Fluorescence Polarization Assays in Signal Transduction Discovery

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Abstract: Fluorescence polarization (FP) has become widely employed for high throughput screening used in pharmaceutical drug discovery. Assays of important signal transduction targets are now adapted to FP. In this review we examine assays for cyclic adenosine monophosphate, phosphodiesterases, and protein kinases and phosphatases using FP competitive immunoassays and a direct enzymatic method called IMAP.

INTRODUCTION

Signal transduction in cells affords several biomolecular targets of screening assays for drug discovery. Protein kinases, which phosphorylate protein or peptide substrates at hydroxy-bearing amino acid residues, are major players in the phosphorylation cascades central to intracellular signaling [1-3]. The human genome is estimated to include hundreds of distinct protein kinases, mostly serine/threonine-specific kinases (STKs) but also including numerous protein tyrosine kinases (PTKs). Kinases have become a major drug discovery target in the past decade [2,3].

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are important second messengers that are involved in the transduction of a variety of extracellular signals, especially the binding of hormones and neurotransmitters to their specific G-protein-coupled receptors [4]. Cellular cAMP and cGMP levels are regulated primarily by the G-protein systems that are positively or negatively coupled to adenylate or guanylate cyclase [5]. Phosphodiesterases (PDEs) provide additional control over cyclic nucleotide levels by catalyzing their hydrolysis to corresponding monophosphate forms. PDEs are of growing interest as drug targets for a broad range of therapeutic areas. Sildenafil citrate, also know as Viagra®, is the first PDE 5 inhibitor that has been commercialized as a drug. Several inhibitors to PDE 4 are in clinical trials as anti-inflammatory drugs for the treatment of diseases such as asthma, and other enzymes in the PDE family are also under study as potential drug targets [6].

Current HTS assays for assessing signal transduction elements include radioactive methods such as filter-binding methods [7], scintillation proximity assays (SPAs) [8,9], and immunoassays that may be based on enzyme-linked immunosorbent assay (ELISA) [10], or on homogeneous methods like homogeneous time-resolved fluorescence (HTRF) [11] and fluorescence polarization [12-15]. The antibody-based homogeneous assay systems have advantages of simplicity and ratiometric signal processing not requiring the use of radioisotopes or other specialized reagents. Commercially available anti-phosphotyrosine antibodies can be used in tyrosine kinase assays for HTS [12-14]. For the larger class of Ser/Thr kinases, however, antibodies are often of low affinity and/or specific for only one or a few kinase targets [15]. In the case of assays for cAMP, availability of sufficiently avid antibodies is not a problem and homogeneous methods based on FP have been described [16].

We recently described a new technology known as IMAP™ [17]. In a typical IMAP assay, a substrate containing a phosphorylation site is provided in fluorescent-labeled form. In phosphorylated form, this species undergoes binding to the surfaces of nanoparticles with immobilized trivalent metal complexes. This results in a change in the molecular mobility of the fluorophore, which can in turn be quantified as a change in fluorescence polarization. The potential applications of IMAP include assays for enzymes such as kinases, phosphatases, and phosphodiesterases , which are involved in reactions that have phosphate-containing substrates or products.

Since the IMAP binding reagent interacts directly with phosphate through metal-ligand coordinate covalent bonds, detection of kinases that phosphorylate Ser, Thr or Tyr residues is equally enabled. This generality of phosphate recognition overcomes a major limitation of kinase assays that employ phosphorylation-selective antibodies. Moreover, this generality allows the same reagent to be used for detecting AMP or GMP, the hydrolysis products generated by PDE action on corresponding cyclic nucleotides.

IMAP shares the advantages of FP assays especially in that there are no wash steps or laborious sample preparation. Inherently highly amenable to HTS applications, FP methods can be scaled up or down for either 96-, 384- or 1536-well plate formats [18].

Experimental

Materials

Unless otherwise specified, all assays were done in Costar^{TM} polystyrene 384-well black plates (Catalog #3710, Corning Inc., Corning, NY). Staurosporine, adenosine 5' triphosphate (ATP), 3-isobutyl-1-methylxanthine (IBMX), Krebs Ringer bicarbonate buffer, forskolin, isoproterenol, and its inhibitor propranolol were purchased from Sigma (St. Louis, MO). The MAPKAP K2 inhibitor (catalog # 385880), an 11-mer peptide, was from Calbiochem (San Diego, CA). Recombinant, bovine cGMP-specific phosphodiesterase (PDE 5) and its inhibitor zaprinast were

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from Calbiochem (San Diego, CA). One unit (U) of PDE 5 is defined as the amount of enzyme that will hydrolyze 1 pmol of cGMP per minute at 25 ºC, pH 7.2. Activator-deficient 3':5'-cyclic nucleotide phosphodiesterase (PDE 1) from bovine brain, its activator (calmodulin) and rolipram were from Sigma (St. Louis, MO). One unit of PDE 1 is defined as the amount of activated enzyme that will hydrolyze 1 μmol of cAMP per minute at 30º C, pH 7.5. PDE 1 was activated according to the instructions from the vendor prior to use in the assay.

The IMAP PDE assay kit for phosphodiesterase, including fluorescein-labeled cAMP and cGMP substrates, reaction buffer, IMAP binding reagent and IMAP binding buffer, was from Molecular Devices Corporation (Sunnyvale, CA)‡. The IMAP reaction buffer contains: 10 mM Tris-HCl, 10 mM $MgCl_2$, 0.1% BSA, 0.05% NaN_3, pH 7.2. The IMAP MAPKAPK2 kit, also from Molecular Devices, contains the same reagents as the PDE kit, except that the substrate is the peptide KKLNRTLSVA labeled on the N-terminus with 5-carboxyfluorescein [19]. In addition, the kit includes recombinant active human MAPKAP-K2 from Upstate Biotechnology (Lake Placid, NY). One unit of enzyme activity is defined as the amount of enzyme that will catalyze incorporation of 1 nmol of phosphate into substrate peptide per minute.

cAMP FP Assay

cAMP assays were performed using the cAMP HEFP kit from Molecular Devices. 20 μL of sample or standard was added to 10 μL of kit antibody, then 10 μL of kit tracer (fluorescein-labeled cAMP) was added. Incubations were carried out at room temperature for two hours and then detected on an Analyst™ multi-mode fluorescence reader (Molecular Devices) using the recommended instrument settings. For assay of cAMP responses in cells, we used T47D cells cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum and penicillin-streptomycin. One day prior to the experiment, T47D cells were sub-cultured in Packard tissue culture grade 96-well plates at a density of 25,000 cells/well in 100 μL of media. After incubation of the cells overnight at 37ºC, the media was gently removed and cells were washed with 250 μL of the Krebs-Ringer bicarbonate buffer. Cells were then incubated for 10 minutes at room temperature in 100 μl of the stimulation buffer (Krebs-Ringer bicarbonate buffer containing 0.75 mM IBMX, prepared fresh prior to use). Induction of cAMP was then initiated by adding 25 μl of 120 μM forskolin or 6 μM of isoproterenol and allowing the cells to incubate for 15 minutes. Twenty microliters of PBS was used as negative control. To characterize the specificity of isoproterenol action, 25 μL of 60 μM of propranolol, was added 15 minutes following the induction. At the end of the incubation, 50 μL of lysis buffer was added to disrupt the cells at room temperature for 10 min. Samples from three replicate wells were pooled and 20μL of the cell lysate was analyzed in the FP assay. Wells containing cell lysates with reaction buffer alone were used as background for FP calculation.

‡ Molecular Devices US Patent Pending

MAPKAP K2 Assay with MAPKAP K2 Substrate

The MAPKAP K2 assay was performed as described in the assay kit. In these experiments, 6 μM ATP was used per reaction. Serial three-fold dilutions of the enzyme (starting from 1.0 U/mL) were made in reaction buffer (no added DTT), mixed 1:1 with a solution of 12 μM ATP and 0.24 μM Fl-MAPKAP K2 substrate in reaction buffer, and 20 μL of this mixture transferred to quadruplicate wells of a 384-well plate. The reaction was allowed to run for 60 minutes. Then binding solution was added to each plate well, and after 30 minutes incubation FP was measured for each well.

MAPKAP K2 IC_{50} Determination with Inhibitors

Into wells of a 96-well plate polypropylene microplate we introduced 25 μL of serial three-fold dilutions of staurosporine, starting at a concentration that would produce 2.4 μM in the final 20 μL reaction. MAPKAP K2 was diluted in assay buffer to give the equivalent of 0.15 U/mL in the final 20 μL reaction. This dilution of MAPKAP K2 was added in 50 μL to separate sets of staurosporine dilution wells and the enzyme and inhibitor were incubated at RT for 10 minutes. Then a solution of Fl-MAPKAP K2 substrate and ATP was prepared such that when 25 μL of substrate / ATP was added to the 96-well plate, the final reaction concentrations were 6 μM ATP and 0.12 μM substrate. Twenty μL of each reaction was transferred to quadruplicate wells of a 384-well plate and allowed to incubate for 60 minutes. Then 60 μL binding solution was added to each well of the 384-well plate, and the FP measured after another 30 minutes of incubation. For the peptide inhibitor, the assay was done essentially the same as the staurosporine assay except four-fold serial dilutions of the 13mer peptide inhibitor (sequence KKKALNRQLGVAA) were made starting at 96 μM final reaction concentration.

Fluorescence Polarization Measurement

FP was measured on Analyst HT™ plate-readers (Molecular Devices) with the following filters: an excitation filter of 485 nm with a bandwidth of 20 nm, an emission filter of 530 nm with a bandwidth of 25 nm and a dichroic filter with a cutoff at 505 nm. An integration time of 100 ms was used and the Z height set at 3 mm. The excitation polarization was set at "static" and emission polarization was set at "dynamic".

The data was analyzed with Excel software (Microsoft, Redmond, WA) unless otherwise noted, and the graphical analyses were done with Prism software (GraphPad, San Diego, CA). The buffer only well intensities were subtracted from each raw data point, and these average background-subtracted parallel and perpendicular intensities were used to calculate each FP value in the usual manner and reported as mP or millipolarization units [20]. In each case the Z' factor or Z factor, dimensionless statistical characteristics widely used for HTS assays, were calculated to evaluate assay quality. The Z' factors were determined from the FP data according to the method of Zhang *et al.* [21].

RESULTS AND DISCUSSION

Determination of cAMP in cell lysates provides a measure of activation of many G-protein coupled receptors.

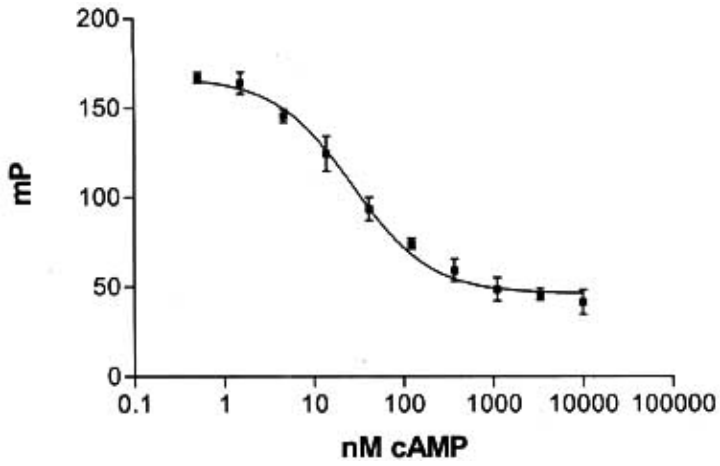

Fig. (1). cAMP calibration curve by FP. Standard concentrations of cAMP ranging from 10,000 to 0.51 nM were prepared in assay buffer. 20 µL of each cAMP standard was added to 10 µL of kit antibody, then 10 µL of kit tracer (fluorescein-labeled cAMP) was added. The average and standard deviation of three replicate samples is shown.

The calibration curve of Fig (**1**) shows that that the assay detects concentrations of cAMP below 5 nM, with a midrange (EC50) response of 30 nM. The total assay range, or ΔmP, is about 125 mP. When applied to assay of cell systems, the FP assay gives results that are consistent with expectations. As shown in Fig (**2**), spiking the cell lysate with 10 µM of unlabeled cAMP represents maximal displacement of the tracer, while lysates from untreated cells are used to generate the maximal binding of tracer to the antibody. Treating the cells with either forskolin or isoproterenol resulted in elevated cAMP level as indicated by the competitive displacement of tracer (i.e., lowered mP values). As expected, propranolol inhibits the agonist activity of isoproterenol, but not that of forskolin (not shown), which stimulates adenylate cyclase activity directly.

As mentioned, IMAP assays afford a new way to exploit the advantages of FP (ratiometric determination, homogeneous format) for certain assays of kinases, phosphatases and phosphodiesterases. The cAMP assay

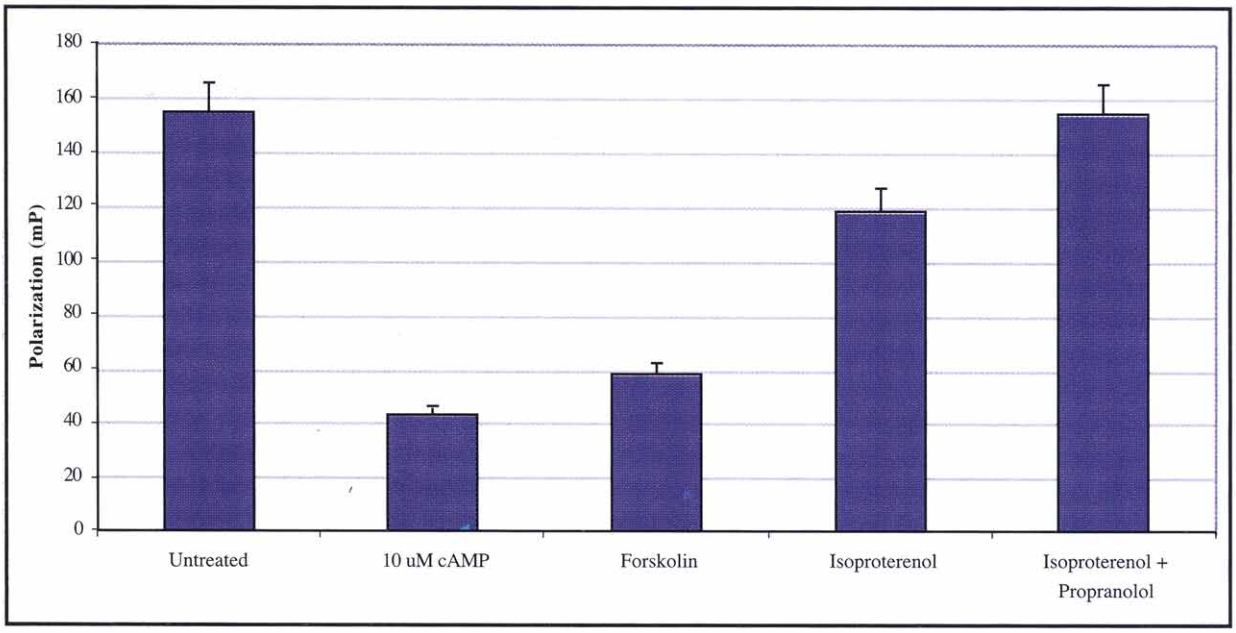

Fig. (2). Induction of cAMP production in adherent T47D cells by forskolin and isoproterenol. Cells (25,000 per well in a 96-well plate) were stimulated and lysed as described in the Experimental section. 20 µL aliquots of lysate were analyzed.

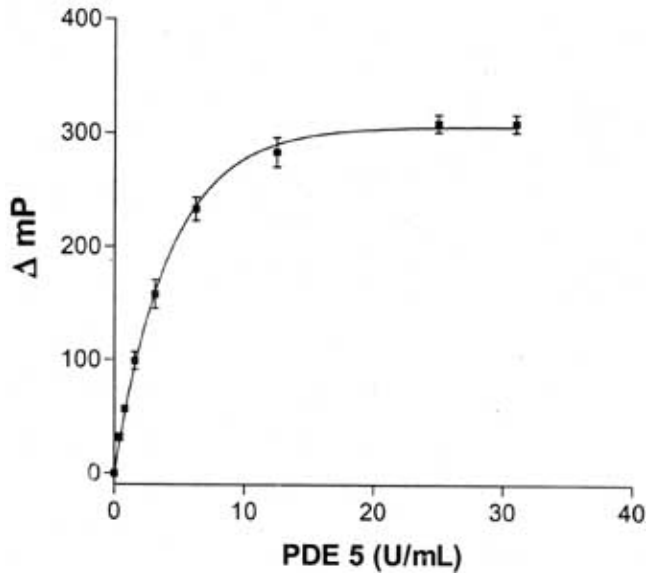

Fig. (3). Titration curve of PDE 5. Fluorescein-cGMP substrate concentration (20 nM) reacted with indicated concentration of PDE 5 in a 20μL reaction volume. Reaction time was 45 min., at which point the reaction was terminated and quantified by addition of 60 μL binding reagent. Error bars represent standard deviations of four replicates; ΔmP is the change from initial polarization value of 39 ± 3 mP. Curve is fit to a "1-phase exponential association" by PRISM 3.0.

mentioned above, like other assays based on competitive inhibition, uses concentrations of fluorescent tracer typically below 5 nM in order to maximize assay sensitivity. In contrast, IMAP assays use fluorescent substrates directly at 20-100 nM or higher concentrations, so that signal levels are very high relative to competitive assays. Two advantages derive from this high signal level. First, the precision of polarization is increased, since the standard deviation of the polarization measurement is roughly inversely proportional to the square root of the number of fluorescent counts [20]. Second, the resistance to spectral interferences of fluorescent and colored compounds is increased, because high counts will swamp out a greater percentage of fluorescent compounds, or will tolerate a great reduction in counts often seen when colored compounds absorb at the fluorescence wavelengths used in the assay.

IMAP PDE assays have been demonstrated for cAMP and cGMP-selective PDEs [17]. Fig. (**3**) shows a typical

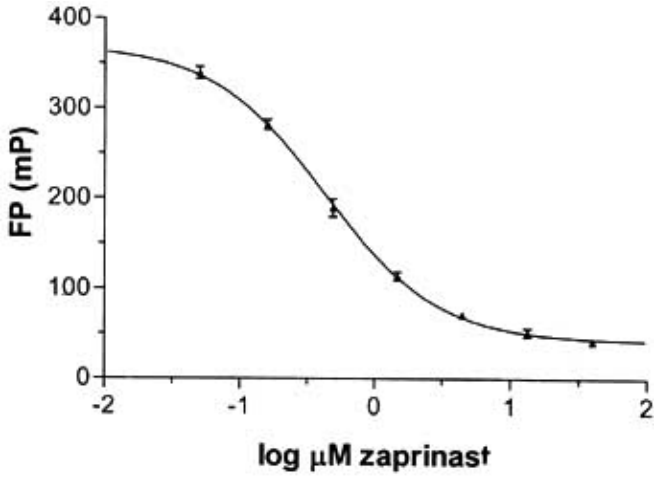

Fig. (4). IC_{50} curve of zaprinast for PDE 5. In the 20 μL reaction volume, the PDE 5 concentration was 10 Units/mL, and the substrate (fluorescein-cGMP) concentration was 20 nM. The reaction was incubated at room temperature for 45 min. and stopped as in Fig. (**3**)**.** The IC_{50} value determined was 0.43 μM. Error bars represent standard deviations of four replicates.

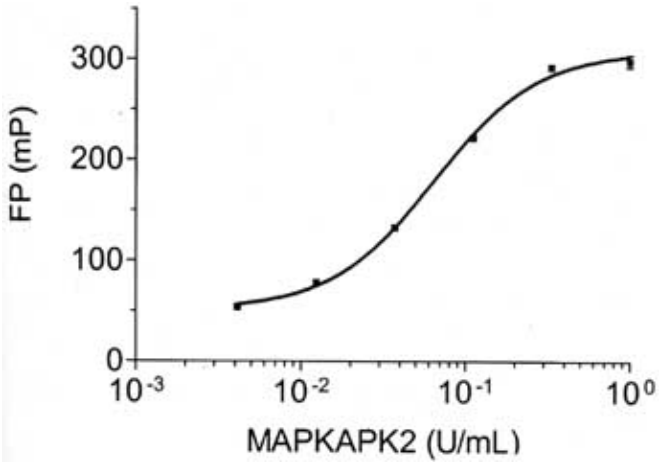

Fig. (5). IMAP FP assay for the Ser/Thr kinase MAPKAP K2. Twenty µL of kinase containing 6 µM ATP and 120 nM Fl-MAPKAP K2 substrate were reacted for 60 minutes as described in the Experimental section. The reaction was terminated and quantified by addition of 60 µL of the IMAP binding reagent. Error bars are standard deviations of four replicates.

titration curve obtained with PDE 5. The FP value increased from below 50 mP without PDE 5 to 350 mP at high PDE 5 concentrations, for a very robust ΔmP of greater than 300 mP. The enzyme titration curve is close to linear at concentrations up to about 10 U/mL. At this concentration of the enzyme, a known inhibitor of PDE 5, zaprinast, was used to validate the assay in an IC_{50} study (Fig. (**4**)). The IC50 value determined from an IMAP assay was 0.43 µM, close to that reported in the literature [22]. Thus the IMAP assay appears well-suited to screening for inhibitors. We have shown already that DMSO concentrations up to 5% in the enzyme reaction step have no effect on the assay results [17].

For the large and important class of kinase assays, IMAP offers many advantages. The IMAP MAPKAP K2 assay, one of 12 kits currently on the market, illustrates typical assay performance. The MAPKAP K2 activity titration curve is shown in Fig. (**5**). The Δ mP was 255 mP and the calculated EC_{50} for the kinase was 0.06 U/mL. At concentrations of 0.04 – 1.0 U/mL MAPKAP K2, the range of Z' factors in the assay was 0.79 – 0.89, indicating a very robust assay [21].

In Figure (**6**), two different inhibitors were titrated with a set concentration of 0.15 U/mL MAPKAP K2, corresponding to approximately 70-80% of the linear part of the enzyme titration curve. IC_{50} values for each inhibitor were then determined. The staurosporine titration is shown in Figure (**6A**), and the MAPKAP K2-specific inhibitor (Calbiochem) was used to generate the results shown in Figure (**6B**). The latter, a selective inhibitor of MAPKAP K2, consists of a peptide thirteen residues in length. Inhibition with respect to the substrate peptide (K_i = 8.1 µM) is competitive as determined by Hayess *et al.* [23]. We calculated the IC_{50} of this inhibitor to be 6.9 µM with the IMAP assay, which closely matches the value reported in the literature.

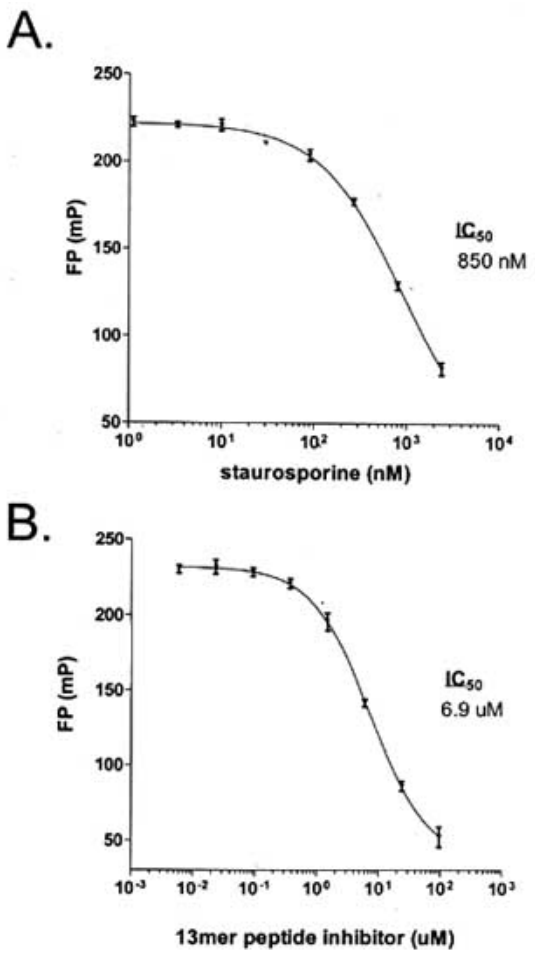

Fig. (6). IC_{50} determination for MAPKAP K2 with inhibitors staurosporine and a 13mer peptide, using the IMAP system. The enzyme concentration was 0.15 U/mL, with 0.12 µM Fl-MAPKAP K2 substrate and 6 µM ATP per 20 µL reaction. The reactions were incubated at RT for 60 min. The IC_{50} values were determined by preparing nonlinear regression curves with Prism software (Graph-Pad Software, San Diego, CA). A four-parameter logistic curve fit was utilized. Error bars represent the standard deviation of four replicates. (**A**) staurosporine inhibition (**B**) titration with a peptide that specifically inhibits MAPKAP K2 activity.

CONCLUSION

Fluorescence polarization assays are now established in the literature for high throughput screening. The "mix and read" convenience of FP, coupled with its ratiometric signal processing produces a high throughput, sensitive, and robust method for many pharmacologic targets like those presented here. The use of cAMP FP assays allows this important second messenger to be assayed in settings where previously only solid phase methods (ELISA-like) or radioactive assays were considered applicable. A recent report describes the use of FP assays of cAMP in receptor membranes, allowing the extension of cAMP screening to systems *in vitro* that can be run without synchronizing for cell culture [16].

IMAP provides a powerful alternative to HTRF, SPA, and other widely used HTS assays that require costly reagents, such as radiolabeled substrates and antibodies. The costs and complication of radioactive waste disposal are well known, as are the limitations of antibody-based assays, which can be difficult to validate and are limited in scope. The IMAP binding reagent, a sol of nanoparticles with immobilized metal (M^{III}) coordination complexes, offers a unique advantage over antibody-based assays: its high affinity enables direct measurement of phosphate. Unlike assays based on antibodies or other binding proteins, IMAP technology can be applied to a broad range of targets, including STKs, PTKs, protein phosphatases and PDEs. We and others have already successfully applied IMAP to over 25 different protein kinases and phosphatases in addition to the assays presented here [24].

IMAP technology does have some limitations. If the enzyme reaction (20 μL volume) requires ATP concentrations above 100 μM, a dilution may be necessary prior to adding the binding reagent. EDTA concentrations greater than 160 μM (or EGTA greater than 40 μM) in the final 80 μL assay (384-well plates) may interfere with phosphopeptide binding to the IMAP nanoparticles. However, EDTA is not needed for its customary role, as the binding reagent (pH 5.5) stops the kinase reaction by lowering the pH and by binding any remaining ATP.

The IC_{50} values obtained with the IMAP assay system for known inhibitors of PDE and MAPKAP-K2 are in agreement with reported values in the literature, and this is also the case for many other kinases not discussed here. The Z' factors calculated for each assay were always greater than 0.5, reflective of the low data variability obtained with the IMAP platform. The IMAP assay system is simple to use, adaptable to many different enzyme targets, and optimized for HTS, including 1536-well formats [17,24].

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