) and incubated for 20 min. After washing, the wells were dried with a stream of cold air and the fluorescence on the dry solid surface was quantified using time-resolved fluorometry on a Cyberfluor 615 Time-Resolved Fluorometer (MDS Nordion, Kanata, Ontario, Canada).

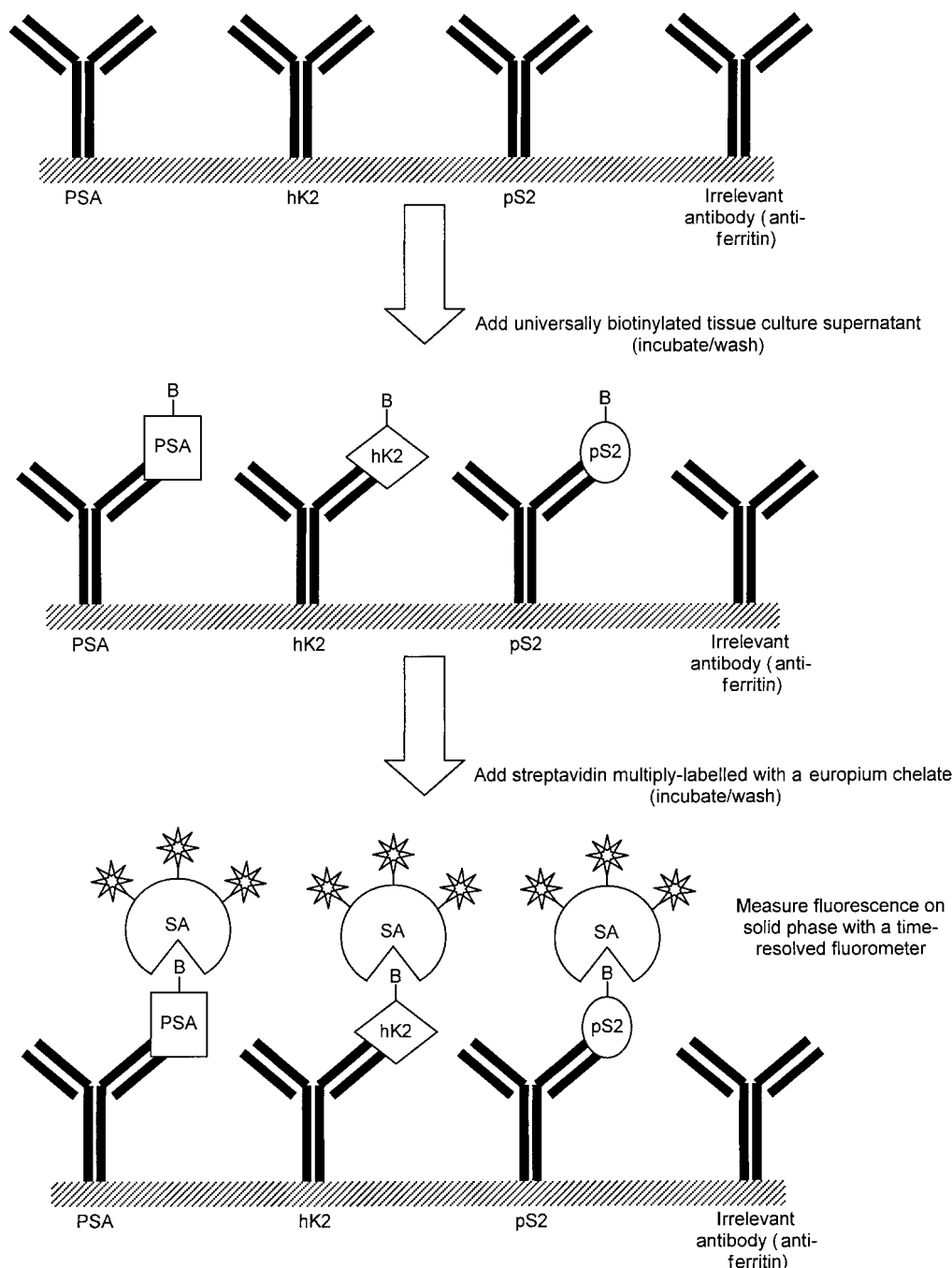


Figure 1. Principle of the experimental procedure. Monoclonal antibodies are immobilized on a polystyrene solid surface and then reacted with a mixture of biotinylated moieties. The bound antigens are then detected with a streptavidin-based universal detection reagent, which is labelled with a europium chelate. Fluorescence is quantified in a time-resolved mode directly on the dry solid surface.

RESULTS

Our experimental procedure is outlined in Fig. 1. An array of four monoclonal antibodies was used in these model experiments, to investigate whether it would be possible to identify the specific induction of PSA, hK2 or pS2 proteins by steroid hormones. It is already known

that PSA expression is upregulated by androgens and progestins but not by oestrogens, hK2 is upregulated by androgens and progestins but not oestrogens, and pS2 protein expression is upregulated by oestrogens but not by androgens or progestins (32). The ferritin antibody was used as a negative control (no regulation by steroid hormones) and alcohol was used to obtain background

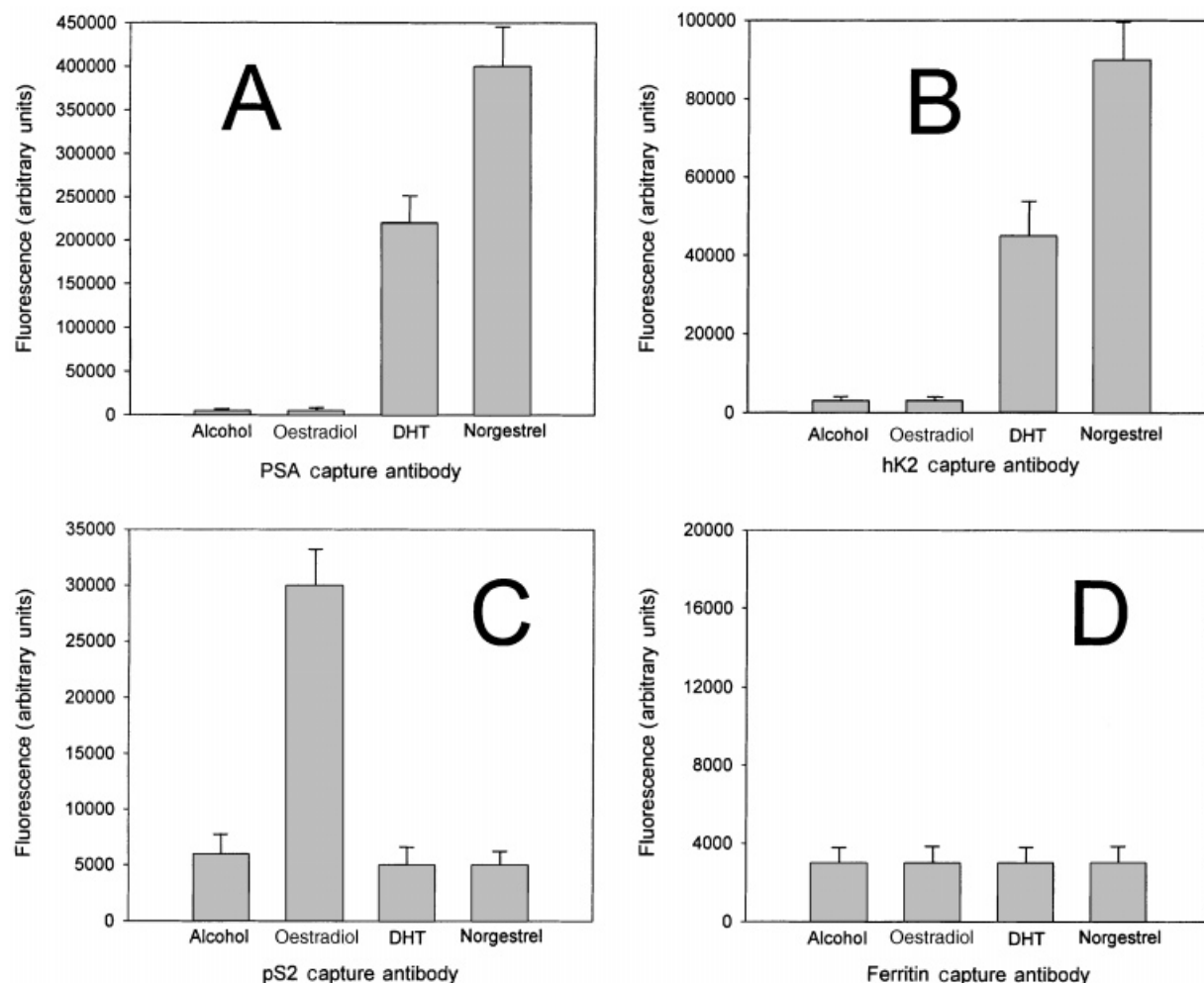


Figure 2. Fluorescence intensity obtained in areas of the microtitre plate coated with four different monoclonal antibodies. This method correctly identifies the induction of PSA production by androgens and progestins, of hK2 production by androgens and progestins, and of pS2 production by oestrogens. The ferritin antibody was used as a control (no induction by steroid hormones). Data represent mean \pm SD ($n = 4$).

expression levels of these proteins. All three proteins are secreted into the culture medium and can be biotinylated with the procedure outlined.

In Fig. 2, we present the data obtained. The monoclonal PSA coating antibody correctly identified PSA upregulation by androgens and progestins but not oestrogens. The same comments apply to the hK2 monoclonal antibody. On the other hand, the pS2 antibody correctly detected the upregulation of pS2 protein by oestrogens, but not by androgens or progestins. The ferritin antibody, as expected, did not detect any changes of these analytes-during the various stimulatory procedures.

DISCUSSION

The advantages of time-resolved fluorometry over conventional fluorescence are well known and have been

described in the literature (16, 17, 21–25). On the other hand, conventional fluorometry is widely used for both cDNA and protein microarrays (1–13). In this preliminary investigation, we have shown that the recently described streptavidin-based universal detection reagent, which is multiply labelled with a fluorescent europium chelate (25, 31), is suitable for detecting solid-phase Eu^{3+} fluorescence with high sensitivity and specificity. We have further shown that it is feasible to universally tag complex protein solutions (such as the tissue culture supernatants described here) with a label (biotin) and then detect various analytes of this mixture, by capturing with a specific monoclonal antibody on a solid phase. In this pilot experiment, we did not use minute volumes (e.g. nL) for technical reasons, but we have previously demonstrated that this technique is applicable at low volumes (31).

By using this experimental procedure, we have correctly identified gene upregulation induced by oestro-

gens, androgens or progestins, through detection of the secreted proteins which were previously biotinylated, using a universal labelling procedure (Fig. 2). Although we here show data for only three specific proteins, the method should be suitable for larger numbers of antigens, present in complex mixtures, provided that monoclonal antibodies or other specific binders, coated in specific areas of polystyrene or other solid phases, are available.

In conclusion, in this preliminary investigation, we demonstrate the applicability of solid-phase time-resolved fluorometric analysis in model multiparametric analysis which, when miniaturized, can form the basis of more complex protein microarrays. By using such methods, dense, parallel protein analysis in microspots may become routinely available.

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