MOLECULAR DEVICES

TECHNICAL NOTE

Establishing and optimizing a fluorescence polarization assay

Purpose

This technical note is designed to provide information to help the user define the optimal experimental conditions for converting existing assays to a robust fluorescence polarization format. The example is a competitive binding assay of the type often used to evaluate receptor-ligand binding. Familiarity with the basic principles of fluorescence polarization is assumed.

Points to consider when designing an FP receptor-ligand binding assay

1. **Maximize size differences between tracer and binder.** When using a usual fluorophore such as fluorescein, Texas Red, Cy 5, BODIPY etc., the tracer should be comparable in size to a peptide of less than 10 kD. The larger binder should be 50 kD or larger. A ten-fold difference in molecular weight is a useful target. However, failure to meet these criteria should not prevent evaluation of the binding pair. The original work on FP used albumin (60 kD) and antibody (160 kD) to achieve publishable results.
2. **Minimize the contribution of other assay materials to non-specific fluorescence polarization.** Quality factors include purity of tracer, purity of binder, buffer intrinsic fluorescence and ability of buffer components such as carrier proteins to bind the tracer. Some microplate materials such as polystyrene can bind free tracer thereby increasing total polarization. Non-binding microplates available from several vendors provide a solution to this problem.

   2a. **Tracer should be >90 % labeled.** Failure to label a high percentage of tracer means that unlabeled tracer will compete for the receptor, changing apparent IC_{50} (the apparent affinity of the interaction and hence affecting the calculated values of IC_{50}). Similarly, failure to purify free fluorophore from tracer means an increased portion of the total fluorescence will not be able to change its polarization.

   2b. **Use highly purified binder.** Because large proteins, cell membranes and cellular debris scatter light, causing a net increase in total polarization, impurities should be minimized. They may be corrected in part by appropriate background subtraction, but it is preferable to minimize the contribution to signal (and hence noise) by using purified receptor. Repeated freezing of receptor preparations may result in increased aggregation, reducing assay performance. Some strategies to remove aggregates include disruption by passing through a narrow gauge syringe and sedimentation/centrifugation to eliminate large material.

   2c. **Minimize buffer contribution to signal.** Increased buffer fluorescence background is due to contaminants that fluoresce at the wavelength of interest. Attention to raw materials, cleanliness of mixing and storage vessels and buffer preparation methods should reduce this to acceptable levels. High background counts due to buffer or non-fluorophore components can seriously affect the signal-noise ratios of an assay as well as the ultimate sensitivity of an assay.

   2d. **Avoid bovine serum albumin.** Buffers for proteins often include carrier proteins such as bovine albumin (BSA). Albumin may bind some fluorophores; this could spuriously increase the baseline polarization, reducing assay range. Solutions include avoiding carrier proteins or using low-binding alternatives such as bovine gamma globulin (BGG). In any case, it is useful to evaluate the contribution of buffer proteins to the net polarization of the tracer, by comparing polarization of the tracer in buffer with and without added protein. Alternatively, reduce the final concentration of BSA to minimize these effects.

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3. **Define format goals.** Microplate type, assay volume, and the relative importance of assay speed versus assay sensitivity/precision should be defined. It is best to conduct feasibility studies under conditions that will approximate final campaign conditions. Assay feasibility may also start with an assay volume greater than the target volume.
4. **Define development criteria.** Assay development and assay validation typically require more replicates and controls than the single wells run during an HTS campaign. Assessment criteria include reagent consumption, read time, incubation time and conditions, signal-to-noise (see below), precision, sensitivity, reproducibility, etc.

Steps for establishing and optimizing an FP receptor-ligand binding assay

1. **Estimate the best settings for your instrument.** The following is a suggested starting point for instrument settings. Start with the PMT setting, Z height, and integration time or flashes per well.
2. **Determine the optimal concentration of free tracer.** This step determines the acceptable range of concentrations of tracer by examining the signal generated and the concentration independence of the polarization. Select the lowest concentration of tracer that still provides a good signal-to-noise ratio. Of course there are biochemical considerations: the concentration of the tracer should be less that the Kd (if known) and less than the concentration of the binder (i.e., the receptor). Comparison of free tracer with free fluorophore (by running free fluorophore in parallel) establishes the suitability of the tracer size. If the tracer mP is significantly greater than that of the free fluorophore, the tracer may be too large for use in FP.

   2a. Make a serial dilution of free tracer using 4 or more replicates (e.g., from 100 nM to 0.1 nM).

   2b. In parallel to the study of the tracer, evaluate free fluorophore. Make a serial dilution of free fluorophore (e.g. the same as used to label the tracer) in groups of four or more (e.g., from 100 nM to 0.1 nM).

   Each series should be established in replicates of at least four points, to allow for subsequent statistical evaluation of background 'noise' levels.

   2c. Include [Buffer only] control to subtract from each of the S and P values. For background subtraction, calculate the mean S and P [Buffer only] values and subtract the mean from individual S and P values of wells containing tracer or free fluorophore.

   2d. Calculate G factor using the assumed theoretical mP (27 for fluorescein and Texas Red) and the results from free fluorophore at a concentration that gives counts well above background for both S and P. $G = P/S * [(1-27/1000)/(1+27/1000)]$. S and P are values for free fluorophore subtracted with buffer only controls ("background subtracted").

   2e. Calculate the mP value for the free tracer: $mP = [(P - S*G)/(P + S*G)]*1000$. Use the calculated G factor from step 2d. S and P are values for background-subtracted free tracer. As a control, the free fluorophore should have a mP value close to the theoretical value. Ideally, the tracer should have a value close to that of the free fluorophore alone, signifying that the size and rate of rotation of the ligand was significantly affected by conjugation with the fluorophore. If the value is much larger, it suggests that the tracer may be sufficiently large to reduce the effective polarization change when complexed to the binder. The acceptable range of concentrations of tracer include all concentrations giving a polarization value (in mP) near to the prescribed 27 mP. In addition, restrict the tracer to concentrations giving counts well above background in the less active channel (typically the P counts). Examine raw signal values: tracer should be at least 3X the signal of buffer only.

   Re-reading the plate in fluorescence intensity mode allows evaluation of the extent of quenching in the free tracer. Quenching effects can affect the ultimate sensitivity of a fluorescence-based assay. Compare the molar fluorescence intensity of the fluorophore-labeled molecule and the fluorophore itself in free solution to determine the degree of quenching caused by the chemical coupling process itself. For example, if there is no quenching, the signal for 1 nM fluoresceinated peptide should be the same as that of 1 nM sodium fluorescein. One would not expect fluorescein coupled to another molecule to be more fluorescent than free fluorescein, so this could indicate that the tracer may have an incorrect concentration assigned. Similarly, tracer with less than 20% of the signal of the same concentration of free label may be very highly quenched, have too low a percent of the tracer labeled, be incorrectly value assigned, or some combination of the above.

   Note that fluorescence polarization often results in the loss of about 10–90% of fluorescence intensity. This in itself may reduce the sensitivity of fluorescence polarization as opposed to direct intensity measurements.

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3. **Titrate binder with appropriate controls.** The purpose of this step is to determine the optimal concentration of binder and tracer. Use of appropriate controls allows accurate estimation of specific polarization. Test multiple concentrations of binder ("Protein"), with and without tracer. Since the binder may contribute to net signal, binder without tracer serves as a proper control; for multiple concentrations of binder, each should have the buffer-only control. Binder should be at a higher concentration than tracer. Consider running more than one concentration of tracer initially. Tracer should be below the Kd. A first study may include rather broad concentration ranges for both tracer and binder, whereas a follow-up test may use only one concentration of tracer and a tightly spaced limited dilution series of the binder. A good starting point is to titrate down the binder from 4X Kd and titrate the tracer down from 1X Kd. For early studies, run at least three replicates; increasing the number of replicates gives a more accurate estimate of assay imprecision. To enable imprecision of the assay to be accurately established, each experimental condition should run at least in triplicate. Specific control groups include:

   3a. [Buffer only]: indicates the contribution of buffer only to the S and P signals, especially when interfering molecules are present in the buffer (e.g. sucrose). This is used as background subtraction for [Tracer only].

   3b. [Tracer only]: S and P background-subtracted values are used for G factor calculation, where G = S/P *[(1-27/1000)/(1+27/1000)].

   S and P are values for free tracer subtracted with [Buffer only] controls. G should be a very stable value and the value calculated in the previous step should be appropriate, but it is worthwhile to confirm the earlier estimate of G at this time.

   Note that a representative value for signal/noise can be calculated from the S value of tracer only. Ideally, signal [Tracer only] to noise [Buffer only] values of at least 10-fold should be targeted.

   3c. [Protein only]: indicates contribution of light scattering by the specific protein binder, especially valuable if it is in a membrane-bound form. This is used as background subtraction for [Protein + Tracer]. Since several concentrations of protein will be used, each should be tested in the absence of tracer, the mean S and P values calculated and subtracted from the individual well S and P values to obtain mP.

   3d. [Protein + Tracer]: determines maximal mP. These include the key groups in this checkerboard study with a titration of the protein against a titration of the tracer to identify optimal concentrations for the protein and the tracer. As mentioned above, a good starting point is to titrate down the protein from 4X Kd and 1X Kd for the tracer.

   If [binder] = Kd, and [tracer] < [binder], then half the tracer should be bound. S and P are values for [Protein + Tracer] subtracted with [Protein only] controls. For background subtraction, calculate the mean S and P [Protein only] values and subtract the appropriate mean from individual S and P values of wells containing tracer, protein and compound/control. Use the calculated G factor from step 2b.

   3e. There are three parameters to evaluate: the background-subtracted mP values, the assay imprecision, and the change in polarization. Imprecision is the standard deviation of the mean of each group of mP values. This should generally be less than 10 mP. Assay range as the change in polarization is calculated by subtracting the mean mP of free tracer from the mean mP of the [Protein + Tracer]. There should be a plateau effect, with supraoptimal concentrations of binder yielding no further increase in mP. The concentration of binder giving imprecision less than 10 mP and the largest change in mP will provide the greatest assay range. However, a concentration of binder which is slightly subplateau gives better performance and consumes less of the precious reagent. Ideally, the net change in polarization should be greater than 70 mP.

   Signal-to-noise may be calculated by dividing the net change by the standard deviation. Thus steps to increase the net polarization change and/or decrease the standard deviations increase the assay performance.

4. **Titer competitor in one or more assay formulations.** Competitor may be unlabeled tracer or other molecules known to inhibit binding of tracer to binder. Use tracer and binder at concentrations determined in step 3 to provide a substantial increase in mP; now addition of a competitor will return the polarization back down to that of free tracer. There should be a sigmoid inhibition curve. Use a 2-, 3-, or 5-fold dilution series of the competitor(s) and cover several logs of dilution to obtain the most descriptive results. The midpoint, giving a net decrease of 50% of polarization, is the IC_{50} (IC means the inhibitory concentration). The values may be compared to those published in the scientific literature, or obtained by other methods in your laboratories. Again, evaluate imprecision and the net change in polarization.

5. **Assay characterization.** At this point it may be useful to consider the number of reagent addition steps in the assay and the possibility of combining the tracer and binder as a single reagent. Competitor(s) such as calibrators, and positive and negative controls should be added in the volume and buffer that compounds will be provided. For example, if compound is to be provided in a buffer with 10% DMSO, then the competitor also should be in this form. Include

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additional control groups to evaluate the sensitivity of the system to solvents (DMSO), to incubation time and temperature, and to reagent storage stability. Other performance criteria may also be evaluated at this phase.

6. **Troubleshooting.** If the imprecision or net polarization change is unacceptable, take steps to evaluate the source of imprecision. Evaluate likely sources of imprecision (pipeting, instrument, buffer, tracer, protein); each component contributes to total imprecision. The major (greatest) source of imprecision should be the most rewarding area for improving assay performance. For inadequate net polarization change, evaluate other tracers or binders, if available. Maximum polarization values of 200 – 300 mP (or higher) are infrequently achieved. A number of factors can contribute to the lowering of the theoretically maximum obtainable polarization value. Some factors that can influence this are quenching of the fluorophore by the molecules themselves, buffer quenching, adsorption onto surfaces, rotational spin (the 'propeller effect') and low affinity of interaction between the components.

   The maximum theoretical mP value obtainable is 500 mP. Hence any experimental value greater than this suggests an artifact within the assay. In such instances, controls should be checked. Occasionally some components give rise to large background intensity values that will mask the polarization effect if not adequately controlled.

   As an additional check on the system, it is advisable to re-read the plate in the fluorescence intensity mode. If the same amount of tracer is present in each well, then there should be equal intensity values across the plate in the fluorescence intensity mode. However, if there are any particular quenching or enhancing effects from some of the sample conditions themselves (such as binding to the protein), or imprecision in reagent addition steps, these may be identified by the variability in intensity measurements

General considerations

- **Controlling for extraneous polarization.** Two methods may be used to control for polarization which is not due to the biochemical binding events of interest. One source of extraneous polarization is the optical surface in the instrument's light path; this is corrected by the G factor. A second source of irrelevant polarization is that contributed by the buffers; this is controlled by background subtraction.
- **Minimal mP.** The polarization value (in mP) is an indication of the rate of molecular tumbling of a fluorescent molecule. It is related to the molecular size and molecular shape under constant temperature and viscosity. For example, a molecule of 3 kD may give a polarization value of 30 mP, whereas a 5 kD molecule may give a value of 60 mP. For a rigid, spherical fluorescein tracer, the mP reaches maximal at about 10 kD. Maximum is 500 mP.
- **Maximal mP.** When a small free tracer is bound to a large molecule, the mP is expected to increase. A good FP assay usually has a mP change of 100 or more.
- **Evaluation of data.** The performance of an assay may be evaluated and expressed in many ways. Two parameters are particularly valuable in describing the performance of an FP-based receptor-binding assay. The first is the net increase in polarization upon addition of receptor. This can be calculated by subtracting the mP of the free tracer from that of the [tracer plus receptor] group. This is the mP change referred to in the above paragraph. A second parameter is the imprecision of the measurement. This is the standard deviation of the signal, given in mP. Because all experimental groups in these FP assays contain the same amount of fluorescence, the signal and the standard deviation of that signal is expected to be independent of the amount or effect of the compounds, protein or controls. A useful way of combining these two parameters is similar to a signal-to-noise value, where the net (or 'delta') mP corresponds to signal and the imprecision (standard deviation) corresponds to the 'noise'.
- **Reagents.** If you lack reagents ready for FP, there are many commercially available kits for binding assays.

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