Fluorescence Spectroscopy and Anisotropy in the Analysis of DNA–Protein Interactions

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Chapter 35

Fluorescence Spectroscopy and Anisotropy in the Analysis of DNA–Protein Interactions

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Summary

Fluorescence spectroscopy can be used as a sensitive non-destructive technique for the characterisation of protein–DNA interactions. A comparison of the intrinsic emission spectra obtained for a protein–DNA complex and for free protein can be informative about the environment of tryptophan and tyrosine residues in the two states. Often there is quenching of the fluorescence intensity of an intrinsic emission spectrum and/or a shift in the wavelength maximum on protein binding to DNA. A step-by-step protocol describes the determination of a DNA-binding curve by measurement of the quenching of the intrinsic protein fluorescence.

Fluorescence anisotropy can also be used to obtain a DNA-binding curve if the molecular size of the protein–DNA complex is sufficiently different from the free fluorescing component. Typically an extrinsic fluorophore attached to one or both 5′ ends of single-stranded or duplex DNA is used, for this increases the sensitivity of measurement.

Fitting of the binding curves, assuming a model, can often yield the stoichiometry and association constant of the interaction. The approach is illustrated using the interaction of the DNA-binding domains (HMG boxes) of mouse Sox-5 and mammalian HMGB1 with short DNA duplexes.

Key words: Fluorescence spectroscopy, Anisotropy, Protein–DNA complex, HMG box.

1. Introduction

Changes in the fluorescence emission spectrum of a protein upon binding to DNA can often be used to determine the stoichiometry of binding and equilibrium-binding constants; in some cases the data can also give an indication of the location of particular residues within the protein. The experiments are generally quick and

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easy to perform, requiring only small quantities of material *(1, 2)*. Spectroscopic techniques allow one to measure binding equilibria (unlike, for example, gel retardation assays and other separation techniques which are strictly speaking non-equilibrium methods). Fluorescence is one of the most sensitive of spectroscopic techniques, allowing the low concentrations (typically in the μM range) required for estimation of binding constants for many protein–DNA interactions. Considerable care, however, needs to be exercised in the experiment itself and in the interpretation of results.

The following methods describe the determination of DNA-binding curves by intrinsic fluorescence quenching and by fluorescence anisotropy with an extrinsic fluorophore. Fluorescence Resonance Energy Transfer (FRET) techniques are becoming useful in the area of protein–DNA interactions particularly if one wants to measure the degree of DNA bending, to triangulate or to measure distances, albeit at a low resolution *(2, 3)*. In this method, two or even three extrinsic fluorophores are attached where the emission light of one fluorophore, the donor, is absorbed by another fluorophore, the acceptor, and becomes part of its emission spectrum. Stopped-flow and time-resolved FRET techniques for kinetic and fluorescent lifetime measurements of DNA bending and binding are also advantageous, though they require sophisticated instrumentation *(4, 5)*.

1.1. Fundamental Principles of Fluorescence

A molecule that has been electronically excited with ultraviolet/visible light can lose some of the excess energy gained by a number of processes as it returns to its ground state. In two of these, fluorescence and phosphorescence, this is achieved by emission of light. Phosphorescence is rarely observed from molecules at room temperature and will not be considered further. Whilst electrons can be excited to a number of higher energy states, fluorescence emission in most cases only occurs from the first vibrational level of the first excited state. This has two implications for the measurement of fluorescence emission spectra. Firstly, some of the energy initially absorbed is lost prior to emission, which means that the light emitted will be of longer wavelength (i.e. lower energy) than that absorbed. This is known as Stoke's shift. Secondly, the emission spectrum and therefore the wavelength of maximum fluorescence will be independent of the precise wavelength used to excite the molecule. Thus for tyrosine, the wavelength of the fluorescence maximum is observed around 305 nm, regardless of whether excitation is at the absorption maximum (~278 nm) or elsewhere in the absorption spectrum (e.g. 230 nm). Of course, the fluorescence intensity will change as a consequence of the difference in the amount of light absorbed at these two wavelengths.

The ratio of the number of photons of light emitted as fluorescence to the number of photons initially absorbed, i.e. the

efficiency of the fluorescence process, is known as the fluorescence quantum yield. The value of the quantum yield for a particular fluorophore will depend on a number of environmental factors such as temperature, solvent, and the presence of other molecules which may enhance or diminish the probability of other processes deactivating the excited state. The deactivation or quenching of fluorescence by another molecule, either through collisional encounters or the formation of excited state complexes, forms the basis of many of the fluorescence studies on protein–DNA interactions.

The study of protein–nucleic acid interactions is greatly simplified by the fact that all detectable fluorescence arises from the protein since all of the naturally occurring bases in RNA and DNA are essentially non-fluorescent. Tyrosine and tryptophan residues account for almost all the fluorescence found in proteins. As a general rule, when both residues are present the emission spectrum will be dominated by tryptophan, unless the ratio of tyrosines to tryptophans is very high. The quantum yield of a tyrosine residue in a protein compared to that observed in free solution is generally very low, illustrating the susceptibility of tyrosine to quenching. Tryptophan residues are highly sensitive to the polarity of the surrounding solvent, which affects the energy levels of the first excited state with the result that the emission maximum for tryptophan can range from 330 nm in a hydrophobic environment to 355 nm in water. Thus in proteins containing only one tryptophan, the general environment surrounding the residue can be ascertained.

In protein–DNA interactions, the fluorescence due to tyrosine residues in a protein may be effectively quenched by energy transfer from the tyrosines to the bases of DNA, there being a large overlap in the absorption (DNA) and fluorescence (tyrosine) spectra. Tryptophan fluorescence, like that of tyrosine, can also be quenched (indirectly) by DNA binding. Unlike tyrosine, the emission wavelength maximum can also change if tryptophan is involved in the interaction and this can also be used to monitor DNA binding *(6)*.

The extent to which the fluorescence of a protein is quenched by DNA is proportional to the concentration of quencher. As quenching is due to the formation of a complex between the protein and the DNA, the extent of quenching is proportional to the amount of bound protein. Thus by determining the extent to which the protein fluorescence is quenched when fully bound to DNA (i.e. at saturation), the fraction of bound and free protein at any point in a titration can be determined. From these data, the stoichiometry and binding constant of the interaction can often be obtained. Note that to establish an accurate stoichiometry, a high concentration of DNA is preferred when titrating into protein (i.e. well above the K_d of the complex) to ensure

stoichiometric binding. To establish the binding constant itself, one should be working at much lower concentrations of protein so that at the stoichiometric point, there is a measurable concentration of unbound protein. In the case of protein–DNA interactions having a low K_d, this may not be possible.

If fluorescence quenching is being used to follow DNA binding, it is vital to take account of sample dilution, as well as the increased absorption of the sample as DNA is titrated in. The latter effect is known as the inner filter effect and arises from the absorption of the excitation beam (and generally to a lesser extent, the emission beam) on passing through the sample (*see* **Fig. 1**). One should aim to keep the absorption of the sample (at the excitation wavelength) as low as possible, although absorbances up

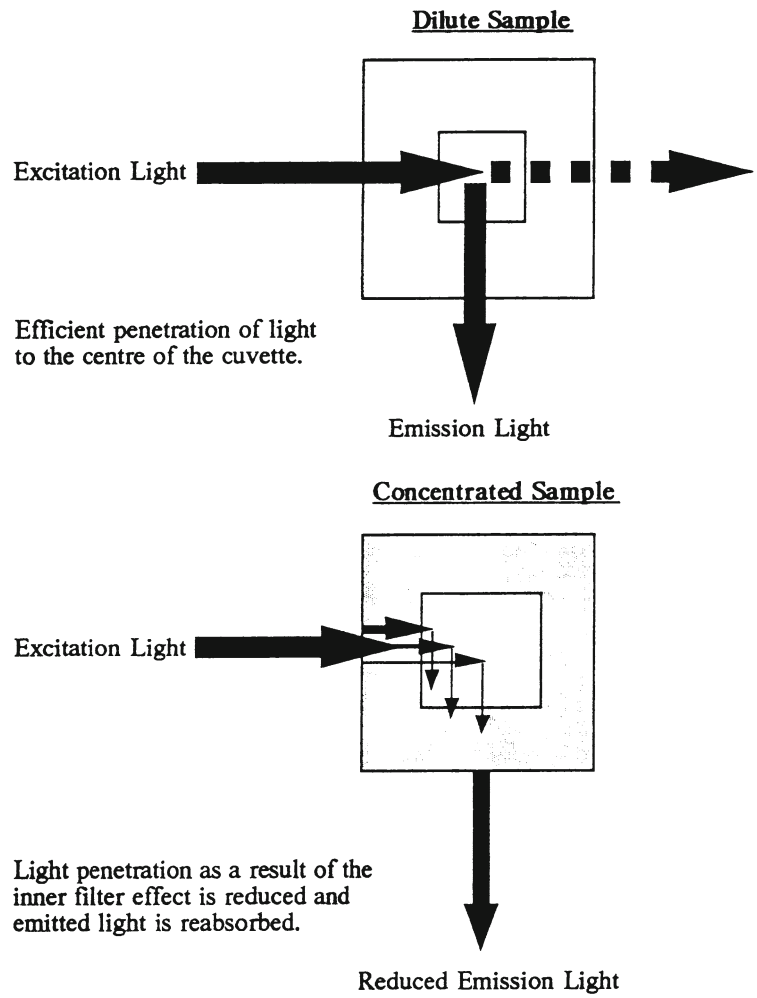

Fig. 1. Schematic representation of the inner filter effect in fluorescence, showing the effect of high concentration on the absorbance of the excitation and emission beams.

to 0.2 can normally be corrected for without too much difficulty. A small pathlength cell will also help. Ideally the absorption of the sample at the excitation and emission wavelengths (A_{ex} and A_{em}) should be measured for each point in the titration (if not, one can calculate these values from the known concentrations of protein and nucleic acid at each point). For normal right-angled geometry of observation, the corrected fluorescence F_{corr} can be obtained from the observed fluorescence F_{obs} by the formula:

$$F_{corr} = F_{obs} \times 10^{(Aex/2 + Aem/2)} \quad (1)$$

Often the value of A_{em} is small enough to ignore (for a detailed treatment of the inner filter correction, *see* **ref.** *7)*. Note that it is equally important to correct for the inner filter effect whether titrating protein into DNA or vice versa.

The use of intrinsic fluorescence, as a method of investigating protein–DNA interactions, is widespread. For example, both binding parameters (K_{obs}) and stoichiometric ratios have been derived for the interaction of the HIV-1 nucleocapsid protein NCp7 with the natural primer $tRNA_3^{Lys}$ and other related RNA molecules *(8)*. Similarly, estimates of binding constants have been determined for the interaction of human replication protein A (hRPA) with single-stranded homopolynucleotides (e.g. poly[dT] and poly[dA]) *(9)*. The interaction of the DNA-binding domain (DBD) of mouse Sox-5, known as the HMG box, with a short DNA duplex *(10)* is used in the text.

1.2. Fundamentals of Fluorescence Anisotropy

Fluorescence anisotropy can be used to measure binding constants if the molecular size of the protein–DNA complex is sufficiently different from the free fluorescing component. Fluorescence emission is not only dependent on the chemical environment of the fluorophore but also on the polarisation of the excitation light used. Fluorescence anisotropy describes the extent of this polarisation of the emission. When excited with plane polarised light a fluorophore often emits light that is also polarised. Rotational diffusion of the fluorophore during the lifetime of the excited state causes depolarisation of the emitted signal. For fluorophores in solution for which the rate of rotational diffusion is much faster than the rate of emission, it follows that the emitted light is fully depolarised and the anisotropy is equal to zero. If the rate of tumbling is much slower than the rate of emission, the emission will have maximal polarisation. If the fluorophore is attached to DNA, the rate of tumbling is such that the emission remains substantially polarised. Binding of a protein to fluorescently labelled DNA will increase the size of the particle and increase the rotational correlation time, i.e. the complex tumbles more slowly in solution than the free DNA. In this case the polarisation of the emitted light will change to give an increase in anisotropy of the

emission. Thus the change in anisotropy can be monitored and subsequently interpreted as a consequence of complex formation. Fluorescence anisotropy, A is defined as:

$$A = \frac{(I_v - I_h)}{(I_v + 2I_h)} \quad (2)$$

where I_v and I_h are the vertically and horizontally polarised emission intensities, respectively, and the value of A is independent of the total intensity of the light emitted. In practice the anisotropy is measured as:

$$A = \frac{I_{vv} - (\mathrm{GF} \times I_{vh})}{I_{vv} + (2 \times \mathrm{GF} \times I_{vh})} \quad (3)$$

and

$$\mathrm{GF} = \frac{I_{hv}}{I_{hh}} \quad (4)$$

where the subscripts h and v in the intensity I indicate vertically and horizontally placed polarisers, the first subscript referring to the excitation polariser position and the second to the emission polariser position. The grating factor, GF, is thus a correction for the polarisation dependence of the instrument transmission, principally the emission monochromator.

Fluorescence anisotropy typically utilizes an extrinsic fluorophore rather than relying on the intrinsic fluorescence emission of the protein. There are several reasons for this. Firstly, to maximise the difference in signal between free and bound states the fluorophore needs to be attached to the smaller of the binding partners: this is usually the DNA. Secondly, extrinsic fluorophores based on fluorescein (known as FAM) or rhodamine derivatives attached to one or both 5′ ends of single-stranded or duplex DNA are available and these have a large extinction coefficient, which increases sensitivity by enabling measurements in the nanomolar concentration range. Thirdly, an extrinsic fluorophore with an emission spectrum in the visible range requires only the use of polarisers constructed of Polaroid rather than the use of quartz polarisers required for emission in the UV from intrinsic Tyr and Trp fluorophores.

Fluorescence anisotropy has been widely used to obtain binding curves for a large number of DNA-binding proteins and domains and include: AT-hooks *(11)*, GCN4-bZIP *(12)*, interferon regulatory factors *(13)*, homeodomains *(14)*, as well as HMG boxes *(15, 16)*. If protein binding results in DNA bending, a double-labelled duplex will display a change in the FRET effect, which can also be used to obtain a binding curve *(15, 16)*. However, unless the bend angle is large, the FRET effect will

be small and this makes the experiment insensitive. However an amplification of small DNA bend angles introduced on protein binding using 'DNA levers' can overcome this to yield binding curves *(17)*.

2. Materials

2.1. Spectrofluorimetry

1. High-quality quartz cuvettes with all four faces polished are required (*see* **Note 1**). A cuvette of square cross-section with light paths of 0.4 and 1.0 cm, a stirring bar, and a close-fitting lid is commonly used (available from Hellma). A sample volume of 1.0–1.5 mL is required.
2. Most commercially available fluorimeters allow scanning by both the excitation and emission monochromators and are suitable for use in these studies. Anisotropic measurements require a fluorimeter with polarisation optics, but since the common fluorophores absorb and emit in the visible range, quartz polarisers are not required and Polaroid is sufficient. Cell sample holders that are thermostatically controlled and contain a magnetic stirrer are essential (*see* **Notes 2** and **3**). We routinely use a Perkin-Elmer LS50B luminescence spectrometer having a Raman S/N ratio of ~1,000. Other more sensitive fluorimeters are available, e.g. the SPEX Fluoromax (from Horiba Jobin Yvon) for work requiring the use of nanomolar concentrations for the determination of very low dissociation constants.
3. To control the spectrofluorimeter and to record fluorescence spectra a computer linked to the fluorimeter is required. The software package FLWinlab running under the WindowsXP operating system is provided for use with the LS50B.

2.2. Reagents and Solutions

1. Reagents used in buffer solutions should be of the highest purity available and the solutions prepared in doubly distilled water. The buffer should have negligible absorbance in the excitation wavelength range (for intrinsic 260–300 nm, for anisotropy 400–500 nm) and should not be used if it shows fluorescence in the emission region (for intrinsic 290–400 nm, for anisotropy 450–600 nm).
2. DNA oligonucleotides were synthesised using phosphoramidite chemistry and purified by reverse-phase HPLC. A 12-bp DNA duplex containing the site-selected AACAAT sequence at its centre *(18)* was used as the Sox-5 HMG box target:

```
5′-GCGAACAATCGG-3′
3′-CGCTTGTTAGCC-5′
```

A 16-bp DNA duplex derived from the Epstein Barr virus BHLF-1 gene promoter and having a TTCAAA core sequence was used as the HMGB1 target *(19)*. For the fluorescence anisotropy experiments, one of the strands was modified at the 5′ end with the fluorescent label 6-FAM:

```
FAM-5′-CGGGTTTCATTAAGGG-3′(FAM-DNAHMG)
     3′-GCCCAAAGTAATTCCC-5′
```

The sequence of the FAM-labelled oligonucleotide is important (*see* **Note 4**). DNA duplexes were constructed by annealing an unlabelled complementary strand at equimolar ratio.

3. The HMG box of mouse Sox-5 (amino acids 182–260 *(18)*) was expressed as a fusion protein in pGEX-2T, using *E. coli* BL21 (DE3) plysS cells. After affinity purification with glutathione-agarose and thrombin cleavage whilst still attached to the column *(20)*, reverse-phase high-performance liquid chromatography (HPLC) was used to purify the protein. Protein was re-dissolved in water and re-folded by extensive dialysis against three changes of 1 L of 100 mM KCl, 10 mM potassium phosphate, 1 mM EDTA (pH 6.0) at 4°C.

   The 100-residue second HMG box of mammalian HMGB1 (amino acids 84–184) and comprising a minimal folded HMG box with a basic C-terminal extension, known as HMGB1-B′ *(21)*, was expressed and purified by anion exchange chromatography and reverse-phase HPLC *(15)*. The minimal second HMG box of HMGB1 (amino acids 92–170), known as HMGB1-B, was expressed as a GST-fusion protein and purified as for Sox-5.

4. Concentrations of all oligonucleotides and DNA duplexes were determined from their UV absorption at 260 nm, after digestion to nucleotides with snake venom phosphodiesterase I (PDE1, from *Crotalus durissus terrificus*, Sigma. (*see* Chapter "Defining the Thermodynamics of Protein/DNA Complexes and Their Components Using Micro-calorimetry"). Account must be made for the contribution to the 260-nm absorption by the fluorescent tag if present (for FAM the extinction coefficient at $\varepsilon_{260\ nm}$ = 28,000 M^{-1} cm^{-1}). Concentrations may also be determined using the fluorophore absorption in the visible region (e.g. for FAM λ_{max} is 496 nm with $\varepsilon_{496\ nm}$ = 78,000 M^{-1} cm^{-1}). Protein concentrations were determined from their UV absorption at 280 nm.

5. Protein and DNA should be dialysed into an identical buffer, in this case 100 mM KCl, 10 mM potassium phosphate, 1 mM EDTA (pH 6.0). Stock solutions of protein and DNA may be prepared in small aliquots and stored at –20°C, assuming this is not detrimental to the protein. Dilution to the working concentration can then be easily made for the fluorescence measurements.

3. Methods

3.1. Intrinsic Fluorescence

The method described in this section assumes that nothing is known concerning the fluorescence properties of the protein or its complex with DNA. Consequently the initial steps described in **Subheading 3.1.1** are concerned with characterising some of the fluorescence properties of the two species such that the optimal conditions for obtaining accurate and reliable data can be obtained. **Subheading 3.1.2** describes a procedure for obtaining data for a protein in which the intrinsic fluorescence is only quenched by DNA on binding, and how these data may be used to obtain information regarding the stoichiometry and dissociation constant of the interaction.

Comparison of the intrinsic emission spectra obtained for protein–DNA complex (at a concentration well above that corresponding to the dissociation constant) and for free protein can be informative. In the case of the Sox-5 HMG box, the complex shows both a quenching of the fluorescence intensity and a shift in the wavelength maxima compared to the free protein (*see* **Fig. 2a**). The difference spectrum having a wavelength maxima at 302 nm is typical of the emission spectrum of tyrosine. This has been interpreted as being due to the fluorescence quenching of three tyrosine residues located close together in the 'minor wing' of the Sox-5 HMG box as they make close approach to the DNA *(10)*. In contrast, fluorescence emission from the two tryptophan residues, located in the hydrophobic protein core and far from the tyrosines in the minor wing, is largely unaffected by DNA binding. A method for obtaining the stoichiometry and dissociation constant of the interaction in which there is fluorescence quenching and a shift in wavelength maxima is outlined in **Subheading 3.1.2.**

3.1.1. Preliminary Experiments

1. Switch on the fluorimeter and allow 10 min for the components to stabilise. Set the excitation and emission slits widths to intermediate values (e.g. 10 nm). Turn on the temperature control.
2. Fill the cuvette with protein solution of ~1 μM concentration (*see* **Note 5**). If the cuvette is rectangular place the smallest pathlength in the excitation beam – this produces the lowest overall absorption. Turn the magnetic stirrer on and allow time for the solution to equilibrate to the temperature of the compartment. To prevent local heating of the solution or possible photodecomposition, the excitation shutter should be kept closed except when taking measurements.
3. If the absorption spectrum of the protein is known set the excitation wavelength to that corresponding to the absorption

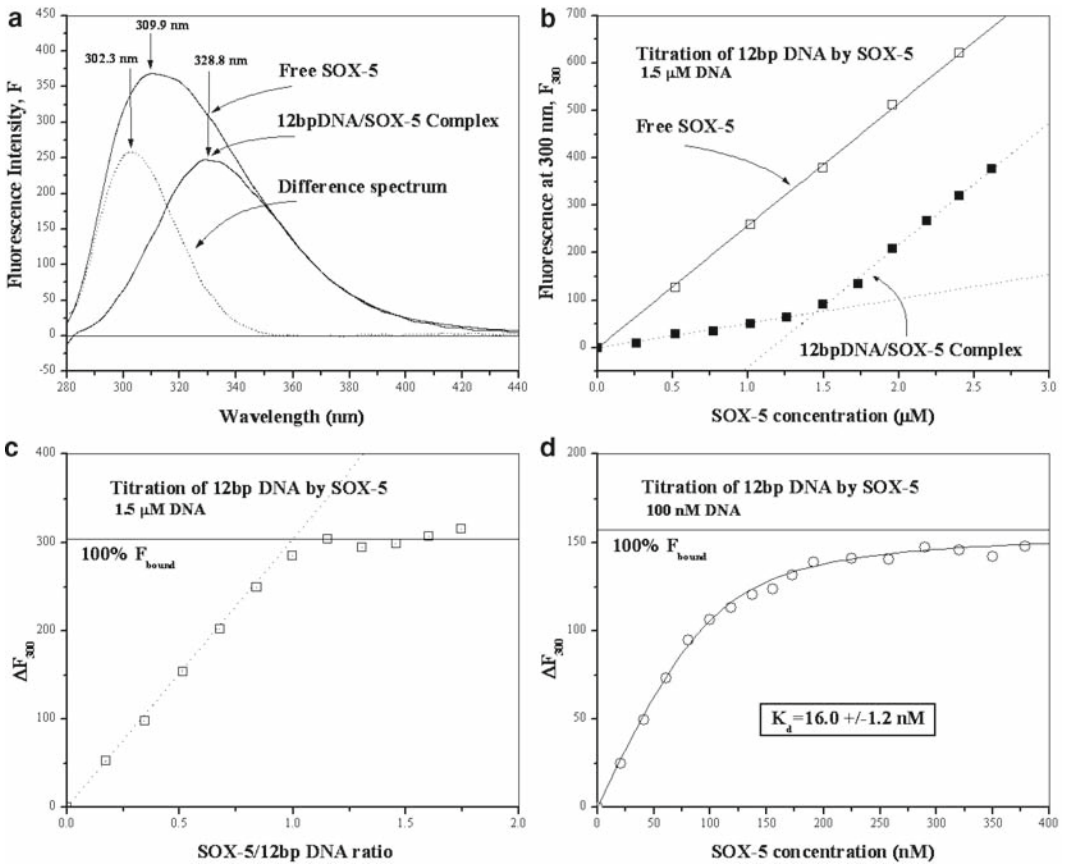

Fig. 2. Titration of the 12-bp DNA duplex with Sox-5 HMG box at 5°C. (**a**) Emission spectra of the free Sox-5, the Sox-5/12-bp DNA complex, and their difference spectrum. The excitation wavelength was 270 nm. The difference spectrum showing a wavelength maxima, λ_{max} of 302 nm, demonstrates that the quenching is of tyrosine fluorescence only. (**b**) Fluorescence intensities at 300 nm for the free Sox-5 and for a titration of 1.50 μM DNA duplex with Sox-5, a concentration well above the dissociation constant K_d. Substantial quenching of the tyrosine fluorescence on binding Sox-5 is evident up to the stoichiometric point (1:1). (**c**) The titration data re-plotted from (**b**) as the difference between the free and bound Sox-5 (ΔF) to show the sharp break at the stoichiometric point. (**d**) The results for a titration of Sox-5 protein into 100 nM DNA duplex, a concentration only 6.25× that of the K_d. The fitted binding curve for a 1:1 interaction is shown. The excitation wavelength was 230 nm to improve sensitivity (though with a larger background).

maximum between 265 and 285 nm; if no such absorption peak exists the protein contains neither tyrosine nor tryptophan residues and it will not fluoresce. If the absorption spectrum is unknown set the excitation wavelength to 280 nm.

4. Open the excitation shutter and quickly scan the emission monochromator between 285 and 400 nm. Identify a wavelength at which there is a maximum value for the intensity. Return the emission monochromator to this wavelength.
5. Find the excitation wavelength maximum between 265 and 285 nm in the same manner, with the emission monochromator

set at the wavelength of maximum fluorescence. Note: The aforementioned FLWinLab software allows for simultaneous scanning of the excitation and emission wavelengths in what is termed a '3D Scan'. This allows both the excitation and the emission wavelength maxima to be determined in one experiment.

6. With both the excitation and the emission wavelengths set at their peak values, adjust the instrument to give a reading corresponding to about 80% of the full scale. Narrow slit widths and a lower amplification (expansion factor, gain) are preferred, and a compromise between the two may have to be found (*see* **Note 6**).
7. Determine the emission spectrum by scanning the emission monochromator over the entire wavelength range over which fluorescence occurs. A scan speed of 100 nm/min is generally suitable.
8. Add a small aliquot of a concentrated DNA solution to the cuvette such that the concentration of DNA is in excess. Mix and immediately check the fluorescence emission at the emission maximum of the protein. Check several times over the next few minutes until a consistent reading is obtained. Allow this time for equilibration in subsequent experiments. Do not adjust the slit widths or the amplification.
9. Obtain an emission spectrum and compare with that obtained for the protein only. If fluorescence quenching is suspected, make sure that allowance for sample dilution has been made. If an inner filter correction is required, measure the absorbance of the sample in a spectrophotometer (in the same cuvette) and correct the observed fluorescence as discussed in the **Subheading 1**.
10. Add aliquots of DNA until there is no further change in fluorescence intensity in the emission spectrum (*see* **Notes 7** and **8**).

3.1.2. Protein–DNA Titrations

1. Examine the emission spectrum of the free protein. If it is characteristic of tyrosine fluorescence check for interference from the Raman band (*see* **Note 9**). If it is characteristic of tryptophan, check for tyrosine contributions which may be masked (*see* **Note 10**).
2. Examine the emission spectrum of the protein bound to DNA. The titration method described in the following passage is particularly applicable when the only change in the spectrum is a change in fluorescence intensity. Several variations of this method are described briefly in **Note 11** including an example where the emission spectrum of the protein shifts on binding DNA.

3. Accurately determine the concentration of protein and DNA solutions, e.g. by UV spectroscopy. As we are titrating DNA into protein try to use a stock concentration of DNA which is at least 20 times the concentration of protein used in the experiment multiplied by the estimated stoichiometric ratio; for example, if the protein concentration used is 10 μM and the stoichiometry estimated to be 1:1 then the DNA concentration should be at least 20 × 10 μM = 200 μM. This would mean that the dilution of the original protein solution will be only 5% at the stoichiometric point.
4. Using the protein solution set up the instrument as described in **steps 1–6** of **Subheading 3.1.1.** If measuring tyrosine fluorescence, use an excitation wavelength near the maximum. This wavelength can also be used for tryptophan excitation if tyrosine fluorescence is insignificant; otherwise, use an excitation wavelength of 295 nm.
5. Run a buffer blank and check that the profile of the emission spectrum is consistent with that previously obtained. Subtract this spectrum from subsequent spectra, if this can be done automatically.
6. Set the emission monochromator to the emission wavelength maximum and ensure that the readout is ~80% of its maximum value. Note down the value.
7. To begin the titration add a small aliquot from the stock DNA solution to the protein in the cuvette. Mix and allow the sample to equilibrate (use the time period determined earlier) before taking a reading. The aliquots should be sufficiently small such that the protein is still greatly in excess and a linear change is observed as more DNA is added.
8. Continue to add the same quantity of DNA for eight to ten points. If changes are still approximately linear at this stage gradually increase the volume of the DNA added, noting the total amount added at each point.
9. When the change in intensity begins to deviate significantly from linearity decrease the size of the aliquot so that more data points are obtained in this region.
10. As quenching approaches the maximum larger aliquots of DNA can be added. Continue until no change in quenching is observed for several points.
11. After the last point check that the emission spectrum of the complex is consistent with that previously obtained for the bound protein.
12. Remove the sample, wash the cuvette thoroughly, and run a blank spectrum consisting of cell plus buffer. This should have negligible or no fluorescence. Subtract any value at the

emission wavelength maximum from the data points, if not already done automatically (*see* **Note 9**).

13. In some cases it may be preferable to titrate protein into DNA, for an example of this method, *see* **ref. *10.*** The procedure is similar to that given here, but in this case the experiment should be repeated by adding protein to the buffer in the absence of DNA as a reference. Subtraction of the two curves should yield a clear binding curve (*see* **Fig. 2**). For a discussion of the merits of whether to titrate protein with DNA or vice versa, *see* **Note 12.**

3.1.3. Data Analysis

1. For each data point calculate the fluorescence quenching as:

$$\Delta F = [F_f - F] \text{ and } Q = 100 \times (\Delta F / F_f) \quad (5)$$

where F is the measured fluorescence intensity and F_f is the fluorescence intensity in the absence of DNA, having made any corrections for inner filter effects and dilution of the sample. Also calculate the DNA and protein concentrations at each point. Then calculate R, the ratio of the concentration of DNA to that of protein.

2. Plot either a graph of Q against R or F against DNA concentration, D. If only one mode of binding is occurring the graph should look like one of the curves shown in **Fig. 3.** If the 'break point' in the titration is sharp, *see* **Fig. 2c**, it indicates a small dissociation constant, K_d (or a large association constant, K_a) compared to the protein concentration. Conversely, weak binding arising from a large dissociation constant (or from too dilute a protein solution) will give a smoothly rising curve with no apparent break point.

3. The stoichiometry of binding is the value of R at which the slope obtained from the initial linear range of the titration crosses the horizontal line defined by Q_{max} (or F_{max}) at which no further change in intensity occurs.

4. Further information can be extracted from the binding curve by fitting to an appropriate model. In the simplest case of a bimolecular interaction ($P + D \Rightarrow PD$), a useful expression to estimate the dissociation constant K_d is:

$$K_d = \frac{(1-\theta) \times \left(D - (\theta \times P_0)\right)}{\theta} \quad (6)$$

where θ is the fraction of bound to total protein at the stoichiometric point and P_0 is the total protein concentration in the cuvette. For a titration in which DNA is added into protein, the fraction of bound protein, θ is given by the analytical function:

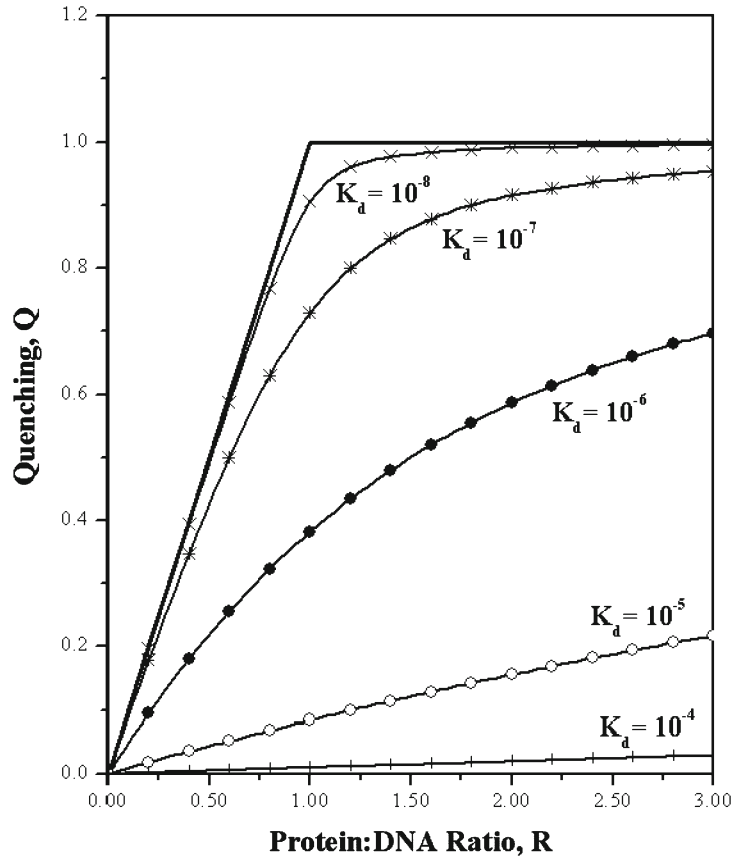

Fig. 3. Graph of fluorescence quenching, Q against protein:DNA ratio, R. The *curves* illustrate the addition of protein to DNA (at 1 μM) for dissociation constants (K_d, M) of 10^{-8} (*times symbol*), 10^{-7} (*asterisk*), 10^{-6} (*filled circle*), 10^{-5} (*open circle*), and 10^{-4} (*plus symbol*), and assuming a bimolecular interaction (1:1 model). The theoretical binding curve for infinitely strong binding (*upper curve*) shows a stoichiometry of one protein bound per DNA.

$$m = 1 + \left(\frac{D}{P_0}\right) + \left(\frac{K_d}{P_0}\right)$$

$$\theta = \frac{\Delta F}{F_f} = \left(0.5m - \sqrt{0.25m^2 - \frac{D}{P_0}}\right) \qquad (7)$$

where D is the DNA concentration at any point in the titration. **Equation** *7* enables fitting of the observed variables by non-linear regression analysis using the Origin fitting/graphical software (*see* **Subheading 3.2.2**).

5. Conversely for a titration in which protein is added into DNA, *see* **Fig. 2**, the fraction of bound DNA, θ is given by the analytical function:

$$m = 1 + \left(\frac{P}{D_0}\right) + \left(\frac{K_d}{D_0}\right)$$

$$\theta = \frac{\Delta F}{\Delta F_b} = \left(0.5m - \sqrt{0.25m^2 - \frac{P}{D_0}}\right) \quad (8)$$

where P is the protein concentration at any point in the titration and D_0 is the total DNA concentration. ΔF_b is the maximum change in fluorescence when all of the DNA is complexed with protein.

6. **Equation 6** also applies to more complex cooperative binding along a linear DNA lattice *(22)*, assuming the cooperativity is sufficiently high, when K_a becomes equal to the apparent binding constant (and approximates to the product of the cooperativity factor and the intrinsic binding constant for one site). For a more extensive discussion of complex DNA-binding equilibria, *see* **ref.** *23.*

3.2. Fluorescence Anisotropy

3.2.1. Anisotropy Experiments

1. With the known working concentration of FAM-labelled-DNA in the cuvette run an emission spectrum using an excitation wavelength of 490 nm (the FAM excitation λ_{max}) over the region 450–600 nm. This serves to check the emission wavelength maxima of ~520 nm and to select slit widths (typically between 2.5 and 12.0 nm) that give a reasonable emission intensity. Slit widths should be varied to increase the signal intensity, depending on the concentration of fluorescently labelled DNA. Use these slit widths in the anisotropy measurements.
2. The anisotropy experiments were performed by titration of protein into DNA. Typically a volume of 5–10 μL of protein is added at each addition into a 1,200-μL volume of FAM-labelled-DNA. Mix the sample by inversion, insert the cuvette into the fluorimeter, and allow the solution to reach equilibrium (~5 min) before measurement of the anisotropy. The sample should be stirred continuously and the sample temperature held constant. DNA concentrations ranging from 30 nM up to 10 μM, depending on the protein construct, were used for the HMGB1-B′ and HMGB1 titrations (*see* **Fig. 4**). Final protein concentrations typically reached 3× that of the DNA. In all cases the dilution factor did not exceed 10%.
3. Fluorescence anisotropy is measured by excitation of the FAM-labelled DNA at 490 nm, setting the excitation path polariser to alternate vertical and horizontal positions. For each position of the excitation polariser, the emission path polariser is also moved alternately to vertical and horizontal positions. At each of the four possible positions the fluorescence intensity is measured at 520 nm to yield I_{vv}, I_{vh}, I_{hh}, and

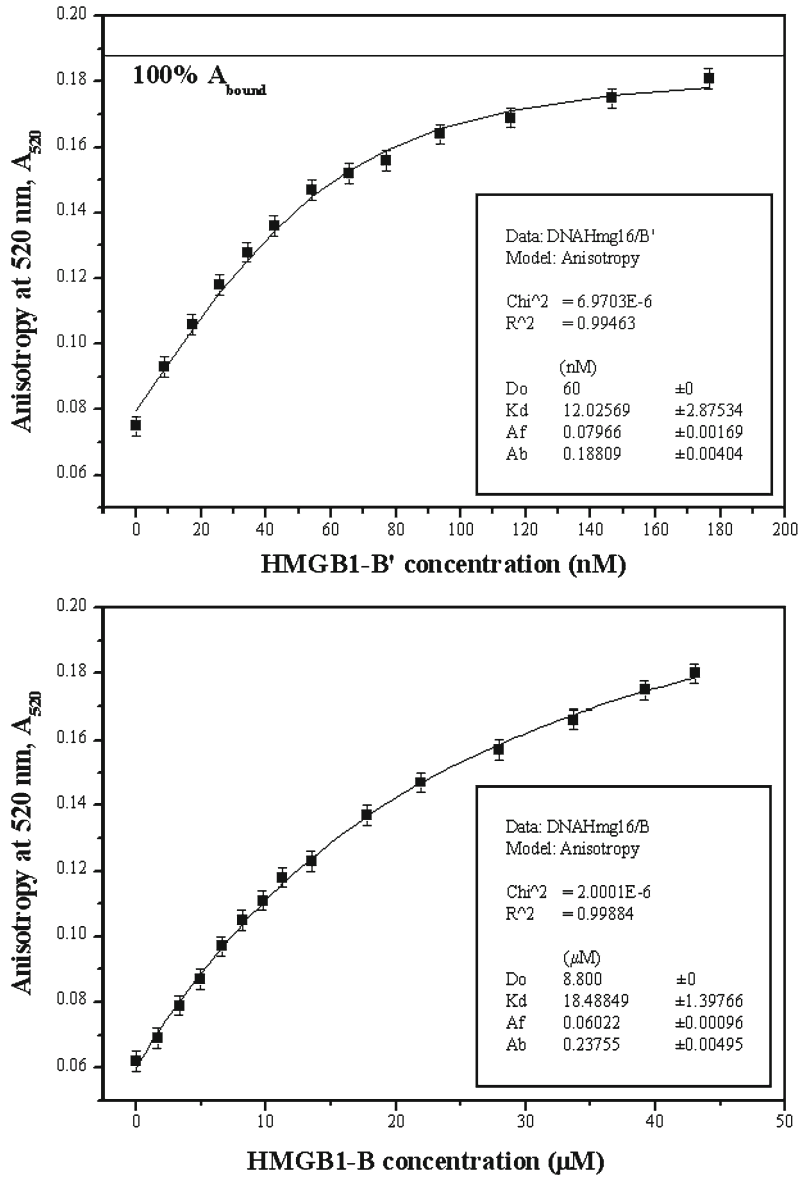

Fig. 4. Plots of the change in fluorescence anisotropy of FAM-DNA^{HMG} upon titration with HMGB1-B′ (**top**) and HMG1-B (**bottom**). Titrations were performed at a number of DNA and protein concentrations; however, the final dataset used for accurate determination of binding constants was recorded with 60 nM FAM-DNA^{HMG} and 1.5 μM HMGB1-B′ (**top**) and 8.8 μM FAM-DNA^{HMG} and 288.0 μM HMGB1-B (**bottom**). The original data for (**a**) are shown in Table 1. The datasets were fitted by non-linear regression to a 1:1 binding model using Origin. The best-fit curves are shown along with the derived K_d values. The anisotropy data fitted to a 1:1 binding model clearly show removal of the very basic C-tail from HMGB1-B′ to generate HMGB1-B raises the K_d from 12 nM to 18.5 μM (i.e. by three orders of magnitude), demonstrating that residues outside the minimal domain help to greatly enhance binding affinity.

I_{hv} (*see* **Subheading 1.2**). Using **Eqs. 3** and **4** the grating factor and anisotropy values can then be calculated for each titration point (*see* **Note 13**).

3.2.2. Data Analysis

1. A tab-delimited text file containing output, the anisotropy values, is read into an Excel spreadsheet (*see* **Table 1**) which can then be directly imported into the 'Data' window of a curve-fitting and graphical software package, such as Origin.
2. A spreadsheet is also useful to calculate the DNA and protein concentrations at each titration point, making any correction for dilution of the sample (*see* **Table 2**). A correction for the inner filter effect could be required at higher FAM-labelled DNA concentrations (at 2.5 μM the absorbance at 496 nm is 0.2). Calculate R, the ratio of the concentration of protein to that of DNA as well. Import into Origin.
3. Plot a graph of anisotropy, A against either protein concentration, P or ratio, R. The graph should be recognisable as a binding curve, similar to that already seen with intrinsic fluorescent quenching data (*see* **Fig. 2** and **Subheading 3.1.3**). The stoichiometry of binding is the value of *R* at which the slope obtained from the initial linear range of the titration crosses the horizontal line defined by the maximum anisotropy, when the DNA is fully bound.

Table 1
Raw data output from a fluorescence anisotropy experiment

Mode:	Anisotropy		Date: ######						
Ex. Slit	Em. Slit	Ex. Wave	Em. Wave	Temp.	I_{vv}	I_{vh}	GF	Int.	Comment
11	11	490	520	20	123.493	104.430	0.951	0.075	60 nM FAM-DNA
11	11	490	520	20	127.373	101.365	0.962	0.093	Plus 7 μL B′
11	11	490	520	20	130.900	99.762	0.967	0.106	Plus 14 μL B′
11	11	490	520	20	133.962	98.694	0.968	0.118	Plus 21 μL B′
11	11	490	520	20	133.221	95.716	0.965	0.128	Plus 28 μL B′
11	11	490	520	20	136.248	95.504	0.970	0.136	Plus 35 μL B′
11	11	490	520	20	136.961	93.429	0.967	0.147	Plus 45 μL B′
11	11	490	520	20	134.135	90.144	0.968	0.152	Plus 55 μL B′
11	11	490	520	20	130.868	86.808	0.971	0.156	Plus 65 μL B′
11	11	490	520	20	123.729	79.592	0.965	0.169	Plus 100 μL B′
11	11	490	520	20	123.142	77.529	0.970	0.175	Plus 130 μL B′
11	11	490	520	20	119.774	74.340	0.969	0.181	Plus 160 μL B′

60 nM FAM-DNA^{HMG} was titrated with successive 7.0 μL aliquots of 1.5 μM HMG1-B′ in 100 mM KCl, 10 mM potassium phosphate buffer (pH 6.0) at 20°C. The excitation wavelength was 490 nm and the emission wavelength was 520 nm, with both slits set at 11 nm. The column labelled 'Int.' is the calculated anisotropy; other column labels are as described in **Subheading 1.2**

Table 2
Spreadsheet example for calculation of protein and DNA concentrations

Titration of 16-bp FAM-HMG DNA with HMGB1-B′					Date: ######	
Ex = 490 nm; Em = 520 nm; Slits = 11.0/11.0 nm; Volume = 1,200 μL						
Buffer:	100 mM KCl, 10 mM Kphosphate (pH 6.0)					
[B′] = 1,500 nM; [F-DNA^{HMG}] = 60 nM						
Add (μL)	Total added (μL)	Total vol. (μL)	Dilution	F-DNA final (nM)	B′ final (nM)	P/F-DNA
0.00	0.00	1,200.00	1.0000	60.0000	0.0000	0.0000
7.00	7.00	1,207.00	1.0058	59.6520	8.6993	0.1458
7.00	14.00	1,214.00	1.0115	59.3081	17.2982	0.2917
7.00	21.00	1,221.00	1.0172	58.9681	25.7985	0.4375
7.00	28.00	1,228.00	1.0228	58.6319	34.2020	0.5833
7.00	35.00	1,235.00	1.0283	58.2996	42.5101	0.7292
10.00	45.00	1,245.00	1.0361	57.8313	54.2169	0.9375
10.00	55.00	1,255.00	1.0438	57.3705	65.7371	1.1458
10.00	65.00	1,265.00	1.0514	56.9170	77.0751	1.3542
15.00	80.00	1,280.00	1.0625	56.2500	93.7500	1.6667
20.00	100.00	1,300.00	1.0769	55.3846	115.3846	2.0833
30.00	130.00	1,330.00	1.0977	54.1353	146.6165	2.7083
30.00	160.00	1,360.00	1.1176	52.9412	176.4706	3.3333

4. A dissociation constant K_d may be obtained by fitting the anisotropy/protein concentration values assuming a binding mode. The equation for a bimolecular (1:1) model is:

$$m = 1 + \left(\frac{P}{D_0}\right) + \left(\frac{K_d}{D_0}\right)$$

$$A = A_f - \left(A_f - A_b\right) \times \left(0.5m - \sqrt{0.25m^2 - \frac{P}{D_0}}\right) \qquad (9)$$

where P is the protein concentration, A is the measured anisotropy upon addition of protein to DNA, A_f is the anisotropy value of the free DNA, A_b is the maximum anisotropy value obtained when the DNA is fully bound to protein, D_0 is the DNA concentration used in the titration, and K_d is the dissociation constant. This function needs to be entered into the fitting software prior to its use in the fitting routine.

5. In the curve-fitting/graphical program the columns of data must be set as follows: the protein concentration P as an independent variable (on the X-axis) and the measured anisotropy A as a dependent variable (on the Y-axis). The DNA concentration (D_0) is a known (fixed) variable. The anisotropy for free and fully bound DNA (A_f and A_b) and the K_d are treated as three unknown variables.
6. The non-linear least squares regression routine is run to best fit to the observed anisotropy/protein concentration data according to the equation for a particular mode of binding (e.g. a 1:1 model, **Eq. 9**). The routine should converge to a solution after a number of iterations, to give values for the unknowns K_d, A_f, and A_b (along with their errors). The curve for the equation representing the best fit of that binding mode may then be plotted, along with the original data (*see* **Fig. 4**). A measure of how well the data are fitted is given by χ^2 and by the regression coefficient r^2. A χ^2 value close to zero and an r^2 value close to one indicate a good fit to the data (*see* **Note 14**).

4. Notes

1. Care should be taken when handling fluorescence cuvettes as both fingerprints and scratches can introduce significant artefacts into the experiment. After use, cuvettes should be thoroughly washed with ethanol or acetone, rinsed with distilled water, and dried thoroughly using a stream of nitrogen or helium gas. If contamination is great, then immerse the cells for several hours in a dilute solution of Hellmanex (from Hellma GmbH), rinse with water, and dry.
2. Changes in temperature affect the viscosity of the solution and hence the number of collisions a fluorophore makes with solvent molecules. Since the viscosity of water is inversely proportional to temperature it follows that there is less collisional quenching and a greater fluorescence at lower temperatures. Both Trp and Tyr fluorescence are highly sensitive to temperature, as is the intrinsic fluorescence of a protein (*see* **ref. *24*** and references therein). For accurate fluorescence measurements temperature control is an essential.
3. A stirred cuvette is similarly required to maintain the thermal equilibrium. If the fluorimeter is not equipped with a magnetic stirrer unit, adequate mixing can usually be achieved by gently drawing the solution in and out through a plastic pipette tip. Avoid the introduction of bubbles into the sample by stirring

or pipetting too fast as this can both denature the protein and cause light scattering.

4. In designing short synthetic oligonucleotides for binding studies, GC base pairs are often added at the end(s) of the DNA duplex to reduce fraying of terminal base pairs. However, the quantum yield of an extrinsic fluorophore attached to the end of a DNA can be affected by the choice of the adjacent (terminal) nucleotide, e.g. in 3′-fluorescein- and 5′-hexachlorofluorescein (HEX)-labelled DNAs an adjacent guanosine nucleotide reduces the quantum yield, i.e. fluorescent intensity by as much as 40% *(25, 26)*. Thus to remove potential quenching effects in fluorescein- (and its derivatives) labelled DNA duplexes but still have a GC terminal base pair, one should design an oligonucleotide with a cytosine nucleotide adjacent to the fluorophore modification, with a base-paired guanosine in the complementary oligonucleotide. Note: 3′-Cy3-labelled DNAs are little affected by the choice of adjacent nucleotide.
5. Fluorescence intensity is only proportional to concentration when the absorbance is no greater than ~0.2 OD units, at the excitation wavelength selected. Remember that the absorption bands for proteins and nucleic acids overlap, and in titrations the contribution of the nucleic acid to the overall absorption must be considered as well as the protein.
6. Whilst it is preferable to have narrow excitation and emission slit widths and a low amplification factor, there may be a need to compromise in order to obtain a stable reading. For proteins displaying tyrosine fluorescence, the small wavelength difference between the excitation and emission maxima suggests that it would be better to maintain narrow slits and increase the signal amplification. For proteins dominated by tryptophan fluorescence the greater the difference between the excitation and emission wavelengths, the greater the feasibility of increasing the slit widths and maintaining a lower amplification. In general, when measuring emission spectra it is better to use a narrow excitation slit width and to widen the emission slit width. For broad banded spectra such as that seen with tryptophan both slits can be widened.
7. If no changes in the emission spectrum of the protein are observed when DNA is added (after inner filter and dilution corrections if necessary), then either the protein is not binding to DNA or binding cannot be detected by this procedure and will need to be assessed by another method such as fluorescence anisotropy or FRET.
8. Note the molar ratio of DNA:protein at which no further changes occur. This will provide a rough guide for future experiments.

9. Tyrosine emission can often be confused with Raman scattering from water molecules which occurs at ~305 nm when an excitation wavelength of 280 nm is used. The presence of the Raman band can be assessed by measuring the emission spectrum using a different excitation wavelength. The fluorescence emission spectrum is independent of excitation wavelength, whereas Raman scattering occurs at a constant wavenumber (=$1/\lambda$ in cm^{-1}) difference from that used for excitation and will shift in the same direction as the change in excitation wavelength. In aqueous solutions this shift is –3,380 cm^{-1}. The contribution of the Raman band to the overall intensity of the signal can be assessed by running an emission spectrum of a buffer blank. Automatically subtract out this spectrum from subsequent spectra where possible. Note: The FLWinLab software includes a 'PreScan Mode' in which the Raman absorption, the Rayleigh scatter, and their second-order peaks are identified automatically.
10. To check the contribution tyrosine may make to a fluorescence emission spectrum dominated by tryptophan, run an emission spectrum using an excitation wavelength of 295 nm. At this wavelength only tryptophan emission will be observed. If the emission spectrum is unchanged then it can be concluded that the contribution from tyrosine residues is negligible. (Of course the intensity will be lower as tryptophan absorption is greater at 280 nm than it is at 295 nm.)
11. In cases where both the emission wavelength maximum shifts and the fluorescence intensity is quenched, the method described can be used provided that an emission wavelength is chosen outside the wavelength region overlapped by the emission spectra of the free and bound protein. Alternatively, the ratio of the intensity of the emission maxima of the free and bound protein can be followed; for an example *see* **ref.** ***6***. The use of a ratio method means that the dilution factor and inner filter correction can usually be ignored, although strictly speaking the ratio is not a linear function of degree of binding.
12. We have found that in some cases different results can be found dependant on the direction of the titration; this can occur when the fluorescence changes observed include contributions from protein–protein interactions accompanying DNA binding in addition to (or instead of) contributions from the interaction with the DNA itself (**ref.** *27)*.
13. In the LS50B the 'Single Read' application of the FLWinLab software automatically controls the change of both polariser positions. Calculation and display of grating factors and anisotropy values is then in real time. All that is required is to enter the excitation and emission wavelengths, with their

respective slit widths and an integration time (typically 1–10 s). The relevant intensities, grating factor, and anisotropy values may be saved into a spreadsheet after each addition of protein (*see* **Table 1**).

14. It can be difficult to decide between different binding modes on the basis of small differences in the fits, e.g. slightly different χ^2 values. One should be careful to not over-interpret, and unless there is good reason the simplest binding model that fits the data should be chosen.

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