Supplementary Information for:

A FRET-based ratiometric redox probe for detecting oxidative stress by confocal microscopy, FLIM and flow cytometry

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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.

ELECTROCHEMISTRY

Electrochemical measurements were acquired using a PGSTAT12 AUTOLAB (Metrohm Autolab B.V., Netherlands) electrochemical analyser. A conventional three-electrode cell configuration was used consisting of a silver wire quasi reference electrode, a platinum gauze auxiliary electrode and a 3 mm diameter glassy carbon disc working electrode. The working electrode was polished with 0.3 μm and 0.05 μm alumina slurry on a BUEHLER Microcloth®, rinsed with Milli-Q water and sonicated in acetonitrile for 30 s. Following sonication, the electrode was rinsed in acetonitrile, dried with a stream of nitrogen. Solutions were prepared at a concentration of 2 mM in a freshly distilled acetonitrile containing 0.1 M tetrabutylammonium hexafluorophosphate ($TBAPF_6$) as a supporting electrolyte. All the potentials were referenced to the ferrocene/ferrocenium couple (1 mM) measured *in situ* as an internal standard All the electrochemical experiments were performed under a nitrogen atmosphere inside a glove box.

SPECTRO-ELECTROCHEMISTRY

Spectro-electrochemical measurements were made using a SEC-C (path length: 1 mm) thin layer quartz glass spectro-electrochemical cell kit (CH Instruments). This consisted of platinum gauze as the working electrode, platinum wire as the counter and Ag/Ag^+ Non-aqueous reference electrode. Photoluminescence spectra (5 nm bandpass, 1 nm data interval, PMT voltage: 700 V) were collected with a Cary Eclipse Spectrofluorimeter (Varian, Australia). The platinum gauze surface faced the detector and the side of the cell aligned with the excitation beam. A potential of -1.3 V was applied and a series of luminescence spectra were recorded at 12 s intervals using a 405 nm excitation.

SPECTROSCOPY

^1H - 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 spectra 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. Fluorescence spectroscopic measurements were performed in 100 mM HEPES adjusted to pH 7.4. Fluorescence measurements were collected using a bench top Perkin Elmer Enspire Multimode Plate Reader at 10 nm slit widths.

QUANTUM YIELDS

The quantum yield (Φ) of the **FCR1** was calculated using fluorescein as a reference for the oxidized form and quinine sulfate as reference for the reduced form. For calculation of quantum yield, five concentrations of the probe and standards were prepared. Quinine sulfate (literature Φ = 0.54) standards were prepared in 0.1 M H_2SO_4 and the fluorescein standards in 0.1 M NaOH. Their fluorescence spectra were recorded at same excitation of 405 nm. The integrated fluorescence intensities (excited at 405 nm) were plotted against the absorbance (at 405 nm) for both the standards and the reduced and oxidized probe. The quantum yield was calculated using the below equation

$$\Phi_x = \Phi_S \ (D_x / D_S)$$

Where Φ is the quantum yield, D is slope, S and X represents the standard and the sample respectively.

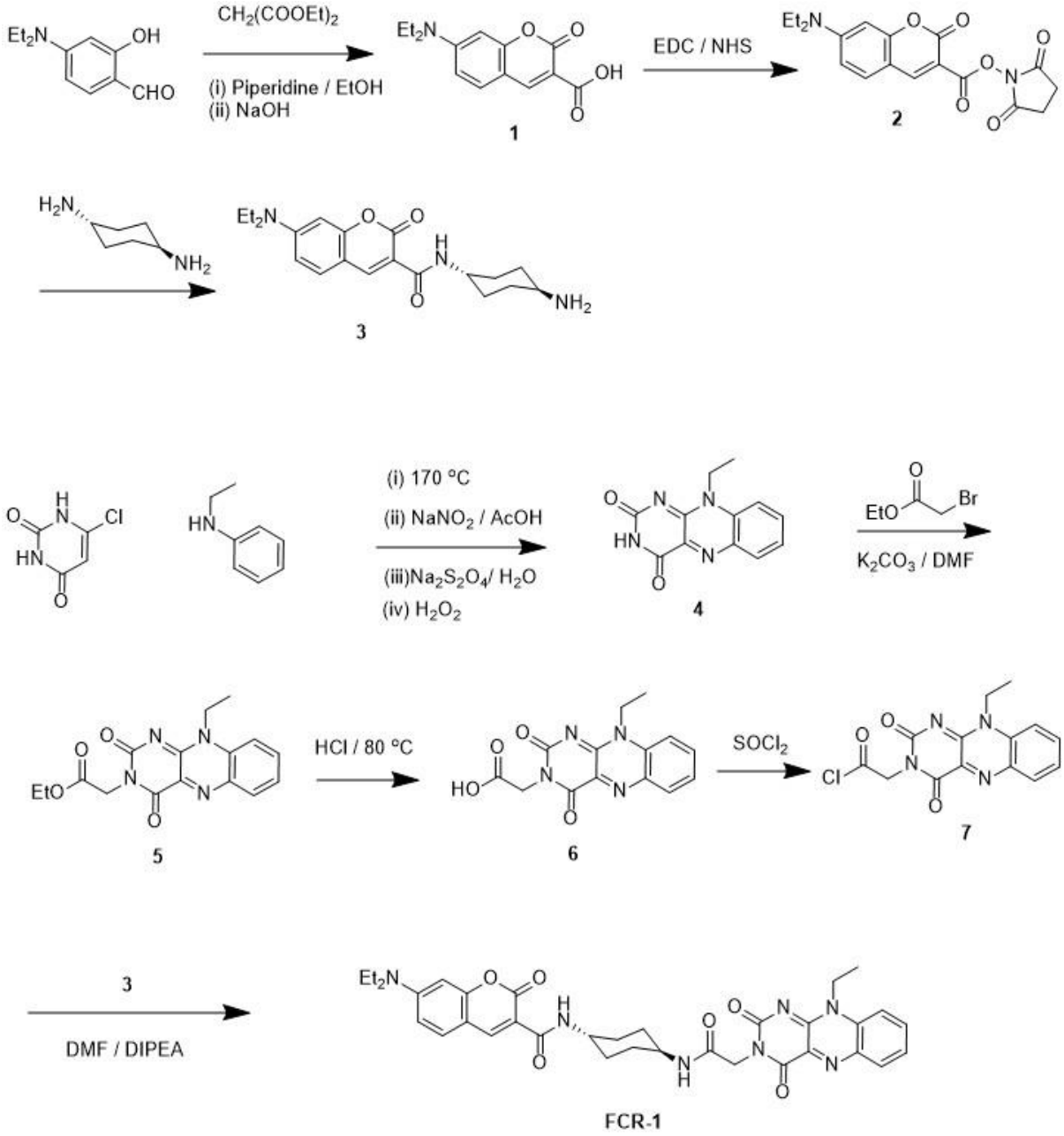
SYNTHESIS OF FCR1

7-(Diethylamino)-2-oxo-2*H*-chromene-3-carboxylic acid (1)

4-Diethylaminosalicylaldehyde (3.86 g, 0.04 mol), diethylmalonate (6.4 g, 0.04 mol) and piperidine (2 mL) were combined in absolute ethanol (60 mL) and stirred for 6 h under reflux conditions. Then 10% NaOH (60 mL) solution was added and the mixture was heated under reflux for 15 min. The reaction mixture was cooled to room temperature and carefully acidified to pH 2 using concentrated hydrochloric acid. The solid obtained was filtered, washed with water, dried and then recrystallized in absolute ethanol to give **1** as bright orange crystals (4.17 g, 80%). ^{1}H -NMR ($CDCl_3$, 400 MHz) δ 12.36 (s, 1H), 8.65 (s, 1H), 7.46 (d, 1H, *J* = 8.8 Hz), 6.73 (d, 1H, *J* = 8.8 Hz), 6.54 (s, 1H), 3.51 (q, 4H, *J* = 6.8 Hz), 1.28 (t, 6H, *J* = 6.8 Hz); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 165.5, 164.4, 158.0, 153.7, 150.2, 131.9, 110.9, 108.5, 105.5, 96.8, 45.3, 12.3.

7-(Diethyl amino)-2-oxo-2*H*-chromene-3-carboxylate succinimidyl ester (2)

To a stirring solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (278 mg, 1.5 mmol), and *N*-hydroxysuccinimide (166 mg, 1.4 mmol) in anhydrous DMF (10 mL), **1** (257 mg, 0.98 mmol) dissolved in anhydrous DMF (5 mL) was added dropwise. The reaction was stirred at room temperature for 48 hours in the dark. The resulting yellow mixture was poured into 150 mL of ice/water slurry. The precipitate which formed was then collected by vacuum filtration, washed with 200 mL water, and dried in air overnight to give **2** as a yellow solid (319 mg, 91%). ^{1}H-NMR (500 MHz, $CDCl_3$): *δ* 8.58 (s, 1H), 7.37 (d, 1H, *J* = 9.7 Hz), 6.64 (d, 1H, *J* = 5.0 Hz), 6.47 (s, 1H), 3.40 (q, 4H, *J* = 5.0 Hz), 2.88 (s, 4H), 1.26 (t, 6H, *J* = 5.0 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): *δ* 169.5, 159.37, 159.1, 157.2, 154.3, 151.3, 132.15, 110.34, 107.88, 102.88, 96.9, 45.5, 25.8, 12.6.



Scheme S1: Synthesis of FCR1

***N*-((*trans*-4-aminocyclohexyl)-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (3)**

A solution of **2** (604 mg, 1.68 mmol) in dry DMF (50 mL) was added dropwise over 24 hours, to a stirring solution of *trans*-1,4-diaminocyclohexane (4 g, 34.0 mmol) in dry DMF (10 mL). The reaction was stirred at room temperature for 24 hours in the dark. The mixture was concentrated under

vacuum and poured into 150 mL of ice-water slurry to precipitate a yellow solid. The solid was then collected by vacuum filtration, washed with 200 mL water, and dried in air to give **3** as a yellow solid (239 mg, 74%). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.62 (1H, s), 7.55 (1H, d, J = 9.0 Hz), 6.82 (1H, dd, J = 9.0, 2.1 Hz), 6.58 (1H, d, J = 2.1), 3.81 (1H, m), 3.52 (4H, q, J = 7.2), 2.69 (1H, m), 1.99 (4H, q, J = 10.1 Hz), 1.37 (4H, m, J = 10.1 Hz), 1.23 (6H, t, J = 6.9 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 162.9, 162.4, 157.7, 152.6, 148.2, 131.2, 110.7, 110.0, 108.6, 96.7, 50.1, 48.1, 45.2, 34.6, 31.7, 12.6.

10-Ethylbenzo[g]pteridine-2,4(3*H*,10*H*)-dione (4)

6-Chlorouracil (0.2 g, 1.3 mmol) and *N*-ethylaniline (0.5 g, 3.9 mmol) were fused at 175 ^{0}C for 20 mins with stirring. The reaction mixture was cooled and crushed in ether. The ether layer was decanted and the solid was vigorously stirred in ether : ethanol (5 : 1) to remove excess aniline, filtered, and dried to obtain 6-(ethyl(phenyl)amino)pyrimidine-2,4(1*H*,3*H*)-dione (0.28 g, 92%) as a colourless solid which was taken forward in synthesis without further characterisation.

6-(Ethyl(phenyl)amino)pyrimidine-2,4(1*H*,3*H*)-dione (0.26 g, 1.1 mmol) was dissolved in 5 ml of acetic acid. Sodium nitrite (0.39 g, 5.5mmol) was added ni one portion and the reaction mixture was stirred at room temperature for 3 h, followed by dilution with 10 mL of water. The precipitate obtained was filtered and washed liberally with ice cold water and dried to give 10-ethyl-2,4-dioxo-2,3,4,10-tetrahydrobenzo[*g*]pteridine-5-oxide as an orange solid (0.25g, 81%) which was taken forward in synthesis without further characterisation.

To a solution of sodium dithionite (0.5 g, 2.91 mmol) in 10 mL water, 10-ethyl-2,4-dioxo-2,3,4,10-tetrahydrobenzo[*g*]pteridine 5-oxide (0.25g, 0.97 mmol) was added and the reaction mixture was allowed to stir at room temperature for 3 h. 2 mL of 30% hydrogen peroxide was added and the reaction mixture was allowed to stand overnight. The precipitate obtained was filtered, washed with ice cold water and dried to give **4** as a yellow solid (0.2 g, 85%) ^{1}H-NMR (300 MHz, DMSO-d_6): δ 11.37 (br. s,1H) 8.15 (d, 1H, J = 9.0 Hz), 7.98-7.95 (m, 2H), 7.66-7.63 (m, 1H), 4.64 (q, 2H, J = 4.2 Hz), 1.33 (t, 3H, J = 4.2 Hz). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 160.4, 156.3, 150.7, 139.3, 135.5, 132.7, 132.4, 132.4 126.5, 116.8, 40.8, 12.4.

Ethyl 2-(10-ethyl-2,4-dioxobenzo[*g*]pteridin-3(2*H*,4*H*,10*H*)-yl)acetate (5)

To a solution of **4** (1.00 g, 4.13 mmol) in 50 mL DMF cesium carbonate (1.60 g, 4.95 mmol) was added and the reaction mixture was stirred under nitrogen at 80^{0}C for 15 min. Ethylbromoacetate (3.45g, 20.65 mmol) was added dropwise and the reaction mixture was allowed to stir at 80^{0}C overnight. After dilution with 100 mL of dichloromethane the reaction mixture was filtered and the solvents were evaporated under reduced pressure. The crude solid was dissolved in 100 mL chloroform and extracted with water (3 x 100 mL) and brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated followed by recrystallization of crude in ethanol to give **5** as

yellow needles (1.05 g, 78%)). ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.21 (d, 1H, *J* = 9.0 Hz), 8.06 - 7.95 (m, 2H), 7.7-7.66 (m, 1H), 4.69 (q, 2H, *J* = 4.2 Hz), 4.64 (s, 2H), 4.14 (q, 2H, *J* = 4.2 Hz), 1.35 (t, 3H, *J* =8.0 Hz), 1.20 (t, 3H, *J* = 8.0 Hz).

2-(10-Ethyl-2,4-dioxobenzo[*g*]pteridin-3(2*H*,4*H*,10*H*)-yl)acetic acid (6)

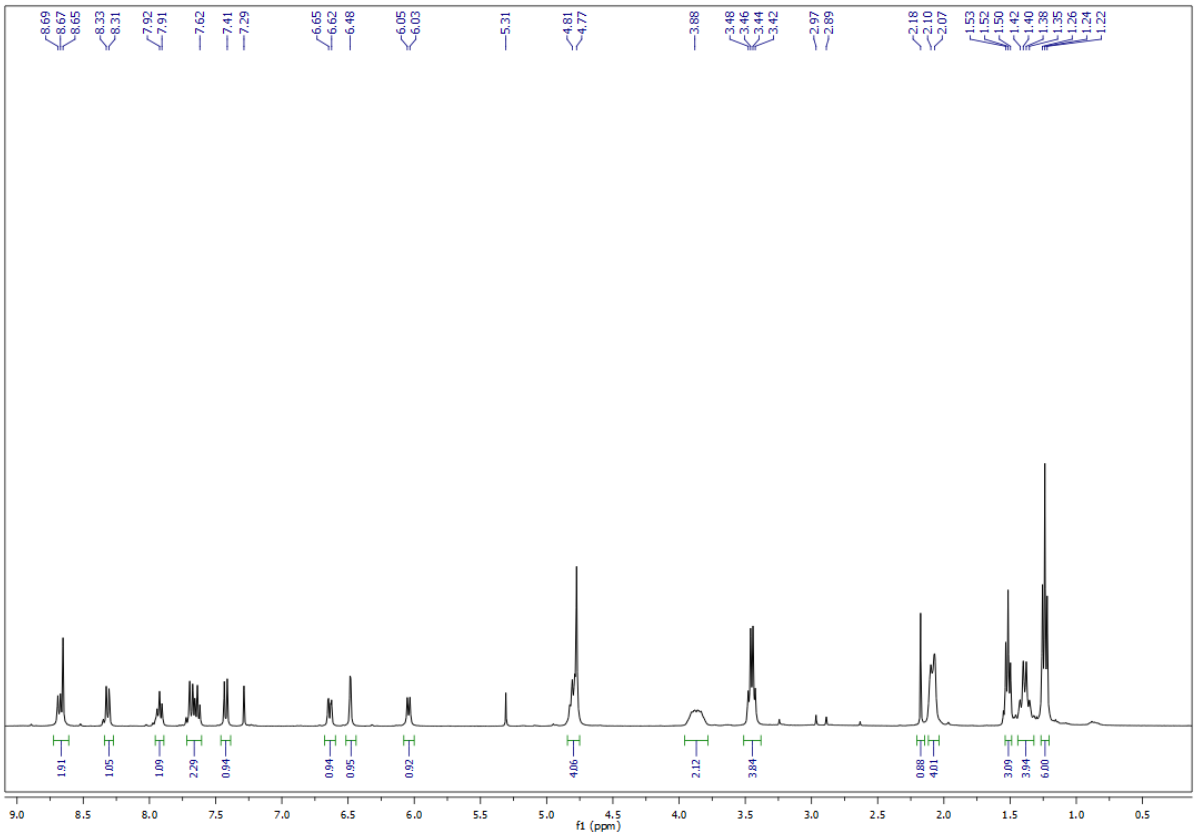
A solution of **5** (1.00 g, 3.04 mmol) in 15 mL of 32% hydrochloric acid was stirred at 85 ^{0}C for 1 h. The reaction mixture was diluted with excess water and cooled in fridge for 2-3 h. The precipitate was filtered, dried and recrystallized in 2 M acetic acid to give **6** as fine yellow needles (0.85 g, 93%) ^{1}H-NMR (500 MHz, DMSO-d_6): δ 8.21 (d, 1H, *J* = 9.0 Hz), 8.06 -7.95 (m, 2H,), 7.7-7.66 (m, 1H,), 4.69 (q, 2H, *J* = 4.2 Hz), 4.64 (s, 2H), 1.35 (t, 3H, *J* = 8.0 Hz).

2-(10-Ethyl-2,4-dioxobenzo[*g*]pteridin-3(2*H*,4*H*,10*H*)-yl)acetyl chloride (7)

6 (0.2 g, 0.66 mmol) was stirred in 1 mL of neat thionyl chloride for 20 min. Excess thionyl chloride was carefully evaporated under reduced pressure. Final traces of thionyl chloride were evaporated by subsequent dissolution of crude in toluene followed by evaporation under reduced pressure. The yellow solid was taken forward in synthesis without further purification.

7-(Diethylamino)-*N*-((1r,4r)-4-(2-(10-ethyl-2,4-dioxo-4,10-dihydrobenzo[g]pteridin-3(2H)-yl)acetamido)cyclohexyl)-2-oxo-2H-chromene-3-carboxamide (FCR1)

To a stirring solution of **7** in 10 mL DMF was added **3** (0.239 g, 0.66 mmol) and DIPEA (0.1 mL, 0.66 mmol). The reaction mixture was stirred for 1 h and concentrated under reduced pressure and triturated into cold ether. The obtained solid was filtered and purified by column chromatography in DCM: MeOH (20:1) to obtain **FCR1** as an orange solid (0.158 g, 38%) APCI-MS: calculated 639.28 for $[M+H]^{+}$ $C_{34}H_{37}N_7O_6$ found 639.00. ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.68 (d, 1H, *J* = 8.0 Hz), 8.65 (s, 1H), 8.32 (d, 1H, *J* = 8.0 Hz), 7.94 -7.91 (m, 1H), 7.70-7.62 (m, 2H), 7.42 (d, 1H, *J* = 8.0 Hz), 6.64 (dd, 1H, *J* = 8.0 Hz), 6.48 (d, 1H, *J* = 4.0 Hz), 6.04 (d, 1H, *J* = 4.0 Hz), 4.79 (m, 4H), 3.80-3.95 (br m, 2H), 3.45 (q, 4H, *J* = 8.0 Hz), 2.08 (m, 4H,), 1.52 (t, 3H, *J* = 8.0 Hz), 1.39 (q, 4H, *J* = 8.0 Hz), 1.24 (t, 6H, *J* = 8.0 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 165.9, 162.6, 162.3, 159.5, 157.6, 155.3, 152.5, 148.7, 148, 137.04, 136.2, 135.8, 133.5, 132.3, 131.1, 126.5, 115.1, 110.3, 109.9, 108.4, 96.6, 48.1, 47.8, 45.0, 44.7, 40.4, 31.51,31.51, 31.45, 31.45, 12.4, 12.3.


8.69
8.67
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8.33
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7.92
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7.62
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6.48
6.05
6.03
5.31
4.81
4.77
3.88
3.48
3.46
3.44
3.42
2.97
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2.18
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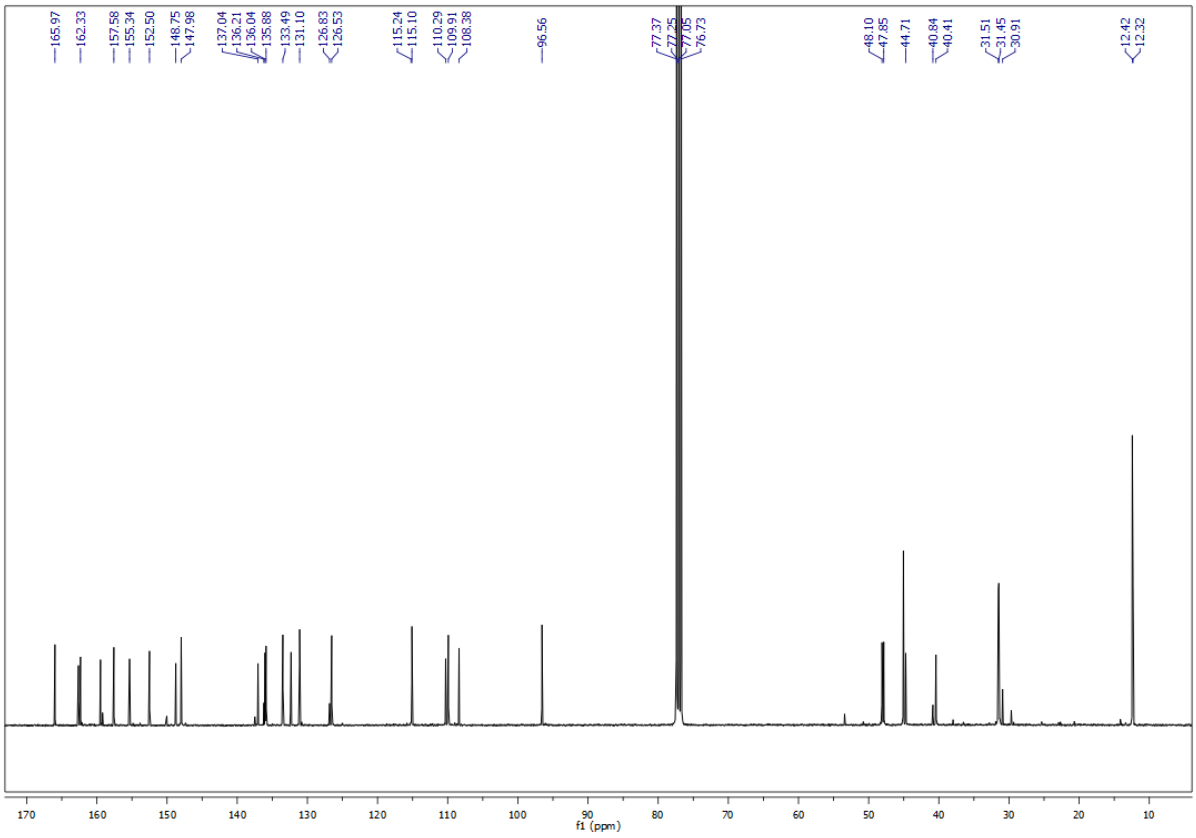
3.84
0.88
4.01
3.09
3.94
6.00
f1 (ppm)


165.97
162.33
157.58
155.34
152.50
148.75
147.98
137.04
136.21
136.04
135.88
133.49
131.10
126.83
126.53
115.24
115.10
110.29
109.91
108.38
96.56
77.37
77.25
77.05
76.73
48.10
47.85
44.71
40.84
40.41
31.51
31.45
30.91
12.42
12.32
f1 (ppm)

CELLULAR EXPERIMENTS

Cell culture

All cellular studies used the HeLa cells. Cells were maintained in exponential growth as monolayers at 37 ºC in 5% CO_2 humidified Dulbecco's modified eagle medium (DMEM) was used supplemented with 2.5 mM glutamine, 10% fetal calf serum and antibiotics as per supplier' s instructions.

Microscopy

Confocal images were acquired using a Leica SP5 II confocal and multi-photon microscope and either a LUCPLFLN 40X air objective lens (NA = 0.60) or a UPLSAPO 63X water-immersion objective lens (NA = 1.20). Excitation light of 820 nm was provided by the Mai Tai DeepSee™ Ti:Sapphire femtosecond pulsed laser. Cells were imaged in a temperature controlled incubator at 37 ºC and CO_2 incubator. Images were collected and processed using Leica Application Suite Advanced Fluorescence Version: 2.8.0 build 7266 viewer software. Image analysis of mean fluorescence intensity was performed using Fiji-ImageJ.

Imaging experiments

HeLa cells were grown as described above. 50,000 cells were seeded and left to attach overnight in glass bottom plates (MatTek Corporation)

For measurement of the oxidative capacity of cells in different environments, cells were first treated by the addition of 50 µL of the stock solution of reducing agent (*N* –acetyl Cysteine) or oxidizing agent (H_2O_2) in phosphate-buffered saline (PBS) to a final concentration of 50 µM in complete media and incubated for 30 minutes before being washed with phosphate-buffered saline (PBS). Control cells were treated with 50 µL of phosphate-buffered saline (PBS) to complete media and incubated for 30 min. The cells were then treated with a 50 mM stock solution of the probe in DMSO to a final concentration of 10 µM probe in the media (final concentration of DMSO in cell media was less than 0.1%) and incubated for 15 minutes 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.

Fluorescence lifetime experiments

Fluorescence lifetime images were collected on a Leica TCS SP5 MP FLIM system containing a tunable Mai Tai Deep See multi-photon laser with a repetition rate of 80 MHz (Spectra-Physics) connected to a Leica DMI6000B-CS inverted microscope. Samples were illuminated with 820 nm laser and emitted light was collected in the de-scanned internal FLIM detectors over the 420-480 nm and 520 - 600 range using a HC PL APO 63x/1.3 (water) objective lens. The data was collected with the aid of the B&H SPCM software and the fluorescence lifetimes were determined using time correlated single photon counting (TCSPC) and analyzed with SPC Image software (version 3.1.0.0). The instrument response function was derived from the decay curve of urea. 512 x 512 pixel images were collected and 3X binning applied for analysis to ensure that at least 10000 photons per pixel were analyzed. Each sample was scanned for 120 s. Analysis of fluorescence lifetimes in cells required individually fitted mono or bi-exponential curves to obtain average χ^2 values closest to 1. The data ware analyzed from 10 different regions of interest from 3 independent experiments. The mean life times were obtained by calculating a decay matrix of each pixel on the 512 x 512 image. The obtained images were false colored using a BGR LUT ranging between 900 ps to 2700 ps.

Flow cytometry

Flow cytometric analyses were performed using BD biosciences LSRFortessa equipped with a 56 mW 405 nm coherent laser. Cells were cultured as detailed above and treated with trypsin for 3 mins to cause detachment from the flask followed by dilution with advanced DMEM and centrifugation. The cells were resuspended and diluted to get 100 000 cells / mL. The cell suspension is then dosed with appropriate treatments followed by the addition of 10 µM of FCR1. Emission intensities were acquired by detectors centered around 450 nm (425 - 475 nm) and 560 nm (550 – 570 nm). Approximately 50, 000 events were collected for each run with appropriate gating applied to isolate healthy and single cells. Data obtained were analyzed using FlowJo software (Tree Star).

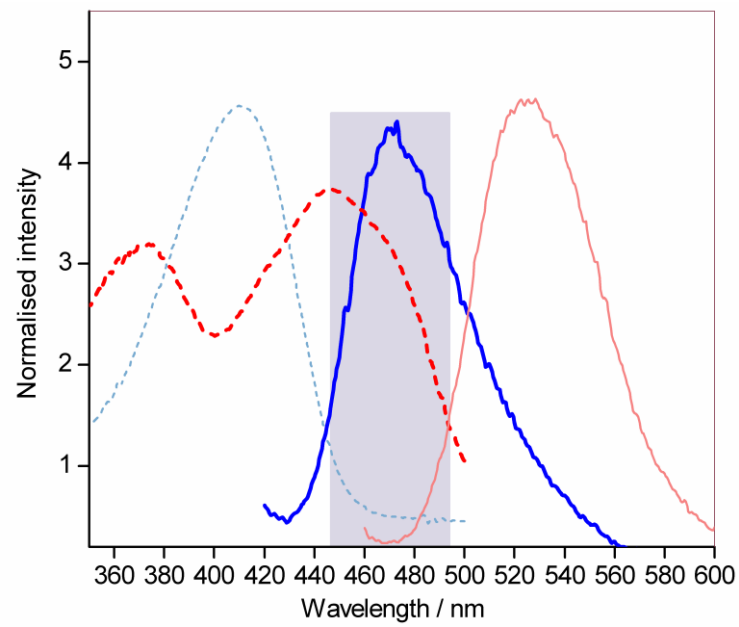


Figure S1: Absorbance and emission spectra of 7-diethylamino coumarin(blue, 10 µM) and *N*-ethyl flavin (red, 10 µM) in (100 mM HEPES buffer, pH 7.4) indicating a significant overlap (grey rectangle) of the emission profile of the coumarin moiety with the absorbance of the flavin.

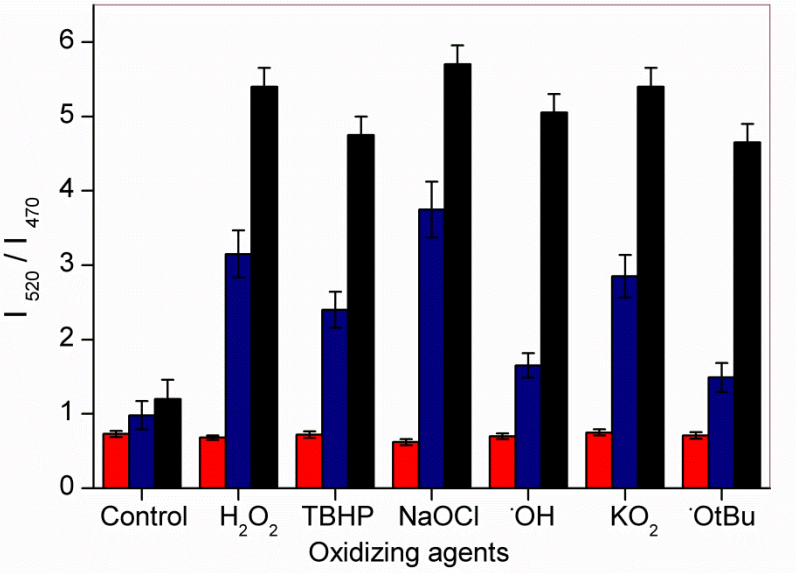


Figure S2: Oxidation of FCR1 with various oxidizing agents. Bars represent the ratio of green to blue fluorescence intensity (520 nm / 475 nm, λ_{ex} = 405 nm) after reduction (red) of FCR1 (10 µM in 20 µM sodium cyanoborohydride) followed by re-oxidation of reduced FCR1 30 mins (blue) and 60 minutes after (black) the addition of 100 µM oxidizing agent.

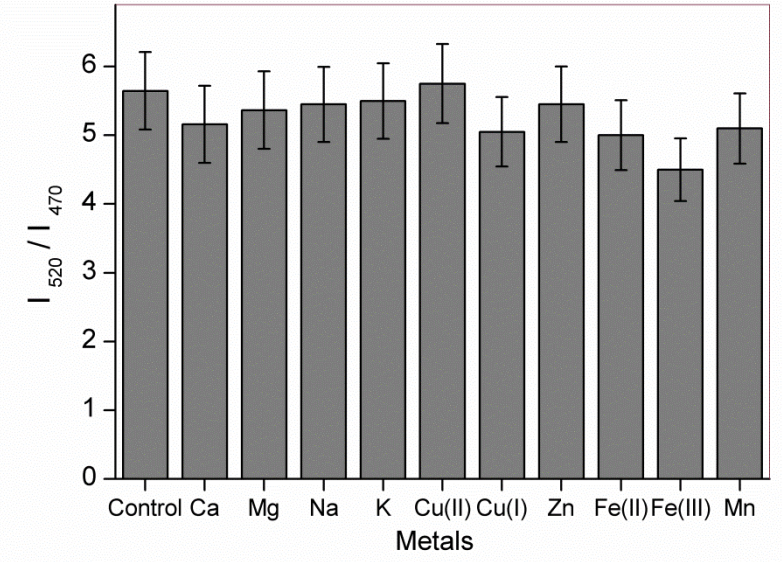


Figure S3: The fluorescence emission from FCR1 (10 μM) in the presence of common metal ions (100 μM). Bars represent the ratio of the fluorescence intensity (520 nm / 475 nm, λ_{ex} = 405 nm) as the mean of three replicates. Error bars represent standard deviation.

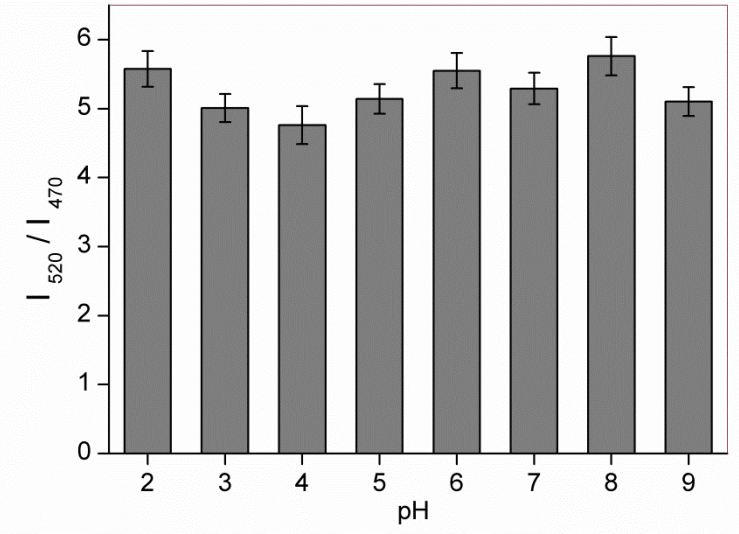


Figure S4: The ratio of fluorescence emission from FCR1 (10 μM) over a range of pH values. Bars represent the ratio of the fluorescence intensity (520 nm / 475 nm, λ_{ex} = 405 nm) as the mean of three replicates. Error bars represent standard deviation.

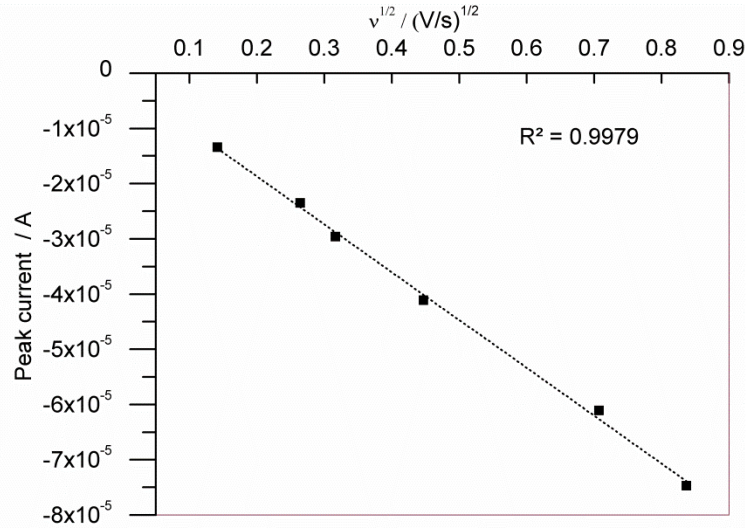


Figure S5: Plot of peak current versus square root of scan rate for the voltammetric reduction of FCR1 (2 mM) in acetonitrile.

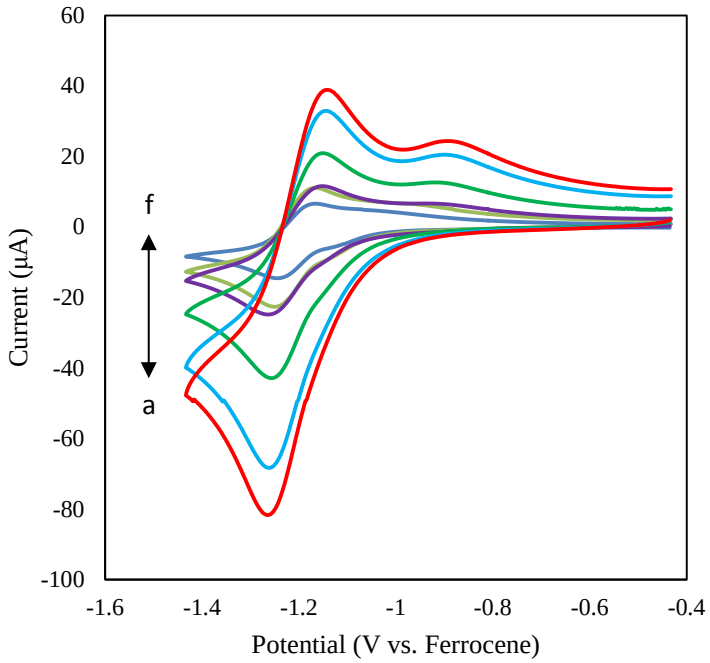


Figure S6: Cyclic voltammograms for FCR1 (2 mM) at various scan rates in acetonitrile containing 0.1 M $TBAPF_6$ as supporting electrolyte. The working electrode was a 3 mm diameter glassy carbon electrode and the scan rates were: (a) 0.02, (b) 0.07, (c) 0.1, (d) 0.2, (e) 0.5 and (f) 0.7 Vs^{-1}.

Table S1: Signal to noise ratios of a range of two photon excitation wavelengths optimized to a laser power of 52.8 mW. Signal to noise ratios are an average of 3 images acquisitions.

Wavelength / nm	Trans / %	Gain / %	Offset / %	S/N ratio
700	64	73	42	240.3
720	35	73	45	251.9
740	22	73	47	243.7
760	22	73	50	249.8
780	27	73	52	261.5
800	33	73	54	337.4
820	43	73	57	359.8
840	50	73	60	317.6
860	50	73	63	210.2
880	56	73	67	168.3
900	49	73	69	171.2
920	68	78	70	149.5
940	79	78	72	104.6
960	88	80	76	74.9
980	97	85	71	98.4

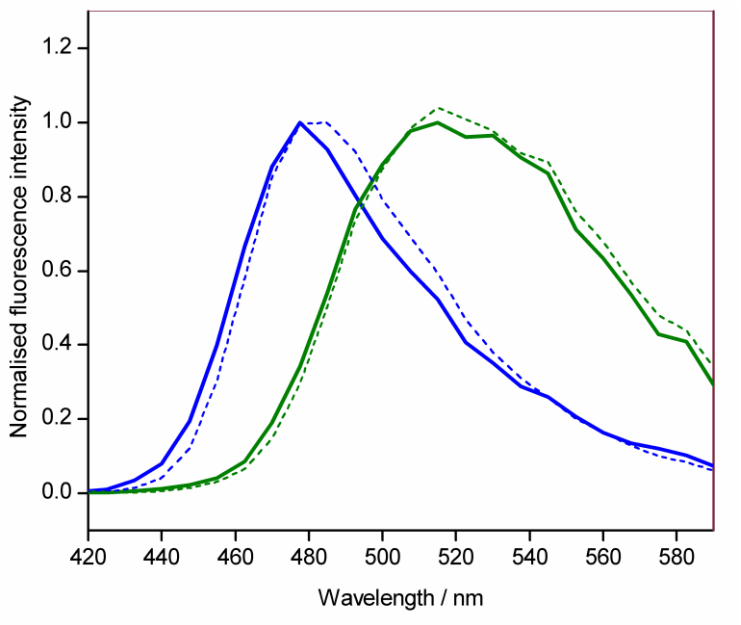


Figure S7: Fluorescent spectra of FCR1 (2 µM) oxidised (green) and reduced form (blue) obtained following excitation at 405 nm (dashed) and 820 nm (Solid). Reduction was achieved by the addition of $NaCNBH_3$ (100 µM).

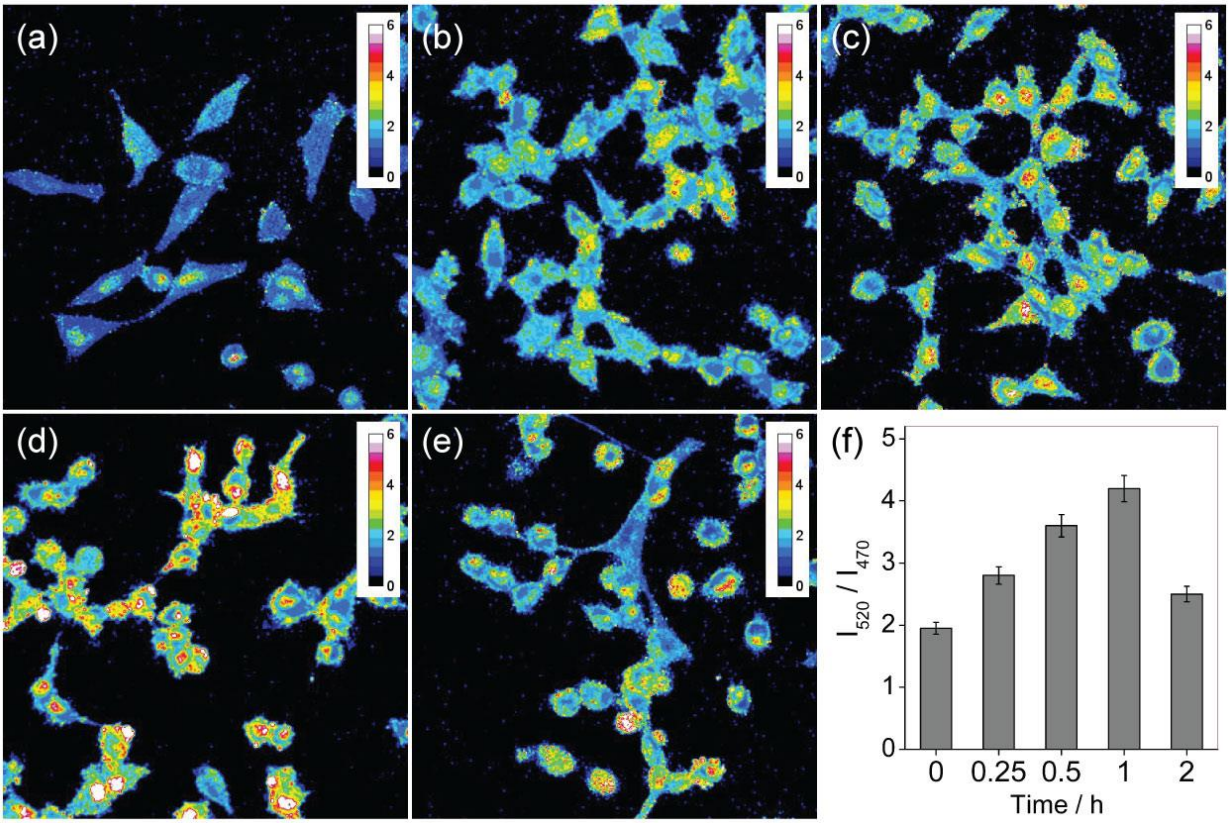


Figure S8: Pseudocoloured ratio images (green/blue) of HeLa cells treated with H_2O_2 (50 µM) for (a) 0 h, (b) 0.25 h, (c) 0.5h, (d) 1 h and (e) 2h, followed by treatment with FCR1 (10 µM) for 15 mins. Average green/blue ratio of individual cells after different durations of H_2O_2 treatmeant (f) are also indicated. Error bars indicate standard deviation.

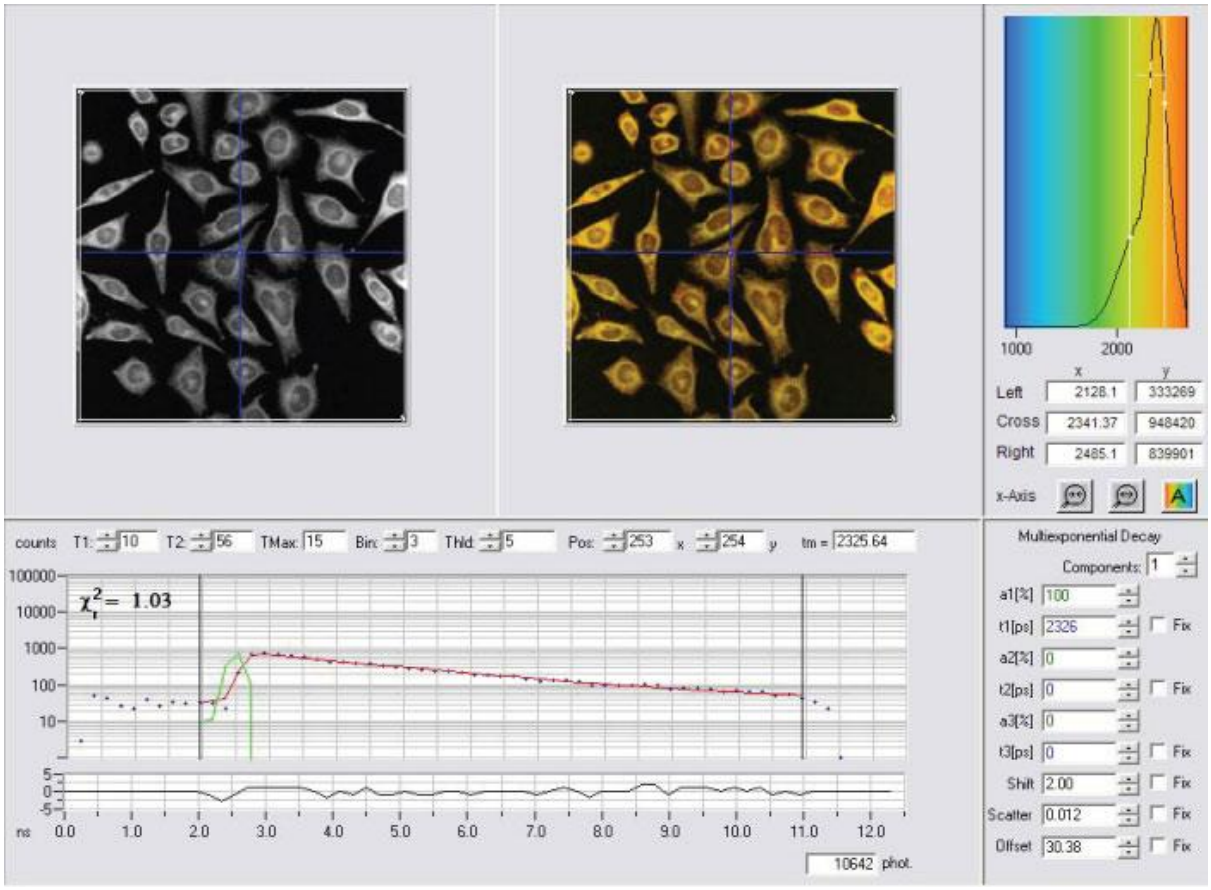


Figure S9(a): Analysis of donor fluorescence lifetimes (420 – 475 nm) of HeLa cells treated with donor only (10 μM, 15 min, λ_{ex} = 820 nm) indicating a single component fit with a lifetime of 2.3 ns. Pixel by pixel lifetime decay map has been color coded from 0.9 ns to 2.7 ns.

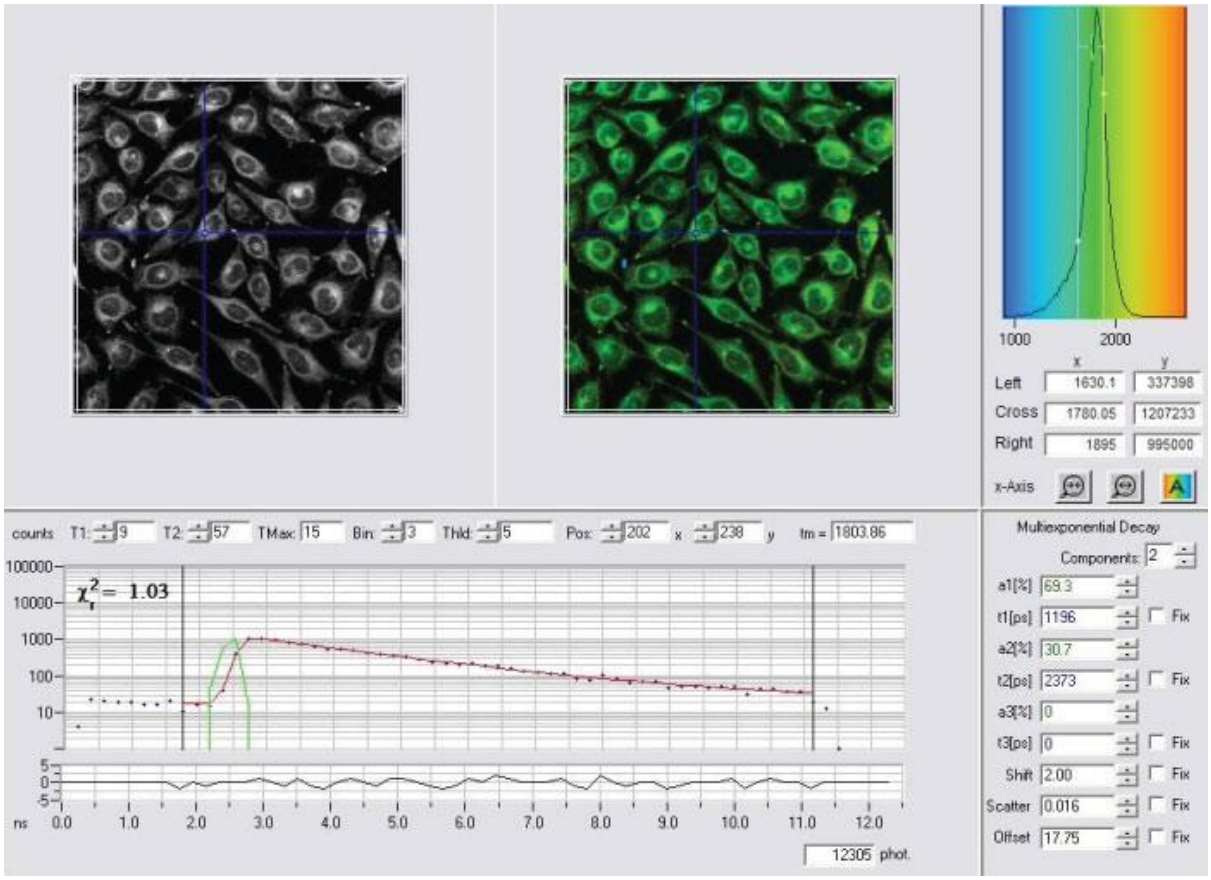


Figure S9(b): Analysis of donor fluorescence lifetimes (420 – 475 nm) of HeLa cells treated with FCR1 (10 μM, 15 min, λ_{ex} = 820 nm) indicating a two component fit with a lifetimes of 1.1 ns (69%) and 2.3 ns (31%). Pixel by pixel lifetime decay map has been color coded from 0.9 ns to 2.7 ns.

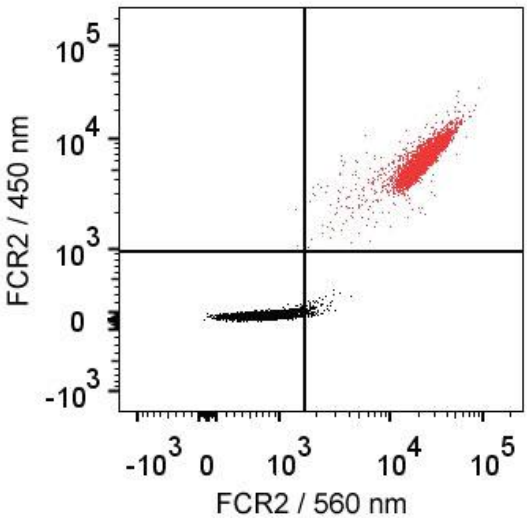


Figure S10: Flow cytometric analysis of HeLa cells treated with vehicle control (black) and FCR1, 10 μM, 15 min, λ_{ex} = 405 nm (red). Dot plots clearly show that cells treated with FCR1 have higher emission intensities in green (560 nm) and blue (450 nm) channels from than those treated with vehicle control.