Fluorescence Polarization (FP) Assays for Monitoring Peptide-Protein or Nucleic Acid–Protein Binding

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ABSTRACT

The technique of fluorescence polarization (FP) is based on the observation that when a fluorescently labeled molecule is excited by polarized light, it emits light with a degree of polarization that is inversely proportional to the rate of molecular rotation. This property of fluorescence can be used to measure the interaction of a small labeled ligand with a larger protein and provides a basis for direct and competition binding assays. FP assays are readily adaptable to a high-throughput format, have been used successfully in screens directed against a wide range of targets, and are particularly valuable in screening for inhibitors of protein-protein and protein-nucleic acid interactions when a small binding epitope can be identified for one of the partners. The protocols in this article describe a general procedure for development of FP assays to monitor binding of such a peptide or oligonucleotide to a protein of interest. *Curr. Protoc. Chem. Biol.* 1:1-15 © 2009 by John Wiley & Sons, Inc.

Keywords: fluorescence polarization • FP • peptides • nucleic acids • proteins • high-throughput screening

INTRODUCTION

This article describes a general procedure for the development of fluorescence polarization (FP) assays that can detect the binding of a small fluorescently labeled peptide or oligonucleotide to a protein of interest based on the property whereby when a fluorescently labeled molecule is excited by polarized light, it emits light with a degree of polarization that is inversely proportional to the rate of molecular rotation. The basic principle of fluorescence polarization is depicted in Figure 1. When a fluorophore that is covalently attached to a small (typically $<$1500 Da) ligand, such as a peptide in solution, is excited by polarized light, the emitted light will be largely depolarized. This is due to reorientation of the fluorophore during the lifetime of its excited state (on the order of nanoseconds for most fluorophores) caused by rapid Brownian molecular rotation of the labeled species. If this labeled ligand is bound to a high-molecular-weight protein (typically $>$10 kDa), the fluorophore reorients to a much smaller degree, due to the significantly reduced rotational speed of the complex. Thus, the emitted light will still be polarized to a significant degree. This property of FP allows it to be used as a technique for measurement of ligand binding, with the observed polarization in a mixture of labeled ligand and receptor being proportional to the fraction of bound ligand (Jameson and Seifried, 1999; Jameson and Croney, 2003). Furthermore, it is straightforward to establish a competition binding assay by measuring the decrease in FP signal produced when an inhibitor of the interaction is added to the mixture of labeled ligand and receptor.

FP has a number of key advantages as an assay technology. It is carried out in solution phase, is nonradioactive, does not require any separation of bound and free ligand, and is readily adaptable to low volumes (on the order of 10 μl). This makes it well suited for high-throughput screening applications, and FP assays have been used successfully

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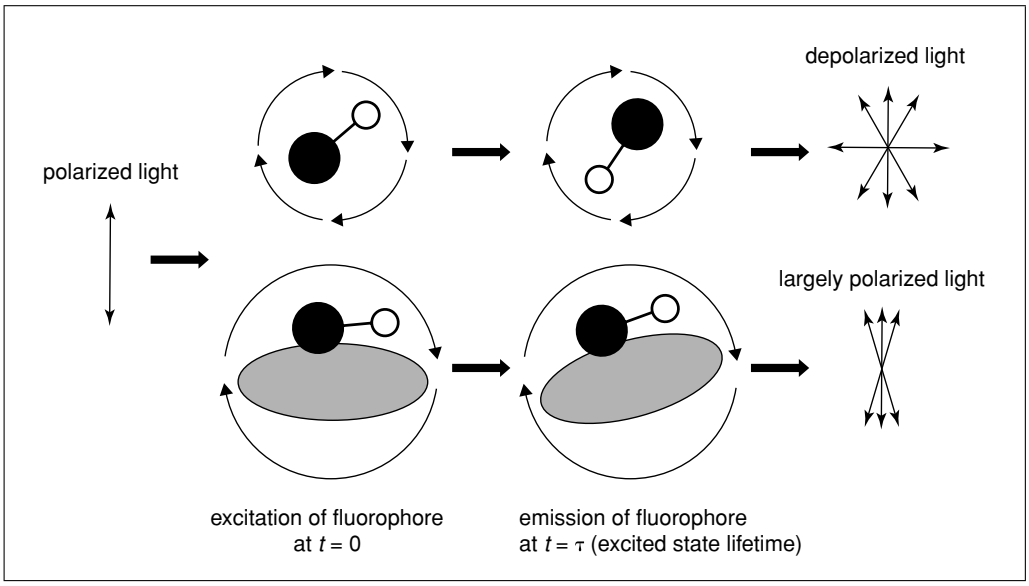

Figure 1 Schematic depicting the basic principle of fluorescence polarization. When a small peptide or nucleic acid ligand (dark circle) with a fluorescent label attached (white circle) is excited by polarized light at the excitation wavelength of the fluorophore, the ligand reorients to a significant degree due to molecular tumbling during the excited state lifetime of the fluorophore. This causes the emitted light to be largely depolarized. If the ligand is bound to a protein (gray ellipse), the resulting complex tumbles much slower, and the emitted light retains its polarization.

to study a wide variety of targets including kinases, phosphatases, proteases, G protein–coupled receptors (GPCRs), and nuclear receptors (Owicki, 2000; Burke et al., 2003). An important application of this type of assay is identification of inhibitors of protein-nucleic acid and protein-protein interactions. Even though these interactions can involve extensive interfaces, in many cases there are "hot spots," small peptide or nucleotide sequences that are disproportionately important for the affinity of the interaction. These features of interfaces can be exploited to design fluorescence polarization probes for competitive binding assays. Novel compounds identified using assays of this type have come to be important not only as tools for basic chemical biology but also as potential drug leads. A significant number of antibiotics and other drugs bind to protein–nucleic acid interfaces (Pommier and Marchand, 2005) and protein-protein interactions have become increasingly recognized as key therapeutic targets (Arkin, 2005).

STRATEGIC PLANNING

Fluorescent Probe Design

Fluorescently labeled peptides and oligonucleotides can be synthesized by a wide range of commercial services and academic core facilities. In the context of polarization experiments, these molecules are typically referred to as "tracers" or "probes." When developing an FP assay to target a protein-peptide or protein–nucleic acid interaction, a number of key strategic considerations are involved in the design of the probe. The first decision is what peptide or oligonucleotide sequence to use. This should correspond to a sequence of one of the binding partners that is known to be necessary for the interaction, and that has a low molecular weight (<1500 Da is typical, although up to 5000 Da can be acceptable if the binding partner is very large). In the best case, a high-resolution crystal or NMR structure of the complex will be available that can guide selection of an appropriate peptide or nucleotide sequence. If this is not the case, site-directed mutagenesis data can be used in defining the probe sequence, and will be useful even if the structure is known. The sequence chosen should be as short as possible (to maximize

the molecular weight difference between the probe and its binding partner, and thus the increase in FP) while still having sufficient binding affinity for the protein (generally in the low micromolar range or better). If available, quantitative measurements (using methods such as isothermal titration calorimetry), or rough estimates of the K_D values of peptides or oligonucleotides, are valuable in deciding between different sequences. A detailed theoretical analysis of binding interactions in FP assays indicates that the higher the affinity of the fluorescent ligand, the wider the range of inhibitor potencies that can be resolved (Huang, 2003). In addition, the ligand concentration used in the assay must not be significantly higher than twice its K_D, to avoid stoichiometric titration of the ligand (which reduces assay sensitivity). Thus, this analysis suggests that the optimal choice is to use a peptide or oligonucleotide with as high an affinity as possible, but not so high an affinity that a concentration lower than twice the K_D would be below the lower detection range of the instrument for the fluorophore used.

Once the probe sequence is chosen, it is necessary to choose a fluorophore and site of labeling. The key parameters for the fluorophore are the quantum yield and the fluorescence excited state lifetime. The fluorescence lifetime has a strong effect on the dependence of fluorescence polarization on molecular weight (Owicki, 2000). Fluorophores with lifetimes around 4 nsec, such as fluorescein and the rhodamines, provide a good separation in FP between small ligands and proteins of 10 kDa and higher. Fluorescein has traditionally been the most commonly used fluorescent label for FP, and suitable filters for its excitation and emission wavelengths are installed on almost all fluorescence plate readers. A number of alternatives to fluorescein with improved fluorescence properties (such as reduced pH dependence and less photobleaching), such as Alexa 488, are also available (Rusinova et al., 2002). Recently, red-shifted dyes such as Cy5 have been used successfully in FP assays as well (Turek-Etienne et al., 2004).

There are a wide variety of different chemistries for covalent linking of a fluorescent label to a peptide or oligonucleotide. A number of fluorophore linking chemistries for both peptides (Brinkley, 1992; Weber et al., 1998; Fischer et al., 2003) and nucleic acids (Proudnikov, 1996) have been described in the literature. These allow one to attach a fluorescent moiety to either end of a peptide or oligonucleotide, or in some cases at an internal position within the molecule. In choosing the site of labeling, the first major consideration is to avoid interference with binding. Thus, for most ligands, the fluorophore will be linked to the peptide N or C terminus (or oligonucleotide 5′ or 3′ end). The other key factor is to avoid attaching the label at a position that will still be highly mobile even when the ligand is bound to the protein. In what is termed the "propeller effect," this local mobility of the fluorophore can reduce the fluorescence polarization measured for the bound state. It is desirable to minimize the length of any linker between the peptide or oligonucleotide and the dye, and avoid attaching the dye to a "floppy end" of the ligand that is still highly flexible in complex with the protein. Structural data are thus extremely valuable in deciding where to attach a probe. In some cases, where little information is available, it may be necessary to try multiple labeling sites.

The protocols in this article describe a general method for the establishment of a competition-based fluorescence polarization assay to monitor the binding of labeled peptide or oligonucleotide to a protein of interest. This can be divided into three basic procedures: establishment of conditions to measure direct binding of the labeled probe to the protein (Basic Protocol 1), demonstration that an unlabeled form of the ligand can competitively displace the labeled probe (Basic Protocol 2), and validation of the performance of the assay in a high-throughput format by determination of a Z' factor (Basic Protocol 3). The protocols described can be generalized to any peptide-protein or nucleotide-protein interaction of interest. As a specific example to illustrate the steps

and results of the first two protocols, an FP assay designed to measure binding of an eIF4G peptide to its interaction partner eIF4E is described (Moerke et al., 2007). The protein:protein interaction between eIF4E and eIF4G is necessary for formation of the eIF4F complex, which is involved in the initiation of translation on capped mRNAs (Gingras et al., 1999; von der Haar et al., 2004). Regulation of this complex plays an important role in cancer and other human diseases, and eIF4F is thus a potential therapeutic target (Sonenberg, 2008).

BASIC PROTOCOL 1

MEASUREMENT OF DIRECT BINDING OF A FLUORESCENTLY LABELED PEPTIDE OR OLIGNUCLEOTIDE TO A PROTEIN BY FLUORESCENCE POLARIZATION

Once a suitable fluorescent probe has been designed and synthesized (see Strategic Planning, above), the first step in assay development is to measure binding to the protein of interest by the increase in FP upon titration of a fixed concentration of the probe with the protein. Although these measurements can be made using a fluorimeter, assuming that the ultimate objective is to develop an assay for high-throughput screening, it is most convenient to use a multiwell plate reader capable of FP measurements. This allows the simultaneous measurement of multiple data points, facilitating the collection of titration curves. A plot of FP versus protein concentration should produce a sigmoidal curve that begins at the baseline polarization for the free ligand (typically ~50 units of millipolarization, mP; described in more detail in step 5 below, and also in Background Information) and rises to plateau at a maximal polarization value that corresponds to complete binding of all labeled ligand (Fig. 2). The titration should be carried out in a buffer in which the protein is known to be stable. If the K_D of the peptide or oligonucleotide that the probe is based on is known, an initial probe concentration close to this value should be chosen for constructing the first binding curve. If desired, subsequent binding curves can be measured at lower probe concentrations until the fluorescence detection limit of the instrument is reached. As long as the FP measurement variability remains constant, it is generally preferable to use as low a concentration of fluorescent probe as possible (as described in the introduction). If the K_D is not known, several binding curves over a range of probe concentrations (from 1 nM to 1 μM) should be measured and evaluated. If desired, the binding data obtained can be used for determination of the K_D of the labeled probe (Roehrl et al., 2004; see Commentary). The following protocol describes the generation of a direct binding curve for a labeled eIF4G peptide to eIF4E, but can be generalized to any FP system. Note that the K_D for eIF4G peptide binding to eIF4E is 3 μM.

Materials

Protein stock solution: 40 μM eIF4E (Moerke et al., 2007) in protein dilution buffer (see recipe for buffer)
Protein dilution buffer (see recipe)
Fluorescently labeled peptide stock solution: 10 μM eIF4E-fluorescein peptide KYTYDELFQLK in protein dilution buffer (see recipe for buffer)

Black opaque 384-well microplates (Corning, cat. no. 3820)
FP-capable plate reader (e.g., Analyst HT, Molecular Devices)
Spreadsheet or graphing software

Prepare triplicate samples for FP measurements

1. Aliquot 180 μl of protein stock solution into a microcentrifuge tube, and make a series of eight two-fold dilutions into protein dilution buffer by sequentially mixing 90 μl of buffer with 90 μl of the previous protein solution in a new microcentrifuge tube.

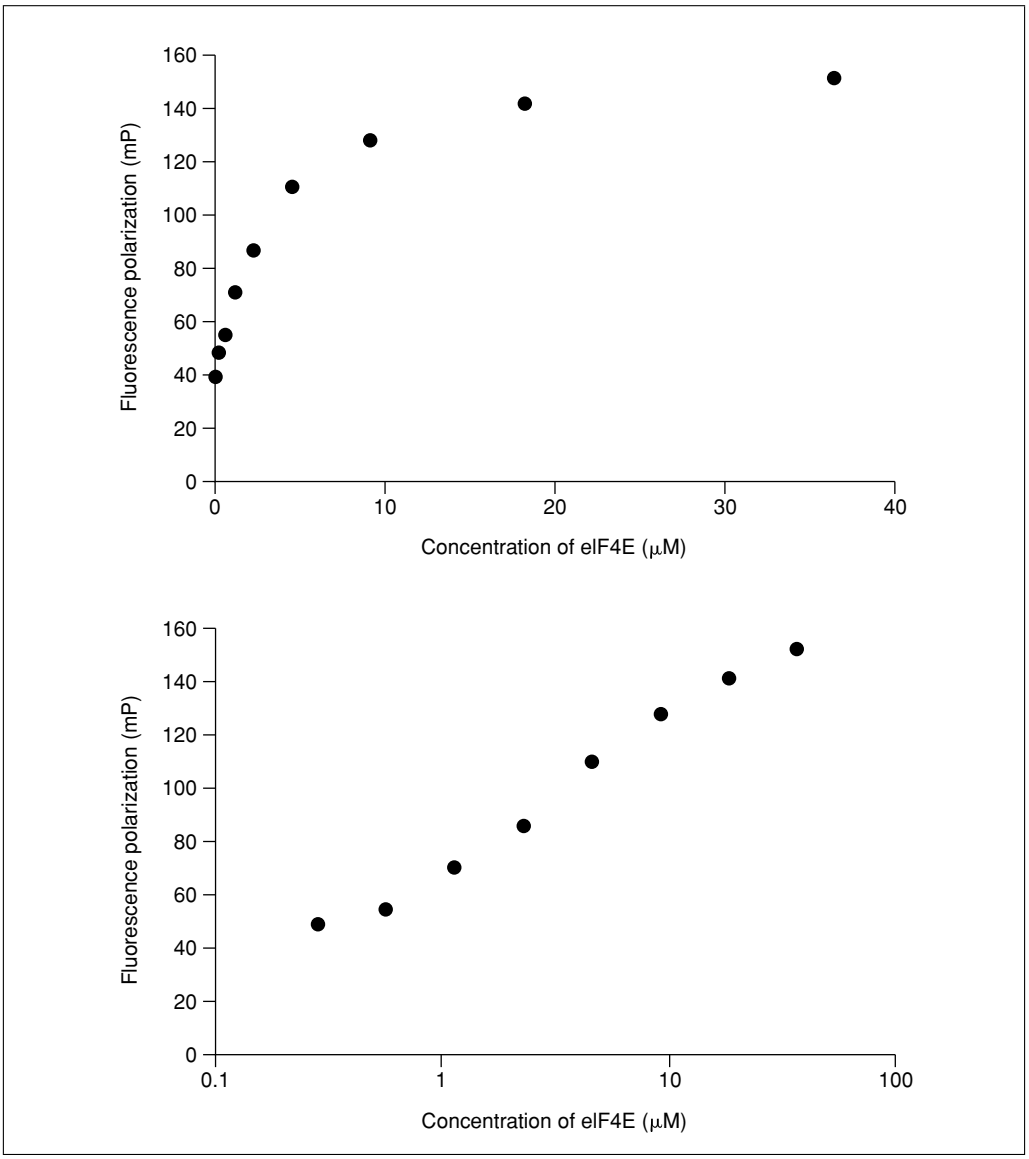

Figure 2 Direct binding of a fluorescent labeled eIF4G peptide to eIF4E. Fluorescence polarization (in units of mP) is plotted as a function of eIF4E concentration using a linear (top) or logarithmic scale (bottom). The concentration of eIF4G peptide is 1 μM.

It is generally desirable to start with as high a protein concentration as possible to make sure the plateau region of the binding curve is well defined. This, of course, is limited by the protein solubility. A typical starting concentration would be in the range of 40 to 100 μM. This should be achievable for most proteins, although in some cases a lower starting concentration will be necessary. It may be necessary to add more dilution points to adequately define the lower part of the curve, especially if the affinity of the labeled probe is very high.

2. Aliquot 10 μl of labeled peptide stock solution into each of the protein dilution tubes, and also to 90 μl of protein dilution buffer as a no-protein data point, for a final concentration of 1 μM labeled probe. Mix the contents of each tube by pipetting or gentle vortexing.

   For a final concentration of the labeled probe other than 1 μM, prepare a 10× stock solution in the protein dilution buffer.

3. Transfer 30 μl of each protein dilution/labeled probe mixture into three wells of a black opaque 384-well plate. Also transfer 30 μl of the no-protein solution and 30 μl of protein dilution buffer (as an assay blank for background fluorescence) into three wells each.

Obtain FP measurements

4. Load the plate into the plate reader, and use the instrument software to determine and set appropriate values for plate dimensions, measurement height, and detector gain.

   The detector gain will need to be reoptimized whenever the labeled probe concentration is changed, to avoid saturation and ensure adequate sensitivity.

5. Read the plate and obtain measurements of fluorescence polarization for each sample.

   For most instruments, the polarization is generally multiplied by 1000 and given in units of millipolarization (mP). The total fluorescence intensity polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the incident light is also generally part of the readout.

   The binding between the labeled tracer and the protein typically reaches equilibrium relatively quickly (on the order of 5 to 20 min), so measurements of FP usually can be made within this time range, after addition of reagents to the plate. The stability of the FP measurements after this time should be verified empirically, as in some cases the binding reaction may take longer (on the order of hours) to reach equilibrium.

Analyze data

6. Export the data and plot the average FP measurement of the three wells for each data point as a function of the protein concentration using standard spreadsheet or graphing software.

   Figure 2 shows representative data for binding of a labeled eIF4G peptide to eIF4E.

7. *Optional:* Correct the polarization measurements for background contributions to the measured intensity by subtracting the parallel and perpendicular intensity readings, from the blank buffer-only wells, from the intensity readings for each data point.

   This is important when the labeled probe concentration is low enough that its signal is on a comparable order of magnitude to the background. For fluorescein, this is typically in the low nM range or below. At higher probe concentrations, this step may not be necessary.

8. *Optional:* Convert the polarization values to anisotropy and fit the data to estimate K_D of labeled probe (see Commentary).

9. If necessary, repeat the experiment with a higher starting protein concentration or more data points to define the lower part of the curve.

BASIC PROTOCOL 2

FLUORESCENCE POLARIZATION MEASUREMENT OF COMPETITIVE BINDING TO A PROTEIN OF AN UNLABELED PEPTIDE OR OLIGONUCLEOTIDE WITH A FLUORESCENTLY LABELED PROBE

Once a fluorescent probe has been designed that binds to a protein of interest, it can be used for a competition binding assay. Such an assay works by measurement of the decrease in FP caused by a ligand that displaces the labeled probe. To establish the specificity of the assay, it is necessary to titrate a mixture of the protein and labeled probe with an unlabeled competitor and demonstrate that the FP decreases to the value observed with the free fluorescent ligand. The "gold standard" for specificity is to titrate with the exact, although unlabeled, peptide or oligonucleotide that was used for the labeled probe. However, other ligands that bind to the same site can be used as well. A concentration of protein should be chosen (based on the binding curve determined in Basic Protocol 1) that produces ~50% to 80% of the increase in FP between the free ligand and the

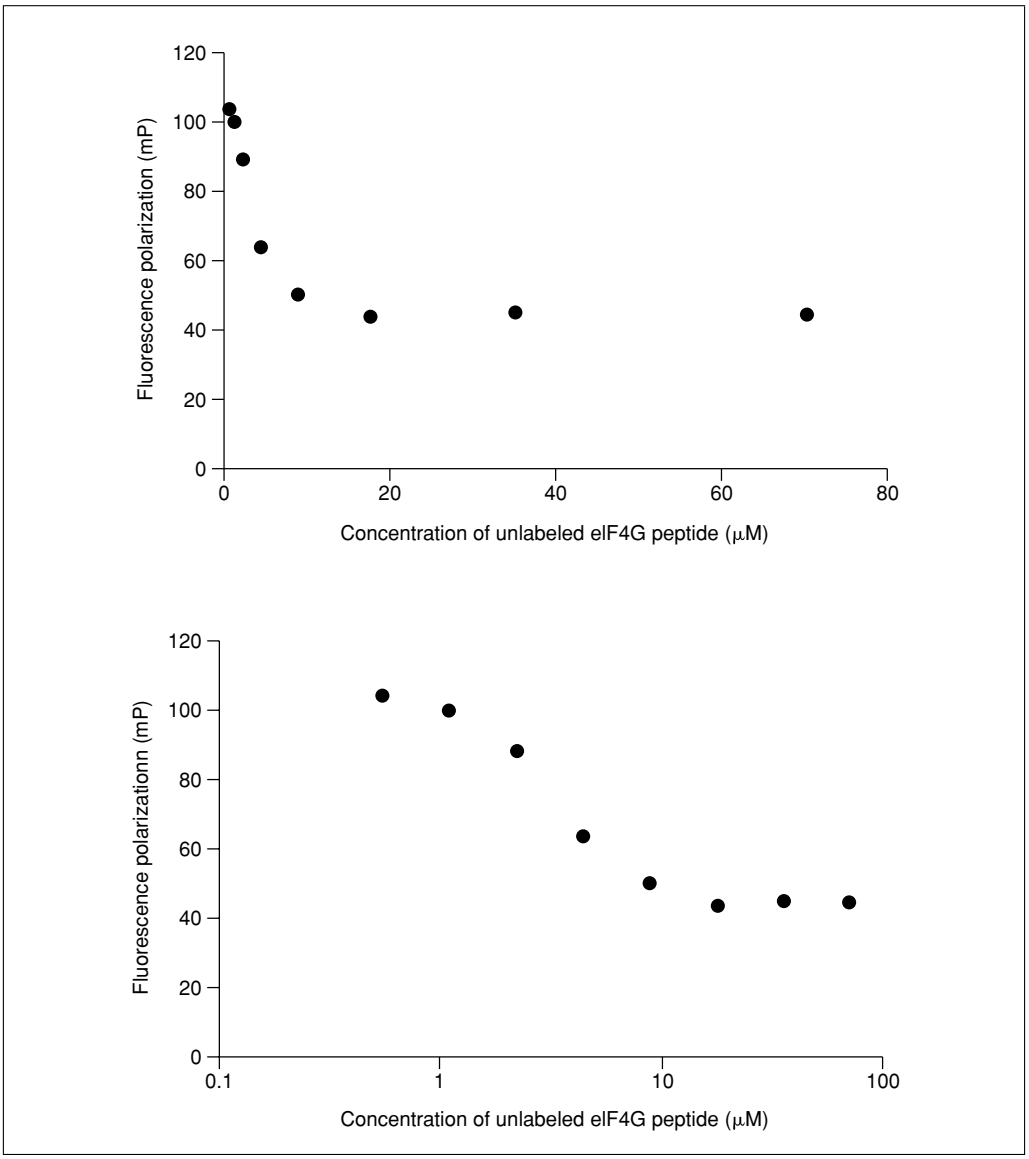

Figure 3 Competitive displacement of a fluorescent labeled eIF4G peptide from eIF4E by an unlabeled eIF4G peptide. Fluorescence polarization (in units of mP) is plotted as a function of unlabeled eIF4G peptide concentration using a linear (top) or logarithmic scale (bottom). The concentration of labeled eIF4G peptide is 1 μM and the concentration of eIF4E is 5 μM.

completely bound state. Higher concentrations of protein will lie in the plateau region of the binding curve and thus be insensitive to the competitor, and lower concentrations will not provide a good assay window for measurement of displacement of the probe. The displacement curve should have a sigmoidal shape, beginning at the bound FP value and decreasing to the FP value of the free labeled probe (Fig. 3). As in Basic Protocol 1, fitting of the binding curve can be used to estimate the K_D of the competitor peptide or oligonucleotide (Roehrl et al., 2004; also see Commentary).

The following protocol describes the generation of a competition binding curve for an unlabeled eIF4G peptide and eIF4E, but can be generalized to any FP system. An eIF4E concentration of 5 μM is chosen for the competition binding assay based on the binding curve generated in Basic Protocol 1. This concentration gives ∼65% of the increase in FP between free and completely bound states, and thus lies in the middle of the recommended range. As before, a concentration of 1 μM labeled peptide is used. Because of problems

with the solubility with the unlabeled form of the first eIF4G peptide, a different peptide that binds to the same site is used as a competitor ligand.

Materials

Unlabeled competitor peptide stock solution: 7 mM eIF4G peptide KKQYDREFLLDFQFMPA in DMSO (see recipe)
Dimethyl sulfoxide (DMSO)
Protein stock solution: 40 μM eIF4E (Moerke et al., 2007) in protein dilution buffer (see recipe for buffer)
Fluorescently labeled peptide stock solution: 10 μM eIF4G-fluorescein peptide KYTYDELFQLK in protein dilution buffer (see recipe)
Protein dilution buffer (see recipe)

Black opaque 384-well microplates (Corning, cat. no. 3820)
FP-capable plate reader (e.g., Analyst HT, Molecular Devices)
Spreadsheet or graphing software

Prepare triplicate samples for FP measurements

1. Aliquot 20 μl of unlabeled peptide stock solution into a microcentrifuge tube, and make a series of eight two-fold dilutions by sequentially mixing 10 μl of DMSO with 10 μl of the previous stock solution in a new microcentrifuge tube.

   It may be necessary to add more dilution points to adequately define the competition curve.

2. Prepare 1 ml of 5 μM protein/1 μM labeled peptide solution by adding 100 μl of 10 μM labeled peptide stock solution and 125 μl of 40 μM protein stock solution to 775 μl protein dilution buffer in a microcentrifuge tube. Mix well by pipetting or gentle vortexing.

3. Aliquot 100 μl of this solution into a separate microcentrifuge tube corresponding to each peptide dilution prepared in step 1, and into an additional tube for a no-competitor control.

4. Pipet 1 μl of each peptide dilution into the corresponding 100-μl aliquot of protein/labeled peptide solution. Add 1 μl DMSO to the 100-μl aliquot for the no-competitor control. Mix all samples well by gentle vortexing or pipetting. Incubate samples for 30 min at room temperature, protected from light.

   In some cases, the complex between the labeled probe and protein may be kinetically very stable (tight binding), and so longer incubation times may be required for the displacement reaction to reach equilibrium.

5. Add 1 μl of DMSO and 10 μl of labeled peptide stock solution to 90 μl of protein dilution buffer to prepare a probe-only control, to assess the completeness of displacement by the unlabeled peptide.

6. Transfer 30 μl of each sample into each of three wells of a black opaque 384-well plate. Also transfer 30 μl of protein dilution buffer into three wells if background correction measurements will be needed (as in the optional step 7, of Basic Protocol 1).

Obtain FP measurements and analyze data

7. Load the plate into the plate reader and use the instrument software to set appropriate values for plate dimensions, measurement height, and gain.

8. Read the plate and obtain measurements of fluorescence polarization for each sample.

9. Export the data and plot the average FP measurement of the three wells for each data point as a function of the peptide concentration using standard spreadsheet or graphing software, optionally correcting the FP values for background fluorescence as in Basic Protocol 1, step 7.

   Figure 3 shows representative data for displacement of a labeled eIF4G peptide to eIF4E by an unlabeled eIF4G peptide.

10. *Optional:* Convert the polarization values to anisotropy, and fit the data to estimate K_D of the competitor (see Commentary).

11. If necessary, repeat the experiment with a different protein concentration or more data points.

ADAPTATION OF A COMPETITION FLUORESCENCE POLARIZATION ASSAY TO HIGH-THROUGHPUT SCREENING FORMAT

BASIC PROTOCOL 3

Once a competition binding assay has been established, it can be adapted to high-throughput format for use in screening for small molecules that inhibit the interaction of interest. This involves choosing labeled probe and protein concentrations for the assay and confirming that the assay will perform adequately under the conditions to be used in screening. This will generally involve filling 384-well plates with the assay solution using automated liquid-handling equipment instead of pipetting, and adding controls and library compounds to the plates by means of pin transfer. A standard metric for evaluating assay quality and robustness is the Z' factor (Zhang et al., 1999), which is given by Equation 1:

$$Z' = 1 - \frac{(3\sigma_{pos} + 3\sigma_{neg})}{|\mu_{pos} - \mu_{neg}|}$$

Equation 1

Here, σ and μ represent the standard deviations and means of the positive and negative controls, respectively. Typically, the Z' factor is calculated based on a single plate that contains approximately one-half positive and one-half negative control wells. An assay with a Z' value of at least 0.5 is recommended for high-throughput screening, and one with a Z' of 0.7 or higher is considered an excellent assay. For a competition-based assay, a positive control should cause a decrease in FP, which is what is expected from a "hit" compound that inhibits the interaction being measured. A negative control should have no significant effect on the FP. Since compound libraries used in high-throughput screening are generally formatted using DMSO as a solvent, the standard negative control is addition (by pin transfer) of a volume of DMSO equivalent to that which will be transferred during the assay (a typical volume would be 100 nl, for a 30-µl assay volume). The standard positive control is the unlabeled peptide or oligonucleotide used for the competition curve in Basic Protocol 2, at a concentration sufficient to completely displace the labeled probe. This should be formulated as a stock in DMSO that will give 1× final concentration when the pin-transfer volume is added to the assay well (so, 300× for transfer of 100 nl for a 30-µl assay volume). A small molecule already known to inhibit the interaction between the probe and the protein may also be used as the positive control.

Materials

Fluorescently labeled peptide or oligonucleotide stock solution (see Basic Protocols 1 and 2)
Protein stock solution (see Basic Protocols 1 and 2)

Unlabeled competitor peptide or oligonucleotide stock solution (see Basic Protocols 1 and 2)
Dimethylsulfoxide (DMSO)
Protein dilution buffer (see recipe)

Polypropylene 384-well compound storage plates (Thermo Scientific, cat. no. AB-1056)
Automated liquid dispenser for multiwall plates (Matrix WellMate, Thermo Scientific)
Black opaque 384-well microplates (Corning, cat. no. 3820)
Pin transfer apparatus (V&P Scientific, *http://www.vp-scientific.com/*)
FP-capable plate reader (e.g., Analyst HT, Molecular Devices)
Spreadsheet or graphing software

Prepare plate for Z′ determination

1. Determine the optimal protein and labeled probe concentrations based on the results of Basic Protocols 1 and 2.
2. Determine the minimum concentration of unlabeled competitor that will completely inhibit binding based on the curve generated in Basic Protocol 2, and prepare 2 ml of a 300× stock in DMSO.
3. Add 10 µl DMSO to one-half of the wells of a polypropylene compound storage plate (negative control) and add 10 µl of the 300× unlabeled competitor stock to the other one-half of the wells (positive control).

   This will be the source plate for pin transfer. This plate can be reused and should be kept at −20°C for long-term storage.

4. Prepare 20 ml of assay solution containing the appropriate concentrations of the protein and labeled probe in protein dilution buffer.

   This is sufficient to fill a single 384-well plate with extra volume to account for the "dead volume" found in most liquid dispensers.

5. Using the automated liquid dispenser, fill an entire black opaque 384-well plate with the assay solution at 30 µl per well (this will be the assay plate). If the measurements of FP will be corrected for background fluorescence, fill one column with protein dilution buffer alone, instead of assay solution.
6. Using the pin transfer apparatus transfer a volume of 100 nl from each well of the source plate to the assay plate.
7. Incubate the assay plate for 30 min at room temperature, covering with foil or an empty plate to protect it from exposure to light.

   The length of the incubation can be increased if necessary for complexes of labeled protein and probe that are kinetically very stable (tight binding).

Obtain FP measurements and analyze data

8. Load the plate into the plate reader, and use the instrument software to set appropriate values for plate dimensions, measurement height, and gain.
9. Export the FP measurement data into a standard spreadsheet program, correct FP values for background fluorescence if necessary, and calculate the Z' score using Equation 1.

REAGENTS AND SOLUTIONS

Use Milli-Q purified water or equivalent in all recipes and protocol steps.

Protein dilution buffer

50 mM sodium phosphate (mix monobasic and dibasic phosphate in appropriate ratio for pH 6.5)
50 mM KCl
Adjust to pH 6.5 with HCl or NaOH
Add DTT fresh from 1 M frozen stock, and filter buffer to remove any precipitated matter before using in assays
Once DTT is added, store up to 1 week at 4°C

Fluorescently labeled peptide stock solution

Dissolve lyophilized peptide (sequence KYTYDELFQLK with fluorescein conjugated to C-terminal lysine; synthesized by Research Genetics, *http://www.researchgenetics.com*) in protein dilution buffer (see recipe) to a concentration of 10 μM. Aliquot peptide solution and store protected from light indefinitely at −20°C or −70°C.

Unlabeled peptide stock solution

Dissolve lyophilized peptide (sequence KKQYDREFLLDFQFMPA; synthesized by Tufts University Core Facility) in DMSO to a concentration of 7 mM. Aliquot peptide solution and store indefinitely at −20°C or −70°C.

COMMENTARY

Background Information

In a typical instrument that measures fluorescence polarization, light at the proper wavelength for excitation of the fluorophore is selected by a band-pass filter. It then passes through an excitation polarizing filter that causes it to be plane polarized. The polarized light is reflected by a dichroic mirror into the wells of a microtiter plate containing the sample of interest where the fluorophore is excited. Emitted light passes back out of the well through the dichroic mirror and goes sequentially through a polarizing filter that has the same orientation as the excitation polarizing filter (the parallel polarizing filter) and a filter oriented at 90° to the excitation filter (the perpendicular polarizing filter). Finally, for both the parallel and perpendicular light, a second band-pass filter selects for the emission wavelength before the light reaches the detector. The fluorescence polarization then is defined by Equation 2:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

Equation 2

Here $I_{\parallel}$ is the intensity of emitted light polarized parallel to the excitation light, and $I_{\perp}$ is the intensity of emitted light polarized perpendicular to the excitation light. An important property of the polarization that emerges from this equation is that it is independent of the fluorophore concentration. Although this equation assumes that the instrument has equal sensitivity for light in both the perpendicular and parallel orientations, in practice this is not the case. A modified version of the polarization equation includes a correction factor, G, that accounts for differential sensitivity, as in Equation 3.

$$P = \frac{I_{\parallel} - G * I_{\perp}}{I_{\parallel} + G * I_{\perp}}$$

Equation 3

The G factor is dependent on the specific optical components used to measure fluorescence polarization in an instrument. Determination of G is necessary for the calculation of absolute polarization values, and for the comparison of data between different instruments. However, it is not necessary for analysis of assay and screening data that are all collected on the same instrument, and if this is the case, it can be ignored (i.e., set to 1) if desired. This is due to the fact that variation in the G factor does not significantly affect the

relative differences between FP measurements in a binding assay, and does not change the assay window. The details of the procedure for measurement of the *G* factor will depend on the fluorophore used and the instrument, and instrument documentation should be consulted for this information.

A closely related property to polarization is fluorescence anisotropy, which is defined by Equation 4 (without *G* factor correction):

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{2P}{3 - P}$$

Equation 4

For historical reasons most instruments give their output in terms of polarization. However, conversion of data to anisotropy is useful in fitting binding curves and displacement curves to equations describing the multiple binding equilibria between the protein, the labeled probe, and the competitor. This can be used to determine K_D values for known ligands and inhibitor compounds, and detailed procedures to do this have been described (Roehrl et al., 2004).

Critical Parameters

Choice of fluorescent label

Before beginning the experiments described in this unit, careful consideration must be given to the choice of fluorescent label to use. The initial choice of fluorophore may, of course, be constrained by the capabilities of the facility used for synthesis of the probe. In addition, it is essential that the available fluorescent plate reader have the correct emission filter, excitation filter, and dichroic mirror (if applicable) for the fluorophore being considered. Once the set of possible labels has been narrowed down, another consideration is the excitation and emission wavelengths. Two major sources of interference in screens using FP assays are fluorescence from library compounds and light scattering due to precipitated compounds (scattered light has a very high polarization). Several studies have found that red-shifted fluorophores suffer from significantly less interference from compound fluorescence than dyes with shorter wavelength maxima (Turek-Etienne et al., 2003; Simeonov et al., 2008). Light scattering is also less of a problem for such fluorophores, as the intensity of scattered light decreases with increasing wavelength. Thus, the use of a red-shifted fluorophore may reduce the percentage of false positives and negatives in a screen. This may be especially advantageous for screens of natural product libraries that have a higher incidence of compound interference (Turek-Etienne et al., 2004).

Reagent purity

As with all fluorescence measurements, the labeled probe should be of the highest available purity to minimize the presence of contaminating fluorescent components. Purification of fluorescently labeled peptide and oligonucleotides is generally performed by HPLC, and the facility may provide HPLC traces and/or mass spectrometry data to confirm purity. The labeled probe should be kept protected from direct light exposure during experiments and in storage. Finally, care must be taken to minimize the presence of precipitated matter in assay solutions that may cause light-scattering interference with FP measurements. Buffers should be filtered, and protein, peptide, or nucleotide solutions should be briefly centrifuged before using.

Multiwell plates

There are a number of choices for black opaque 384-well plates to be used in these experiments. It is essential that the specific plates used be validated for the plate reader and other automation that will be used. If there is concern about nonspecific interactions of the protein or labeled probe with the surface of wells, it can be worthwhile to start out using plates with a hydrophilic, nonbinding surface (NBS). Also, if the protein used in the assay is difficult or expensive to purify, low-volume assay plates for fluorescence polarization are available (with a typical working volume of 5 to 40 μl) that can minimize protein consumption. Such plates are available from many microplate suppliers, e.g., Corning and Greiner. Fluorescent plate readers differ in the precise methods used in setting plate dimensions, plate measurement height, detector gain, and signal integration time used in FP measurements for a specific plate and fluorophore concentration. Careful attention must be paid to these parameters in order to maximize assay sensitivity.

Troubleshooting

An overall flowchart of the protocols described in this unit with appropriate troubleshooting steps at various points is shown in Figure 4.

Probe binding

There may be a lack of increase in FP in the titration of Basic Protocol 1 due to the fluorescent label interfering with the interaction with

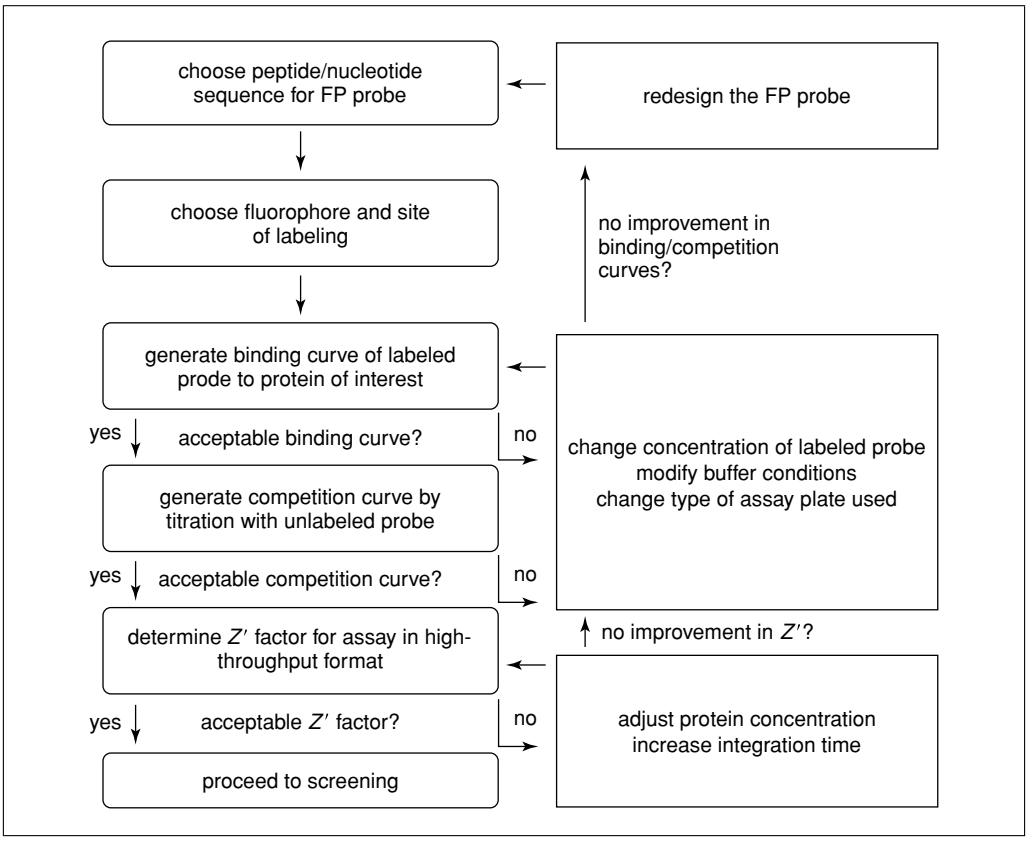

Figure 4 Flowchart describing the stages of development of an FP assay for high-throughput screening, with recommended troubleshooting procedures for each stage.

the protein. If another binding assay exists for the protein of interest, this can be used to confirm whether this is the case, and, if so, it will be necessary to redesign the labeled probe.

Nonspecific binding

Another source of potential error in fluorescence polarization measurements is nonspecific binding of the protein of interest to the labeled probe, possibly due to interactions with the fluorescent label. In Basic Protocol 1, this may cause an abnormal binding curve, possibly with a linear rather than sigmoidal shape. There may be a lack of a clear plateau region, with the FP continuing to increase with increasing protein concentration. Another strong indicator of nonspecific binding is an inability to achieve complete displacement of the labeled probe in Basic Protocol 2. If this type of nonspecific binding is suspected, one troubleshooting step is to reduce the labeled probe concentration as much as possible while still maintaining adequate sensitivity of detection. In addition, nonspecific interactions can often be reduced or eliminated by adding proteins such as BSA or γ globulin and/or detergents such as Tween-20 or Triton X-100 to the buffer. These should be carefully titrated so as to avoid disrupting the specific interaction between the protein and the labeled probe. If a labeled peptide containing free cysteines is being used, inclusion of reducing agents such as DTT may also be helpful. If these measures fail to eliminate nonspecific binding, it may be necessary to redesign the probe with a different fluorophore or site of labeling.

It is also possible to have nonspecific binding of the labeled probe or the protein to the surface of the wells of the plates being used. This can cause there to be no increase, or a smaller-than-expected increase in FP upon titration of the labeled probe with protein in Basic Protocol 1, or also an abnormally high FP for the labeled probe alone. Again, this can potentially be reduced by changing buffer conditions to reduce nonspecific interaction. In addition, the use of NBS plates designed to minimize hydrophobic interactions can often eliminate the problem.

Z′ factor optimization

When determining the Z' factor in Basic Protocol 3, the two factors affecting this value are the precision of FP measurements and the size of the assay window, or difference in mP between the free or displaced labeled

probe and the bound probe. The assay window is dependent on the concentration of protein used, and so carefully increasing this (without moving into the plateau region of the binding curve) can improve the Z' factor. For many plate readers, it is possible to improve the precision of FP measurements by increasing the signal integration time. As the effective probe concentration can be reduced by nonspecific binding to the surface of wells, changing buffer conditions or switching to an NBS plate may also improve the precision of measurements and the Z' factor. For these changes in conditions, one should repeat the binding and displacement curves in Basic Protocols 1 and 2 to confirm that the assay is still functioning as before.

Anticipated Results

Basic Protocols 1 and 2 will generate one or more FP binding curves for the labeled probe and one or more competition curves for the unlabeled form of the probe (or other appropriate peptide or oligonucleotide). In Basic Protocol 3, these data will be used to establish initial optimal conditions for a high-throughput FP assay, which are tested by determination of the Z' factor. Once an adequate Z' factor has been achieved (possibly after one or more rounds of optimization of the initial conditions), the assay can be considered to be ready for high-throughput screening.

Time Considerations

Once all necessary reagents are in hand, the procedures described above could be completed within a day of work or less, although in practice up to a week is often needed to develop and validate the assay if one or more steps need to be optimized.

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Internet Resources

http://www.ncgc.nih.gov/guidance/manual_toc.html

National Center for Chemical Genomics Assay Guidance Manual. This provides an overview of FP assays in high-throughput screening and a comparison to other assay methods.