SSB–DNA Binding Monitored by Fluorescence Intensity and Anisotropy

ARTICLE *in* METHODS IN MOLECULAR BIOLOGY (CLIFTON, N.J.) · SEPTEMBER 2012

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Chapter 4

SSB–DNA Binding Monitored by Fluorescence Intensity and Anisotropy

Alexander G. Kozlov, Roberto Galletto, and Timothy M. Lohman

Abstract

Fluorescence methods have proven to be extremely useful tools for quantitative studies of the equilibria and kinetics of protein–DNA interactions. If the protein contains tryptophan (Trp), as is often the case, and there is a change in intrinsic Trp fluorescence of the protein, one can use this change in signal (quenching/enhancement) to monitor binding. One can also attach an extrinsic fluorophore to either the protein or the DNA and monitor binding due to a change in fluorescence intensity or a change in fluorescence anisotropy. Such equilibrium studies can provide important quantitative information on stoichiometries (occluded site size, number of binding sites) and energetics (affinities and cooperativities) of the interactions. This information is needed to understand the mechanisms of protein–DNA interactions. A critical aspect of such approaches for systems that have non-unity stoichiometries (e.g., a protein that binds multiple ligands) is knowledge of the relationship between the change in fluorescence signal (intensity or anisotropy) and the average extent of binding. Here we describe procedures for using fluorescence approaches to examine the stoichiometries and equilibrium binding affinities of *Escherichia coli* single-stranded DNA-binding protein (SSB) and *Deinococcus radiodurans* SSB with long polymeric ssDNA to determine an occluded site size. We also provide examples of studies of SSB binding to shorter oligonucleotides to demonstrate analysis and fitting of the data to an appropriate model (monitoring fluorescence intensity or anisotropy) to obtain quantitative estimates of equilibrium binding parameters. We emphasize that the solution conditions (especially salt concentration and type) can influence not only the binding affinity, but also the mode by which an SSB oligomer binds ssDNA.

Key words: EcoSSB, DrSSB, ssDNA binding, Fluorescence equilibrium titrations, Intrinsic tryptophan quenching, SSB–ssDNA thermodynamics, Anisotropy

1. Introduction

If a change in the fluorescence properties (e.g., intensity or anisotropy) accompanies the binding of two species, this can be used to quantitatively monitor the binding interaction and determine both the stoichiometry and energetics (affinity and thermodynamics) of binding using equilibrium titration methods. Such approaches

James L. Keck (ed.), *Single-Stranded DNA Binding Proteins: Methods and Protocols*, Methods in Molecular Biology, vol. 922,
DOI 10.1007/978-1-62703-032-8_4,

have been used extensively to examine numerous protein–DNA interactions in solution including many SSB–ssDNA interacting systems. Generally, if the molecule undergoes a change in its intrinsic fluorescence (e.g., tryptophan (Trp) in a protein), then this species is titrated with the nonfluorescent molecule (e.g., DNA) and the change in fluorescence signal (intensity or anisotropy) is monitored as a function of total DNA concentration. Alternatively, if the changes in the intrinsic fluorescence of the protein are not sufficient to reliably monitor the interaction, an extrinsic fluorophore can be attached to one species (either protein or DNA) to study the interaction. However in this case, it is often observed that the modification affects the energetics of the interaction. Independent of the nature of the fluorescence signal that is monitored (intensity or anisotropy), one needs to know (or determine) how the change in fluorescence signal relates to $<X>$, namely, the average number of ligands (in this case, DNA) that bind to the macromolecule (in this case, protein). We emphasize this point since for macromolecules that bind multiple ligands, the average fractional fluorescence signal change is often not directly proportional to $<X>$. However, once this relationship is established, then one can obtain an equilibrium binding isotherm that can be analyzed to determine stoichiometries (number of binding sites), equilibrium binding constants (K), and cooperativities of protein–DNA interactions. Equilibrium experiments performed at different temperatures and different solution conditions (salt, pH) allow one to examine how the binding is thermodynamically linked to these variables (1). Such linkage analysis can yield information on the binding enthalpy (linkage to temperature) and whether ions or protons bind preferentially to the protein–DNA complex or the free protein and/or DNA (2–6). This provides information about the thermodynamic forces that drive the formation of the complex and more practically how solution conditions (pH, salt concentration and type, etc.) can influence these interactions. This information is needed to understand the energetics of the protein–DNA interaction and allows predictions of their behavior under conditions that either have not been examined or are not as easily accessed experimentally.

Fluorescence equilibrium titrations have been used extensively to study interactions of single-stranded DNA-binding proteins (SSBs) with ssDNA. The *Escherichia coli* SSB (*Eco*SSB) and the *Deinococcus radiodurans* SSB (*Dr*SSB) are used as examples in this chapter and are also discussed in the chapter describing isothermal titration calorimetry (ITC) methods; hence the reader should refer to that chapter for more introductory comments about these protein–DNA systems.

Here we briefly review the equilibrium fluorescence titration studies that have been performed to examine the interactions of *Eco*SSB and *Dr*SSB with ssDNA. We first remind the reader that in solution, *Eco*SSB and *Dr*SSB are stable homotetramers and

homodimers, respectively. As a result of the multiple ssDNA-binding sites (4 OB folds each), these SSB proteins can bind to long polymeric ssDNA in different binding modes in vitro that depend on solution conditions, particularly salt concentration and type (7). The *Eco*SSB tetramer shows dramatic changes in its mode of binding to ssDNA as a function of salt concentration, type, and valence. On poly(dT) *Eco*SSB binds in its fully wrapped $(SSB)_{65}$ mode at [NaCl] >0.2 M in which ssDNA interacts with all four subunits of the SSB tetramer with an occluded site size of 65 nts. However, at lower [NaCl] (≤10 mM) it binds in its highly cooperative $(SSB)_{35}$ mode, in which ssDNA binds to an average of only two subunits of the tetramer with an occluded site size of ~35 nts (2, 3, 7). At intermediate salts (40–100 mM NaCl), *Eco*SSB forms its $(SSB)_{56}$ binding mode on poly(dT). Equilibrium fluorescence titrations have been used extensively to study the effects of various solution conditions on its different binding modes with polynucleotides and on the energetics of ssDNA binding. Effects of salt concentration (2, 3), anion and cation type (4–6), pH (4, 6), polyamines (8), binding density (4), and base composition (5, 9) have been examined. Statistical thermodynamic models describing the binding of SSB tetramers to long ssDNA in its highly cooperative $(SSB)_{35}$ mode at low salt conditions (<0.02 M NaCl) (10) and in its low cooperativity $(SSB)_{65}$ mode at higher salt concentrations (>0.2 M NaCl) (11) have also been developed.

Oligodeoxynucleotides of different lengths have also been used to examine binding of ssDNA to the individual tetramer. Four molecules of $(dX)_{16}$, two molecules of $(dX)_{35}$, or one molecule of $(dX)_{70}$ can bind per SSB tetramer. However, the shorter oligodeoxynucleotides, $(dX)_{16}$ or $(dX)_{35}$, display negative cooperative binding to an individual SSB tetramer such that ssDNA binds with high affinity to the first two subunits of the tetramer and with much lower affinity to the second two subunits. In addition, this negative cooperativity is highly salt dependent, decreasing with increasing [NaCl] or [$MgCl_2$], although high salt does not eliminate the negative cooperativity (12). In fact, this salt-dependent negative cooperativity provides part of the explanation for the salt-dependent change in the SSB–ssDNA binding modes observed with poly(dT) (12–14). Studies of the *Eco*SSB binding to oligodeoxynucleotides of lengths close to the occluded site size ($n = 65$) have been used to estimate the binding enthalpies and have shown that the unusually high negative heat capacity change associated with the binding of $(dA)_{70}$ is due to the temperature-dependent stacking of adenine within the ssDNA and the loss of these stacking interactions upon binding SSB (15, 16). The energetics of cooperative binding of two SSB tetramers to $(dA)_{70}$ has also been examined at low salt conditions (17). More limited fluorescence binding studies have been performed with the *Dr*SSB dimer (18) and yeast *sc*RPA hetero-trimer (19).

We note that in all of the studies mentioned above the intrinsic Trp fluorescence quenching of the protein has been used to monitor binding, although recent studies (20) have used *Eco*SSB that has been extrinsically labeled with fluorophores that undergo fluorescence intensity changes upon binding ssDNA. Generally these extrinsic fluorophores also change the binding affinities and properties and thus care should be taken to characterize these labeled proteins rather than assume that their behavior mirrors the unlabeled protein.

The energetics and kinetics of *Eco*SSB binding to single-stranded oligodeoxynucleotides have also been studied by monitoring the change in fluorescence intensity of fluorescently labeled nucleic acid (21–23) as well as SSB (20, 24). Although changes in the fluorescence anisotropy of a labeled ssDNA can also be utilized to monitor binding, these have not been extensively used to study SSB–ssDNA interactions. Changes in the fluorescence anisotropy of *Eco*SSB have been used to study the self-assembly of a His-55 to Tyr mutant of SSB (SSB-1) that destabilizes the tetramer to form monomers (25, 26).

In the following sections we describe procedures for performing and analyzing titration experiments monitoring changes in either fluorescence intensity or anisotropy. These experiments were performed using a PTI QM-2000 spectrofluorometer (Photon Technologies, Inc., Lawrenceville, NJ) in which the quenching of the intrinsic Trp fluorescence of the protein or the change in fluorescence intensity or anisotropy of an ssDNA labeled with an extrinsic fluorophore (fluorescein) was used to monitor binding.

2. Materials

2.1. Buffer Solutions

All solutions were prepared with reagent grade chemicals and glass distilled water that was subsequently treated with a Milli Q (Millipore, Bedford, MA) water purification system. The buffer used is Buffer T (10 mM Tris (tris(hydroxymethyl) aminomethane), pH 8.1, 0.1 mM EDTA) with different NaCl concentrations as indicated in the text for each particular experiment. Buffers were prepared as described in the ITC Chapter.

2.2. Proteins and ssDNA

1. *Eco*SSB (27) and *Dr*SSB (28) proteins were expressed, purified, and stored as described in the ITC Chapter. Protein concentrations were determined spectrophotometrically in buffer T containing 0.20 M NaCl using the extinction coefficients, $\varepsilon_{280} = 1.13\times10^5\ M^{-1}$ (tetramer) cm^{-1} for *Eco*SSB and $\varepsilon_{280} = 8.2\times10^4\ M^{-1}$ (dimer) cm^{-1} for *Dr*SSB (see Note 1).
2. The single-stranded oligodeoxythymidylates, $(dT)_{70}$, $(dT)_{50}$, $(dT)_{35}$, and F-$(dT)_{50}$ (labeled with fluorescein at the 5′ end),

were synthesized and stored as described in the ITC Chapter of this book. Poly(dT) has an average length ~1,100 nucleotides and was purchased from Midland Certified Reagents Co. (Midland, TX). The concentration of poly(dT) is expressed in nucleotides and was determined spectrophotometrically in buffer *T* (pH 8.1), 0.1 M NaCl using the extinction coefficient, $\varepsilon_{260,\mathrm{dT}} = 8.1\times10^3\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$. Concentrations of oligodeoxythymidylates were determined using extinction coefficients (per mole of ssDNA molecule) at 260 nm calculated according to the expression $\varepsilon_{260} = N\times\varepsilon_{260,\mathrm{dT}} + \varepsilon_{260,\mathrm{F}}$, where *N* is the number of nucleotides and $\varepsilon_{260,\mathrm{F}} = 2.096\times10^4\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ is the extinction coefficient for fluorescein (Glen Research) (see Note 1).

3. Methods

3.1. Equilibrium Binding Models Used for Analysis of Titration Data

For the binding of a ligand (*X*) to a one-site macromolecule (*M*), the equilibrium can be defined as in Scheme 1,

$$M + X \overset{K_{obs}}{\rightleftharpoons} MX$$

Scheme 1

where $K_{\mathrm{obs}} = [MX]/[X][M]$ is the equilibrium association constant and $[MX]$, $[X]$, $[M]$ are the equilibrium concentrations of each species in solution. The expression for the average degree of binding $<X>$ (average number of ligands bound per macromolecule) can be obtained using the mass conservation equation $[M]_{\mathrm{tot}} = [M] + [MX]$ and is given in Eq. 1

$$<X> = \frac{[MX]}{[M]_{\mathrm{tot}}} = \frac{K_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]} \tag{1}$$

If the macromolecule has n identical, independent sites the expression for $<X>$ is given in Eq. 1a (1)

$$<X> = \frac{[MX]}{[M]_{\mathrm{tot}}} = \frac{nK_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]} \tag{1a}$$

The equilibrium binding model in Scheme 1 was used to fit the titration curves for *Dr*SSB binding to $(\mathrm{dT})_{50}$ shown in Figs. 2a and 3a, b (see Subheading 10).

Scheme 2 defines the equilibrium for the binding of two molecules of ligand to a macromolecule,

$$M + X \underset{}{\overset{K_{1,obs}}{\rightleftharpoons}} MX + X \overset{K_{2,obs}}{\rightleftharpoons} MX_2$$

Scheme 2

where $K_{1,\mathrm{obs}} = [MX]/[X][M]$ and $K_{2,\mathrm{obs}} = [MX_2]/[X][MX]$ are the macroscopic (see Note 2) equilibrium binding constants for binding of the first and second ligands, respectively. The expression for $<X>$ for this system is given in Eq. 2 (1):

$$<X> = \frac{\mathrm{d}\ln P}{\mathrm{d}\ln X} = \frac{K_{1,\mathrm{obs}}[X] + 2K_{1,\mathrm{obs}}K_{2,\mathrm{obs}}[X]^2}{1 + K_{1,\mathrm{obs}}[X] + K_{1,\mathrm{obs}}K_{2,\mathrm{obs}}[X]^2} \qquad (2)$$

This model was used to fit the data in Fig. 2b for the binding of two molecules of $(\mathrm{dT})_{35}$ to *Eco*SSB (see Subheading 10).

3.2. Monitoring Binding by a Fluorescence Intensity Change

The total fluorescence intensity of a mixture of "i" fluorescent species can be defined as in Eq. 3:

$$F = \sum F_i[X]_i \qquad (3)$$

where F_i is the molar fluorescence intensity of species i and $[X]_i$ is its concentration. For a macromolecule (M) with fluorescence intensity, $F_{\mathrm{M}} = F_0$, that upon formation of a 1:1 complex (MX) undergoes a quenching of its fluorescence intensity, $F_{\mathrm{MX}} = F_{\min}$, the observed change in relative fluorescence quenching is described by Eq. 4:

$$Q_{\mathrm{obs}} = Q_{\max}\frac{K_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]} \qquad (4)$$

where $Q_{\mathrm{obs}} = (F_0 - F_{\mathrm{obs}})/F_0$ is the observed fluorescence quenching and $Q_{\max} = (F_0 - F_{\min})/F_0$ is the fluorescence quenching at saturation of the macromolecule with ligand, where $<X>$ and K_{obs} are as defined above. The concentration of free ligand, $[X]$, can be determined from the mass conservation Eq. 4a:

$$[X]_{\mathrm{tot}} = [X] + <X>[M]_{\mathrm{tot}} = [X] + \frac{K_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]}[M]_{\mathrm{tot}} \qquad (4a)$$

For a macromolecule with n independent and identical sites, Eqs. 4 and 4a transform into Eqs. 5 and 5a:

$$Q_{\mathrm{obs}} = Q_{\max}\frac{nK_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]} \qquad (5)$$

$$[X]_{\mathrm{tot}} = [X] + <X>[M]_{\mathrm{tot}} = [X] + \frac{nK_{\mathrm{obs}}[X]}{1 + K_{\mathrm{obs}}[X]}[M]_{\mathrm{tot}} \qquad (5a)$$

The expression describing the binding of two ligands to a macromolecule according to Scheme 2 is given by Eq. 6:

$$Q_{\text{obs}} = \frac{Q_1 K_{1,\text{obs}}[X] + Q_2 K_{1,\text{obs}} K_{2,\text{obs}}[X]^2}{1 + K_{\text{obs}}[X] + K_{1,\text{obs}} K_{2,\text{obs}}[X]^2} \tag{6}$$

where $K_{1,\text{obs}}$ and $K_{2,\text{obs}}$ are the macroscopic equilibrium binding constants (see Note 2) for binding of the first and the second ligands, X, to the macromolecule M, and Q_1 and Q_2 are the fluorescence quenching upon binding of one and two molecules of ligand, respectively. In Eq. 6 the concentration of free ligand ($[X]$) can be determined from the mass conservation Eq. 6a:

$$[X]_{\text{tot}} = [X] + <X>[M]_{\text{tot}} \tag{6a}$$

where $<X>$ is defined in Eq. 2.

3.3. Monitoring Binding by Fluorescence Anisotropy Changes

SSB–DNA binding can also be monitored by the change in fluorescence anisotropy of an extrinsic fluorophore attached to either the SSB or the DNA. Fluorescence anisotropy (r) reflects the extent of depolarization of the fluorescence emission of a species in solution when excited with polarized light. It is directly related to the rotational diffusion of the species and is low for small and flexible molecules (higher depolarization), and increases when larger complexes are formed (slower rotational movement, smaller depolarization). Therefore, the change in fluorescence anisotropy can generally be used to monitor a binding event (see Note 3). This is particularly convenient for systems where no change in fluorescence intensity is observed between the free and bound states. For one-channel spectrofluorometers (L-format) the anisotropy is defined in Eq. 7 (29):

$$r = \frac{I_{\text{VV}} - G I_{\text{VH}}}{I_{\text{VV}} + 2 G I_{\text{VH}}} \tag{7}$$

where I_{VV} is the fluorescence emission intensity measured for vertically polarized excitation and vertically polarized emission. I_{VH} is the intensity measured for vertically polarized excitation and horizontally polarized emission, and G is the correction factor (see Note 4). We note that in many studies polarization (p) changes have been used directly to quantitatively analyze interacting systems rather than anisotropy (r). This will result in an incorrect binding isotherm and incorrect binding constant and should be avoided (see Note 5).

The anisotropy of a mixture of "i" fluorescent species is given by Eq. 8:

$$\bar{r} = \sum r_i f_i \tag{8}$$

where $f_i = X_i F_i / \sum X_i F_i$ is the fractional contribution to the observed fluorescence intensity of component i. It is important to

note the differences in the weighting factors in Eq. 3 versus Eq. 8. In Eq. 3, the observed fluorescence intensity is the sum of the fluorescence intensities of each species weighted by their concentrations, whereas in Eq. 8, the observed anisotropy is the sum of the anisotropies of each species weighted by the fractional contribution of each species to the total fluorescence. Based on Eq. 8 the expression for r for a 1:1 binding system is given by Eq. 9:

$$r = r_0 + (r_{\max} - r_0)\frac{K_{\text{obs}}[X]c}{1 + K_{\text{obs}}[X]c} \tag{9}$$

where r_0 and $r_{\max}$ are the limiting values of the anisotropy for (M) and (MX), respectively, $[X]$ is the free protein concentration, and K_{obs} is as defined above. The factor $c = F_{\text{bound}}/F_{\text{free}} = F_{\text{MX}}/F_{\text{M}}$ is the ratio of the fluorescence of the bound and free macromolecule (DNA) (assuming that it is labeled with fluorophore).

3.4. Amounts of SSB and ssDNA Required for Fluorescence Titrations and the Design of the Experiment

In general, preliminary information on the system (such as the oligomerization state of the protein and its stability) should be obtained prior to performing titration experiments (see Note 6). *Eco*SSB (7) is a stable tetramer and *Dr*SSB (28) is a stable dimer under the conditions of the experiments presented here. Any preliminary information on the value of binding affinities (if available from literature) can also be useful in designing the experiments and to estimate the amounts of SSB and DNA needed. For example, for most of the experiments presented in Fig. 1 (except the titrations with *Dr*SSB in 1.0 M NaCl) the affinity of the SSB for ssDNA is so high that binding is "stoichiometric" (i.e., all DNA added is bound until the "stoichiometric" point n is achieved) and the reaction is completed at a total DNA-to-protein ratio, $R = 1$ (mole $(\text{dT})_{\text{L}}$/mole SSB) in the case of oligonucleotides (Fig. 1c, d, 0.2 M NaCl) or $R \approx n$ for poly(dT) (Fig. 1a, b, 0.2 M NaCl), where n is the occluded site size in nucleotides (poly(dT) concentration is expressed in moles of nucleotides and not molecules). For such experiments the spectroscopic signal changes linearly with titrant concentration until the stoichiometric point is reached and does not change further upon addition of more titrant. A two- to three-fold excess of $[\text{DNA}]_{\text{tot}}$ over $[\text{SSB}]_{\text{tot}}$ is usually sufficient for these titrations while generally obtaining 20–25 points in the titration. The amounts of SSB and DNA required for a single titration can be estimated using the formula in Eq. 10:

$$R \cdot V_{\text{SSB,cell}} \cdot C_{\text{SSB,cell}} = V_{\text{DNA}} \cdot C_{\text{DNA}} \tag{10}$$

where R is the final total DNA-to-total protein ratio desired in the spectrophotometric cell at the end of titration, $V_{\text{SSB,cell}}$ is the minimum volume of protein solution in the cell (usually ~2 mL), $C_{\text{SSB,cell}}$ is the concentration of SSB in the cell (usually varying from 0.1 to 1 μM), and V_{DNA} is the volume of the titrant (DNA solution,

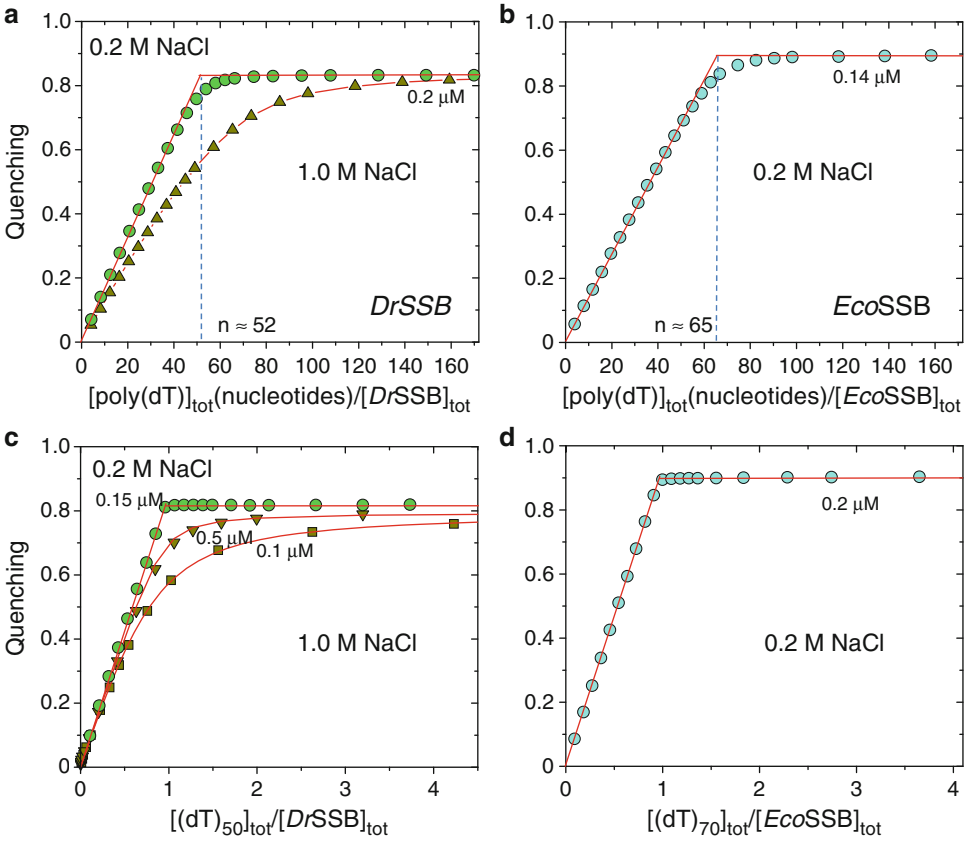

Fig. 1. Determination of the occluded site size of *Dr*SSB (**a**) and *Eco*SSB (**b**) on poly(dT) under stoichiometric conditions monitoring change in intrinsic Trp fluorescence. (**a**) Fluorescence titrations of *Dr*SSB (0.2 μM) with poly(dT) (829 μM (nucleotides)) under stoichiometric conditions (0.2 M NaCl, buffer T, pH 8.1, 25°C) are shown in *green circles*. The results are presented as a change in the extent of fluorescent quenching relative to free protein fluorescence versus the ratio of total poly(dT) concentration (nucleotides) to total concentration of the *Dr*SSB dimer in the cell. The linear increase in relative quenching indicates stoichiometric binding of the protein to poly(dT) until the point of saturation (plateau value) is reached (~83% quenching). The occluded site size (≈52 nucleotides per *Dr*SSB dimer) was determined by extrapolation of the linear part of the titration curve to the point of intersection with the corresponding plateau value after saturation (shown in *red*). For 1.0 M NaCl (*yellow triangles*) the titration is not stoichiometric, so a determination of occluded site size is not possible (in this case the concentrations used in the experiment were 0.2 μM *Dr*SSB and 817 μM of poly(dT)). (**b**) Fluorescence titration of *Eco*SSB (0.14 μM) with Poly(dT) (495 μM (nucleotides)) under stoichiometric conditions (0.2 M NaCl, buffer T, pH 8.1, 25°C). For this titration the occluded site size determined from the intersection point (as described in *panel A*) is ~65 and maximum quenching is ~90%. (**c**) Fluorescence titrations of *Dr*SSB with $(dT)_{50}$ in buffer T (pH 8.1, 25°C) under stoichiometric (0.2 M NaCl, *green circles*) and nonstoichiometric conditions (1.0 M NaCl, *yellow squares* and *triangles*). The titration isotherms are presented in the form of the dependence of relative fluorescence quenching versus the ratio of total $(dT)_{50}$ to total protein concentration. The concentrations of *Dr*SSB (dimer) used in the titrations are shown under corresponding plots. (**d**) Fluorescence titration of *Eco*SSB (0.1 μM tetramer) with $(dT)_{70}$ in buffer T (0.2 M NaCl, pH 8.1, 25°C) is indicative of 1:1 stoichiometric binding of *Eco*SSB tetramer to ssDNA.

usually ~100 μl), which generally does not exceed 5% of the solution volume in the cell (see Subheading 7). The concentration of the titrant (C_{DNA}) required for the experiment can then be determined from Eq. 10. For example, in the stoichiometric titrations

Table 1
Experimental Excel spreadsheet design for the titration of *Eco*SSB (0.14 μM, 1,800 μL total volume) with 495 M of poly(dT) in buffer T, 0.2 M NaCl (see Fig. 1b)

#	Titrant conc. (μM)	Titrant aliquot (μL)	Total titrant volume (μL)	Titrant conc. in cell (μM)	$[Poly(dT)]_{tot}$ per $[EcoSSB]_{tot}$
1	495	2	2	0.55	4
2	495	2	4	1.10	8
3	495	2	6	1.64	12
4	495	2	8	2.19	16
5	495	2	10	2.73	20
6	495	2	12	3.28	24
7	495	2	14	3.82	28
8	495	2	16	4.36	31
9	495	2	18	4.90	35
10	495	2	20	5.44	39
11	495	2	22	5.98	43
12	495	2	24	6.51	47
13	495	2	26	7.05	51
14	495	2	28	7.58	55
15	495	2	30	8.11	59
16	495	2	32	8.65	63
17	495	2	34	9.18	67
18	495	4	38	10.23	75
19	495	4	42	11.29	83
20	495	4	46	12.33	90
21	495	4	50	13.38	98
22	495	10	60	15.97	118
23	495	10	70	18.53	138
24	495	10	80	21.06	157

shown in Fig. 1b ($C_{SSB,cell} = 0.14$ μM, $R \approx 160$) and in Fig. 1d ($C_{SSB,cell} = 0.10$ μM, $R \approx 4$) the suggested titrant concentrations of poly(dT) and $(dT)_{70}$ are ~450 μM and ~8μM, respectively. These experiments are performed by adding equal-volume aliquots of the titrant to the protein solution as shown in Table 1 (see Note 7) for the titration of *Eco*SSB (0.14 μM) with poly(dT) (Fig. 1b).

Since the only information that can be obtained from such a titration is the occluded site size on poly(dT) (or the stoichiometry of SSB binding to a $(dT)_L$) and the maximum fluorescence change at saturation (Q_{max}) these data are generally plotted in the form of relative fluorescence quenching ($Q = (F_0 - F_i)/F_0$) versus the ratio of total DNA concentration to total protein concentration as shown in Fig. 1, where F_0 is the initial protein fluorescence before the addition of DNA and F_i is the fluorescence for the ith addition of DNA (see Subheading 5 for more details).

The results presented in Fig. 1a, c for *Dr*SSB binding to poly (dT) and $(dT)_{50}$ in the presence of 1.0 M NaCl indicate that the binding affinity is not high enough to be stoichiometric under these conditions (see Note 8) and therefore larger ratios of DNA to protein are required to achieve saturation, $R = [\text{poly(dT)}]_{tot}/[DrSSB]_{tot} > n$ (Fig. 1a) and $R = [(dT)_{50}]_{tot}/[DrSSB]_{tot} \gg 1$ (Fig. 1c). In this case, it is better to plot the data as $(Q = (F_0 - F_i)/F_0)$ versus log [DNA] (see Fig. 2) since for accurate analysis (see Subheadings 9 and 10) more experimental points should be obtained at low and high DNA concentrations. For example, in the titrations presented in Fig. 2a the concentration range of $[(dT)_{50}]_{tot}$ is varied from 1 nM to ~4 μM. In designing these experiments four different titrant stock concentrations with varying volumes of added titrant were used to achieve an even distribution of the experimental points over a logarithmic scale of titrant concentration in the cell. This is exemplified in Table 2 (see Note 7) for the particular titration of 0.5 μM of *Dr*SSB with $(dT)_{50}$ (see Fig. 2a). In general, for this type of experiment we recommend preparing a concentrated stock of the titrant (~200 μM) (see Note 9), which can be diluted to the desired concentrations to perform the experiments.

The following procedures describe the preparation of SSB and DNA samples used in performing 2–10 fluorescent titration experiments with 0.1–0.5 μM of SSB or F-$(dT)_{50}$ in the cell.

3.5. Dialysis of Proteins and ssDNA

We recommend dialyzing protein and DNA samples prior to performing titration experiments since the storage conditions for DNA (water or Tris buffer) and SSB (20 mM Tris (pH 8.3), 50% (V/V) glycerol, 0.50 M NaCl, 1 mM EDTA, and 1 mM BME) are very different from the typical experimental conditions.

1. Prepare ~1–1.5 mL of an approximately 2 μM solution of either *Dr*SSB or *Eco*SSB by diluting an aliquot of the concentrated SSB stocks into the buffer to be used for dialysis. For titrations of fluorescein-labeled DNA (e.g., F-$(dT)_{50}$) make 500 μL of an ~15 μM *Dr*SSB solution.
2. Prepare ~100–200 μL of an approximately 200 μM solution of $(dT)_L$ or a 500–800 μM solution of poly(dT) by adding the

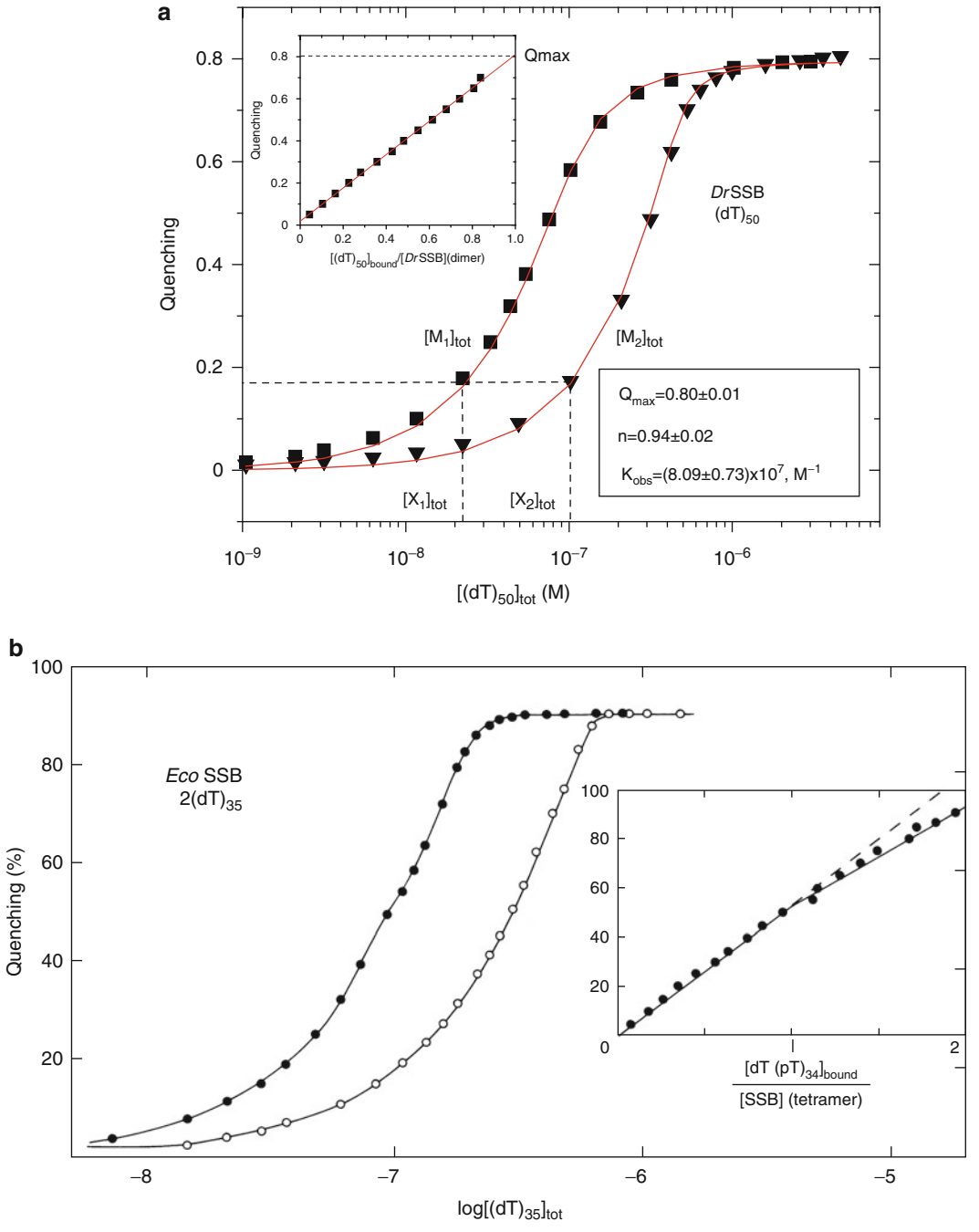

Fig. 2. The analysis of titration isotherms of SSB binding to oligo(dT) using model-independent approach and fitting the data to a particular model. (**a**) The titration isotherms from Fig. 1c for $(dT)_{50}$ binding to *Dr*SSB (0.2 M NaCl, pH 8.1, 25°C) at two protein concentrations, 0.1 μM dimer (*squares*) and 0.5 μM dimer (*triangles*), shown in the form of relative fluorescence quenching versus total concentration of the $(dT)_{50}$. The insert shows the results of MBDF analysis of the data (see text for details) indicative that relative change in fluorescence is directly proportional to the extent of DNA binding (binding density) and that the stoichiometry of the formed complex is 1 mol of $(dT)_{50}$ per mole of *Dr*SSB dimer (the maximum quenching ~80% is reached when the binding density $<X> = 1$). Smooth curves through the data points

appropriate volume of a concentrated DNA stock solution to dialysis buffer.

3. Place the SSB and DNA solutions in dialysis bags of appropriate size (MWCO: 10,000 Spectra/Por) and proceed with the dialysis as described in ITC Chapter.
4. After dialysis equilibrium has been reached (3 changes of 300 mL volume), remove the contents from the dialysis bag and determine the concentrations of protein and ssDNA (see Note 1).

3.6. Titration Protocol

Here we describe the experimental procedure for titration of the fluorescent species in the cell (SSB) with nonfluorescent ssDNA in which binding is monitored by quenching of the intrinsic Trp fluorescence of SSB. The procedure is essentially the same if the fluorescent species in the cell is DNA (e.g., $(dT)_{50}$ labeled with fluorescein) that is titrated with SSB while monitoring the change in fluorescein intensity or anisotropy (see Subheadings 10 and 11). We assume that the experimenter is familiar with the principles of operation of the fluorometer.

1. After starting the instrument allow the lamp to warm up for at least 15 min. Fluorescence intensity is temperature sensitive, as are the equilibrium binding constants for protein–DNA interactions (see Note 10). Hence, the temperature of the sample cell should always be controlled precisely when performing fluorescence experiments. Turn on the water bath or peltier block that controls the temperature of the cuvette holder, and set to the desired temperature, in this case, 25°C.
2. Place a magnetic stir bar in a 3 mL (10 mm pathway) quartz cuvette and dilute SSB directly in the cell to the desired concentration with dialysis buffer such that the total volume is 1.9–2 mL (see Note 11). Make certain that no bubbles are stuck to the stir bar. Prepare a reference cell by adding the same volume of dialysis buffer into another cuvette (also with a stir bar). Prepare solutions of the appropriate concentrations and volumes of the titrant by diluting the DNA stock with dialysis buffer.
3. Place cuvettes with protein and buffer into the cuvette holder of the instrument (see Note 12) and open the "Time base"

Fig. 2. (continued) represent a global fit of the two experimental data sets to the model of n-independent and identical sites (see Eqs. 5 and 5a and text for details) with the following parameters: $Q_{max} = 0.80 \pm 0.01$; $n = 0.94 \pm 0.02$; and $K_{obs} = (8.09 \pm 0.73) \times 10^7\ M^{-1}$. (**b**) Titration curves representing fluorescence titrations of the *Eco*SSB tetramer with $(dT)_{35}$ in buffer T (0.2 M NaCl, pH 8.1, 25°C) at two protein concentrations, 0.1 μM tetramer (*closed circles*) and 0.32 μM tetramer (*open circles*) (14). The insert shows the results of an MBDF analysis of the data (as described for Fig. 1a), which indicates that two molecules of $(dT)_{35}$ bind to *Eco*SSB tetramer upon saturation. The binding of the first one is characterized by the fluorescence quenching of ~50% and the second one by fluorescence quenching of ~40% (~90% quenching for the final complex having 2 mol of $(dT)_{35}$ bound per mole of *Eco*SSB tetramer).

Table 2
Experimental Excel spreadsheet design for the titration of *Dr*SSB (0.5 μM, 1,900 μL total volume) with $(dT)_{50}$ in buffer T, 1.0 M NaCl (see Figs. 1c and 2a)

#	Titrant conc. (μM)[a]	Titrant aliquot (μL)[b]	Total titrant volume (μL)	Titrant conc. in cell (M)	$[(dT)_{50}]_{tot}$ per $[DrSSB]_{tot}$
1	1	2	2	1.1×10^{-9}	0.002
2	1	2	4	2.1×10^{-9}	0.004
1	1	2	6	3.1×10^{-9}	0.006
4	1	6	12	6.3×10^{-9}	0.013
5	10	1	13	1.2×10^{-8}	0.023
6	10	2	15	2.2×10^{-8}	0.044
7	10	5	20	4.8×10^{-8}	0.097
8	100	1	21	1.0×10^{-7}	0.202
9	100	2	23	2.0×10^{-7}	0.413
10	100	2	25	3.1×10^{-7}	0.623
11	100	2	27	4.1×10^{-7}	0.834
12	100	2	29	5.1×10^{-7}	1.044
13	100	2	31	6.2×10^{-7}	1.255
14	100	3	34	7.7×10^{-7}	1.571
15	190	2	36	9.7×10^{-7}	1.971
16	190	6	42	1.6×10^{-6}	3.171
17	190	10	52	2.5×10^{-6}	5.171
18	190	10	62	3.5×10^{-6}	7.171
19	190	10	72	4.4×10^{-6}	9.171

[a]Concentrations and [b]aliquots of titrant ($[(dT)_{50}]_{tot}$) are varied in a way that results in even distribution of experimental points over the logarithm of concentrations of the titrant in the cell

mode in the software, which allows one to monitor the fluorescence emission at a particular wavelength (for a given excitation wavelength) as a function of time. Set the excitation (296 nm) and emission (350 nm) wavelengths (see Note 13) and slit widths (usually corresponding to a 2 nm bandpass for excitation and a 4–8 nm bandpass for emission, depending on SSB concentration (see Note 14)). Specify a number of acquisition points and the averaging time for each point (we usually set 8 points with an averaging time of 1 s and then take average of these 8 points to obtain a single data point for the fluorescence intensity, $F_{obs,i}$).

4. Take the first measurement for the protein solution ($F_{obs,0}$) and then for the buffer ($F_{ref,0}$). Repeat the measurements a few times waiting 2–3 min between measurements to make sure that the signal is stable (see Note 15) and record the observed intensity values for the sample and the buffer.
5. Begin the titration by adding the same-volume aliquots of the titrant (DNA) into the sample cell and the reference cell containing buffer using a micropipette (see Note 16). Wait for 2–3 min for equilibration and take measurements ($F_{obs,1}$ and $F_{ref,1}$). Repeat acquisition after 2–3 min to make sure that the signal intensity is no longer changing indicating that the system has reached equilibrium (if a slow change in fluorescence is observed a separate kinetic experiment should be performed to determine the equilibration times required between additions of titrant). Record the fluorescence intensity values for both cuvettes.
6. Proceed with the serial addition of aliquots of the titrant as described in the previous step while recording $F_{obs,i}$ and $F_{ref,i}$ for each addition until saturation is reached (i.e., the fluorescence intensity is no longer changing). Further additions of the titrant (DNA) may still be needed (see Note 17).
7. After finishing the titration redetermine the concentration of the DNA solution used as the titrant (see Note 18).

3.7. Correction of Experimental Data for Background Signal, Dilution, Inner Filter Effects, and Photobleaching

Prior to analysis of the data it is necessary to correct the observed fluorescence intensities and concentrations for dilution, photobleaching, and inner filter effects (30, 31).

1. First the concentrations of the species in the cell need to be corrected for dilution. The total concentration of the SSB in the cell (P) after addition of the ith aliquot of titrant DNA (D) solution can be calculated using Eq. 11:

$$P_{tot,i} = \frac{P_0 \cdot V_0}{(V_0 + V_i)} \tag{11}$$

where P_0 is the initial concentration in the cell, V_0 is the initial sample volume, and V_i is the total volume of titrant added up to the point i in titration. The total concentration of the titrant in the cell at each point i in the titration is calculated using Eq. 12:

$$D_{tot,i} = \frac{D_0 \cdot V_i}{(V_0 + V_i)} \tag{12}$$

where D_0 is the concentration of the titrant (see Note 19).

2. The first correction to obtain the true fluorescence intensities, F_i, is to subtract the background contribution to the signal due to any light scattering or impurities in the DNA or buffer. This background signal, $F_{ref,i}$, is obtained from the titration with

DNA of the reference cuvette containing buffer, yielding $F_i = F_{\mathrm{obs},i} - F_{\mathrm{ref},i}$. Next, the experimentally observed intensities, F_i, must be further corrected for dilution, photobleaching, and inner filter effects (31). Photobleaching is the excitation-dependent loss of fluorescence intensity, whereas inner filter effects can result when the absorbance spectrum of the sample overlaps either the fluorescence excitation or emission wavelength, such that some of the excitation or emission intensity is re-absorbed ("filtered") by the sample, resulting in attenuation of the fluorescence signal (30). These factors are multiplicative, so the corrected fluorescence for each titration point can be expressed as in Eq. 13:

$$F_{i,\mathrm{corr}} = \left(\frac{(V_0 + V_i)}{(V_0)}\right)\left(\frac{1}{C_i}\right)\left(\frac{f_0}{f_i}\right) \tag{13}$$

where the first term accounts for dilution, the second term is for inner filter effects (see Note 20), and the third term is for photobleaching, where f_0 and f_i are the fluorescence intensities measured before and during the experiment for a sample which was not titrated, but exposed to the excitation light for the same periods of time as a titrated sample (see Note 15).

3. The actual fluorescence signal depends on the concentration of the fluorescent species and will vary in different experiments. Hence, the change in fluorescence is usually expressed as a quenching (Q_i) or an enhancement (En_i), which normalizes the signal relative to the initial fluorescence of the sample before the start of the titration, i.e., $Q_i = (F_{\mathrm{corr},0} - F_{\mathrm{corr},i})/F_{\mathrm{corr},0}$ or $En_i = (F_{\mathrm{corr},i} - F_{\mathrm{corr},0})/F_{\mathrm{corr},0}$.

3.8. Determination of the Occluded Site Size of SSB on Poly(dT) Under Stoichiometric Conditions

Titration of SSB proteins with polynucleotides under stoichiometric conditions can be used to determine the occluded site size of the protein on the DNA. The occluded site size is defined as the average number of nucleotides made inaccessible to other proteins by the binding of one protein (32). This differs from the number of nucleotides that are physically contacted by the protein, which is usually a lower number. Figure 1a, b shows the results of titrations of *Dr*SSB and *Eco*SSB with poly(dT) at 0.2 M NaCl, a condition under which binding of these proteins to ssDNA is stoichiometric. Under these conditions, an accurate determination of the equilibrium binding constant (affinity) is not possible; however, this allows for an accurate determination of the occluded site size. In these titrations the intrinsic Trp fluorescence of the protein in the cell is monitored as a function of total added ssDNA. The data are plotted as the relative fluorescence change versus the ratio of the total poly(dT) concentration (in nucleotides) to total protein concentration. Such a plot allows one to determine the occluded site size as the point of intersection of a linear extrapolation of the linear part of the titration curve with the

plateau value of the fluorescence at saturation. The occluded site sizes, n, and maximum fluorescence quenching, Q_{max}, differ for *Dr*SSB ($n \sim 52$ nucleotides, $Q_{max} = 82\%$) and *Eco*SSB ($n \sim 65$ nucleotides, $Q_{max} = 90\%$). To ensure that binding is stoichiometric, multiple titrations should be performed at a few different SSB concentrations. If binding is stoichiometric, then plots of the type shown in Fig. 1a obtained for different SSB concentrations should overlay.

Upon increasing the [NaCl], the binding isotherm for *Dr*SSB becomes non-stoichiometric (less sharp) (see Fig. 1a) indicating a decrease in affinity (lowering of the equilibrium binding constant, K_{obs}). In contrast, the binding of *Eco*SSB to poly(dT) remains stoichiometric up to 3 M NaCl (14). This does not mean that increasing [NaCl] does not reduce the value of K_{obs} for the *Eco*SSB–DNA interaction, but rather that K_{obs} remains above the value (generally ~10^9 M^{-1}) needed for binding to appear "stoichiometric". In each case an increase in [NaCl] does not affect the maximum quenching suggesting no change in the mode of DNA binding. On the other hand, as the [NaCl] decreases to ~0.010 M NaCl, the occluded site size and maximum fluorescence quenching change for both *Eco*SSB (2, 7) and *Dr*SSB (18). For *Eco*SSB this change is more dramatic ($n = 35$ and $Q = 50\%$) and indicates a transition from its $(SSB)_{65}$ mode to its $(SSB)_{35}$ mode of DNA binding in which on average only two instead of four subunits of SSB tetramer are bound to ssDNA (7, 33). For *Dr*SSB the change is less dramatic ($n = 45$ and $Q = 76\%$) (18) but still represents a change in ssDNA binding mode (18). For this reason one should always examine the effects of solution conditions on the occluded site size, rather than assume that a site size measured under one condition is applicable to all conditions.

3.9. Model-Independent Analysis of Titration Data Using the Macromolecule Binding Density Function Method

A model-independent analysis of two or more titrations performed at different macromolecule concentrations can be used to determine the stoichiometry of binding and to construct a true binding isotherm (34–36). This is most useful for systems in which one species binds multiple copies of the other. For this discussion, the macromolecule is defined as the species that binds multiple ligands. This analysis is also needed when the relationship between the average extent of ligand binding ($<X>$ = ligands bound per total macromolecule) and the spectroscopic signal change (in this case fluorescence intensity or anisotropy) is not known a priori. Application of the following analysis to two or more titrations performed at different SSB concentrations as shown in Fig. 2a, b results in a true isotherm, i.e., a plot of $<X>$ versus free or total ligand concentration (34–36). The two titrations need to be performed under conditions such that the total protein concentration is high so that the free ligand and total ligand concentrations differ significantly throughout the titrations.

The basis for this analysis is the thermodynamic constraint that for a macromolecule that does not undergo self-association, the average number of ligands bound per macromolecule, $<X>$, is determined solely by the free ligand concentration (strictly the chemical potential of the ligand) (34–36). Moreover, it is important to recognize that for a given set of solution conditions the relative fluorescence change ($Q_i = (F_{corr,0} - F_{corr,i})/F_{corr,0}$) is an intrinsic property of the system and is determined by the average degree of saturation of the binding sites, which in turn is determined solely by the free ligand concentration. Therefore, if two titrations are performed at two different total macromolecule (SSB) concentrations, $[M_1]_{tot}$ and $[M_2]_{tot}$, and plotted as a function of the total ligand concentration $[X]_{tot}$ (DNA), then whenever the fluorescence quenching is the same for the two titrations (e.g., for the horizontal line in Fig. 2a, where $Q = 0.18$), it follows that the free ligand concentration must be the same at that same fluorescence quenching value (e.g., $Q = 0.18$) (34–36). As such, one can calculate the value of $<X>$ and the free ligand concentration, $[X]$, from knowing the pair of $[M_1]_{tot}$ and $[M_2]_{tot}$ and $[X_1]_{tot}$ and $[X_2]_{tot}$ that are needed to attain the same value of Q as indicated in Eqs. 14 and 15:

$$<X> = \frac{[X_2]_{tot} - [X_1]_{tot}}{[M_2]_{tot} - [M_1]_{tot}} \tag{14}$$

$$[X] = \frac{[M_1]_{tot}[X_2]_{tot} - [M_2]_{tot}[X_1]_{tot}}{[M_1]_{tot} - [M_2]_{tot}} \tag{15}$$

By repeating this calculation at multiple values of the observed Q for the two titrations, the titration curves can be analyzed as shown in Fig. 2a to obtain the dependence of Q_{obs} on $<X>$ ($[(dT)_{50}]_{bound}/[DrSSB]_{tot}$ or $[(dT)_{35}]_{bound}/[EcoSSB]_{tot}$; see inserts in Fig. 2a, b, respectively) and to construct true binding isotherm of $<X>$ versus $[X]$ or $[X]_{tot}$ (not shown).

The model-independent analysis of multiple titrations of a fluorescent ligand at different concentrations with a nonfluorescent macromolecule ("reverse titrations") can also be performed to obtain the same information, although the analysis is a bit more complex (35, 36).

MBDF analysis of two titrations of *Dr*SSB (at 0.1 and 0.5 μM) with $(dT)_{50}$ shown in the insert to Fig. 2a indicates that the observed fluorescence quenching is directly proportional to the average degree of binding ($<X>$), as expected for a 1:1 binding interaction, and that the binding stoichiometry for this system is 1:1 (as follows from linear extrapolation of the dependence of Q on $<X>$ to the value of $Q_{max} = 0.8$). Another example of an MBDF analysis for a system where the macromolecule binds multiple ligands is shown in Fig. 2b for the binding of *Eco*SSB to $(dT)_{35}$ (14). Two titrations were performed at two protein concentrations, 0.10 and 0.32 μM (buffer T, pH 8.1, 0.2 M NaCl, 25°C). The analysis (see insert in Fig. 2b) shows

that two molecules of $(dT)_{35}$ can bind to an *Eco*SSB tetramer at saturation (~90% quenching); however binding of the first and the second molecule is characterized by different fluorescence quenching values of ~50% and ~40%.

3.10. Fitting the Data to a Particular Model to Obtain the Equilibrium Binding Parameters

Model-independent analysis of the titrations in Fig. 2a shows that a 1:1 complex is formed upon interaction of *Dr*SSB with $(dT)_{50}$. The binding isotherm shown in Fig. 2a can be directly fit to the expression for an "*n*" site binding model given in Eqs. 5 and 5a (see Subheadings 1 and 2). A global fit of the data for two titrations performed at *Dr*SSB concentrations of 0.1 and 0.5 μM (see Fig. 2a and see Note 21) is consistent with this model and yields the following binding parameters: $n = 0.94 \pm 0.02$; $Q_{max} = 0.80 \pm 0.02$; and $K = (8.09 \pm 0.73) \times 10^7\ M^{-1}$.

One can also examine binding by monitoring the fluorescence intensity or anisotropy of a DNA molecule if it is labeled with an extrinsic fluorophore. To demonstrate this we show in Fig. 3 the results of a titration of F-$(dT)_{50}$ ($(dT)_{50}$ labeled with fluorescein at its 5′ end) with *Dr*SSB performed at the same solution conditions as for the experiment in which the *Dr*SSB Trp fluorescence was monitored in Fig. 2a (buffer T, pH 8.1, 1.0 M NaCl, 25°C). In this case, binding is accompanied by a quenching of the fluorescein fluorescence (0.2 μM F-$(dT)_{50}$ in the cell, $\lambda_{ex} = 494$ nm, $\lambda_{ex} = 520$ nm) upon binding protein. The data have been corrected as described above for titrations of the SSB with DNA and the resulting binding isotherm shown in Fig. 3a was fitted to the expression for an "*n*" independent and identical sites model given in Eqs. 5 and 5a, where *Dr*SSB is now considered the ligand (X) and F-$(dT)_{50}$ is the macromolecule (M). The fit describes the data well and provides the following binding parameters: $n = 0.98 \pm 0.01$; $Q_{max} = 0.55 \pm 0.01$; and $K = (2.42 \pm 0.35) \times 10^8\ M^{-1}$. The results indicate that the affinity of $(dT)_{50}$ when labeled with fluorescein is increased threefold compared to the binding of an unlabeled $(dT)_{50}$. This is likely due to additional interactions of the fluorescein with the protein. Hence, although the use of extrinsically labeled protein or DNA is convenient, the extrinsic probes will generally influence the energetics of the interaction. In fact, this is common for SSB interactions with DNA labeled with different fluorescent dyes. For example, the affinity of *Eco*SSB binding to $(dT)_{70}$ (2.0 M NaBr) increases three- and tenfold, when the DNA is labeled with fluorescein or Cy3, respectively (22). However, one can always use the equilibrium of the SSB with the labeled DNA in a competition experiment to probe the interaction of SSB with an unlabeled DNA (37, 38).

The data in Fig. 2b (14) can be fit to the equilibrium model presented in Scheme 2 using Eqs. 6 and 6a. Under the conditions of this experiment (buffer T, 0.2 M NaCl) the following parameters were obtained (12): $Q_1 = 0.50 \pm 0.02$; $Q_2 = 0.90 \pm 0.01$; and

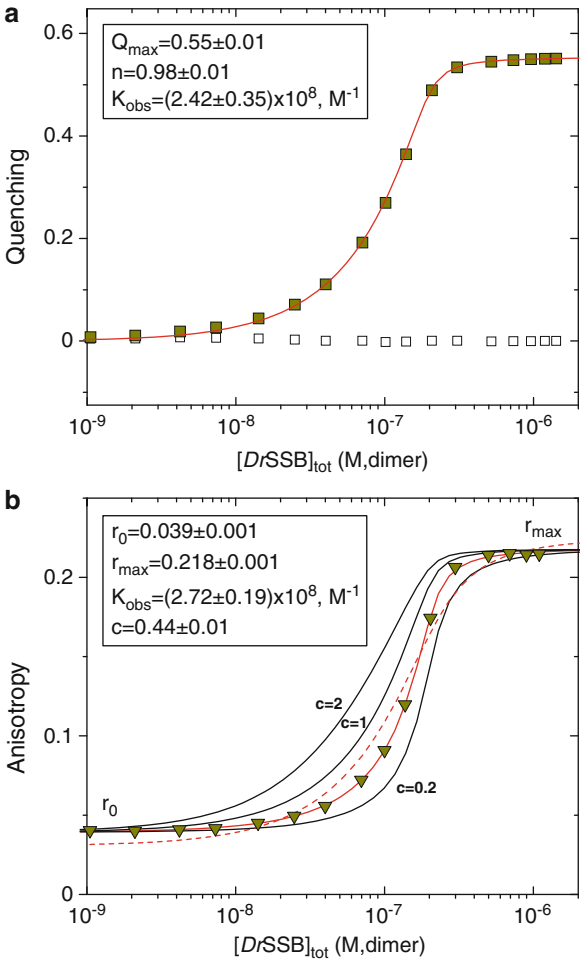

Fig. 3. Titrations of fluorescein-labeled DNA (F-$(dT)_{50}$) with SSB monitoring (**a**) quenching of total fluorescence intensity or (**b**) fluorescein anisotropy. (**a**) Binding isotherm for the titration of 0.2 μM of F-$(dT)_{50}$ with *Dr*SSB (buffer T, pH 8.1, 0.2 M NaCl, 25 °C) plotted as relative fluorescence quenching versus total concentration of *Dr*SSB (dimer). The smooth curve through the data points represents a fit of the data to an n-independent and identical sites model (see Eqs. 5 and 5a and text for details) with the binding parameters indicated. The residuals for the fit are shown as open squares. (**b**) Binding isotherm for the titration of 0.2 μM of F-$(dT)_{50}$ with *Dr*SSB (buffer T, pH 8.1, 0.2 M NaCl, 25 °C) plotted as anisotropy versus total concentration of *Dr*SSB (dimer) (*yellow triangles*). The *dashed red line* shows the best nonlinear least squares fit of the data to a 1:1 binding model (Eq. 9), assuming no fluorescence change between the bound and free states of DNA (i.e., $c = F_{bound}/F_{free}$ was constrained to equal 1) and demonstrates that the fit is not adequate. The *solid red curve* shows the best nonlinear least squares fit of the same data to Eq. 9 when c is allowed to float yielding $c = 0.44 \pm 0.01$ and $K_{obs} = (2.72 \pm 0.19) \times 10^8$ M^{-1} (see insert), which are in excellent agreement with the binding parameters obtained for the same experiment when total fluorescence quenching is monitored (Fig. 3a) (see text for more details). The titration curves shown in *solid black lines* are simulations showing the effect of different values of c (different extents of fluorescence quenching/enhancement) on the shape and position of the titration curves. The simulations were performed using Eq. 9 with $K_{obs} = 2.4 \times 10^8$ M^{-1} and different values of c ($c = 1$, no fluorescence change), ($c = 2$, 100 % fluorescence enhancement), and ($c = 0.2$, 80 % fluorescence quenching).

$K_{2,obs} = (3.3 \pm 0.3) \times 10^8\ M^{-1}$. Determination of $K_{1,obs}$ was not possible at this salt concentration due to the fact that the binding affinity is too high ($K_{1,obs} > 10^{10}\ M^{-1}$). These data show that an *Eco*SSB tetramer can bind two $(dT)_{35}$ molecules, but with negative cooperativity. Similar titrations performed over a range of salt concentrations (12) indicate that this negative cooperativity decreases as the salt concentration increases and contributes to the fact that SSB displays salt-dependent binding modes (12).

3.11. Analysis of Titration Data When Monitoring a Change in Fluorescence Anisotropy

If a change in fluorescence anisotropy of one species accompanies binding of a second species, then this can also be used to monitor binding. In fact, this approach has become increasingly popular due to the ease with which DNA can be labeled with an extrinsic fluorescence probe. However, there are some important caveats to be considered when using this approach. These include the likelihood that the binding energetics will be influenced by the addition of the extrinsic fluorophore, as shown above for F-$(dT)_{50}$. In addition, if there is also a change in fluorescence intensity of the probe, this must be considered in the analysis of the data.

As an example of a binding system that is accompanied by a fluorescence anisotropy change we show results for the binding of *Dr*SSB to F-$(dT)_{50}$ (where F is fluorescein). This experiment is identical to that shown in Fig. 3a, in which the change in fluorescein fluorescence intensity of F-$(dT)_{50}$ was monitored. However, in Fig. 3b we plot the change in fluorescein fluorescence anisotropy as a function of added *Dr*SSB (see Note 22). For this system, the fluorescence anisotropy for the free F-$(dT)_{50}$ is $r = 0.039$, whereas $r = 0.218$ for the SSB–DNA complex. (We note that the maximum fluorescence anisotropy for a system is $r = 0.4$; see also Note 3).

We have chosen this system as an example because it allows us to demonstrate the need for caution when using fluorescence anisotropy to analyze a binding equilibrium if there is also a change in fluorescence intensity that accompanies binding. For this system the fluorescence intensity of the bound F-$(dT)_{50}$ is quenched by 55% relative to the free F-$(dT)_{50}$ ($F_0 - F_{min}/F_0 \approx 0.55$) as shown in Fig. 3a. Since the observed anisotropy is a sum of the individual species' anisotropies weighted by the fractional fluorescence contribution (see Eq. 8), this will influence the measured values of the anisotropies (29, 39) and thus the shape of the titration curve, a problem that seems not to be widely appreciated. Indeed, a direct comparison of the titration curves monitoring fluorescence intensity (Fig. 3a) and fluorescence anisotropy (Fig. 3b) shows that the titration curve obtained by monitoring the anisotropy change is steeper and shifted to higher total protein concentrations compared to the titration curve obtained by monitoring the fluorescence intensity change. As a result, an attempt to fit the anisotropy titration curve to a simple 1:1 binding model without accounting for the fluorescence intensity change fails (red dashed curve in Fig. 3b) in the direction that would suggest

some positive cooperativity, which is impossible for a simple 1:1 binding system such as this.

To analyze the anisotropy titration curve correctly, one must account for the change in fluorescence intensity of the free and bound DNA. This is taken into account in Eq. 9 by the factor $c = F_{\text{bound}}/F_{\text{free}} = F_{\text{MX}}/F_{\text{M}}$ (in this case we consider the DNA as the macromolecule (M) and *Dr*SSB as the ligand (X)).

The experimental data shown in Fig. 3b are well described by a simple 1:1 binding model Eq. 9 as long as the correction factor ($c = 0.44 \pm 0.01$) is included and the resulting best fit equilibrium binding parameters ($r_0 = 0.039 \pm 0.001$; $r_{\max} = 0.218 \pm 0.00$; $K_{\text{obs}} = (2.72 \pm 0.19) \times 10^8\ \text{M}^{-1}$) are in excellent agreement with the parameters obtained by directly fitting the fluorescence intensity titration curve in Fig. 3a ($n = 0.98 \pm 0.01$; $K_{\text{obs}} = (2.42 \pm 0.35) \times 10^8\ \text{M}^{-1}$; and $Q_{\max} = 0.55 \pm 0.01$) (recall that $Q_{\max}$ in Fig. 3a is related to c in Fig. 3b by $c = 1 - Q_{\max}$).

In order to further illustrate the effect on the observed anisotropy titration curve when a fluorescence quenching/enhancement accompanies binding, we also show several simulations for a 1:1 binding system where $K_{\text{obs}} = 2.4 \times 10^8\ \text{M}^{-1}$ but with different values of c ($c = 1$, no fluorescence change), ($c = 2$, 100% fluorescence enhancement), and ($c = 0.2$, 80% fluorescence quenching). The solid lines in Fig. 3b show the resulting shifts in apparent binding affinity and shape of the isotherms. As a result, significant errors in binding parameters and even conclusions about cooperativity can result if these corrections are not considered. This also points out that if there is a significant change in fluorescence intensity of the labeled DNA it is simpler to use that signal directly to monitor binding rather than the anisotropy change.

4. Notes

1. Precise values of the extinction coefficients for the protein and ssDNA are required for determination of their concentrations which, in turn, are needed for accurate analysis of the titration data. The methods for the determination of extinction coefficients of proteins and ssDNA are described in detail in Note 5 of the ITC Chapter.
2. The macroscopic binding constants in Eqs. 2 and 6 do not contain the statistical factors and can be converted to microscopic binding constants, k_i, which account for statistical factors as follows: $k_1 = K_1/2$ and $k_2 = 2\ K_2$ (1).
3. For this reason in designing the anisotropy titration experiments for SSB–ssDNA binding we recommend labeling ssDNA with the fluorophore (r values usually range from 0.04 to 0.1) allowing one to observe a detectable increase in

anisotropy upon protein binding. We also note that the theoretical upper limit for r is 0.4 (40); hence apparent experimental values of r that exceed this value likely indicate experimental problems such as aggregation resulting in an increase in light scattering.

4. The G-factor accounts for the differential sensitivities of the detection system for vertically and horizontally polarized light (29) and can be determined experimentally by measuring horizontally and vertically polarized emission with the excitation polarizer in the horizontal position, I_{HH} and I_{HV}, respectively, and then calculated as $G = I_{HH}/I_{HV}$. It is important to note that the G factor is dependent upon emission wavelength and to some extent the bandpass of the monochromator. Therefore, the G factor must be determined for each particular experimental setup for the wavelengths being used at least once by measuring I_{HH} and I_{HV} for the fluorescent species in the cell before the titration is started.
5. The polarization (p) is defined by Eq. 16:

$$p = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \tag{16}$$

Although the polarization and anisotropy contain the same information, use of the anisotropy for studies of interacting systems is preferred because it is normalized to the total fluorescence intensity $I_T = I_{VV} + 2\ GI_{VH}$. This simplifies its use in defining the average anisotropy of a mixture of fluorescent species $\bar{r} = \sum r_i f_i$ which was used for the derivation of Eq. 9 (see Subheading 3). The corresponding expression using p becomes very cumbersome (29). When data are obtained as polarization, Eq. 17 should be used to convert to anisotropy:

$$r = \frac{2p}{3 - p} \tag{17}$$

6. To obtain information about the assembly state(s) and relative stability of the protein under study (particularly for a new system) the protein should be characterized using both sedimentation velocity and sedimentation equilibrium methods over a range of protein concentrations under the same solution conditions that are to be used for the titrations.
7. We recommend that such titration tables are prepared in the process of designing a titration experiment. This can be easily accomplished using Excel spreadsheet that can also be used for recording the experimentally observed fluorescence intensities corresponding to each titration point.

8. An important test to determine if the binding is stoichiometric under the conditions and for the protein and DNA concentrations used is to perform a second titration at a lower or higher concentration of the species in the cell. If the binding is stoichiometric no change in the shape of the binding isotherm should be observed, whereas for non-stoichiometric binding the isotherm becomes more shallow as seen in Fig. 1c for the titration with $(dT)_{50}$ performed at a fivefold lower *Dr*SSB concentration in 1.0 M NaCl.
9. Preparation of concentrated solutions of the titrant (DNA) as suggested here will certainly leave some extra DNA unused, although any unused DNA can be safely stored at 4°C for long periods of time and used in future studies.
10. *Eco*SSB–ssDNA interactions are characterized by a very large and temperature-dependent enthalpy of binding (41). Hence, even small changes in ambient temperature will generally influence the binding affinity. Moreover, the fluorescence intensity itself is also temperature dependent; hence it is critical to maintain control of the temperature throughout the titration.
11. The use of a stir bar is recommended for thorough mixing of the solution in the cuvette. However, the stirring speed should be maintained as low as possible while maintaining a stable fluorescence signal. We use "Tube" (or cylindrical) type stir bars (http://www.stirbars.com) specifically designed to fit in 10 mm cuvettes. If the cuvette holder does not have a built-in stirring plate, mixing can be achieved by gently redistributing the cell solution back and forth using micropipette; however, this approach generally leads to poorer results.
12. If your instrument has only one cuvette holder, the reference titration should be performed after completing the experimental titration, but without changing any of the instrument settings. Subtraction of the buffer background signal arising from light scattering can be important when the fluorescence intensity of the sample is low.
13. For a new system we recommend that both excitation and emission spectra are run on the fluorescent sample first to determine the optimal excitation and emission wavelengths. This is also important for choosing an excitation wavelength to minimize any inner filter effects (see Subheading 7 and Note 20). For example, when monitoring Trp fluorescence, we typically use a higher excitation wavelength (e.g., 296 nm), rather than the maximum excitation wavelength of 280 nm, to reduce the inner filter effect.
14. The fluorescence signal should be directly proportional to the concentration of the fluorophore. It can deviate from linearity if the photon count reaches the saturation limit of the PMT

(for our instrument this limit is 10^6 s^{-1}). Therefore, before starting the experiment it is necessary to ensure that the signal change during the titration will stay within the appropriate range. This can be adjusted by increasing or decreasing the slit width (preferably for the emission monochromator). For a new system we recommend using an intermediate value at the beginning of the titration.

15. Some proteins are more susceptible to photobleaching than others. Photobleaching occurs when the protein sample is exposed to the excitation light for an extended period of time. Therefore, it is useful to close the excitation shutter and not expose the protein sample to the excitation beam unless a reading is to be taken. Use of a smaller excitation slit width will also reduce photobleaching. In addition, the signal can decrease if protein has a tendency to stick to the cuvette walls. The latter situation is observable at low concentrations of the protein (usually <0.1 μM) (this is typically not observed for DNA labeled with external dye). Both phenomena lead to a decrease in fluorescence intensity. Therefore, before starting an experiment you should determine if the fluorescence signal of your sample is stable or changes with time. A slight decrease in fluorescence intensity due to photobleaching can be corrected (see Subheading 7) by monitoring the fluorescence of a protein solution in a separate cuvette when exposed to the excitation light for the same periods of time as during the titration experiment. Sticking of a nonself-associating protein to the cuvette walls can generally be detected by titrating increasing protein concentrations into buffer while monitoring the fluorescence intensity. If the fluorescence intensity is not directly proportional to protein concentration (e.g., if the molar intensity is lower at lower protein concentrations), then this suggests that protein is sticking to the cuvette walls. If either of these effects is severe it is possible that the system cannot be studied using fluorescence. One alternative is to change the design of the experiment. For example if the protein can be labeled with an external fluorophore that can be excited at a wavelength above 300 nm, then one can add BSA to the solution (usually ~0.1 mg/mL) to prevent sticking of the labeled protein to the cuvette (38). However, any such labeling may affect the DNA binding properties of the protein (20). If this is the case, then one can examine the binding of the unlabeled protein using competition methods (37, 38).
16. For our titrations we use 2, 10, and 20 μl Gilson micropipettes with disposable plastic tips.
17. It is sometimes difficult to determine from the raw titration data when saturation of the signal has been achieved, especially if binding is weak or shows negative cooperativity. In fact, this is

often only determined after all of the corrections to the data have been made and the data plotted. In that case, one knows that when a second titration is performed higher DNA concentrations need to be achieved to approach saturation. One example is the case of a much weaker binding of a second molecule of $(dT)_{35}$ to *Eco*SSB (13).

18. It is important to know the precise values of the concentrations of SSB and DNA used in the experiment for meaningful quantitative analyses of the data. Mistakes can be made when diluting the samples to the experimental concentrations. An accurate determination of the titrant concentration (DNA) and SSB in the cell is of particular importance under stoichiometric binding conditions (see Fig. 1) since it is used for determination of the stoichiometry or occluded site size.
19. If a few different titrant stock concentrations are used during a titration (e.g., as in Fig. 2a, see also Table 2) Eq. 12 can be modified to account for this. It is also recommended that the total volume of the added titrant (V_i) at the end of the titration not exceed ~5–10% of the initial volume (V_0) in the cell.
20. The inner filter correction factor, C_i, can be calculated for absorbances $A_i < 0.3$ (which is usually the case for the experiments described here) using the formula in Eq. 18 (30, 31):

$$C_i = \frac{1 - 10^{-A_i}}{2.303\ A_i} \tag{18}$$

where A_i is the absorbance at titration point i determined using Eq. 19:

$$A_i = \varepsilon_D D_{\mathrm{tot},i} + \varepsilon_P P_{\mathrm{tot},i} \tag{19}$$

where $D_{\mathrm{tot},i}$ and $P_{\mathrm{tot},i}$ are the concentrations of DNA and SSB at particular titration point and ε_D and ε_P are defined in Eqs. 20a and 20b:

$$\varepsilon_D = \varepsilon_{D,\mathrm{ex}} + \varepsilon_{D,\mathrm{em}} \tag{20a}$$

$$\varepsilon_P = \varepsilon_{P,\mathrm{ex}} + \varepsilon_{P,\mathrm{em}} \tag{20b}$$

where $\varepsilon_{D,\mathrm{ex}}$, $\varepsilon_{D,\mathrm{em}}$, $\varepsilon_{P,\mathrm{ex}}$, and $\varepsilon_{P,\mathrm{em}}$ are the extinction coefficients for DNA and protein at excitation and emission wavelengths. These can be easily determined from the corresponding absorbance spectra if the extinction coefficient at one wavelength is known. For higher absorbances more complicated expressions taking into account geometric factors of the fluorescence cell compartment need to be taken into account or the inner filter correction can be determined empirically (30).

21. Nonlinear least squares fitting of the data was performed using the nonlinear regression package in Scientist (MicroMath Scientist Software, St. Louis, MO). This software allows implicit solutions of equations of the type shown in Eqs. 5 or 6 to be used to fit multiple data sets simultaneously.
22. The experiment was performed essentially as described in Subheading 3.6; however F-$(dT)_{50}$ in the cuvette was titrated with *Dr*SSB with the excitation and emission polarizers in place and I_{VV} and I_{VH} were measured for each titration point. The measurements of I_{HH} and I_{HV} were performed before the experiment was started and after completion of the experiment and yielded the same value of $G = 0.66$.

Acknowledgements

This work was supported in part by grants to T.M.L. from NIH (GM030498 and GM045948) and R.G. from NIH (GM098509).

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