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Understanding the role of oxidative stress in disease requires real time monitoring of redox status within a cell. We report a FRET-based, ratiometric redox probe which can be applied to monitor cellular oxidative capacity using three different modalities – confocal microscopy, fluorescence lifetime imaging and flow cytometry.

The oxidative capacity of a cell is a consequence of the ratio of the pro-oxidants and anti-oxidants that it contains. Pro-oxidants, which include reactive oxygen species (ROS), are vital for physiological functions such as signal transduction and phagocytosis, in sub-nano-molar concentrations.[1–3] Transient increases in pro-oxidant concentrations are balanced by anti-oxidants in healthy cells. However, uncontrolled production of pro-oxidants results in a chronically elevated cellular oxidative capacity commonly called oxidative stress.[4,5] While oxidative stress is thought to play a role in many pathologies such as Alzheimer's disease, cardiovascular disease, obesity, diabetes and arthritis,[6–9] the underlying mechanisms and pathways linking oxidative stress and disease are far from understood.

To date, attempts to quantify oxidative stress have focused on imaging production of individual ROS[10–12] or their effects on common redox couples that exist in the cell (such as GSH/GSSG),[13] but there is a need for tools to provide an overall picture of the cell's oxidative capacity. This can be best understood by measurement of the cellular redox state.

A limited number of fluorescence-based redox sensors exist based on both simple organic fluorophores and fluorescent proteins.[14–18] These probes are intensity-based, with redox events eliciting changes in the emission intensity of a single fluorescence peak.[19] As a result, application of such probes in quantifying oxidative stress is limited by variations in probe concentration, probe environment (pH, transition metals) and instrumental factors (such as inconsistencies in the excitation source and emission inefficiencies). These effects can be nullified by the use ratiometric probes, in which two different emission signals respond differently to the condition being sensed. The ratio of these two intensities therefore reports on the condition, independent of any background effects.[20,21] One well established method of developing ratiometric probes is by Förster Resonance Energy Transfer (FRET) - a distance dependent energy transfer mechanism that operates between two fluorophores, wherein the emission profile of one fluorophore (referred to as the donor) shows a significant overlap with the excitation profile of the other fluorophore (the acceptor).[11,22,23]

While most attention in the application of fluorescent probes has centred on confocal microscopy, valuable and complementary information can be given by other imaging modalities. Fluorescence lifetime imaging is a robust modality that is unaffected by concentration and inner filter effects, spurring recent interest in the provision of probes for lifetime imaging.[24] This technique is commonly utilised to investigate FRET processes. Furthermore, accurate quantitative information about individual cells in a large cell population can be acquired using flow cytometry.[25]

To this end, we report the synthesis of a novel ratiometric fluorescent redox sensor, flavin coumarin redox sensor 1 (**FCR1**) and demonstrate its utility in reporting on cellular redox state *via* confocal microscopy, fluorescence lifetime imaging microscopy and flow cytometry. **FCR1** was designed as a FRET sensor of redox state. The two fluorophores in the FRET pair were chosen to be a non-redox-responsive coumarin donor molecule and a redox-responsive flavin acceptor (Figure 1). In order to ensure a well-defined and consistent energy transfer distance, we included a rigid linker to tether the two fluorophores. Flavin is particularly interesting as a redox responsive group due to its involvement in several subcellular redox processes, ensuring that its reduction lies well within biologically-relevant potentials. We and others have utilised the fluorescence properties of flavin in intensity-based fluorescent probes.[14,16]

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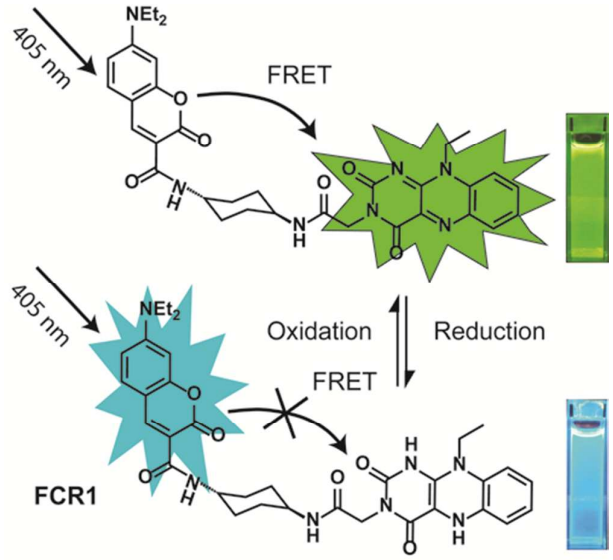

Figure 1: Design of **FCR1**, showing FRET processes in oxidised form. Inset: photographs of cuvettes of **FCR1** in oxidised and reduced forms under 365 nm excitation.

Coumarin was chosen as the donor molecule because of a significant overlap of its emission with the absorbance profile of the oxidised flavin molecule (Figure S1). For the oxidised probe, therefore, there will be FRET between the coumarin and the flavin, resulting in green emission from the flavin acceptor. In the reduced form, flavin adopts a bent conformation that is colourless and non-fluorescent,[26] therefore decreasing the spectral overlap between coumarin and flavin, interrupting the energy transfer. The reduced probe will therefore exhibit blue emission from the coumarin.

FCR1 was synthesised by the reaction of *N*-ethyl flavin and coumarin across a central cyclohexane linker (Scheme S1). Condensation of 4-diethylaminosalicylaldehyde with diethylmalonate gave 7-diethylaminocoumarin-3-carboxylic acid, which was then activated to its *N*-hydroxysuccinimide ester which, upon reaction with trans-1, 4-diaminocyclohexane, gave the corresponding amide. *N*-Ethyl flavin was synthesised using the standard 6-chlorouracil method.[27] Alkylation of *N*-Ethyl flavin with bromoethylacetate gave the *N*3- alkylated flavin which was then hydrolysed to carboxylic acid and activated with thionyl chloride to give the corresponding acid chloride. Condensation of this acid chloride with the coumarin cyclohexylamide in the presence of a base gave **FCR1** in moderate yield.

Photophysical characterisation of **FCR1** was performed in HEPES buffer (100 mM, pH 7.4). In the oxidised form, excitation of **FCR1** at 405 nm resulted in a green fluorescence with maximum emission at 525 nm (Φ = 0.242). The absence of the blue donor emission is consistent with an efficient FRET interaction between the two fluorophores in this state. Treatment of **FCR1** with a mild reducing agent, sodium cyanoborohydride, reduced the flavin. This resulted in a decrease in the intensity of green fluorescence accompanied by a simultaneous increase in the blue fluorescence band centred at 475 nm. (Figure2). This is consistent with a decrease in FRET interaction between the donor and acceptor fluorophores. The ratio of flavin to coumarin emission intensities (I520 / I475) upon excitation of **FCR1** at 405 nm decreased approximately 6-fold upon reduction (Figure 2b). The reduction of **FCR1** can also be achieved by other mild reducing agents such as sodium cyanoborohydride, dithiothreitol (DTT) and glutathione (GSH). Re-oxidation of **FCR1** could be achieved by mild oxidizing agents (Figure S2) although this re-oxidation process was much slower. Control experiments confirmed that the ratio of the emission intensities in oxidised and reduced forms were unaffected by the presence of common metal ions (Figure S3) and remained constant over the pH range 2 – 9 (Figure S4).

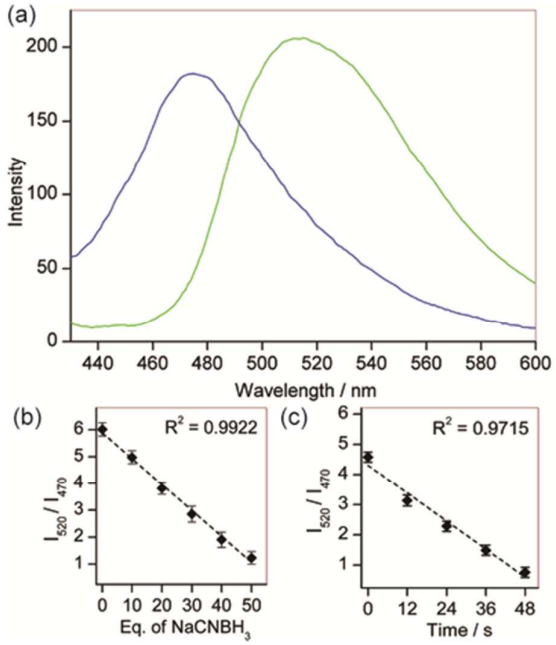

Figure 2: (a) Fluorescence behaviour of **FCR1** (10 μM, λ_{ex} = 405 nm) in the oxidised (green) and reduced (blue) form upon addition of NaBH3CN; ratio of green/ blue emission of **FCR1** (10 μM) (b) with incremental addition of $NaBH_3CN$ in 100 mM HEPES buffer and (c) over time after the application of a potential of -1.3 V to **FCR1** (100 μM) in MeCN.

Electrochemical studies by cyclic voltammetry confirmed the electrochemical reversibility of the probe, with a reduction potential of -1.15 V vs ferrocene (Fc) in acetonitrile, which is similar to that reported for riboflavin (-1.18 V vs. Fc),[28] thus confirming that addition of the second fluorophore has not altered the redox potential of the probe. To provide a sufficient potential window for electrochemistry, acetonitrile was used as the solvent for all electrochemical measurements. The peak current (I_p) for the main process varies linearly with the square root of scan rate (Figure S5) showing that the electro-reduction of **FCR1** is a normal diffusion-controlled process under these conditions. The asymmetry in the cathodic and anodic branches (Figure S6) suggests a degree of chemical irreversibility of the molecule, which also explains the slow re-oxidation observed.

Spectro-electrochemical studies were performed, in which the probe was reduced by applying a potential of -1.3 V and its fluorescence spectrum was measured every 12 s. As suggested by

the voltammetric analysis in Figure S6, the product of the electrochemical reduction seems to be less stable in acetonitrile compared to aqueous media (HEPES buffer). Despite this, Figure 2(c) shows that it was possible to observe a similar trend in the change of the ratio of fluorescence response at 520 nm over that at 475 nm.

FCR1 was also tested for its cytotoxicity in HeLa cells by an MTT assay. The IC_{50} value was found to be 80 µM over 24 h. Having demonstrated the redox sensitivity, ratiometric response and non-cytotoxic behaviour of the probe, we next sought to test its ability to respond to changes in the oxidative capacity of cultured cells using multi-photon confocal microscopy. A two photon excitation wavelength of 820 nm was used, as it gave the best signal to noise ratio (Table S1). Furthermore, the spectroscopic behaviour of the reduced and oxidised forms of **FCR1** with 820 nm two-photon and 405 nm single photon excitation were not significantly different (Figure S7). HeLa cervical cancer cells treated with **FCR1** (10 µM, 15 min) showed significant fluorescence in both blue (420-470 nm) and green channels (520-600 nm), while untreated control cells showed no noticeable fluorescence in either region. The concentration of **FCR1** used in these experiments is far below the IC_{50} value. Figure 3 clearly shows that the cells sequentially treated with **FCR1** and the reductant *N*-acetyl cysteine (NAC) demonstrated a lower intensity ratio when compared to cells treated with probe alone, while cells oxidised with H_2O_2 showed a higher ratio, in agreement with the FRET based ratio changes in solution.

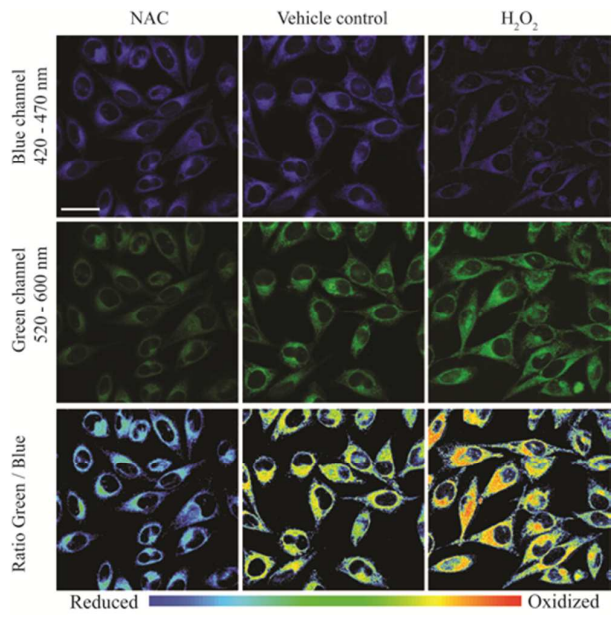

Figure 3: Two photon - confocal microscopy imaging of HeLa cells treated with **FCR1** (10 µM, 15 min, λ_{ex} = 820 nm) and (a) *N*-acetyl cysteine (50 µM, 30 min), (b) vehicle control and (c) H_2O_2 (50 µM, 30 min) in blue and green channels. The pseudo colour ratio images indicate the ratio of emission intensity in the green channel to blue channel. Scale bar represents 20 µm.

In addition, the oxidative capacity of peroxide-treated HeLa cells was analysed at different time points (Figure S8). These studies showed that, with increasing peroxide treatment times up to 1 h, there was an increase in the average intensity ratio of **FCR1** and thereby the cells' oxidative capacity. A much lower intensity ratio after 2 h of peroxide treatment highlights the cells' ability to restore its redox homeostasis.

The FRET efficiency of **FCR1** was examined using fluorescence lifetime imaging microscopy (FLIM) of HeLa cells treated with either the 7-diethylaminocoumarin donor or with **FCR1**. In the absence of acceptor, the donor lifetimes were found to fit a single component decay curve with a lifetime of 2.3 ns (Figure S9(a)), whilst in the presence of the acceptor, a two component fit with a lifetime of 1.1 ns (69%) and 2.3 ns (31%) was obtained (Figure S9(b)), indicating a 36% FRET efficiency between the coumarin donor and flavin acceptor moieties in **FCR1**. In addition to the ratio images obtained from confocal microscopy, the mean fluorescence lifetimes of the donor fluorophore can also be used to report cellular redox state. As seen in Figure 4, reduced cells have higher T_m (2.0 ns) in comparison to normal (1.7 ns) and oxidised cells (1.3 ns).

The redox status of cells can also be followed by flow cytometry, which enables collection of data from a large population of cells. HeLa cells treated with **FCR1** were interrogated by flow cytometry with excitation at 405 nm, and emission collected with windows centred at 450 and 560 nm. Cells treated with **FCR1** showed considerably greater fluorescence in both windows than untreated cells (Figure S10). Populations of cells treated with either H_2O_2 or NAC could be clearly distinguished from control cells on the basis of the fluorescence ratio (Figure 4(b)).

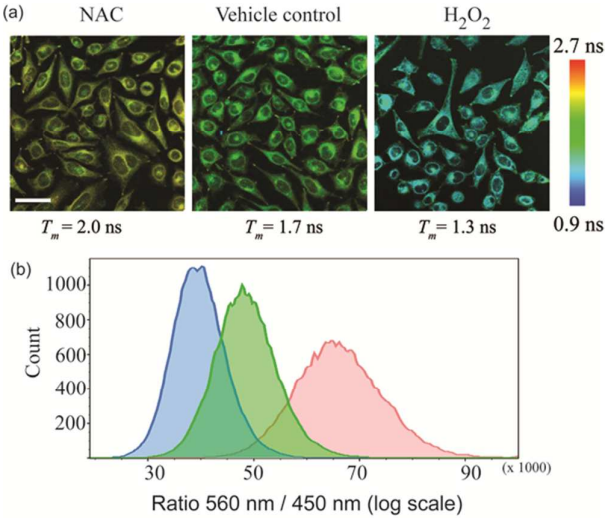

Figure 4: (a) Fluorescence lifetimes of the donor (420 – 470 nm) in HeLa cells treated with **FCR1** (10 µM, λ_{ex} = 820 nm) and *N*-acetyl cysteine, vehicle control and H_2O_2. Pseudo-colour images represent mean lifetime. Scale bar represents 50 µm. (b) Flow cytometric studies of HeLa cells treated with **FCR1** (10 µM, λ_{ex} = 405 nm) showing the fluorescence ratio (560 / 450 nm) when treated with *N*-acetyl cysteine (blue), vehicle control (green) and H_2O_2 (red).

In conclusion, **FCR1** shows great promise as a tool to study oxidative stress in biology. With its ratiometric output, the probe can be used to observe changes in oxidative capacity without interference from background effects such as probe concentration. As well as demonstrating the utility of **FCR1** in detecting changes by confocal microscopy, we have shown that it is a useful probe for both FLIM and flow cytometry. No doubt, ratiometric sensors of overall

cellular oxidative capacity such as **FCR1**, will provide a valuable tool for the future study of oxidative stress in biology.

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Notes and references

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