SUPPORTING INFORMATION

A highly sensitive fluorometric assay for the determination of glutathione reductase activity

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1 Supporting Experimental Data

1.1 Materials

Di-*tert*-butyldicarbonate and 3,3'-dithiodipropionic acid were obtained from Fluka (Switzerland). Acetonitrile, ammonium chloride and hydrochloric acid were obtained from BDH/Merck (Germany). DMSO was obtained from Riedel-de-Haën (Germany). Bulk solvents were obtained from Fronine (Australia). NHS-fluorescein was obtained from Pierce (USA). All other reagents were obtained from Aldrich (USA). Ethanol, ethyl acetate and methanol were distilled from

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anhydrous potassium carbonate. Acetonitrile and dichloromethane were distilled under nitrogen from calcium hydride. Chloroform was distilled under nitrogen from phosphorus pentoxide and stored in a dark bottle over silver wire. DMSO was dried over microwave-activated 4 Å molecular sieves. All other solvents and reagents were used without further purification.

1.2 Equipment

NMR spectra were recorded in 5 mm Pyrex tubes (Wilmad, USA) on a DPX-400 400 MHz spectrometer (Bruker, Germany). IR spectra were recorded on a Paragon PE1000 FTIR spectrometer (Perkin Elmer, USA). UV-Vis spectra were recorded on a Cary 1-Bio spectrophotometer (Varian, USA) in 1 cm matched quartz cuvettes (Selbys Scientific, Australia). Melting points were determined using a digital melting point apparatus (Gallenkamp, UK) in open glass capillaries, and are uncorrected. High resolution ESI mass spectrometry was performed on an APEXII Fourier transform ion cyclotron resonance spectrometer (Bruker, Germany). HPLC was performed using a 600E multisolvent delivery system and a 490 programmable multiwavelength detector (Waters, USA), with a Gemini 250 × 10 mm (semi-preparative) C18 HPLC column (Phenomenex, Australia). Small-scale distillation was performed by Kugelrohr (Büchi, Switzerland). Water was purified using a Milli-Q Ultrapure Water Purification System (Millipore, USA).

1.3 Synthesis

1.3.1 *tert*-Butyl-2-aminoethylcarbamate (Boc-en-NH_2)

A solution of di-*tert*-butyldicarbonate (9.1 g, 42 mmol) in DCM (50 mL) was added dropwise, with vigorous stirring, to a solution of ethylenediamine (2.5 g, 42 mmol) in DCM (50 mL), during which time a white precipitate formed. The precipitate was collected at the pump and washed with ice-cold DCM (25 mL), yielding crude Boc-en-Boc as a white powder, which was not further purified (3.5 g, 64%). m.p. 137–138 °C (Lit.[1] 139–140 °C). ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C) δ 6.69 (bs, 2H, 2 × NHCO), 2.90 (s, 4H, 2 × CH_2), 1.34 (s, 18H, 6 × CH_3).

A solution of the crude Boc-en-Boc (3.0 g, 12 mmol) and TFA (1.0 mL, 13 mmol) in DCM (50 mL) was refluxed gently for 18 h. The solution was then extracted with aqueous ammonium chloride (1 M; pH 5; 3 × 25 mL) and the combined aqueous layers adjusted to pH 13 (NaOH) and extracted with DCM (3 × 25 mL). The combined organic layers were dried over anhydrous magnesium

sulfate and reduced to dryness *in vacuo* to give a cloudy yellow oil. The oil was purified by Kügelrohr distillation (95–100 °C; 0.1 mmHg) yielding Boc-en-NH_2 as a colourless, viscous oil (1.6 g, 80%). ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C) δ 5.06 (bs, 1H, NHCO), 3.10 (q, 2H, C$\underline{H_2}$NHCO), 2.73 (t, 2H, C$\underline{H_2}NH_2$), 1.39 (s, 9H, 3 × CH_3), 1.22 (bs, 2H, NH_2).

1.3.2 3,3'-Dithiodipropionic acid 2-*tert*-butoxycarbonylaminoethylamide (1)

A solution of 3,3'-dithiodipropionic acid (100 mg, 0.476 mmol), *tert*-butyl-2-aminoethylcarbamate (152 mg, 0.951 mmol), EDC (182 mg, 0.951 mmol) and TEA (100 mg, 0.990 mmol) in chloroform (50 mL) was stirred at room temperature for 18 h. The reaction mixture was then washed with hydrochloric acid (0.05 M; 2 × 50 mL) and water (1 × 50 mL), dried over anhydrous magnesium sulfate and reduced to dryness *in vacuo*, yielding Boc-en-S-S-en-Boc **1** as a white powder (200 mg, 85%). m.p. 139–140 °C. ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C) δ 6.77 (bs, 2H, 2 × NH), 5.19 (bs, 2H, 2 × NH), 3.38 (q, 4H, 2 × CH_2N, J = 5.0 Hz), 3.28 (q, 4H, 2 × CH_2N, J = 5.0 Hz), 2.99 (t, 4H, 2 × CH_2S, J = 7.0 Hz), 2.58 (t, 4H, 2 × CH_2CO, J = 7.0 Hz), 1.44 (s, 18H, 6 × CH_3). ^{13}C-NMR (100 MHz, $CDCl_3$, 25 °C) δ 171.7 (C=O), 40.6 (CH_2), 40.2 (CH_2), 35.9 (CH_2), 34.6 (CH_2), 28.4 (CH_3). Mass spectrum (HRESI+) *m/z*: 517.2119 ([M+Na]$^+$), calcd. for $C_{20}H_{38}N_4O_6S_2Na$ 517.2130. λ_{max} (MeCN) 203, 251 nm, ε 15700, 430 L.mol^{-1}.cm^{-1}. ν_{max} (KBr disc) 3362(s), 3312(s), 3084(w), 2978(m), 2940(m), 1688(s), 1648(s), 1536(s), 1447(m), 1366(m), 1282(s), 1254(s), 1174(s), 877(m) cm^{-1}.

1.3.3 3,3'-Dithiodipropionic acid (5/6-fluoresceinylcarbonylaminoethyl)-amide (2)

A solution of **1** (10 mg, 20 μmol) and TFA (1 mL) in anhydrous chloroform (5 mL) was stirred at room temperature for 30 min and then reduced to dryness under a stream of nitrogen, yielding the di-TFA salt of H_2N-en-S-S-en-NH_2 as a colorless oil (10 mg, 100%), which was used immediately and not characterized further.

A solution of NHS-fluorescein (19 mg, 40 μmol; mixture of 5- and 6- isomers) and TEA (10 mg, 100 μmol) in ethanol (10 mL) was added to the oily residue and the solution was stirred at room temperature for 18 h. The reaction mixture was then reduced to dryness under a stream of nitrogen and the resulting sticky orange residue was triturated with ethyl acetate (5 × 1 mL), yielding crude **2** as a pale orange powder (16 mg, 82%). A small quantity (2 mg) of the crude product was further

purified by HPLC (Semi-prep C18 column, 3 mL/min, gradient from 10–100% acetonitrile: water+0.1% TFA over 40 min), with a large UV-active peak (254 nm) eluting after 29 min. This peak was collected and reduced to dryness under a stream of nitrogen, yielding pure FSSF **2** as a mixture of isomers. ^{1}H-NMR (400 MHz, DMSO-d_6, 25 °C) δ 8.84 (bs, NH), 8.71 (bs, OH), 8.44 (m, Ar-H), 8.21 (m, Ar-H), 8.13 (m, Ar-H), 8.05 (m, Ar-H), 7.63 (s, Ar-H), 7.35 (d, Ar-H), 6.66 (m, Ar-H), 6.56 (m, Ar-H), 6.54 (m, Ar-H), 3.35 (m, CH_2N), 3.26 (m, CH_2N), 3.22 (m, CH_2N), 3.15 (m, CH_2N), 2.84 (m, CH_2S), 2.43 (m, $CH_2C(O)$). ^{13}C-NMR (100 MHz, DMSO-d_6, 25 °C) δ 134.3 (Ar), 129.1 (Ar), 124.9 (Ar), 124.3 (Ar), 123.4 (Ar), 122.3 (Ar), 112.8 (Ar), 102.1 (Ar), 39.0 (CH_2N), 38.0 (CH_2N), 34.8 ($\underline{C}H_2C(O)$), 33.4 (CH_2S). Mass spectrum (HRESI+) *m/z*: 1033.2021 ($[M+Na]^+$), calcd. for $C_{52}H_{42}N_4O_{14}S_2Na$ 1033.2037. λ_{max} (MeOH) 240, 474, 500 nm, ε 77000, 51000, 120000 $L.mol^{-1}.cm^{-1}$. ν_{max} (KBr disc) 3410(br), 1740(m), 1646(s), 1608(s), 1548(s), 1504(m), 1461(s), 1388(m), 1322(m), 1249(m), 1180(m), 1113(m) cm^{-1}.

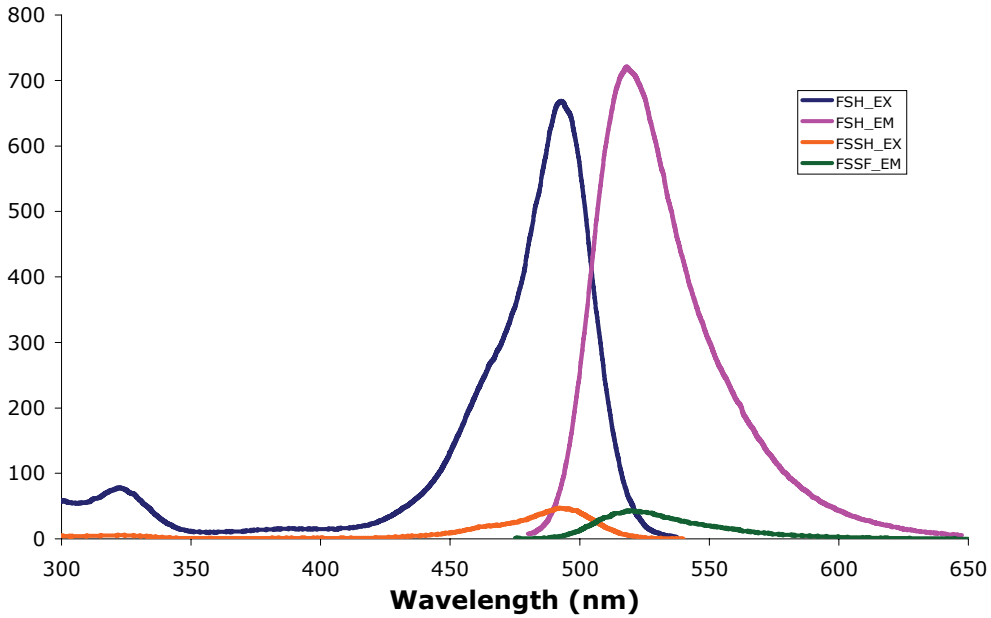

Figure S-0: Fluorescence excitation (orange) and emission (green) spectra for 100 ng/mL of FSSF (**2**) in phosphate buffered saline (pH 7.5). After addition of 10 μg/mL DTT, the excitation (blue) and emission (magenta) spectra for the same sample measured again.

The FRET reduction in intensity for FSSF was measured to be ~94% over two molecules of FSH. This figure correlates well with the theoretical efficiency of energy transfer given by equations (1) and (2). The rate of energy transfer (k_T) over a distance (r), is dependent on the fluorescence lifetime (τ_D) and the Förster radius (R_0). The efficiency of energy transfer, expressed as a fraction of full fluorescence is then given by (2).

$$k_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \quad \text{..... (1)}$$

$$E_T = \frac{k_T(r)}{\gamma + k_T(r)} \text{ where } \gamma = 1/\tau_D \quad \text{..... (2)}$$

For fluorescein/fluorescein donor/acceptor pairs, the Förster radius is 44 Å (http://probes.invitrogen.com/handbook/boxes/0422.html) and the fluorescence lifetime is ~ 4 ns (http://probes.invitrogen.com/handbook/boxes/1572.html). Thus for a separation of 30 Å (Figure S-1) a rate of transfer of 2.5 ns^{-1} is calculated. The efficiency of energy transfer is thus 0.91, i.e. 9% of a single fluorescein molecule, which is in good agreement with the observed RET of 0.94. The observed quenching corresponds to an average distance of 24 Å, which is completely reasonable considering that the linker is flexible but the amides and disulfide are likely to spend most of their time in a *trans*-orientation.

1.4 Molecular Modeling

A geometry optimized molecular model of FSSF (Figure S-1) was created using the Chem3D software package (Cambridgesoft, USA) running under Microsoft Windows XP. Energy minimization was performed using molecular mechanics (MM2 force field) with a conjugate gradient convergence method (minimum RMS gradient = 0.100).

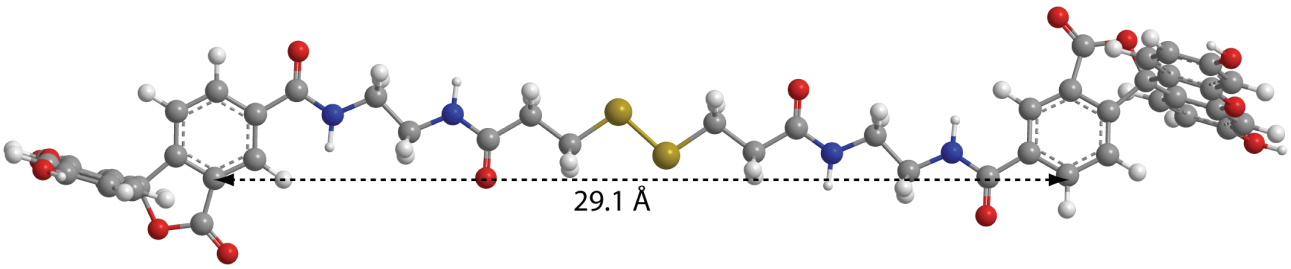

Figure S-1 – Geometry optimized (MM2) model of FSSF (**2**) showing that the maximum distance between the two fluorophores is less than 30 Å when the disulfide-containing linker is fully extended.

1.5 Non-Linear Fitting of Progress Curves

All non-linear least squares regressions in this study were performed using the Dynafit[2] program (Biokin). Dynafit characterizes reacting system in terms of symbolic, or stoichiometric, equations. The names of chemical species are entirely arbitrary and can be freely chosen by the investigator. Our general approach was first to fit the data to the simplest model possible and then to introduce more complex terms, using the previously determined constants as starting points, to try and improve the fit. This led ultimately to the description of the FRET based GR assay as a five-component system.

All input data for DynaFit are simple text files in the ASCII format arranged in columns (independent variable vs. dependent variable). Script files contain the description of the reaction mechanism, initial estimates of fitting parameters, and the location of experimental data files on the disk. Parameters followed by '??' are allowed to vary and are optimized by the least squares regression algorithm, while all other parameters are treated as constants. The "delay" parameter for each reaction refers to the difference (in seconds) between t = 0 and the time when the reagents were mixed together to start the reaction. The "ConcError" parameter allows the concentrations of reagents to vary by a certain percentage to account for pipetting errors, which are unavoidable with small volumes, thereby improving the overall fit of the model.

The Dynafit "response factor" for a particular reagent refers to the change in signal (fluorescence at 520 nm in this case) observed when 1 μM of that reagent is formed. For our analyses, we used two separate response factors – one for FSSF and one for FSH. The response factor for FSSF was calculated by measuring the fluorescence of a 1 μM solution of FSSF at 520 nm, while the response factor for FSH was determined by fitting the progress curve obtained from the cleavage of a 1 μM solution of FSSF with 10 mM DTT to an exponential equation and extrapolating out to infinity (Figure S-2). The response factors obtained vary slightly between experiments as different receiver gain settings were used on the fluorimeter on different days.

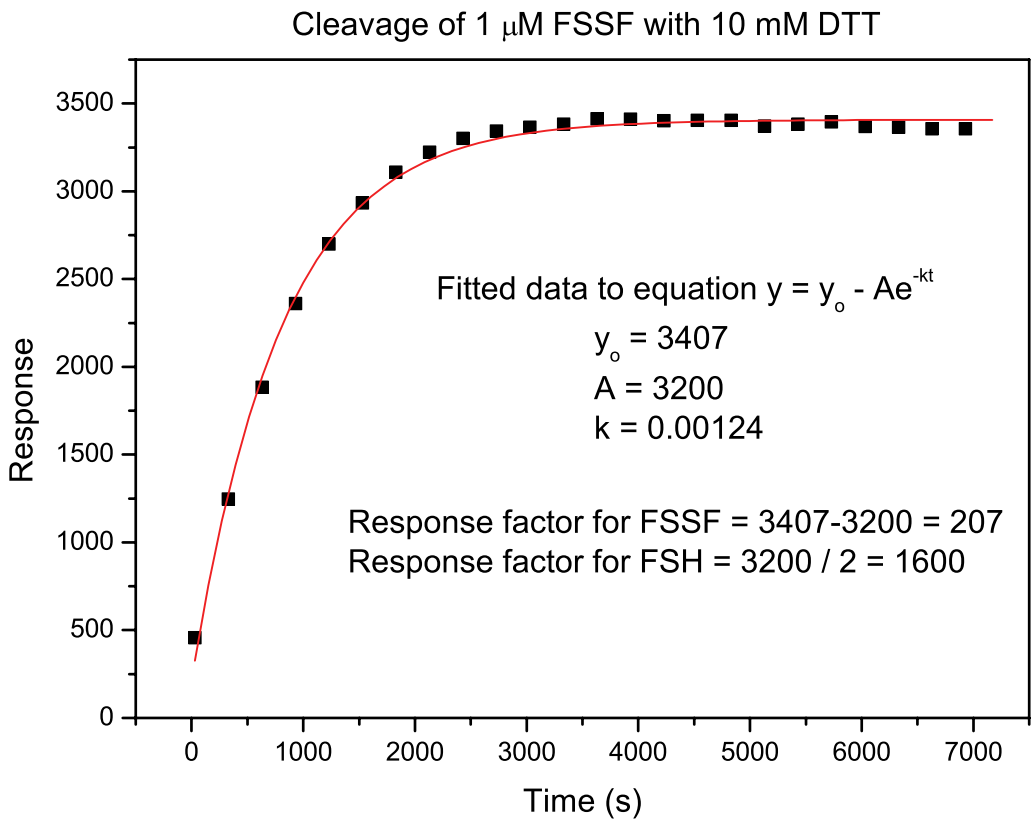

Figure S-2. Calculation of Dynafit response factors for FSSF and FSH.

1.5.1 Photobleaching of FSSF

Photobleaching occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage. In this study, both the probe (FSSF) and product (FSH) were susceptible to photobleaching and hence the rates of photobleaching of both these species had to be determined and accounted for in subsequent Dynafit models. This can be observed in Figure S-2 as a slight drop in fluorescence after 4000 seconds. To calculate the rate of photobleaching of FSSF, solutions of FSSF (5, 10 and 20 μM) in 1 mM GSSG, 100 μM NADPH, 100 mM K_3PO_4, 1 mM EDTA, pH 7.5 were prepared and the fluorescence intensity of each solution was measured at 10 min intervals for a total of 18 h (the same as for the GR experiments). The data obtained were then fitted to a first order reaction model using non-linear least squares regression with Dynafit and a rate constant (k_{pb2}) for the photobleaching of FSSF was calculated. The rate constant for photobleaching of FSH (k_{pb1}) was determined separately by cleaving FSSF with DTT and will be discussed on subsequent pages.

1.5.1.1 Dynafit Script for Non-Linear Least Squares Regression Analysis of FSSF Photobleaching

```
[task]
        data = progress
        task = fit

[mechanism]
        FSSF   --->     photobleached-FSSF   : kpb2

[constants]
        kpb2 = 2e-006 ??                        ;       Initial estimate

[responses]
        FSSF = 207 ?

[progress]
        directory ./glutathione/GR/data
        extension txt

        file 25uMFlu01UGR0uL        | conc. FSSF = 25 ?    | delay = 90
        file 25uMFlu001UGR0uL       | conc. FSSF = 25 ?    | delay = 420
        file 10uMFlu01UGR0uL        | conc. FSSF = 10 ?    | delay = 90
        file 10uMFlu001UGR0uL       | conc. FSSF = 10 ?    | delay = 420
        file 5uMFlu01UGR0uL         | conc. FSSF = 5 ?     | delay = 90
        file 5uMFlu001UGR0uL        | conc. FSSF = 5 ?     | delay = 420

[output]
        directory ./glutathione/GR/kpb2/output

[settings]
        <Marquardt>
        Interrupt = 1000

        <Constraints>
        ConcError = 0.15        ;Concentrations allowed to vary up to 15% to account for pipetting errors

[end]
```

Table S-1. Fitted parameters from Dynafit analysis of FSSF photobleaching

Set	*Parameter*	*Initial*	*Fit*	*Error*	*%*
	k_{pb2}	2e-006	1.9705e-006	4.06e-008	2.1
	r_{FSSF}	207	178.424	0.153	0.1
1	[FSSF]	25	21.3756	0.02325	0.1
2	[FSSF]	25	23.8448	0.03152	0.1
3	[FSSF]	10	9.90829	0.01848	0.2
4	[FSSF]	10	11.048	0.0235	0.2
5	[FSSF]	5	4.79654	0.01788	0.4
6	[FSSF]	5	5.43827	0.01552	0.3

Table S-2. 99% confidence intervals for fitted parameters from Dynafit analysis of FSSF photobleaching

k_{pb2}	*lower*	*upper*
standard error	1.9299e-006	2.0111e-006
linear approximation	1.8656e-006	2.0753e-006
exact intervals	1.9407e-006	2.0003e-006

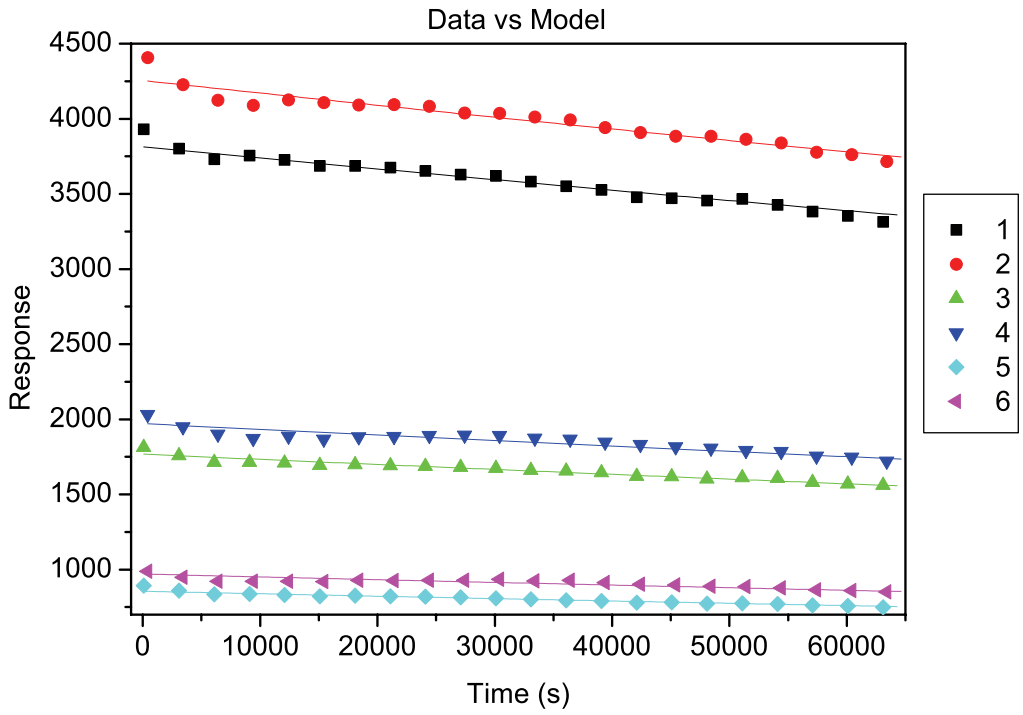

Figure S-3. Data vs Model plot from Dynafit analysis of FSSF photobleaching. Symbols represent measured data points, while lines represent the fitted model. Numbers in the legend refer to the data set numbers in Table S-1.

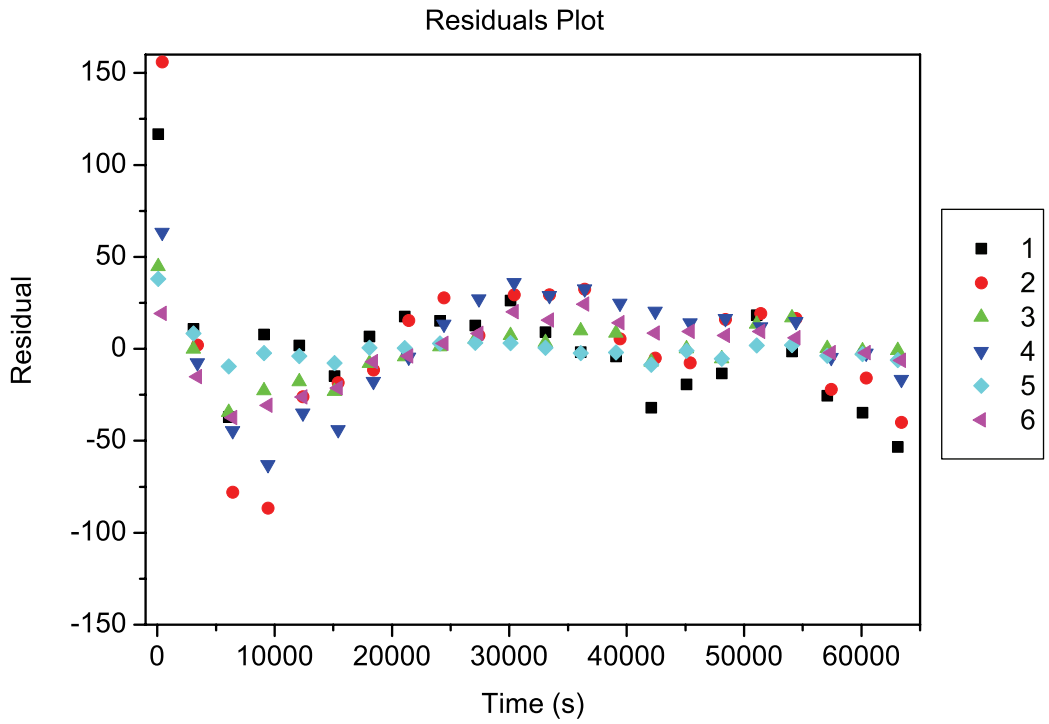

Figure S-4. Residual plot from Dynafit analysis of FSSF photobleaching. Numbers in the legend refer to the data set numbers in Table S-1.

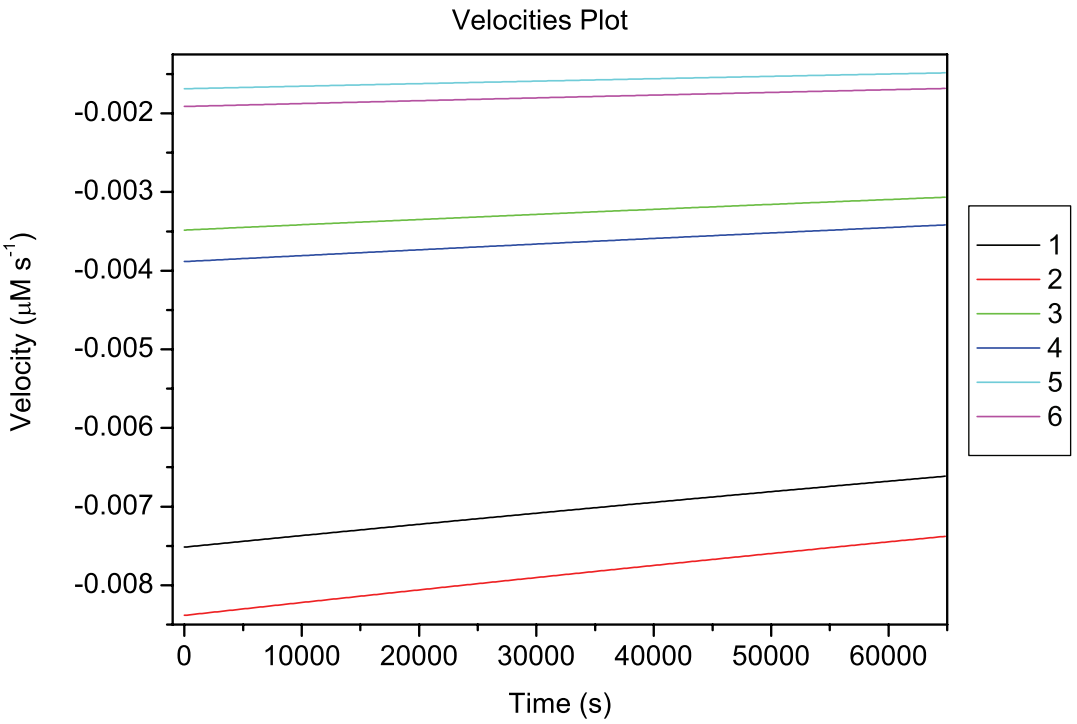

Figure S-5. Velocities plot from Dynafit analysis of FSSF photobleaching. Numbers in the legend refer to the data set numbers in Table S-1.

1.5.2 Cleavage of FSSF by DTT

Initially, a two-reaction model was used to fit the data obtained from the cleavage of FSSF by DTT – one reaction describing the actual cleavage of FSSF by DTT (k_1) and the other reaction accounting for the photobleaching of FSSF (k_{pb2}). The value of k_{pb2} was fixed to that obtained in the previous photobleaching experiment while the value of k_1 was allowed to vary. Once an initial estimate for k_1 was obtained, a third reaction was added to the model to account for photobleaching of FSH (k_{pb1}) and both k_1 and k_{pb1} were allowed to vary to improve the overall fit.

This produced a much better fit for the reaction between our probe and DTT. Thus Figure S-6 has residuals an order of magnitude smaller than those of Figure S-2 (data not shown) where a simple one-phase exponential is used to calculate the response factor.

1.5.2.1 Dynafit Script for Non-Linear Least Squares Regression Analysis of FSSF Cleavage by DTT

```
[task]
        data = progress
        task = fit

[mechanism]
        FSSF + DTT  -->  FSH + FSH + oxidized-DTT      : k1
        FSH         -->  photobleached-FSH             : kpb1
        FSSF        -->  photobleached-FSSF            : kpb2

[constants]
        k1      = 1e-007??
        kpb1    = 1e-005??          ;  estimate based on rate of photobleaching of FSSF
        kpb2    = 1.9705e-006       ;  fixed to value obtained from photobleaching experiment

[responses]
        FSH     = 1600?
        FSSF    = 207?

[progress]
        directory ./glutathione/DTT/data
        extension txt
        file 10uMFlu10mMDTT     | conc. FSSF = 10?,    DTT = 10000?      | delay = 35
        file 10uMFlu1mMDTT      | conc. FSSF = 10?,    DTT = 1000?       | delay = 45
        file 10uMFlu01mMDTT     | conc. FSSF = 10?,    DTT = 100?        | delay = 60
        file 1uMFlu10mMDTT      | conc. FSSF = 1?,     DTT = 10000?      | delay = 30
        file 1uMFlu1mMDTT       | conc. FSSF = 1?,     DTT = 1000?       | delay = 45
        file 1uMFlu01mMDTT      | conc. FSSF = 1?,     DTT = 100?        | delay = 60
        file 01uMFlu10mMDTT     | conc. FSSF = 0.1?,   DTT = 10000?      | delay = 30
        file 01uMFlu1mMDTT      | conc. FSSF = 0.1?,   DTT = 1000?       | delay = 45
        file 01uMFlu01mMDTT     | conc. FSSF = 0.1?,   DTT = 100?        | delay = 60
        file 001uMFlu10mMDTT    | conc. FSSF = 0.01?,  DTT = 10000?      | delay = 30
        file 001uMFlu1mMDTT     | conc. FSSF = 0.01?,  DTT = 1000?       | delay = 45
        file 001uMFlu01mMDTT    | conc. FSSF = 0.01?,  DTT = 100?        | delay = 60

[output]
        directory ./glutathione/DTT/output

[settings]

        <Marquardt>
        Interrupt = 1000

        <Constraints>
        ConcError = 0.15        ;  Concentrations allowed to vary up to 15% to account for pipetting errors

[end]
```

Table S-3. Fitted parameters from Dynafit analysis of FSSF Cleavage by DTT

Set	*Parameter*	*Initial*	*Fit*	*Error*	*%*
	k_1	1e-007	1.11089e-007	7.974e-011	0.1
	k_{pb1}	1e-005	9.07381e-006	4.684e-008	0.5
	r_{FSSF}	207	231.565	0.6776	0.3
	r_{FSH}	1600	1609.01	0.262	0.0
1	[FSSF]	10	9.83172	0.00161	0.0
1	[DTT]	10000	10719.3	12.29	0.1
2	[FSSF]	10	10.4941	0.003616	0.0
2	[DTT]	1000	1059.88	0.5668	0.1
3	[FSSF]	10	10.9925	0.01525	0.1
3	[DTT]	100	110	0.423	0.4
4	[FSSF]	1	1.1	0.002145	0.2
4	[DTT]	10000	10171	107	1.1
5	[FSSF]	1	1.1	0.003081	0.3
5	[DTT]	1000	1095.3	5.109	0.5
6	[FSSF]	1	1.1	0.01199	1.1
6	[DTT]	100	110	3.361	3.1
7	[FSSF]	0.1	0.10705	0.002191	2.0
7	[DTT]	10000	10200	1120	11.0
8	[FSSF]	0.1	0.11	0.003067	2.8
8	[DTT]	1000	1100	51.19	4.7
9	[FSSF]	0.1	0.11	0.01196	10.9
9	[DTT]	100	110	33.63	30.6
10	[FSSF]	0.01	0.011	0.002567	23.3
10	[DTT]	10000	11000	13360	121.4
11	[FSSF]	0.01	0.011	0.003069	27.9
11	[DTT]	1000	1100	512.7	46.6
12	[FSSF]	0.01	0.011	0.01121	102
12	[DTT]	100	110	293.4	266.7

Table S-4. 99% confidence intervals for fitted parameters from Dynafit analysis of FSSF Cleavage by DTT

k_1	*lower*	*upper*
standard error	1.11009e-007	1.11168e-007
linear approximation	1.10883e-007	1.11294e-007
exact intervals	1.10975e-007	1.11223e-007

k_{pb1}	*lower*	*upper*
standard error	9.02698e-006	9.12065e-006
linear approximation	8.95301e-006	9.19462e-006
exact intervals	8.9779e-006	9.1743e-006

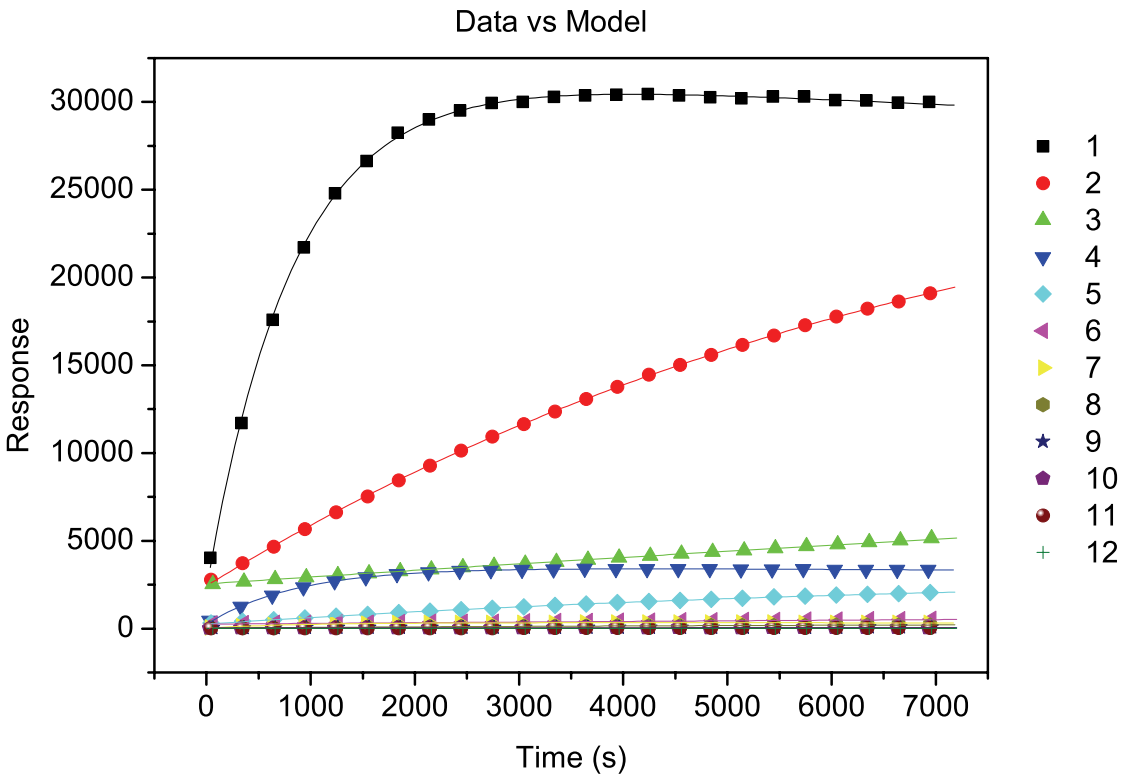

Figure S-6. Data vs Model plot from Dynafit analysis of FSSF cleavage by DTT. Symbols represent measured data points, while lines represent the fitted model. Numbers in the legend refer to the data set numbers in Table S-3.

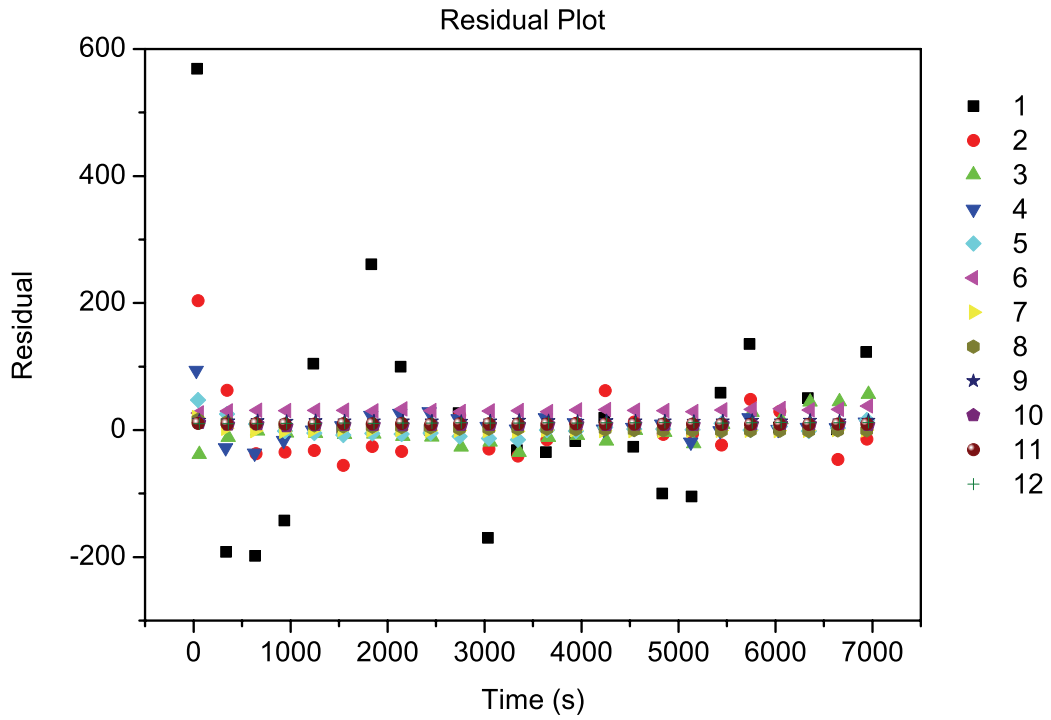

Figure S-7. Residual plot from Dynafit analysis of FSSF cleavage by DTT. Numbers in the legend refer to the data set numbers in Table S-3.

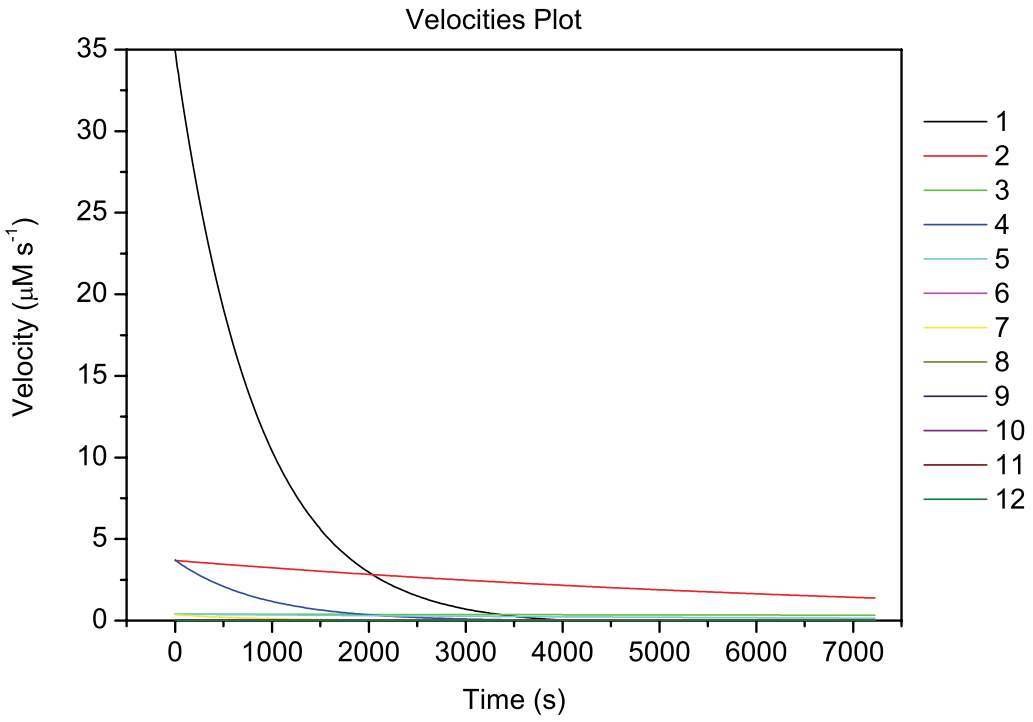

Figure S-8. Velocities plot from Dynafit analysis of FSSF cleavage by DTT. Numbers in the legend refer to the data set numbers in Table S-3.

1.5.3 Calculation of initial estimate for k_2

The reaction of GSH (released by the action of GR on GSSG) with FSSF produces FSSG and FSH, both of which have equal fluorescence. FSH then immediately reacts with the excess of GSSG present to form a second molecule of FSSG, although this has no net effect on fluorescence. This is a complex process but can be adequately modeled as a pseudo-first order process that simply converts GSSG to two moles of FSSG. An initial estimate for k_2 was obtained by using high concentrations of GR to generate a large excess of GSH, effectively eliminating the effect of enzyme concentration (k_{cat}) from the calculations (Figure S-9).

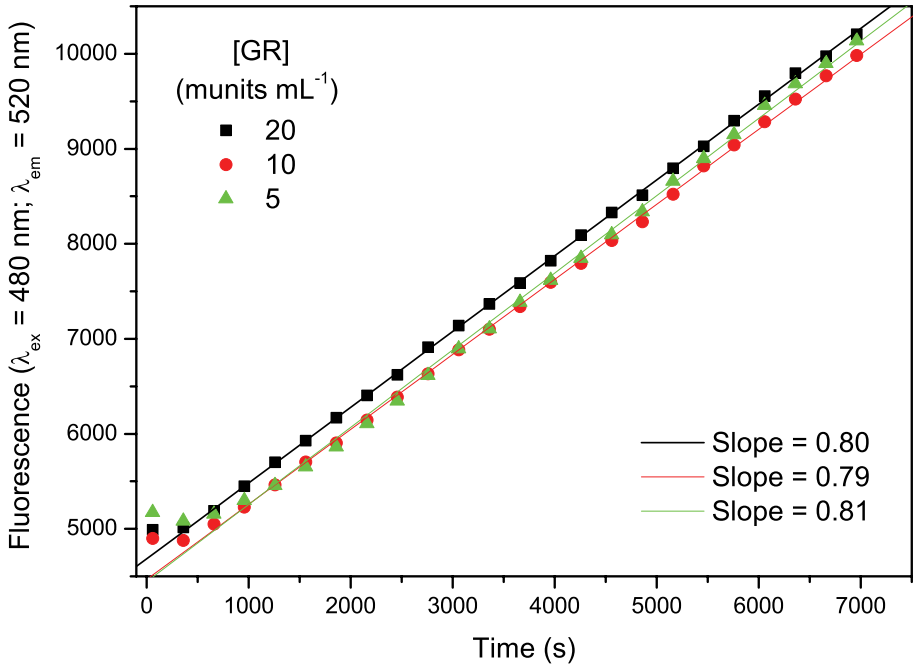

Figure S-9. Fluorescence intensity of 25 µM FSSF, 1 mM GSSG, 100 µM NADPH in 100 mM K_3PO_4, 1 mM EDTA, pH 7.5, after addition of 5, 10 and 20 munits mL^{-1} of GR at RT.

The average slope of the linear part of the curves = 0.8 fluorescence units per second. As the formation of 1 µM FSH generates 1600 fluorescence units, the reaction rate = 5×10^{-4} µM s^{-1}

[GSH] = 2 × [GSSG] = 2000 µM

[FSSF] = 25 µM

Rate = $k_2[GSH]^2[FSSF]$

$5 \times 10^{-4} = k_2(2000)^2(25)$

$k_2 = 5 \times 10^{-12}$ L^2 µmol^{-2} s^{-1}

1.5.3.1 Dynafit Script for Non-Linear Least Squares Regression Analysis of GR Assay

```
[task]
        data = progress
        task = fit

[mechanism]
        GSSG + GR               --->  GSH + GSH + GR          : kcat
        GSH + GSH + FSSF        --->  FSSG + FSSG             : k2
        FSSG                    --->  photobleached-FSSG      : kpb1
        FSSF                    --->  photobleached-FSSF      : kpb2
        GR                      --->  denatured-GR            : kden

[constants]
        kcat    = 11 ??
        k2      = 5e-012 ??             ;  Initial estimate determined from Figure S-9
        kpb1    = 9.07381e-006          ;  Fixed to value obtained from DTT experiment
        kpb2    = 1.9705e-006           ;  Fixed to value obtained from photobleaching experiment
        kden    = 3e-005 ??             ;  Enzyme inactivation optimized with 99% confidence interval

[responses]
        FSSG = 1600?,    FSSF = 207?

[concentrations]
        GSSG = 1000

[progress]
        directory ./glutathione/GR/data
        extension txt
        file 5uMFlu001UGR0uL            | conc. FSSF = 5?, GR = 0                 | delay = 420
        file 5uMFlu001UGR5uL            | conc. FSSF = 5?, GR = 0.000002 ?        | delay = 315
        file 5uMFlu001UGR10uL           | conc. FSSF = 5?, GR = 0.000004 ?        | delay = 225
        file 5uMFlu001UGR20uL           | conc. FSSF = 5?, GR = 0.000008 ?        | delay = 150
        file 5uMFlu01UGR5uL             | conc. FSSF = 5?, GR = 0.00002 ?         | delay = 45
        file 5uMFlu01UGR10uL            | conc. FSSF = 5?, GR = 0.00004 ?         | delay = 15
        file 5uMFlu01UGR20uL            | conc. FSSF = 5?, GR = 0.00008 ?         | delay = 0
        file 10uMFlu001UGR0uL           | conc. FSSF = 10?, GR = 0                | delay = 420
        file 10uMFlu001UGR5uL           | conc. FSSF = 10?, GR = 0.000002 ?       | delay = 315
        file 10uMFlu001UGR10uL          | conc. FSSF = 10?, GR = 0.000004 ?       | delay = 225
        file 10uMFlu001UGR20uL          | conc. FSSF = 10?, GR = 0.000008 ?       | delay = 150
        file 10uMFlu01UGR5uL            | conc. FSSF = 10?, GR = 0.00002 ?        | delay = 45
        file 10uMFlu01UGR10uL           | conc. FSSF = 10?, GR = 0.00004 ?        | delay = 15
        file 10uMFlu01UGR20uL           | conc. FSSF = 10?, GR = 0.00008 ?        | delay = 0
        file 25uMFlu001UGR0uL           | conc. FSSF = 25?, GR = 0                | delay = 420
        file 25uMFlu001UGR5uL           | conc. FSSF = 25?, GR = 0.000002 ?       | delay = 315
        file 25uMFlu001UGR10uL          | conc. FSSF = 25?, GR = 0.000004 ?       | delay = 225
        file 25uMFlu001UGR20uL          | conc. FSSF = 25?, GR = 0.000008 ?       | delay = 150
        file 25uMFlu01UGR5uL            | conc. FSSF = 25?, GR = 0.00002 ?        | delay = 45
        file 25uMFlu01UGR10uL           | conc. FSSF = 25?, GR = 0.00004 ?        | delay = 15
        file 25uMFlu01UGR20uL           | conc. FSSF = 25?, GR = 0.00008 ?        | delay = 0

[output]
        directory ./glutathione/GR/output

[settings]
        <Marquardt>
        Interrupt = 1000
        <Constraints>
        ConcError = 0.15        ;Concentrations allowed to vary up to 15% to account for pipetting errors

[end]
```

Table S-5. Fitted parameters from Dynafit analysis of GR Assay

Set	*Parameter*	*Initial*	*Fit*	*Error*	*%*
	k_{cat}	11	11.787	0.03504	0.3
	k_2	5e-012	4.3078e-012	3.939e-015	0.1
	k_{den}	3e-005	3.05799e-005	9.979e-008	0.3
	r_{FSSF}	207	185.537	0.6688	0.4
	r_{FSSG}	1600	1252.49	0.4896	0.0
1	[GR]	8e-005	6.8e-005	2.919e-006	4.3
1	[FSSF]	25	21.5275	0.04466	0.2
2	[GR]	4e-005	3.4e-005	5.503e-007	1.6
2	[FSSF]	25	21.3396	0.0333	0.2
3	[GR]	2e-005	1.94802e-005	1.095e-007	0.6
3	[FSSF]	25	21.8753	0.02156	0.1
4	[GR]	8e-006	8.25097e-006	2.228e-008	0.3
4	[FSSF]	25	21.25	0.02148	0.1
5	[GR]	4e-006	4.20997e-006	1.031e-008	0.2
5	[FSSF]	25	22.7022	0.03148	0.1
6	[GR]	2e-006	2.3e-006	7.7e-009	0.3
6	[FSSF]	25	24.1363	0.05208	0.2
7	[FSSF]	25	22.9304	0.1273	0.6
8	[GR]	8e-005	6.8e-005	8.34e-006	12.3
8	[FSSF]	10	10.011	0.06464	0.6
9	[GR]	4e-005	3.4e-005	1.072e-006	3.2
9	[FSSF]	10	10.2263	0.03478	0.3
10	[GR]	2e-005	1.71399e-005	1.478e-007	0.9
10	[FSSF]	10	10.7597	0.02104	0.2
11	[GR]	8e-006	8.21207e-006	4.024e-008	0.5
11	[FSSF]	10	9.24953	0.02174	0.2
12	[GR]	4e-006	4.15684e-006	1.768e-008	0.4
12	[FSSF]	10	10.3055	0.03107	0.3
13	[GR]	2e-006	2.3e-006	1.407e-008	0.6
13	[FSSF]	10	11.5	0.05049	0.4
14	[FSSF]	10	10.6244	0.08378	0.8
15	[GR]	8e-005	6.8e-005	1.928e-005	28.3
15	[FSSF]	5	4.8683	0.07326	1.5

16	[GR]	4e-005	3.4e-005	1.757e-006	5.2
16	[FSSF]	5	5.08412	0.02902	0.6
17	[GR]	2e-005	1.7e-005	2.641e-007	1.6
17	[FSSF]	5	5.25433	0.01871	0.4
18	[GR]	8e-006	8.59071e-006	8.4e-008	1.0
18	[FSSF]	5	4.43675	0.02014	0.5
19	[GR]	4e-006	4.13491e-006	3.363e-008	0.8
19	[FSSF]	5	5.02587	0.02977	0.6
20	[GR]	2e-006	2.3e-006	6.362e-008	2.8
20	[FSSF]	5	5.75	0.1072	1.9
21	[FSSF]	5	5.2297	0.06843	1.3

Table S-6. 99% confidence intervals for fitted parameters from Dynafit analysis of GR assay

$\mathbf{k_{cat}}$	*lower*	*upper*
standard error	11.7519	11.822
linear approximation	11.6966	11.8773
exact intervals	11.5878	11.5878

$\mathbf{k_2}$	*lower*	*upper*
standard error	4.30386e-012	4.31174e-012
linear approximation	4.29765e-012	4.31795e-012
exact intervals	4.29555e-012	4.31688e-012

$\mathbf{k_{den}}$	*lower*	*upper*
standard error	3.04801e-005	3.06797e-005
linear approximation	3.03226e-005	3.08372e-005
exact intervals	2.9211e-005	3.1106e-005

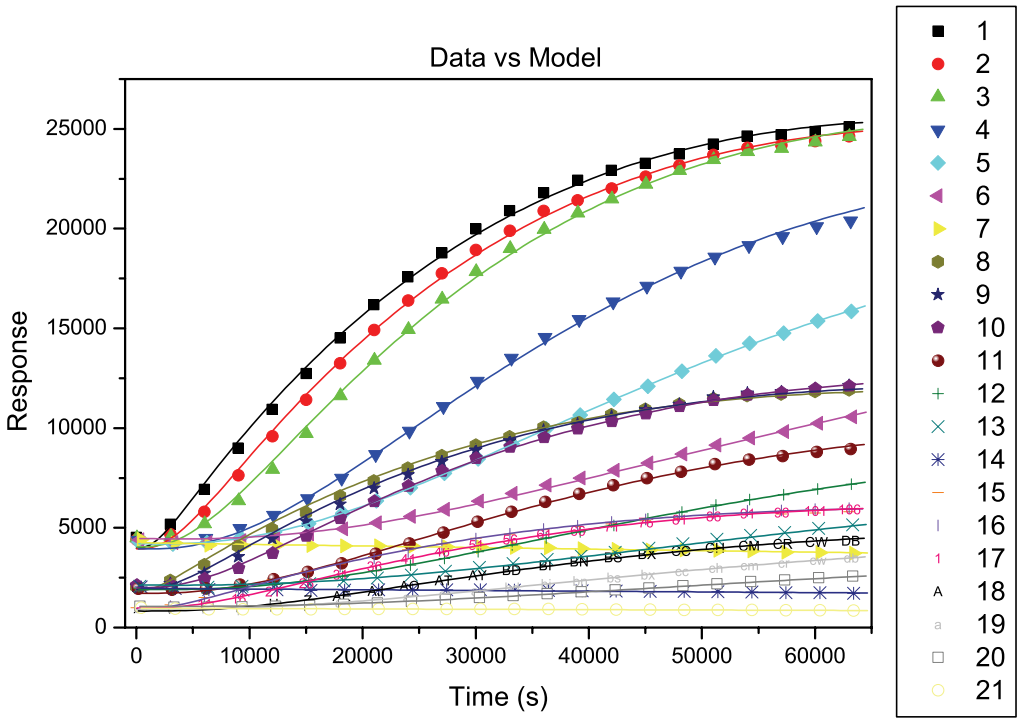

Figure S-10. Data vs Model plot from Dynafit analysis of GR assay. Symbols represent measured data points, while lines represent the fitted model. Numbers in the legend refer to the data set numbers in Table S-5.

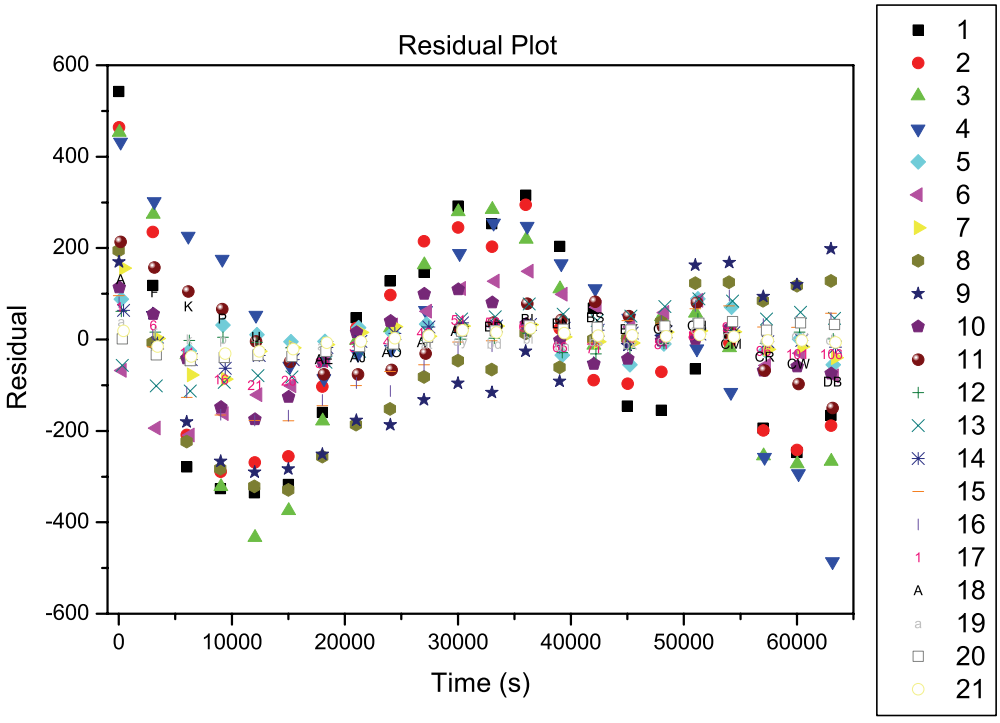

Figure S-11. Residual plot from Dynafit analysis of GR assay. Numbers in the legend refer to the data set numbers in Table S-5.

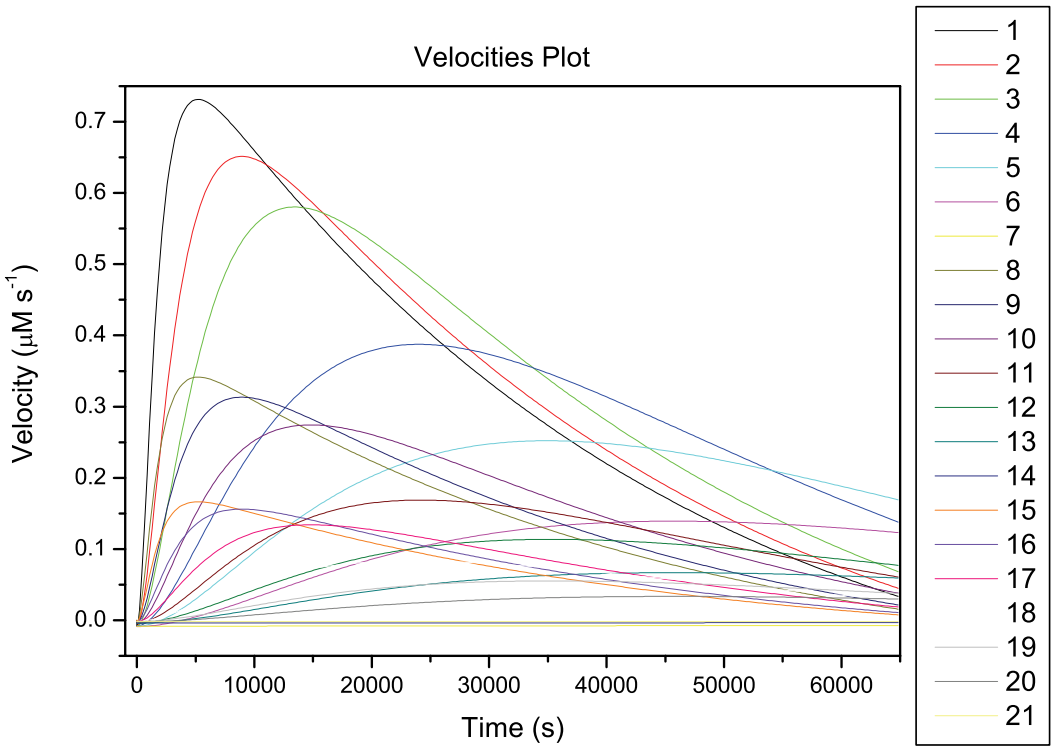

Figure S-12. Velocities plot from Dynafit analysis of GR assay. Numbers in the legend refer to the data set numbers in Table S-5.

1.5.4 Comparison of fluorometric assay with commercial colorimetric (DTNB) GR assay

To compare our fluorometric GR assay with the commonly used colorimetric assay based on DTNB, a commercially available assay kit from Sigma-Aldrich (USA) was used to construct a calibration plot for GR concentrations between 1–10 munits mL^{-1} (Figure S-13). The assay was performed exactly as described in the instructions supplied with the kit. The kit claims the concentration dependent enzymatic reaction is linear between 3 and 30 munits mL^{-1}, while our fluorometric assay was linear between 0.05 and 2 munits mL^{-1}, making our assay at least 60 times more sensitive and having a dynamic range at least one order of magnitude more than the colorimetric assay. The fluorometric assay is complementary to the Sigma-Aldrich assay in that it is useful in the lower range of GR activity but is also competitive in that GR assays suitable for the colorimetric assay can be diluted by up to 1000× and run on our fluorometric assay thereby diluting out possible interfering compounds by the same amount.

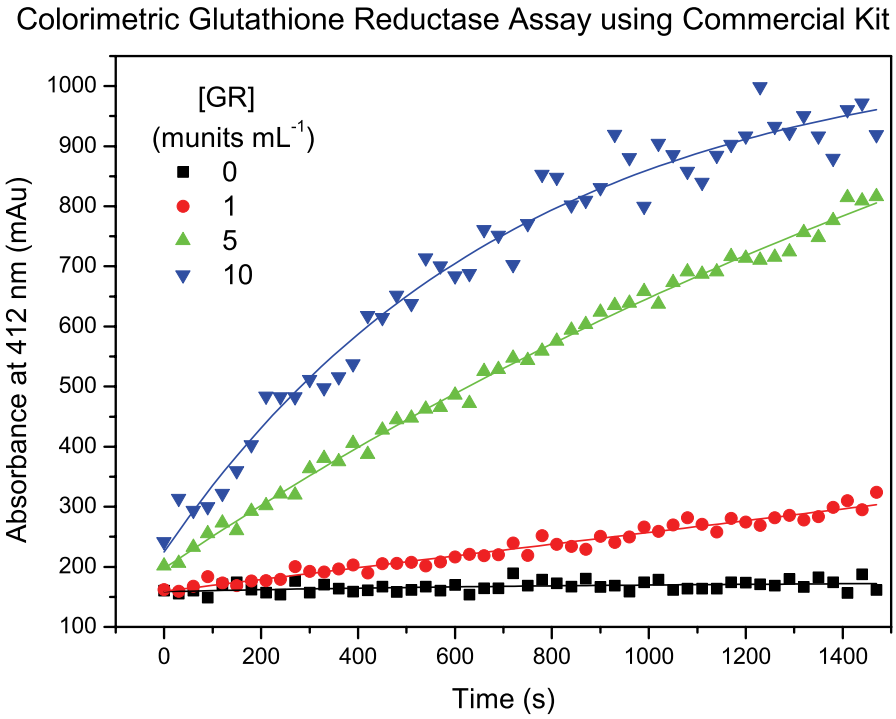

Figure S-13. Progress curves obtained from commercial colorimetric GR assay

1.6 References

(1) Spivak, D.; Shea, K.J. *J. Org. Chem.* **1999**, *64*, 4627-4634.

(2) Kuzmic, P. *Anal. Biochem.* **1996**, *237*, 260-273.