Supplementary Material for:

Illuminating the reaction pathway of the FokI restriction endonuclease by fluorescence resonance energy transfer

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References for Supplementary Material, numbered from the main text:

[27] Catto,L.E., Ganguly,S., Milsom,S.E., Welsh,A.J. and Halford,S.E. (2006) Protein assembly and DNA looping by the FokI restriction endonuclease. *Nucleic Acids Res.*, **34**, 1711-1711.

[30] Bath,A.J., Milsom,S.E., Gormley,N.A. and Halford,S.E. (2002) Many type IIs restriction endonucleases interact with two recognition sites before cleaving DNA. *J. Biol. Chem.*, **277**, 4024-4033.

[31] Halford,S.E., Johnson,N.P. and Grinsted,J. (1980) The EcoRI restriction endonuclease with bacteriophage lambda DNA. Kinetic studies. *Biochem. J.*, **191**, 581-592.

[34] Lakowizc,J.R. (2006) *Principles in Fluorescence Spectroscopy* (3rd ed.). Kulwer Academic/Plenum Press, New York, New York.

Supplementary Figure 1

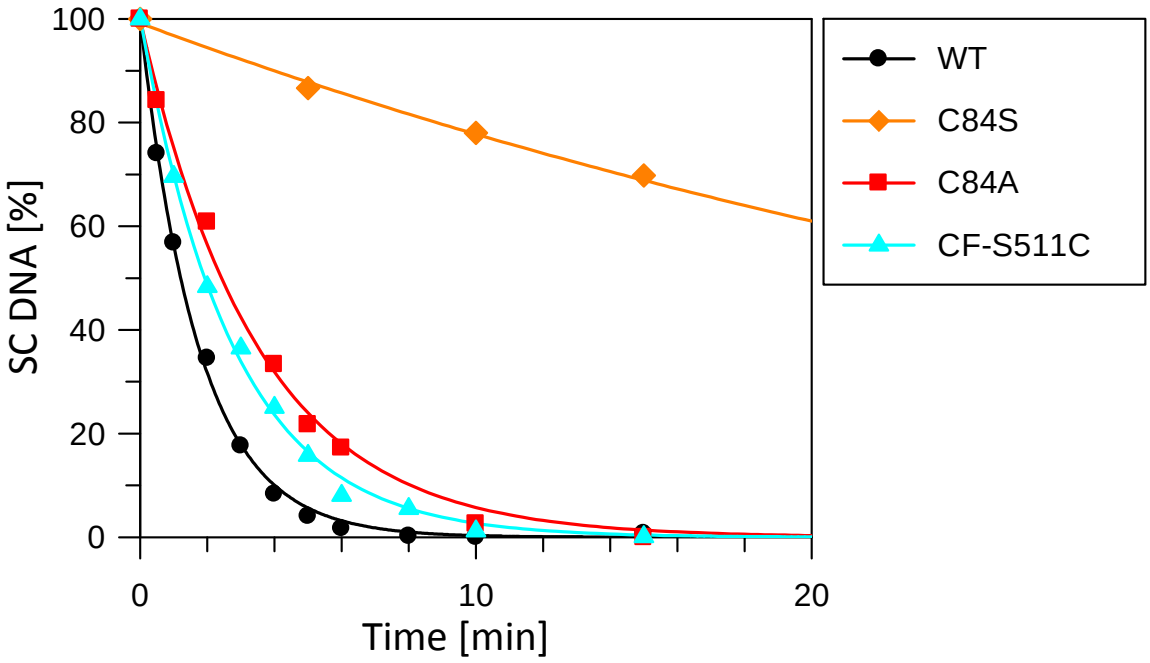

Figure S1. Catalytic activities of FokI mutants. The variants of the FokI endonuclease constructed here were tested for their ability to cleave the plasmid pIF190, a supercoiled (SC) DNA with two FokI sites in inverted orientation 190 bp apart (27). [WT FokI cleaves plasmids with two recognition sites much more readily than those with one site (30), and pIF190 is cleaved even more rapidly than many other two-site substrates (27).] The reactions contained 2 nM FokI protein and 10 nM pIF190 DNA (^{3}H-labelled) in 200 µl M-Buffer at 37°C. Samples were removed from the reactions at timed intervals after adding the FokI protein, mixed immediately with an EDTA stop mix and processed as described previously (27,30). At each time point, the amount of the DNA still present as the SC form was measured and is given on the *y*-axis as a % of that present at zero time before adding the enzyme. Shown here are the reactions with the following forms of FokI: WT FokI, black circles; C84S, orange diamonds; C84A, red squares; CF-S511C (CF denotes the cysteine-free form of FokI carrying both the C84A and the C541T mutations), cyan triangles. The decline in the concentration of the SC substrate during the reaction with each form of FokI was fitted to an exponential decay: the best fits are shown, in the same colour as the data points.

Supplementary Table S1

	FokI variant	Relative k_{cat}/K_m values (% of WT)	
		Before labelling	After labelling with Alexa Fluor 594
Native FokI enzyme	WT	100	
Mutations at C84	C84S	14	-
	C84T	2	-
	C84L	8	-
	C84A	57	-
Mutations at C541	C541S	12	-
	C541T	170	-
	C541I	53	-
	C541A	76	-
The cysteine-free (CF) double mutant	C84A-C541T	73	
Mutations in the CF protein	CF-S446C	51	NR
	CF-S511C	84	67
	CF-R570C	22	6
Dimerisation-defective variant of CF-S511C	ddCF-S511C	NR	

Reactions were carried out as in Supplementary Figure S1 on all of the variants of FokI noted above: the protein was always at 2 nM and the DNA at 10 nM. For the variants that could be fully labelled with the maleimide derivative of Ax594, activities of the labelled protein were also measured. [Post-labelling activities of the single mutants at either C84 or C541 are not listed because the extent of labelling of these proteins never exceeded 50% and even that level required prolonged incubation with the dye in DMSO, conditions that denature FokI.] In all cases where cleavage was observed (NR indicates no detectable reaction), the concentration of the supercoiled substrate declined exponentially with time, from which a first-order rate constant (k_{obs}) was evaluated. A multiple-turnover reaction containing excess substrate over enzyme can follow an exponential progress curve only if the initial substrate concentration [S_0] is far lower than the K_m (31). In this situation, the Michaelis-Menten equation, $v = (V_{max} \times [S])/(K_m + [S])$, can be written as $dS/dt = (k_{cat} \times [E_0])/K_m) \times [S]$. Hence, the first-order rate constant for the exponential decline in [S] is equal to $(k_{cat}/K_m) \times [E_0]$. The values for k_{cat}/K_m determined by this approach are listed above as a percentage relative to the value of 4.25×10^6 $M^{-1}s^{-1}$ for WT FokI. Differences from the WT value by <2-fold are unlikely to be significant.

Supplementary Figure 2

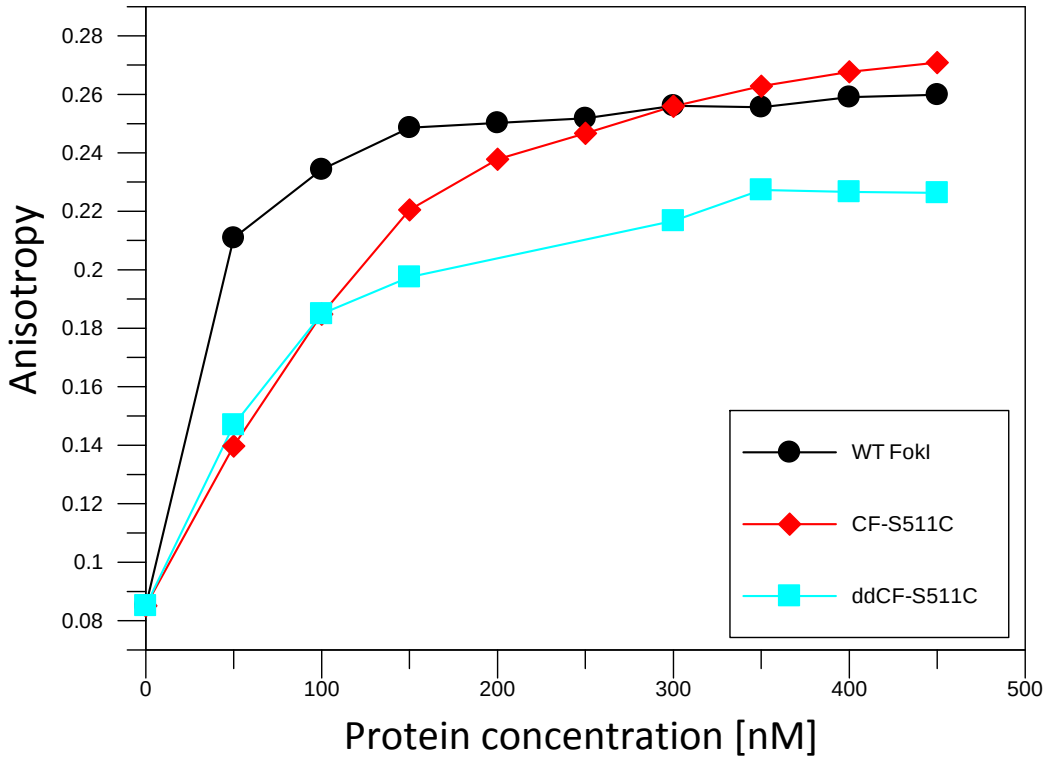

Figure S2. Equilibrium binding by fluorescence anisotropy. The fluorescence anisotropy from a solution of the HEX-24s oligoduplex (20 nM), in C–buffer at 25°C, was measured using the automated polarisation functions on the Horiba JY Fluorolog-Tau3 spectrofluorimeter: excitation was at 537 nm and emission observed in L-format at 552 nm. For each anisotropy value, three measurements were taken - with 0.5 s integration times - for each of the following settings: H/H, H/V, V/H and V/V. The readings at each setting were averaged and then used to calculate the anisotropy values (34). Subsequently, increasing amounts of the following variants of FokI were added to the duplex to give the protein concentrations indicated on the *x*-axis: WT FokI, black circles; CF-S511C, red diamonds; ddCF-S511C, blue squares. After each addition, the anisotropy was measured as above. Representative records from individual experiments are shown. [The HEX-labelled DNA may carry any of the following states of the FokI protein(s) and each of these complexes may result in a distinct change in the anisotropy of the emission from the HEX moiety: a monomer of FokI in its compact configuration, with juxtaposed catalytic and recognition domains; the FokI monomer in an extended configuration, with its catalytic domain positioned on the scissile bond; dimeric assemblies with two monomers. The overall anisotropy is the sum of the anisotropy for each species multiplied by the concentration of that species. Without knowing the anisotropy for each individual species, K_D values cannot be evaluated from this data. In addition, the protein concentrations on the *x*-axis denote total protein, from A_{280} measurements, rather than the concentration of enzyme in its native state so even the protein concentration that gives 50% of the total signal is not an indicator of the K_D value.]

Supplementary Figure 3

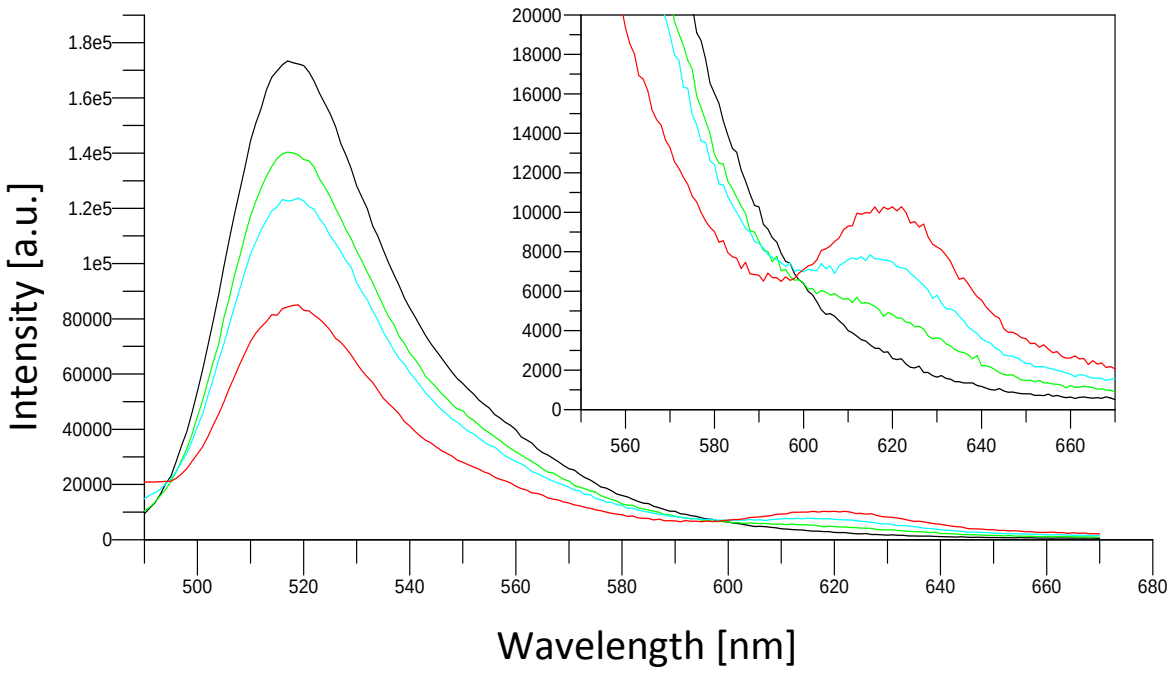

Figure S3. Fluorescence spectra. The Fluorolog-Tau3 spectrophofluorimeter was used to excite the fluorescence from solutions, in C-buffer at 25°C, containing 150 nM 24s-Ax488 and either no FokI protein (black trace) or the CF-S511C-Ax594 variant of FokI at one of the following concentrations: 50 nM, green trace; 150 nM, cyan trace; 300 nM, red trace. Excitation was at 465 nm and the emission recorded at 0.5 nm steps from 490 to 670 nm, with 0.5 nm slits on both beams. The intensity of emission noted on the *y*-axis shows the total photon count at each step, integrated over 0.5 s. The insert shows the spectra from 550 to 670 nm on a magnified scale. [Excitation was at 465 nm rather than the maximum for Ax488, at 492 nm, in order to minimise the direct excitation of Ax594.]