RedoxiFluor: A microplate assay to quantify protein thiol redox state in percentages and moles

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Abstract

An accessible, time- and cost-efficient microplate assay to quantify protein thiol redox state in percentages and moles relative to the thiol proteome (i.e., context) and other targets (i.e., array mode) would be invaluable for understanding how protein thiols regulate essential biological processes. RedoxiFluor achieves several key benefits (i.e., percentages, moles, context, array mode) in a microplate format. After robustly validating RedoxiFluor, comparative analysis reveals that key benefits are intractable to other immunological techniques. Moles is an unprecedented achievement. Proof-of-concept studies illuminating fundamental redox principles (i.e., specificity, context, and heterogeneity) through measurement alone demonstrate how RedoxiFluor can advance understanding. For example, target specific protein thiol redox state changes are: (1) context specific (i.e., redox stimulus dependent); (2) selective (i.e., redox stimuli oxidise select targets); and (3) heterogenous (i.e., target responses vary markedly). RedoxiFluor is a powerful new tool for advancing a far-reaching and influential field: protein thiol redox biology.

Introduction

Protein thiol redox switches regulate essential biological processes by changing protein activity, locale, interactome, lifetime, and phase\(^1\)\(^-\)\(^4\). Flipping a redox switch by changing target protein thiol specific redox state (i.e., increasing reversible oxidation) is central to understanding what Reactive Oxygen Species (ROS) do and how they do it (e.g., ROS regulate protein phosphorylation by oxidising phosphatases)\(^5\)\(^-\)\(^7\). Biological context dictates whether target specific protein thiol redox state changes are beneficial (i.e., redox signalling) or harmful (i.e., oxidative stress)\(^8\).

From a measurement perspective, understanding protein thiol redox biology relies on: (1) quantifying redox state changes in percentages; (2) quantifying redox state changes in moles; (3) contextualising redox state changes relative to the thiol proteome; and (4) multiplexing proteins. Placing target thiol redox state along a set spectrum 0-100% reversibly oxidised is essential\(^9\). Consider two redox regulated enzymes: A and B. If A is 50% oxidised and B is 2% oxidised, a thiol reductant should only impact the activity of A. Assuming [A] and [B] are equal and similar ROS reactivity (i.e., kinetics), B should be more sensitive to a thiol oxidant since reversible oxidation occupancy can increase by 98 compared to 50%. Moles are an important redox modifiable functional parameter: more of less of a [protein] can be biologically significant\(^10\). Moles rationalise percentage changes. If [A] is 50 pM and [B] is 100 pM and a redox stimulus increases the amount of reversibly oxidised protein by 5 pM, it would translate to a percent increase of 10 and 5%, respectively. Differential protein content produces a 50% difference in percentage change magnitude. Together moles and percentages can unravel threshold gating (i.e., how much of a target needs to be oxidised to exert a biologically meaningful functional effect) and rationalise the extent to which target specific protein thiol redox state should be chemically and genetically manipulated. Context is key. Comparing target specific protein thiol redox to the bulk thiol proteome is essential (i.e., it can reveal whether a target is highly oxidised)\(^11\). Finally, array mode is needed to
simultaneously screen the redox state of multiple proteins to account for biological complexity\textsuperscript{12}. For example, multiple redox switches distributed across several proteins regulate growth factor signalling\textsuperscript{13–15}.

No redox proteomic or immunologically technique achieves percentages, moles, context, and array mode. Global redox proteomics achieves percentages, context, and by definition array mode\textsuperscript{9}. Targeted redox proteomics can achieve molar quantification using deuterated peptides at the expense of context and by definition limits the number of proteins measured\textsuperscript{16}. Coverage is often limited to abundant proteins—detecting hydrophobic and/or weakly expressed proteins is challenging\textsuperscript{17}. Context is limited to what can be detected (i.e., global measures are derived from mass spectrometry detectable cysteines). Even targeted redox proteomics cannot detect many potential redox regulatory thiols (e.g., 30\% in the redox regulated phosphatase PTP1B\textsuperscript{16,18}) because they reside in difficult to digest sequences or poorly ionisable peptides\textsuperscript{19}. Pressingly, access to redox proteomics can be rate-limiting. While readily accessible immunological techniques report the global target specific protein thiol redox state (i.e., all modifiable thiols are detected), they often cannot measure the target at all. If a target is detectable, redox analysis is usually qualitative or semi-quantitative at best\textsuperscript{20}. When quantitative analysis is possible, it is limited to fold changes\textsuperscript{21}. Immunological techniques lack percentages, moles, context, and array mode.

To quantify target specific protein thiol redox state in percentages and moles relative to the thiol proteome (i.e., context) and other proteins (i.e., array mode), we present RedoxiFluor. RedoxiFluor can quickly and inexpensively deliver actionable results (i.e., a change in target specific protein thiol redox state) in a widely accessible microplate format. The microplate format affords specific high-throughput and automated plate reader analysis, as well as, the ability to readily calibrate the assay using known protein and redox standards. The proof-of-principles studies reported herein demonstrate the value of achieving key benefits (i.e., percentages, moles, context, and array mode) by deriving biological insights from RedoxiFluor alone.

**Results**

**RedoxiFluor**

RedoxiFlour uses two thiol-reactive fluorescent reporters and a capture antibody functionalised solid support to measure target protein thiol redox state in percentages and moles in a microplate (Figure 1). Reduced thiols are labelled with a fluorescent maleimide (F-MAL1) reporter via a thioether bond. Reversibly oxidised thiols (RS\textsubscript{OX}) are reduced to the maleimide reactive sulphydryl state (RSH/RS\textsuperscript{-}) using 1,4-dithiothreitol (DTT) and decorated with a spectrally distinct fluorescent maleimide reporter (F-MAL2). Optionally, a specific chemotype (e.g., sulfenic acids) can be labelled using selective reductants or a direct reactivity strategy\textsuperscript{22,23}. Direct reactivity strategies can be used to label DTT irreducible sulfenic and sulfonic acids\textsuperscript{24}. A capture antibody functionalised solid support is used to bind the target protein thiol from a biological sample (e.g., cell lysate). The thiol redox state encoded F-MAL reporters enable one to
quantify protein thiol redox state in percentages (i.e., protein A mode). A biotin-conjugated detector antibody and recombinant protein standard curve (i.e., ELISA mode) enable one to calculate target specific protein thiol redox state in percentages and moles. Global mode RedoxiFluor (i.e., untargeted F-MAL1/2 analysis) can contextualise target specific protein thiol redox state relative to the thiol proteome. Array mode RedoxiFluor can quantify the redox state of several proteins in percentages (i.e., protein A mode). Follow-up ELISA mode RedoxiFluor can confirm and extend (i.e., percentages and moles) array mode findings. RedoxiFluor achieves percentages, moles, context, and array mode in a widely accessible microplate format.

**RedoxiFluor can quantify target specific protein thiol redox state in percentages and moles**

Mixing equimolar F-MAL1/2 standards to construct artificial redox states from 90 to 10% reversibly oxidised in L-cysteine buffer to exact any thiol dependent turn-on fluorescence confirmed that RedoxiFluor can calculate redox state in percentages (supplementary Figure 1). Experiments with fully F-MAL1/2 labelled recombinant bovine serum albumin (BSA) show that RedoxiFluor can discern between different redox states from 100, 75, 50, 25, to 0% reversibly oxidised (supplementary Figure 1). No effect of fluorophore labelling order is observed: the same result is obtained if F-MAL1 or F-MAL2 is used first (supplementary Figure 1). Unbound and protein conjugated F-MAL experiments validate RedoxiFluor.

To validate RedoxiFluor using a capture antibody to bind a target from a complex biological sample, we selected the catalytic subunit (i.e., PPP2CA, uniport ID: P67775) of the serine/threonine protein phosphatase PP2A: a strategically important redox regulated target. To measure PP2A, we bound a capture antibody to a protein A derivatised microplate. Protein A mode RedoxiFluor accurately (e.g., the mean observed redox difference from the standard was 0.8%) and reproducibly (e.g., the mean CV value between samples was 2.9%) quantifies the redox state of PP2A in 10-90% reversible oxidised standard samples (Figure 2A and Table 1). Accurate and reproducible redox state reporting confirms the suitability of our labelling protocol. RedoxiFluor is specific: no discernible F-MAL signals were observed when samples (50% F-MAL1/ 50% F-MAL2) were incubated with rabbit immunoglobulin (IG) control or blank (i.e., protein A only) wells and immunodepleting PP2A abolished the signal (supplementary Figure 2). In protein A mode, RedoxiFluor consumed minimal sample (0.5-1 ug), antibody (0.1 ug per well), and rapidly delivered actionable results (i.e., 3-4 h). For context, we performed comparative macroscale and microscale immunological analysis. Click-PEG wherein clickable polyethylene glycol (PEG) payloads are used to detect reversibly oxidised thiols as mass shifted bands by immunoblot cannot report PP2A redox state (Figure 2B). PEG decorated PP2A is undetectable. While ALISA works, it cannot compute PP2A redox state in percentages (Figure 2C). RedoxiFluor can quantify target protein thiol specific redox state in percentages—a feat that proved intractable to other immunological assays.

ELISA mode RedoxiFluor can accurately (e.g., the mean observed redox difference from the standard was 1.3%) and reproducibly (e.g., the mean CV value between samples was 4.1%) (Figure 2D and Table 1) measure the redox state of PP2A in 10-90% reversible oxidised sample standards. Specificity is evidenced by the positive biotin-conjugated detector antibody dependent signal in sample standards and lack of any
discernible signal in the immunodepleted and IG controls, as well as, blanks (supplementary Figure 2). The recombinant protein standard curve evidences an ability to detect PP2A in the picogram range (i.e., from 8,000 to 125 pg/ml, supplementary Figure 2). To illustrate the significance, we generated new 20 and 40% reversibly oxidised redox state standards. The recombinant protein standard curve, can convert percentages into picomoles of reduced and oxidised protein. Picomoles of reduced PP2A are significantly greater in the 20 compared to the 40% reversibly oxidised redox state; as confirmed by a corresponding decrease in picomoles of reversibly oxidised PP2A (Figure 2E-F). ELISA mode RedoxiFluor consumed minimal sample (0.5-1 ug), antibody (0.1 ug per well), and delivered actionable results in 12-16 h (i.e., extra time is needed to bind the capture antibody and block the plate). RedoxiFluor can quantify target specific protein thiol redox state in percentages and moles.

Table 1. Measured PP2A specific RedoxiFluor standard values in protein A and ELISA mode. Values reported are mean (M), standard deviation (SD), the mean observed difference from the standard (i.e., 2% if a mean of 48 was registered for the 50% standard), and co-efficient of variation (CV) between standards. All values are reported in percentages. All CV values within standards (i.e., values from triplicate readings of the same sample) were all less than 5%. All standards were derived from *Xenopus laevis* lysates (see methods).

| Mode | Protein A | ELISA |
|------|-----------|-------|
|      | M          | SD    | Difference | CV1 | M | SD | Difference | CV |
| 90   | 90.2       | 1.1   | 0.2        | 1.7 | 89.1 | 0.48 | 0.9 | 0.5 |
| 80   | 81.1       | 1.1   | 1.1        | 1.2 | 81.2 | 2.9 | 1.2 | 3.6 |
| 70   | 71.5       | 0.9   | 1.5        | 0.7 | 70.7 | 1.4 | 0.7 | 2   |
| 60   | 60.6       | 1.1   | 0.6        | 1.8 | 62.1 | 1.2 | 2.1 | 1.9 |
| 50   | 48         | 1.2   | 2          | 2.6 | 52.6 | 0.3 | 2.6 | 0.6 |
| 40   | 41.1       | 2.1   | 1.1        | 5.1 | 39.2 | 1.6 | 0.8 | 4   |
| 30   | 30.3       | 0.52  | 0.3        | 3   | 32  | 1.5 | 2   | 4.8 |
| 20   | 19.9       | 2.9   | 0.1        | 2.9 | 20.4 | 2   | 0.4 | 9.6 |
| 10   | 10.3       | 0.8   | 0.3        | 7.3 | 11.3 | 1.1 | 1.3 | 9.7 |
| Mean | n/a        | 1.3   | 0.8        | 2.9 | n/a | 1.4 | 1.3 | 4.1 |

Redox immunology part 1: Protein A and ELISA mode RedoxiFluor reveal a dramatic LPS induced increase in IRAK1 specific reversible thiol oxidation.
Having established the ability of RedoxiFluor to measure the redox state of calibrated standards (i.e., knowns), we determined the redox state of experimental samples (i.e., unknowns) in a topical and translationally important immunology context. We measured the redox state of interleukin-1 receptor-associated kinase 1 (IRAK1, uniport ID: P51617) in unstimulated and lipopolysaccharide (LPS, 100 ng/ml for 30 min) stimulated human monocytes. LPS activates the innate immune response via toll-like receptors (TLR)\textsuperscript{28}. IRAK1 helps activate TLR in response to LPS\textsuperscript{29}. We report a potential link between the LPS induced increase in ROS and the redox state of IRAK1: a strategically important and contextually relevant protein kinase\textsuperscript{30}. Protein A mode RedoxiFluor reveals that LPS markedly increases IRAK1 specific reversible thiol oxidation by 33.7% from 41.05 to 74.75% (Figure 3A-C). Note, IG controls are challenging in THP-1 cells: they express Fc binding proteins. ELISA mode RedoxiFluor confirms the dramatic (+48%) increase in IRAK1 specific reversible thiol oxidation in LPS stimulated compared to unstimulated monocytes (Figure 3D-F). Since both modes accurately report protein thiol redox state (Table 1), we attribute the greater increase in ELISA compared to protein A mode RedoxiFluor to baseline variability in unstimulated cells. While IRAK1 protein content remains at ~1.8 pM (Figure 3G), reversibly oxidised IRAK1 increased by 0.9 pM in LPS stimulated compared to unstimulated controls (Figure 3H, supplementary Figure 3). Analogous to redox proteomics and immunological techniques, the change in target specific protein thiol redox state reflects differential oxidation formation and/or repair\textsuperscript{3}. Despite a profound (mean change = 40.9%) increase in IRAK1 specific reversible thiol oxidation, the thiol proteome remains highly reduced (i.e., ~85%) irrespective of LPS (supplementary Figure 4). A 15% reversibly oxidised value is consistent with previous literature\textsuperscript{11,31} and shows that our lysis protocol limits \textit{ex vivo} oxidation\textsuperscript{32}. Global RedoxiFluor—a standalone assay—confirms the specificity of the LPS response by ruling out a global proteome wide oxidative shift. It also highlights the greater than the sum of their parts value of combining a global and specific redox measure. RedoxiFluor discovered a new molecular feature of the innate immune response by quantifying IRAK1 redox state in percentages and picomoles.

\textbf{Redox immunology part 2: Array mode RedoxiFluor reveals LPS increases PP2A, PTP1B, SHP1, and CD45 specific reversible thiol oxidation.}

IRAK1 RedoxiFluor realises three key benefits (i.e., context, percentages, and moles). To achieve array mode RedoxiFluor we configured a microplate to simultaneously measure the redox state of several redox regulated phosphatases in unstimulated and LPS stimulated human monocytes (supplementary Figure 5 and supplementary Table 1). To do so, we used a cost- and time-efficient protein A microplate format. In unstimulated cells, protein phosphatase redox state ranged from 75.6 (i.e., PTP1B) to 88.2% (i.e., SHP2) reduced, with most targets clustering (i.e., within 3%) around 85%—the global protein thiol redox state. PTP1B and PP2A were, however, displaced from 85% by 10 and 6%, respectively. Array mode RedoxiFluor revealed no significant difference in SHP2, calcineurin, and PTEN redox state—they remained highly reduced (i.e., ~90-85%) in unstimulated and LPS stimulated cells (Figure 4, supplementary Figure 6-7). Consistent with both the specificity of LPS induced redox changes and heterogeneity within an enzyme class (i.e., phosphatases), array mode RedoxiFluor reveals a significant LPS induced increase in PTP1B (+4.8%), SHP1 (+10.4%), CD45 (+10.1%), and PP2A (+8.4%) specific reversible thiol oxidation.
Within the responsive phosphatases, the magnitude of the LPS induced change ranged from 4.8% to 10.4% (Figure 4, supplementary Figure 6-7). Aside from PTP1B (~75% to 70%), LPS decreased their redox state from between 88 to 80% to 78-70% reduced. To validate one of the “hits”, we selected PP2A for follow-up ELISA mode RedoxiFluor. ELISA mode RedoxiFluor confirmed the LPS increase in PP2A specific reversible thiol oxidation (+6% from 19 to 25%, supplementary Figure 8). After confirming PP2A content remains at ~44 pM, ELISA mode RedoxiFluor extended the protein A screen by revealing that the percentage change corresponds to a 3.6 pM increase in the amount of reversibly oxidised PP2A from 8.4 to 11 pM (supplementary Figure 8). RedoxiFluor can screen how multiple strategically important targets respond to a fundamental and translationally relevant redox stimulus.

**Developmental biology: Macroscale RedoxiFluor reveals a fertilisation induced increase in PTEN specific reversible thiol oxidation**

In the final worked example, we studied a fundamental process: fertilisation in *Xenopus laevis* (X. laevis). How fertilisation impacts PTEN redox state is unknown, so we measured PTEN redox state in unfertilised eggs and 1-cell zygotes (i.e., 30 min post fertilisation). Protein A mode RedoxiFluor reveals a significant fertilisation induced increase (+20%) in PTEN specific reversible thiol oxidation (Figure 5, supplementary Figure 9). In line with specificity, we observed no background F-MAL signal in IG compared to blank wells (supplementary Figure 9). When matched pair antibodies are unavailable, it can be useful to confirm microscale findings in macroscale mode to visually verify PTEN. Macroscale RedoxiFluor is valuable because it measures a redox modifiable functional property: protein interactome. To study intermolecular disulfide bonds, one should covalently bind the capture antibody and complete the F-MAL labelling following elution (i.e., to preserve the bond and eliminate antibody thiols). After verifying successful PTEN “pull-down” by immunoblot (supplementary Figure 9), we measured the aggregated redox state of the PTEN interactome in a microplate (i.e., measuring the redox state of eluent aliquots). The collective redox state of the six co-eluting proteins (i.e., the PTEN interactome) is impervious to fertilisation (Figure 5, supplementary Figure 9). However, band 2 is only present in zygotes and is largely oxidised. Consistent with the microscale protein A finding, PTEN specific reversible thiol oxidation, as measured by analysing the eluent from the excised 55 kDa band in a plate reader, is increased (+14%) in 1-cell zygotes compared to unfertilised eggs (Figure 5, supplementary Figure 9). Target specific redox state can change without a corresponding redox interactome change. PTEN redox state is displaced from the thiol proteome, but this result and the accompanying redox proteomic data will be published separately in due course. One could test the redox theory of development by determining whether fertilisation induced redox changes inactivate PTEN to promote PI3K signalling. PTEN, an LPS unresponsive phosphatase, highlights the importance of biological context. RedoxiFluor can operate at extremes of the analytical spectrum: from the micro to macroscale.

**Discussion**
RedoxiFluor achieves hitherto intractable benefits: percentages, moles, context, and array mode. Seldom have so many key benefits been achieved in one step or in a widely accessible microplate format. Percentages, moles, context, and array mode are inaccessible to other immunological techniques\textsuperscript{20}. Recent technical developments enable percentage cysteine residue redox state to be quantified on a proteome wide scale (i.e., array and context)\textsuperscript{9,35}. RedoxiFluor measures all target specific redox modifiable thiols; which, for many targets is a breakthrough achievement (i.e., proteomics has yet to detect 94 and 50\% of the thiols in IRAK1 and PP2A, respectively). The molar benefit is unprecedented. Redox proteomic molar quantification is unrealised and isn’t yet possible without sacrificing array and context. RedoxiFluor complements redox proteomics. Combined they can report the redox state of the target at the global (i.e., RedoxiFluor) and individual (i.e., redox proteomics) level. Combinatorial analysis could prove decisive. For example, if no redox state change occurs at mass spectrometry detectable individual thiols, RedoxiFluor can report whether undetectable residues respond. Reciprocally, if there is no global difference, redox proteomics can report whether individual thiols responded. RedoxiFluor can report redox modifiable functional parameters: lifetime (i.e., moles), interactome (i.e., macroscale); locale (i.e., fractionate a sample), phase (i.e., isolate condensates), and activity (i.e., inferred from surrogate like phosphorylation). RedoxiFluor should, therefore, prove instrumental for advancing protein thiol redox biology.

The insight derived from measurement alone demonstrates how RedoxiFluor can advance protein thiol redox biology. RedoxiFluor reveals that: (1) some proteins (i.e., ITAK1) are highly oxidised (i.e., heterogeneity principle); (2) target thiol redox state is displaced from the bulk thiol proteome (i.e., specificity principle); (3) proteins, even closely related ones (i.e., SHP1 and SHP2), respond differently to the same redox stimulus (i.e., heterogeneity and specificity principles); (4) target responsiveness is stimulus dependent (i.e., context principle); (5) global thiol proteome redox state is unresponsive to a fundamental redox stimulus (i.e., specificity and preservation of redox homeostasis principles); and (6) a target specific redox state change can occur without a change in redox state of its interactome (i.e., local specificity principle). RedoxiFluor also confirms a redox homeostasis principle: the thiol proteome is highly reduced\textsuperscript{36}. RedoxiFluor suggests target specific redox codes\textsuperscript{37}: unique biological meaning might be encoded within the global response of a target protein to a redox stimulus. Consistent with different residues being oxidised between tissues and as a function of ageing\textsuperscript{9}.

Quantitative protein thiol redox biology is insightful. The difference in the magnitude of the PP2A compared to IRAK1 LPS response (i.e., 6-8 vs 40\%) might reflect a difference in protein content (i.e., \~44 vs. 2 pM). The absolute increase in reversibly oxidised PP2A is 2.5 pM greater (i.e., 3.6 vs. 0.9 pM); that there is more [PP2A] might raise (i.e., buffer) the response threshold. Moles also mean two very different percent changes (i.e., 32\% difference) might both be functionally significant. Selecting a target to manipulate from percentages alone might be misleading. Moles are also instrumental for understanding threshold gating. If there is less of a protein in one cell compared to another, then the percent oxidised values might differ while the absolute pico moles of reversibly oxidised protein remains the same. Practically, the percent and molar terms rationalise the extent to which target thiol redox state should be
manipulated. Chemically increasing target protein thiol redox state by 40% is inappropriate if the maximal response to a redox stimulus is 20%. Finally, quantitative protein thiol redox biology is useful for mapping where target and context specific oxidative eustress ends and distress starts.8

Array mode RedoxiFluor can develop data-led redox signatures of particular states (e.g., ageing) or processes (e.g., cell cycle). A redox proteomic screen to expand the survey by 1-4 orders of magnitude9,12 can be used to rationally design and validate array mode RedoxiFluor to measure a state specific redox signature (e.g., a redox signature of COVID-19) in a microplate. Developing multiparametric state and process specific redox signatures could have far-reaching significance. For example, disease specific redox state signatures could rapidly screen how large numbers of patients respond to a treatment; which might make it easier to evaluate the therapeutic value of next-generation antioxidants.38

Ultimately, RedoxiFluor should prove invaluable for unravelling the vital role protein thiol redox state changes play in understanding redox functionality—what ROS do and how they do it—by quickly and inexpensively delivering actionable results in a widely accessible microplate format.

Methods

Samples: Xenopus laevis (X. laevis)

Following ethical approval (#ETH2021-0222), unfertilised eggs and 1-cell zygotes collected 30 min post-fertilisation were harvested from three different adult females in groups of 20 (i.e., each X. laevis sample represents the weighted mean of 20 eggs/zygotes). Samples were immediately lysed in immunoprecipitation (IP) buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.1) supplemented with a protease inhibitor tablet (Sigma, UK, #11697498001) and 1 mM F-MAL (F-MAL1: Fluorescin-5-maleimide, ThermoFisher, UK, #F150; or F-MAL2: AlexaFluor™647-C2-maleimide, ThermoFisher, UK; #A20347).

Samples: THP-1 human monocytes

THP-1 human monocytes (ECACC, #88081201) were cultured (95% air, 5% CO₂ at 37°C) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2000 mg/L glucose, 2 mM L-glutamine and 1% penicillin/streptomycin at a density of 5 x 10⁵ cells. Unstimulated or LPS stimulated (100 ng/ml for 30 min, SigmaAldrich, UK, #L6529) cells were lysed in IP buffer supplemented with 1 mM F-MAL. LPS (100 ng/ml, SigmaAldrich, UK, #L6529) and processed for RedoxiFluor (see below).

Antibodies

The antibodies used for RedoxiFluor were as follows: PP2A (Abcam, UK, #ab226790), PP2A matched pair ELISA (Abcam, UK, #ab218174), IRAK1 matched pair ELISA (Abcam, UK, #ab210071), SHP1 (Abcam, UK, #ab227503), SHP2 (Abcam, UK, #ab30283), CD45 (Abcam, UK, #ab208022), PTEN
Sample processing: Thiol labelling procedure

Samples were incubated with 1 mM F-MAL1 or 2 (i.e., depending on the labelling order) for 30 min on ice and centrifuged at 14,000 \( g \) for 5 min at 4\(^\circ\)C. Soluble supernatants were passed through a 6000 kDa spin column (Bio-Rad, UK, #7326222) to remove excess F-MAL1. Flow-throughs were treated with 5 mM DTT (ThermoFisher, UK, #RO861) for 30 min on ice. After removing excess DTT with a spin column, samples were treated with 1 mM F-MAL1 or 2 for 30 min on ice. Unreacted F-MAL1 or 2 was removed with a spin column. To prepare assay calibrants, samples were lysed in 5 mM DTT for 30 min and centrifuged at 14,000 \( g \) for 5 min at 4\(^\circ\)C. After removing excess DTT with a spin column, samples were incubated with 1 mM F-MAL1 or 2 for 30 min. Unreacted F-MAL1 or 2 were removed with a spin column. Fully labelled F-MAL1/2 standards were mixed as appropriate to produce the 10-90% reversibly oxidised redox states (e.g., for a 10 ul final volume, 9 ul of F-MAL1 was mixed with 1 ul of F-MAL2 to prepare the 90% reversibly oxidised standard). To prepare BSA standards, 50 ug BSA was reduced with 1 mM DTT for 15 min at room temperature (RT). Excess DTT was removed with a spin column and flow-throughs were treated with 0.5 mM F-MA1 or 2. After removing excess F-MAL1/2, the desired redox states were prepared by mixing the standards as appropriate. Samples were protected from ambient light throughout.

Isolated fluorophore and recombinant BSA experiments

For the isolated fluorophore experiments described in supplementary Figure 1, 1 mM of each fluorophore was incubated in L-cysteine buffer (25 mM Tris, pH 7.2, 2 mM L-cysteine) for 30 min at RT in the dark to capture any thiol dependent turn-on fluorescence. To construct the 90 to 10% standard curve, appropriate amounts of fluorophore were mixed to a final volume of 10 ul. Aliquots (1 ul) were analysed in triplicate in a plate reader (see RedoxiFluor analysis). BSA standards (prepared as described above) were analysed in the same way. For the gel experiments, BSA standards (1 ug) were resolved by SDS-PAGE and analysed as described below.

Protein A mode RedoxiFluor

To assess a single target (i.e., PP2A, PTEN, or IRAK1), 0.1 ug of capture antibody in binding buffer (50%: 0.05% Tween-20 in phosphate buffer saline [PBST]; 50% Superblock [ThermoFisher, UK, #37580) was added to each well of a black protein A derivatised microplate (ThermoFisher, UK, #15155) for 1 h at RT at 350 rpm on a plate shaker. Unbound capture antibody was removed by washing (3 x 2 min PBST washes at 400 rpm), before assay calibrants (i.e., 10-90% reversibly oxidised standards), controls (i.e., immunodepleted, see below), or samples were added in duplicate and incubated in the dark for 2 h at RT. For X. laevis, 5 ul of sample/assay calibrant was diluted in 95 ul PBS. For THP-1 cells, 10 ul of sample was diluted in 90 ul PBS. After removing unbound sample, wells were washed (3 x 2 min PBST washes at 400 rpm), rinsed in PBS to remove excess Tween-20, and incubated with denaturing buffer (4% SDS, 50
mM Tris, pH 7.1) for 15 min at RT with vigorous shaking (500-700 rpm). After measuring F-MAL/2 fluorescence in a plate reader, target specific protein thiol redox state was calculated (see below).

**ELISA mode RedoxiFluor**

To measure PP2A and IRAK1 redox state in ELISA mode matched pair antibodies were used. Black MaxiSorp immuno microplates (ThermoFisher, UK, #437111) were incubated with 50 ul of 2 ug/ml capture antibody overnight at 4°C in binding buffer (35 mM NaHCO$_3$, 15 mM Na$_2$CO$_3$, pH 9.6) on a plate shaker at 350 rpm. Unbound capture antibody was removed by washing (3 x 2 min PBST washes at 400 rpm), before wells were blocked (50% PBST, 50% Superblock) for 2 h at RT at 350 rpm and washed (3 x 2 min PBST washes at 400 rpm). The recombinant protein standards, assay calibrants (i.e., 10-90% reversibly oxidised), assay controls (i.e., immunodepleted sample, and samples (diluted as above to a final volume of 50 ul) were added in duplicate and incubated for 2 h at RT at 350 pm in the dark. Excess sample was removed and wells were washed (3 x 2 min PBST washes at 400 rpm). Next, 0.5 ug/ml biotin-conjugated detector antibody was added for 1 h at RT at 350 rpm in the dark. After a wash step, 0.05 ug/ml of HRP-conjugated streptavidin (Abcam, UK, #ab210901) was added for 1 h at RT at 350 rpm in the dark. After a final wash step, wells were incubated with QuantaBlu™ (ThermoFisher, UK, #15169) prepared according to the manufacturer's guidelines for 10 min at RT at 400 rpm in the dark. QuantaBlu signal was measured at 325 (excitation) and 425 (emission) nm for 100 ms on a plate reader. To stop the HRP reaction and unmask the F-MAL1/2 signal wells were incubated in denaturing buffer (4% SDS, 50 mM Tris, pH 7.1) for 15 min at RT at 500-700 rpm. After measuring F-MAL/2 fluorescence in a plate reader, target specific protein thiol redox state was calculated (see below).

**Assay controls**

For the PP2A and PTEN microplate experiments in *X. laevis* described, sample aliquots (diluted as above) were also added to rabbit isotype control wells in protein A and ELISA mode to assess non-specific binding. To check specificity in the case of PP2A, samples were incubated with a PP2A capture antibody functionalised protein A magnetic beads (see below) overnight at 4°C with gentle rotation. The PP2A immunodepleted sample was added to a second capture antibody functionalised magnetic bead for 1 at RT before being added to the protein A or ELISA microplate. Sample specific (i.e., THP-1 for IRAK1) calibrated standards (e.g., 10-90% reversibly oxidised) were used in every RedoxiFluor experiment.

**Array mode RedoxiFluor**

To assess the redox state of multiple targets in array mode, 0.1 ug of SHP1 (row B), SHP2 (row C), PTP1B (row D), PP2A (row E), PTEN (row F), CD45 (row G), and calcineurin (row G) capture antibodies (see Table 3) were added in binding buffer (50%: PBST; 50% Superblock) to a black protein A derivatised microplate for 1 h at RT at 350 rpm on a plate shaker. Row A was reserved as a blank well. Unstimulated (lanes 1-3 & 7-9) and LPS stimulated (lanes 4-6 & 10-12) samples (diluted 1:10) were added for 2 h at 350 rpm in the dark. Thereafter array mode experiments were identical to protein A mode RedoxiFluor.
Global thiol proteome redox state

To measure the redox state of the thiol proteome, samples (1 μl diluted in 199 μl PBS) were measured in triplicate in a plate reader.

Macroscale: RedoxiFluor

Aliquots (10 μg) of PTEN capture antibody or rabbit isotype control (ThermoFisher, UK, #31235) were incubated with 50 μl protein A derivatised magnetic beads for 1 h at RT. Beads were magnetised to remove excess capture antibody and incubated with undiluted *X. laevis* samples (~500 μg protein) overnight at 4°C. Beads were magnetised to remove unbound sample, washed in PBST (0.025% Tween-20), and resuspended in denaturing buffer (4% SDS, 200 mM Tris, 20% glycerol, pH 7.1). To prevent any potential heating induced fluorescence artefacts, PTEN was eluted from the capture antibody by chemically breaking the antibody apart: the disulfide bonds required to structure the antibody for target binding were reduced with 10 mM DTT for 15 min at RT. This procedure proved effective as magnetic beads were negative for F-MAL1-2 following elution. Note, glycine elution is incompatible with RedoxiFluor (the low pH destroys the fluorescence). Eluents were isolated by magnetising the beads. To assess PTEN interactome redox state, triplicate eluent aliquots (2 μl) were measured in a plate reader. To identify the interacting proteins, eluents were resolved by SDS-PAGE using a pre-cast 4-15% gradient gel (Bio-Rad, UK, #4561085) and F-MAL1/2 signals were captured on a gel scanner for 1 s using the appropriate filters. To assess PTEN redox state, eluents were resolved by SDS-PAGE and the specific PTEN band (as confirmed by immunoblot, see below) was manually excised and passively eluted in distilled water (dH₂O) for 24 h at RT with vigorous agitation. Passive elution was successful because the bands were F-MAL1/2 negative after 24 h. For PTEN specific redox analysis, no gel scanner imaging was performed to eliminate potential photo-toxicity interfering with microplate analysis. Passively eluted PTEN redox state was measured using a plate reader.

RedoxiFluor analysis

Irrespective of the RedoxiFluor mode (i.e., protein A mode), target redox state was assessed by measuring F-MAL1 (494-518 nm) and F-MAL2 (651-671 nm) with a 5 nm bandwidth for 100 ms on a plate reader. After blank subtraction, F-MAL1 and F-MAL2 signals (v) were corrected using the following equation: \( v = \frac{v}{E} \) or a derivative thereof were E and q represent the extinction coefficient and quantum yield, respectively. Corrected values were totalled and percent reduced or reversibly oxidised protein was calculated (i.e., reduced = (reduced/total)*100). In ELISA mode RedoxiFluor, pg/ml protein content values computed from the recombinant protein standard curve were converted to picomoles by dividing them by the molecular weight of the target protein (i.e., picomoles = picogram / target molecular weight). The amount of reduced and reversibly oxidised target protein in picomoles could then be calculated (i.e., pM reduced = total pM*reduced%).

Immunoblot
After resolving samples by SDS-PAGE, they were transferred to a 0.45 uM 100% methanol activated PVDF membrane for 1 h. Membranes were blocked for 1 h in 5% non-fat-dry milk (NFDM) and incubated with a primary PTEN antibody overnight (1:1000) at 4°C with gentle agitation. To avoid interference from the eluted capture antibody light and heavy chains, we incubated the PTEN primary antibody aliquot with 100 uM DyLight755 fluorescent conjugated N-hydroxysuccinimide ester (F-NHS, ThermoFisher, UK, #62278) for 30 min at RT. Unreacted F-NHS was quenched with 1 mM Tris pH 7 for 15 min at RT. Fluorescence was assessed on a gel scanner.

**ALISA**

PTEN primary antibody aliquots (0.1 ug) were incubated with an epoxy group functionalised plate (PolyAn, Germany, #00695251) in binding buffer (150 mM Na₂HPO₄, 50 mM NaCl, pH 8.5) overnight at 4°C at 350 rpm on a plate shaker. Unreacted epoxy groups were quenched (100 mM Tris, pH 9) for 2 h at RT, washed in PBST, and incubated with samples labelled with a single F-MAL for 2 h at RT with gentle agitation. After removing unbound sample, wells were washed in PBST (3x), resuspended in PBS, and incubated with 350 uM F-NHS for 30 min. Excess F-NHS was removed and wells were washed in PBST (3x), and resuspended in denaturing buffer for 1 h. Eluent redox state was assessed in new microplate. After blank subtraction, target specific redox state was calculated as: F-MAL/F-NHS.

**Click-PEG**

Catalyst-free inverse electron demand Diels Alder chemistry Click-PEG wherein trans-cyclooctene (TCO) labelled reversibly oxidised thiols are conjugated to 6-methyltetrazine derivatised PEG 5 kDa was performed as previously described^{27,39}. Click reacted samples were analysed by PP2A immunoblot.

**Statistics**

Data-set normality was assessed using Shapiro-Wilk and Kolmogorov-Smirnov testing. For within sample redox state (i.e., reduced vs. reversibly oxidised), data were analysed using a paired t-test or non-parametric equivalent. For between samples redox state (i.e., %reversibly oxidised), data were analysed with an unpaired t-test or non-parametric equivalent. When comparing multiple proteins in the array to determine how their reversible oxidation set-points differed, a one-way ANOVA was used. In all cases, alpha was set to $P > 0.05$ and tests were performed on GraphPad Prism Version 9 (https://www.graphpad.com). Figure legends report exact $P$ values and statistical tests. Data are presented as Mean (M) and Standard Deviation (SD).

**Declarations**

**Conflict of interest**

J.N.C. has filed a UK patent application for RedoxiFluor.

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**Figures**
Figure 1

General microscale RedoxiFluor scheme. Block and wash steps are omitted for clarity throughout (see methods). Step 1. Bind the capture antibody to a microplate. Step 2. Bind the fluorescent maleimide (F-MAL) decorated target. In two colour mode, one spectrally distinct reporter labels reduced thiols (e.g., F-MAL1, green groups) and another reversibly oxidised thiols (e.g., F-MAL2, red groups). Step 3. The target is eluted from the capture antibody to unmask the F-MAL reporters. Step 4. Target specific F-MAL1/2 signals are measured in a plate reader. Step 5. Target specific protein thiol redox state is calculated in percentages. Molar quantification is possible if a biotin-conjugated detector antibody and recombinant protein standard curve is used (i.e., ELISA mode, see main text).

Figure 2
RedoxiFluor can quantify protein thiol redox state in percentages and moles. A. Protein A mode RedoxiFluor can accurately and reproducibly discern between different PP2A redox states ranging from 10 to 90% reversibly oxidised (n = 3 per standard, see methods). B. Catalyst-free Click-PEG cannot detect PP2A redox state, as evidenced by the loss of signal in the “PEGylated” lanes (3-5) compared to lysates (lane 1) and the PEG-free clickable maleimide handle only control (lane 2). C. ALISA can detect a significant difference (unpaired t-test, P = 0.0192, n = 3) between the 20 and 40% redox state. D. ELISA mode RedoxiFluor can accurately and reproducibly discern between different PP2A redox state ranging from 10 to 90% reversibly oxidised (n = 3 per standard, see methods). A separate PP2A ELISA mode standard experiment quantifying the significant differences (unpaired t-tests, P = < 0.0001 in panels E-G, n = 6) between the 20 (n = 6) and 40% (n = 6) reversibly oxidised states in percentages (E) and picomoles of reduced (F) and reversibly oxidised (G) protein. All standards and samples were derived from Xenopus laevis lysates (see methods).

Figure 3

Redox immunology part 1: Protein A and ELISA mode RedoxiFluor reveal a dramatic LPS induced increase in IRAK1 specific reversible thiol oxidation. A. In protein A mode, percent reduced is significantly
(paired t-test, \(P = 0.0419, n = 6\)) greater than percent oxidised IRAK1 in unstimulated (control) human monocytes. B. In protein A mode, percent oxidised is significantly (paired t-test, \(P = < 0.0001, n = 6\)) greater than percent reduced IRAK1 in LPS stimulated human monocytes. C. Protein A mode RedoxiFluor reveals a significant (unpaired t-test, \(P = < 0.0001, n = 6\)) LPS induced increase in percent oxidised IRAK1 in LPS stimulated compared to unstimulated human monocytes. D. In ELISA mode, percent reduced is significantly (paired t-test, \(< 0.0001, n = 6\)) greater than percent oxidised IRAK1 in unstimulated human monocytes. E. In ELISA mode, percent oxidised is significantly (paired t-test, \(P = < 0.0001, n = 6\)) greater than percent reduced IRAK1 in LPS stimulated human monocytes. F. ELISA mode RedoxiFluor reveals a significant (unpaired t-test, \(P = < 0.0001, n = 6\)) LPS induced increase in oxidised IRAK1 in LPS stimulated compared to unstimulated human monocytes. G. No significant difference (unpaired t-test, \(P = 0.9159, n = 6\)) in picomoles of IRAK1 in unstimulated compared to LPS stimulated human monocytes. H. A significant (unpaired t-test, \(P = < 0.0001, n = 6\)) LPS induced increased picomoles of oxidised IRAK1 in LPS stimulated compared to unstimulated human monocytes.
**Figure 4**

Redox immunology part 2: Array mode RedoxiFluor reveals LPS increases PP2A, PTP1B, SHP1, and CD45 specific reversible thiol oxidation. No significant difference (all unpaired t-tests and n = 6) in PTEN (P = 0.1871), SHP2 (P = 0.3054), and calcineurin (P = 0.2780) specific reversible thiol oxidation (i.e., percent oxidised protein) in unstimulated (control) and LPS stimulated human monocytes as determined by array mode RedoxiFluor. Array mode RedoxiFluor reveals significant (all unpaired-tests and n = 6) LPS induced increases in PP2A (P = 0.0181), SHP1 (P < 0.0001), PTP1B (P = 0.0483), and CD45 (P = 0.0018) specific reversible thiol oxidation in unstimulated and LPS stimulated human monocytes.

**Figure 5**

Developmental biology: Micro and macroscale protein A mode RedoxiFluor reveals a fertilisation induced increase in PTEN specific reversible thiol oxidation. A. Protein A mode RedoxiFlour reveals a significant (unpaired t-test, P = 0.0002 n = 6) increase in PTEN specific reversible thiol oxidation in 1-cell zygotes compared to unfertilised eggs in *X. laevis*. B. Macroscale RedoxiFluor finds no significant unpaired t-test, P = 0.2592, n = 3) difference in PTEN interactome redox state in 1-cell zygotes compared to unfertilised eggs in *X. laevis*. C. A representative SDS-PAGE gel image showing the PTEN (highlighted) and the redox state (reduced = green channel; oxidised = red channel) of its interactome (arrows 1-6 corresponding to proteins with a molecular weight of ~100, 75, 60, 37, 25 and 10 kDa respectively) in unfertilised eggs (E) and zygotes (Z). D. Quantifying the redox state of the PTEN specific band manually excised and eluted from (C) in unfertilised eggs and 1-cell zygotes in *X. laevis* confirms the significant (unpaired t-test, P = 0.0020 n = 3) fertilisation induced increase in PTEN specific reversible thiol oxidation.

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