Supplementary Information for:

Correlating calmodulin landscapes with chemical catalysis in neuronal nitric oxide synthase using time-resolved FRET and a 5-deazaflavin thermodynamic trap.

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Table of contents

1. Experimental Section

1.1 Materials.

All reagents were of analytical grade and were purchased from Sigma-Aldrich (Gillingham, Dorset, UK), unless otherwise stated. 5-deazariboflavin (5-dRF) was synthesized by Salford Ultrafine Chemicals and Research Limited (Manchester, UK). [4(R)-2H]NADPH (pro-R NADP2H) and [4(S) 2H] NADPH (pro-S NADP2H) were prepared and characterized as described previously.[1] Alexa Fluor 555 C2 (A555) and Alexa 647 C2 (A647) maleimide were purchased from Thermo Fisher Scientific (Loughborough, UK). Pre-cast Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were purchased from Bio-Rad (Hempstead, UK).

1.2 Cloning, expression, and purification of tcRFK.

A synthetic gene encoding FAD synthetase from C. ammoniagenes[2] was obtained from GenScript. A truncated version encoding the C-terminal riboflavin kinase domain (tcRFK)[3] was amplified using the oligonucleotide primers tcRFK_fw (5'-GGGATTCATATGTTTTATGTTACCGGTCCG-3') and rFS-His_rv (5'-CACACTCGAGTGATTCGGCTTGCAGAAAG-3'), and cloned between the NdeI and XhoI restriction sites of the pET22b(+) vector (Novagen, Prudhoe, UK) resulting in plasmid pET-tcRFK-His. *Escherichia coli* BL21(DE3) cells harbouring the pET-tcRFK-His plasmid were grown in 1 litre cultures of Luria-Bertani medium supplemented with 50 μg mL^{-1} carbenicillin at 37°C until and OD_{600} of ~ 0.6 was reached. Protein expression was induced by the addition of 0.3 mM isopropyl-d-1-thiogalactopyranoside (IPTG), and incubation was continued at 25°C for 16 h before harvesting the cells by centrifugation. The cell pellet was re-suspended in 50 mM Tris-HCl (pH 8.0) supplemented with 200 mM NaCl and 10 mM imidazole, and the cells were broken by ultra-sonication. The lysate was cleared by centrifugation and loaded onto a Ni-IDA Agarose (Generon, Maidenhead, UK) column equilibrated with lysis buffer. The column was washed with 75 mM imidazole in the same buffer, and the tcRFK protein was eluted with 250 mM imidazole. The purified protein was dialysed against 50 mM Tris-HCl (pH 8.0) supplemented with 200 mM NaCl and stored at -20°C. Protein concentrations were determined using a calculated molar extinction coefficient (ε_{280}) of 14.44 mM^{-1} cm^{-1} at 280 nm.

1.3 Expression and purification of native NOS.

Native His_6-tagged rat neuronal NOS was overexpressed in *E. coli* and purified by Ni-IDA Agarose affinity chromatography and 2',5'-ADP-Sepharose (GE Healthcare, Little Chalfont, UK) affinity chromatography as described previously.[4,5] Protein concentrations were determined at 444 nm in the presence of carbon monoxide (CO), using a molar extinction coefficient of 74 mM^{-1} cm^{-1} ($A_{444} - A_{500}$) for the ferrous heme-CO adduct.[6] Immediately prior to kinetic experiments, native NOS was incubated with 0.2 mM FAD, 0.2 mM FMN, 0.2 mM tetrahydobiopterin (H_4B) and 2 mM DTT for ~30 min on ice. Next, a few grains of potassium ferricyanide were added and excess reagents were removed by applying the protein solution onto a Econo-Pac DG10 gel filtration column (Bio-Rad, Hempstead, UK) equilibrated in 40 mM HEPES (pH 7.6) buffer supplemented with 10 % glycerol and 150 mM NaCl. For anaerobic experiments, the final desalting step was performed in an anaerobic glove box (Belle Technology, Weymouth, UK) under nitrogen atmosphere in which oxygen levels were kept below 5 ppm.

1.4 Cloning, expression, and purification of native and T34C/T110C calmodulin (CaM).

The CaM variant T34C/T110C was constructed using the wild-type CaM containing pCOLADuet-1 plasmid template by using the 'QuickChange' method (Agilent, Stockport, UK). Both recombinant mammalian CaM and the T34C/T110C were expressed in *E. coli* BL21(DE3) and purified using a single Phenyl-Sepharose hydrophobic interaction chromatography (GE Healthcare, Little Chalfont, UK) step as described before.[4,7] Protein concentrations were determined at 276 nm using an extinction coefficient of ε_{276} = 3.006 mM^{-1} cm^{-1}.

1.5 Biosynthesis of 5-deaza-FMN.

For the biosynthesis of 5-deaza-FMN (5-dFMN), a solution containing 0.5 mM 5-dRF, 1 mM ATP, 10 mM $MgCl_2$ and 10 µM tcRFK in 50 mM Tris-HCl (pH 8.0) was incubated at 37°C for 120 min while shaking. The reaction was stopped by incubating the solution for 5 min at 95°C, and any insoluble material was removed by centrifugation. The reaction products were identified by thin-layer chromatography (TLC). Samples taken during the reaction, and the reference compounds FAD, FMN, riboflavin, and 5-dRF were applied onto a silica gel 60 F_{254} TLC plate (Merck Millipore, Nottingham, UK). Butanol:acetic acid:water (12:3:5) was used as mobile phase and fluorescent spots were visualized by UV illumination.

1.6 Circular dichroism (CD) measurements.

Circular dichroism was performed on an Applied Photophysics (Leatherhead, UK) Chirascan qCD spectrometer at 25 °C using a sealed cuvette with a 0.1 mm path length. Measurements were performed with 5 µM native or 5-dFMN nNOS in 40 mM HEPES (pH 7.6), supplemented with 150 mM NaCl and 10 % glycerol. Mean residual ellipicities (MRE, $[\Theta]_{MR}$) were calculated using eq 1. Where Θ is degrees of ellipicities, l is the cuvette path length in cm and C_{MR} is the mean residue concentration. C_{MR} is calculated using eq. 2 where n is the number of amino acids and c is the concentration of the protein in molar.

$$[\theta]_{MR} = 100\theta/(C_{MR}l) \qquad \text{(eq S1)}$$

$$C_{MR} = nc \qquad \text{(eq S2)}$$

1.7 Static fluorescence measurements.

Fluorescence emission spectra were recorded on an Edinburgh Instruments (Livingston, UK) FLS920 fluorometer equipped with double excitation and emission monochromators, a red-sensitive cooled photo-multiplier detector, and a 450 W xenon arc lamp. Spectra were recorded using 0.5 nm excitation and 5 nm emission slit-widths in 1 mL fluorescent quartz cells (Starna Scientific Ltd, Hainault, UK) with a 10 mm excitation path length. Fluorescence emission data were collected at 25 °C in 40 mM HEPES (pH 7.6), 150 mM NaCl and 10 % glycerol, unless otherwise stated. For Ca^{2+} free measurements a calcium sponge (Molecular Probes, Thermo Fisher Scientific – Loughbourough, UK) was used to remove the divalent ion from all buffers.

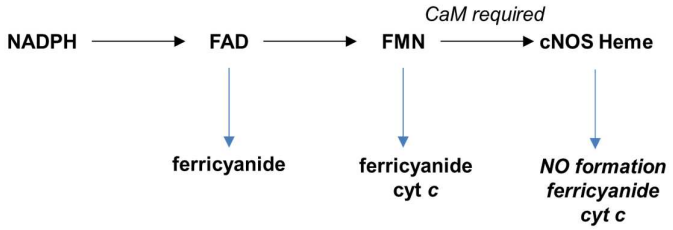
1.8 Steady-state activity measurements.

The steady-state turnover of native- and 5-dFMN reconstituted-nNOS was determined at 10 ^{o}C on assay mixtures containing catalytic amounts of NOS, 10 mM L-arginine, 0.5 mM $CaCl_2$, and 0.1 mM NADPH, in the presence or absence of 7 μM CaM in assay buffer. Steady-state NADPH oxidation rates were determined at 340 nm (ε_{340} = 6.22 mM^{-1} cm^{-1}), cytochrome *c* reduction at 550 nm ($\Delta\varepsilon_{550}$ = 21.1 mM^{-1} cm^{-1}) in the presence of 10 μM bovine heart cytochrome *c*, ferricyanide reduction at 420 nm (ε_{420} = 1.04 mM^{-1} cm^{-1}) in the presence of 1 mM potassium ferricyanide, and NO formation was monitored at 401 nm ($\Delta\varepsilon_{401}$ = 38 mM^{-1} cm^{-1}) in the presence of 10 μM oxyhemoglobin. All steady state assays were conducted in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl, 10 % glycerol.

Results

2.1 Steady State Turnover

2.1.1.



Scheme S1. Electron flow through the NOS di-flavin oxidoreductase to intrinsic and extrinsic electron accepting partners. Black and blue arrows are representative of electron flow through nNOS and from nNOS to redox partners, respectively.[8] CaM is required for cross-monomer electron transfer from nNOS FMN to heme.[9,10] CaM along with all electron accepting partners that require CaM-nNOS binding are shown in italics.

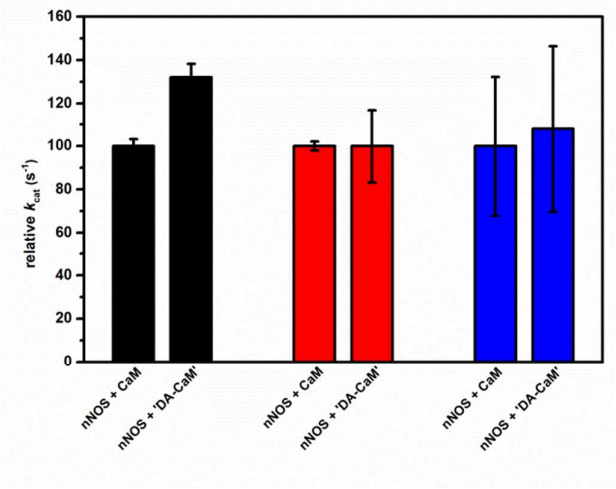


Figure S1. Comparison between native- and DA-T34C/T110C-CaM ('DA-CaM') bound nNOS steady-state turnover numbers. Black, red and blue bars represent NADPH consumption, NO formation and cyt *c* reduction assays, respectively. Data are normalized to the native nNOS assay turnover numbers.

Table S1. Steady state k_{cat} values for nNOS turnover assays.

NOS	FeCN reduction (s^{-1})	*cyt *c* reduction (s^{-1})	NADPH consumption (s^{-1})	NO formation (s^{-1})
Native	32.8 (1.1)	0.4 (0.03)	0.05 (0.00)	ND
Native + CaM	32.5 (0.7)	1.6 (0.6)	0.27 (0.01)	0.09 (0.002)
5-dFMN	28.2 (0.4)	ND	0.003 (0.000)	ND
5-dFMN nNOS + CaM	28.2 (0.4)	ND	0.003 (0.000)	ND

All data are recorded at 10 ^{o}C. *cyt *c* was shown to react with NADPH without nNOS present, so steady-state cyt *c* reduction rates were calculated by subtracting rates of solution reaction from those with enzyme present. Estimated errors are given in parenthesis. ND, not detected.

2.2 Static Fluorescence Measurements.

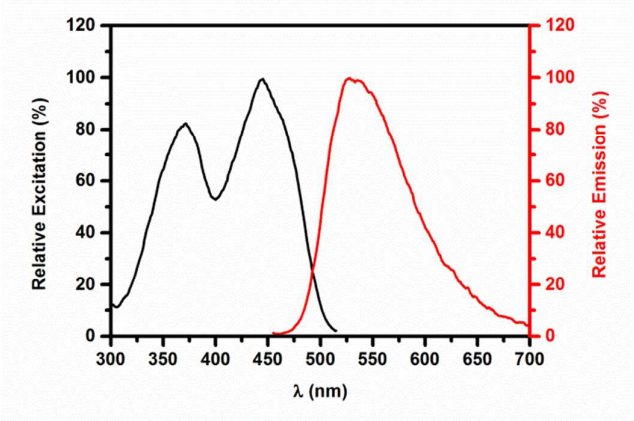


Figure S2. Fluorescence excitation and emission spectra of oxidized nNOS. Excitation wavelengths used in fluorescence experiments (555 and 645 nm) to monitor nNOS-bound CaM dynamics are red shifted from intrinsic nNOS flavin chromophore excitation spectra (black). nNOS emission was recorded with a 450 nm excitation wavelength while excitation data was recorded with a 525 nm emission wavelength. All 1 μM nNOS samples were in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol in the presence of 5 μM H_4B. Data are normalized to λ-max values for both excitation and emission spectra. The appearance of nNOS-blue semiquinone (which has absorbance features between 550 and 650 nm) is minimal during single-turnover stopped-flow measurements of nNOS flavin reduction thus there are no complications from direct excitation of nNOS semiquinone flavin species.[11]

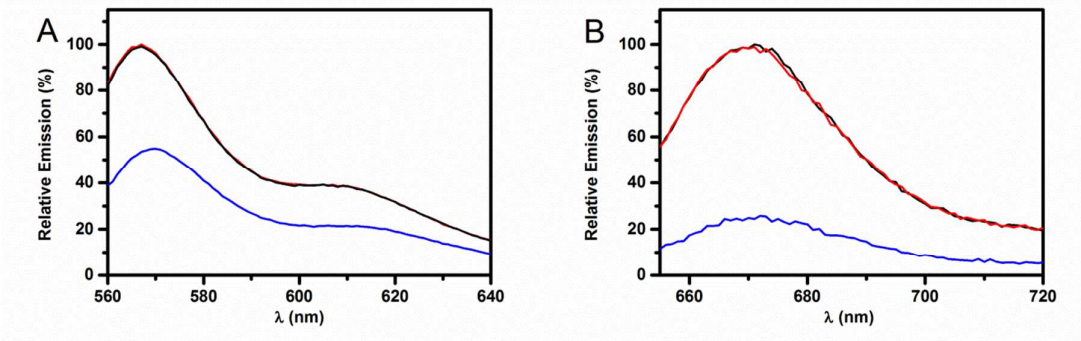


Figure S3. Fluorescence emission changes associated with the binding of A) 0.3 µM D-T34C/T110C-CaM or B) 0.3 µM A-T34C/T110C-CaM (black) with Ca^{2+} (red) or both Ca^{2+} and 0.3 µM nNOS (blue). Data show both a quenching in the emission as well as a ~3 nm red-shift in the λ-maxima of both donor and acceptor fluorophores when fluorophore labelled T34C/T110C-CaM binds to nNOS in the presence of Ca^{2+} relative to the Ca^{2+} free or Ca^{2+} bound fluorophore labelled T34C/T110-CaM. D-T34C/T110C- and A-T34C/T110C-CaM samples were excited at 555 nm and 645 nm, respectively. Data are plotted relative to the emission maxima of the D/A-T34C/T110C CaM only samples (black) which was normalized to 100 %. All samples were in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol in the presence of 5 µM H_4B.

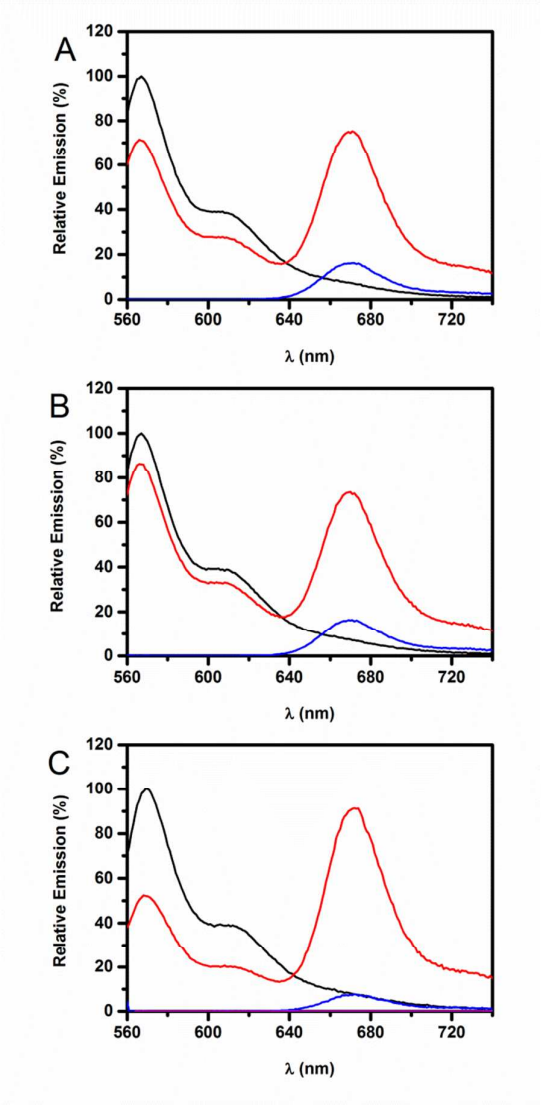


Figure S4. Fluorescence emission spectra of donor and acceptor fluorophores bound to T34C/T110C-CaM. A), B) and C) are representative of apo-CaM, Ca^{2+}-bound CaM and nNOS with equimolar Ca^{2+}-bound CaM, respectively. Black is 0.15 µM donor only labelled sample (D-T34C/T110C-CaM), red is 0.3 µM donor-acceptor labelled CaM (DA-T34C/T110C-CaM) and blue is 0.15 µM acceptor labelled CaM (A-T34C/T110C-CaM). In C) the magenta line is 0.3 µM nNOS bound to 0.3 µM unlabelled T34C/T110C-CaM, excited at 555 nm. All data are plotted relative to the emission maxima (~ 570 nm) of the donor only sample (black). All data were recorded using a 555 nm excitation wavelength and all samples were in 40 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol in the presence of 5 µM H_4B.

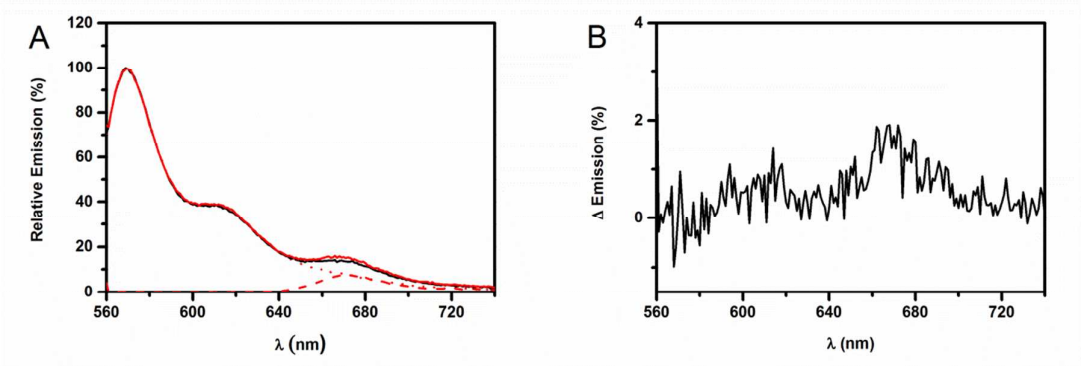


Figure S5. No Inter-CaM FRET across the nNOS dimer. The black spectrum in A) shows the fluorescence emission from 0.15 µM of D-T34C/T110C CaM and 0.15 µM A-T34C/T110C CaM bound to 0.3 µM oxidized nNOS (black). The red spectrum in A) show the emission from 0.15 µM single labelled D-T34C/T110C CaM bound to one equivalent of nNOS (dotted) as well as the emission spectrum of 0.15 µM A-T34C/T110C sample bound to equimolar nNOS (dash red). The sum of the two aforementioned spectra in A) is shown as a solid red line. All data in A) are normalized to the concentration of donor fluorophore in the D-T34C/T110C CaM bound nNOS containing sample (dotted red line). B) shows the difference in relative fluorescence emission between the solid red and black spectra shown in A) (red spectrum – black spectrum) to demonstrate that with using the A555-A647 fluorophore pair (R_0 = 47 Å) there is no recordable FRET between the two calmodulins bound to the nNOS dimer. All data were recorded using a 555 nm excitation and all samples were in 40 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol in the presence of 5 µM H_4B.

2.3 Stopped-flow UV-Vis Measurements.

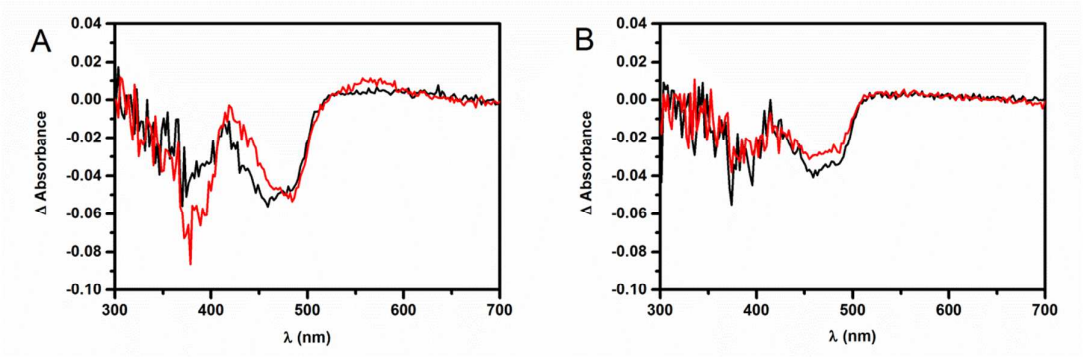


Figure S6. Absorbance changes, recorded by a photodiode array (PDA) stopped-flow instrument, 10 s after mixing of 5 µM (final concentration) of A) native or B) 5-dFMN-reconstituted NOS with 20-fold excess NADPH in the presence (red) and absence (black) of CaM. All experiments were conducted at 10 ^{o}C in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol with 1 mM $CaCl_2$.

Table S2 Observed KIE values for NADPH-driven nNOS flavin reduction

NOS	NADPH	k_1	k_2	k_3	k_4
Native	Pro-*R* $NADP^2H$	8.9 (2.8)	7.6 (5)	2.7 (1.1)	2.3 (1.4)
Native +CaM	Pro-*R* $NADP^2H$	4.3 (0.6)	2.4 (4.6)	6 (8.5)	1.7 (1.3)
Native	Pro-*S* $NADP^2H$	1.1 (0.6)	1.1 (1.1)	1.4 (0.6)	1.8 (1.0)
Native +CaM	Pro-*S* $NADP^2H$	1.4 (0.2)	2.1 (4.1)	2.7 (3.8)	1.1 (1.0)

Estimated errors are given in parenthesis.

2.4 Stopped-flow FRET measurements.

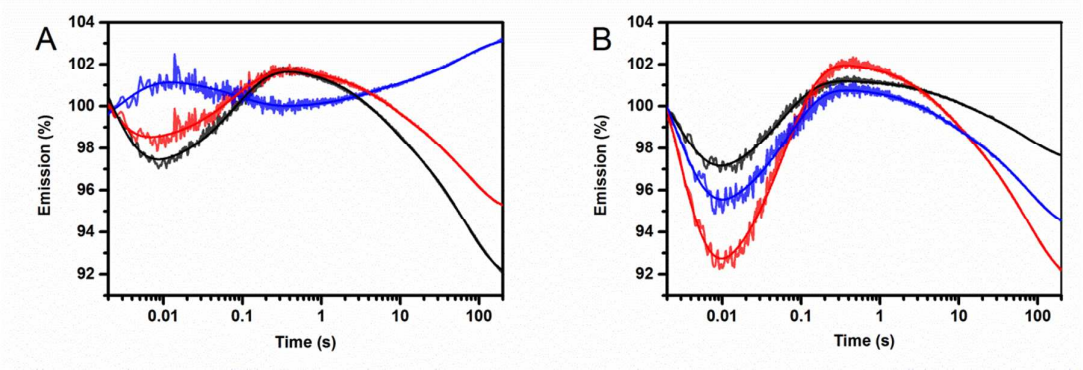


Figure S7. Time-dependent changes in fluorescence emission of fluorophores bound to the 0.3 µM single-labelled (donor or acceptor labelled - black) or 0.3 µM double-labelled (donor and acceptor labelled – red) T34C/T110C CaM-oxidized nNOS complex upon reduction with excess NADPH (100 µM, final concentration). No emission changes were recorded in the dead-time of the stopped-flow instrument and all data were normalized to percentage of emission change, relative to the emission at 0.002 seconds. The difference between the fluorophore emissions in the single- (donor or acceptor labelled T34C/T110C CaM) and double-labelled samples (donor and acceptor labelled T34C/T110C CaM) were normalized to percentage emission change at time 0.002 seconds and is shown as the blue transient. Donor emission changes are monitored using a 600 nm (+/- 10 nm) band width pass (BWP) with a 555 nm excitation wavelength and are shown in panel A). Acceptor emission changes are followed using a 650 nm long-pass (cut-on) filter, data collected for the acceptor only single labelled sample (A-T34C/T110C CaM-nNOS) were collected using a 645 nm excitation wavelength while the emission changes for the acceptor in the double labelled sample were collected with a 555 nm excitation wavelength. All fluorescence changes associated with the acceptor are shown in panel B). All experiments were conducted in 40 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol with 5 µM H_4B. Transients shown here are averages of 8 traces.

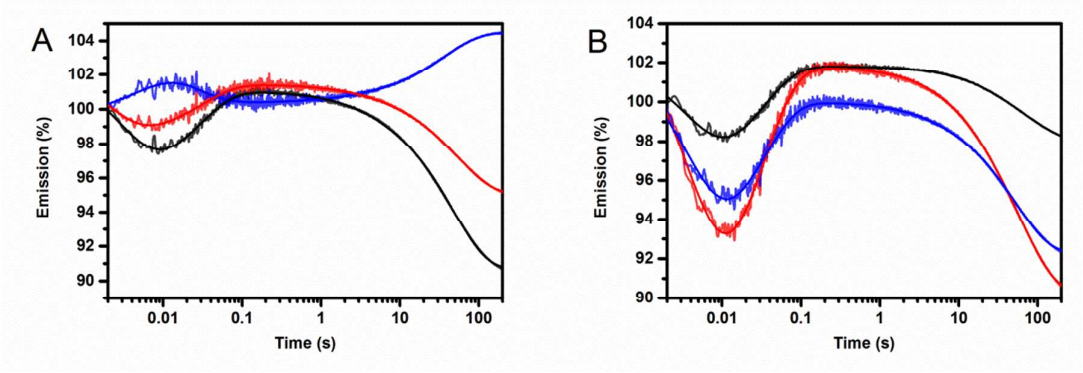


Figure S8. Time-dependent changes in fluorescence emission of fluorophores bound to the 0.3 µM single-labelled (donor or acceptor labelled - black) or 0.3 µM double-labelled (donor and acceptor labelled – red) T34C/T110C CaM-oxidized 5-dFMN nNOS complex upon reduction with excess NADPH (100 µM, final concentration). No emission changes were recorded in the dead-time of the stopped-flow instrument and all data were normalized to percentage of emission change, relative to the emission at 0.002 seconds. The difference between the fluorophore emissions in the single- (donor or acceptor labelled T34C/T110C CaM) and double-labelled samples (donor and acceptor labelled T34C/T110C CaM) were normalized to percentage emission change at time 0.002 seconds and is shown as the blue transient. Donor emission changes are monitored using a 600 nm (+/- 10 nm) band width pass (BWP) with a 555 nm excitation wavelength and are shown in panel A). Acceptor emission changes are followed using a 650 nm long-pass (cut-on) filter, data collected for the acceptor only single labelled sample (A-T34C/T110C CaM-5-dFMN nNOS) were collected using a 645 nm excitation wavelength while the emission changes for the acceptor in the double labelled sample were collected with a 555 nm excitation wavelength. All fluorescence changes associated with the acceptor are shown in panel B). All experiments were conducted in 40 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol with 5 µM H_4B. Transients show here are averages of 8 traces.

Table S3. Donor and acceptor fluorophore emission changes extracted from fitting to transients seen in Figure 4A and 4C (de-convoluted FRET) and Figure S7 (native) and S8 (5-dFMN).

NOS		k_1 (s^{-1})	ΔC_1	k_2 (s^{-1})	ΔC_2	k_3 (s^{-1})	ΔC_3	k_4 (s^{-1})	ΔC_4	y0
native	SL Donor	504 (86)	9.8 (3.7)	11.7 (1.3)	-4.9 (0.8)	0.19 (0.03)	2.8 (0.3)	0.016 (0.001)	7.3 (0.1)	91.3 (0.8)
	DL Donor	663 (262)	3.5 (2.3)	11.6 (1.8)	-3.5 (0.7)	0.19 (0.01)	1.7 (0.1)	0.014 (0.001)	4.9 (0.1)	95.4 (0.9)
	Donor (FRET)	259 (125)	-2.9 (0.7)	14.2 (5.2)	1.8 (1.0)	0.25 (0.16)	-0.9 (0.5)	0.018 (0.003)	-2.5 (0.2)	103.6 (1.3)
	SL Acceptor	438 (116)	9.5 (2.8)	17.2 (1.2)	-5.1 (0.4)	0.13 (0.02)	1.0 (0.1)	0.013 (0.001)	2.8 (0.2)	98.0 (1.0)
	DL Acceptor	417 (68)	21.6 (4.5)	15.8 (1.2)	-11.5 (1.2)	0.15 (0.01)	2.8 (0.3)	0.013 (0.001)	7.7 (0.2)	92.1 (1.2)
	Acceptor (FRET)	297 (61)	8.1 (1.9)	15.1 (2.4)	-6.4 (1.2)	0.17 (0.02)	1.7 (0.2)	0.014 (0.002)	5.0 (0.3)	94.3 (2.3)
5-dFMN	SL Donor	239 (68)	6.3 (1.3)	38.8 (6.0)	-5.9 (2.0)	0.22 (0.1)	1.1 (0.2)	0.021 (0.001)	9.2 (0.2)	90.3 (1.2)
	DL Donor	295 (116)	3.4 (1.1)	37.2 (12.2)	-3.7 (0.9)	0.61 (1.2)	0.7 (0.2)	0.018 (0.001)	5.7 (0.2)	95.0 (1.2)
	Donor (FRET)	240 (79)	-2.7 (1.3)	28.9 (20.1)	1.7 (1.1)	1.13 (1.14)	-0.4 (0.1)	0.030 (0.004)	-3.7 (0.2)	104.7 (1.3)
	SL Acceptor	201 (39)	5.7 (1.0)	31.6 (4.2)	-6.1 (1.0)	0.04 (0.02)	1.2 (0.5)	0.010 (0.002)	2.8 (0.4)	97.6 (0.9)
	DL Acceptor	189 (19)	15.7 (1.6)	32.5 (2.1)	-14.9 (1.6)	0.09 (0.04)	1.8 (0.6)	0.016 (0.001)	9.8 (0.6)	90.6 (2.2)
	Acceptor (FRET)	186 (42)	10.1 (1.4)	33.4 (6.0)	-8.8 (1.2)	0.18 (0.08)	0.9 (0.3)	0.019 (0.002)	7.0 (0.4)	93.0 (1.9)

Estimated errors are given in parenthesis. SL, single labelled; DL, double labelled.

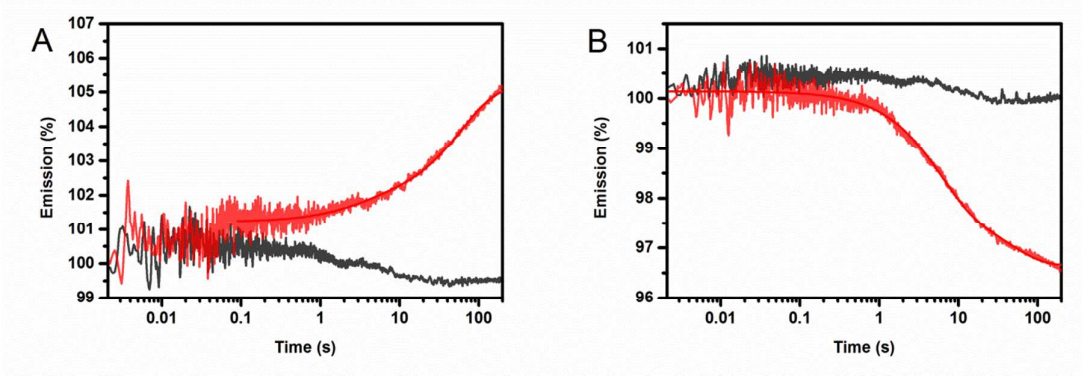


Figure S9. Time-dependent changes in fluorescence emission of fluorophores bound to the 0.3 µM single-labelled (donor or acceptor labelled - black) or 0.3 µM double-labelled (donor and acceptor labelled – red) T34C/T110C CaM-oxidized nNOS complex upon reduction with excess $NADP^+$ (500 µM, final concentration). No emission changes were recorded in the dead-time of the stopped-flow instrument and all data were normalized to percentage of emission change, relative to the emission at 0.002 seconds after mixing. The difference between the fluorophore emissions in the single- (donor or acceptor labelled T34C/T110C CaM) and double-labelled samples (donor and acceptor labelled T34C/T110C CaM) were not calculated since little changes were observed in the single labelled samples (black) which are attributed to photo-bleaching (*see results and discussion*). Donor emission changes are monitored using a 600 nm (+/- 10 nm) band width pass (BWP) with a 555 nm excitation wavelength and are shown in panel A). Acceptor emission changes shown in B) were followed using a 650 nm long-pass (cut-on) filter, data collected for the acceptor only single labelled sample (A-T34C/T110C CaM-nNOS) were collected using a 645 nm excitation wavelength while the emission changes for the acceptor in the double labelled sample were collected with a 555 nm excitation wavelength. All fluorescence changes associated with the acceptor are shown in panel B). All experiments were conducted in 40 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM $CaCl_2$, 10 % glycerol with 5 µM H_4B. Transients show here are averages of 8 traces.

Table S4. Donor and acceptor fluorophore emission changes extracted from fitting exponential functions to transients seen in Figure 6 and Figure S9.

	k_1 (s^{-1})	ΔC_1	k_2 (s^{-1})	ΔC_2	y0
SL D	ND	ND	ND	ND	ND
DL D	1.24 (1.2)	-0.7 (0.2)	0.017 (0.003)	-3.9 (0.7)	105.0 (1.1)
Donor (FRET)	1.24 (1.2)	-0.7 (0.2)	0.017 (0.003)	-3.9 (0.7)	105.0 (1.1)
SL A	ND	ND	ND	ND	ND
DL A	0.53 (0.97)	1.7 (1.8)	0.055 (0.057)	1.8 (1.1)	97.8 (2.5)
Acceptor (FRET)	0.53 (0.97)	1.7 (1.8)	0.055 (0.057)	1.8 (1.1)	97.8 (2.5)
Donor/Acceptor	0.43 (0.56)	-0.034 (0.012)	0.020 (0.011)	-0.052 (0.013)	1.10 (0.02)

Estimated errors are given in parenthesis. SL, single labelled; DL, double labelled. ND, not determined.

2.5 Circular dichroism (CD) measurements.

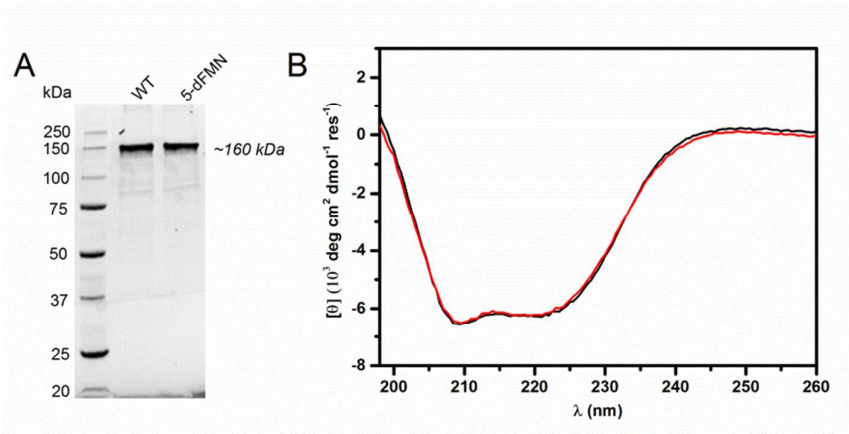


Figure S10. A) SDS-PAGE gel of native and 5-dFMN nNOS showing no alteration (proteolysis) in nNOS monomer after unfolding treatment with potassium bromide and subsequent protein refolding (*see Experimental Section*). B) Circular dichroism of native (black) and 5-dFMN nNOS (red) showing secondary structure is identical between the two enzymes. CD experiments were performed on 5 µM of both native and 5-dFMN nNOS in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl and 10 % glycerol.

2.6 Absorbance Measurements

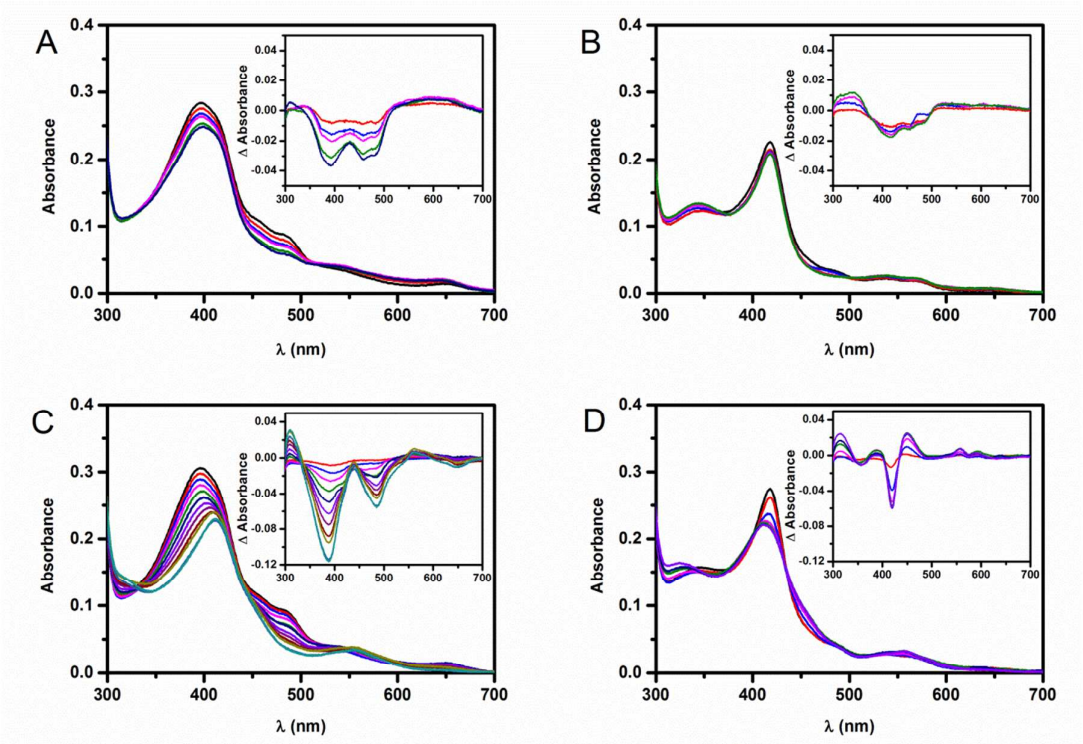


Figure S11. Reductive titration of native and 5-dFMN reconstituted NOS. NOS (~3 µM) was anaerobically titrated with NADPH (~1 µM aliquots) and dithionite (DT) in the presence of 5 mM L-arginine and 5 µM H_4B in 40 mM HEPES (pH 7.6) buffer supplemented with 10% glycerol and 150 mM NaCl. Spectra were taken after each addition. A) Native NOS titrated with NADPH, B) 5-dFMN NOS titrated with NADPH, C) Native NOS titrated with dithionite, and D) 5-dFMN substituted NOS titrated with DT. The insets show the observed difference spectra upon addition of reductant. Spectra were recorded at ambient room temperature on a Cary UV-Vis spectrometer with 10-20 µM of flavin samples.

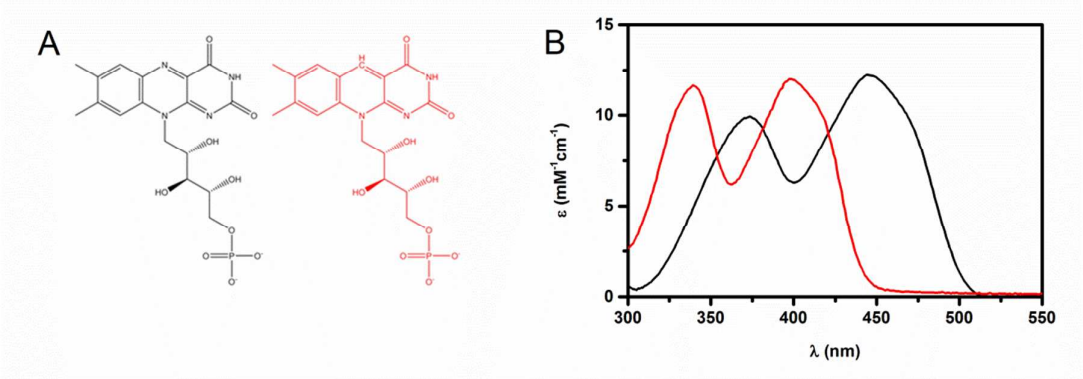


Figure S12. A) Structures of FMN (black) and 5-dFMN (red). B) The absorbance spectra of flavin mononucleotide (FMN; black) and 5-deazaflavin mononucleotide (5-dFMN; red) in 40 mM HEPES (pH 7.6) supplemented with 150 mM NaCl and 10 % glycerol. Spectra were recorded at ambient room temperature on a Cary UV-Vis spectrometer with 10-20 µM of flavin samples. Spectra in B) are normalized to molar extinction coefficient.

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