Quantification of intracellular HNO delivery with capillary zone electrophoresis

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none

Abstract

Redox signaling, wherein reactive and diffusible small molecules are channeled into specific messenger functions, is a critical component of signal transduction. A central principle of redox signaling is that the redox modulators are produced in a highly controlled fashion to specifically modify biotargets. Thiols serve as primary mediators of redox signaling as a function of the rich variety of adducts, which allows initiation of distinct cellular effects. Coupling the inherent reactivity of thiols with highly sensitive and selective chemical analysis protocols can facilitate identification of redox signaling agents, both in solution and in cultured cells. Here, we describe use of capillary zone electrophoresis to both identify and quantify sulfinamides, which are specific markers of the reaction of thiols with nitroxyl (HNO), a putative biologically relevant reactive nitrogen species.

Keywords

nitroxyl, sulfinamide, glutathione, thiol, capillary zone electrophoresis, fluorescence

Abbreviations

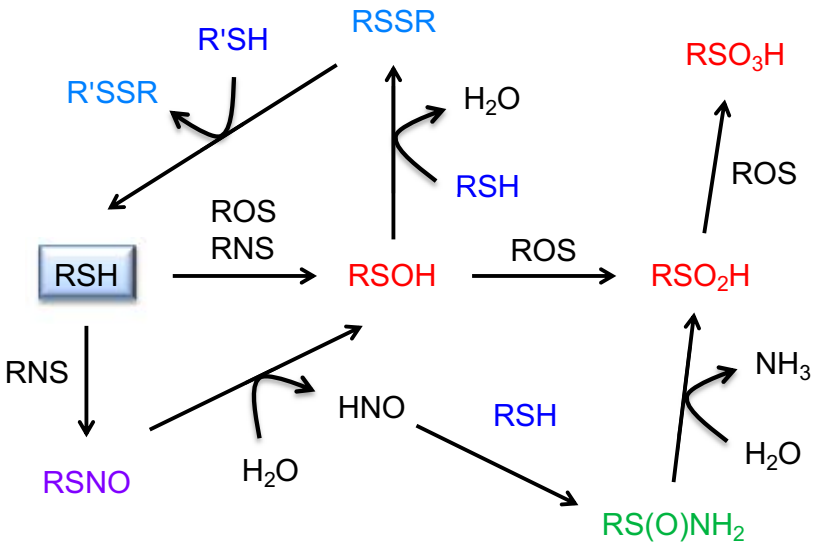
CZE-LIF	capillary zone electrophoresis with laser induced fluorescence detection
DCFL	dichlorofluorescein
DTPA	diethylenetriaminepentaacetic acid
EDRF	endothelium-derived relaxing factor
EOF	electroosmotic flow
FITC	fluorescein isothiocyanate
GSH	glutathione
GSNO	S-nitrosoglutathione
$GS(O)NH_2$	glutathione sulfinamide
GSSG	oxidized glutathione
HNO	nitroxyl
IPA/NO	isopropylamine diazeniumdiolate
NBD-Cl	4-chloro-7-nitrobenzofurazan
NDA	naphthalene-2,3-dicarboxaldehyde
NEM	N-ethylmaleimide
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
ROS	reactive oxygen species
RNS	reactive nitrogen species
RSH	thiol

RSNO	S-nitrosothiol
RSOH	sulfenic acid
RSO_2H	sulfinic acid
RSO_3H	sulfonic acid
$RS(O)NH_2$	sulfinamide
RSSR	disulfide
sGC	soluble guanylyl cyclase
SOD	superoxide dismutase
TCEP	tris(2-carboxyethyl)phosphine

Introduction

Redox signaling is a key component of signal transduction and contributes broadly to both physiological and pathophysiological processes. Evolving from the seminal discoveries of superoxide dismutase (SOD) [1,2] and nitric oxide (NO) biosynthesis (e.g., [3]), redox signaling is now known to be mediated by a variety of small molecules. Since the 1980s, many experimental tools have been designed to study these reactive and short-lived signaling agents, including chemical donors and scavengers, enzymatic inhibitors and fluorogenic indicators. Such tools have greatly simplified, expanded and standardized analysis. Nonetheless, investigation of the intracellular production of small molecular bioregulators continues to be challenging, often due to low sensitivity or specificity of the available assays.

To enhance specificity, we have been inspired by the intrinsic mechanisms that evolved to channel readily diffusible and highly reactive molecules into signaling functions. Cellular specificity is largely achieved by spatial, temporal and stoichiometric control of biosynthesis coupled with specific chemical modifications of target molecules, as dictated by both thermodynamic and kinetic factors [4]. Among the existing reactive sites, cysteine is of particular interest given the diversity of possible modifications, including alteration of the sulfur oxidation state, addition of a variety of small functional groups, coordination to metals, hypervalency and deprotonation. Modification of thiols by reactive nitrogen or oxygen species is shown in Scheme 1.



Scheme 1. Modification of thiols by reactive nitrogen species (RNS) or reactive oxygen species (ROS). RSH, thiol; RSSR, disulfide; RSOH, sulfenic acid; RSO_2H, sulfinic acid; RSO_3H, sulfonic acid; RSNO, *S*-nitrosothiol; $RS(O)NH_2$, sulfinamide.

While cysteine modification is an attractive basis for assay development, the broad range of potential reactive products necessitates high chemical selectivity. We have recently developed an assay that combines the sensitivity of fluorogenic detection with low-volume, high-resolution separation to both identify and quantitate modifications of the ubiquitous tripeptide glutathione (GSH) [5]. This method was originally designed to be both selective and sensitive enough to detect endogenous production of the elusive nitrogen oxide nitroxyl (HNO) through its unique sulfinamide product ($GS(O)NH_2$). It has now been modified to detect other thiols and thiol derivatives.

Capillary zone electrophoresis with laser induced fluorescence detection (CZE-LIF) is a high-resolution separation technique that is capable of detecting sub-nanomolar concentrations of biomolecules from sample volumes as small as a single cell (ca. 1 pL) [6] in time windows as short

as 5 s [7,8]. A variety of species are detectable by CZE, including amino acids, nucleic acids and peptides such as GSH.

In addition to short separation times and high sensitivity, separation allows clear identification of chemical species with closely related structures. For example, *S*-nitrosoglutathione (GSNO) and $GS(O)NH_2$ are respective markers of NO and HNO, and only differ by two hydrogen atoms. Nonetheless, these species can clearly be distinguished by CZE-LIF with a sensitivity in the ~1 nM range [5]. Currently, our CZE-LIF method is sufficiently sensitive to detect GSH and GSH-derivatives in heterogeneous cells grown in standard culture plates or flasks.

While highly sensitive and selective, CZE inherently requires sample processing, and thus prohibits dynamic *in vivo* detection in living cells. However, the quantitative recovery of standards indicates minimal sample loss during workup, suggesting that this approach is useful for detecting reactive species formed in cells and other samples. CZE-LIF instruments are commercially available, making this a broadly accessible approach for monitoring reactive species. The sensitivity of custom versus commercial CZE-LIF instruments differs by 1-2 orders of magnitude. Commercial CZE-LIF instruments yield detection limits in the low to mid-nM range, which may affect the minimum sample size that can be used for analysis using this approach.

This review briefly discusses HNO as a potential signaling molecule and then describes the evolution of our CZE-LIF assay, beginning with detection of GSH. The procedure to detect HNO through formation of $GS(O)NH_2$ is then provided in detail, including measurement of $GS(O)NH_2$ within cells.

HNO: biology and chemistry

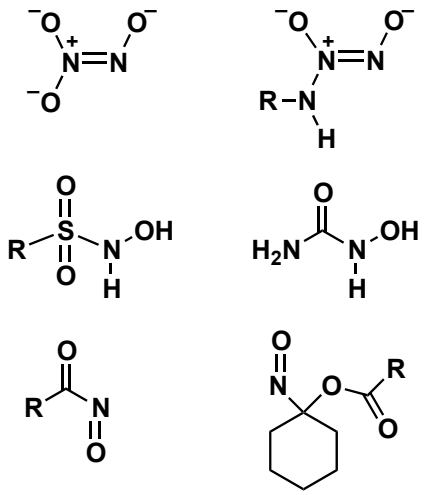
Interest in HNO as a bioregulator arose in the initial analysis of the endothelium-derived relaxing factor (EDRF) [9]. Although nitric oxide (NO) was identified as the primary stimulator of soluble guanylyl cyclase (sGC), HNO can induce moderate activity [10]. In fact, NO synthase (NOS) has been suggested to produce HNO under hypoxic or low cofactor conditions or by oxidative degradation of the intermediate N^{ω}-hydroxy-L-arginine [11-19].

While HNO and NO form a redox couple, direct interconversion is likely limited (see [20]). As such, the possible physiological ramifications of HNO production are significant. For instance, the possibility of biosynthesis of HNO by NOS offers intriguing oxygen-dependent response pathways. For instance, Colton and colleagues [21,22] showed that HNO and NO uniquely impact glycine-independent desensitization of the NMDA receptor. While both NO and HNO potentiate glutamate-mediated channel activation, hypoxia augments the NO-mediated response, resulting in enhanced neuronal toxicity. In contrast, hypoxia attenuates the effect of HNO on calcium influx, potentially offering a degree of protection toward ischemia/reperfusion injury in the brain.

Multiple comparisons have shown that the responses to NO and HNO are generally discrete (see [20,23,24]), due to distinct chemical modifications. Although HNO is more reactive than NO, the main targets for both species are heme proteins and thiols [20,24]. Consistent with the redox relationship, the oxidation state of the heme iron is a key factor, with HNO primarily interacting with ferric heme while NO binds to ferrous heme, in both cases producing a ferrous nitrosyl complex. Inversely, both HNO and NO can modify thiols, but through unique mechanisms and products (Scheme 1).

As with NO, HNO can be produced either metabolically or spontaneously by a variety of donors (Scheme 2) [25,26]. To date, Angeli's salt is the most commonly used HNO donor,

although hydroxylamine derivatives, which can be oxidatively metabolized to produce HNO, are predominant in clinical use. For example, cyanamide (NH_2CN) is used as an aversive agent in the treatment of alcohol abuse, through inhibition of a critical thiol in aldehyde dehydrogenase, and hydroxyurea reduces the incidence of painful crisis in sickle cell disease [27-29].



Scheme 2. Donors of HNO in order as shown: Angeli's dianion, primary amine diazeniumdiolates, N-hydroxylsulfonamide derivatives, hydroxylamine and acyl and acyloxy nitroso compounds.

Angeli's salt is an oxygen-based diazeniumdiolate that has proven to be resistant to modification. In contrast, amine-based diazeniumdiolates are readily functionalized, which imparts diversity in both the rates and mechanisms of decomposition [30-32]. Angeli's salt and primary amine diazeniumdiolates (decomposition mechanisms described in [33]) as well as hydroxylamine derivatives can produce HNO or NO depending on pH. For example, isopropylamine diazeniumdiolate (IPA/NO) produces 60 and 35% HNO at pH 7.4 and 7.0, respectively [34,35].

There are many advantages to use of diazeniumdiolates, including employing the same scaffold to produce NO, HNO or both. However, as with other donor classes, the existence of

multiple condition-dependent decomposition mechanisms is clearly a complicating factor in data interpretation.

In addition to clinical use of HNO donors in treatment of alcohol abuse and sickle cell crisis, pharmacological studies [32,36,37] have led to phase IIB clinical trials of one class of HNO donor for treatment of congestive heart failure. More recently, efficacy of HNO donors in treatment of cancer [38] and pain [39,40] has also been indicated. Although HNO can induce mild vasodilation, intravenous administration of HNO donors does not result in hypotension [36], which is a significant advantage over NO donors. Such studies have established the feasibility of clinical application of HNO donors in a broad spectrum of conditions and consequently support a role for this reactive nitrogen species in physiological regulation.

Detection of HNO

While much evidence points to classification of HNO as a redox mediator, confirmation of biosynthesis for such reactive species are generally challenging. Unusual difficulties arise when working with HNO. Foremost is the fact that in contrast to the free radical NO, HNO rapidly and irreversibly dimerizes [41,42] (Eq. 1; 8×10^6 M^{-1} s^{-1}, pH 7.4, 22 °C) [43]). This self-consumption pathway severely complicates detection and necessitates *in situ* generation of HNO.

$$2HNO \rightarrow [HONNOH] \rightarrow N_2O + H_2O \quad (1)$$

Initially, a variety of different indirect methods, including demonstration of *in vivo* hemodynamic effects, collectively inferred the presence of HNO [32]. A number of more efficient detection assays for HNO have since been designed (see [34,44]). Two of particular note exploit the electrophilicity of HNO to produce unique modifications of thiols and phosphines [34,35].

While there are advantages to both exogenous and endogenous trapping agents, we focused on GSH based on its high intracellular concentration.

Reaction of HNO with GSH

The reaction of HNO with thiols is rapid (Eq. 2; 2×10^6 M^{-1} s^{-1} for GSH [24]) and produces a unique product [45].

$$RSH + HNO \rightarrow [RSNHOH] \rightarrow RS(O)NH_2 \quad (2)$$

At relatively high concentrations of thiol, a second pathway becomes kinetically competitive (eq. 3), but sulfinamide remains the major product [5].

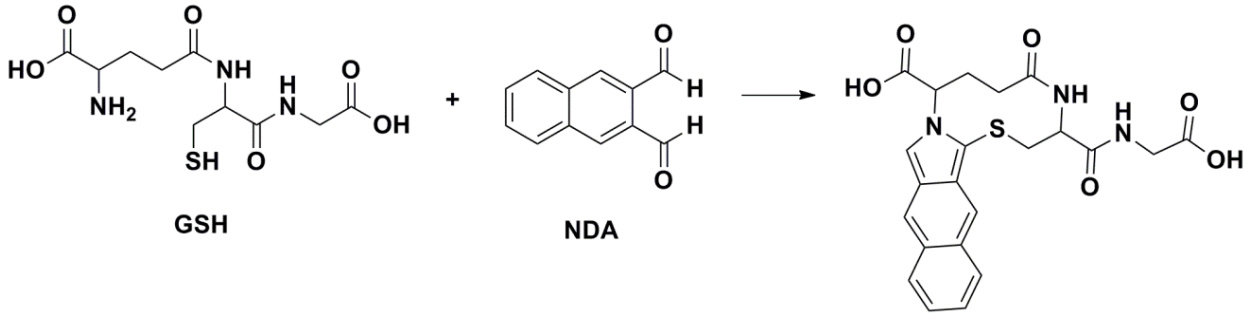
$$RSH + [RSNHOH] \rightarrow RSSR + NH_2OH \quad (3)$$

These two pathways lead to irreversible and reversible thiol modification, respectively, which may be beneficial for signaling [46,47].

Glutathione sulfinamide ($GS(O)NH_2$) can be produced by heating a solution of GSNO and solid sodium cyanoborohydride in a boiling water bath [45]. The reaction of GSH with Angeli's salt [34] leads to nearly quantitative conversion of GSH to $GS(O)NH_2$, with nitrite as a byproduct. Such solutions, while not pure, serve well as standards based on the assumption of complete modification of GSH. Reduction of GSNO, for example by NADH [48] or GSH [49-52], can lead to formation of HNO, which can the react with GSH to form $GS(O)NH_2$. The reduction of GSNO by NADH is also catalyzed by an alcohol dehydrogenase, with $GS(O)NH_2$ being the major product [53]. Whether this is a concerted reaction or involves free HNO requires further investigation.

Detection of GSH and derivatives

A number of assays have been developed to measure GSH, but we sought a simple, high throughput assay based on a thiol specific modification with a fluorescent label. While a large catalog of fluorescent and fluorogenic derivatizing agents is available, of particular utility is a suite of amine reactive probes. This includes naphthalene-2,3-dicarboxaldehyde (NDA), which provides rapid, high sensitivity, quantitative labeling of amino acids and other compounds when used in excess [54]. Due to the amine and thiol side groups of GSH, the reaction with NDA produces a unique aminothiol–aromatic dialdehyde adduct (Eq. 4).



(4)

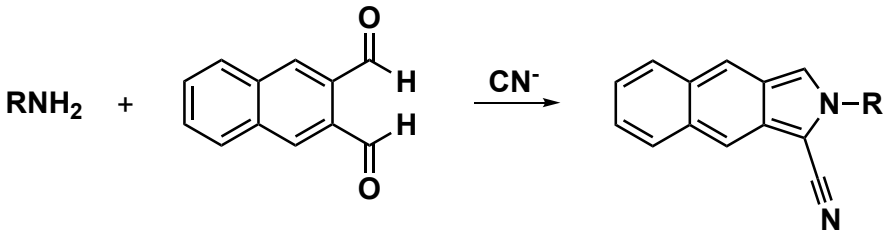
Labeling with NDA is complete in 15 min, and the resulting adduct is stable much longer than necessary for analysis with a plate reader [34]. The signal intensity (λ_{max} 460 nm) for NDA-labelled GSH is linear and reproducible, such that a single calibration curve can be used to quantitate GSH over many days. The limit of detection was determined to be 2.7 ± 0.3 nM (see data in reference 34).

Derivatized GSH does not react directly with NDA since the thiol is no longer available. The ratio of unmodified to modified GSH in a sample can then be readily determined from the loss of signal compared to unreacted sample. While exposure to both HNO and NO modifies GSH, this simple assay can distinguish these nitrogen oxides because the HNO reaction is direct while the

NO reaction requires autoxidation of NO to N_2O_3. Addition of azide to scavenge N_2O_3 removes the NO-based pathway (see data in reference 34), such that only HNO reactivity is assessed.

Given Eqs. 2 and 3, to distinguish the sulfinamide from the disulfide in a sample of GSH exposed to HNO, matched analysis can be carried out with or without tris(2-carboxyethyl)phosphine (TCEP), which reduces GSSG back to GSH. Such processing before NDA labeling demonstrated that 100% of HNO was trapped by excess GSH in an Eppendorf tube [34].

While this assay is quite useful for quantitation of HNO, for example from donor molecules, the sulfinamide itself must be measured to indicate biosynthesis. Free amines have been detected at low nanomolar concentrations using NDA labeling with cyanide to produce isoindole derivatives (Eq. 5), which fluoresce around 490 nm [55-57]. We thus, sought to develop such an assay for GSH and its derivatives.

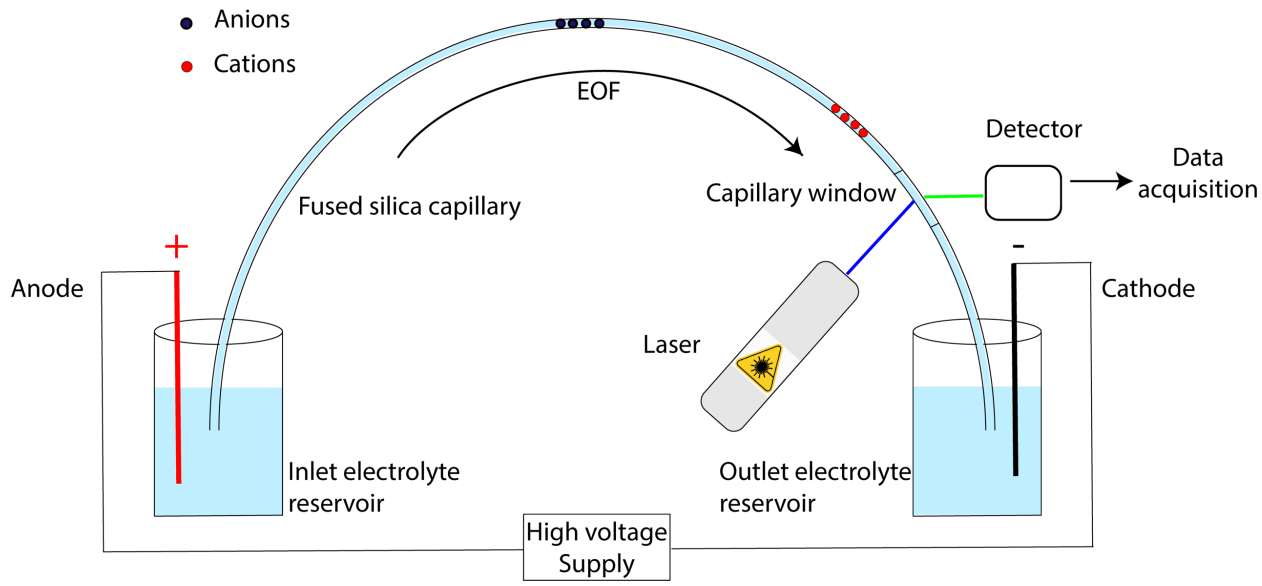


(5)

CZE-LIF

Due to the unique mechanism of separation, CZE allows rapid separation and high-resolution detection of both cations and anions (Scheme 3) [58,59]. Since very small sample volumes are used, CZE is ideal for analysis of biological samples with limited volumes [60,61]. CZE is compatible with a wide range of biological analytes, including carbohydrates, proteins, peptides, amino acids, DNA and other small molecules [62]. To be detected by CZE-LIF, analytes must be fluorescent. Species that lack native fluorescence in the desirable wavelength range can

by labeled with fluorescent or fluorogenic probes. Alternative methods such as UV detection coupled CZE can be used for non-fluorescent analytes, but the detection limits are relatively poor.



Scheme 3. Simple diagram of a CZE-LIF setup where a fused silica capillary is stretched between two reservoirs filled with an electrolyte. The sample is introduced at the inlet end of the capillary, which travels to the outlet due to a combination of the electroosmotic flow (EOF) and electrophoretic mobility. The differences in electrophoretic mobilities causes the analytes to separate into discrete zones and migrate towards the detector placed near the outlet reservoir.

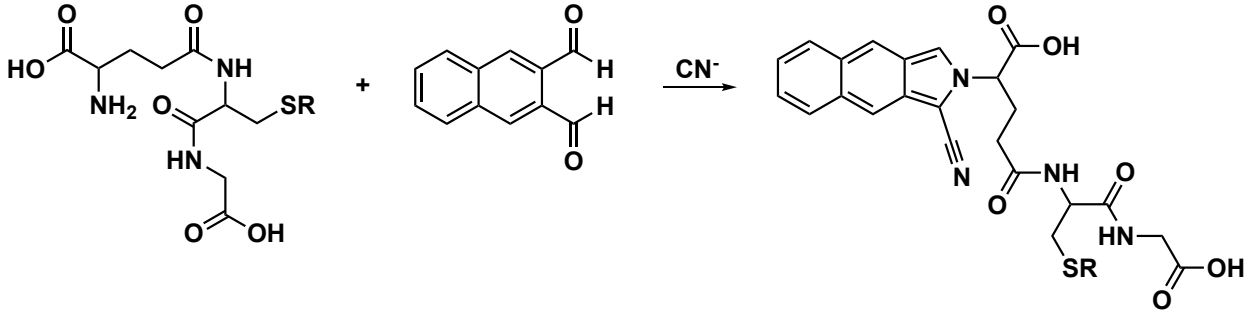
Proteins and amino acids are routinely labelled using a broad catalog of fluorescent or fluorogenic dyes such as NDA, fluorescein isothiocyanate (FITC) and 4-chloro-7-nitrobenzofurazan (NBD-Cl). NDA is a fluorogenic compound, which provides rapid, quantitative labeling of primary amine containing analytes. Due to the fluorogenic nature, addition of excess NDA will not increase the overall fluorescence background enabling large excesses of dye to be used to increase labeling efficiency. Once fluorescent samples are obtained, separation conditions

can be optimized to obtain the desired signal and resolution based on the nature and the complexity of the sample.

The resulting NDA-derivatized analytes are fluorescent in readily accessible wavelength ranges and enable low-nM detection limits. Though NDA is the labeling reagent used in our assay, in principle, any fluorescent labeling reagent can be utilized. Should fluorescent labeling reagents with different structural or optical properties be desired, the reaction time and reactant concentrations will need to be optimized on an individual basis. The use of CZE-LIF for this assay is somewhat forgiving when higher concentrations of fluorescent labels are used since they will be inherently separated from the analytes, but increased backgrounds may result in lower sensitivity.

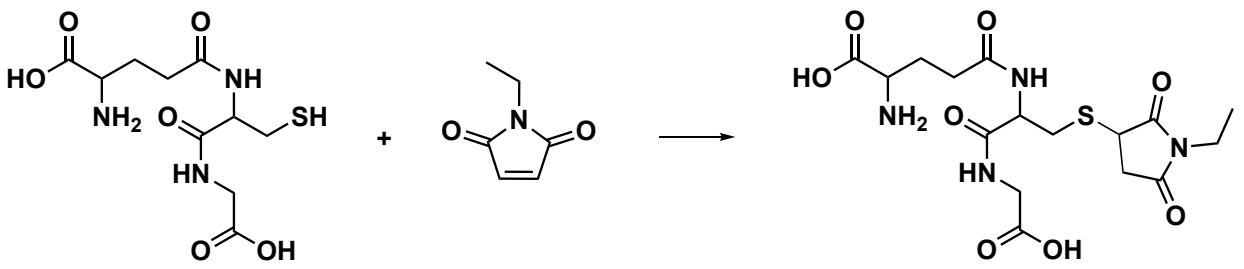
Detection of $GS(O)NH_2$

Labelling of modified GSH with NDA in the presence of cyanide occurs by Eq 6.



(6)

Residual GSH can also be modified with N-ethylmaleimide (NEM) prior to derivatization (Eq. 7, such that only a single labelling process is necessary.



(7)

Separation of labelled GS(O)NH_2 and GSH-NEM by CZE is complete in 3 min (Figure 1), compared to 30 min by HPLC [63]. GSH appears as a split peak in the electropherogram. This is explained by formation upon labelling with NEM of diastereomers, which can be partially resolved under the separation conditions. The response is linear (see reference 5 for data), and the limits of detection are ca. 1 nM. These parameters are sufficient for measurement of both species in cells. In contrast, the signal for GSSG, while apparent, is quenched due to labeling of two amines in close proximity [64]. While GSSG then cannot be quantified directly, concentrations can be determined indirectly through GSH levels in matched samples with or without TCEP treatment.

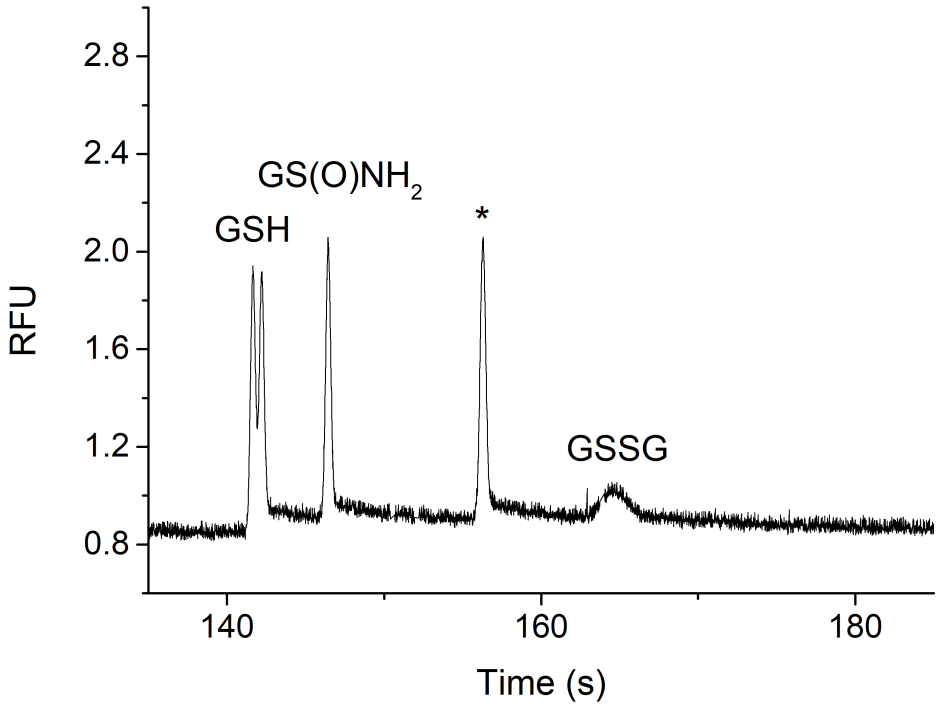


Figure 1. Electropherogram of NDA/CN^--labeled derivatives of GSH. Concentrations of species in the sample: GSH (130 nM, NEM labelled), $GS(O)NH_2$ (150 nM) and GSSG (100 nM) with fluorescein (5 nM; *) as the internal standard.

Labeling of cells lysates with NDA/CN^- and subsequent separation by CZE leads to detection of numerous species with free amines. Fortunately, GSH elutes late among this mixture, such that identification is straightforward (Figure 2). Treatment of cells with exogenous HNO donors leads to a single new signal after that of GSH, which corresponds to the migration time of $GS(O)NH_2$. The signal was found to be specific for treatment to HNO [5], thereby demonstrating the selectivity of the assay. Furthermore, this experiment clearly established that sulfinamides have significant lifetimes within a cellular environment.

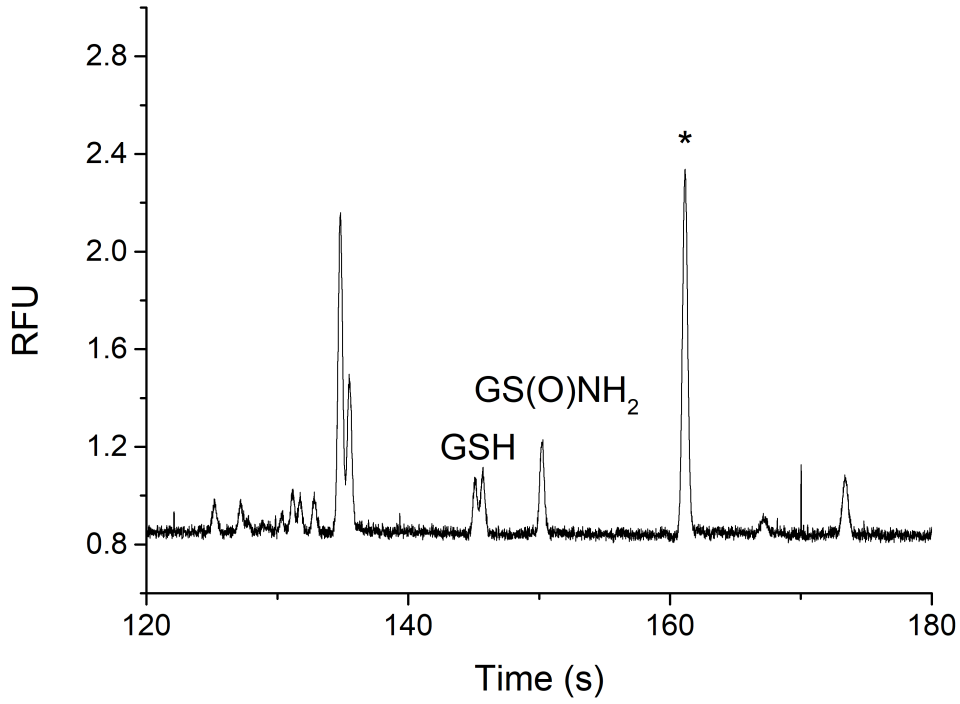


Figure 2. Electropherograms of MDA-MB-231 cell lysate with addition of 250 nM $GS(O)NH_2$ and 5 nM fluorescein (*) as the internal standard. The GSH peak represents intracellular GSH.

Detailed Procedure

A step-by-step protocol to obtain data similar to that shown above is provided here.

Buffer preparation

- Borate buffer (20 mM) is prepared using boric acid (Spectrum) in nanopure water (resistivity of 18.2 MΩ.cm ; Barnstead™ E-Pure™ Ultrapure Water Purification System) and adjusted to pH 9.2 using NaOH. Prior to use, the buffer is filtered using 0.2 μm membrane filters, photobleached for at least 30 min using a UV lamp, and deaerated for at least 15 min by sparging with argon or nitrogen.
- The assay buffer is phosphate buffered saline (PBS, pH 7.4), which is prepared to have final concentrations of potassium dihydrogen phosphate (KH_2PO_4, 1.5 mM), disodium

hydrogen phosphate (Na_2HPO_4, 8.10 mM), potassium chloride (KCl, 2.7 mM), and sodium chloride (NaCl, 137 mM). The metal chelator diethylenetriaminepentaacetic acid (DTPA, 50 μM) is added to reduce adventitious redox reactions.

Capillary conditioning

- A transparent window is introduced to flexible fused silica capillary tubing (25 μm i.d., Polymicro brand, molex; 47 cm long, 37 cm to the detector) by burning the outer polyimide coating (1 cm length).
- The capillary is then flushed with HCl (0.1 M) for 5 min using a syringe pump with a flow rate of 0.1 mL/min. After the acid wash, the capillary is regenerated for 5 min by successive flushes using the same flow rate with nanopure water, NaOH (0.1 M), water, and finally borate buffer (20 mM, pH 9.2) which is used as the electrophoresis running buffer.
- The capillary is inserted into the capillary holder and aligned to obtain maximum signal intensity.

Reagent preparation

- Angeli's salt ($Na_2N_2O_3$, sodium trioxodinitrate) is used as the HNO donor. Stock solutions (>10 mM) are prepared in NaOH (10 mM), and the concentration is determined directly before use by the absorbance at 250 nm ($\varepsilon = 8000\ M^{-1}\ cm^{-1}$). Such solutions can be stored at -20 °C for several weeks, with concentration determined upon thawing. Dilutions of stock solutions are made with NaOH (10 mM) immediately before use at 100× of final reaction condition (e.g., add 10 μL of an Angeli's salt solution to a final reaction volume of 1 mL). Diluted solutions are stored on ice while in use and are not utilized for longer than 1 h.
- Solutions of GSH (>10 mM) are prepared daily in nanopure water and stored on ice.
- Solutions of NDA (4 mM; Sigma) are made in DMSO (Fisher Scientific) and can be stored at 4 °C for several weeks when protected from light.
- Fluoresceine, or derivatives such as dichlorofluorescein (DCFL), is used as the internal standard. Solutions (1 mM) are prepared in DMF weekly and are stored protected from light at 4 °C. Prior to use, the stock is diluted in assay buffer to 500 μM.

- Aqueous solutions of NEM (50 mM; TCI), potassium cyanide (80 mM) and TCEP (50 mM) are prepared daily.

Labeling of GSH

- In preparation for analysis by CZE-LIF, GSH (50 μM, 1 mL) is treated with NEM (50 mM, 10 μL) for 15 min at room temperature to block the thiol group.
- The resulting solution of GSH-NEM (50 μM, 100 μL) is labeled with NDA (4 mM, 12 μL) and KCN (80 mM, 1 μL) in assay buffer (887 μL) in the dark at 4 °C
- The sample is kept on ice for 30 min in the dark, and a color change is evident (Figure 3). Fluorescent labeling is also readily apparent when the sample is illuminated with a laser (Figure 4).
- To produce a standard curve, this stock solution is diluted to final concentrations of 50, 100, 150, 200, and 250 nM (1 mL) using borate running buffer.

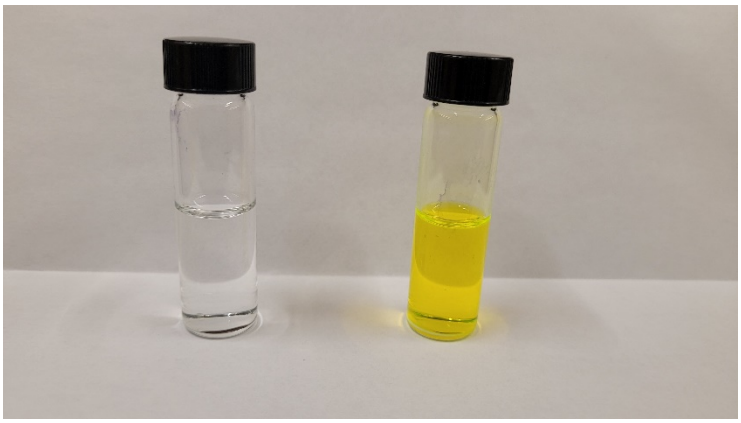


Figure 3. GSH-NEM stock solutions before (left) and after labeling with NDA/CN^- (right).

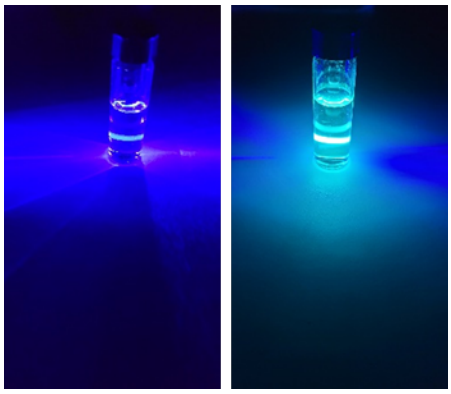


Figure 4. GSH-NEM stock solutions before (left) and after labeling with NDA/CN^- (right) when illuminated with a laser (457 nm).

Preparation and Labeling of $GS(O)NH_2$

- Originally, samples of $GS(O)NH_2$ were prepared by adding aliquots of Angeli's salt to GSH to minimize formation of GSSG [34]. The procedure is as follows: to 10 mL of 50 µM GSH, a total of 100 µL of a 20 mM stock solution of Angeli's salt was added in 20 µL aliquots in 10 min increments while incubating at 37 °C (four-fold excess). The mass spectrum of the sample was consistent with $GS(O)NH_2$.
- Upon development of the CZE-LIF method, it was determined that reaction of GSH (from 500 nM to 50 µM) with two-fold excess Angeli's salt for 1 h at 37 °C produced a single signal consistent with $GS(O)NH_2$. For example, $GS(O)NH_2$ (50 µM) is produced from 1 h reaction of GSH (10 mM, 5 µL) and AS (10 mM, 10 µL) in assay buffer (985 µL).
- To prepare a standard curve, a stock solution of $GS(O)NH_2$ (50 µM, 100 µL) is labeled with NDA (4 mM, 12 µL) and KCN (80 mM, 1 µL) in assay buffer (887 µL) in the dark at 4 °C. The observed color changes are similar to those in Figures 3 and 4.
- The stock solution is then diluted to final concentrations of 10, 20 40, 60, 80, 110 and 150 nM (1 mL) using borate running buffer, followed by addition of the internal standard (500 µM, 10 µL).
- The above conditions are used to analyze the reactions of varied amounts of GSH and Angeli's salt. As necessary, samples are treated with TCEP (5 µL of a 50 mM stock solution for 15 min at room temperature) to reduce GSSG to GSH.

CZE-LIF

- Given the sensitivity of the detector, samples generally must be diluted before loading onto the capillary. For example, $GS(O)NH_2$ (5 µM, 30 µL) is diluted with running buffer (960 µL).
- Before separation, to compensate for any variability in injection volume, an internal standard is added (e.g., in 1 ml sample to give 5 nM internal standard, 10 µL of 500 µM fluoresceine is added).
- Each sample is measured in triplicate, and a minimum of three separate experiments are carried out in total.
- For detection, the 457 nm line of an Ar^+ laser (Innova 70C series, Coherent, Santa Clara, CA, USA), is focused into the center of the separation capillary.
- One capillary end is immersed in the inlet glass vial filled with the running buffer and connected to the high voltage supply using a platinum electrode (Figure 5).

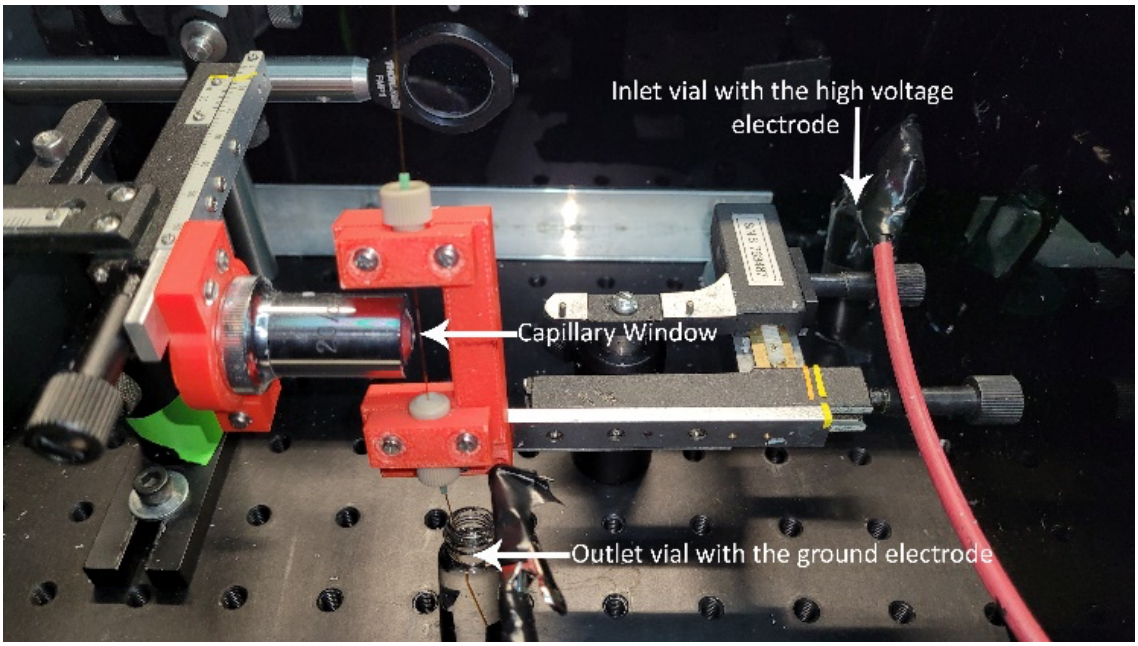
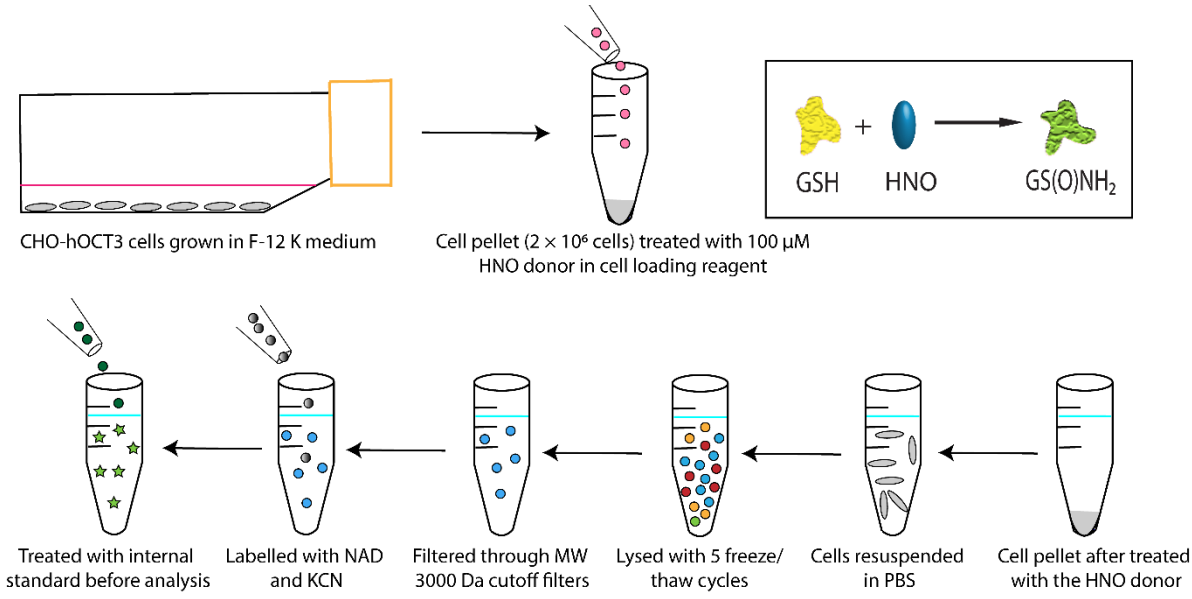


Figure 5. Instrument arrangement for CZE-LIF showing the inlet and outlet buffer vials with the immersed capillary ends and a well aligned capillary window for fluorescence detection.

- The end closer to the capillary window is immersed in the outlet glass vial, which is filled with running buffer and grounded to complete the electrical circuit.
- The laser is turned on, and the capillary window is aligned to maximize the signal and thus to result in high sensitivity.
- Beam power is set to 25 mW, and the fluorescence signal is collected and passed through a 480 nm longpass filter. Emission is detected by a photomultiplier tube (H8249-101, Hamamatsu Photonics, Bridgewater, NJ, USA), current from which was amplified (10^6 V/A) and low pass filtered (at 10 Hz) using a current amplifier (Model 950L8L, Frequency Devices, Inc., Ottawa, IL, USA).
- Hydrodynamic injection is used to introduce the analyte samples into the capillary. Briefly, the capillary end is lifted 10 cm from the inlet vial and dipped inside the analyte vial for 5 s. The end is then again placed in the inlet vial.
- Electric fields are applied using a 30 kV power supply (CZE-1000, Spellman High Voltage Corporation, Hauppauge, NY, USA) with a potential of 25 kV applied across the capillary.
- Signal is collected by an A/D converter using software written in LabVIEW.
- CZE-LIF can successfully resolve unreacted GSH from $GS(O)NH_2$, and even small amounts of other fluorescence impurities and byproducts can be observed as shown in Figure 2. GSH and $GS(O)NH_2$ standards are used to verify peak positions.
- The area under the peak for GSH, $GS(O)NH_2$ and the internal standard is evaluated for each sample using software (for example, Origin 2019b, OriginLab Corporation, Northhampton, MA). The peak area ratio for GSH derivatives and internal standard is then calculated, to provide normalized values.
- Linear calibration curves generated with normalized peak area vs. known GSH or $GS(O)NH_2$ concentration as described above are then used to determine the concentrations in other samples.

Cellular preparations

The workflow is shown in Scheme 4 and described stepwise below.



Scheme 4. Schematic diagram of the workflow for detection of HNO in cells. Following addition of an HNO donor to a cell pellet, cells are lysed and filtered to remove cell debris. The sample is labeled with NAD and KCN, spiked with an internal standard and then analyzed by CZE-LIF.

- The human breast cancer cell line MDA-MB231 (ATCC, Manassas, VA) is maintained in RPMI 1640 (Gibco) supplemented with HEPES (10 mM; Fisher Scientific), 10% fetal bovine serum (Gibco), 1 × Glutamax-I (Life Technology), 1 mM sodium pyruvate (Life Technology), 1% penicillin- streptomycin (50 U/ml, Life Technology). Cells are maintained at 37 °C in an atmosphere of 95% room air and 5% CO_2, and the media is changed twice weekly.
- Experiments are performed in triplicate from three culture flasks.
- After 72 h growth in 250 mL culture flasks, cells are harvested by trypsinization (0.05% trypsin-EDTA, Life Technology) and spun down to cell pellets. The cell pellets are rinsed twice with cold PBS buffer and then resuspended in 8 mL of PBS buffer. Cells are counted with a bright line hemocytometer (Sigma), then repelleted.
- The cell pellets are resuspended and diluted to 6×10^5 cell/mL with pre-chilled PBS/DTPA. The cell suspension (1 mL) is transferred to a 1.5 mL tube for centrifugation at 1200 rpm for 2 min, and the supernatant is decanted and discarded. The cells are resuspended in 400

μL of ice-cold lysis buffer (containing 400 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl and protease inhibitors, in PBS buffer, pH 8.0).

- Cells are lysed using 5 × freeze-thaw cycles. Briefly, dry ice is mixed with isopropanol to carry out the freeze cycle, and warm water at 37 °C is used for the thaw cycle. Tubes containing the cell suspension are immersed in each bath for 10 min.
- After the fifth thaw, cell lysate is centrifuged at 5000 rpm for 20 min to spin down insoluble cell debris.
- Supernatants are transferred to the top of a clean 3kDa MWCO filter, for centrifugal filtration (14000 rpm, 30 min, 4 °C) to remove large proteins and micelles.
- The flow through cell lysate (100 μL) is transferred into a 1.5 mL tube, then is supplemented with $GS(O)NH_2$ (50 μM, 10 μL) and assay buffer (892 μL).
- Free thiols are blocked with NEM (50 mM, 1 μL) for 15 min at room temperature, followed by fluorescent labeling using NDA (4 mM, 6 μL) and KCN (80 mM, 1 μL) in the same tube for 30 min at 4 °C protected from the light.
- The labeled cell lysate is filtered with a 0.22 μm PVDF syringe-filter.
- Labeled cell lysate (50 μL) is transferred to a 1.5 mL tube containing internal standard (500 μM, 10 μL) and running buffer (940 μL).

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