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Original Articles

Duplexed, Bead-Based Competitive Assay for Inhibitors of Protein Kinases

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Background: Many cellular signal transduction cascades have protein kinases as critical components. Small molecule protein kinase inhibitors can be effective as laboratory probes and drugs. Methods that allow two or more kinases to be evaluated simultaneously for inhibition by a small molecule would allow unequivocal tests of specificity and selectivity of action of the small molecule.
Methods: Two hexahistidine-tagged activin receptor-like kinases were expressed in *E. coli*, purified, and bound to nickel beads. A fluorescent kinase ligand (F-KL) that binds to the ATP-binding site of these kinases with nanomolar affinity was developed. Binding of F-KL with kinase on the bead made the beads bright, and inhibitors decreased the brightness.
Results: A test panel of 17 nonfluorescent kinase inhibitors, spanning two orders of magnitude affinity for the kinases, gave K_d values for the kinases that correlated well with a fluorescence polarization assay. Results were obtained for the kinases in duplex, using an autosampler to send beads from a 96-well plate to a flow cytometer in a format suitable for high throughput screening.
Conclusions: Inhibitors of kinases can be measured in duplex in a high throughput format by flow cytometry, if a suitable fluorescent ligand is available. © 2007 International Society for Analytical Cytology

Key terms: protein kinase; kinase inhibitor; high throughput; bead-based; ALK4; ALK5

Protein kinases, hereafter termed merely kinases, transfer the terminal phosphate of ATP to other proteins, which then display altered activity. This regulation of activity is one of the most widespread mechanisms by which mammalian cells regulate their responses to a changing environment. There are 518 human kinases, and every active kinase phosphorylates a different set of substrates (1). The first member of the transforming growth factor β (TGF-β) superfamily of ligands was discovered about 20 years ago. The plasma membrane receptors for these soluble ligands consist of Type I receptors (TβRIs, also known as activin receptor-like kinases, or ALKs) and Type II receptors (TβRIIs). Ligand binding induces TβRIIs to phosphorylate and activate TβRIs, or ALKs (2). Activated ALKs phosphorylate a subset of downstream signalling molecules. Specific small molecule inhibitors of ALK4 and ALK5 have been developed for analyzing these complex signal transduction pathways in normal and diseased states, and as lead compounds for pharmacological treatments (3–7).

Many types of assays for the determination of the activity of protein kinases have been developed. There are several different kinase assay formats in use for high throughput screening of small molecule compounds for drug discovery (8–10). Radiochemical methods are less preferable because of handling and disposal problems. Assay formats that do not need separation of signal from background (homogeneous assays) are preferred. High throughput screening (HTS) methods generally refer to those that can be developed to read 100,000 assays in 24 h. This level of throughput enables the testing of large

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collections of small molecules (>500,000 compounds) in a timely and cost-effective manner for drug discovery. Fluorescence polarization (FP) and fluorescence resonance energy transfer (FRET) are commonly used biophysical approaches for characterizing small molecule inhibitors. Fluorescence based approaches are readily amenable to miniaturization to a small assay volume ($\leq$10 μl) enabling HTS and reducing the need for large quantities of target protein and various detection reagents. FP and FRET have the further advantage of being solution assays where kinase activity and detection of phosphorylated substrate occur in solution phase. FP and FRET kinase assays often rely on antiphospho-antibodies for detection of phospho-substrate. The lack of high affinity anti-P-Ser/Thr antibodies limits the utility of FP and FRET assays for the characterization of this family of kinases.

Fluorescently tagged small molecules, developed to bind to kinases of interest, are useful tools in building new assays and for identifying and characterizing kinase inhibitors. Because of the common mechanism of action of kinases and high sequence homology within a family of kinases, identifying small molecule inhibitors specific to a given kinase requires testing molecules against large panels of kinases. To gain selectivity information within a given family requires the generation of multiple assays. Multiplexing approaches would significantly benefit kinase drug discovery. Multiplexing with FP or FRET would require multiple fluorescent ligands or fluorescent tagged antibodies, which can be difficult to generate. The bead-based system we describe here uses a fluorescently tagged ligand that binds to ALK4 and ALK5, and can be multiplexed so that two kinases can be probed in the same reaction. This allows direct comparison of kinases, for example using one kinase as an internal standard, or to ensure specificity of small molecule inhibitors of interest.

HyperCyt® (HC) is a plate-based flow cytometry system which can measure the fluorescence of particles from microplate wells at rates up to 100 samples/min (11). It is composed of an autosampler, tubing, and a peristaltic pump to move suspensions of cells or particles to a flow cytometer. Microliter sized samples are separated by air bubbles, such that the data from a plate comprises a single flow cytometry kinetic data file. It has been used for analysis of cellular fluorescence (12), and we use it here for analysis of microsphere fluorescence, based on the activity of small molecule inhibitors of ALK4 and ALK5 in a duplexed, high throughput mode.

MATERIALS AND METHODS

Materials and Protein Expression

All chemicals were obtained from Sigma–Aldrich (Saint Louis, MO), and were of analytical grade, unless otherwise mentioned. The fluorescent kinase ligand (F-KL) and the small molecule kinase inhibitors were from Biogenidec. BL21(DE3) bacteria expressing hexahistidine-tagged enhanced (for 488 nM excitation) green fluorescent protein (GFP) were obtained from J. Nolan. An overnight culture of these bacteria was diluted 100-fold into 20 ml fresh LB broth with kanamycin and grown at 37°C until an absorbance of 0.6 was obtained, then 0.25 mM IPTG was added and the culture was grown at 30°C for 4–5 h. The bacteria were centrifuged and resuspended in 2 ml of water, then aliquotted into 0.5 ml amounts and quick frozen in a −80°C freezer. A green aliquot was thawed and subjected to 10 pulses of 0.5 s from a Branson Sonifier 250 with a microtip set at 3 power, carefully, so as not to foam. The suspension was centrifuged at ~14,000*g* for 10 min, and the supernatant was applied to a 0.2 ml column of nickel agarose (Invitrogen, Carlsbad, CA) in 0.01% dodecyl maltoside, 30 mM HEPES hemisodium salt, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$ (buffer A). The column was washed with 0.5 ml buffer A, then the visible green was eluted by eye from the column using 40 mM imidazole in buffer A. The affinity-purified, colored eluate was concentrated 10-fold and diluted back to the original volume four times using a Microcon YM-30 membrane (Milipore, Billerica, MA) to reduce imidazole, and stored at 4°C in the dark in buffer A plus 0.02% sodium azide. Concentration was determined in a fluorimeter using carboxyfluorescein (fluorescein) as a standard, with 10-nm bandpass filters centered at 490 and 520 nm.

Synthesis of Nickel Beads

Briefly, the amino beads were epoxy-activated, a chelator containing an amino group was attached, and Ni^{2+} was added. Polystyrene beads (5.8 μm) with amino groups were obtained from Polysciences (Warrington, PA) as a suspension at 3×10^5 beads/μl. The aminobeads were resuspended in 200 μl of 0.01% dodecyl maltoside (DM) in 0.6 M NaOH, and subjected to bath sonication for 10 s. To this was added 200 μl of butanediyl diglycidyl ether (formerly named butanediol diglycidyl ether) and the suspension was mixed for 3 h at 40°C. The suspension was diluted to 1 ml with ethanol and centrifuged, the supernatant was removed, the beads were washed four times with 800 μl of 0.01% dodecyl maltoside, resuspended in 360 μl of 0.2 M Na_2CO_3, pH 11.3, and sonicated. Forty microliters of 250 mM bis(carboxymethyl)-L-lysine, titrated to pH 11, and 2 μl of 10% dodecyl maltoside were added, and the beads were mixed gently overnight at 40°C. The suspension was brought to 1 ml with DM, centrifuged, washed twice with 1 ml of DM, and resuspended in 400 μl of DM. Four microliters of 1 M $NiCl_2$ were added, the suspension was mixed for 5 min, and the beads were washed with 400 μl of DM six times. The nickel beads were resuspended and stored at 4°C in 400 μl of buffer A with 0.02% NaN_3 added, giving $\sim 8 \times 10^4$ beads/μl. These beads were characterized by their binding to 1–50 nM hexahistidine tagged GFP for 2 h at 4°C in buffer A with 10,000 beads in 10 μl; we obtained a B_{max} of 1.2 million H6-GFP per bead (data not shown), with a K_d of 6 nM, similar to the K_d value of 10–20 nM observed previously for different nickel chelate beads (13).

Texas Red Labeling of Nickel Beads

One hundred and twenty microliters of the nickel bead suspension was centrifuged, the supernatant was re-

moved, and the beads were resuspended in 200 μl of 50 mM sodium phosphate, pH 7.5, 0.01% dodecyl maltoside, then 2 μl of 10 mM Texas Red, N-hydroxysuccinimidyl ester, in dimethyl sulfoxide was added, and the suspension was mixed for 2 h at 22°C. The beads were centrifuged, the supernatant was removed, and the beads were washed once with 50% ethanol, twice with ethanol, once with 50% ethanol, and once with DM. The beads were resuspended in 120 μl of buffer A plus 0.02% NaN_3 and stored at 4°C.

Coating Nickel Beads with Hexahistidine-Tagged Proteins

Detailed effects of buffer composition and mixing technique were not investigated. Hexahistidine-tagged kinase (20 pmol) was gently mixed with 10^7 nickel beads in 40 μl of 0.01% dodecyl maltoside, 30 mM HEPES hemisodium salt, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, for 30 min at 4°C. The beads were centrifuged, the supernatant was removed, and the beads were resuspended in 200 μl of 0.1% Tween-20, 30 mM HEPES hemisodium salt, pH 7.5, 100 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 1% dimethyl sulfoxide (buffer B). These beads were routinely aliquotted and flash frozen by placing in a −80°C freezer, which resulted in about 10% loss of binding activity. The suspension was thawed and diluted 10-fold in buffer B, and 2 μl, or 10,000 coated beads, were used per assay. If all the kinase remained with the beads, this would give 0.02 pmol/10 μl in the assay, or 2 nM kinase in the assay. With 1.2 million Ni sites/bead and 10,000 beads/10 μl assay, using a K_d of 6 nM for the hexahistidine to Ni interaction, one expects 0.42 nM to be on the bead and 1.58 nM to be in solution. This can also be expressed as 0.02 pmol kinase/10^4 beads, which is 2 attomol/bead, or 1.2×10^6 kinase molecules/bead total; if distributed according to the K_d above, it would give 490,000 kinase molecules bound per bead.

Manual Kinase Binding Assay

The standard assay in 10 μl of buffer B consisted of 2 μl of kinase-coated beads (10^4 beads), 2 μl of 50 nM fluorescent ATP analog (F-KL), and 6 μl of buffer without or with an additional compound. The 10-μl assay was routinely carried out in a well of a 96-well plate with a V-shaped bottom (Corning Costar 2897; Corning Inc.; Corning, NY) at 4°C with moderate mixing using a vortex mixer for 2 h. The assays were diluted with 90 μl of buffer B just before determination of bead fluorescence by flow cytometry, using a FACScan cytometer (Becton-Dickinson, Franklin Lakes, NJ), counting 500 gated events. The residence time of the F-KL was long enough that no significant decrease in fluorescence was observed during the 5–10 s necessary to obtain 500 gated events. Bead fluorescence was collected using Cellquest software (Becton-Dickinson) as mean channel fluorescence (MCF), then was converted to mean equivalents of soluble fluorescein molecules (MESF) by reference to standard beads supplied by Bangs Laboratories (Fishers, IN). Data analysis was performed using Prism (Graphpad Software, San Diego, CA).

HyperCyt Automated Kinase Assay

Twenty-microliter assays were carried out as above, then 15 μl of each suspension was transferred to 96-well Imp@ct plates (Greiner Bio-one, Monroe, NC). (The wells of these plates are too shallow to keep the suspensions inside during the moderate mixing necessary to keep the beads from aggregating, but the bottoms of these plates are machined accurately enough to allow automated removal of microliter volumes.) The plate was then sampled, typically for 1 s or 500 beads, using an autosampler connected via plastic tubing and a peristaltic pump to a FACScan. The cytometer was controlled by a laptop computer, and collected the 96 clusters of beads in one kinetic file of CellQuest. This entire system is named HyperCyt (HC) (14). Software (IDLQuery) computed parameters such as mean channel fluorescence or median channel fluorescence for each of the clusters of beads (assay wells), and output them to typical analysis and graphing programs, such as Excel or Prism.

Fluorescence Polarization Assays

The reactions were carried out in 100 μl of buffer B in a ThermoLabsystems black 96-well microfluor 2 plate no. 7205 (Waltham, MA), using 0.5 and 1 nM F-KL and ALKs as shown in Figure 2, and 25 nM F-KL and 4.5 nM ALK4 or ALK5 otherwise. Competitor was added at various concentrations and incubated at room temperature for 60 min, then read on a fluorescent plate reader (LJL Analyst, Molecular Devices; now MDS; Toronto, Canada) with 10-nm bandpass filters set at 490 and 530 nm. Measurements are reported in milli-polarization (mP). K_d measurements were performed in triplicate and are representative of four independent measurements. Data analysis was performed using Prism with quadratic and hyperbolic fits. K_d values and fits were comparable by both methods and data from a hyperbolic fit is shown. IC_{50} measurements were performed as 10-point curves with 3× dilution series where each point was measured in duplicate. Each IC_{50} value is representative of three independent measurements. K_i values were obtained by Cheng–Prusoff conversion from the measured K_d for F-KL and a given ALK, and the IC_{50} value obtained for a given test inhibitor. Data analysis was performed by Prism with a four-parameter fit. All binding reactions were performed in a 96-well plate format, with control wells to define nonspecific binding containing 1 mM ATP.

RESULTS

Nickel Beads Bind Hexahistidine-Tagged GFP

We have previously reported the use of all-dextran nickel beads to couple hexahistidine-tagged proteins to the bead surface (15,16). We report here the use of polystyrene-amino-nickel beads (hereafter, nickel beads), which are smaller and sink more slowly than the all-dextran beads, and are compatible with automated liquid handling and, thus, determination of bead fluorescence. Proof of principle for the use of nickel beads is shown in Figure 1. Twenty thousand nickel beads were incubated

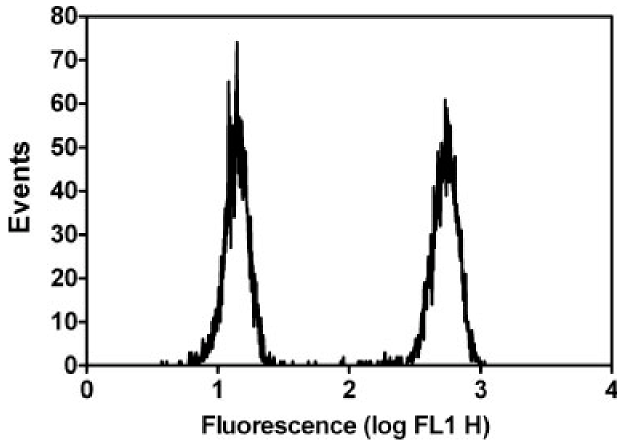
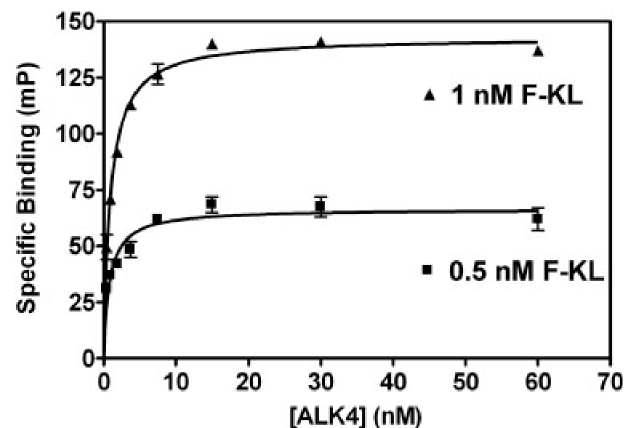


Fig. 1. Polystyrene-amino-nickel beads bind hexahistidine-tagged GFP. Ten thousand nickel beads were incubated in 10 μl of 10 nM hexahistidine-tagged GFP without and with 10 mM EDTA for 2 h in buffer A with moderate mixing, as described in the Materials and Methods section. Bead fluorescence was determined by diluting the beads in 90 μl of buffer A immediately before cytometry. The two populations are shown overlaid; the left population included 10 mM EDTA, and is a measure of nonspecific binding.

with 10 nM hexahistidine-tagged GFP without and with 10 mM EDTA for 2 h at 4°C (10 μl reaction) as described in the Materials and Methods section. The beads were then diluted with 90 μl of buffer immediately before cytometry to determine bead fluorescence. The decrease of fluorescence due to dilution was insignificant over the time necessary to obtain 3,000 events. The histograms of these two populations are shown overlaid in Figure 1. The two bead populations are narrowly distributed, and the nonspecific binding, in the presence of EDTA, was less than 3% of the total binding. Analysis of data according to a single site binding model showed that at least 1 million hexahistidine-tagged GFP molecules could bind to the beads with a K_d of ~6 nM (data not shown), similar to the 10–20 nM K_d observed by others (13).

F-KL Binds ALK4 and ALK5

F-KL is a fluorescent small molecule that binds to ALK4 and ALK5 with high affinity. Binding affinity was measured by fluorescence polarization (Fig. 2). K_d values were measured using various concentrations of F-KL and ALK4 (Fig. 2A) or ALK5 (Fig. 2B). Values obtained for ALK4 with 0.1, 0.25, 0.5, and 1.0 nM ligands were comparable; K_d = 1.2 ± 0.13 nM. K_d values for ALK5 with 0.1, 0.25, 0.5, and 1.0 nM ligand were comparable; K_d = 0.34 ± 0.01 nM. F-KL binds to both ALK4 and ALK5 with high affinity and appears to bind ALK5 with ~3× greater affinity. Affinity measurements were also made with manual cytometry in a duplexing mode (Fig. 3). Uncolored nickel beads were coated with ALK4, while red nickel beads were coated with ALK5, including a simple centrifugation and resuspension to eliminate the majority of unbound kinase, as described in the Materials and Methods section. The binding of F-KL to the kinases on these coated beads was determined by incubating 10^4 of each of these coated beads in a well with different concentrations of F-KL for 2 h at 4°C with moderate mixing, and determining the green fluorescence for each bead type after dilution by flow cytometry. Beads were incubated with F-KL alone to obtain total binding, and with F-KL and 10^{-3} M ATP to obtain nonspecific binding (Fig. 3A). The nonspecific binding is linear with concentration of F-KL, and is less than 15% of the total bound at the highest concentration. Specific binding is plotted in Figure 3B, from which the K_d of F-KL for ALK4 and ALK5 were determined. From three similar experiments, the K_d of F-KL for ALK4 was 10.8 nM, and the K_d of F-KL for ALK5 was 2.8 nM. F-KL binding to ALK4 and ALK5 by flow cytometry shows about one tenth the affinity shown by FP, and ~3× higher affinity for ALK5 compared to ALK4, as shown by FP.



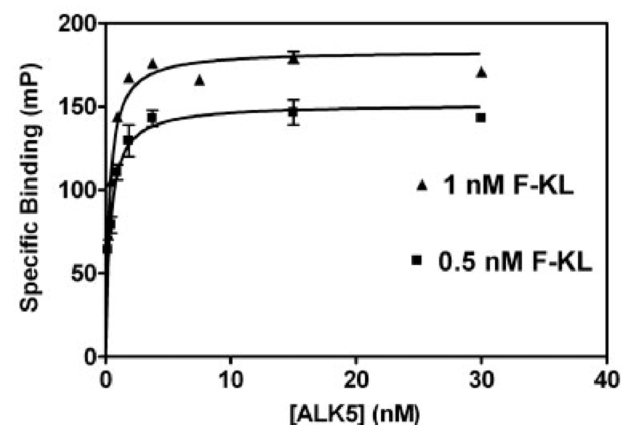


Fig. 2. Fluorescence polarization dissociation constant measurements. Multiple concentrations ALK4 (**A**) and ALK5 (**B**) protein were incubated with various concentrations of F-KL. Binding measurements and curve fits were performed, as described in the Materials and Methods section. Measurements were performed in triplicate, and each curve is representative of four independent measurements. Dissociation constants of 1.28 and 0.34 nM were obtained for ALK4 and ALK5, respectively.

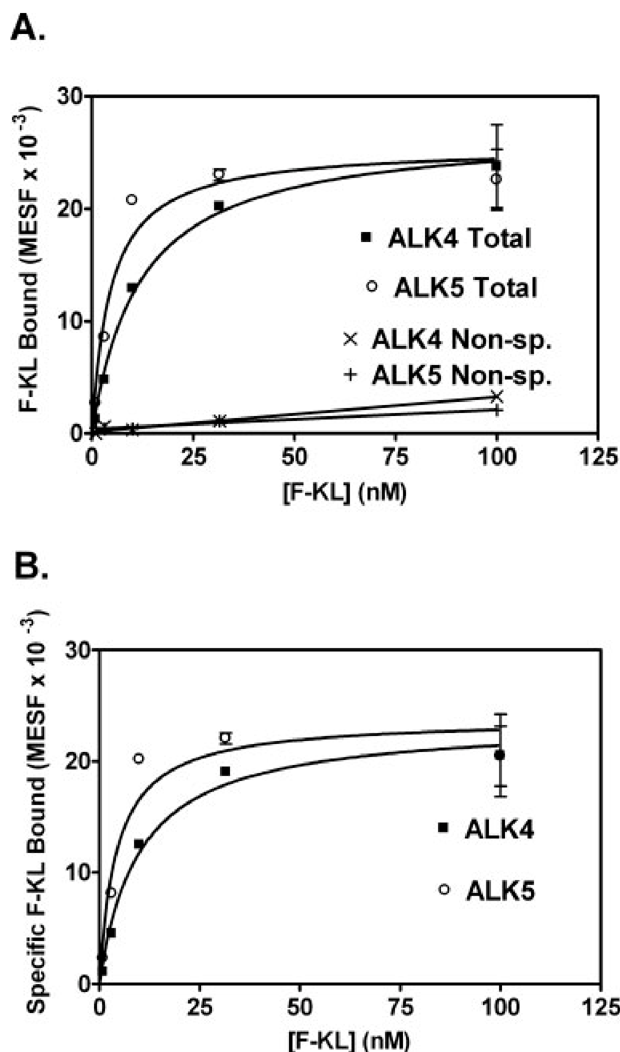


Fig. 3. Bead-based dissociation constant measurements. (**A**) ALK4-coated colorless beads and ALK5-coated red beads were used. Ten thousand beads of each type were placed together in a given well of a plate with the shown amount of F-KL; control wells included 1 mM ATP as a measure of nonspecific binding. The beads were mixed as described in the Materials and Methods section, then 90 μl of buffer was added immediately before determination of bead fluorescence by flow cytometry. (**B**) The specific binding was obtained and analyzed to give dissociation constants of 12.3 nM for ALK4 and 2.8 nM for ALK5 for this data set.

ATP Competes with F-KL for Binding to ALK4 and ALK5

HC and FP assays were used to measure displacement of F-KL by ATP (Fig. 4). Beads were coated with ALK4 and ALK5 as described in the Materials and Methods section. Both bead types were incubated with 10 nM F-KL and increasing amounts of ATP, as shown in Figure 4A. The K_i of ATP for ALK4 was determined to be 260 nM and the K_i of ATP for ALK5 was 93 nM, using the Cheng–Prusoff approximation and the K_d values obtained earlier (Fig. 3). These duplexed values were consistent with K_i values obtained similarly by FP measurements of competition by ATP with F-KL binding to ALK4 or ALK5, as shown in Figure 4B. K_i values obtained by FP were 126 and 51 nM for ALK4 and ALK5, respectively. ATP binds to ALK4 and ALK5 with comparable affinity, which is expected because there is 100% amino acid sequence homology between the ALK4 and ALK5 ATP-binding pockets. Differences in calculated K_i values between assays are within a factor of 3 and are likely due to assay variation than true affinity differences.

Automated Competition Curves

A panel of 17 small molecule ALK inhibitors was tested by HC and FP assays to (i) assess assay robustness and performance and (ii) evaluate results between the assay formats. Compounds were chosen based on potency values over 2 logs and chemical properties such as solubility and stability. Since HC assays were performed in duplex

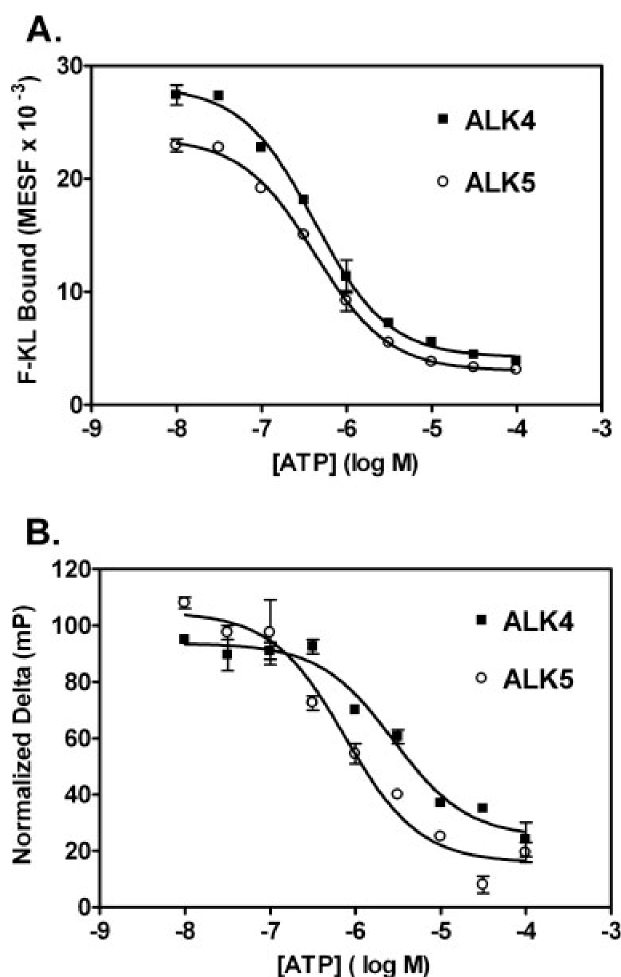


Fig. 4. F-KL and ATP compete for ALK4 and ALK5. ALK4- and ALK5-coated beads were incubated with 10 nM F-KL and various concentrations of ATP as shown, then analyzed by manual flow cytometry (**A**). Fluorescence polarization was used as described in the Materials and Methods section (**B**). Inhibition constant values of 260 and 93 nM for ALK4 and ALK5, respectively, were obtained by flow cytometry. Inhibition constant values of 126 and 51 nM for ALK4 and ALK5, respectively, were obtained by fluorescence polarization.

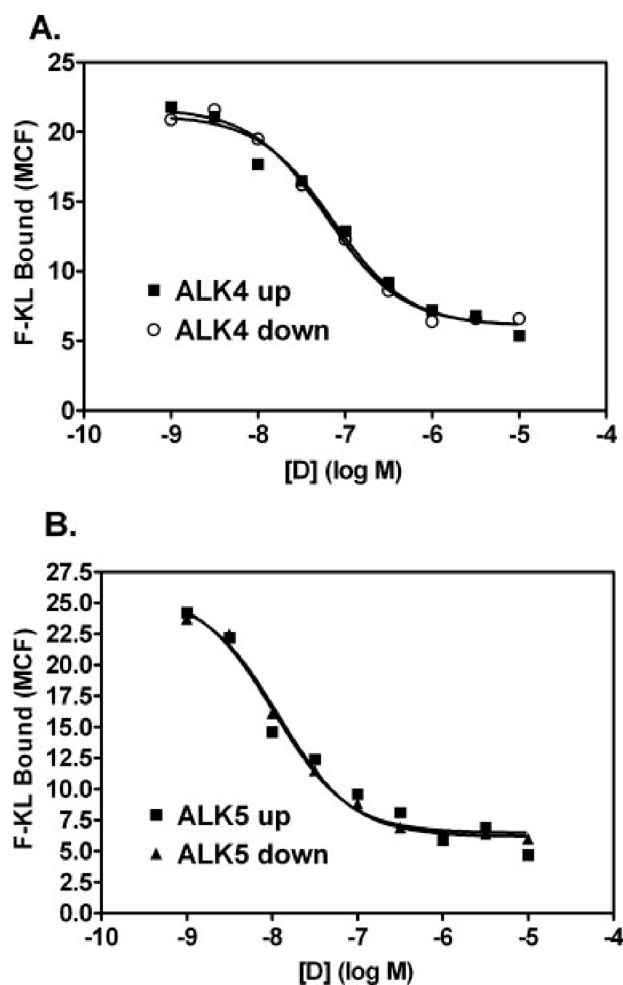


Fig. 5. HC competition curves and compound plating. Test compounds (n = 17) were plated in wells of a 96-well plate such that the concentration of inhibitor increased or decreased in successive wells. Both ALK4 (**A**) and ALK5 (**B**) coated beads were incubated in each well with various concentrations of compound D as shown. The plates were incubated and read as described in the Materials and Methods section.

mode, potential compound carryover issues between the wells of the 96-well plate were addressed. Compound plates were set up such that in a row of twelve wells, the first two wells contained no competitor, the next nine wells contained 10^{-9} M competitor to 10^{-5} M competitor (in alternate rows, from 10^{-5} to 10^{-9} M competitor) in one half log intervals, and the last well contained 10^{-4} M ATP as a positive control. Following incubation with 10 nM F-KL without or with competitor, as described in the Materials and Methods section, the bead fluorescence was determined as the uncalibrated mean channel fluorescence. An example of two rows of test compound in wells of a 96-well plate, with the concentration of the test compound D rising in one row and decreasing in the next row, is shown in Figure 5 for ALK4 (Fig. 5A) and ALK5 (Fig. 5B). Both sets of beads were in the same well for a given concentration of competitor. There is little evidence of carryover of F-KL between wells of the assay plate: the curves obtained with increasing compound concentration are nearly superimposable upon curves obtained with decreasing compound concentrations. K_i values of 37 and 2.5 nM for ALK4 and ALK5, respectively, were obtained by both dilution plating methods, indicating that HC assays are sufficiently robust to proceed to compound testing.

Comparison of Assays for ALK4 and ALK5

K_i values generated by solution phase FP and bead-based HC are shown in Table 1. The $\log_{10}$ of each K_d (in nM) for the compound in each assay was calculated and the log values for one assay result versus the other assay result were compared. The agreement between the assay results was assessed by a correlation plot. K_i values obtained by duplexed HC against ALK4 and ALK5 was plotted against FP results (Fig. 6). The correlations were good, as quantified by r^2 = 0.81 and 0.66 for ALK4 and ALK5, respectively. The data do not fall along the line of equivalence, as evidenced by the shift in potency values obtained by FP to the left. Nonetheless, a positive cor-

Table 1
Summary of K_i Values for Test Inhibitor Panel Against ALK4 and ALK5 by FP and HC

Compound ID	ALK4 FP (in nM)	ALK4 HC (in nM)	ALK5 FP (in nM)	ALK5 HC (in nM)
A	2.6	17	1.5	4.6
B	6.7	15	4.2	2.7
C	4.9	31	5.1	11.7
D	1.2	37	0.4	2.5
E	3.1	34	0.8	4.8
F	0.4	11	0.2	2.8
G	1.4	20	0.7	9.3
H	0.6	21.5	0.2	1.4
I	1	5.9	0.7	1.5
J	30.1	176	10.3	41
L	4	33	1.2	0.7
M	0.2	4	0.06	1.3
N	0.2	3	0.13	0.8
O	6.9	98	1.9	4.2
P	2.8	48	0.5	3.2
Q	19.5	134	1.9	7.2
R	21.5	100	1.8	6.1

The different ligand displacement binding assays generated K_i values for ALK4 and ALK5: one used fluorescence polarization (FP); the other used bead-based HyperCyt® (HC) in duplexed mode.

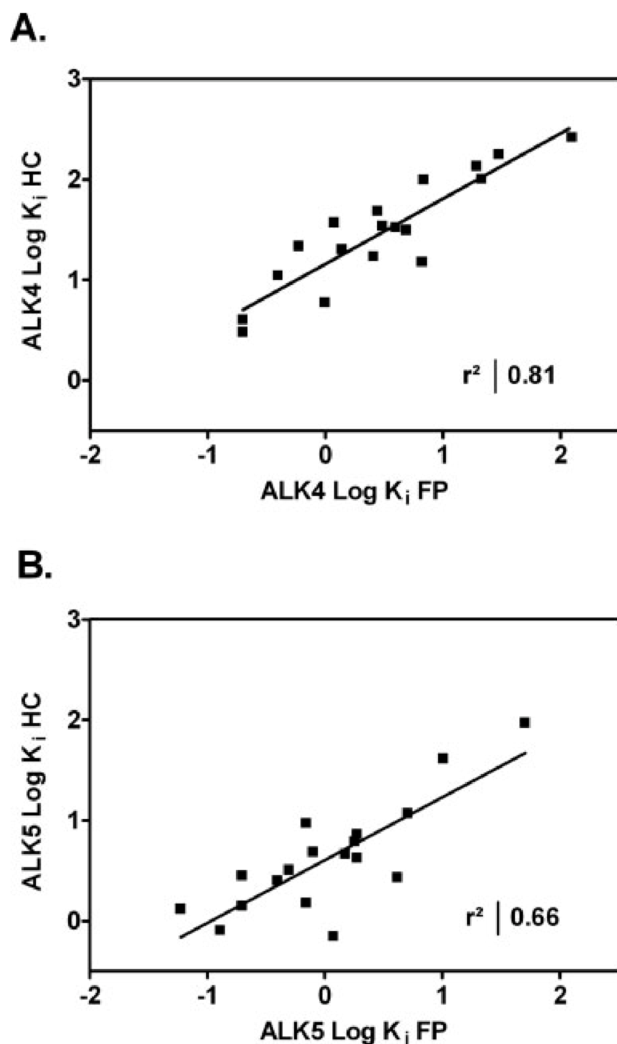


FIG. 6. Correlation plots of inhibition constants measured by FP and HC assays. Correlation of inhibition constants for a panel of small molecule inhibitors ($n = 17$) by FP and HC (duplexed) against ALK4 and ALK5; $r^2 = 0.81$ and 0.66, respectively. The units are in log K_i, with K_i expressed as micromolar, for ALK4 (A) and ALK5 (B).

relation is observed between the two assay results. The correlation plots suggest that the potency measurements by either HC or fluorescence polarization are predictive of the relative affinity of a given test compound. Thus, the duplexed HC values are comparable in their measurement of a compound's activity against ALK4 and ALK5 as individual measurements of activity in the fluorescence polarization assays.

DISCUSSION

We have validated a new duplexed HTS approach for determining the binding characteristics of compounds to protein kinases. The system consists of (i) beads with an epitope-binding moiety; (ii) epitope-tagged protein kinases of interest; (iii) a fluorescently tagged ligand with high affinity for the selected kinases; (iv) an autosampler that delivers bead suspensions from a multiwell plate to a cytometer; (v) cytometer; and (vi) software to collect the data from a 96-well plate in a single file. We have validated the bead-based assay format by comparison with potency measurements from a fluorescence polarization assay, a well established approach for measuring binding affinity of protein:ligand interactions and of kinase inhibitors. The HC system results (potency values) were comparable to fluorescence polarization results. The data were generated in a reaction volume of 10 μl, indicating that the present system is suitable for high throughput screening of small molecule inhibitors in a duplexed mode. A significant advantage of the HC system is the ability to multiplex. We show data corresponding to the duplexing of two kinases with a single fluorescently tagged ligand that binds to both kinases with high affinity. Because many kinase inhibitors bind multiple kinases, it is conceivable that a single fluorescent ligand will have high affinity against multiple kinases, and HC can be used to generate quantitative binding data against a panel of kinases in a HTS, multiplexed mode.

During assay development of the HC system, several steps were optimized prior to achieving the final duplexed, HTS assay. Detergents are present in small molecule biochemical binding assays to reduce nonspecific binding to surfaces and prevent aggregation of test small molecules. The binding of F-KL to ALK4 was poor in 0.1% dodecyl maltoside, intermediate in 0.1% BRIJ 35, and optimal in 0.1% Tween-20. It is likely that buffer conditions will have to be surveyed for each protein:fluorophore combination used to obtain robust assay conditions.

The linearity of the response of a fluorophore illuminated by the probe beam in the flow cytometer (both soluble and bead-associated) depends upon the dynamic range of the response. Experimental conditions were evaluated to assure that the background signals did not saturate the phototube, leading to a falsely low estimate of bead fluorescence. Thus, low concentrations of the fluorescently tagged ligand are desired. One approach to reduce this problem is by manual reading of bead fluorescence, where the 10-μl sample is diluted to 90 μl immediately prior to measuring fluorescence. Bead fluorescence must stay constant for the 5–10 s necessary to collect 500 data points, and a high affinity binding interaction is more likely to meet this requirement (as in Fig. 3A). A second approach to addressing the problem is to reduce the voltage on the photomultiplier. In this case, the voltage was lowered to the point that the nonspecific binding was linear with concentration of the unbound fluorophore. This approach allowed us to measure bead fluorescence accurately even in the presence of 500 nM F-KL (data not shown). However, at very high probe concentrations, when the nonspecific binding becomes 50% or more of the total signal, these assay conditions are no longer viable for HTS because the Z' score, a measure of the quality of the assay (the variability of the signal compared to the background), is too low.

Automating the bead-based assay provided some unexpected challenges. We found that the particles did not

transit the tubing connecting the autosampler to the flow cytometer efficiently. The inclusion of 0.1% BSA in the automated HC assay buffer increased the number of beads sampled per well, decreased the scatter of beads sampled per well, decreased the carryover of F-KL, and did not affect the K_d of ALK4 or ALK5 for F-KL. It is likely that 0.1% BSA will always improve the first three of these aspects of measurement, but care must be taken with BSA. Serum albumins, which bind small molecules, can change the apparent affinity for the targets by factors greater than 100 (17).

Another technical problem was caused by ions. The presence of phosphate in sheath fluid makes small precipitates with divalent cations that appear in forward scatter versus side scatter plots: 1 mM Mg^{2+} is acceptable, while 1 mM Mn^{2+} is poor for 6 μm particles in the manual mode as well as with HyperCyt. This can also be avoided by the use of nonphosphate-based sheath fluid. The inclusion of 2% dimethyl sulfoxide (DMSO) in the buffer did not affect the K_ds of ALK4 or ALK5 for F-KL. However, protein and assay tolerance must be established for each high throughput HC assay.

Binding affinity (K_d) for F-KL, ATP competition, and the K_i values from a series of test compounds ($n = 17$) were used in two different assays to determine the overall correlation of measurements of ALK4 and ALK5 activity. Both assays used inhibition of F-KL binding, one monitored by fluorescence polarization, the other monitored by bead fluorescence, and the correlations between these two assays for each enzyme were good. While the relative values obtained were comparable, the absolute values measured by FP and HC did differ. The source of lower K_d values by FP than those measured by HC may be due to differences in the lower limits of detection of each assay. Overall, the bead-based assay in a duplexed, high throughput screening format gave results that were comparable to results obtained by the fluorescence polarization assay.

Solution-based fluorescence assays such as FP and bead-based fluorescence assays such as HyperCyt have distinct strengths and weaknesses. In polarization, the protein concentration is typically in the vicinity of the K_d for the fluorescent ligand. This assures that a significant fraction of the ligand is bound, giving rise to a polarization signal. Assay sensitivity can be improved by increasing the fluorescently tagged ligand concentration. In the bead-based assay, the protein concentration is established to give tens of thousands of copies of the protein per bead, to assure that the binding event is detected. The sensitivity is a function of the total protein concentration (~nM). However, because the assay volume is small, and the total protein concentration is not raised to the K_d of the binding interaction, the bead-based assay may be constructed to use less protein than an FP assay. A Z' factor is a measure of the quality of an assay, and a Z' factor >0.5 is considered acceptable for a HTS assay (18). We observed a Z' factor of 0.75 in duplexing mode with HC. Fluorescence polarization routinely achieves a Z' score of >0.8. The benefit of multiplexing could outweigh the cost of early efforts to develop a robust HC assay.

We did not measure directly the extent of transfer of ALK4 molecules from the colorless beads to the red beads (or vice versa) during the assay. However, the fact that the relative K_d and K_i values were comparable for ALK4 and ALK5 by both FP and HC suggests this was not an issue. That is, the results indicated that distinct values for ALK4 and ALK5 were obtained. In most cases, the differences between the two kinases were larger in HC than FP, so it is unlikely that "bead-hopping" contributed to the results. On the other hand, the level of chelated nickel on the beads may be increased, increasing the residence time of hexahistidine-tagged proteins (19), and minimizing potential exchange. Alternatively, different epitope tags to bead surfaces can be used (20).

There are a number of issues that are worthy of consideration for future development of the analysis of molecular assemblies by flow cytometry. These possibilities have been reviewed previously (21,22). The overriding consideration is the association of molecules in solution (as in the FP assay) versus the association of molecules from solution with molecules bound to a surface. In our experimental situation, the starting concentration of kinase was typically ~2 nM. Because of ligand depletion effects, FP binding constants of below 2 nM would both have been reported as 2 nM in the flow cytometry assay, if there were no other factors involved. Our binding constants of ~3 and ~12 nM suggest that ligand depletion is not responsible for the observed values.

In these current studies, we observe roughly an order of magnitude difference between the K_d values for F-KL obtained by flow cytometry and fluorescence polarization (FP), which in turn result in roughly an order of magnitude difference in the calculated K_i values for the competitive ligands, using the Cheng–Prusoff approximation. As far as we know, the apparent binding affinity of the hexahistidine-tagged kinases for the nickel beads (~10 nM), which creates a less than ideal detection situation, does not in itself affect the measurement.

While the differences between these two approaches have not been evaluated systematically, we examined the kinetics of binding of the fluorescent ligand in new pilot studies conducted after completion of the original studies. We observed that the binding half-times as a function of concentration at 4°C for the fluorescent ligand were analogous to the half-times for the binding of Fab to cells reported in Ref. 23. These binding half-times appeared to be an order of magnitude more than that expected, based on molecular size. For example, using 9 nM F-KL, the half time for association was 10 min, while using 1 nM F-KL, the half time was more than 30 min, as compared to the 2 h equilibration used for the assay. Thus, immobilization slowed the ligand association rate and could contribute to the difference in K_d for the F-KL.

There are, in addition, multiple possibilities for surface effects that have been discussed elsewhere, particularly with respect to the applications of the BIAcore technology (24). Rebinding of molecules to surfaces when surface concentrations of the binding partner are high would alter the dissociation rate, while steric restriction of access to

the binding site through hindered diffusion, orientation, or even deformation of the binding partner could alter the association rate. The case of the nickel beads is interesting for the fact that such surfaces offer the possibility of specific binding via the hexahistidine tag and nonspecific binding via other histidine residues.

It is worth noting that accurate K_d values have been reported in flow cytometry for some other binding interactions. For example, when soluble β2-adrenergic receptors were bound to a ligand (dihydroalprenolol) displayed on beads, the micromolar affinities of native ligands for the binding pocket by competition were accurate compared to those found in solution (16). In yet another case (25), solution measurements of FLAG peptide binding to anti-FLAG antibody reported a K_d of 9 nM compared to bead-based measurements of 4 nM; here, the antibody was biotinylated and associated with the surface through multiple high affinity streptavidin binding sites. Finally on GST beads, where rebinding stabilizes the interaction between GST-tagged proteins and the bead, we are finding comparable binding affinities in competition assays between fluorescent GTP and guanine nucleotides and low molecular G proteins compared to literature values for binding to the protein (data not shown).

We have validated a bead-based assay format for duplexing kinase assays by comparing assay results from HC against results generated by an FP assay format, which is a widely used biophysical approach for measuring binding affinities. Direct binding measurements (K_d) of a fluorescently tagged ligand to two different members of the ALK family (ALK4 and ALK5), and K_i measurements of ATP and a panel of test compounds, generated comparable results using FP and HC. With the use of bead arrays sold by various particle manufacturers, a higher degree of multiplexing is possible if a fluorescent kinase ligand is found to interact with more kinases than the two shown here.

The HC approach enables multiplexing with numerous bead types against a single fluorescently tagged ligand, which is an advantage over many traditional methods of kinase measurements. While multiplexing may not be practical for a comprehensive panel of kinases (518 known human kinases), it may be useful for characterizing selectivity within key family members for lead identification and optimization in drug discovery.

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