Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information for:

A Novel Flavin Derivative Reveals the Impact of Glucose on Oxidative Stress in Adipocytes

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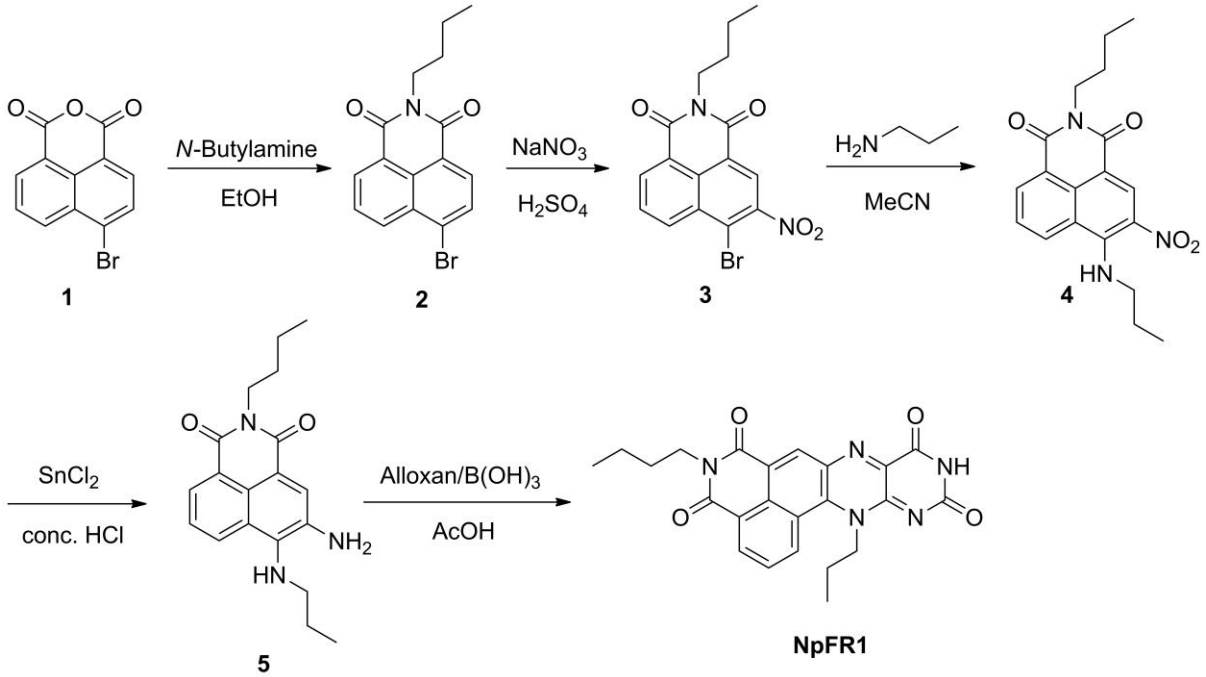
GENERAL EXPERIMENTAL METHODS

All solvents used were laboratory grade and were dried over appropriate drying agents when required. MilliQ water was used to prepare all aqueous solutions. Merck 230-400 mesh Kieselgel 60 was used for column chromatography and Merck Kieselgel 60 0.25 mm F254 precoated sheets were used for analytical thin layer chromatography. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Alfa Aesar and Combi Blocks and used as received. Electrochemical measurements were performed using a BAS 100B/W Electrochemical analyser. Cyclic voltammograms were collected at room temperature at a scan rate of 20 mV/s; using a glass carbon working electrode, a platinum auxiliary electrode and Ag/AgCl reference electrode. Compounds were dissolved in acetonitrile containing 0.1 M tetrabutylammonium bromide and ferrocene (as an internal standard) to a final concentration of 5 mM. All solutions were degassed with argon for ten minutes prior to measurement.

SPECTROSCOPIC METHODS

^{1}H - NMR spectra were collected in commercially available deuterated solvents (Cambridge Isotope Laboratories, Cambridge, MA) at 25 ºC on a Bruker DRX 300 or Bruker Ascend 400 and 500 spectrometer at the School of Chemistry NMR Facility at the University of Sydney. ^{13}C - NMR were recorded on Bruker Ascend 400 and 500 spectrometers at frequencies of 100 MHz and 125 MHZ. All chemical shifts are reported in the standard δ notation of parts per million using the peak of the residual solvent proton signals as an internal reference. J values are given in Hertz. Low-resolution mass-spectral analyses were performed by liquid chromatography-mass spectrometry (Finnigan LCQ quadrupole ion trap mass spectrometer). Spectroscopic measurements were performed in 100 mM HEPES, pH 7.4. Fluorescence measurements were collected using a bench top Perkin Elmer Enspire Multimode Plate Reader at 10 nm slit widths.

SYNTHESIS OF NpFR1



6-Bromo-2-butyl-1H-benzo[de]isoquinoline-1,3(2H)-dione (2)

According to the method reported by Zhang *et al.*,[1] to a solution of 4-bromo-1,8-naphthalic anhydride (3.0 g, 10.8 mmol) in EtOH (100 mL) was added *N*-butylamine (1.17 mL, 11.8 mmol). The mixture was heated to reflux for 17 hours before the solvent was removed under vacuum and the product recrystallised in EtOH to give yellow crystals of **1** (2.60 g, 7.83 mmol, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.65 (d, *J* = 8.4, 1H), 8.55 (d, *J* = 8.5, 1H), 8.40 (d, *J* = 7.9, 1H), 8.03 (d, *J* = 7.8, 1H), 7.84 (t, *J* = 7.6, 1H), 4.17 (t, *J* = 7.7, 2H), 1.72 (quin, *J* = 7.6, 2H), 1.44 (sex, *J* = 7.6, 2H), 0.98 (t, *J* = 7.5, 3H) ppm.

6-Bromo-2-butyl-5-nitro-1H-benzo[de]isoquinoline-1,3(2H)-dione (3)

According to the procedure reported by Zeng *et al.*,[2] to a solution of **1** (750 mg, 2.26 mmol, 1 equiv.) in sulfuric acid (20 mL), sodium nitrate (0.22 g, 2.6 mmol, 1 equiv.) was added and the solution stirred for 30 minutes at -10 ^{o}C and then for 3 hours at room temperature. The mixture was added slowly to ice-water (200 mL) and the suspension filtered, washed with water and recrystallised from ethanol to give **2** as a pale yellow solid (732 mg, 1.94 mmol, 86%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.81 (s, 1H), 8.77 (d, *J* = 7.3, 2H), 8.01 (t, *J* = 7.8, 1H), 4.19 (t, *J* = 7.7, 2H), 1.72 (m, 2H), 1.46 (m, 2H), 0.99 (t, *J* = 7.2, 3H) ppm.

2-Butyl-5-nitro-6-(propylamino)-1H-benzo[de]isoquinoline-1,3(2H)-dione (4)

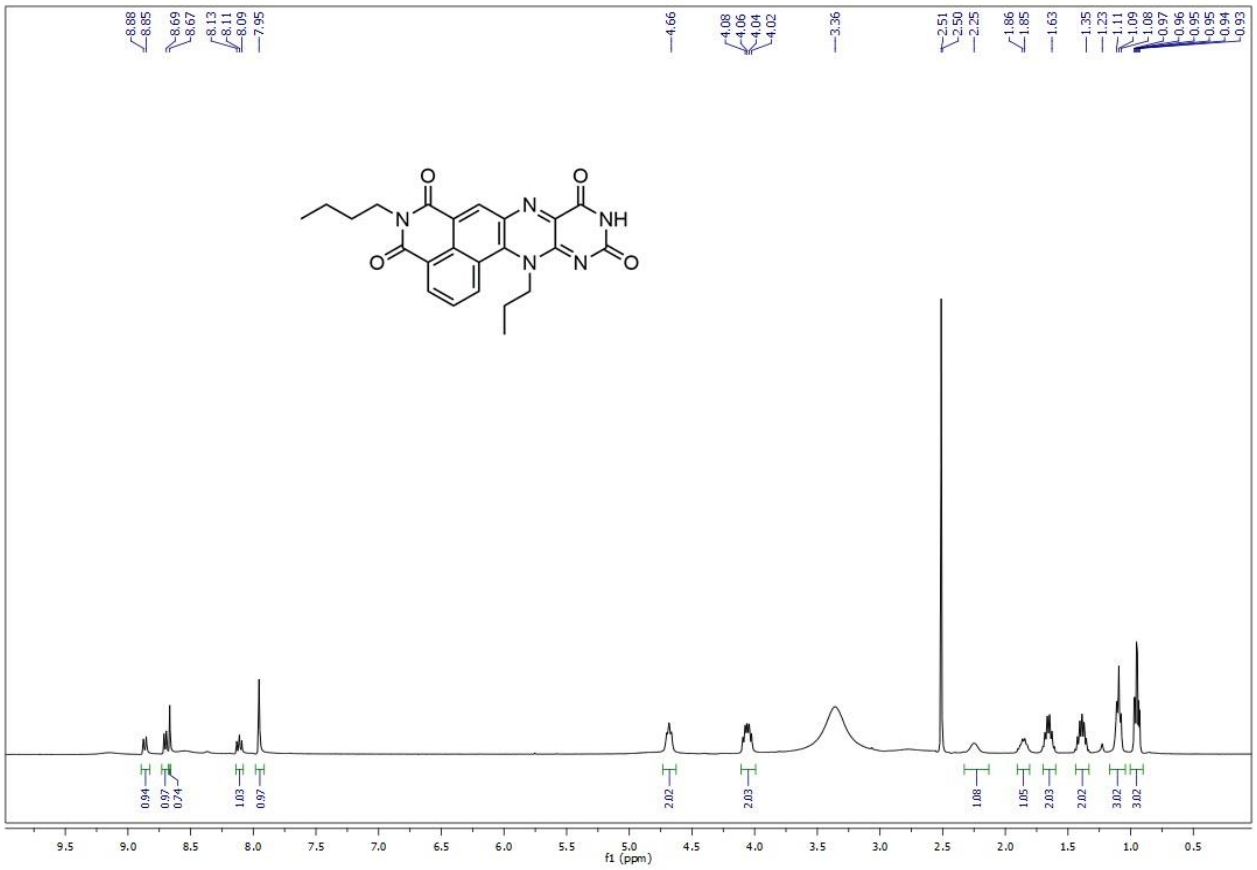
N-propylamine (0.47 g, 7.9 mmol, 3 equiv.) was added to a solution of **2** (1.00 g, 2.65 mmol, 1 equiv.) in MeCN (50 mL). The mixture was stirred for 1 hour at room temperature under nitrogen before the solvent was evaporated under vacuum and the residue recrystallised in EtOH to give **3** as a yellow crystalline solid (0.710 g, 2.00 mmol, 75%).

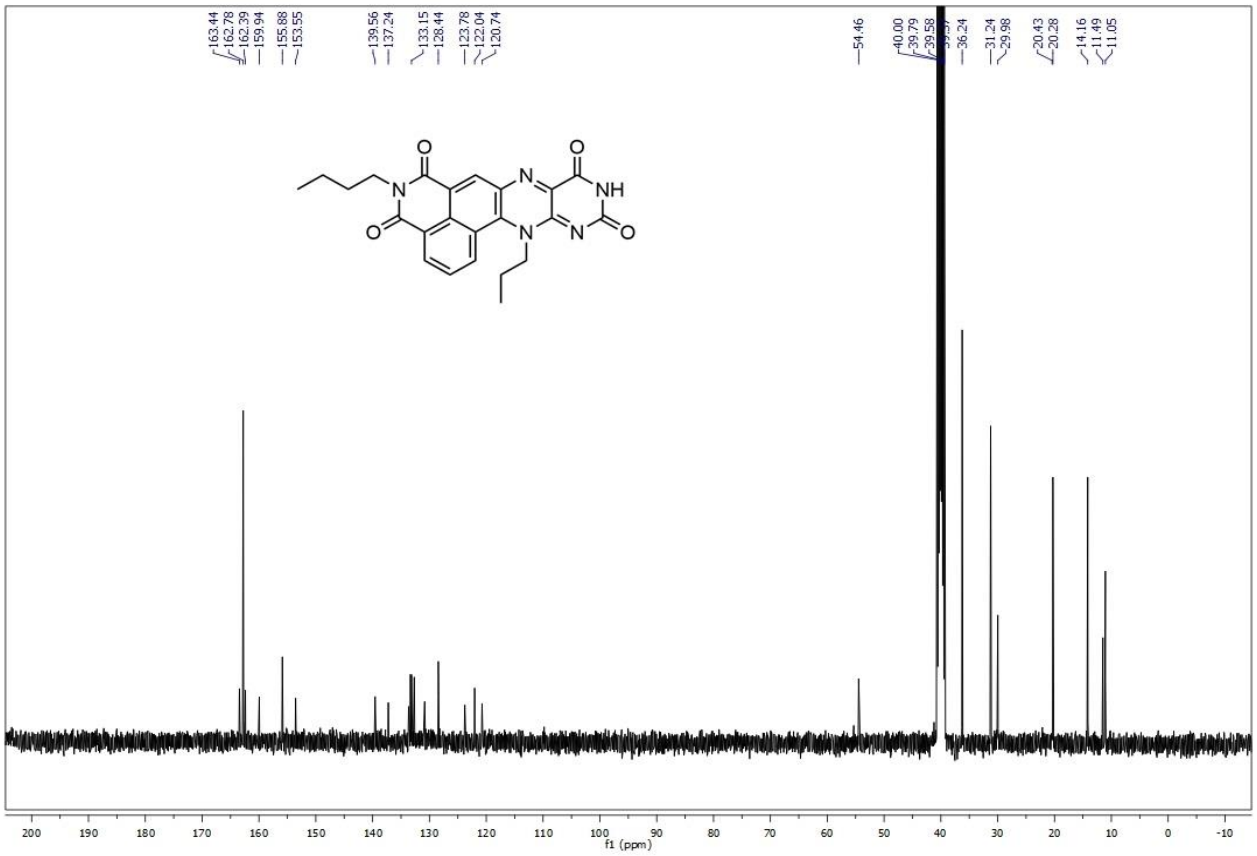
^{1}H NMR (400 MHz, $CDCl_3$): δ 9.90 (br s, 1H), 9.24 (s, 1H) 8.65 (m, 2H), 7.68 (t, *J* = 8, 1H), 4.13 (t, *J* = 7.6, 2H), 3.94 (t, *J* = 7.5, 2H), 1.90 (sex, *J* = 7.2, 2H), 1.69 (quin, *J* = 7.2, 2H), 1.43 (sex, *J* = 7.6, 2H), 1.11 (t, *J* = 7.2, 3H), 0.97 (t, *J* = 7.6, 3H) ppm; APCI-MS: calculated 355.15 for $[M]^+$ $C_{19}H_{21}N_3O_4$, found 356.00

5-Butyl-13-propyl-4H-benzo[4,5]isoquinolino[7,6-g]pteridine-4,6,9,11(5H,10H,13H)- tetraone (NpFr1)

Stannous chloride dihydrate (0.51 g, 2.2 mmol, 8 equiv.) was added to a suspension of **3** (120 mg, 0.34 mmol, 1 equiv.) and 32% HCl (3 mL) under a nitrogen atmosphere. The suspension was refluxed for 3 hours before being allowed to stir at room temperature overnight. The mixture was poured onto deionised ice (5 g) and the solution adjusted to pH11 with 5 M NaOH. The suspension was extracted with CH_2Cl_2 (3 x 50 mL) and washed sequentially with water, saturated $NaHCO_3$ solution and brine. The combined organic extracts were dried over Na_2SO_4 and evaporated to dryness to give **5** (104 mg) as a yellow solid which was used in the next step immediately, without further purification. Alloxan monohydrate (54.1 mg, 0.34 mmol, 1.1 equiv.) and boric acid (24.7 mg, 0.40 mmol, 1.3 equiv.) were added to a stirred solution of **5** (100 mg, 0.31 mmol, 1 equiv.) in glacial acetic acid (5 mL). The solution was stirred for 3 hours under nitrogen, filtered and washed with water. The crude solid was purified by preparative TLC using DCM : MeOH (20:1) as eluent to give **5** as a bright orange solid (96 mg, 0.22 mmol, 32 %). ^{1}H NMR (500 MHz, d_6-DMSO): δ 8.86 (d, *J* = 12, 1H), 8.70 (d, *J* = 8 Hz,1H), 8.67 (s, 1H), 8.11 (t, *J* = 8.0 Hz,1H), 4.66 (t, *J* = 7.2, 2H), 4.05 (t, *J* = 7.5, 2H), 2.25 (m, 1H), 1.86 (m, 1H), 1.63 (sex, *J* = 7.4, 2H), 1.35 (sex, *J* = 7.4, 2H), 1.09 (t, *J* = 6.8, 3H), 0.95 (t, *J* = 4, 3H). ^{13}C NMR (125 MHz, d_6-DMSO): δ 163.4, 162.8, 162.4, 159.9, 155.9, 153.5, 138.6, 137.2, 133.1, 128.4, 123.8, 122.0, 120.7, 54.5, 36.2, 31.2, 29.1, 20.5, 20.3, 14.2, 11.5, 11.0. ; APCI-MS: calculated 432.17 for $[M+H]^+$ $C_{23}H_{22}N_5O_4$ found 432.00

^{1}H and ^{13}C NMR spectra of **NpFR1**





CELLULAR EXPERIMENTS

Cell lines

All cellular studies used the mouse preadipocyte cell line 3T3-L1. Cells were maintained in exponential growth as monolayers at 37 ºC in 5% CO_2 Dulbecco's modified eagle medium (DMEM) supplemented with 2.5 mM glutamine, 10% fetal calf serum and antibiotics as per suppliers instructions.

Cells were differentiated by the method outlined by Lee *et al*.,[3] in which preadipocytes were first allowed to grow to confluence. Two day post-confluent cells (designated as day 0) were then incubated in DMEM supplemented with 1 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 1 µg/mL insulin, 2.5 mM glutamine, 10% fetal calf serum and antibiotics for two days. Cells were then washed with phosphate buffered saline (PBS) and maintained in DMEM supplemented with 1 µg/mL insulin, 2.5 mM glutamine, 10% fetal calf serum and antibiotics.

Microscopy

Brightfield images were taken using a Nikon ECLIPSE TE200 inverted light microscope and an ANMO Dino-eye microscope eyepiece camera. Confocal images were acquired using an Olympus Fluoview FV1000 inverted light, fluorescence and confocal microscope and either a LUCPLFLN 40X air objective lens (NA = 0.60) or a UPLSAPO 60X water-immersion objective lens (NA = 1.20). Excitation light of 405 nm was provided by LD405 nm laser. Cells were imaged in a temperature controlled incubator at 37 ºC. Images were collected and processed using FV10-ASW viewer software v1.7 (Olympus). Image analysis of mean fluorescence intensity was performed in ImageJ.

Imaging experiments

3T3-L1 preadipocytes were grown and differentiated in 6-well glass bottom plates (MatTek Corporation) as described above. Cells were treated with a solution of 50 µM probe in DMSO (final concentration of DMSO in cell media was less than 1%) and incubated for two hours before being washed with phosphate-buffered saline (PBS) and maintained in DMEM (without phenol red) supplemented with 2.5 mM glutamine, 10% fetal calf serum and antibiotics for the duration of imaging.

For measurement of the oxidative capacity of cells maintained in different glucose conditions, cells were maintained for 24 hours in DMEM containing either 25 mM or 10 mM glucose and supplemented with 2.5 mM glutamine, 10% fetal calf serum and antibiotics as per suppliers instructions.

For comparison of the oxidative capacity of preadipocytes vs. mature adipocytes, cells were differentiated as described above. Microscopy was performed just as lipid droplets became visible (day 5) while a second set was left to accumulate lipid and imaged once multiple, large lipid droplets had formed (day 11).

References

1. Zhang, Z.; Chen, Y.; Xu, D.; Yang, L.; Liu, A., A new 1,8-naphthalimide-based colorimetric and "turn-on" fluorescent Hg2+ sensor. *Spectrochimica acta. Part A, Molecular and biomolecular spectroscopy* **2013,** *105*, 8-13.
2. Zeng, X.; Zhang, X.; Zhu, B.; Jia, H.; Li, Y., A highly selective wavelength-ratiometric and colorimetric probe for cysteine. *Dyes and Pigments* **2012,** *94* (1), 10-15.
3. Lee, H.; Lee, Y. J.; Choi, H.; Ko, E. H.; Kim, J. W., Reactive oxygen species facilitate adipocyte differentiation by accelerating mitotic clonal expansion. *The Journal of biological chemistry* **2009,** *284* (16), 10601-9.

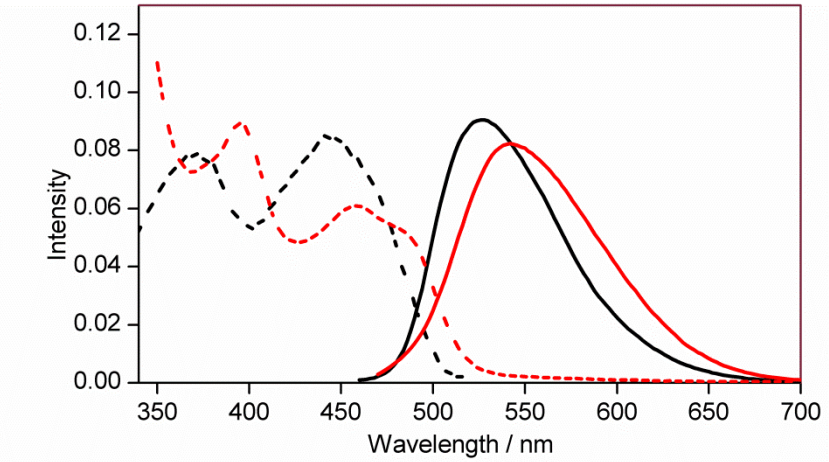


Figure S1: Absorption and fluorescence spectra of NpFR1 (Red) and riboflavin (Black). Excitation was provided at 405 and 465 nm respectively. Spectra were acquired in HEPES buffer (100 mM, pH 7.4).

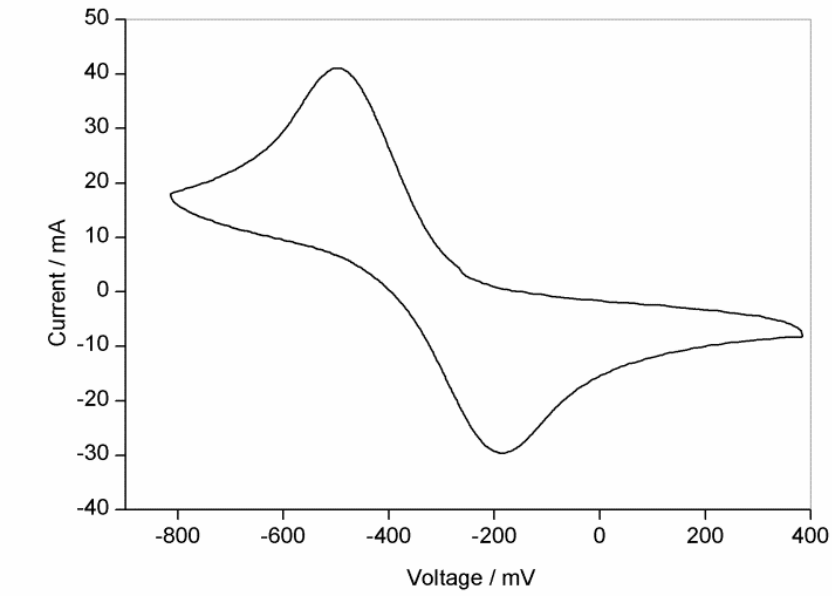


Figure S2: Cyclic voltammogram of NpFR1 (5 mM concentration) in (MeCN with Ferrocene) at 25 °C with a scan rate of 20 mV/s.

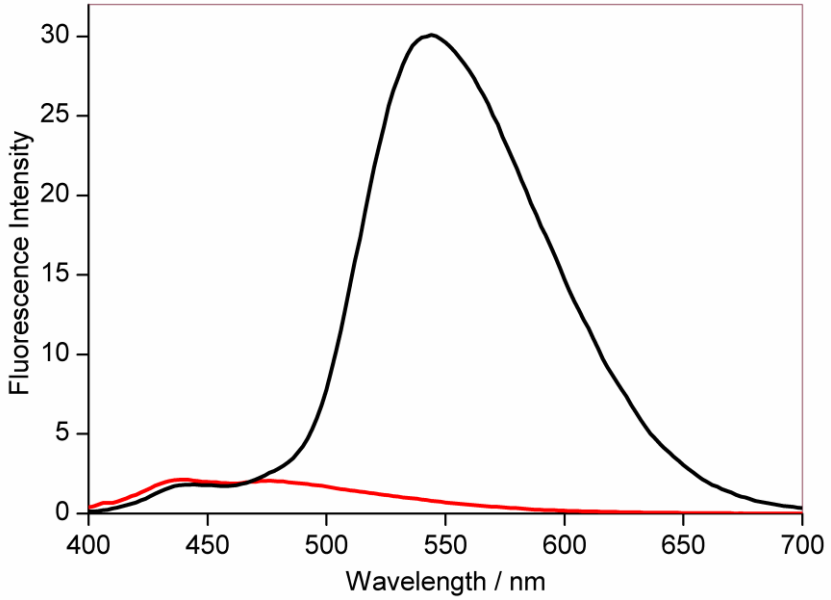


Figure S3: Emission spectra of NpFR1 in oxidised (black) and reduced (red) form following excitation at 380 nm. Spectra were acquired in HEPES buffer (100 mM, pH 7.4).

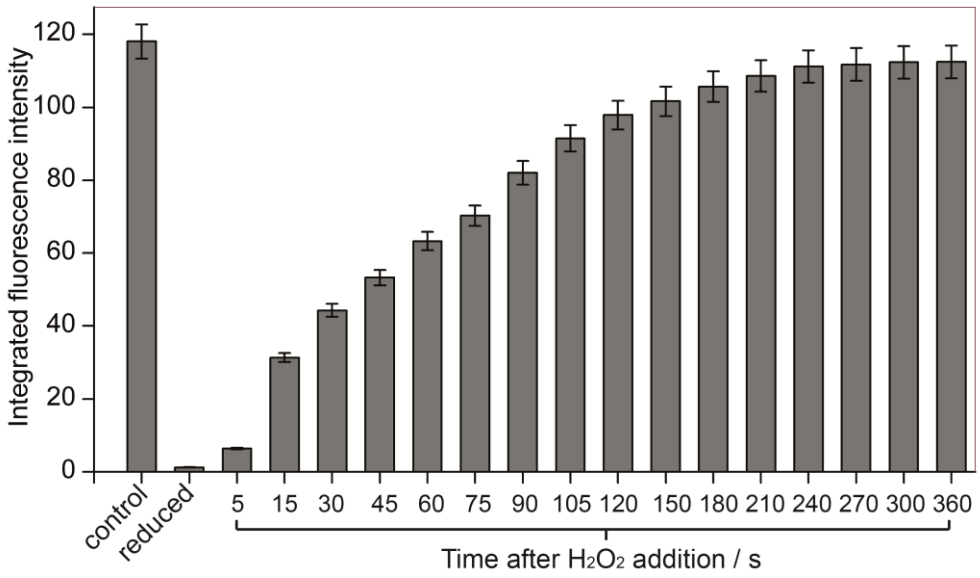


Figure S4: The fluorescence emission from **NpFR1** (5 µM) over time. Bars represent the integrated emission intensity (420 to 650 nm, λ_{ex} = 405 nm). **NpFR1** was reduced with sodium dithionite (100 µM) and re-oxidised with hydrogen peroxide (250 µM). All spectra were acquired in HEPES buffer (100 mM, pH 7.4).

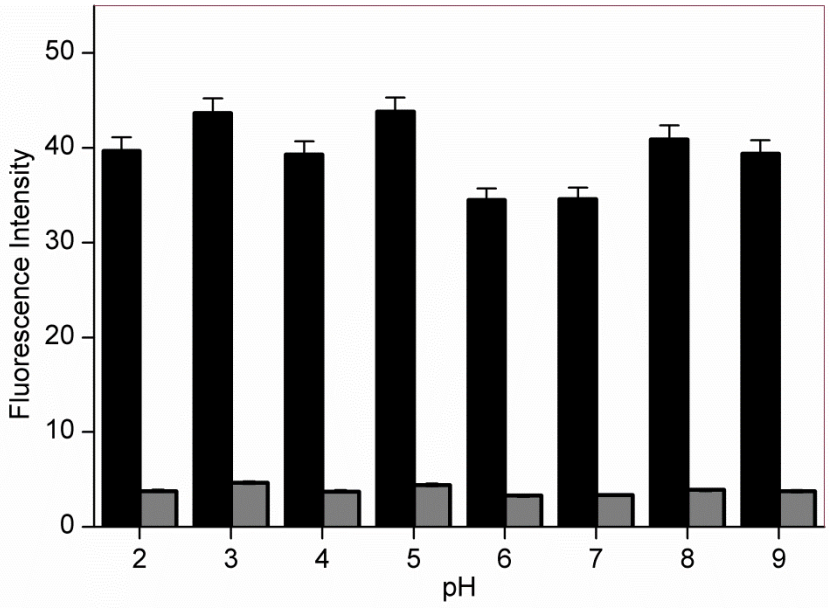


Figure S5: The fluorescence emission from NpFR1 (5 µM) over a range of pH values. Bars represent the integrated emission intensity (420 to 650 nm, λ_{ex} = 405 nm) for oxidised (black) and reduced (grey) forms.

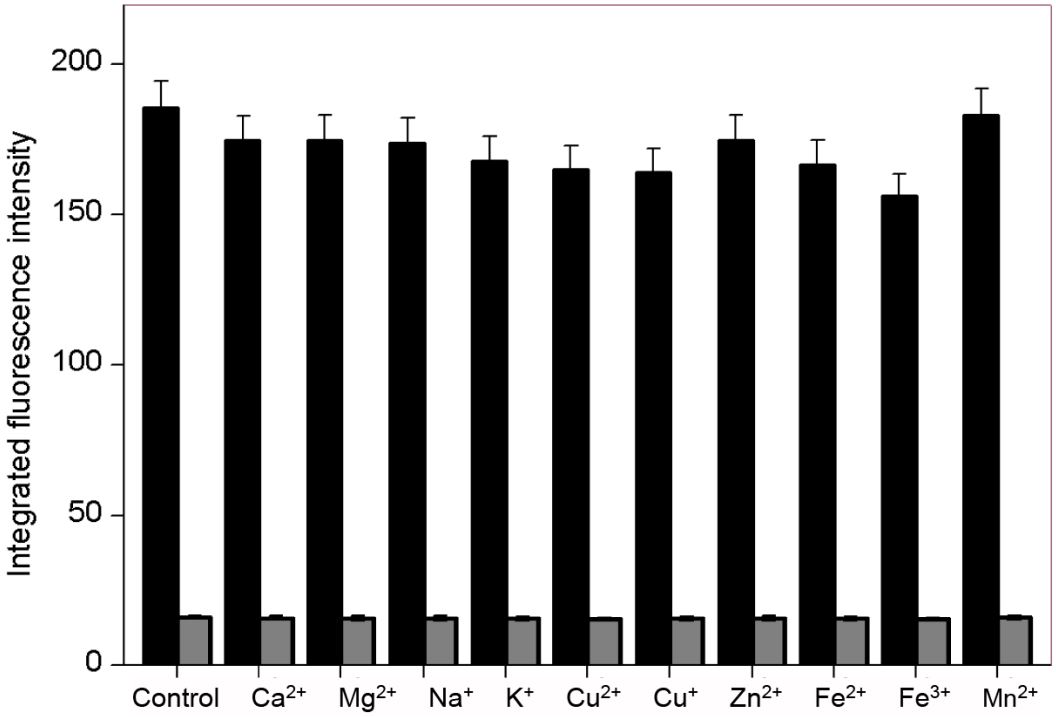


Figure S6: The fluorescence emission from NpFR1 (5 μM) in the presence of common metal ions (100 μM). Bars represent the integrated emission intensity (420 to 650 nm, λ_{ex} = 405 nm) for oxidised (black) and reduced (grey) forms.

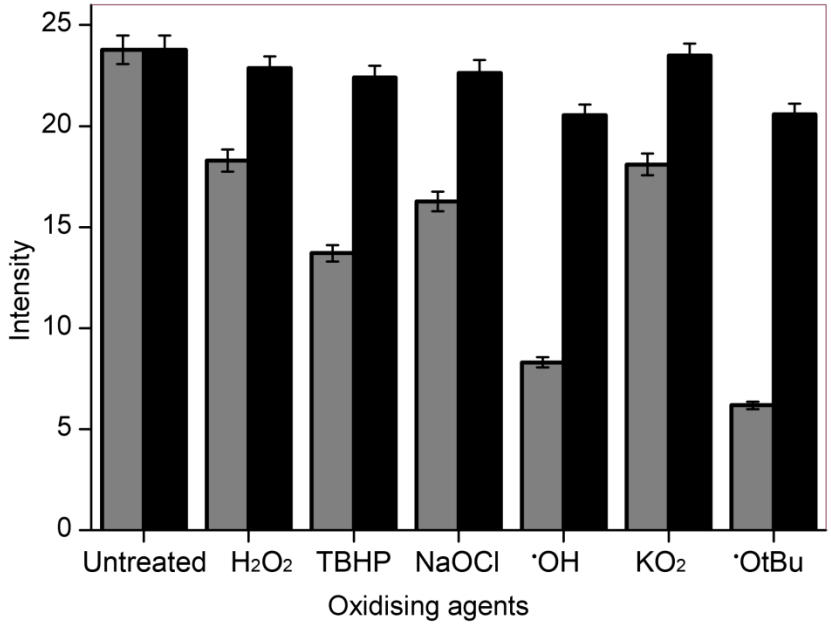


Figure S7: Oxidation of NpFR1 with various oxidising agents. Bars represent the increase in integrated emission intensity (420 to 650 nm, λ_{ex} = 405 nm) upon re-oxidation of reduced NpFR-1 (10 µM in 50 µM sodium dithionite) immediately (grey) and 60 minutes after addition of 100 µM of oxidising agent.

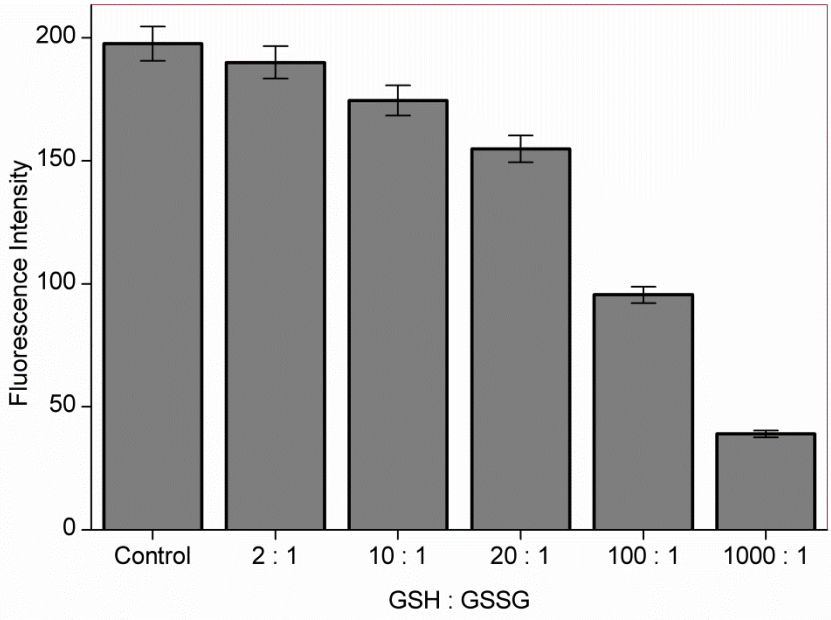


Figure S8: Integrated emission intensity (420 to 650 nm, λ_{ex} = 405 nm) of a 5 µM solution of NpFR1 in the presence of biologically relevant GSH: GSSG ratios (where the total concentration of GSH + GSSG equals 50 µM in each case) in HEPES buffer (100 mM, pH 7.4).

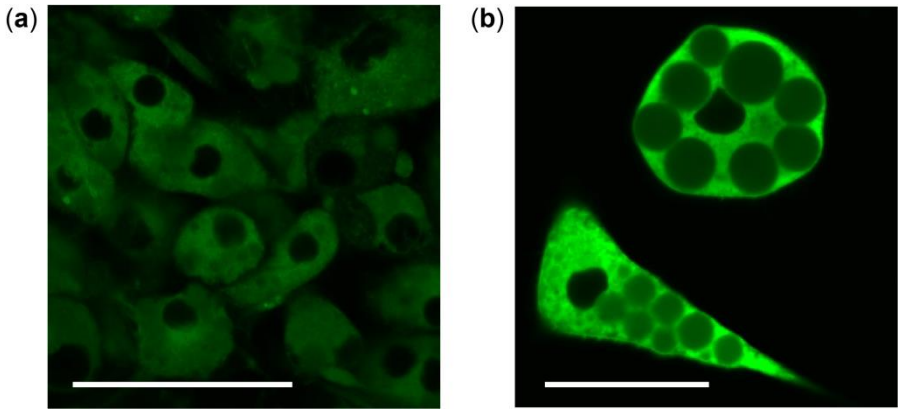


Figure S9: Confocal microscopy images 3T3-L1 adipocytes incubated with NpFR1 (50 µM, 2 hours). (a) Small, recently differentiated adipocytes, showing small lipid droplets and (b) swollen, mature adipocytes with large lipid droplets. Fluorescence intensity increases as adipocytes mature. Scale bars represent 50 µm.