

## [1] Fluorescence Anisotropy Assays for Analysis of ISWI-DNA and ISWI-Nucleosome Interactions

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Fluorescence anisotropy is a rapid, sensitive, and quantitative technique that is well suited to the analysis of protein-protein and protein-DNA interactions in solution. Fluorescence anisotropy is a measure of the depolarization of emitted fluorescence intensity obtained after excitation by a polarized light source, and depends directly on the relative rate of fluorescence emission versus the rate of tumbling in solution. The concept is simple: if a fluorescent molecule (or, more typically, a molecule to which a fluorescent probe has been attached) tumbles slowly in solution relative to the lifetime of fluorescence emission, then the light emitted in response to polarized excitation will remain highly polarized. However, if the molecules tumble rapidly in comparison to the emission lifetime, then, prior to emitting, they will have tumbled sufficiently so as to have “forgotten” their orientation at the moment of excitation, thus depolarizing (randomizing the polarization of) the emitted light.

Fluorescence anisotropy is applicable for analysis of macromolecular interactions because there is a good match between typical fluorescence lifetimes and typical macromolecular tumbling times. For approximately spherical molecules, the tumbling time scales as the molecular volume, that is, as the molecular weight. Thus, binding of an unlabeled macromolecule can make a significant change to the tumbling time of the molecule to which the fluorescent probe is attached, and hence to the measured anisotropy. For the studies described in the following, we utilize DNA molecules labeled at one end with the fluorescent dye fluorescein (these DNA molecules may be “naked DNA” or they may be incorporated into nucleosomes), and we use fluorescence anisotropy to monitor the binding of the *Drosophila* ISWI chromatin remodeling protein<sup>1-3</sup> to the labeled DNA or nucleosomes.

Fluorescence anisotropy is especially useful because of its high inherent sensitivity. Dyes such as fluorescein allow quantitative analysis of emission polarization from sub-nanomolar concentrations. Since dissociation constants are typically nanomolar or greater, this allows experiments to be

<sup>1</sup> T. Tsukiyama, C. Daniel, J. Tamkun, and C. Wu, *Cell* **83**, 1021 (1995).

<sup>2</sup> P. D. Varga-Weisz *et al.*, *Nature* **388**, 598 (1997).

<sup>3</sup> G. Längst and P. B. Becker, *J. Cell Sci.* **114**, 2561 (2001).

set up with the probe concentration  $\ll K_d$ ; consequently the free concentration of the added macromolecule (ISWI, in our case), which is generally either difficult to measure or is completely unknown, will be approximately equal to the total concentration, which can be definitively measured, thus greatly simplifying the analysis of the binding measurements. Another important benefit of the sensitivity of the anisotropy measurement is that it preserves precious reagents. Measurements can be made in small volumes, and samples can be recovered and reused if desired.

Finally, as discussed later, the experiment can be carried out using inexpensive conventional fluorimeters such as are found at most biochemical or chemical research laboratories, or, alternatively, using an inexpensive instrument specialized for the fluorescence anisotropy experiment.

Investigators planning to carry out such studies should study two particularly useful references, one on fluorescence theory and methodology in general<sup>4</sup> and one focused on fluorescence approaches to analysis of protein-DNA interactions in particular.<sup>5</sup> These references nicely define and explain the set of four fluorescence intensity measurements that go into a single measurement of fluorescence anisotropy; we will not duplicate this important topic here, but rather refer readers to these other sources.

### Fluorescein-Labeled DNA

We use DNA sequences labeled with fluorescein attached at the 5'-end through a C6 linker. Relatively short sequences are purchased as a pair of complementary oligonucleotides, one containing 5'-fluorescein. These are annealed, and the resulting duplex purified away from any remaining single strand by reverse-phase HPLC on a Zorbax-10 column using a gradient of 10–20% acetonitrile in 0.1 M triethanolamine-acetate, pH 7.0, 0.1 mM EDTA, developed over 10 min at 1 ml min<sup>-1</sup>. When longer sequences (e.g., nucleosome-length DNAs) are required, direct synthesis is not practical. Instead we use preparative scale PCR, with one of the two primers again containing 5'-fluorescein. The resulting PCR product is purified by gel electrophoresis in 1% agarose gels with standard TAE buffer, and extracted from the gel using Ultra-DA (Millipore) gel extraction kits.

DNA concentrations are quantified by UV absorbance.

<sup>4</sup> J. R. Lakowicz, "Principles of Fluorescence Spectroscopy," 2nd Ed. Kluwer Academic/Plenum Press, New York, 1999.

<sup>5</sup> J. J. Hill and C. A. Royer, *Meth. Enzymol.* **278**, 390 (1997).

## Preparation of Nucleosomes

Nucleosomes are formed by salt gradient dialysis using purified histone octamer and DNA, and the resulting nucleosomes are purified by sucrose gradient ultracentrifugation, as described.<sup>6-8</sup> We typically label a small amount of the fluorescein-labeled DNA with [ $\gamma$ -<sup>32</sup>P] ATP (at the 5' end that does not have a fluorescein) using T4 polynucleotide kinase to facilitate following the sample throughout preparation and purification. Reconstitution reactions typically contain 300 ng of (<sup>32</sup>P, fluorescein) double-labeled DNA, 15  $\mu$ g of fluorescein-only labeled DNA, 3  $\mu$ g of histone octamer, in a 300  $\mu$ l volume of 2.5 M NaCl, 0.5  $\times$  TE (TE is 10 mM Tris, pH 8.0, 1 mM Na<sub>3</sub> EDTA) with 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM benzamidine (BZA) added as protease inhibitors. The reconstitution reactions are loaded onto  $\sim$ 12 ml 5–30% sucrose gradients in 0.5  $\times$  TE and centrifuged at 4 $^\circ$  in an SW41 rotor (Beckman) at 41,000 rpm for 22–24 h. (We aim for substoichiometric reconstitution of histone octamer onto DNA, as this eliminates the possibility of overloading nucleosomes with excess histones<sup>9</sup> while providing useful diagnostics for the reconstitution and markers for the subsequent sucrose gradient purification.) Gradients are fractionated from the bottom in 0.5-ml fractions; fractions containing nucleosomes are identified by scintillation counting, pooled and exchanged into 0.5  $\times$  TE buffer on Centricon-30 concentrators, and analyzed by native polyacrylamide gel electrophoresis. Nucleosome concentrations are measured by UV absorbance at 260 nm. Reconstituted nucleosomes are stored at concentrations of 50 nM or greater, on ice in 0.5  $\times$  TE, and are used within 2 weeks.

## Instrumentation and Technical Considerations

We use a conventional photon counting steady-state fluorometer (ISS PC-1, L-format) with rotatable polarizers in the excitation and emission paths. Alternatively, the Panvera Corporation markets a sensitive and relatively inexpensive instrument dedicated specifically to fluorescence anisotropy measurements. We generally increase the sensitivity of the PC-1 by removing the emission monochromator and use instead a set of optical filters chosen to pass a desired broad band of fluorescence emission wavelengths, as described later.

<sup>6</sup> K. J. Polach and J. Widom, *J. Mol. Biol.* **254**, 130 (1995).

<sup>7</sup> P. T. Lowary and J. Widom, *J. Mol. Biol.* **276**, 19 (1998).

<sup>8</sup> J. D. Anderson, A. Thåström, and J. Widom, *Mol. Cell. Biol.* **22**, 7147 (2002).

<sup>9</sup> G. Voordouw and H. Eisenberg, *Nature* **273**, 446 (1978).

### *Sample Cleanliness*

As with any sensitive experimental method in analytical biochemistry, care must be taken in certain matters to avoid potential pitfalls.

It goes without saying that both buffers and samples must be free from significant fluorescent contaminants. Fluorescence from the buffer alone and from unlabeled samples should be checked and shown to be negligible in comparison to the fluorescence obtained at the desired concentration of labeled sample. In our experience this has never proven to be a problem using dilute buffers supplemented with approximately physiological concentrations of salts and  $Mg^{2+}$  and small amounts of glycerol; nevertheless, it should be checked, especially in case of problems with the water or with contaminated glass- or plastic-ware.

### *Scattered Light*

Even when samples are free of contaminants, one must take care to eliminate certain additional potential artifacts due to scattered light. Scattered light is particularly problematic in anisotropy measurements because it is generally perfectly polarized, and hence will systematically distort measurements of fluorescence anisotropy from the sample.

Two chief types of scattered light need to be considered in anisotropy measurements: elastic (Rayleigh) scattering and inelastic (chiefly Raman) scattering. Elastic scattering is a process in which excitation light is scattered in all directions, unshifted in wavelength, by interaction of the excitation light with molecules in the sample. Even pure solvents scatter light elastically. Such scattering is weak, yet may nevertheless be significant in comparison to the faint fluorescence from a very dilute fluorophore. Macromolecules in solution greatly increase the intensity of scattered light, in proportion to their concentration and molecular weight. Solutions containing a high molecular weight species such as nucleosomes can result in a scattering intensity that greatly exceeds the intensity of fluorescence emission from a dye attached to that same macromolecule.

If excitation and emission monochrometers were "perfect," then elastic scattering would present no problem: one could simply set the excitation and emission monochrometers to different wavelengths (e.g., the excitation and emission maxima, respectively), and there would be no leakage of scattered excitation light through the emission monochrometer. In fact, however, the finite resolution of the monochrometers, together with optical imperfections that allow light of colors outside the assumed bandpass to pass through, albeit at reduced intensity, are such that there can be significant excitation intensity at the color chosen for emission measurement, even though these colors may differ by 30 nm or more. In fact, this leakage

can in practice be so great that, when combined with a relatively strong elastic light scattering from a macromolecular sample, the intensity of scattered excitation light reaching the emission detector may be significant in comparison to the intensity of fluorescence.

Raman (inelastic) scattering occurs when excitation light is scattered by solvent molecules (water, in biochemical applications) with concomitant vibrational excitation of the solvent molecules. The Raman scattered light is thus shifted in color toward the red relative to the excitation color by an amount corresponding to the vibrational energy change. This is a fixed amount in energy terms ( $3600\text{ cm}^{-1}$  for water), but corresponds to a varying wavelength change because of the reciprocal relationship between energy and wavelength ( $E = hc/\lambda$ ,  $h$  = Planck's constant,  $c$  = velocity of light,  $\lambda$  = wavelength). For excitation at 280 nm, the Raman peak occurs at 311 nm, whereas for excitation at 490 nm (our typical choice for fluorescein), the Raman peak occurs at approximately 595 nm. The width of the Raman peak will be identical to that of the excitation light (measured on an energy axis, not on a wavelength axis). The Raman intensity is generally low relative to elastic scattering, but may nevertheless become significant when sample concentrations are low.

#### *Excitation Path Filter*

Both kinds of scattered light are readily eliminated with appropriate optical filters, with or without the use of an emission monochrometer. We use a bandpass interference filter in the excitation path, placed between the excitation monochrometer and the sample, to eliminate any remaining light at colors other than the desired excitation wavelength that happens to pass through the excitation monochrometer. This filter is chosen such that its wavelength of maximum transmission matches the excitation monochrometer wavelength setting, and the bandwidth of the filter is chosen to be comparable to that of the excitation monochrometer, so as to minimize unnecessary loss of excitation intensity.

#### *Emission Path Filters*

The combination of excitation monochrometer and bandpass filter in the excitation path together ensures that the excitation light is adequately clean. There remains, however, the possibilities that either light elastically scattered (at the excitation color) by the sample or Raman scattered excitation light may make it through the emission path and be counted by the emission detector.

We use a cut-on filter in the emission path to reject elastically scattered excitation light. Such filters absorb or reflect short wavelengths, while passing

longer wavelengths with high transmittance, with a steep rise in transmittance occurring over a relatively narrow wavelength range. In general, colored glass filters work well for this purpose, and are available in a closely spaced series of cut-on wavelength ranges. One picks a filter that has essentially zero transmittance over the full bandpass of the excitation light, but that has high transmittance over much of the width of the fluorescence emission spectrum. In certain cases, if other constraints dictate that there will be only small shifts in color between excitation and emission wavelengths, it can be beneficial to use specialized multilayer dielectric filters, which can achieve much steeper cut-on characteristics.

Finally, one must remember to eliminate also the Raman scattered light, taking into account its full spectral width. Depending on the excitation wavelength and the fluorophore, the Raman scatter may be blue-shifted or red-shifted relative to the fluorescence emission. Even if the Raman scattering is superimposed on the fluorescence emission spectrum, the fluorescence emission spectrum will generally be much broader than the Raman band, so that it will be possible to choose filters that pass either the blue-side or the red-side of the fluorescence emission while rejecting the Raman.

The particular situation dictates the choice of filters to be used. When the Raman scatter is to the blue of the fluorescence emission, cut-on filter can be chosen to reject both elastic and Raman scattered light. When the Raman band is to the red of the fluorescence, one may need to supplement the cut-on filter with a cut-off filter. If an emission monochromator is used, this itself may serve effectively as a cut-off filter (because of the lower intensity of Raman scattering compared to elastic scattering). Alternatively, or in addition, cut-off (short-pass) filters may be used. The selection of colored glass cut-off filters is much less extensive than for cut-on. In general, one will need to use specialized multilayer dielectric filters instead.

Once a filter combination has been chosen (whether or not an emission monochromator will be used for the actual experiment), it is wise to verify that the filter combination works as planned by recording fluorescence emission spectra of buffer alone, of unlabeled sample, and of labeled sample (at the concentration that will be used for anisotropy measurement), scanning the emission monochromator from below the excitation wavelength to above the fluorescence emission range and Raman band. Both buffer and unlabeled sample spectra should show negligible intensity, in comparison to the intensity from the labeled sample, at all wavelengths that will be monitored during the anisotropy measurement.

### Filter Set for Use with Fluorescein

We find the following combination of filters to be highly effective for use with fluorescein, whether an emission monochromator is present or not. We choose 490 nm as the center wavelength for excitation.

Excitation interference filter	Coherent/Ealing #35-3482 interference filter, center wavelength = 490.0 nm, bandpass = 7.3 nm (full width at half-maximum transmission)
Emission cut-on (long-pass) filter	Coherent/Ealing #26-4333 OG-515 colored glass

Figure 1 shows excitation and emission spectra for fluorescein in panel A, compared to the transmission characteristics of the three filters in panel B. The excitation filter is well matched to the excitation maximum for

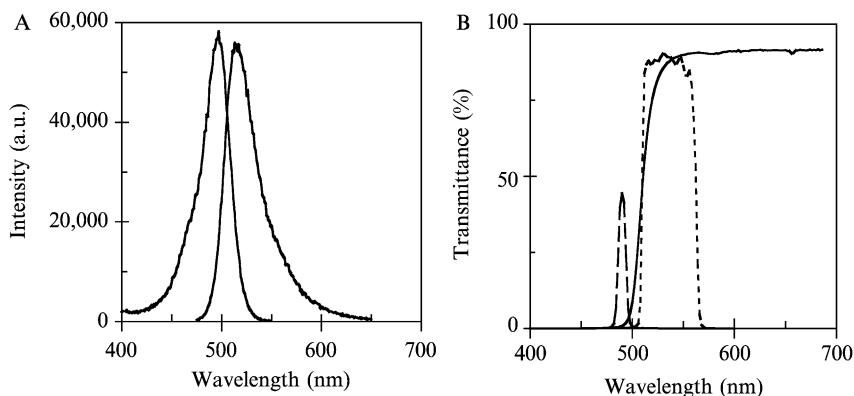


FIG. 1. Fluorescence spectra of fluorescein compared to transmission spectra of the optical filters used for measurement of fluorescein anisotropy. (A) Fluorescence excitation and emission spectra. Left-hand curve: fluorescein excitation spectrum, obtained monitoring emission at  $\lambda = 520$  nm; 0.5 nM fluorescein-labeled oligonucleotide in a buffer containing 20 mM HEPES-KOH, pH 7.6, 80 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 5% glycerol. Right-hand curve: the corresponding emission spectrum, with excitation at  $\lambda = 490$  nm. (B) Long-dashed curve: transmission characteristics of the 488.8 nm interference filter used in the excitation path in conjunction with the excitation monochromator. Solid and short-dashed curves, transmission characteristics of the cut-on and bandpass filters, respectively, which are used in the emission path, typically with no emission monochromator. The excitation filter matches the excitation maximum for fluorescein. The cut-on and leading (short wavelength) edge of the bandpass filters both strongly reject any elastically scattered excitation light; the falling (long wavelength) edge of the bandpass filter strongly rejects any Raman scattered light, which is centered at  $\sim 595$  nm.

fluorescein. The colored glass cut-on filter rejects most of the excitation bandpass, while passing the majority of the emission spectrum. The specialized cut-off filter nicely rejects any Raman scatter, which is centered at 595 nm, while further strongly reducing any elastically scattered excitation light. (The cut-off [bandpass] filter also strongly rejects elastically scattered light on its own; however, the combination of the two filters gives far better blocking at the excitation bandpass than either one on its own. This improved scatter rejection is important in strongly scattering/weakly fluorescing samples.)

The performance of this filter set can be appreciated from the emission spectra in Fig. 2. Panel A shows the ability of the filter set to suppress both elastically scattered light ( $>10,000$ -fold reduction) and Raman scattering to undetectably low levels, while panel B shows that this huge reduction in background scattering intensity comes at a modest cost (2- to 3-fold) in the collectible intensity of fluorescence emission.

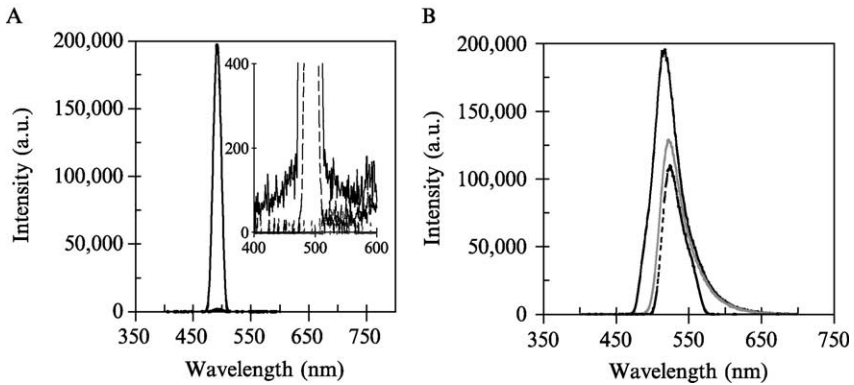


FIG. 2. Performance of the filter set for fluorescein anisotropy measurement. (A) Rejection of elastically scattered light and Raman scatter. Emission spectra recorded from a scattering solution ( $10 \mu\text{g/ml}$  BSA in buffer), with excitation at 490 nm using the excitation monochromator plus the 488.8 nm interference filter. Solid line: no filters in the emission path. Long-dashed line: 515 nm cut-on filter. Short-dashed line (essentially invisible): both 515-nm cut-on plus bandpass filters. When no filters are used in the emission path, note the strong peak of scattering intensity detected when  $\lambda$  emission =  $\lambda$  excitation. Inset: same spectra plotted on a 500-fold more sensitive scale. The emission filter combination reduces the scattering signal by  $>10,000$ -fold, rendering it immeasurably low. Similarly, there is negligible remaining intensity at the Raman scattering wavelength ( $\sim 595$  nm). (B) Fluorescence emission from fluorescein with no filter in the emission path (solid black line), 515-nm cut-on filter only (grey line) or both cut-on and bandpass filters (dashed line). The  $>10,000$ -fold reduction in scattering intensity comes at a cost of only  $\sim 2$ -fold in emission intensity at the emission maximum, and only  $\sim 3$ -fold in total emission intensity integrated over all wavelengths (such as could be measured by a detector in the emission path if no emission monochromator were present).

### *pH Sensitivity*

Fluorescein has a  $pK_a$  of  $\sim 6.5$ , and its fluorescence intensity is dependent on the charge state. Consequently, it is important to control the pH of the solution, preferably at a  $pH \geq 7.5$ , so that unanticipated small changes in pH will affect the fluorescence intensity only negligibly.

### *Intensity Changes*

Two additional phenomena concerning the sample fluorescence intensity bear mention. First, fluorescein is sensitive to photobleaching; thus, prolonged exposure of sample to excitation light, or even room light, can significantly reduce the fluorescence intensity. Since anisotropy is an inherent property, independent of concentration, modest amounts of sample bleaching occurring during binding titrations need not be problematic, provided that there is negligible bleaching during the time required for any given anisotropy measurement. This can be checked by monitoring the total intensity during the four individual measurements that make up an anisotropy measurement.

In certain cases, however, the binding of a protein to a fluorescein-labeled DNA can affect the fluorescence intensity directly. For example, the bound protein might happen to quench (or enhance) the fluorescence quantum yield, or perhaps shift the emission color, so that the intensity measured over a given wavelength range may increase or decrease. Such effects invalidate the usual interpretation of the anisotropy changes. If the intensity changes, the likelihood is that the fluorescence lifetime too is changing; and if the lifetime changes, then the anisotropy is affected even if molecular tumbling time were to remain constant. Consequently, it will no longer be possible to assign a linear relationship between a measured anisotropy change and a probability of binding site occupancy.

Actually, such cases can be a blessing in disguise: one can often monitor the binding process simply by measuring the intensity change directly. In any event, it is necessary to pay attention to the total intensity when carrying out anisotropy measurements. Systematic changes in intensity that correlate with binding invalidate the standard interpretation of the anisotropy experiment.

### *Cuvettes*

Samples used in fluorescence experiments may be precious, and one may wish to minimize the volume of sample used. We routinely use samples of 75–100  $\mu\text{l}$  in quartz ultramicro-cuvettes (Hellma, black walled, #105.251-QS) having a sample chamber of  $3 \times 3$  mm with a 5-mm tall

aperture, that is, a 45- $\mu\text{l}$  illuminated volume. These cuvettes are easy to fill and clean, and fit in the 1.25-cm square cuvette holders that are standard in most fluorimeters. These cuvettes are available with the sample chamber placed at various heights above the base of the cuvette. It is important to pay attention to the height of the optical axis of the fluorometer, and to make certain that the height of the sample chamber of the cuvette matches that of the fluorometer.

### Experimental Design for Binding Titrations

We use selected high-affinity nucleosome positioning sequences<sup>7,10,11</sup> to simultaneously provide a high degree of homogeneity in nucleosome positioning while also enhancing the stability of the nucleosomes against dissociation by mass action despite the dilute nucleosome concentrations used.

ISWI is an ATP-dependent nucleosome remodeling factor that induces nucleosome sliding on nicked DNA.<sup>12</sup> It is expressed in *Escherichia coli* and purified by gel-filtration to near homogeneity.<sup>13</sup> Non-specific interactions of ISWI with DNA alone and with DNA at its entry into the nucleosome have been described qualitatively.<sup>3</sup> Fluorescence anisotropy measurements permit a quantitative description of these interactions.

Binding buffer for ISWI-DNA or ISWI-nucleosome binding reactions contains: 20 mM HEPES-KOH, pH 7.6, 80 mM KCl, 2 mM  $\text{MgCl}_2$ , 5% glycerol, 1 mM DTT, supplemented when desired with ATP, ADP, or other nucleotides or analogs. We typically make up a distinct sample for each ISWI to be investigated. This allows the DNA or nucleosomes to remain constant as the ISWI is varied over a titration. Binding reactions are 100  $\mu\text{l}$  final volume, typically with 1 nM fluorescein-labeled DNA or 5 nM nucleosomes, and the desired ISWI. ISWI protein is diluted into binding buffer as appropriate, such that accurately measurable volumes are added into the 100- $\mu\text{l}$  final binding reaction volumes. Samples are incubated at room temperature for 30 min prior to measurement of fluorescence anisotropy to ensure that binding reactions are well equilibrated (control studies show that binding appears to equilibrate essentially instantaneously, as judged by the absence of further changes in anisotropy, hence the 30-min equilibration time is more than sufficient). Samples are placed in quartz ultramicro-cuvettes as described earlier. We use an excitation wavelength of 490 nm, no emission monochromator, and the

<sup>10</sup> A. Thåström *et al.*, *J. Mol. Biol.* **288**, 213 (1999).

<sup>11</sup> J. Widom, *Q. Rev. Biophys.* **34**, 269 (2001).

<sup>12</sup> G. Längst and P. B. Becker, *Mol. Cell* **8**, 1085 (2001).

<sup>13</sup> D. F. Corona *et al.*, *Mol. Cell* **3**, 239 (1999).

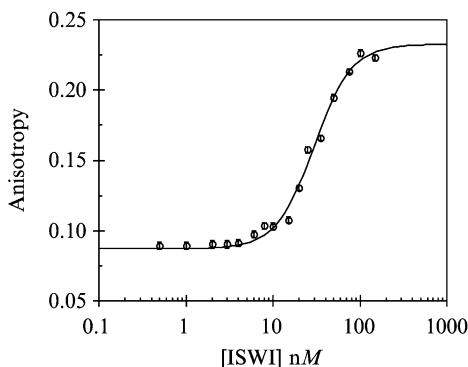


FIG. 3. Raw fluorescence anisotropy data from ISWI binding to naked DNA. Titration of a 5 nM 5'-fluorescein-labeled 35-bp long DNA with increasing concentrations of ISWI protein. (See text for buffer conditions.) The curve superimposed on the data represents a fit to a cooperative binding model.

combination of the cut-on and bandpass cut-off filter in the emission path described previously.

We always include a sample prepared with no ISWI ( $ISWI = 0$ ) to establish the experimental “baseline” for each titration, and we extend the titrations to sufficiently high ISWI to allow accurate determination of the anisotropy corresponding to complete binding (complete occupancy of binding sites by bound ISWI). Note that, whatever method is used to monitor binding processes, it is important to carry titrations through the full range of the binding process. A good practice is to use “direct” plots<sup>14</sup> of the measured signal (anisotropy, in our case) versus the titrant concentration (ISWI, in our case) plotted on a log scale to allow representation of the wide range of titrant necessary to explore the full range from fraction bound  $\sim 0$  (no binding) to fraction bound  $\sim 1$  ( $\sim 100\%$  binding).

We use Kalaidagraph software to fit raw binding data to desired binding models.

## Results of Binding Experiments

Typical raw data resulting from such an experiment are shown in Fig. 3, for a 5'-fluorescein-labeled 35-bp long DNA, used at 5 nM. Aficionados of binding studies will recognize immediately from the raw data that the

<sup>14</sup> I. M. Klotz, “Ligand-Receptor Energetics: A Guide for the Perplexed.” Wiley, New York, 1997.

binding curve as plotted in this manner is too “steep” to correspond to simple 1:1 binding of ISWI to DNA, implying positive cooperativity in the binding of ISWI to DNA. The titration midpoint for these given conditions ( $EC_{50}$ ) is 30 nM. The concentration of fluorescein-labeled tracer is small in comparison and hence may safely be neglected (or alternatively quantitatively accounted for,<sup>14</sup> yielding small corrections that convert  $[ISWI]_{total}$  to  $[ISWI]_{free}$ ). If binding were simple (1:1 ISWI:DNA complex, noncooperative binding curve), then this measured  $EC_{50}$  would also be the thermodynamic  $K_d$ .

In any experimental analysis of binding processes, it is helpful to reduce the number of adjustable parameters in the curve fitting. In our anisotropy studies, we directly measure both the lower and upper “baselines” for the titrations, and hold these quantities fixed at their measured values during the curve fitting procedure. We set the anisotropy for the 0-nM ISWI baseline (the lower baseline for the curve fitting) equal to the average of several measurements on the same DNA sample lacking any ISWI, and we set the upper baseline equal to an average of the results for (replicate measurements on) the last couple or few titration points, having taken care to extend the titrations to the point at which any further increases in ISWI do not result in any further significant changes in measured anisotropy. For a given fixed assumed stoichiometry of the binding process, this leaves only one free parameter—the apparent affinity or  $K_d$ —to be determined by curve fitting. Alternatively, one may allow both the apparent affinity and the cooperativity (or “molecularity”) to be simultaneously fit.

Figure 4 shows results for a negative control, in which the DNA (18-bp duplex, 5'-end labeled with fluorescein, 1 nM concentration) proves to be too short to allow high affinity binding of the ISWI. Plainly, even the raw data resulting from the anisotropy experiment can distinguish samples in which binding does occur from samples in which it does not. The  $EC_{50}$  ( $K_d$ , if 1:1 ISWI:DNA complex) for ISWI binding to this DNA is  $\gg 100$  nM.

Figure 5 shows the results an experiment monitoring binding to 177-bp-containing nucleosomes (again, 5'-end labeled with fluorescein, 5 nM concentration). The raw data are rescaled along the ordinate to represent the fraction of DNA with bound ISWI, simply by linearly rescaling the measured anisotropies from 0 (experimentally measured lower baseline) to 1 (measured upper baseline). The titration midpoint for this particular dataset ( $EC_{50}$ ) is 15 nM, slightly lower (i.e., higher affinity) than for the 35-mer DNA of Fig. 3. An average over many datasets (data not shown) suggests that this small apparent difference in affinity between DNA and nucleosome is not statistically significant. Evidently, ISWI binds to nucleosomes with an affinity that is close to its affinity for long naked DNA.

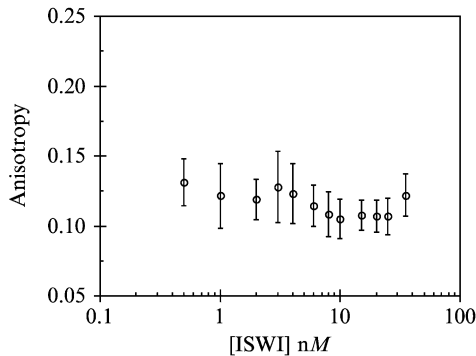


FIG. 4. Raw anisotropy data when no binding occurs. A 1 nM solution of a 5'-fluorescein-labeled 18-bp long (double-stranded) DNA is titrated with increasing concentrations of ISWI protein. ISWI has negligible affinity for such short DNAs.

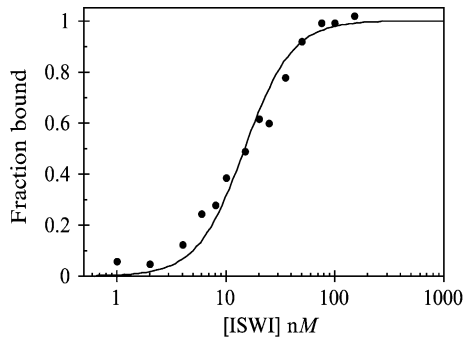


FIG. 5. ISWI binding to a nucleosomal DNA. A 5-nM solution of a 5'-fluorescein-labeled 177-bp DNA, assembled into nucleosomes, is titrated with increasing concentration of ISWI. Raw fluorescence anisotropy data are scaled to fraction bound (fraction of DNAs having ISWI protein bound): anisotropy data obtained in the absence of any ISWI protein establish the anisotropy for fractional occupancy = 0; the averaged anisotropy from the highest several titration points (where the signal appears to have plateaued) defines the fractional occupancy = 1. The curve represents a least-squares fit to a cooperative binding model. The DNA template used includes a 147-bp selected nucleosome positioning sequence together with an additional 30 bp of DNA extending beyond one end; a single fluorescein is attached at the 5'-end of this 30-bp extension. The DNA is assembled into nucleosomes and purified as described (see text).

Finally, in Fig. 6 we study the binding of ISWI to DNA (5'-fluorescein, 1 nM concentration) in the presence of 1 mM ADP. The titration midpoint for this dataset ( $EC_{50}$ ) is 28 nM, very close to the measured 30 nM  $EC_{50}$  for binding to naked DNA in the absence of nucleotide (Fig. 3). Evidently, the

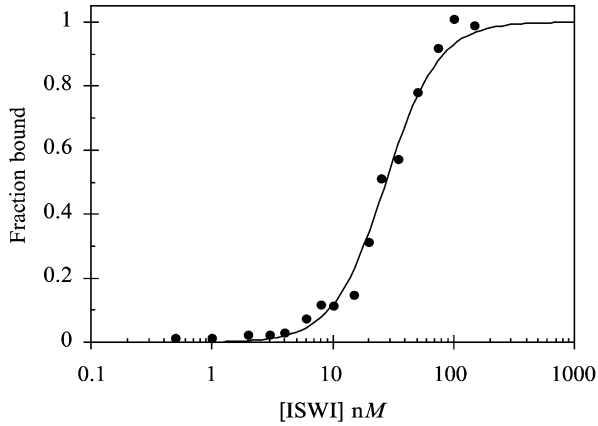


FIG. 6. ISWI titration in the presence of added nucleotide. Titration of a 1 nM 5'-fluorescein-labeled 150-bp long DNA with increasing concentrations of ISWI protein, in the presence of 1 mM ADP. The raw anisotropy data are scaled to fraction bound. Nucleotides or other cofactors or binding partners can be included in the reactions without difficulty.

affinity of ISWI for naked DNA is not influenced by the presence of high concentrations of ADP. This figure highlights the utility of anisotropy measurements to monitor binding in solutions containing other additives such as nucleotides. In contrast, it is difficult or risky to carry out such studies using a gel electrophoretic mobility shift approach, for example, since prohibitively costly amounts of nucleotide analogs might be required, and moreover these compounds may actually electrophorese. In that case, their concentrations around the complexes during a gel separation would be undefined, rendering the experiments uninterpretable.

## Conclusions

Fluorescence anisotropy is well known to be useful for analysis of protein-DNA interactions, and it seems likely to be particularly useful for analysis of nucleosome remodeling factors because it is rapid, quantitative, highly sensitive (conserving precious reagents), suitable for use in the presence of cofactors such as ATP, readily measured even during rapid kinetic experiments, and very broadly applicable. It will allow analysis of the interactions of remodeling factors or their individual proteins or domains with any other species that can be specifically labeled.