Half-time report

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Title of doctoral project:

Fluorogenic probes for single cell redox signalling

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Co-supervisor: Oliver Thorn-Seshold

Half-time
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Venue: Biomedicum, B0812
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Simon Elsässer
Vicente Pelechano

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CONTENTS

List of relevant publications and manuscripts

Felber JG, Zeisel L, Poczka L, **Scholzen K**, Busker S, Maier MS, Theisen U, Brandstädter C, Becker K, Arnér ESJ, Thorn-Seshold J, Thorn-Seshold O. Selective, Modular Probes for Thioredoxins Enabled by Rational Tuning of a Unique Disulfide Structure Motif. J Am Chem Soc. 2021 Jun 16;143(23):8791-8803. doi: 10.1021/jacs.1c03234. Epub 2021 Jun 1. PubMed PMID: 34061528.

Felber JG, Poczka L, **Scholzen KC**, Zeisel L, Maier MS, Busker S, Theisen U, Brandstädter C, Becker K, Arnér ESJ, Thorn-Seshold J, Thorn-Seshold O. Cyclic 5-membered disulfides are not selective substrates of thioredoxin reductase, but are opened nonspecifically. Nat Commun. 2022 Apr 1;13(1):1754. doi: 10.1038/s41467-022-29136-4. PubMed PMID: 35365603.

Zeisel L, Felber JG, **Scholzen KC**, Poczka L, Cheff D, Maier MS, Cheng Q, Shen M, Hall MD, Arnér ES, Thorn-Seshold J. Selective cellular probes for mammalian thioredoxin reductase TrxR1: rational design of RX1, a modular 1, 2-thiaselenane redox probe. Chem. 2022 May 12;8(5):1493-517. doi: 10.1016/j.chempr.2022.03.010. PMID: 35936029

Cheff DM, Huang C, **Scholzen KC**, Gencheva R, Ronzetti MH, Cheng Q, Hall MD, Arnér ES. The ferroptosis inducing compounds RSL3 and ML162 are not direct inhibitors of GPX4 but of TXNRD1. Redox Biology. 2023 Jun 1;62:102703. doi: 10.1016/j.redox.2023.102703. PMID: 37087975

Zeisel L, Felber JG, **Scholzen KC**, Schmitt C, Wiegand AJ, Komissarov L, Arnér ES, Thorn-Seshold O. Piperazine-Fused Cyclic Disulfides Unlock High-Performance Bioreductive Probes of Thioredoxins and Bifunctional Reagents for Thiol Redox Biology. J Am Chem Soc. 2024 Feb 15;146;5204-5214. doi: 10.1021/jacs.3c11153.

Literature review
A critical assessment of fluorescent probes as activity reporters for the Trx system enzymes

1. Introduction

The "redox state" of a cell is a not well-defined concept of redox regulation within the cell, consisting of several redox pairs, such as GSH/GSSG or NADPH/$NADP^+$, occurring and fluctuating within more or less controlled steady state levels[1]. A constant regulation of these redox pairs is necessary, to keep a balance, preventing any over oxidation or reduction of biomacromolecules. Numerous studies, both in early years as well as recently, illuminate the importance of redox control in health and disease, with major impact of dysregulated, cellular oxidation within development of cancer, cardiovascular disease and neurodegenerative diseases[2–6]. Recent approaches to understand redox signalling better, have improved the understanding of its importance in diverse pathways, besides oxidative stress and cancer development[7–11].

A tight regulation of the complex connected pathways within mammalian cells keeps the redox state consistent. The crosstalk and distinct functions between the Glutathione (GSH) and Thioredoxin (Trx) systems are necessary and crucial for sustaining cellular functions[12–16]. Both of these two major redox regulatory systems consist of an upstream reductase (Glutathione reductase (GR) and Thioredoxin reductase (TrxR) respectively) and the effector proteins (Glutaredoxins (Grx) and Trxs) that transfer the reduction equivalents collected from NADPH onto the targeted molecules[17,18] (Figure 1). TrxR is a selenoprotein, with a unique selenocysteine in its active site, making the whole Trx system selenium dependent[19–22]. The enzyme is seen as a target for cancer therapy, due to its upregulation in cancer progression[5,23,24]. There are three known isoforms of TrxR, TrxR1 which is mainly cytosolic, TrxR2 which is the mitochondrial form and TR3, primarily found in testis[25,26]. This review will be focussed on the cytosolic form and its probes.

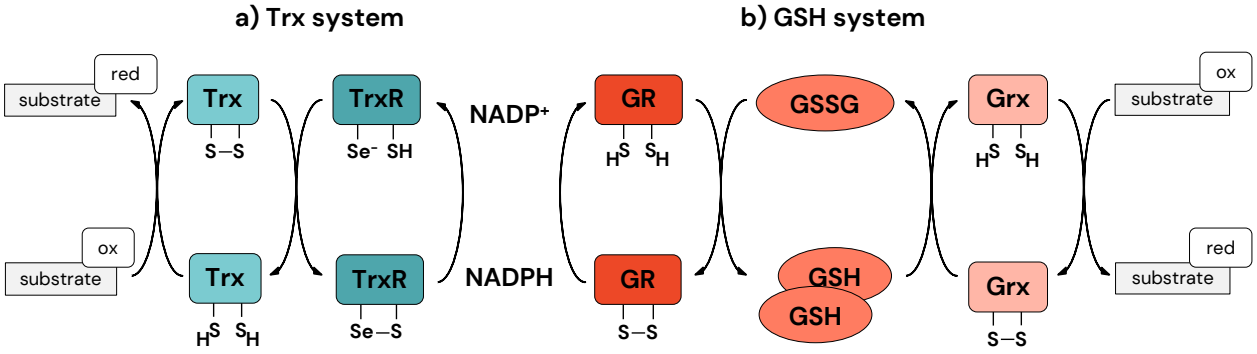


Figure 1 A simplified overview of the Trx and GSH systems and their respective reductive pathways, both depending on the availability of NADPH.

All major players within these pathways have been shown to interact with different factors, influencing the proliferation, transcription, and apoptosis of the cell and have been highly discussed[2,3,6,27–32]. Classical approaches for enzyme level or activity determinations, such as DTNB assay, Insulin assay and western

blots, are laborious and cannot be performed in live cells over time, but rather work as endpoint assays using cell lysates[33–37]. In this review, some recently developed fluorescent tools for measuring enzyme activities of the Trx system will be presented and shortly evaluated in the context of previous and recent studies.

1.1 Visualisation of redox biology in live cells

The close relation and structural similarities of Trx and Grx system enzymes is a major factor in evaluation of probes, measuring their activities, since a distinguishable measurement of a respective enzyme activity is a crucial aspect of the utility of these probes. A reliable and specific measurement of their activity is key to further understanding their role in redox biology and disease progression and differentiation between these two major redox systems. Because of their importance and impact in health and disease, efforts in several laboratories during recent years brought up a number of tools and methods, to directly measure the activity of the involved redox active enzymes. Fluorescent reporters are often in favour over other possible reporter methods, offering a few advantages. Due to its spectral properties, it has a high selectivity and, if a fluorophore is chosen wisely, a good signal to noise ratio. Additionally, the reporting method is minimally invasive if it can be used with live cells and typically does not interfere with cellular or *in vivo* processes, on top of being very convenient[38–40]. The broad range of commonly available fluorophores and detection methods, such as plate readers for intensity reading, flow cytometry for single cell analyses, fluorescent microscopy for different applications and other, further simplifies the usage of fluorescent probes. The range of fluorescent tools for redox biological research spans from small molecules to fluorescent proteins, with distinct features. A fascinating part of these are the genetically encoded fluorescent indicators that act as sensors for small molecules. Metabolites or small molecules introduce changes within a domain, resulting in a structural re-organization and a consecutive fluorescent signal, thereby giving the possibility of direct measurement of environmental states *in vivo*. There are excellent reviews highlighting the different utility and application approaches of genetically encoded fluorescent biosensors for redox biology related research and these will not be further discussed herein[41–46].

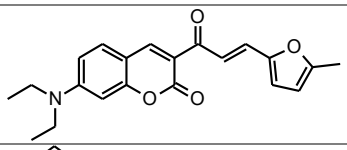
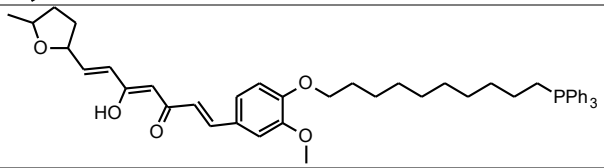
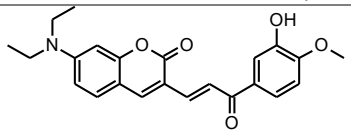
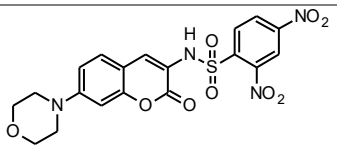
2. Small molecules as Trx or TrxR probes

Several aspects need to be considered during the development and validation of Trx or TrxR selective probes. Besides resistance to known bioreductants, such as GSH, NADPH, Cysteine, or other amino acids, they must withstand other redox active enzymes and a general high background of nonspecific thiols, if considered as possibly cellularly compatible with a noticeable selectivity for the intended target. Small molecular probes for live cells are also required to be cell-permeable, have low toxicity and report withing a reasonable timeframe, to be applicable.

Due to the special chemistry of the selenocysteine-containing and easily accessible active site of TrxR's, a number of covalently bound markers were developed[47–49]. These can be fairly selective and label TrxR fluorescently in live cells. While the strategy works well to label and visualize TrxR's abundance and

possible localization, it does not reflect directly on the actual activity of the enzyme, acting effectively as fluorescent inhibitors. Such probes remove the TrxR molecule as an active reductase and can thereby not reflect intrinsic activity fluctuations, more importantly it could dramatically affect downstream effects of TrxR inhibition[11,30,49–53]. Although chemically interesting, this class of small molecules, probing TrxR for labelling purposes in live cells, will therefore not be further discussed within this review. However, see table 1 for a short summary of these probes and original references for further reading.

Table 1 Overview of probes which covalently bind to the selenocysteine of TrxR and report fluorescently consequently

PROBE	ORIGINAL REFERENCE	STRUCTURE
TR-green	Huang, Lei, et al. *Chemical Communications* 50.53 (2014): 6987-6990.[47]	
TPP2a	Liang, Baoxia, et al. *ACS chemical biology* 11.2 (2016): 425-434.[54]	
Xn-2	Wang, Yu, et al. *Bioorganic Chemistry* 95 (2020): 103530.[53]	
probe 1	Ma, Junyan, et al. *Materials Chemistry Frontiers* 5.23 (2021): 8108-8117.[49]	

2.1 Substrate mimicking fluorescent probes

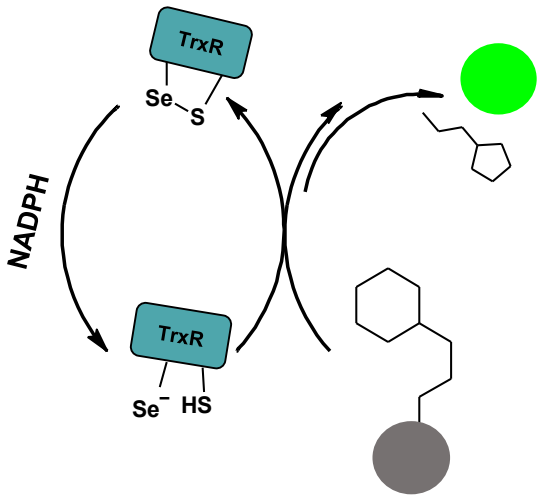


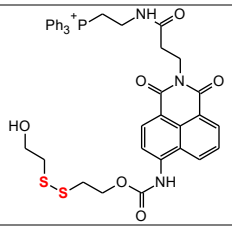
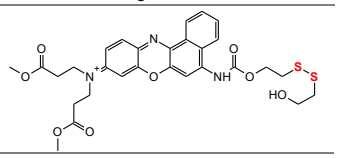
Figure 2 schematic overview of TrxR driven activation of fluorophore releasing probes, with structural reorganization upon reduction by TrxR's active site, resulting in the release or enhancement of the fluorophore and an inactive side product.

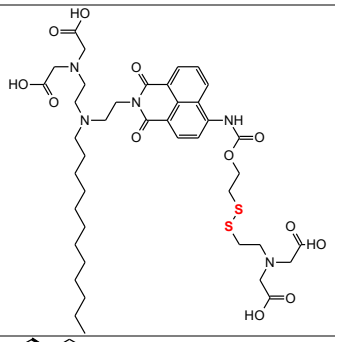
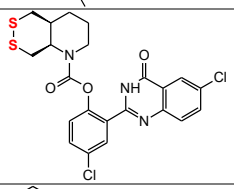
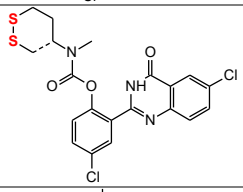
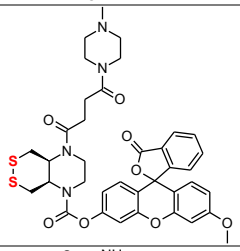
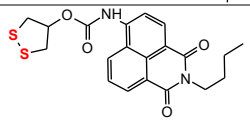
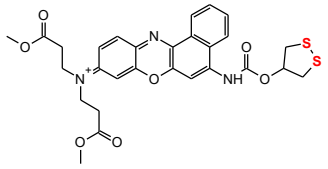
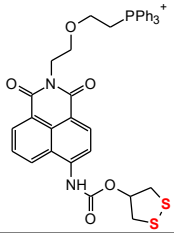
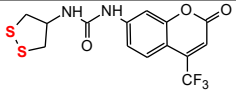
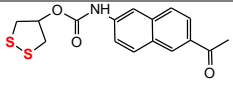
An optimal way to visualise the activity of an enzyme, is to use small molecular probes that report on the turn-over of said enzyme. The active structure of the probe can mimic a substrate, thereby being reduced by the enzyme and diffusing from it subsequently, releasing a fluorophore or enhance the existing fluorophore. Such molecules should consist of at least two distinct functional parts, one containing the enzyme-interactive motif, and another part to act as a reporter (fluorophore e.g.). Upon reduction of the target structure, a fluorescent signal becomes detectable by release of a

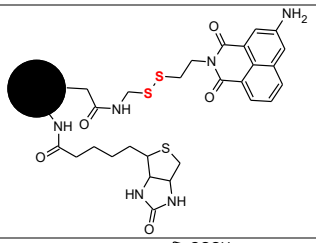
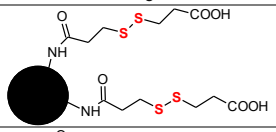
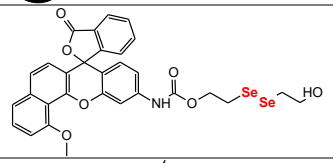
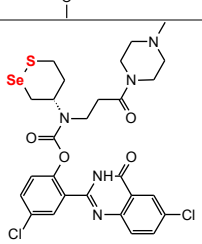
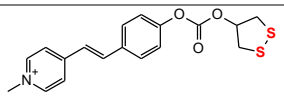
fluorophore or enhancement of a given signal. In this case, the redox active enzyme itself is not inhibited or labelled, but its activity correlates with fluorescence. Although the probe can act as a substrate competitor with native substrates and thereby decrease the turn-over of the enzyme, the inhibitory effect would most likely be less acute than binding to the active site. Some reviews aimed to discuss the chemical design of such probes in detail have been published[55,56], however not fully commenting on practicality, methodology and actual selectivity of these probes, which will be further focus in this review. The probes that will be discussed at detail herein are summarized in table 2.

The complex nature of intracellular redox homeostasis involves several redox active structures which might interfere or react with small redox active molecules. Although TrxR with its unique Sec residue has a high reactivity and turnover towards its substrates, other endogenous enzymes and reductants must be considered in terms of claiming TrxR selectivity for a given probe aimed to report at the activity of this enzyme, especially if built upon natural substrate similarity. Trx is one of the main substrates of TrxR, however the closely related Grx1 is not a substrate of TrxR but is reduced by GSH[15], while the isoform Grx2 is also a substrate for TrxR[57], showing the complicated interaction of the two system. GSH is a redox active small molecule and has a high concentration in cells, within low mM range[58]. Possible cross-reactions with these and other molecules should be considered, when developing TrxR or Trx selective structures. Considering enzymatic activity and cellular processes, it is also important to include all necessary components of cycling enzymatic systems, when checking for possible cross reactivity, to ensure well imitated cellular conditions.

Table 2 Overview of different substrate mimicking probes for the Trx system, probing Trx or TrxR, including the validation methods to confirm selectivity. The red coloured site in the structure is proposed to be the recognition site for the enzyme. Enzymatic or non-enzymatic cell-free activation depends on whether the assay included all necessary components for a cycling enzymatic system (Trx/TrxR/NADPH or TrxR/NADPH for the respective enzymes)

PROBE	ORIGINAL REFERENCE	TARGET ENZYME	ACTIVE SITE	VALIDATION METHODS		STRUCTURE
				cell-free	in cells	
Mito-Naph	Lee et al, J. Am. Chem. Soc. 2012, 134, 41, 17314–17319	Trx2	linear disulfide	– Trx titration (not enzymatic) – bioreductants – GSH instabile – not desalted Trx before assay after DTT reduction	– Trx inhibitor (PX-12)	
NBL-SS	Jia et al, Anal. Chem. 2019, 91, 13, 8524–8531	Trx	linear disulfide	– Trx titration (not enzymatic) – bioreductants	– Trx inhibitor (PX-12) – Trx knockdown	

Memb-Trx	Lee et al, J. Am. Chem. Soc. 2014, 136, 23, 8430–8437	Trx	linear disulfide	- Trx titration (not enzymatic) - ions and bioreductants - not stable against thiols	- Trx inhibitor (PX-12)	
SS66C-PQ	Felber et al, J. Am. Chem. Soc. 2021, 143, 23, 8791–8803	Trx	annelated 1,2-dithiane	- enzymatic Trx driven activation - cell free enzymatic screen - bioreductants		
SS6O-PQ	Felber et al, J. Am. Chem. Soc. 2021, 143, 23, 8791–8803	Trx	1,2-dithiane	- enzymatic Trx driven activation - cell free enzymatic screen - bioreductants		
C-DiThia	Zeisel et al, J. Am. Chem. Soc. 2024	Trx	Piperazine-annelated 1,2-dithiane	- enzymatic Trx driven activation - cell free enzymatic screen - bioreductants	- TrxR inhibitor (TRi-1) - TrxR knock-out - selenium supplementation - GSH synthesis inhibitor (BSO)	
TRFS-green	Zhang et al, J. Am. Chem. Soc. 2014, 136, 1, 226–233	mammalian TrxR bacterial Trx and Grx	1,2-dithiolane	- enzymatic TrxR titration - Cys mutant - bioreductants, - lysate with TrxR inhibitor (DNCB)	- TrxR inhibitor (DNCB)	
TRFS-red	Ma et al, Chem. Commun., 2016,52, 12060-12063	TrxR	1,2-dithiolane	- enzymatic TrxR driven activation - Cys mutant - bioreductants - lysate with TrxR inhibitors (Auranofin and DNCB)	- TrxR inhibitor (Auranofin and DNCB)	
Mito-TRFS	Liu et al, Chem. Commun., 2016,52, 2296-2299	TrxR2	1,2-dithiolane	- enzymatic TrxR1 driven activation - bioreductants	- colocalization with MitoTracker - TrxR inhibitor (Auranofin)	
Fast-TRFS	Li et al, Nat Commun 10, 2745 (2019)	mammalian TrxR bacterial Trx and Grx	1,2-dithiolane	- enzymatic TrxR driven activation - bioreductants - Cys mutant - lysate with TrxR inhibitors (Auranofin)	- TrxR inhibitor (Auranofin) - TrxR knockdown in HeLa cells	
TP-TRFS	Zhao et al, Chem. Commun., 2020,56, 14075-14078	TrxR	1,2-dithiolane	- enzymatic TrxR driven activation - bioreductants - Cys mutant	- TrxR inhibitor (Auranofin) - TrxR knockdown in HeLa cells	

				- lysate with TrxR inhibitors (Auranofin)		
Biotin-CD-Naph	Sidhu et al, ACS Appl. Mater. Interfaces 2017, 9, 31, 25847−25856	TrxR	linear disulfide	- enzymatic TrxR driven activation - bioreductants	- cellular activation	
fCDs-Cu2+	Sidhu et al, Analyst, 2018,143, 1853-1861	TrxR	linear disulfide	- enzymatic TrxR driven activation - bioreductans	- cellular activation	
probe 1a	Mafireyi et al, Angew. Chem. Int. Ed. 2020, 59, 15147.	TrxR	Linear diselenide	- enzymatic TrxR driven activation - bioreductants	- TrxR inhibitor (DNCB)	
RX1	Zeisel et al., Chem 8, 1493–1517, 2022	TrxR	1,2-thiaselenane	- enzymatic TrxR driven activation - cell-free enzymatic screen - bioreductants - Cys mutant - Other selenoproteins	- TrxR inhibitor (TRi-1) - TrxR knock-out - selenium supplementation	
DSMP	Yang et al, Anal. Chem. 2022, 94, 12, 4970−4978	TrxR2	1,2-dithiolane	- enzymatic TrxR driven activation - bioreductants - only 100 µM of GSH tested	- cellular activation - colocalization with Mito-Tracker	

2.1.1 Probing Trx

One of the earliest attempts to visualize the activity of a redox active enzyme with small molecular probes was by Kim and coworkers in 2012[59]. **Mito-NAPH** is a disulfide-based trigger structure with a naphthalimide fluorophore, which was claimed to be selectively reduced by mitochondrial Trx. The triphenylphosphonium (TPP; Ph_3P^+ in chemical structure) group guides the probe to mitochondria[60,61], where the fluorophore is released upon reduction of the disulfide, supposedly by mitochondrial Trx. To understand its selectivity, the interesting part is the target structure, a linear disulfide in this case, which mimics a Trx substrate. Although the opening mechanism and fluorophore release influences the probe and its characteristics as well, the understanding of its selectivity is largely based on the target structure. The probe was screened in cell free conditions with other amino acids, ions, GSH and homocysteine, where only GSH gave minor activity. Although slow, there is a background of activation with GSH observed, while Trx driven reduction is clearly favoured. Important to note here is that the cell free assays were performed with single turn-over reactions, a cycling enzymatic system was not implemented (e.g. GR or TrxR together with NADPH as upstream reductants were not included). This means that cellular conditions were not well displayed in this selectivity screen. Another problematic part of the general design of this probe is the location of the

mitochondrial localization part. It is attached to the fluorophore and not to the trigger structure, meaning that the reduction of the probe can happen anywhere in the cell and the fluorophore is consequently transported to the mitochondria, independent on where it was reduced. Thus, the probe does not necessarily rely on mitochondrial reductive enzymes.

The probe was activated in cellular conditions, and an inhibitor assay using PX-12 as Trx inhibitor was used to confirm further selectivity. Pre-treatment of cells with PX-12 decreased the fluorescent intensity of the probe. It is important to note here that although PX-12 shows a tendency towards Trx inhibition, it is not a selective mitochondrial Trx inhibitor, but also reacts with cytosolic Trx[62,63], as well as TrxR[64], thus affecting the redox state of the cell more broadly.

PX-12 is an irreversible Trx inhibitor, which has been included in clinical trials as a therapeutical agent for cancer[62,65]. It is a disulfide-based molecule, which alkylates the active site Cys of Trx irreversible, thereby inhibiting its function[63]. However, it is most likely not a truly selective Trx inhibitor, since there are studies suggesting additional targets of PX-12 besides Trx, such as tubulin or Cys dependent proteases; thus, PX-12 may be targeting thiols in a more general sense in cells[66].

Based on this approach, Kim and coworkers published another Trx labelling probe, **Memb-Trx** in this case for membrane-localized Trx[67]. Previous studies suggest a correlation of membrane associated Trx and inflammation, proposing it as an inflammatory marker[17,68]. The probe should therefore be able to selectively monitor membrane associated Trx activity and act as a chemical marker for inflammation. The Trx targeting structure is again a linear disulfide which connects hydrophilic carboxylic acid groups with the fluorophore. The hydrophilic group prevents membrane diffusion and thereby hinders the uptake of the probe in the cell. However, membrane associated Trx can cleave the disulfide, which results in a removal of the hydrophilic group, a possible uptake of the probe and consecutive fluorescent signal within the cell. The selectivity was screened in a similar attempt as previously, concluding a selective pattern towards Trx.

Following this, Fang and coworkers presented an approach on cytosolic Trx activity measurement. **NBL-SS** showed a stability towards bioreductants in cell free conditions and was rapidly activated by Trx1[69]. However, although the rate of GSH was slower than with Trx, it did not show a full stability towards GSH. Upon reduction of the disulfide, the fluorophore is released and therefore acts as a direct reporter. In cellular settings, the previously used Trx inhibitor PX-12 was used to show Trx selectivity in cells (which is likely an unspecific Trx inhibitor, as discussed above).

These Trx probes all used linear disulfides for compartmental specific Trx activity monitoring; however, the literature has an ambivalent view on linear disulfides as a selective target. Ellman's reagent, DTNB, a known reporter for thiol quantification, is based on a linear disulfide and reacts rapidly and generally with any thiols[70,71]. Other probes based on linear disulfides have been reported to measure thiol content in cells, not selectively reduced by redox active enzymes[72,73].

We, together with Thorn-Seshold, recently presented a set of Trx probes, using a modular concept, including **SS66C-PQ** and **SS60-PQ**[74], which have been

evaluated with an extensive, cell-free screening against other redox active effector proteins (such as Grx1, Grx2 and TRP14, under catalytically cycling conditions), their respective upstream reductases as well as chemoreductants, showing little cross-reactivity. Several possible trigger structures were considered and included in the screen, covering a linear disulfide as well. Within that study, the linear disulfide did not show any selectivity towards Trx but was rather reduced by several reductants, including enzymes from both, the Trx and Grx system, as well as monothiols such as GSH. This in-depth screen with several bio- and chemoreductants, including physiological relevant enzymes, molecules, and concentrations, should hopefully set an example for future probe development approaches, as necessary in order to claim selectivity towards single enzymes. However, none of these probes were active in cells, due to the slow probe activation and can therefore not be evaluated on their cellular performance. It is possible that the two presented probes by Kim and coworkers show a selective pattern due to their compartmental localization, excluding several possible cross reactions. However, the linear disulfide motif is most probably ineligible for a truly selective probe targeting Trx, independent of compartments.

Following this study, Thorn-Seshold together with us, developed a new set of probes, based on similar trigger structure as before[75]. According to the modular design as presented in the previous study, two main probes were designed and evaluated, which are based on piperazine-annelated 1,2-dithianes, **C-DiThia** and **T-DiThia**. Interestingly, their different stereochemistry (*cis* vs *trans*) revealed a different reactivity towards thiols and therefore a different selectivity pattern. While C-DiThia was mainly reduced by enzymatically driven Trx, to a certain extend also Grx, but was stable towards GSH and other monothiols, T-DiThia was rapidly reduced by both mono- and disulfides. A major key point of the study was the massively improved kinetical behaviour, meaning a faster activation of the probes. Thus, an evaluation in cellular models was made possible. Due to the lack of selective Trx inhibitors, we modified the general Trx system activity by modulating TrxR's activity with selenium supplementation/starvation, chemical inhibition (TRi-1[76]) or genetic knockout. The activation of T-DiThia was not affected by these modifications, whereas C-DiThias activation was to up to 70% affected. On the other hand, the GSH synthesis inhibitor buthionine-sulfoximine (BSO) strongly affected T-DiThias activation, whereas C-DiThia was not affected, supporting the hypothesis of T-DiThia mainly being activated by cellular GSH and C-DiThia mainly being activated by dithiols, especially Trx. Although C-DiThia is not a truly selective probe targeting Trx, the validation methods, in both cell-free and cellular models, allow a thorough evaluation of the performance of the probe.

2.1.2 Probing TrxR

Evaluating probes to report on the activity of mammalian TrxR, the most commonly used motif is 1,2-dithiolanes, a cyclic 5-membered disulfide, which is inspired by lipoic acid, a known TrxR substrate[77]. As a first probe for TrxR **TRFS-green** was published by Fang and coworkers in 2014[78]. The disulfide is cleaved and upon reduction releases a fluorophore, resulting in spectral change and a different fluorescent state, for a direct readout of TrxR activity. To test its

dependency on TrxR, the authors tested the probe activation by some bioreductants (e.g. GSH, L-Cys), however did not include any screens with other redox active enzymes such as the GSH system (Grx/GSH/GR). In cells, the activation was decreased upon treatment with DNCB, a TrxR inhibitor[79].

Based on the reactive structure, several additional probes were developed, all characterised through the cyclic 5-membered disulfide. Due to different chemical modifications, additional characteristics were introduced, however the validation methods as well as the general chemical properties were kept consistent. A brief presentation of these probes follows below. Nevertheless, an important evaluation of TRFS-green must be mentioned before further discussion of its characteristics. Fang and co-workers recently published a follow-up study, using TRFS-green as a sensor for bacterial Trx and Grx. Since prokaryotic TrxR does not contain a Sec in its active site, TRFS-green cannot be reduced by such a motif in bacteria, yet the complete Trx or Grx system (TrxR/Trx/NADPH or Grx/GSH/GR/NADPH respectively) could reduce and therefore activate TRFS-green also in bacteria[80], thus clearly questioning its possible selectivity for the mammalian selenoprotein TrxR.

With an additional TPP moiety added to localize the probe to mitochondria, **Mito-TRFS** was developed and presented by Fang and coworkers[81]. The fluorophore and selectivity structure are the same as in TRFS-green, thereby the same selectivity pattern was expected and observed, using similar validation methods. A direct compartmentalisation, by including a TPP structure, to mitochondria was intended to result in a measurement of TrxR2 activity. However, given the different reactivities of TrxR1 and TrxR2 to substrates, especially kinetically[82], it would have added valuable information to include a cell free evaluation of the probe with TrxR2, while only TrxR1 was used in the study. Looking at the design of Mito-TRFS, it is suffering from the same design flaw as Mito-Naph, i.e. the positioning of the mitochondrial localization sequence is on the fluorophore (see discussion above). Due to its connection to the fluorophore, the probe can be activated in the cytosol and the thereby released fluorophore could be transported to the mitochondria guided by TPP, thus actually not displaying mitochondrial TrxR dependency even if the fluorophore is imaged and localized within the mitochondria.

A red shifted probe named **TRFS-red** was published soon after, showing a similar selectivity pattern while demonstrating improved spectral properties, as a near infrared (NIR) fluorescent probe[83]. A major drawback of the TRFS probes was identified as the slow release kinetic, taking hours for release of the fluorophore. The ‘first-generation’ TRFS-probes are fluorophore release probes, meaning that a reduction of the disulfide site results in an intramolecular cyclization and further release of the fluorescent molecule. To tackle this problem, Fang published a new probe named **Fast-TRFS** as the equivalent of the previous probes but with improved response kinetics[84]. It does not rely on a release mechanism upon reduction but rather a direct switch-on of the fluorophore after disulfide cleavage, thereby showing faster activation, with full activation in 5 minutes or less. Selectivity of the probe was shown with several known bioreductants such as GSH, Cys or Sec, where no activation was observed. They also showed a Sec dependency of TrxR, since the U498C mutant did not activate the probe. However, the focus was on GSH stability. Cellular assays were

performed with DNCB and Auranofin as TrxR inhibitors to show a decrease of probe activation with these probes. Later on this probe was also used to report on bacterial Trx and Grx system, in a study similar to the one used for TRFS-green[85] (see discussion above; this questions selectivity for the mammalian selenoprotein).

As an alternative probe for usage *in vivo* and tissue, a two-photon fluorescent probe **TP-TRFS** was more recently introduced by Fang and coworkers[86]. The advantages are based on different spectral properties, making the probe suitable for *in vivo* imaging and in deeper tissue. The general structure of the TrxR selective site is the known cyclic 5-membered disulfide, connected via a carbamate linker to a fluorophore with two-photon absorption properties. A proof-of-concept study showed a decrease of TrxR activity in brain of mice after stroke, using TP-TRFS[86].

Another probe, which was published in 2022 and is based on a cyclic 5-membered disulfide, is **DSMP**[87]. It works as a ratiometric sensor; upon reduction of the disulfide the emission of the fluorophore shifts towards longer wavelengths. The ratio of the native probe (blue) and reduced probe (green) thereby reports upon the activity of TrxR. Within localization experiments, the authors found a colocalization with MitoTracker, therefore argued that DSMP mainly shows the activity of mitochondrial TrxR. The activation of the probe is a two-step mechanism though, one step to reduce the disulfide and then one step to activate the fluorophore. The disulfide reduction can thus also happen in the cytosol, with subsequent localization to the mitochondria, and can thereby also react to cytosolic TrxR. Due to the enzymatic differences of the two isoforms, especially the higher turn-over of cytosolic TrxR, a clear mitochondrial TrxR dependency cannot be concluded[82]. However, looking at possible TrxR selectivity experiments, the authors used cell free bioreductants, but used GSH only with 100 µM as highest concentration, which does not represent physiological conditions well, and omitted other redox active enzymatic reactions. Nevertheless, the authors showed an activation of the probe in different cell lines as well as mouse brain tissue, suggesting possible biological applications.

Cyclic 5-membered disulfides, 1,2-dithiolanes, have been a recurrent topic within redox biological studies. Early studies indicating an instability towards a number of chemoreductants as well as possible polymerization[77,88–90] or as uptake mediating reagents[91]. We together with Thorn-Seshold discussed this inconsistent literature and performed cell-free and cellular screens with probes, based on cyclic 5-membered disulfides[92]. A clear TrxR dependency in its reduction pattern is not observed, in fact all probes were also activated by Trx, Grx and TrxR in physiological concentrations within cell-free assays, suggesting a careful re-evaluation of previous studies performed with probes, based on this motif. Additionally, the probes were also activated in TrxR1 knockout cells, indicating cross activation besides TrxR1. These findings are somewhat consistent with the additional studies by Fang and Lu and coworkers, showing a reactivity of TRFS-green and Fast-TRFS with bacterial Trx and Grx systems[80,85]. Due to the similarities between mammalian and bacterial thioredoxins and glutaredoxins, all studies together suggest that the initially suggested

mammalian TrxR selectivity for several of the published probes (including the TRFS series as well as DSMP) might not be complete.

A different attempt was followed by Strongin to develop a TrxR selective turn-on probe, **probe 1a**[93]. A linear diselenide probe was introduced which showed stability towards other bioreductants but was rapidly activated by TrxR1 in cell free assays. They considered different sulfur species, in different oxidative states, as well as GSH, Cys and Homocysteine. Cellular studies, based on the TrxR inhibitor DNCB, revealed a DNCB dependency, suggesting TrxR selective characteristics. A linear diselenide is an interesting structure, which has not been discussed intensively so far in the background of redox biological tools, whereby future studies of this probe could show promising characteristics.

Exploring a new trigger structure, Thorn-Seshold in collaboration with us recently presented **RX1**, which is a fluorogenic probe, based on a 1,2-thiaselenane recognition moitey[94]. Following the study about 1,2-dithiolanes, we implemented several cell-free validations, such as screening against other redox active enzymes and enzymatic systems (GR/Grx/GSH/NADPH, TrxR/Trx/NADPH) including different isoforms and additional selenoproteins (GPx1/GR/GSH). We found very little cross-activation and confirmed these results with cellular assays, based on selenium supplementation or starvation to increase or decrease TrxR's cellular activity, genetic depletion of TrxR (knock-out cell lines) and chemical inhibition (TRi-1 as the most selective TrxR inhibitor known[76,95]). RX1 is the most in-depth validated probe thus far, and has been shown to be highly selective for TrxR. We also included a linear disulfide as an unselective control, of which the activation was not influenced by the modifications of TrxR's activity in cells. Within the study, we also showed the possibility of applying RX1 in high-throughput screenings for drug discovery. Remarkably, the results of this screen agreed highly with a previous screen performed only with pure enzymes instead of in a cellular context, which was used to identify TRi-1 as the most selective TrxR1 inhibitor currently known[76]. While the previous screen was based on a cell-free enzyme activity assay, RX1 was hence applied in live cells but still showed excellent agreement with the in vitro findings, highlighting the usability and reproducibility of this probe.

Another attempt by Sidhu and coworkers of developing TrxR selective fluorescent probes is based on a FRET pair, coupled to carbon dots via a linear disulfide linker[96]. Under non-reduced conditions, the carbon dots act as an energy donor and the coupled naphthalimide fluorophore as an acceptor, with an emission at λ_{em} = 565 nm when excited at λ_{ex} = 360 nm, resulting in the final sensor **Biotin-CD-Naph**. The disulfide is reducible by TrxR and then interrupts the FRET pair and the fluorescent emission. A number of other chemoreductants only reduces the sensor to a minor part, whereas Trx can rapidly activate it as well.

Following this approach, Sidhu continued on carbon dot probes for TrxR, developing, **fCDs-Cu^{2+}** [97]. In an attempt to optimize fluorescent characteristics, the emission of the functionalized carbon dots (fCD) is quenched upon addition of Cu^{2+} via its coordination by a chelator on the surface of the fCDs. The chelator is connected to fCDs via a linear disulfide linker, which is cleaved after addition of TrxR, restoring the initial fluorescence by removing the Cu^{2+} complex. This results in a turn-off probe by addition of Cu^{2+} and subsequent turn-on by TrxR.

However, Cu^{2+} is known to be cytotoxic, which is observed in a lowered proliferation in cells after addition of fCDs-Cu^{2+}. Selectivity for TrxR should be based on the disulfide site and was claimed based on cell free assays with GSH, Cys and other bioreductants, that showed a lower turn-on rate of the probe compared to TrxR.

2.2 Possible applications and limitations of enzyme activity probes

Developing small chemical molecules to act as fluorescent reporters for enzymatic activity in live cells or *in vivo* is aiming to provide additional tools for fundamental biological research. However, depending on the performance and applicability of these tools, some probes might not actually find any use. Thereby asking whether the tools are developed for the purpose of developing tools, or for the purpose to be applicable, may be an unwanted question. Although the field of small molecule fluorescent probes has been expanded a lot very recently, only some of the probes have yet been in use in different biologically relevant studies, leading to new insights. Despite most of the probes have been shown to be applicable in cellular models, few have been used in biological studies besides the original publication.

TRFS-green as the most prominent one and commercially available, has been used in 35+ studies, investigating either TrxR inhibitors in live cells or the upregulation of the TrxR system in different mammalian cell types upon treatment (e.g. [98–101]). As discussed above, however, noteworthy is to mention that TRFS-green has also been shown to report on bacterial glutaredoxins and thioredoxins[80], as well as being labile towards mammalian oxidoreductases besides TrxR[92], suggesting careful evaluation and interpretation of results using this probe. Fast-TRFS, which has also been reported to be a sensor for bacterial glutaredoxins and thioredoxins[85], has been used to investigate the effect of compounds treated cell lysate on its TrxR activity[102]. The newest TrxR activity reporter, RX1 was recently applied to evaluate an inhibitor effect in live cells, thereby validating the RSL3 and ML162 compounds as inhibitor of TrxR1[103]. NBL-SS as a Trx probe has been reported to be used in a study, to assess the inhibitory effect of compounds on Trx's activity in HepG2 cells[104].

However, many of the presented probes need a long incubation time, of several hours, to adequately evaluate the probes performance, which clearly limits some possible applications, beyond assessment of inhibitors, which is clearly represented in the given studies, using these probes.

Using newly developed tools, which are less established, should require additional assessments, careful validation efforts, and well-defined limitations of a given tool. Most studies using activity probes for enzymes, focus on inhibitor evaluations, which works fairly well, as seen above. However, it is important to note that, independent of the actual specificity of a given probe, an inhibitor is not automatically specific to a certain enzyme even if an effect is observed. It merely means that the enzyme is one of the targets in cells.

3. Conclusion

Here we have discussed recent approaches of monitoring Trx and TrxR activity in cells using fluorescent activity probes and carefully evaluated the

given data and the respective experimental efforts to validate selectivity, as well as different approaches to use the given tools in studies. Several structures were considered and evaluated within the last year of probe development, to close this gap in redox biology. A major problem is that incomplete selectivity screens do not necessarily translate well to cellular settings. Very few of the presented probes were tested with other redox active enzymes in fully cycling enzyme assays or with other selenoproteins under cell free conditions, although these could yield possible cross reactivity in cells. Different isoenzymes were barely considered or tested. Additionally, cellular bioreductants were often not used in physiological concentrations, within the cell-free evaluation.

Cellular assays are the central node of these evaluations, showing possible future application and underlining selective pattern. However, inhibitor screens with small redox active structures always hold the problem of possible cross reactivity between the inhibitor and the probe, possibly deactivating the probe. A commonly used TrxR inhibitor Auranofin is on the one hand not very selective for TrxR and is on the other hand a highly reactive gold compound, suggesting possible interactions between thiols and Auranofin, making it a difficult model inhibitor for TrxR to show selectivity, if incubated together with disulfides[95,105,106]. The lack of highly selective Trx inhibitors complicates the cellular validation further, and together with incomplete cell-free validations, the often-claimed selectivity is difficult to fully interpret. This hinders a full evaluation and assessment of the presented probes. The different motifs that are investigated to be selective for specific redox active enzyme appear with different characteristics in different studies throughout the literature, e.g. linear disulfides which is claimed to be a selective substrate imitating probe for either Trx or TrxR in different studies[59,67,69,96,97], but has been shown to report unselectively in others[74,92,94]. This is clearly difficult to put in a comprehensive context, without further investigation. The selectivity claims in several of the presented studies should also be seen as problematic without further validation.

To conclude, there are several promising studies about possible selective target structures for Trx or TrxR activity monitoring in live cells. The recent years brought up a major interest in this topic, resulting in several probes which are continuously being improved. However, a standardized set of selectivity screens is not established yet, so the assessment of some of these probes is complex and requires additional effort.

4. Future perspectives

While there is a great development of new tools for redox biological research, it would be attainable to communicate flaws and limitations of existing tools more transparently to simplify approaches to use the tools in future studies. This should include a careful scrutiny of the actual selectivity of a given probe, also offering the possibility to other researchers to evaluate the performance of the respective probes without being experts in redox biology.

The toolbox for redox biological research has been greatly improved in the past years, which reflects the increased interest in redox biology, for fundamental as well as applied biomedical research. Some clear limitations have been identified and discussed in this review, notably unclear selectivity patterns of several, general structural design misses, e.g. concerning the positioning of a

localization or targeting structure, as well as the limitation of long incubation times. While a selective trigger motif has been identified for probing TrxR in RX1, Trx has not yet been successfully selectively probed. Additional efforts are necessary to achieve a truly selective probe for Trx over Grx, and other Trx-like proteins. It should be expected that a thorough effort will be needed to validate any newly designed probe, such as selectivity screens against other redox active enzymes, including different isoenzymes. Moving towards new tools, a set of highly selective probes, targeting different redox enzymes in different compartments would be highly attainable. Including different fluorophores this would provide a toolbox to simultaneously probe different redox enzymes, helping to understand the interplay and regulation of such enzymes better. Additionally, if there could be a way to decrease the incubation time of the probes, it would most likely be beneficial to easier interpret native enzymatic fluctuations.

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