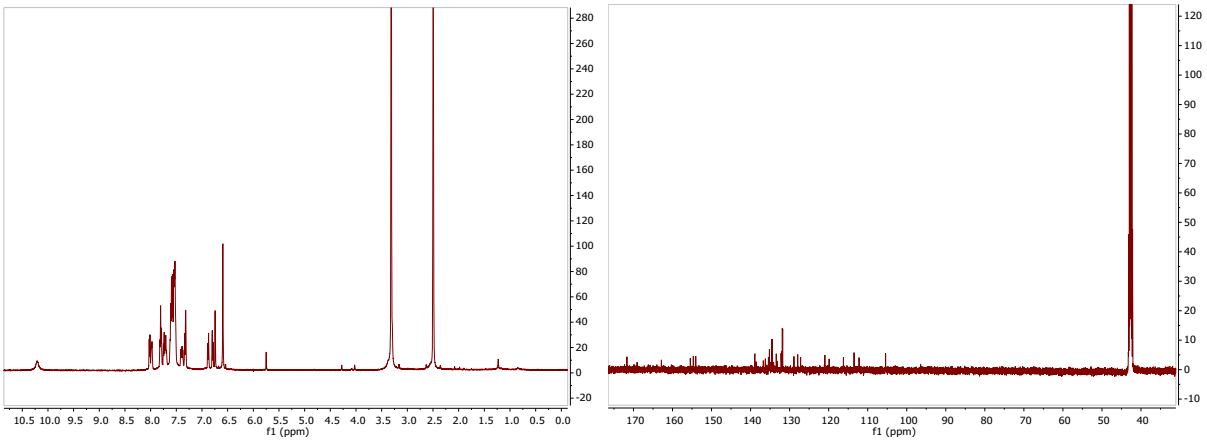
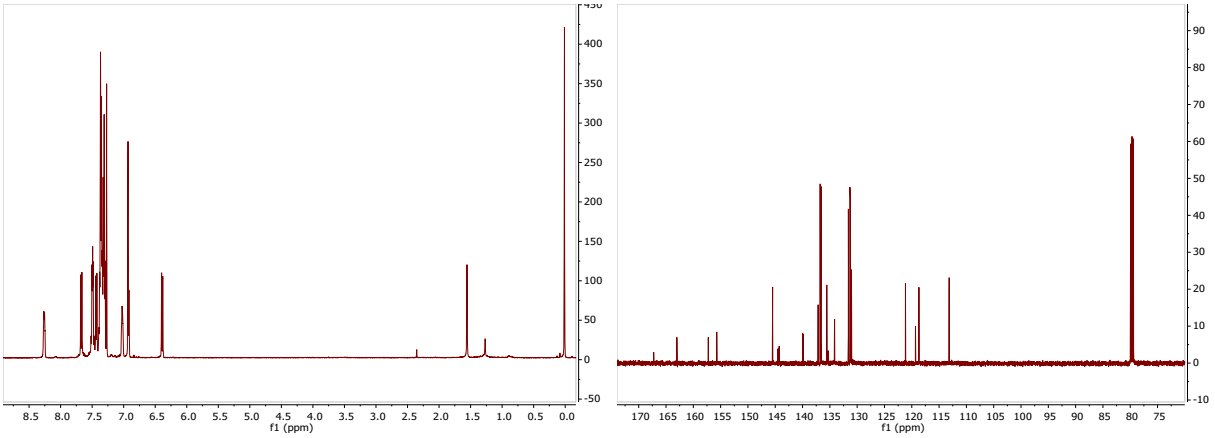
Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

SUPPLEMENTARY DATA

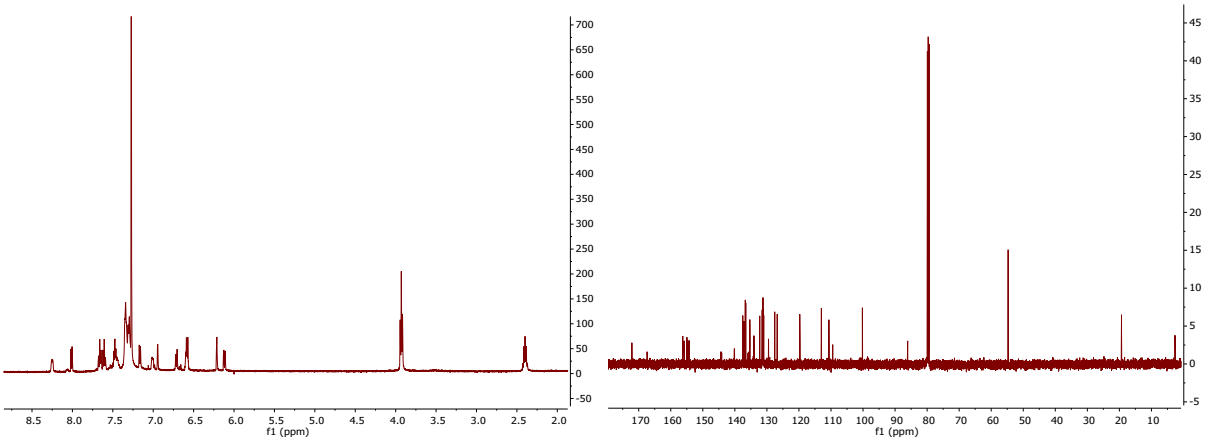
NMR of **sensor 1**



NMR of **sensor 2**

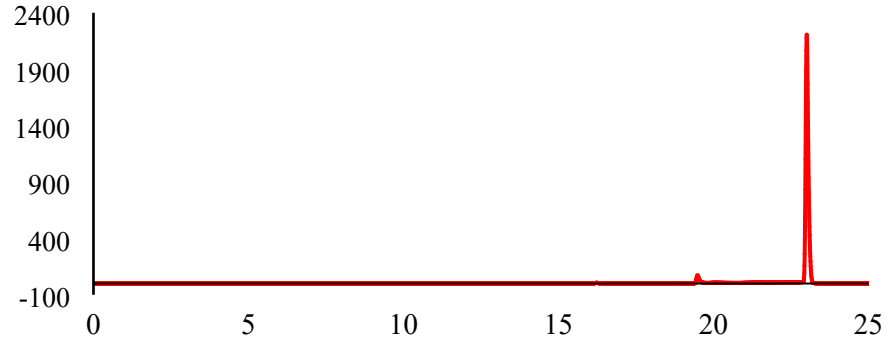


NMR of **sensor 3**

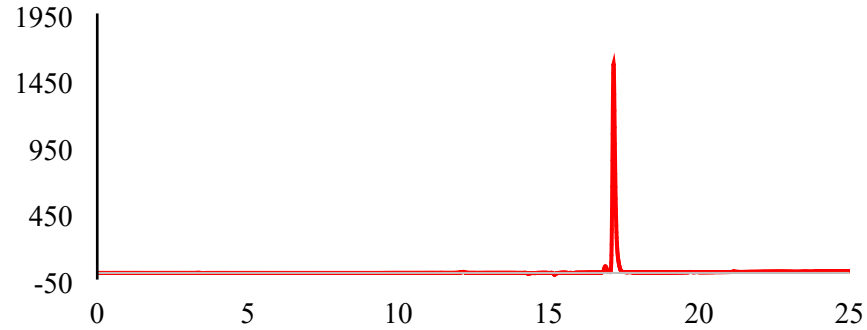


Supplementary Figure S1 Sensor 1 - 3 NMR traces

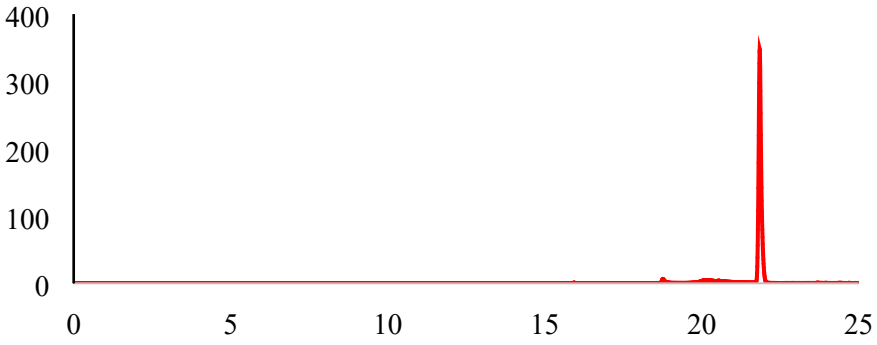
HPLC trace (λ = 220 nm) of **sensor 1**



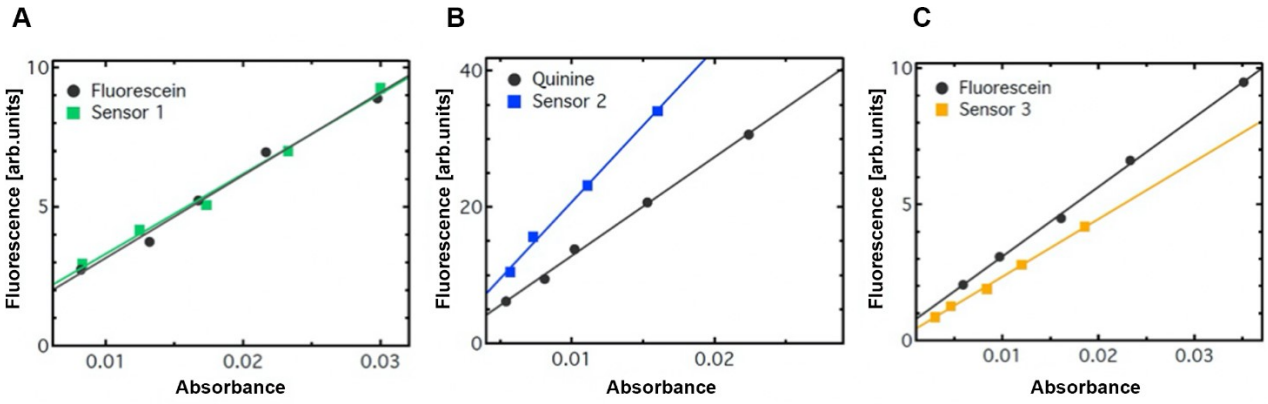
HPLC trace (λ = 220 nm) of **sensor 2**



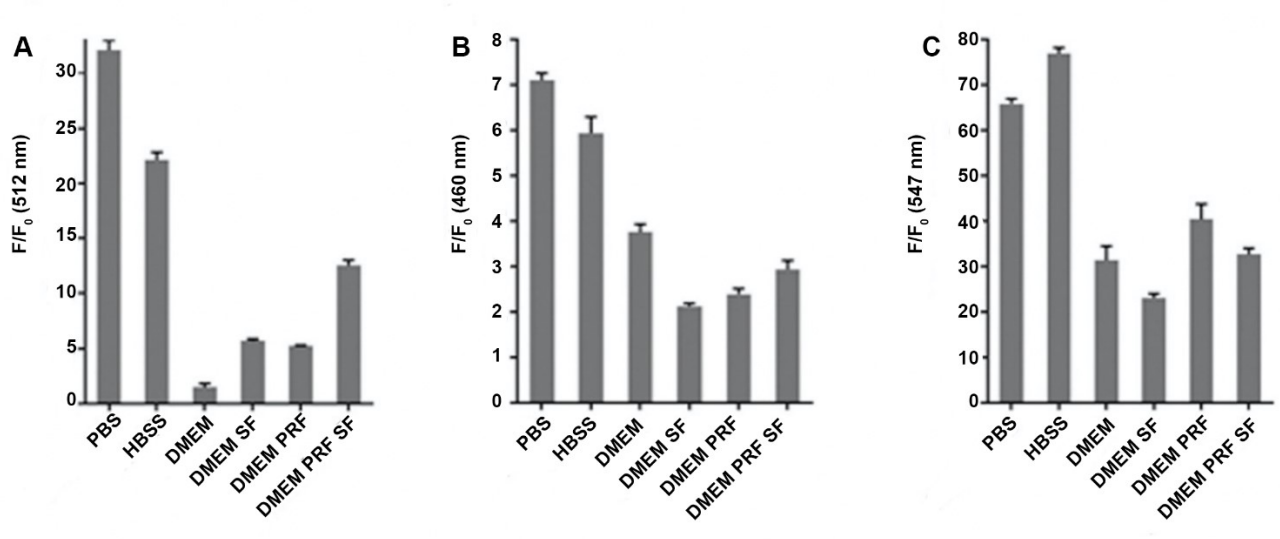
HPLC trace (λ = 220 nm) of **sensor 3**



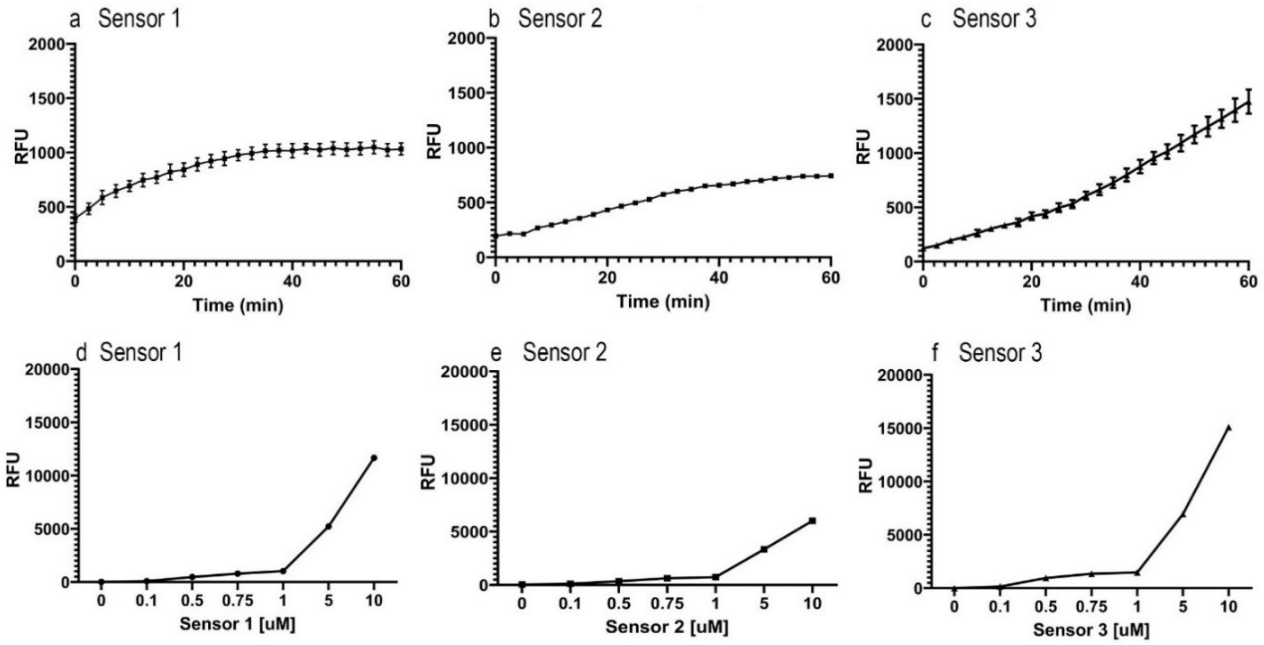
Supplementary Figure S2 Sensor 1 - 3 HPLC traces



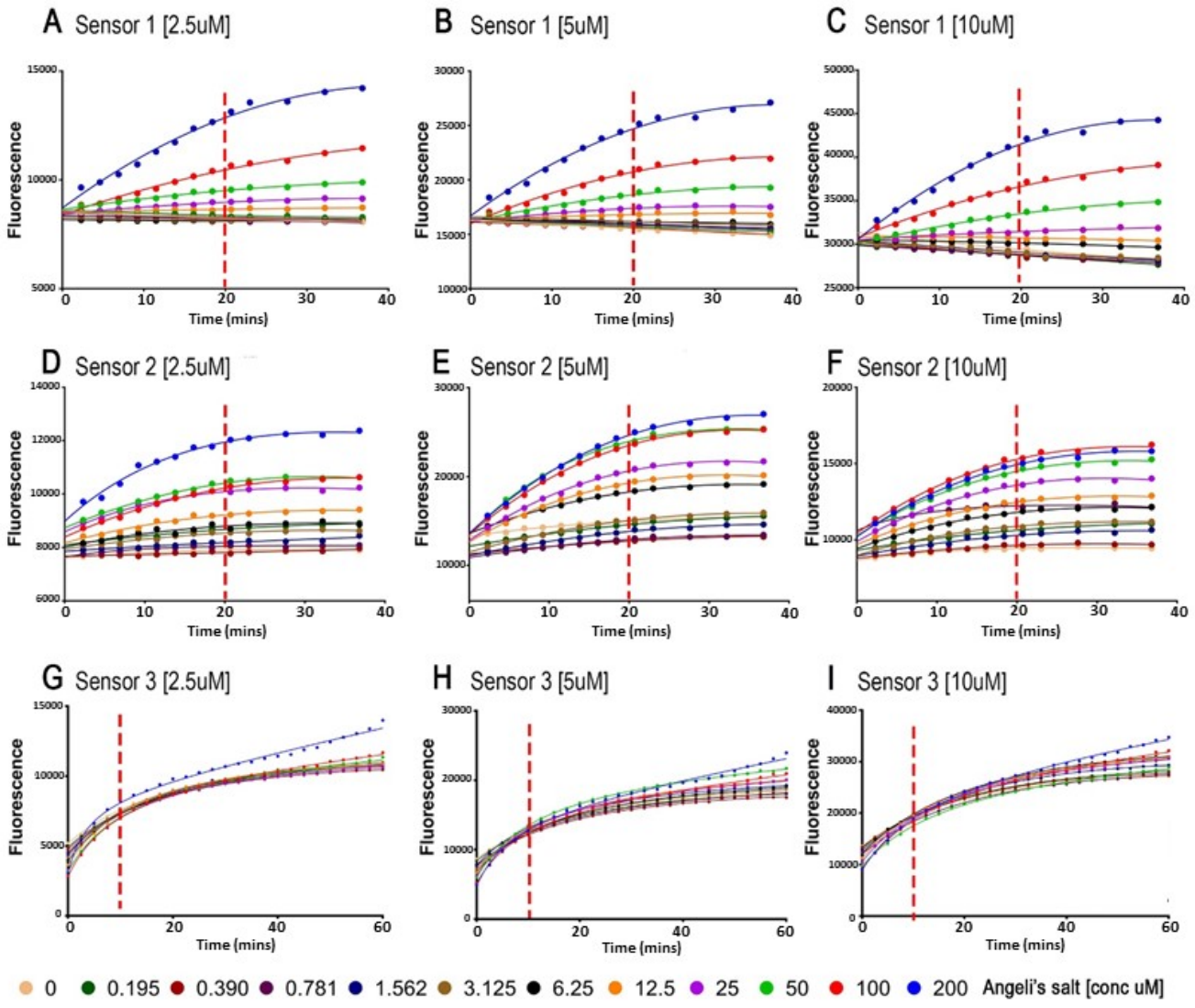
Supplementary Figure S3 - **Quantum yield**
Sensor 1 give highest quantum yield of all 3 sensors.
The integrated fluorescence depends linearly on the absorbance for (A) Fluorescein (black circles) and Sensor 1 (green squares) (ΦSensor 1 = 0.9). (B) Quinine (black circles) and Sensor 2 (blue squares) (ΦSensor 2 = 0.85) and (C) Fluorescein (black circles) and Sensor 3 (yellow squares) (ΦSensor 3 = 0.77).



Supplementary Figure S4 - Fluorescence (buffers & media)
Sensor 3 shows highest fluorescent signal in biological buffers and media following AS.
Fluorescence of sensors 1 (G), 2 (H) and 3 (I) in buffers (PBS pH 7.2, HBSS pH 7.2) and cell media (DMEM, DMEM SF (Serum free), DMEM PRF (Phenol Red free), DMEM SF PRF (Serum and Phenol Red free) with 100 μM AS.

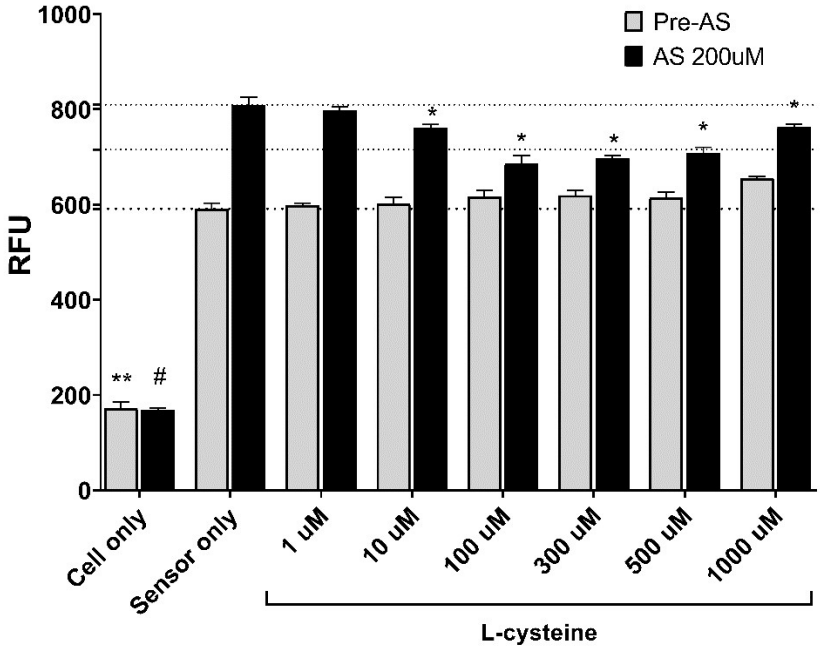


<u>Supplementary Figure S5</u> Sensor 1 - 3 kinetics in the presence of Angeli's salt in HBSS solution.
Data showing the time it takes for each sensor 1 (a), 2 (b) and 3 (c) to saturate fluorescence in the presence of Angeli's salt (200 µM). At a concentration of 1 µM, Sensor 1 reached saturation of fluorescent signal by 10 min, Sensors 2 and 3 took longer than 10 min to reach saturation. The fluorescence at 1 hour following the addition of Angeli's salt (200 µM) for increasing concentrations of Sensor 1 (d), Sensor 2 (e) and Sensor 3 (f). At a concentration of 1 µM, Sensor 1 showed a 67-fold increase in fluorescence compared to 0 µM, Sensor 2 showed a 19-fold increase in fluorescence and Sensor 3 showed the highest fluorescence increase with a 368-fold increase.



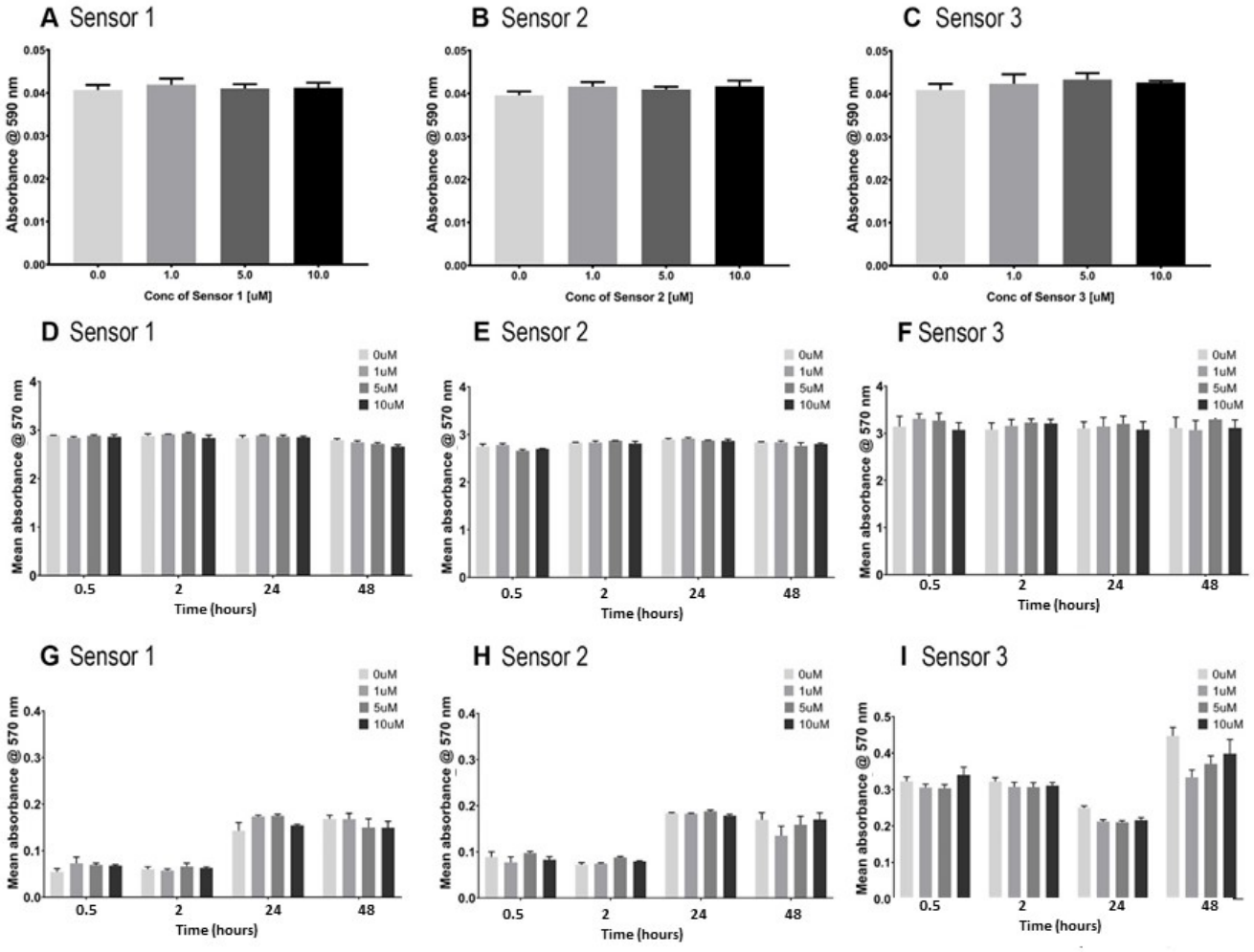
<u>Supplementary Figure S6</u> Fluorescence relationship between HNO Sensor concentration (1 – 3) and HNO donor, Angeli's salt in buffer.

A-C) Fluorescence intensity of Sensor 1 (concentrations; 2.5, 5 and 10 μM) following a 40 min incubation with increasing amounts of AS (0 – 200 μM). D-F) Sensor 2 following a 40 min incubation with increasing amounts of AS (0 – 200 μM). G-I) Sensor 3 following a 60 min incubation with increasing amounts of AS (0 μM; light pink line – 200 μM; light blue line), all in Hank's balanced salt solution (HBSS). Peak signal time shown by red dotted line.



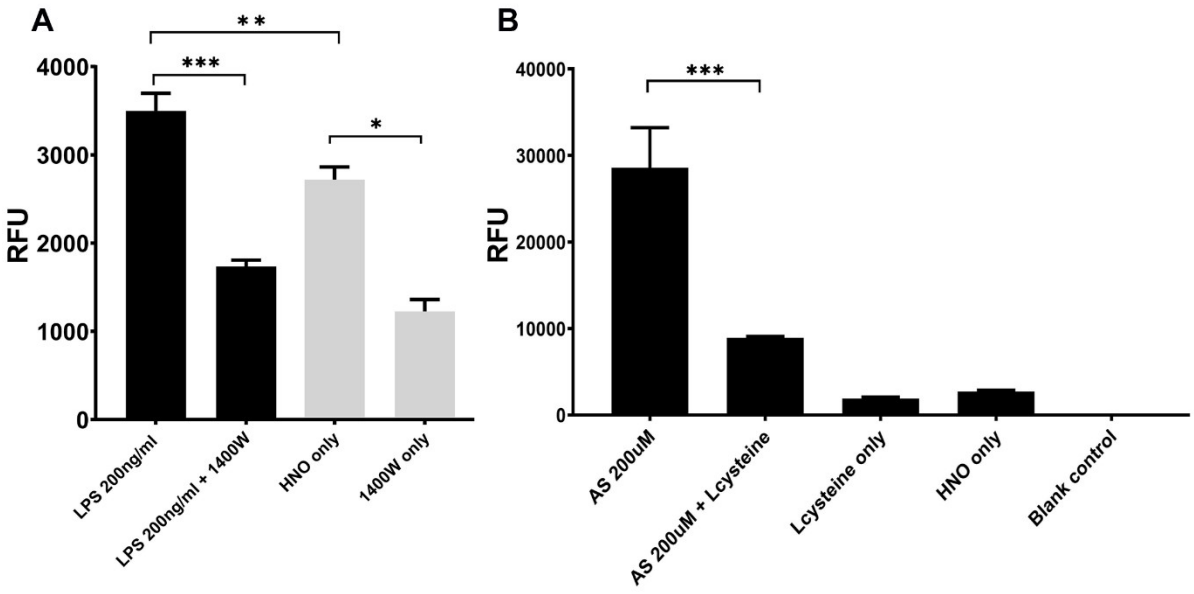
Supplementary Figure S7: Effect of L-cysteine on scavenging AS derived HNO signal in cell free media

The mean RFU (relative fluorescence unit) of Sensor 3 in cell free culture media (MesoEndo Cell Growth Medium) before and after the addition of 200 μM Angeli's salt in the presence or absence of different concentrations of HNO scavenger, L-cysteine (n = 3). The presence of L-cysteine [10 – 1000 μM] reduces the AS derived HNO fluorescent signal in cell free media when compared to sensor only (black bars; *$p < 0.0001$). The HNO signal is higher in the sensor only cell free media of both pre-AS (grey bar) and AS 200 μM (black bar) when compared to media only controls (**$p < 0.0001$; $^{\#}p < 0.0001$).

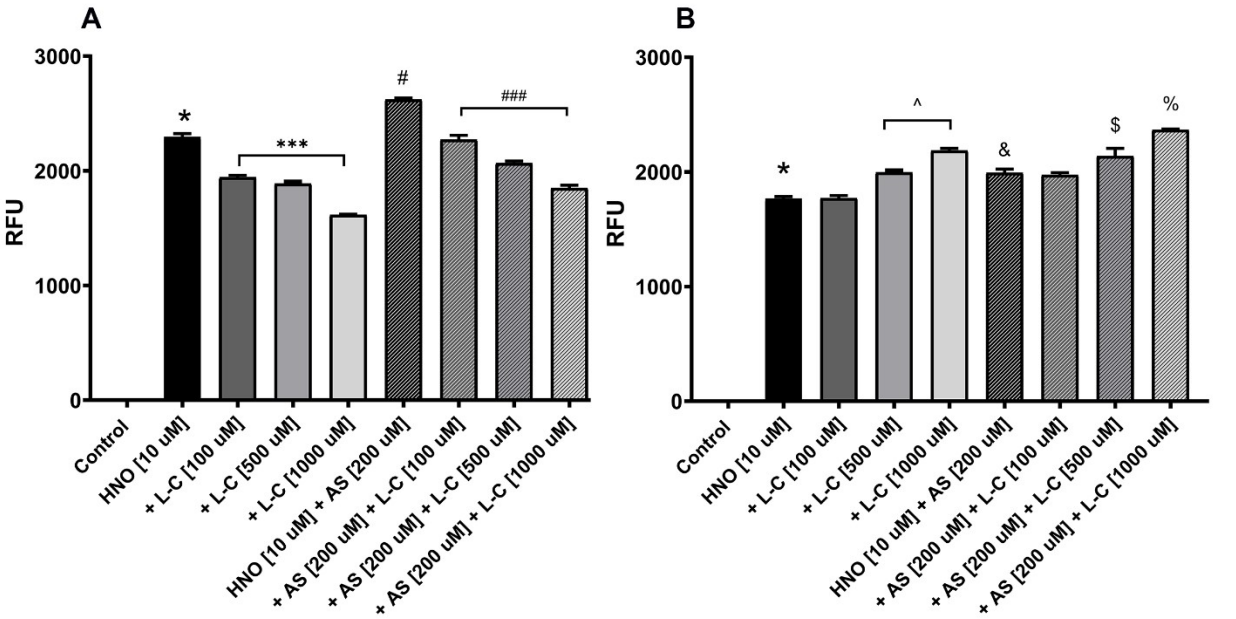


<u>Supplementary Figure S8 -</u> Dose and incubation time testing of Sensors 1, 2 & 3; effects on cell –viability and metabolic activity

Trypan Blue Trypan Blue dye exclusion test of sensor 1 (a), 2 (b) and 3 (c) with increasing concentrations following a 1.5-hour incubation in BV2 cells. There was no toxic effect observed of the sensors at any of the concentrations following the incubation. There also was no effect on cell viability (change in cell metabolism) as assed by the MTT assay in the presence of increasing concentrations of either sensor in either BV2 (D; sensor 1, E; sensor 2 or F; sensor 3) or HCAEC cells (G; sensor 1, H; sensor 2 or I; sensor 3) for up to 48 hours (n = 3).



Supplementary Figure S10 Sensor 3 can detect HNO levels in rat blood.
Bar graphs showing the RFU from spectrophotometer readings of Sensor 3 in BV2 cells (n = 3) following 24-hour incubation of LPS (black bars) or vehicle (light grey bars) (A). LPS treatment (500 ng/ml) significantly increased Sensor 3 RFU compared to control (HNO only) (**$p < 0.05$). However, pre-treatment with iNOS inhibitor 1400W, showed reduced RFU compared with LPS alone (***$p < 0.001$). Pre-incubation with 1400W significantly attenuated Sensor 3 fluorescent signal when compared to control conditions (light grey bars) (*$p < 0.01$) (n = 3). Graph B shows that the signal derived from Sensor 3 is HNO specific due to the signal increase following application of Angeli's salt (HNO donor: 200 μM) (***$p < 0.001$) which could be scavenged by L-cysteine (HNO scavenger: 1 mM).



Supplementary Figure S10 Sensor 3 can detect HNO levels in rat blood.

Bar graph showing the mean fluorescence intensity of rat blood samples taken via cardiac puncture and incubated with Sensor 3 [10 μM] for 20 minutes, in the absence (A) or presence (B) of EDTA solution [0.5M]. Data show that a higher mean fluorescence was observed in both serum (without EDTA) and plasma (with EDTA) derived from Sensor 3 containing blood, compared to the 'Control' without (*$p < 0.0001$). After the initial read (HNO 10 μM: black bars), the addition of AS further increased the fluorescent signal ($^{\#}p < 0.0001$, $^{\&}p < 0.001$). The control experiments done in the presence of the HNO scavenger L-cysteine, showed that the presence of the common anti-coagulant, EDTA, interfered with L-cysteine's ability to scavenge HNO. A) Increasing concentrations of L-cysteine gave a step wise reduction in mean fluorescence signal when compared to Sensor 3 (HNO 10 μM: black bar) (***$p < 0.0001$) or Sensor 3 with AS (dark grey hatch bar) ($^{\#\#\#}p < 0.0001$), in blood samples that did not contain EDTA. However, in blood samples containing EDTA (B), L-cysteine produced an increased mean fluorescent signal at the higher concentrations (500, 1000 μM), when compared to Sensor 3 alone (HNO 10 μM: black bar) (^$p < 0.0001$) or Sensor 3 with AS (dark grey hatch bar) ($^{\$}p < 0.05$, $^{\%}p < 0.0001$).

SUPPLEMENTARY METHODS – Chemistry

Synthesis of sensors 1-3

3'-hydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 2-(diphenylphosphanyl)benzoate ***(sensor 1)***. To a solution of fluorescein (5) (1 g, 3.00 mmol) in anhydrous DMF (20 mL) under N_2 was added 2-(diphenylphosphino)benzoic acid (6) (760 mg, 2.48 mmol), DIC (520 mg, 4.12 mmol) and DMAP (126 mg, 2.80 mmol). The mixture was stirred under N_2 at room temperature for 21 h before H_2O (50 mL) was added. The aqueous phase was extracted with ethyl acetate (3 * 50 mL) and the combined organic layer was washed with H_2O (50 mL) and brine (100 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give the crude product as a yellow oil (2.093 g). The mixture was purified by flash column chromatography to afford pure sensor 1 as a yellow solid (845 mg, 44%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.21 (s, 1H), 8.02 (d, J = 7.8 Hz, 1H), 7.98 (d, J = 7.9 Hz, 1H), 7.83 – 7.78 (m, 2H), 7.77 – 7.68 (m, 2H), 7.62 – 7.49 (m, 10H), 7.39 (dd, J = 13.0, 7.8 Hz, 1H), 7.35 – 7.30 (m, 2H), 6.88 (d, J = 8.7 Hz, 1H), 6.79 (d, J = 8.7 Hz, 1H), 6.74 (s, 1H), 6.59 (s, 2H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.7, 162.8, 155.5, 154.7, 154.0, 138.9, 136.7, 136.2, 135.2, 134.5, 133.4, 132.0, 128.9, 127.9, 121.0, 119.9, 116.2, 113.5, 112.2, 105.4. HRMS (m/z) for $[C_{39}H_{25}O_6P]^+$ calculated 620.1389, found 620.1389.

2-oxo-2H-chromen-7-yl 2-(diphenylphosphanyl)benzoate ***(sensor 2)***. To a solution of 7-hydroxycoumarin (7) (91 mg, 0.56 mmol) in anhydrous DCM (20 mL) under N_2 was added 2-(diphenylphosphino)benzoic acid (6) (205 mg, 0.67 mmol), DIC (92 mg, 0.73 mmol) and DMAP (61 mg, 0.50 mmol). The mixture was stirred under N_2 at room temperature for 21 h before H_2O (20 mL) was added. The aqueous phase was extracted with ethyl acetate (3 * 20 mL) and the combined organic layer was washed with H_2O (20 mL) and brine (50 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give the crude product as a brown oil (130 mg). The mixture was purified by flash column chromatography to afford pure sensor 2 as a white solid (70 mg, 28%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.29 – 8.23 (m, 1H), 7.67 (d, J = 9.6 Hz, 1H), 7.55 – 7.46 (m, 2H), 7.43 (d, J = 8.0 Hz, 1H), 7.39 – 7.28 (m, 10H), 7.06 – 6.98 (m,

1H), 6.95 – 6.86 (m, 2H), 6.39 (d, *J* = 9.5 Hz, 1H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.3, 163.0, 157.3, 155.7, 145.5, 144.5, 139.9, 136.6, 135.6, 135.2, 134.2, 131.6, 131.3, 121.2, 119.3, 118.7, 113.2. HRMS (m/z) for $[C_{28}H_{19}O_4P]^+$ calculated 450.1021, found 450.1015.

3'-(azetidin-1-yl)-6'-hydroxy-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (4). To a dry solution of fluorescein (5) (1 g, 3.00 mmol) in anhydrous DMF (7.5 mL) was added phenyl triflimide (9) (1.08 g, 3.00 mmol). The mixture was stirred under N_2 and DIPEA (1.56 mg, 12.04 mmol) was added dropwise. The mixture was further stirred under N_2 for 48 h at room temperature and acidified to pH 1 with 1 M HCl. The mixture was extracted with ethyl acetate (3 * 50 mL) and the combined organic layer was washed with H_2O (50 mL), dried over $MgSO_4$, and concentrated in vacuo to give crude product white solid (800 mg), which was used without further purification. To a solution of compound 8 (150 mg, 0.32 mmol), Pd_2dba_3 (30 mg, 0.03 mmol), XPhos (46 mg, 0.1 mmol) and Cs_2CO_3 (295 mg, 0.9 mmol) in anhydrous 1,4-dioxane (2.5 mL) under N_2 was added azetidine (44 mg, 0.78 mmol). The mixture was stirred under N_2 at 100 °C for 20 h and diluted with MeOH (20 mL). The volatiles were removed in vacuo to give the crude product as a dark red oil (849 mg). The mixture was purified by column chromatography to give rhodol 4 as a red solid (88 mg, 73%). H NMR (500 MHz, Methanol-d_4) δ 8.11 (m, 1H), 7.83 – 7.64 (m, 2H), 7.27 (d, *J* = 7.5 Hz, 3H), 6.86 – 6.75 (m, 2H), 6.67 (d, *J* = 2.4 Hz, 1H), 6.41 (dd, *J* = 4.6, 2.6 Hz, 2H), 4.11 (m, 4H), 2.48 (p, *J* = 7.4, 2H).

3'-(azetidin-1-yl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 2-(diphenylphosphanyl)benzoate **(sensor 3)**. Rhodol 4 (88 mg, 0.24 mmol), 2-(diphenylphosphino)benzoic acid (73 mg, 0.24 mmol), EDC (110 mg, 0.71 mmol) and DMAP (29 mg, 0.24 mmol) was dissolved in anhydrous THF (2 mL). The mixture was stirred under N_2 at room temperature for 20 h. The mixture was acidified to pH 1 with 1 M HCl and extracted with DCM (2 * 20 mL). The combined organic layer was washed with H_2O (20 mL) and brine (50 mL), dried over Na_2SO_4 and concentrated in vacuo to give the crude product as a pink oil (97 mg). The crude mixture was purified by rp-HPLC to afford sensor 3 as a pale pink solid (12 mg, 8%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 –

8.16 (m, 1H), 8.01 (d, *J* = 7.5 Hz, 1H), 7.63 (m, 2H), 7.47 (m, 2H), 7.38 – 7.22 (m, 10H), 7.17 (d, *J* = 7.5 Hz, 1H), 7.05 – 6.92 (m, 2H), 6.71 (d, *J* = 8.7 Hz, 1H), 6.61 – 6.54 (m, 2H), 6.21 (s, 1H), 6.12 (d, *J* = 8.7, 1H), 3.93 (t, *J* = 7.3 Hz, 4H), 2.40 (p, *J* = 7.3 Hz, 2H).^{13}C NMR (126 MHz, $CDCl_3$) δ 172.1, 167.4, 156.3, 155.8, 155.0, 154.7, 154.3, 144.3, 144.1, 140.2, 140.1, 137.5, 137.1, 136.8, 136.7, 135.7, 135.3, 134.0, 132.2, 131.5, 131.5, 131.3, 131.2, 131.2, 131.1, 131.0, 129.5, 127.6, 126.8, 119.7, 113.0, 110.7, 109.5, 100.2, 86.1, 54.7, 19.3. HRMS (m/z) for $[C_{28}H_{19}O_4P]^+$ calculated 659.1862, found 659.1860.

Fluorescence of sensors 1-3 with and without added AS.

A solution of sensors 1-3 (2 μM for sensors 1 and 3 and 5 μM for sensor 2) in 1% DMSO in water (2 mL) was prepared in a sealed container and purged with N_2 for 30 min. Varying concentrations of AS ([AS] = 0–100 μM) in 10 mM NaOH (20 ul) was added and the mixture was incubated in the sealed container for 10 min in the dark at room temperature. All concentrations of sensors 1-3 and AS reported are final concentrations of the solution after mixing. The resultant fluorescence (sensor 1: λ_{ex} = 488 nm; sensor 3: λ_{ex} = 512 nm) spectra of sensors 1 and 3 with each concentration of GSH was recorded on a Cary Eclipse Fluorescence Spectrophotometer. The fluorescence spectra of sensor 2 (λ_{ex} = 380 nm) was similarly measured on a microplate reader. The maximum fluorescence intensity of each spectrum was plotted against AS concentration in μM to produce a standard curve of calibration for sensors 1-3. A linear trendline was fitted to the plot by GraphPad Prism 7.0.

Limit of detection of sensor 3.

A solution of sensor 3 (1 μM) in 1% DMSO in water (2 mL) was prepared in a sealed container and purged with N_2 for 30 min. AS (10 μM) in 10 mM NaOH (20 uL) was added and the mixture was incubated in the sealed container for 10 min in the dark at room temperature. The resultant fluorescence (λ_{ex} = 512 nm) spectrum was recorded on a Cary Eclipse Fluorescence Spectrophotometer.

Fluorescence of sensors 1-3 with and without added AS in buffers and cell media.

A solution of sensors 1-3 (2 μM for sensors 1 and 3 and 5 μM for sensor 2) in 1% DMSO in various buffers (PBS and HBSS) and cell media (DMEM, DMEM serum-free (SF), DMEM phenol-red free (PRF) and DMEM SF PRF) (2 mL) was prepared in a sealed container and purged with N_2 for 30 min. AS (100 μM) in 10 mM NaOH (20 μL) was added and the mixture was incubated in the sealed container for 10 min in the dark at room temperature. The resultant fluorescence (sensor 1: $\lambda_{ex/em}$ = 488 nm/512 nm; sensor 2: $\lambda_{ex/em}$ = 380 nm/460 nm; sensor 3: $\lambda_{ex/em}$ = 512 nm/550 nm) was measured on the microplate reader (Figure S2). The intensity was plotted in GraphPad Prism 7.0 as bar graphs. The experiment was conducted in triplicate.

Selectivity of sensors 1-3.

In a black 96-well plate, sensors 1-3 (2 μM for sensors 1 and 3 and 5 μM for sensor 2) was separately mixed with solutions (100 μM) of various biologically relevant species (NO, NO_2^-, NO_3^-, N_3^-, $ONOO^-$, Cys, Arg, ascorbate, H_2S, GSH, GSNO, H_2O_2 and $^{\cdot}OH$) in 1% DMSO in H_2O. The mixtures were incubated in the dark for 30 min before fluorescence (sensor 1: $\lambda_{ex/em}$ = 488 nm/512 nm; sensor 2: $\lambda_{ex/em}$ = 380 nm/460 nm; sensor 3: $\lambda_{ex/em}$ = 512 nm/550 nm) of each mixture was measured on the plate reader. The experiments were carried out in triplicate.

Quantum Yield Calculations

The integrated fluorescence intensity is calculated as a sum of the intensities over the emission spectra for each sample. The integrated fluorescence intensity from a blank was subtracted and the plot of the integrated fluorescence intensity as a function of absorbance should show a linear relationship. The slope of the linear fit for the standards, a_r, is used to calculate the quantum yield of the fluorescent protein, Φ_S. According to the equation:

$$\Phi_S = \Phi_R \frac{a_s}{a_r}\left(\frac{n_s}{n_r}\right)^2 \qquad (1)$$

where a_r is the quantum yield of the standard (Fluorescein; Φ = 0.93 (1), Quinine; Φ = 0.55 (2)), a_s is the slope of the linear fit for the integrated fluorescence intensity of the

fluorescent protein as a function of absorbance, and n_s and n_r are the refractive indices of the fluorescent protein and the standard solutions, respectively. Graph pad Prism was used for all linear fitting and calculations.

SUPPLEMENTARY METHODS – Biological validation

Chemicals and Sensors

LPS (Lipopolysaccharides from Escherichia coli O111:B4, L2630) and L-cysteine (L-cysteine Hydrochloride; C7477) were supplied by Sigma-Aldrich and Angeli's Salt (AS; 82230), carboxy-PTIO (cPTIO; 81540) and 1400W (1400W hydrochloride; 81520) from Cayman Chemicals and MTT (dimethylthiazol-diphenyltetrazolium bromide; M6494) from Life Technologies Australia. HNO sensors 1, 2 & 3 was synthesised and provided by Dr. Xiaozhou Zhang, School of Sciences, University of Adelaide. Details provided in 'Synthesis of sensors 1 – 3 section of this thesis manuscript.

Cell culture

Immortalized BV2 cells from a murine microglial cell line (BV2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher-Scientific), 100 IU/ml penicillin, 100 µg/ml streptomycin, (Penicillin-streptomycin; Sigma-Aldrich) 2 mM L-glutamine (Gibco, ThermoFisher-Scientific) and 100 µg/ml Normocin (InvivoGen). Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2 and used for assays when 75-80% confluent. To prepare samples for spectrophotometer or confocal experiments, 10,000 cells/ml of BV2 cells were seeded in a 96 well plate in 200 µl of media per well. Cells were grown to approximately 75-80% confluency prior to carrying out experiments. On the day of experimentation Sensors were added to wells and allowed to incubate for 15 – 30 min prior to the addition of stimulus (unless otherwise specified below).

Primary HCAEC (human coronary arterial endothelia cells) were cultured using MesoEndo Cell Growth Medium (Sigma-Aldrich) in a cell incubator at 37˚C with humidified 5% CO_2. Primary HCAECs from passages 3 and 4 were used to detect HNO. To prepare samples for spectrophotometer or confocal experiments, 2.5 x 10^4 cells/ml

of cells were seeded (quadruplicates) in a 96 well cell culture plates (in 200 µl of media) or 4 well ibidi slides (in 600 µl of media) and allowed to adhere overnight at 37 °C. Cells were grown to approximately 75-80% confluency prior to carrying out experiments.

H9C2 (rat cardiomyocyte) cells were cultured in ATCC-formulated Dulbecco's Modified Eagle's Medium (ATCC, Australia), supplemented with 10% fetal bovine serum (FBS; Gibco, ThermoFisher-Scientific) and maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO2 and used for assays when 75% confluent. To prepare cells for experimentation, 2.5 x 10^4 cells/ml were seeded (quadruplicates) either in 96 well cell culture plates (in 200 µl of media) or 4 well ibidi slides (in 600 µl of media) and allowed to adhere overnight at 37 °C.

Cell viability and function in the presence of Sensors 1, 2 and 3.

A trypan blue spectrophotometric assay (3) was used to measure the effect of 1.5 hours exposure to Sensors 1, 2 and 3 on BV2 cell death, before using them for HNO detection. Briefly, BV2 cells were exposed to 4 concentrations [0, 1, 5 & 10 µM] of each Sensor (1, 2 or 3) for 1.5 hours before removing media and replaced with a 0.05% Trypan blue solution in PBS (0.01M) and incubated (37°C) for 15 minutes. Cells were then gently washed 3x with ice cold PBS (0.01M) before adding 200 µl of 1% SDS solution (sodium dodecyl sulfate made in PBS) and contents gently triturated. Finally, 175 µl of the SDS/trypan solution was transferred into a clean 96 well culture plate and the absorbance read at 590 nm on a spectrophotometer.

Cytotoxicity of Sensors 1, 2 & 3 were further assessed using MTT (dimethylthiazol-diphenyltetrazolium bromide; Life Technologies Australia) assay. For the cytotoxicity experiments, 3 x 10^4 cells/ml BV2 cells or 2.5 x 10^4 cells/ml HCAEC cell, were seeded into a 96 well culture plate and incubated for 24 hours at 37°C with 5% CO_2 until cells were 75-80% confluent. Four time points of exposure were used: 30 minutes, 2 hours, 24 hours and 48 hours in the presence of 4 concentrations [0, 1, 5 & 10 µM] of each Sensor. At the end of the incubation, the supernatant was removed and 100 µl of MTT solution (0.25mg/ml) was quickly added to each well and the plate incubated for 2 hours

at 37 °C with 5% CO_2. After the incubation period, the MTT solution was removed and 100 µl of DMSO (dimethyl sulfoxide) added to each well for 10 minutes and the plate gently agitated. The absorbance was then measured on a spectrophotometer at 570 nm. The experiments were carried out in triplicate.

Detecting HNO in cell free HBSS buffer

The following series of experiments were all measured using a BioTek SynergyMx spectrophotometer. Measurements were taken at appropriate excitation/emission wavelengths for each Sensor (Sensor 1 $\lambda_{ex/em}$ = 470/512 nm, Sensor 2 $\lambda_{ex/em}$ = 387/456 nm, Sensor 3 $\lambda_{ex/em}$ = 518/550 nm). The relationship between Sensor concentration and HNO concentration was assessed in HBSS (Gibco, ThermoFisher-Scientific) buffer. Three concentrations of each Sensor [2.5, 5 & 10 µM] were assessed with increasing concentrations of AS were added [0, 0.195, 0.39, 0.78, 1.562, 3.125, 6.25, 12.5, 25, 50, 100 & 200 µM] and measurements taken every 2.5 minutes for 40-60 minutes total time. In a separate series of experiments, spectrophotometric fluorescent measurements for each Sensor [at 1 µM] were assessed in HBSS over 1 hour in the presence of 200 µM Angeli's salt. In another series of experiments, the limit of detection for each Sensor in the presence of Angeli's salt [200 µM] was determined in HBSS. Angeli's salt was added increasing concentrations of each Sensor [0, 0.1, 0.5, 0.75, 1, 5 & 10 µM] and measured after 1-hour incubation time.

Detecting HNO in cell free MesoEndo culture media

MesoEndo Cell Growth Medium (Sigma-Aldrich) was used to demonstrate the response of Sensor 3 to AS and L-Cysteine. Equal volumes of media were added to a 96 well plate together with different concentrations of L-Cysteine (0, 1, 10, 100, 200,500 and 1000 µM). Then 200 µM of Angeli's Salt was added for 15 minutes at room temperature and the plate was read between 580-640nm using Promega GloMax spectrophotometer.

Spectrophotometer experiments

LPS / 1400W experiments

LPS is known to induce increased iNOS enzyme expression and subsequent NO and other reactive nitrogen species production in BV2 cells (3) and was used as potential driver of endogenous HNO production in this study. BV2 cells (triplicates) were incubated with LPS (500 ng/ml) for 24 hours. In parallel experiments, cells were pre-incubated for 2 hours with 1400W (10 μM) prior to the addition of LPS for 24 hours, to demonstrate the reduction of fluorescence in the presence of an iNOS inhibitor. Control cells were incubated with 200 μM Angeli's salt, with and without 1 mM L-cysteine following the addition of Sensor 3, to demonstrate the increase and reduction of fluorescence in the presence of HNO donor and scavenger, respectively. Negative control wells included cells without any stimuli or absence (HNO Sensor only) of Sensor 3 (blank control). Following the 24-hour LPS incubation, Sensor 3 (2 μM) was added to each well (except blank control wells) and incubated for 30 minutes prior to measuring the unwashed cells on the spectrometer using a top-down spiral read.

H9C2 hypoxia experiments

Hypoxia-reoxygenation injury is a commonly used cell-based model of ischemia-reperfusion injury. Hypoxia is induced by incubating the cells with hypoxic gas mixture/buffers, causing energetic failure due to the lack of oxygen and driving anaerobic metabolism and can lead to oxidative stress (5). The replacement of hypoxic conditions, back to that of normoxic is akin to the reperfusion of oxygenated blood *in vivo* and can cause an immediate and significant spike in NO levels which is thought to drive the myocardial tissue injury (6). H9C2 cells (2.5×10^4 cells/ml; quadruplicates) were seeded in 96 well culture plates and allowed to adhere overnight at 37°C. Cells were exposed to either media or hypoxic buffer for 2 hours at 37°C. Samples of the supernatant (50 μl) were taken and added to 5 μM of Sensor 1 in a second 96 well culture plate, the remaining supernatant was removed and replaced with either media or normoxic buffer (Table 1) and left to incubate for 1-2 minutes before a second sample

(50 µl) was transferred to 5 µM of sensor 1 and read using a spectrophotometer at $\lambda_{ex/em}$ = 470/512 nm. The values of the second sample were subtracted from the first value to determine the difference in fluorescence signal. The experiments were carried out in triplicate.

Table 1

Combo	**Treatment 1**	**Treatment 2**
1	Media	Media
2	Hypoxic buffer	Media
3	Hypoxic buffer	Normoxic buffer
4	Media	Normoxic buffer

Confocal experiments

Acute LPS exposure experiments

To prepare samples for confocal imaging, BV2 cells (40,000 cells/ml) were seeded in 8 well ibidi slides (Cat: 80826) in 300 µl of media per well. Cells were grown to approximately 75-80 % confluency prior to carrying out experiments. Sensors 3 (1 µM) was added to each well and incubated for 30 minutes prior to imaging. A continuous time-lapse series was set-up to capture both fluorescence, 514nm/543nm (excitation/emission) wavelength and DIC images for 10-15 minutes. Following 1 min of recording baseline either an endogenous stimulant of HNO (LPS; 500 ng/ml), an exogenous donor (AS; 200 µM) or vehicle control (0.01M NaOH) was added to the well, followed by a further 15 minutes of recording. LPS is an inflammatory mediator known to increase ROS levels in BV2 cells (Kim et al., 2004). Control conditions included the addition of HNO donor, Angeli's salt (200 µM) with or without the HNO scavenger, L-cysteine (1 µM) or Sensor 3 only. Images for this series of experiments were captured

using a live cell heated and oxygenated system on an Olympus FV3000 inverted scanning confocal microscope, using a 40x magnification objective and 1024 x 1024-pixel image size. All images were taken at the same gain for mean pixel intensity comparisons. The experiments were carried out in triplicate.

HCAEC hypoxia experiments

For HCAEC confocal images experiments, cells were seeded in 2 x 4 well Ibidi slides (Cat: 80426) in 600 µl of media per well and allowed to adhere overnight. The media was then changed, and one slide was placed in a normoxic incubator (5% O_2) and the second slide placed in a hypoxic incubator (1.2% O_2) for 24 hours. Cells were approximately 95% confluency at this stage of the experiment. Cells were imaged (1024 x 1024) on a Leica TCS SP8X/MP Confocal Microscope using 40x objective. After the 24-hour treatment, z-stack fluorescence (514nm/543nm; excitation/emission wavelength) and DIC images were captured (baseline) immediately prior to the addition Sensor 3 (1 µM) with further z-stack images captured every 3 min for a total of 15 minutes following the addition of Sensor 3 for both normoxic and hypoxic slides. Control conditions included adding Angeli's salt (200 µM, 10 min) to cells incubated with Sensor 3 (15 min). All images were taken at the same gain for mean pixel intensity comparisons. The experiments were carried out in triplicate.

H9C2 hypoxia experiments

For H9C2 confocal imaging experiments, cells (2.5 x 10^4 cells/ml) were seeded on 4 well Ibidi slides (Cat: 80426) in 700 µl of media per well and allowed to adhere overnight at 37°C. Sensor 1 (2 µM) was added to the cells for 30 minutes in the presence of the first treatment. After the incubation period a z-series 60x magnification image was captured on SP5 Leica scanning confocal system, with a 512 x 512-pixel image size. The Treatment **1** buffer was then removed and replaced with the second Treatment **2** solution and allowed to incubate for 1-2 minutes before a second confocal image was captured of the same field of view for comparison (Table 2). Sensor 1 was detected using 512 nm/ 488 nm (excitation/emission) wavelength. The experiments were carried out in triplicate.

Table 2

Combo	Treatment 1	Treatment 2
1	Media	Media
2	Normoxic buffer	Media
3	Media	Normoxic buffer
4	Hypoxic buffer	Normoxic buffer

Localisation experiments

For co-localisation experiments, BV2 cells (40,000 cells/ml) were seeded in 8 well ibidi slides (Cat: 80826) and allowed to adhere overnight. The cells were incubated with Sensor 3 (2 μM) in media for 15 minutes then washed with HBSS before incubating for a further 15 minutes with 100 nM Mitotracker Deep Red (Cat# M22426; ThermoFisher-Scientific). The cells were washed with HBSS and incubated with Angeli's salt (200 μM) for 20 minutes and 1024 x 1024 z-stack images taken on an Olympus FV3000 scanning confocal microscope using a 60x objective. Sensor 3 was detected using a 514nm/543nm (excitation/emission) wavelength and Mitotracker Deep Red was detected using a 644nm/665nm (excitation/emission) wavelength. DIC filter was used to detected cells on the dish.

Image analysis

The mean pixel intensity of either Sensor 1 (488 nm emission) or Sensor 3 intracellular fluorescence (543 nm excitation channel) of 20 randomly selected cells, were analysed using Image J (FIJI version 1.52d) and compared between control and treatment conditions as outlined below. To determine the change in fluorescence signal, 20

randomly selected BV2, H9C2 or HCAEC cells were outlined using ImageJ (v1.52p, National Institute of Health, USA) from the DIC image, then the mask applied to the 515 nm channel and the mean pixel intensity (expressed as Random Fluorescent Units: RFU) was measured for those cells. The baseline values (1 min pre-treatment time) were subtracted from the brightest frame in the captured series at approximately the 10-minute time point and the data expressed as the percentage change in fluorescence (reported as 'fold change from baseline') from the baseline frame.

Blood collection and sensor experiments

All animal care and handling procedures were approved by Animal Ethics Committee of the University of Adelaide. All procedures were performed in accordance with the Australian Code for the Care and Use of Animals for Scientific Purpose (2013), ethics application M-2017-005. Sprague Dawley, male rats were bred in-house at the Laboratory Animal Services facility at University of Adelaide. Adult male rats were deeply anesthetized with intraperitoneal sodium pentobarbitone (60mg/kg). After thoracotomy, blood was directly drawn from the heart in to a 1 mL syringe containing 1 of 2 series of treatment conditions. Treatment 1 was EDTA (0.5M)/PBS solution and either; 500 µl of 20 µM Sensor 3 in PBS, 500 µl of 100 µM Sensor 3 with L-cysteine (HNO scavenger; 100, 500 or 1000 µM) in PBS or 500 µl PBS as the vehicle control without Sensor 3. Treatment 2 was as described above without EDTA present. The drawn blood was left to incubate in the dark for 30-minute at RT before centrifuging at 3000 rpm for 10 minutes then 100 µl of either serum (samples without EDTA) or plasma (samples with EDTA) was extracted, snap frozen in a 96 well (quadruplet) and stored in -80 °C for 24 hour and thawed prior to reading. After the first read, HNO donor AS was added to the specified wells, incubated in the dark for 30 mins, before a second read was carried out. The samples were processed in three groups and all reading were done on the SynergyMx Microplate Reader at λex = 518 nm and λem = 550 nm.

Statistical analysis

Data were analysed using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA,

USA). Shapiro-Wilk or Kolmogorov-Smirnov normality test was used to test the normality of data distribution. Paired or unpaired t-test was used where relevant to compare the fluorescence counts under different conditions and the two-tailed *p*-value for significance was < 0.05. One-way ANOVA was used to compare three or more groups and the *p*-values for significant differences were derived from a relevant post-hoc test for multiple comparisons. All data are reported as mean ± standard error of the mean (SEM), along with the individual data points where relevant to demonstrate the biological variability.

Supplementary References

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