Published in final edited form as:

Methods Enzymol. 2008 ; 450: 253–272. doi:10.1016/S0076-6879(08)03412-5.

Using Fluorophore-labeled Oligonucleotides to Measure Affinities of Protein-DNA Interactions

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Abstract

Changes in fluorescence emission intensity and anisotropy can reflect changes in the environment and molecular motion of a fluorophore. Researchers can capitalize on these characteristics to assess the affinity and specificity of DNA-binding proteins using fluorophore-labeled oligonucleotides. While there are many advantages to measuring binding using fluorescent oligonucleotides, there are also some distinct disadvantages. Here we describe some of the relevant issues for the novice, illustrating key points using data collected with the F plasmid relaxase domain and a variety of labeled oligonucleotides. Topics include selection of a fluorophore, experimental design using a fluorometer equipped with an automatic titrating unit, and analysis of direct binding and competition assays.

Introduction

DNA-binding proteins perform or regulate myriad cellular activities. To fully appreciate the function of these proteins, we need to determine their affinities and specificities for DNA. Numerous methods have been developed and employed to characterize the interactions between proteins and DNA. For example, in a filter-binding assay, varying protein concentrations are combined with a radiolabeled oligonucleotide, and bound oligonucleotide is separated from free by filtration through nitrocellulose disks. In the conceptually similar electrophoretic mobility shift assay (EMSA), bound and free DNA are separated owing to their differing mobilities in a nondenaturing polyacrylamide gel. Using isothermal titration calorimetry (ITC), binding is observed by heat release or absorption occurring when protein and DNA are combined. While these methods are still widely used, researchers are increasingly following binding by observing changes in fluorescence emission intensity and anisotropy. The attraction is due in part to the diversity of available fluorophores, and the decreased cost of commercial syntheses.

Fluorescence as a technique to measure binding is powerful and useful, but also has limitations and some inherent complications. These potential problems may be obvious to the experienced fluorescence spectroscopist, but they could be troublesome for the unsuspecting novice. Here we hope to point out some of the strengths, weaknesses, and pitfalls of these methods for those starting out. Those desiring more detailed discussions of the topics in fluorescence introduced here are referred to *Principles of Fluorescence Spectroscopy* (Lakowicz, 2006).

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Definitions of fluorescence anisotropy and intensity

Frequently, the binding of protein to a fluorophore-labeled oligonucleotide is monitored by changes in fluorescence anisotropy. For anisotropy measurements, the solution is excited by a polarized beam of light at an appropriate wavelength. Use of a polarized excitation beam photoselects the subset of the fluorophores that is oriented properly in solution relative to the incoming light. If the excitation beam is vertically polarized, the vertically ($I_{\parallel}$) and horizontally ($I_{\perp}$) polarized emissions are measured and used to calculate the anisotropy, r , according to:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (1)$$

There is a directionality to the fluorescent emission as well as the excitation, and the directionality of the excitation and emission are related. Therefore, a fluorophore that rotates slowly in solution relative to its fluorescent lifetime (τ , the time required for the excited population to decay to $1/e$ of its initial value) will have a large $I_{\parallel}$ value and small $I_{\perp}$ value and therefore a relatively large anisotropy. For fluorophores that tumble rapidly relative to their fluorescent lifetime, $I_{\parallel}$ and $I_{\perp}$ will be similar and the anisotropy will be near 0. Fluorescence anisotropy is a useful measure for binding because the rotation of biological macromolecules in solution occurs on the same nanosecond time scale as the fluorescent lifetime of many commonly used fluorophores. Frequently the binding of a protein to a fluorescently-labeled oligonucleotide will increase the mass sufficiently to significantly decrease the rotational diffusion of the fluorophore. Thus, binding can be followed by the change in anisotropy. One disadvantage to using anisotropy to follow binding is that the use of polarimeters reduces the intensity of the excitation beam, and higher concentrations of the fluorophore-labeled oligonucleotide may be required to obtain sufficient signal.

The fluorescence emission intensity of a fluorophore-labeled oligonucleotide also can change upon binding of a protein. The fluorescent properties of dyes can vary considerably with changing environment, and a proximally bound protein can enhance or decrease fluorescence emission. The change in emission intensity as protein is titrated into the solution can be a convenient measure of protein binding, but it can also complicate anisotropy measurements. The conditions that cause these intensity changes frequently change the fluorescent lifetime of the fluorophore. Anisotropy and fluorescent lifetime are related, as is evident in the Perrin equation:

$$r = \frac{r_0}{1 + (\tau/\theta)} \quad (2)$$

where r is expected anisotropy, r_0 is anisotropy in absence of rotational diffusion, τ is the fluorescent lifetime and θ is the rotational correlation time. One assumption inherent in the analysis of binding curves is that the change in signal is proportional to the change in the distribution of molecules between the unbound and bound states. When binding is accompanied by changes in both fluorescence anisotropy and emission intensity, this proportionality starts to break down. Although this effect may be minor in most cases, it can be dealt with by fitting both intensity and anisotropy data with the program SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b). Use of this program has the added benefit that each calculated binding constant results from fitting two data sets, potentially improving the quality of the fits. Alternatively, if the change in fluorescence intensity upon binding is sufficient, intensity changes alone can be used to follow binding.

In summary, fluorescence anisotropy is expected to increase when a protein binds a fluorophore-labeled oligonucleotide. Fluorescence emission intensity may also change, and if used in the analysis, this additional data can be considered a bonus. Alternatively, the intensity data may be used alone. The effect of binding on both intensity and anisotropy should be assessed as part of the characterization of the system.

Advantages of fluorescence measurements

There are several advantages of fluorescent measurements over those using radioactivity. Unlike use of radioactivity, use of fluorophores does not require special licensing and incurs no costs for disposal of radioactive waste. The two-week half-life of ^{32}P limits the useful lifetime of an end-labeled oligonucleotide, which may necessitate multiple costly and time-consuming labelings. In contrast, a commercially synthesized and purified fluorophore-labeled oligonucleotide can be aliquoted and stored indefinitely in a freezer (repeated freeze-thaw cycles, however, should be avoided). In addition, although these syntheses generate only nmol quantities of the oligonucleotides, the sensitivity of fluorescence and fluorometers is sufficient to allow the yield to last for dozens of measurements.

Using fluorescence, affinities can be measured in solution without a step such as electrophoresis or filtration designed to separate free and bound oligonucleotide. Thus fluorescence measurements may yield true equilibrium binding constants. Furthermore, results from solution-based assays may be more accurate for measurements of lower affinity interactions, which are more likely to be affected by dissociation during the separation step than high affinity interactions. Another advantage is that studies in which the influence of pH or salt concentrations on binding are examined to explore the intermolecular forces involved may be easier to perform because there is no worry that the conditions in the binding reaction and in the separation step differ meaningfully. As discussed later, however, these studies may be complicated by the behavior of some fluorophores as solution conditions are altered.

ITC, like fluorescence intensity and anisotropy, may be used to follow equilibrium binding in solution without the need for a separation step. ITC has the added benefit that it also provides information on the entropic and enthalpic contributions to binding (Oda and Nakamura, 2000). Calorimetric titrations, however, often require considerably more material and higher concentrations than comparable fluorescence experiments. These requirements can present particular problems if the protein under study is difficult to purify or has low solubility. In addition, the high concentrations used in ITC experiments can make it difficult to accurately measure binding when dissociation constants drop much below the micromolar range.

Fluorescence has considerable advantages when the kinetics of binding are being measured. Many fluorometers, even the most basic models, can collect intensity data in a kinetic mode, allowing continuous collection of data points. Scores or hundreds of time points can be collected over the course of a binding reaction, yielding well-defined binding curves. In contrast, other methods can be adapted to collect kinetic data, but are not nearly as amenable to collecting large data sets.

Disadvantages of fluorescence measurements

Fluorescence measurements have several disadvantages relative to electrophoretic mobility shift assays (EMSA) using ^{32}P -end-labeled oligonucleotides. One is the cost of equipment. One can obtain useful information from an EMSA with a nondenaturing gel, buffers, a power supply, a gel dryer, film and developer. In contrast, fluorescence measurements require a comparatively expensive fluorometer. Another disadvantage is the lower

sensitivity of fluorescence, relative to radioactivity. Picomolar concentrations of ^{32}P -labeled oligonucleotides provide ample signal for an EMSA, while nanomolar concentrations of a fluorophore-labeled oligonucleotide may be necessary for a suitable signal-to-noise ratio. This can present a problem for high affinity ($K_D \sim$ subnanomolar) reactions because essentially all protein titrated into the reaction will bind the oligonucleotide, thus making it difficult to obtain a useful binding constant from the binding isotherm. If this poses a problem, however, binding can be measured under different conditions (for example, higher ionic strength) that will reduce the affinity to a more accessible range.

Another problem with fluorescence measurements is that they may not report well on other than 2-state reactions. Fluorescence measurements work well when observing a single transition between the unbound and bound states that is accompanied by a substantial change in anisotropy or intensity. Fluorescence also can work well for more complex systems, at least in some cases. If, for example, there are well-separated transitions between different bound states, the situation should be immediately apparent and an appropriate binding model developed. If multiple protein molecules bind with high cooperativity, this situation may be evident by poor fitting of a simple (stoichiometry 1:1) binding model to the data, with the slope of data points through the transition being greater than the slope of the fit through the region. If, however, there are multiple bound states and poorly separated transitions, it is likely that the binding model selected for analysis will be too simple. Unfortunately, it may still be possible to obtain reasonable quality fits to these data with a simple binding model, leaving the investigator with few indications that the studies are flawed. In contrast, EMSA data can provide additional information in the form of shifted bands. The presence of multiple shifted bands indicates that different binding stoichiometries are present, and these are useful in determining an appropriate binding model. If an appropriate gel imaging system is available, such as a GE Healthcare Typhoon 9410, and an appropriate fluorophore is used, a researcher can use the same oligonucleotide for solution and EMSA studies, with the complementary approaches providing additional insight into the protein-DNA interaction.

Another disadvantage of using fluorophore-labeled oligonucleotides is that the fluorophores are large enough to potentially interfere with or otherwise influence binding (Wang et al., 1998). In contrast, ITC experiments can be performed with unmodified DNA oligonucleotides, and EMSA experiments can use oligonucleotides end-labeled with ^{32}P phosphate, a comparatively minor modification. To check whether the fluorophore is affecting binding, routine characterization of an oligonucleotide should include comparing the measured K_D of binding with the measured K_I of an unlabeled version of the same oligonucleotide measured by competition experiments (Stern and Schildbach, 2001; Titolo et al., 2003b). (Competition assays are discussed later in this review.) Agreement between the K_D and K_I values indicates that the fluorophore has little effect on the binding.

In summary, while measuring binding using fluorophore-labeled oligonucleotides requires access to a fluorometer, the method can measure binding in solution using less material than ITC and without the radioactive waste generated by EMSA. EMSA, however, can more directly report on stoichiometry of binding through the presence of multiple bands that indicate different bound species. To make sure an appropriate binding model is selected, an investigator should pay careful attention to any systematic deviation of the fit from the data points, and if possible should use multiple experimental approaches to confirm any proposed model.

Designing the oligonucleotide

In the last decade or so, preparing fluorophore-labeled oligonucleotides has progressed from a largely do-it-yourself endeavor involving fluorescein or rhodamine or one of their derivatives to one where an oligonucleotide labeled with one of dozens of different fluorophores can be commercially synthesized, purified, and delivered for a reasonable price. Available fluorophores have a variety of excitation and emission spectra with maxima above 500 nm, well above the absorbance maxima of soluble protein and DNA, reducing the possibility of inner filter effect (interference caused by light absorbance by the solutions). Many of these fluorophores are relatively insensitive to photobleaching, have large extinction coefficients (reflecting their ability to absorb photons) and have reasonable quantum yields (the fraction of photon emitted per photon absorbed), all of which are beneficial.

While several fluorophores that can be linked to oligonucleotides are available, their differing properties may make one optimal for one assay and a poor choice for another. To illustrate this point, we provide curves generated by the binding of TraI36 to single-stranded DNA (ssDNA) oligonucleotides 3'-end-labeled with different fluorophores. TraI36, a 36-kDa N-terminal fragment of the TraI protein of the conjugative bacterial plasmid F factor, binds with sequence-specificity and subnanomolar K_D to a single-stranded oligonucleotide containing a plasmid sequence (Stern and Schildbach, 2001). All oligonucleotides were synthesized and HPLC-purified by Integrated DNA Technologies. While we believe that the curves presented are useful examples, the reader should keep in mind that the results are to some extent situational and cannot necessarily be extrapolated to another experimental system.

Figure 1 depicts the changes in fluorescence anisotropy and intensity that occur upon binding of the TraI36 protein to 17-base ssDNA oligonucleotides labeled with various fluorophores. All three fluorophores show significant increases in fluorescent anisotropy, with the increase of an indocarbocyanine-3 (Cy3)-labeled oligonucleotide being the greatest. All three also show changes in fluorescence emission intensity, but the intensity of the Cy3-labeled oligonucleotide falls, while the intensities of carboxytetramethylrhodamine (TAMRA) and 6-carboxyfluorescein (6-FAM)-labeled oligonucleotides rise.

The linker used to connect the fluorophore to the oligonucleotide can also affect results. Shown in Figure 2 is a comparison of two 22-base oligonucleotides 3'-end-labeled with TAMRA, one synthesized using a TAMRA-modified CPG (controlled pore glass) column and the other linked through a succinimidyl (NHS) ester. The CPG-derived oligonucleotide, which has the fluorophore attached via a linker to a modified thymine, shows significantly greater increases in fluorescence emission intensity and anisotropy than the NHS oligonucleotide. In addition, the NHS-linked oligonucleotide, which has the fluorophore attached to the 3'-phosphate of the oligonucleotide via a 6-carbon linker, also yields a significantly sloped upper baseline for both intensity and anisotropy plots. The combination of small signal change and sloped upper baseline makes for data to which it is difficult to fit a model.

Sometimes the sequence of the oligonucleotide can affect the properties of the attached fluorophore. Time-resolved fluorescence studies of various tetramethylrhodamine (TMR)- or TAMRA-labeled oligonucleotides demonstrate that the fluorophore can have multiple fluorescent lifetimes, suggesting that the probe exists in multiple environments (Edman et al., 1996; Eggeling et al., 1998; Harley et al., 2002; Vamosi et al., 1996). One of these lifetimes may involve an interaction between fluorophore and DNA bases, which can potentially quench fluorescence emission or otherwise affect the measured values (Sauer et

al., 1995; Seidel et al., 1996; Sevenich et al., 1998). Fluorophore-nucleic acid interactions have also been proposed for Cy3 and fluorescein (Nazarenko et al., 2002; Nelson et al., 1993). Because these interactions can influence behavior of the fluorophore, two labeled oligonucleotides having the same recognition sequence placed within different contexts may have significantly different properties. Some fluorophores, notably TMR and TAMRA, which are more sensitive to their environment than other dyes, may show a greater context dependence than others.

The length of the oligonucleotide, and more specifically the distance between the fluorophore and the bound protein, can affect the results (Titolo et al., 2003a). We found that TraI36 binds 3'-TAMRA-labeled 17-base and 22-base ssDNA oligonucleotides (which contains an extra 5 bases at the 3' end) with similar affinities, but the shorter oligonucleotide exhibits greater increases in fluorescence anisotropy and intensity upon binding (Harley et al., 2002). This is a general phenomenon in our system, with 17-base oligonucleotides 3'-labeled with Cy3, 6-FAM, and NHS or CPG-linked TAMRA showing greater changes in intensity and anisotropy upon TraI36 binding than 22-base 3'-labeled oligonucleotides (Figure 3 and data not shown). The differences in anisotropy can be explained by noting that anisotropy is a composite of the individual motions of the fluorophore and the global rotational diffusion of the macromolecule to which it is conjugated. A greater length of highly flexible ssDNA between dye and bound protein likely translates into a greater contribution of local motions to anisotropy in both bound and unbound states, and a smaller change in anisotropy upon binding. To what degree these observations with single-stranded DNA can be generalized to double-stranded DNA (dsDNA) is not clear, especially given the more rigid nature of dsDNA relative to ssDNA.

As mentioned previously, one advantage to using fluorescence to follow formation of protein-DNA complexes is that it is possible to determine the effect of changing pH or salt conditions on binding without worrying whether the conditions for the binding reaction can be maintained while bound and free DNA are separated. Properties of fluorophores, however, are often sensitive to pH and salt. For example, the fluorescence intensity of a TAMRA-labeled oligonucleotide can decrease with increasing NaCl concentrations, while its fluorescence anisotropy increases (Vamosi et al., 1996). Both fluorescence intensity and anisotropy of a TAMRA-labeled oligonucleotide decrease with increasing temperature (Vamosi et al., 1996). The fluorescence emission intensity of fluorescein falls dramatically below pH 7. As shown in Figure 4 and previously (Stern et al., 2004), while the fluorescence anisotropy of 22-base ssDNA oligonucleotides 3'-labeled with Cy3 or TAMRA generally increases upon binding by TraI36, at pH 10.5 and above the anisotropy decreases. Provided that binding causes a significant change in fluorescence anisotropy or emission intensity, the effect of pH or salt or temperature on the behavior of the fluorophore probably will not affect data interpretation in most cases. It is possible, however, that a reduction in signal relative to noise could reduce the quality of data fits and, in the worst case, bury the transition in the noise.

Fluorophores can potentially interact with the protein, altering its fluorescent properties. Often this is advantageous, causing a greater increase in anisotropy or a change in intensity than would have occurred in absence of the fluorophore-protein interaction. The situation, however, can complicate analysis when examining the effects of amino acid substitutions. As part of a mutagenesis study designed to examine the contributions of various amino acids to TraI36 DNA recognition, we generated a series of protein variants with Ala substituting for contact residues (Harley and Schildbach, 2003; Larkin et al., 2005). Usually, the effects of variant proteins on anisotropy and intensity of a 3'-TAMRA-labeled 22-base oligonucleotide were similar to those caused by binding of the wild-type protein. Two exceptions, R150A and E187A, affect fluorescence differently than the wild-type protein

and other variants. As shown in Figure 5, R150A causes a smaller fluorescence anisotropy change when it binds, and E187A causes a larger change, than the change caused by binding of the wild-type protein. In addition, both variant proteins show a steeply sloped upper baseline on the anisotropy plot, complicating analysis. Because R150 and E187 have opposite charges and Ala substitutions for them cause distinct effects on the fluorophore's behavior, one possibility is that altered electrostatic interactions between fluorophore and protein may explain the altered fluorescent characteristic. An examination of the TraI36 structure (Datta et al., 2003), however, shows that residues 150 and 187 are located nearly 30 Å apart, making a model involving direct charge-charge interactions seem less likely.

Given the differing properties of the fluorophores and the effects that altered conditions can have on them, how should an oligonucleotide be selected for binding studies? First, proceed with caution. Time and money spent at the beginning in an effort to select and characterize a fluorophore is well spent. Second, the greater information available for certain dyes may give them an edge. For example, David Lilley and colleagues determined the NMR structure of a Cy3-labeled oligonucleotide (Norman et al., 2000). They found that that 5'-labeled Cy3, attached by a 3-carbon-tether, stacked over the terminal C-G pair. This interaction may account for the stabilizing effect that Cy3 can have on an oligonucleotide duplex (Moreira et al., 2005). This Cy3 characteristic may benefit anisotropy studies because if the local motions of the fluorophore are limited, such as by stacking over the terminal base pair of the oligonucleotide, then the anisotropy will more directly report on the binding by the protein and potentially will lead to greater changes in anisotropy. Another advantage is that if the fluorophore is stacked over the terminal base pair, it is less likely to be interacting directly with the protein and thus causing unexpected results. Because the behavior of Cy3 is better understood than many fluorophores, Cy3 is one of the more attractive choices of fluorophore. Finally, the equipment may in part dictate the fluorophore used. If one wishes to characterize the binding by both fluorometer and gel imaging system, the fluorophore has to be compatible with both systems. The allowed excitation wavelengths may be restricted by the light sources in, or the filters available for, the equipment to be used.

Designing the experiment

Once the oligonucleotide is designed and the protein purified, a binding curve can be generated. Prior to collecting data, though, a number of parameters should be assessed. Perhaps the first item to check is the absorbance of the protein solution and binding buffer at the excitation and emission wavelengths. If the solution absorbs significantly (>0.1 optical density units at the excitation wavelength), inner filter effect can interfere with measurements. If binding is to be followed by observing a change in fluorescence emission, emission spectra of unbound and bound oligonucleotide should be collected to determine the optimal emission wavelength for the experiment. In addition, simple measurements of the association and dissociation kinetics should be made to determine reasonable equilibration periods that should elapse between addition of protein to the fluorometer cell and collection of data.

Regarding the data collection itself, an accurate fit requires well-defined upper and lower baselines, in addition to several data points through the transition. If performing a manual titration, such as those depicted in Figures 1–5, a decent curve can be collected given a bit of planning, a rough estimate of affinity, and a pair of pipettors. If using a fluorometer outfitted with an automatic titrator, such as one in the Hamilton Microlab 500 series, the task is a little more complicated. While automatic titrators deliver the protein to the fluorometer cell with greater precision than is possible manually and save the user considerable time, the simple models are not particularly well designed to deliver protein over the three to four logs of concentration required to generate a good curve. Again, though, with some planning,

generating a high-quality binding curve is possible. Shown in Figure 6 is a binding curve of a TraI36 variant binding to a ssDNA sequence from the R100 plasmid (4 nM oligonucleotide, $K_D = 7$ nM). Using a titrator equipped with a 50 μ l syringe, a protein solution of 250 nM, and a program using three concentration step sizes (0.08 nM, 3 nM, 65 nM), we can cover the entire binding curve well. Titration schemes usually have to be optimized for each protein studied to make sure that the concentration jumps that occur when step sizes change do not occur at positions that would cause a less accurate fit, such as at the beginning of the transition.

Once the data are collected, a model may be fit to them to yield the binding constant. If fluorescence anisotropy was measured, the values may be used in a fit without further manipulation. If fluorescence emission intensity data were collected, the intensity values must be corrected for volume changes during the course of the experiment. As protein is titrated, the total volume increases and the fluorophore concentration, and thus emission intensity, decreases. To correct for the dilution, simply multiply the intensity value at protein concentration P by the ratio of the total volume in the fluorometer cell at P to the initial volume.

For the simple reaction having a 1:1 stoichiometry

where P is protein, D is DNA, and PD is the complex, the dissociation constant, K_D , is defined

$$K_D = \frac{[P_F][D_F]}{[PD]} \quad (4)$$

where $[P_F]$ and $[D_F]$ are the concentrations of free protein and free DNA respectively. Because the total DNA concentration ($[D_T]$) is the sum of $[D_F]$ and $[PD]$, and $[P_T]$ is the sum of $[P_F]$ and $[PD]$,

$$K_D = \frac{[P_F][D_F]}{[PD]} = \frac{([P_T] - [PD])([D_T] - [PD])}{[PD]} \quad (5)$$

Multiplying through and rearranging terms yields

$$[PD]^2 - ([PD] \times ([D_T] + [P_T] + K_D)) + ([D_T][P_T]) = 0 \quad (6)$$

Solving for $[PD]$ using the quadratic equation

$$[PD] = \frac{(D_T + P_T + K_D) - \sqrt{(D_T + P_T + K_D)^2 - 4[D_T][P_T]}}{2} \quad (7)$$

Using this definition of [PD] we can fit data with the formula

$$\theta = ((B_U - B_L) \times \frac{[PD]}{[D_T]}) + B_L \quad (8)$$

where θ is fraction DNA bound, B_U is the upper baseline and B_L is the lower baseline.

Competition assays for determining specificity

Frequently, determining the specificity of a protein-DNA interaction is as important as defining the affinity of the interaction. To measure specificity, one could, in principle, synthesize (or, more likely, pay to have synthesized) a series of variant oligonucleotides, all similarly fluorescently labeled, and measure the affinity of the protein for each. The expense of a large panel of labeled oligonucleotides, however, is prohibitive, limiting the sequence space that can be examined.

Alternatively, unlabeled oligonucleotides may be used in competition assays. One common method is to combine protein and labeled wild-type oligonucleotide, then titrate unlabeled variant oligonucleotide to compete away binding of the labeled oligonucleotide. This approach has the advantage that less expensive unlabeled oligonucleotides, instead of fluorophore-labeled oligonucleotides, can be used to define binding specificity. We have had some problems with this approach, however. Even if we convert the IC_{50} value (concentration of unlabeled competitor oligonucleotide required to compete away 50% of the binding of labeled oligonucleotide) obtained in the competition experiment to a K_I value (the equivalent of a K_D for an inhibitor) using the method of Cheng and Prusoff (Cheng and Prusoff, 1973), the method underestimates our high affinity interactions. We have also observed changes in fluorescence emission intensity of our labeled oligonucleotide in the presence of high concentrations ($\sim 1 \mu M$) of an unlabeled competitor oligonucleotide, perhaps the result of interactions between the oligonucleotides. The resulting shift in the baseline makes fitting a model to the data challenging. While these apparent oligonucleotide interactions may be a problem specific to our system, or one that is more likely to plague ssDNA oligonucleotides than dsDNA oligonucleotides, they still are potential obstacles.

We have had greater success with a second competition method that assesses the binding of the protein to a labeled oligonucleotide in the presence of unlabeled competitor oligonucleotide. We then calculate the affinity of the protein for the competitor oligonucleotide based on the apparent affinity of the protein for the labeled oligonucleotide. The approach has several advantages. It requires less competitor oligonucleotide than a complete competition with the competitor, even if it does require more protein. If, as in our case, interactions between oligonucleotides at high concentration are a problem, these interactions may be minimized by carefully selecting the concentrations of oligonucleotides. Finally, because the assay does not require all binding of the labeled oligonucleotide to be competed away to obtain a complete curve and a good fit to the data, it may be more suitable for low affinity interactions.

Zhi-Xin Wang published an expression that can be fit to data produced by this type of competition assay (Wang, 1995). (Because the derivation is detailed, we refer the reader to the paper rather than recapitulate it here.) We have found that using this method in test cases, the binding constant measured for an unlabeled competitor correlates well with the binding constant for the labeled version of that oligonucleotide. The method has limitations, however. Because the assay is indirect, it requires that the concentration of unlabeled competitor be accurately known. Well-defined baselines and numerous points through the

transition are essential to obtaining a high quality fit to the data. Finally, because two oligonucleotides with potentially different binding characteristics are used in the reaction, the equilibration times must be chosen carefully to ensure that true equilibrium measurements are made.

Conclusions

Using fluorophore-labeled oligonucleotides to measure the affinity and specificity of DNA recognition has many advantages. If a researcher has access to appropriate equipment, especially if that equipment is a fluorometer outfitted with an automatic titrator, a relatively modest investment in set-up time can yield a wealth of binding data. As for any measurement, however, understanding the behavior of the system is crucial for subsequent successful measurements. In addition to carefully selecting the length and sequence of the DNA to be used in the assay and to determining the stoichiometry of the complex, a suitable fluorophore must be selected. As shown here, different fluorophores can demonstrate dramatically different behavior when attached to the same oligonucleotide, underscoring the importance of initial characterization to successful measurements.

Acknowledgments

The authors thank Professor Lenny Brand, and Drs. Dima Topygin and Mike Rodgers for their generosity with their time, equipment and expertise. This manuscript is based on work supported by National Institutes of Health grant GM61017 to J.F.S.

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Figure 1.

Binding of TraI36 to a fluorophore-labeled single-stranded oligonucleotide has different effects on different fluorophores. The changes in fluorescence intensity (top) and anisotropy (bottom) are shown for a 17-base oligonucleotide (5'-TTTGCGTGGGGTGTGTG-3') 3'-labeled with TAMRA (open circles), 6-FAM (filled triangles), or Cy3 (filled squares). The solid lines depict simultaneous fits to intensity and anisotropy data using SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b) as described (Stern and Schildbach, 2001).

Figure 2.

The linkage between fluorophore and DNA can affect fluorescent properties. TraI36 titrations into solutions of 22-base (5'-TTTGCGTG GGGTGTGTGCTTTT-3') single-stranded oligonucleotide 3'-labeled with TAMRA using either a CPG (open circles) or NHS (filled diamonds) linkage are shown. The solid lines depict simultaneous fits to intensity and anisotropy data using SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b) as described (Stern and Schildbach, 2001).

Figure 3.

The proximity of fluorophore to bound protein can affect fluorescent properties. Single-stranded 22-base (5'-TTTGCGTGGGGTGTGTGCTTTT-3'; open squares) or 17-base (5'-TTTGCGTGGGGTGTGTG-3'; filled squares) oligonucleotides were synthesized with a 3'-6-FAM probe. Intensity (top) or anisotropy (bottom) changes occurring upon titration of TraI36 are shown. The solid lines depict simultaneous fits to intensity and anisotropy data using SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b) as described (Stern and Schildbach, 2001).

Figure 4. Changing solution conditions can alter fluorescent behavior of probes. TraI36 binding to 22-base oligonucleotides 3'-labeled with TAMRA (open circles) or Cy3 (filled squares) at pH 10.5 causes less significant changes to intensity (top) and decreases instead of increases in anisotropy (bottom) (compare to Figure 1). The solid lines depict simultaneous fits to intensity and anisotropy data using SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b) as described (Stern and Schildbach, 2001).

Figure 5.

Amino acid substitutions in TraI36 can alter fluorophore behavior. Shown are the increases in fluorescent anisotropy of a 3'-TAMRA-labeled 22-base oligonucleotide upon binding of wild type TraI36 (open circles) or the TraI36 variant proteins E187A (filled squares) or R150A (filled diamonds). The solid line depicts simultaneous fits to intensity and anisotropy data using SPECTRABIND (Toptygin and Brand, 1995a; Toptygin and Brand, 1995b) as described (Stern and Schildbach, 2001).

Figure 6.

Titration scheme using an automatic titrator. Shown is a curve for the binding of an F TraI36 variant protein to an oligonucleotide containing a sequence from the R100 plasmid. For an interaction with $K_D = 7$ nM and fluorescent oligonucleotide concentration of 4 nM, three step sizes (0.08 nM step size to 0.96 nM, 3 nM step size to 30 nM, 65 nM step size to 400 nM) were used to generate this binding curve. Note the breaks at the point at which the step size changes. The titration scheme has to be adjusted to prevent these from occurring at points in the curve where they could reduce the quality of the fit. The solid line depicts a fit to the data using KaleidaGraph as described (Larkin et al., 2007).