

TECHNICAL ADVANCE

Quantitative *in vivo* measurement of glutathione in *Arabidopsis* cells

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Summary

A new, non-destructive assay is described to quantify cytoplasmic glutathione (GSH) levels *in vivo* in single cells or populations of cells from *Arabidopsis* suspension cultures. Cytoplasmic GSH was labelled with monochlorobimane (MCB) *in situ* to give a fluorescent GSH-bimane (GSB) conjugate. At low (10–100 μM) concentrations of MCB, labelling was mediated by a glutathione S-transferase, which confers specificity for GSH. HPLC analysis of MCB-labelled low molecular-weight thiols showed that the assay measures the total GSH pool, including the oxidized glutathione. The progress curve for the labelling could be described using Michaelis–Menten kinetics with an apparent K_M of 40 μM and V_{max} of 470 $\mu\text{mol l}_{\text{cyt}}^{-1} \text{min}^{-1}$. There was no evidence for *de novo* synthesis of GSH during the labelling period of 2 h, suggesting that control of GSH synthesis is not mediated by feedback control of γ -glutamylcysteine synthetase in this system. The total cellular level of GSH was calculated from the plateau value of the progress curve, after appropriate calibration, as 830–942 $\text{nmol g}^{-1} \text{FW}$. The volume fraction of cytoplasm was measured from serial optical sections of bimane-labelled cells collected by confocal laser scanning microscopy (CLSM) with excitation 442 nm, or two-photon laser scanning microscopy (TPLSM) with excitation 770 nm. A value of $42 \pm 3\%$ cytoplasm was determined by manual segmentation, and a value of $37 \pm 2\%$ using stereological techniques. Using these figures, values for cytoplasmic [GSH] were estimated to be between 2.7 ± 0.3 and $3.2 \pm 0.3 \text{ mM}$ for cell populations. In addition, measurement of GSH levels in individual cells using CLSM and TPLSM gave values of 3.0 ± 0.5 and $3.5 \pm 0.7 \text{ mM}$, respectively.

Keywords: glutathione, monochlorobimane, glutathione S-transferase, fluorimetry, laser scanning microscopy, stereology, *Arabidopsis*.

Introduction

The tripeptide glutathione (GSH) is the major cytoplasmic pool of non-protein reduced sulphur in most organisms. It has a number of key metabolic functions and plays a central role in anti-oxidant defences, heavy metal tolerance and xenobiotic detoxification (reviewed by May *et al.*, 1998a; Noctor and Foyer, 1998; Noctor *et al.*, 1998). It appears that a complex set of interactions regulate the steady-state level of GSH (May *et al.*, 1998b; Noctor *et al.*, 1996; Xiang and Oliver, 1998), and GSH levels are known to vary during plant development and in response to a wide

array of stimuli such as atmospheric pollutants, biotic stress and light (Alscher, 1989; May *et al.*, 1998a). The importance of GSH in plant physiology is underlined by a number of current attempts to genetically modify levels of GSH in plants to enhance their environmental stress tolerance (Creissen *et al.*, 1999; Noctor *et al.*, 1996; Strohm *et al.*, 1995).

GSH levels in plants or tissue extracts are routinely measured by HPLC analysis of low molecular-weight thiols after derivatization with monobromobimane (MBB)

(Newton *et al.*, 1981), or by absorption measurement after oxidation by 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB) (Anderson, 1985) or following conjugation to 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of glutathione *S*-transferase (GST) (Hermsen *et al.*, 1997). Although specific and sensitive, these methods require extraction of the tissue and provide an average estimate for all cells in the tissue. Results are typically expressed on a fresh weight or protein basis rather than in terms of cytoplasmic concentrations. To complement these approaches, we have been developing fluorescent imaging techniques to measure GSH in intact plant tissues, with particular emphasis on mapping cell-specific differences in cytoplasmic GSH concentrations following labelling with monochlorobimane (MCB) (Fricker *et al.*, 2000; Gutiérrez-Alcalá *et al.*, 2000; Meyer and Fricker, 2000; Sánchez-Fernández *et al.*, 1997). MCB is a cell-permeant and essentially non-fluorescent probe, which reacts with GSH to form a cell-impermeant and fluorescent GSH-bimane (GSB) conjugate (Haugland, 1999). It has been shown that MCB is a valid fluorescent probe to trace the detoxification of xenobiotics by conjugation to GSH in cells of carrot, maize and barley (Coleman *et al.*, 1997), and that GSB is transported into two different types of vacuole in barley aleurone cells (Swanson *et al.*, 1998). MCB also labels GSH in intact tissues, and the level of fluorescence can be quantified using confocal laser scanning microscopy (CLSM) or two-photon laser scanning microscopy (TPLSM) within individual cells in their correct tissue context (Fricker and Meyer, 2001; Fricker *et al.*, 2000; Gutiérrez-Alcalá *et al.*, 2000; Meyer and Fricker, 2000; Sánchez-Fernández *et al.*, 1997). This type of analysis has highlighted the variability of GSH levels in different cell types, and can also provide information on the activities of glutathione *S*-transferase (GST) and glutathione *S*-conjugate pump (GS-X-pump) activities *in vivo*. However, it requires complex and time-consuming calibration and correction procedures, and is therefore not appropriate for large numbers of samples.

There is considerable interest in developing routine assays for physiological parameters, including GSH, both to help dissect out their own underlying control network (e.g. Vernoux *et al.*, 2000), and also to use as indirect assays for uptake and metabolism of factors, such as xenobiotics and heavy metals, that can be followed through their effect on GSH levels. Ideally, to keep pace with the major genome and proteome initiatives such assays should be scaleable for high-throughput screening. In this paper we describe a simple, rapid and quantitative assay to measure $[GSH]_{\text{cyt}}$ in suspension-culture cells across a range of measurement platforms, from single cells imaged by CLSM or TPLSM to populations of cells measured by fluorimetry in stirred cuvettes or multi-well plates.

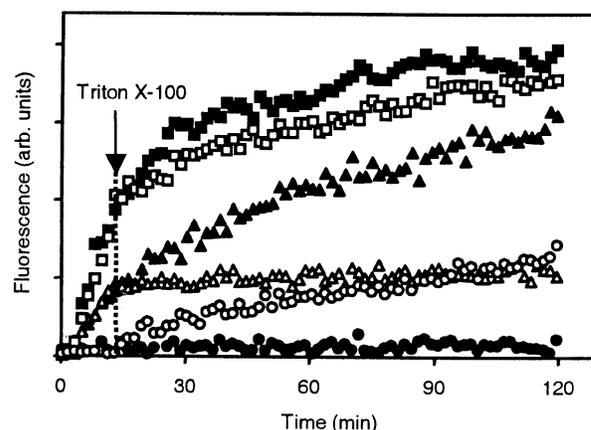


Figure 1. Typical progress curves for *in vivo* labelling of *Arabidopsis* suspension-culture cells with different bimanes and effect of cell lysis on the rate of GSB formation.

10 μM MCB (\blacktriangle , \triangle); MBB (\blacksquare , \square); or QBB (\bullet , \circ) were added to MS medium at pH 7.2. For full progress curves (closed symbols) cells were added to the medium 3 min after start of the time course ($\lambda_{\text{ex}} = 442 \text{ nm}$; $\lambda_{\text{em}} = 477 \text{ nm}$). Labelling rapidly increased to a plateau in the presence of MBB and more slowly in the presence of MCB. No fluorescence increase was observed in the presence of QBB. After cell lysis by addition of Triton X-100 9 min after the start of labelling, progress curves continued with altered rates reflecting different rate constants for non-enzymatic conjugation of different bimanes (open symbols).

Results

In vivo labelling of Arabidopsis cells with bimanes

Monochlorobimane (MCB) and the more reactive monobromobimane (MBB) are both uncharged and cell-permeant, whilst the quaternary ammonium ion bimane-derivative monobromotrimethylammoniumbimane (QBB) is charged and therefore membrane impermeant. When cells were labelled with 10 μM MBB, there was an increase in fluorescence and the progress curve for the reaction ended up at a plateau after about 90 min (Figure 1). Labelling with 10 μM MCB was slower, and was still increasing slightly after 120 min (Figure 1). There was no significant increase in fluorescence intensity over a 2 h period with QBB (Figure 1), indicating that no GSH was present in the medium. In addition, sampling of the suspension medium after separation of cells showed that no significant release of GSH from the cells occurred during the time course of the experiment ($n = 4$; data not shown).

Triton X-100 was then added during the course of the assay to lyse the cells in a medium comparable to the ionic composition of the cytoplasm at pH 7.2. After lysis, an increase in fluorescence was observed with QBB, reflecting the non-enzymatic second-order reaction at this pH for nucleophilic attack on the bimane by thiols released from the cells (Figure 1). Conjugation with MBB also continued after lysis at a similar rate to that observed with QBB; however, labelling with MCB was dramatically reduced.

Rate constants for this non-enzymatic reaction were determined to be $0.81 \pm 0.17 \text{ M}^{-1} \text{ sec}^{-1}$ ($n = 5$) for the conjugation of GSH to MBB, and $0.15 \pm 0.03 \text{ M}^{-1} \text{ sec}^{-1}$ ($n = 6$) for the conjugation of GSH to MCB. When the pH of the medium was adjusted to the normal pH of the culture medium (pH 5.8), no conjugation was observed after cell lysis, as the thiol groups are predominantly protonated and relatively unreactive at this pH (data not shown).

Specificity of bimane labelling

To determine which cellular constituents were labelled by MCB and MBB, cell extracts were separated by size-exclusion chromatography on Sephadex G-25 columns. The main peak, following labelling with both MCB and MBB, corresponded to low molecular-weight soluble compounds (Figure 2a). In addition, MBB at concentrations higher than $100 \mu\text{M}$ also labelled a fraction that co-eluted with the main protein peak (Figure 2a). No protein labelling was detected with MCB at a comparable concentration (Figure 2a), and only limited labelling was observed when the concentration was raised to $300 \mu\text{M}$ MCB (data not shown).

To determine which soluble thiols were labelled, extracts were separated by HPLC. *In vivo* labelling with $100 \mu\text{M}$ MCB for 1 h showed one major peak for GSH and two minor peaks for cysteine and γ -glutamylcysteine (γ -EC) (Figure 2b). The amount of labelled cysteine was 6–7% of the labelled GSH when cells were incubated with MCB concentrations at $100 \mu\text{M}$ or higher (Table 1). To further test the specificity of the labelling, cells were pre-incubated with CDNB for 5 min to deplete the GSH pool. After the CDNB treatment, MCB labelling resulted only in a minor GSB peak equivalent to $14 \text{ nmol GSH g}^{-1} \text{ FW}$ or 1.7% of GSB in the control ($839 \text{ nmol g}^{-1} \text{ FW}$) (Figure 2b, Table 1) and there was essentially no γ -EC or cysteine labelling. Labelling with MBB *in vitro* gave similar results for GSH, but slightly less cysteine labelling. In this case the cysteine pool was about 4.5% of the size of the reduced GSH pool, and the γ -EC pool was about 2.5% of the reduced GSH pool. Additionally, the amount of oxidized glutathione (GSSG) was measured *in vitro* to be $35 \text{ nmol g}^{-1} \text{ FW}$, or 4.2% of the reduced GSH pool.

To test whether the entire glutathione pool was labelled, cells were ground after *in vivo* labelling with MCB, and then remaining thiols reacted with the conventional *in vitro* MBB derivatization method. After *in vivo* labelling with $10 \mu\text{M}$ MCB there was still additional *in vitro* labelling with MBB as expected, as the assay had not run to completion, but for 100 and $300 \mu\text{M}$ MCB no further labelling of GSH was observed by subsequent *in vitro* labelling with MBB (Table 1).

As labelling reaches a stable plateau, we infer that no new GSH synthesis takes place during the assay. In

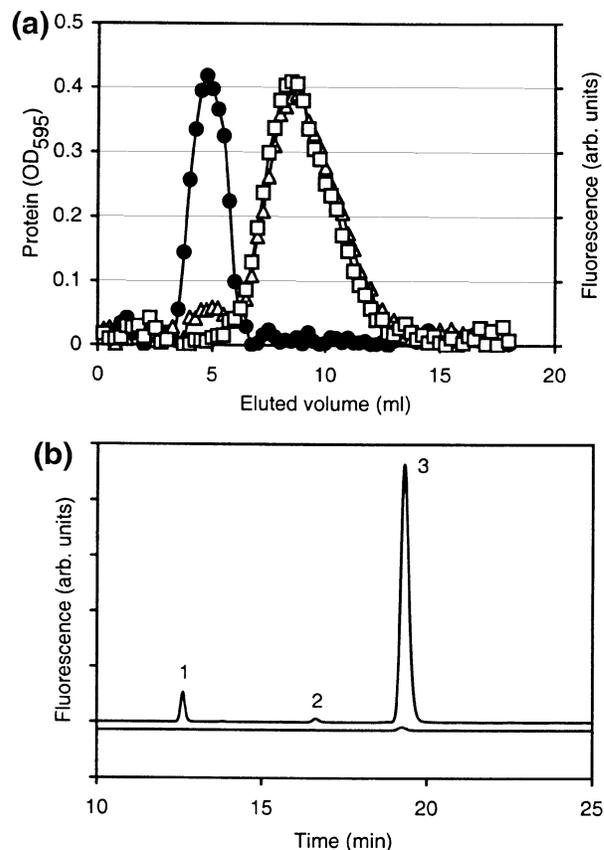


Figure 2. Separation of bimane-labelled proteins and low molecular-weight thiols after labelling *Arabidopsis* suspension-culture cells with different concentrations of monobromobimane and monochlorobimane. (a) Typical elution profile of bimane-labelled thiols on a Sephadex G-25 column after *in vivo* labelling of cells with $100 \mu\text{M}$ MCB (\square) or $100 \mu\text{M}$ MBB (Δ), respectively. The elution profile for fluorescently labelled thiols is overlaid with the elution profile for proteins (\bullet) to identify fractions containing bimane-labelled proteins. (b) Typical HPLC elution profile of low molecular-weight thiols after *in vivo* labelling of *Arabidopsis* suspension-culture cells with $100 \mu\text{M}$ MCB. (i) Labelled thiols after incubation of cells with MCB for 2 h without CDNB (upper chromatogram); (ii) labelled thiols after pre-incubation of cells with 5 mM CDNB for 5 min and subsequent incubation with MCB for 2 h (lower chromatogram). 1 = cysteine S-bimane; 2 = γ -glutamylcysteine S-bimane; 3 = glutathione S-bimane.

support of this, the progress curves for labelling of cells simultaneously incubated with buthionine sulfoximine (BSO), an inhibitor of GSH synthesis, during the measurement were not different from the controls across a range of MCB concentrations (Figure 3).

Kinetics of bimane labelling and quantification of GSH levels

If labelling with MCB is enzyme-catalysed, the progress curves of labelling at different MCB concentrations should saturate, and the plateau values should reflect the total

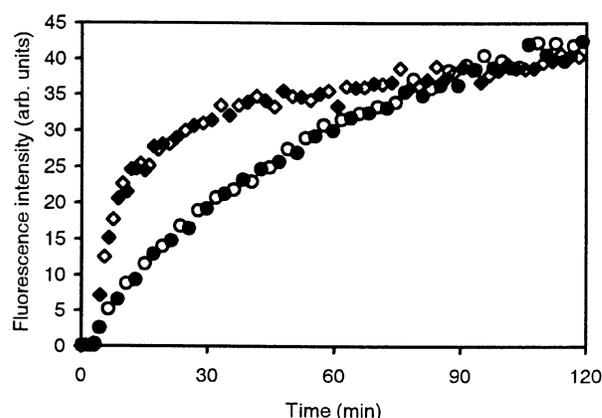
Table 1. Comparison of *in vivo* and *in vitro* labelling of low molecular-weight thiols with bimanes

Labelling	Dye concentration	GSH ^a	GSSG ^a	Cysteine ^a	γ-EC ^a
<i>In vivo</i>	10 μM MCB	586 ± 59	nd	39 ± 5	8 ± 2
	100 μM MCB	839 ± 68	nd	51 ± 7	14 ± 3
	300 μM MCB	826 ± 46	nd	57 ± 8	15 ± 3
<i>In vitro</i>	3 mM MBB pH 8.2	848 ± 52	35 ± 13	39 ± 8	32 ± 7
<i>In vivo</i> plus subsequent <i>in vitro</i>	10 μM MCB	827 ± 61	15 ± 3	50 ± 4	19 ± 9
	100 μM MCB	856 ± 92	nd	53 ± 9	16 ± 3
	300 μM MCB	861 ± 83	nd	62 ± 8	18 ± 5
<i>In vivo</i> after pre-treatment with 5 mM CDNB	100 μM MCB	14 ± 2	nd	1.1 ± 0.3	0.5 ± 0.1

^aAll nmol g⁻¹ FW. All values are mean ± SD (*n* = 5). For *in vivo* labelling cells were subjected to monochlorobimane for 2 h. nd, Not detected.

glutathione pool in the cells. An increase in fluorescence to a stable plateau value was observed for *Arabidopsis* suspension-culture cells treated with varying concentrations of MCB. For example, the half-time to reach the plateau was about 60 min with 10 μM MCB, whilst at 50 μM MCB this figure was reduced to 25 min and to 10 min at 100 μM MCB (Figure 4a). Although the rate of GSB formation increased with increasing concentrations of MCB, the final fluorescence intensity attained was progressively reduced. Addition of Triton X-100 was sufficient to lyse the cells at the end of the experiment, and resulted in a partial recovery of fluorescence at high MCB concentrations, suggesting that an amount of self-quenching of the fluorescence was occurring inside the cells. The remainder of the reduction in signal could be attributed to self-absorption in the cuvette in accordance with the Lambert–Beer Law, as both MCB and GSB absorb over a broad range extending from the UV to blue ($\lambda_{\text{max}} = 395 \text{ nm}$, $\epsilon = 6000 \text{ cm}^{-1} \text{ M}^{-1}$) (Haugland, 1999; Kosower and Kosower, 1987). In cuvette measurements, this inner filter effect caused marked deviations from linearity in the calibration curve measured with excitation at 395 nm, and at concentrations above 100 μM the intensity of the emitted light dropped significantly (Figure 4b). To reduce the contribution of self-absorption, measurements were routinely made at 442 nm, the wavelength also used for CLSM measurements, where the absorption coefficient is lower. At this wavelength, although less sensitive, the calibration deviated from linearity by only 4% up to 60 μM GSB (Figure 4b), which is well above the typical concentration of GSB in the cuvette following cell lysis.

The free dye in the medium was also found to attenuate the fluorescence signal from GSB formed within the cells. To determine the correction factors for this attenuation, the effect of free MCB on the fluorescence from a given concentration of GSB was measured (Figure 4b, inset). The fluorescence excited at 442 nm in a 3 ml cuvette decreased by 30% when 100 μM MCB was added, and by 60% in the presence of 300 μM free MCB.

**Figure 3.** Effect of simultaneous incubation with BSO on the progress curve for labelling *Arabidopsis* cells with MCB.

Cells were incubated with either 10 μM (●, ○) or 100 μM (◆, ◇) MCB in the absence (closed symbols) or presence (open symbols) of BSO.

To calibrate the GSB signal, fluorescence intensities were corrected by background subtraction and for inner filter effects using the empirically determined correction factor (Figure 4b, inset). After correction, the fluorescence intensity at the plateau was similar for MCB concentrations up to 300 μM (Figure 4c).

In the plate reader, cells were allowed to settle and the fluorescence measured using fibre-optic sensors from the bottom of each well. Although the path length through the free dye was minimal during the experiment, with correspondingly little effect of self-absorption, the cells had to be lysed with Triton X-100 at the end of the experiment to allow comparison with the free dye which is homogeneously distributed in the calibration solutions. In this system, cell lysis results in a reduction in fluorescence as the dye redistributes from the cells throughout the well (Figure 5a). The corresponding calibration curve for the plate reader is shown in Figure 5(b).

The advantage of the plate reader system over single cuvette assays is shown in Figure 5(a), in which replicate

progress curves for a range of GSB concentrations were simultaneously measured to determine the kinetic parameters for the GST-catalysed conjugation reaction *in situ*. The initial rate of GSB formation was dependent on the concentration of MCB. This relationship was non-linear, but could be described by Michaelis–Menten kinetics (Figure 5c). The apparent K_M (K_{app}) for the GST operating in intact cells was $40 \mu\text{M}$, and the V_{max} was $470 \mu\text{mol l}_{cyt}^{-1} \text{min}^{-1}$.

Determination of cytoplasmic GSH concentration

To convert the total GSH level measured into the cytoplasmic GSH concentration ($[\text{GSH}]_{cyt}$) requires an

estimate of the volume of cytoplasm used in the assay. The total cell volume was between 10 and 20 μl in a 3 ml assay, depending on whether cells were used 3 or 4 days after subculture. The volume of cytoplasm in these cells was measured from serial optical sections collected by laser scanning microscopy using two different approaches. In the first method, either the cytoplasm (Figure 6a) or the vacuole (Figure 6b) was manually delineated in each section and summed to give the total volume of the respective compartment. This approach resulted in an estimate of 42% cytoplasm (Table 2). The second approach involved stereological analysis of cells using the Cavalieri estimator of volume, and gave a cytoplasmic volume fraction of 37% (Figure 6c–e, Table 2). Based on these different values for the volume of the cytoplasm, cytoplasmic GSH concentrations between $2.8 \pm 0.3 \text{ mM}$ (segmentation) and $3.2 \pm 0.3 \text{ mM}$ (Cavalieri) were calculated from the fluorimeter data, and $2.7 \pm 0.4 \text{ mM}$ (segmentation) and $3.1 \pm 0.4 \text{ mM}$ (Cavalieri) from the plate reader data (Table 2).

Direct measurement of GSH in single cells

Direct measurement of GSH as its bimane derivative in single cells by TPLSM overcomes the problem with the inner filter effect in the cuvette-based system, because with $\lambda_{ex} = 770 \text{ nm}$ there is essentially no absorption outside the focal volume. The calibration of the fluorescence against GSB standards was linear in this case (data not shown). After addition of MCB to the cells the fluorescence increased, initially in the cytoplasm, and followed a

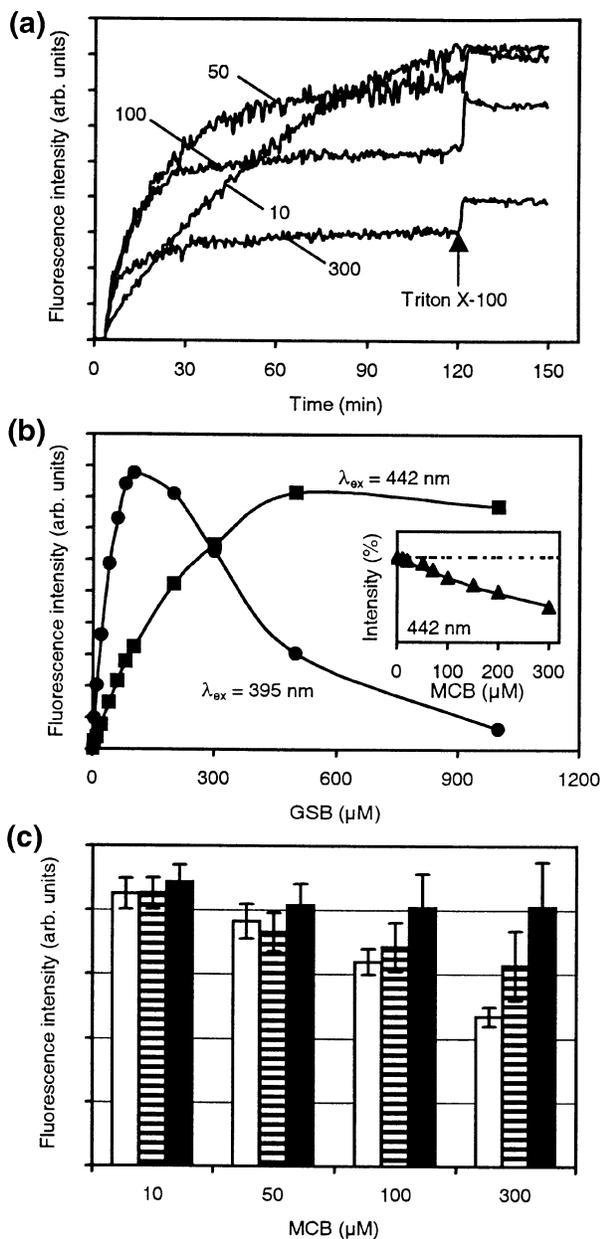
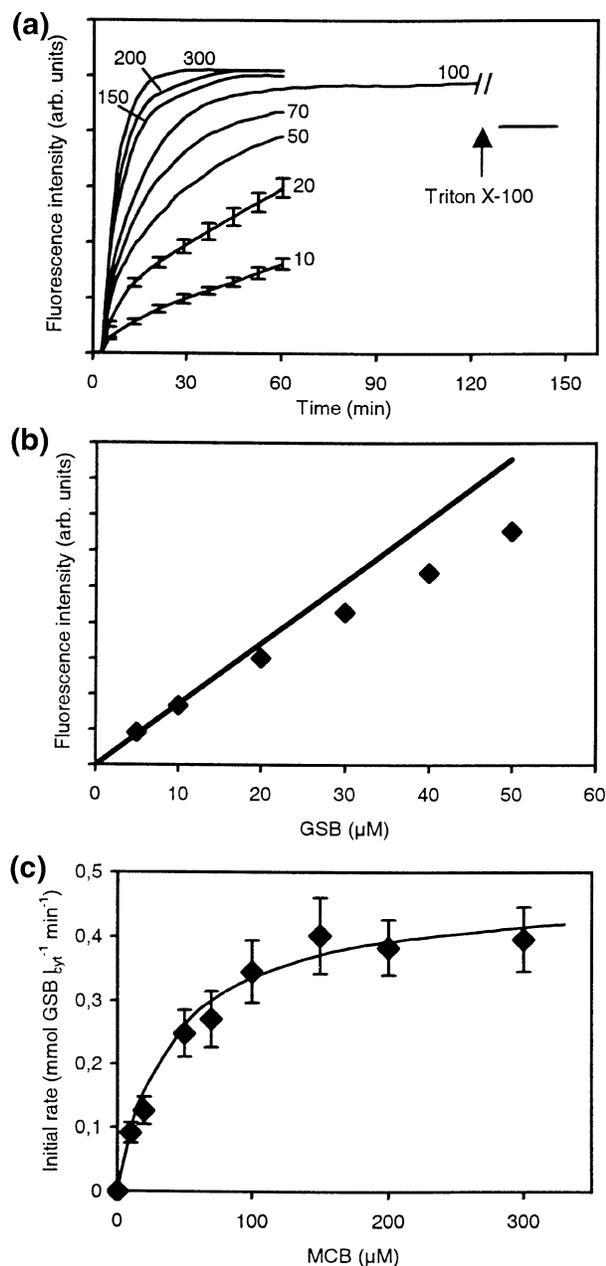


Figure 4. Quantitative *in vivo* measurement of GSH in *Arabidopsis* suspension-culture cells using a fluorimeter.

(a) Typical progress curves for the *in vivo* labelling of *Arabidopsis* suspension-culture cells with different concentrations of MCB. Cells were added to MS medium containing different concentrations of MCB at $t = 3 \text{ min}$. At $t = 120 \text{ min}$, cells were lysed by addition of 2% Triton X-100. Numbers indicate the concentration of MCB used in μM .

(b) GSB calibration curves for fluorimetry. Different concentrations of GSB were added to a medium comparable to the ionic composition of the cytoplasm buffered at pH 7.2 in a 3 ml cuvette. Fluorescence was excited at 395 ± 2.5 or $442 \pm 2.5 \text{ nm}$, with emission recorded at $477 \pm 2.5 \text{ nm}$. The apparent decrease in fluorescence with excitation at 395 nm reflects self-absorption by bimane in the cuvette. This effect is less pronounced with excitation at 442 nm, although the fluorescence yield is lower. Inset shows decrease in fluorescence of GSB with increasing concentrations of free MCB. Horizontal line gives 100% fluorescence. The graph allows calculating the correction factors for *in vivo* measurements of GSB in the presence of free MCB in the medium.

(c) Correction of plateau values of fluorescence intensity for intrinsic fluorescence of free MCB, absorption of excitation light by free dye, and quenching of GSB fluorescence by high concentrations of free dye. Measured fluorescence intensities (white bars) were corrected for absorption of excitation light by free dye in the medium (horizontally striped bars). Black bars show corrected fluorescence intensities after cell lysis. All values mean \pm SD ($n = 6$).



progress curve similar to that measured by fluorimetry (data not shown). Imaging also revealed that transport of GSB into the vacuole started immediately after labelling was initiated, and after 2 h very little fluorescence remained in the cytoplasm (Figure 6b). To quantify the total amount of GSB formed after 2 h of labelling, the fluorescence in both cytoplasm and vacuole was measured and converted to concentrations by calibration of the fluorescence signal against a series of solutions with known amounts of GSB that were imaged under identical instrument settings. The GSB concentrations in cytoplasm and vacuole were then summed and calculated on the basis of cytoplasmic volume, taking into account the volume ratio between cytoplasm and vacuole as measured either by segmentation of confocal images, or by the Cavalieri estimator for the TPLSM images. From this the cytoplasmic GSH concentration measured by CLSM was 3.0 ± 0.5 mM and the concentration measured by TPLSM was 3.5 ± 0.7 mM (Table 2).

Discussion

The aim of the present work was to determine whether MCB could be used as an *in vivo* probe to directly measure cytoplasmic GSH concentrations in intact *Arabidopsis* cells

Figure 5. Quantitative *in vivo* measurement of GSH in *Arabidopsis* suspension-culture cells using a fluorescence plate reader and multi-well plates.

(a) Typical progress curves for labelling cells with different concentrations of MCB on a multi-well plate. Numbers next to the graphs indicate the concentration of MCB used in μM . Because cells settled at the bottom of the wells, fluorescence was not significantly reduced by self-absorption. However, for correct calibration cells had to be lysed at the end of the time course to compare fluorescence with the fluorescence from standard GSB solutions. The progress curves shown are averaged from five different wells on the same plate. Standard deviations are given as examples for every third data point obtained with 10 and 20 μM MCB.

(b) Calibration curve for GSH measurements on a fluorescence plate reader. Fluorescence was excited at 390 nm and recorded at 460 nm. Deviation from linearity at concentrations above 30 μM is due to self-absorption by GSB.

(c) Kinetic analysis of concentration dependence of labelling of *Arabidopsis* cells with MCB.

All values, mean \pm SD ($n = 5$).

Figure 6. Optical sections of live *Arabidopsis* cells taken by two-photon laser scanning microscopy with $\lambda_{\text{ex}} = 770$ nm.

Cells were stained with MCB to label GSH (green) and propidium iodide as a counter-stain for the cell walls and as an indicator of plasma membrane integrity (red). Bars = 20 μm .

(a) Median optical section from cells labelled with 100 μM MCB on ice for 30 min. Cold treatment inhibited vacuolar sequestration of GSB.

(b) Median optical section from cells labelled with 100 μM MCB for 2 h. Note the red colour of chloroplasts was due to chlorophyll autofluorescence rather than propidium staining.

(c) Same cells as in (b), stack of 31 optical sections throughout the cells. Sections were collected with a distance of 1 μm between single sections; the stack is displayed from the side as a maximum projection with a tilt angle of 90°.

(d) Six sections out of the stack presented in (c). Images are displayed as a maximum projection with a tilt angle of 75° and are stretched out along the vertical axis by a factor of 2 to allow the features in each slice to be seen more clearly.

(e) Single section out of the stack shown in (c,d) overlaid with a grid used for stereological determination of volumes for cytoplasm and vacuole. Each pixel at the intersections of the counting grid was allocated to cytoplasm, vacuole or medium.

in different technical set-ups that enable quick screening of the GSH status in either single cells or populations of cells. MCB has been widely used as a specific *in vivo* probe for GSH in different mammalian cell types (Barhoumi *et al.*, 1995; Fernández-Checa and Kaplowitz, 1990; Shrieve *et al.*,

1988; Young *et al.*, 1994). In some cases, however, MCB is not specific for GSH because cells lack an appropriate GST to catalyse the conjugation of MCB to GSH (van der Ven *et al.*, 1994). The results presented here show that the conjugation of bimanes to GSH was catalysed by a GST in

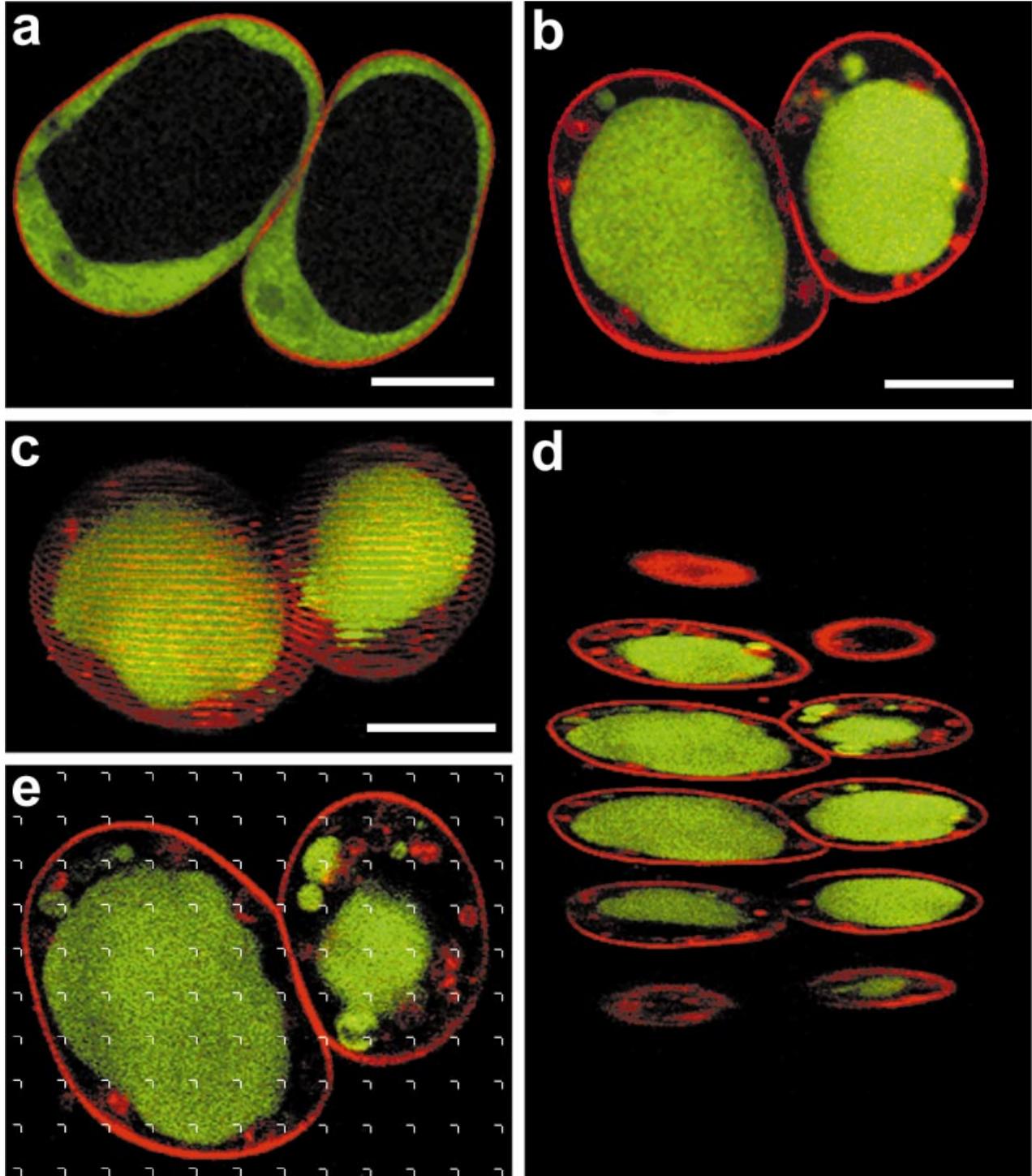


Table 2. Cytoplasmic GSH concentrations

	Percentage cytoplasm	Fluorimetry		Plate reader		Confocal microscopy		Two-photon microscopy	
		[GSH] _{cyt} (mM)	nmol GSH g ⁻¹ FW	[GSH] _{cyt} (mM)	nmol GSH g ⁻¹ FW	[GSH] _{cyt} (mM)	nmol GSH g ⁻¹ FW	[GSH] _{cyt} (mM)	nmol GSH g ⁻¹ FW
Segmentation	42 ± 3 (n = 43)	2.8 ± 0.3 (n = 10)	830 ± 78 (n = 10)	2.7 ± 0.4 (n = 8)	816 ± 105 (n = 8)	3.0 ± 0.5 (n = 7)	889 ± 148 (n = 7)	nd	nd
Cavalieri:	37 ± 2 (n = 9)	3.2 ± 0.3 (n = 10)	942 ± 88 (n = 10)	3.1 ± 0.4 (n = 8)	933 ± 120 (n = 8)	nd	nd	3.5 ± 0.7 (n = 5)	1030 ± 206 (n = 5)

Concentrations determined by fluorimetry, plate reader, confocal laser scanning microscopy at $\lambda_{\text{ex}} = 442$ nm, and two-photon laser scanning microscopy with $\lambda_{\text{ex}} = 770$ nm, respectively, based on different approaches to measure the cytoplasmic volume. All values mean ± SD. Manual segmentation of cytoplasm was done on 43 separate cells. The Cavalieri estimator of volume was applied to nine separate stacks of optical sections throughout the whole sample chamber, which always included several cells. nd, Not detected.

Arabidopsis suspension-culture cells. This confers specificity for GSH if the spontaneous reaction of bimanes with other cellular thiols is minimized.

Specificity of GSH labelling

The non-enzymatic rate constant for MCB (0.15 M⁻¹ sec⁻¹) was five- to sixfold lower than for MBB when measured at 25°C, and was in good agreement with the rate constant of 0.33 M⁻¹ sec⁻¹ determined at the higher temperature of 37°C (Shrieve *et al.*, 1988). Thus, to maintain specificity for GSH *in vivo* the non-enzymatic conjugation was minimized by using MCB rather than MBB, and by keeping the [MCB] low (10–100 µM). This contrasts with the conditions used to derivatize all cellular thiols *in situ* for HPLC analysis using millimolar concentrations of MBB (Newton and Fahey, 1995). An MCB concentration of 100 µM was found to be sufficient to label the GSH pool entirely without causing changes in pool size during the course of measurement, and did not give any detectable protein labelling. In unstressed plants the total glutathione pool consists of more than 90% reduced GSH (Noctor *et al.*, 1998). A comparison of *in vivo* with *in vitro* labelling and a combination of both methods showed that, at the plateau level of the labelling kinetics, the entire GSH pool was labelled. This means that the cells kept reducing GSSG while GSH levels were declining due to conjugation. Therefore the *in vivo* assay described here results in labelling of the total glutathione pool (GSH + GSSG).

HPLC analysis of MCB-labelled thiols showed additional peaks for cysteine and γ-EC. These might arise from non-specific reaction between MCB and the thiols; however, it is also possible that the additional peaks are derived from breakdown of GSB following transport into the vacuole, as has been suggested for other GSH conjugates (Wolf *et al.*, 1996). In support of this latter hypothesis, the amount of cysteine labelling was higher with MCB than MBB, although the ratio of cysteine/GSH in the latter case is in

good agreement with cysteine/GSH ratios found in other cell types (Noctor *et al.*, 1996). In addition, very little cysteine and γ-EC labelling was detected following pre-treatment with CDNB. CDNB is a known model substrate for GSTs (Cummins *et al.*, 1998), and is able to deplete the entire GSH pool very quickly. Therefore only a minor fraction of GSH was left for labelling with MCB, and hence only minute amounts of the GSB degradation products were found. We have not yet tested whether labelling of the cysteine and γ-EC peaks still occurs if vacuolar sequestration is prevented. As the plateau remains stable for an extended period, we have no evidence that additional metabolism of the biman group takes place in the vacuole. Thus further degradation of the biman conjugates, and especially the biman moiety itself, does not appear to take place.

Measurement of cytoplasmic GSH concentrations

The combination of direct visualization of GSH with MCB as a specific fluorescent probe *in vivo* and measurement of subcellular volumes allowed direct determination of the cytoplasmic GSH concentration in live cells. GSH levels given in the literature are usually expressed on the basis of g FW. To determine the cytoplasmic concentration of GSH, the volume of the cytoplasm was measured from serial optical sections. Two approaches were used during this work: manual delineation of each compartment, or stereological analysis, and both gave similar results. However, digital stereology provided a much quicker, easier and statistically robust way of measuring subcellular volumes (Howard and Reed, 1998).

To compare the figure of 2.7–3.5 mM GSH in the cytoplasm of *Arabidopsis* cells with other values in the literature, values were converted to the amount of GSH g⁻¹ FW. These values equate to ≈800–1000 nmol GSH g⁻¹ FW. These values were in good agreement with the figures measured in control experiments after conven-

tional labelling of cell extracts and subsequent HPLC analysis. However, these values were significantly higher than previously reported estimates. GSH levels in unstressed wild-type plants determined by HPLC vary between 70 and ≈ 700 nmol g⁻¹ FW depending on the species, the tissue and seasonal variation in thiol contents (Arisi *et al.*, 1997; Bergmann and Rennenberg, 1993; Xiang and Oliver, 1998). It is possible that suspension-culture cells in their exponential phase of growth genuinely have a higher level of GSH than cells in intact plants. This would agree with observations by Vernoux *et al.* (2000) that the G₁-to-S phase transition in tobacco cell-suspension cultures requires an adequate level of GSH. Alternatively, the direct measurement of GSH in live cells used here circumvents the potential loss of GSH during extraction of tissue necessary for HPLC analysis, which might underestimate the true level.

Effects of GSH depletion on GSH synthesis

One interesting result in the present study is the absence of GSH synthesis despite the complete depletion of the GSH pool. The biosynthetic pathway of GSH consists of two steps catalysed by γ -glutamylcysteine synthetase (γ -ECS) and GSH synthetase. The importance of γ -ECS in the control of GSH biosynthesis has been shown by several groups (reviewed by May *et al.*, 1998a; Noctor *et al.*, 1998), and feedback control of γ -ECS by GSH levels has been shown to occur *in vitro* (Hell and Bergmann, 1990). However, the extent to which this feedback control operates *in vivo* has not yet been resolved. Thus feedback control is not particularly important in transgenic poplars overexpressing γ -ECS (Arisi *et al.*, 1997; Herschbach *et al.*, 1998; Noctor *et al.*, 1996), but may be significant in *Arabidopsis* (Xiang and Oliver, 1998). In this study, the fact that the labelling reached a plateau value, and the absence of an effect of BSO on the labelling kinetics, are both consistent with no feedback control of γ -ECS by GSH. Under these conditions, γ -ECS may be regulated by post-transcriptional activation (May *et al.*, 1998b) with a slower time course. Alternatively, the extent of feedback control of γ -ECS could be masked by limitations earlier in the pathway. The importance of cysteine availability has been pointed out by several groups (Farago and Brunold, 1994; Noctor *et al.*, 1996; Strohm *et al.*, 1995). This might suggest there is only a limited capacity to synthesize the relevant precursors for GSH synthesis in unstressed *Arabidopsis* cells and the synthesis capacity is not increased within 2 h of severe stress. Further experiments with external supply of precursors should give a more detailed indication of which step is rate-limiting for GSH synthesis.

In conclusion, we have shown first, that MCB was a reliable probe to measure cytoplasmic concentrations of

GSH in populations of *Arabidopsis* cells using fluorimetry or single cells using CLSM or TPLSM; second, that the assay provides information on the kinetics of GSTs operating *in vivo*; and third, that analysis of the cellular response to GSH depletion provides an indication of flux through the GSH synthesis pathway. Measuring the GSH status of suspension-culture cells in multi-well plates enables high-throughput screening for induced changes in the cellular GSH pool. We are currently extending the assay to measure the effects of xenobiotics and heavy metals on cytoplasmic GSH, through competition with MCB labelling at both population and single-cell levels.

Experimental procedures

Plant material

Arabidopsis thaliana cell-suspension cultures were maintained as described previously (May and Leaver, 1993). Cells were used during the logarithmic phase 3 or 4 days after subculture. Unless otherwise stated, all experiments were carried out in MS basal medium (M 5519, Sigma, Poole, UK) supplemented with 30 g l⁻¹ sucrose at pH 5.8.

Fluorescent dyes

Stocks of MCB (100 mM in DMSO), MBB (100 mM in DMSO), and QBB (100 mM in DMSO) were stored at -20°C in 10 μ l aliquots. Aliquots were thawed and diluted immediately prior to use. Propidium iodide (PI) was used as a cell-wall stain. Additionally, PI labels the nuclei of dead cells and thereby was a good indicator of cell viability. PI was prepared as a 5 mM stock solution in water and used at a final concentration of 50 μ M. All dyes were purchased from Molecular Probes (Eugene, OR, USA).

Fluorimetry

Excitation and emission spectra and labelling kinetics were measured using a luminescence spectrometer (LS50B, Perkin-Elmer, Beaconsfield, UK) equipped with a four-position temperature-controlled cell holder for 3 ml cuvettes. 100 μ l suspension culture was added to 2.9 ml MS medium containing different concentrations of MCB or other bimane-derivatives. The cell suspension in the cuvettes was continuously stirred and fluorescence intensity was measured over time with $\lambda_{\text{ex}} = 442 \pm 2.5$ nm, $\lambda_{\text{em}} = 477 \pm 2.5$ nm at 25°C.

Calibration standards were made by serial dilution from a 10 mM GSB stock solution prepared by a reaction of 10 mM MCB with excess of GSH in the presence of GST (Rabbit Liver GST, G 8216, Sigma).

Plate reader experiments

50 μ l cell suspension and 50 μ l fresh medium were placed in wells on 96-well microtitre plates with flat, clear bottom (Falcon, Becton Dickinson, Oxford, UK). Plates were then placed in a fluorescence plate reader (Fluostar, BMG, Aylesbury, UK) set up to excite and read fluorescence from the bottom of the cells. Fluorescence intensity was measured with $\lambda_{\text{ex}} = 390$ nm and $\lambda_{\text{em}} = 460$ nm at 25°C in 3 min intervals. Labelling reaction was started by injection

MCB via the built-in injectors into the wells. Plates were not agitated between the measurements, so that cells sedimented and formed a thin layer at the bottom of the wells. At the end of the time course, cells were lysed by addition of Triton X-100 in order to calibrate fluorescence against standard GSB solutions at concentrations between 5 and 50 μM .

Gel filtration chromatography of bimane labelled compounds

Suspension-culture cells were labelled with varying concentrations of MCB or MBB for 1 h, washed twice in MS medium to remove free dye, and pelleted in Eppendorf tubes (325 g, 5 min). The medium was removed and cells were resuspended in 1 ml ice-cold extraction buffer (100 mM Tris KOH pH 8.0, 2 mM MgCl_2 , 1 mM EDTA) and thoroughly ground in a chilled mortar with addition of 0.1 g PVPP (polyvinylpyrrolidone, MW 40 000). The extract was centrifuged for 10 min at 25 000 g. The supernatant was then fractionated on Sephadex G-25 (PD10 columns, Supelco, Bellefonte, PA, USA) with extraction buffer as eluent. 250 μl fractions were collected and fluorescence was measured on the luminescence spectrometer (LS50B, Perkin-Elmer) equipped with a plate reader with excitation 395 nm and emission 477 nm. Protein levels in each fraction were measured according to Bradford (1976).

HPLC analysis of bimane-labelled thiols

After *in vivo* labelling of cells with 10–300 μM MCB or MBB, respectively, cells were ground with ice-cold 200 mM methanesulfonic acid in a chilled mortar. Cell extracts were centrifuged (12 000 g, 10 min) and supernatants stored at -20°C until analysis. Bimane-labelled thiols were separated by HPLC (Hichrom 5C18, 300 \times 4.6 mm, Hichrom, Reading, UK) using 0.25% (v/v) acetic acid (pH 3.9) as solvent A and methanol as solvent B. The elution protocol employed a linear gradient from 92% A to 85% A in 10 min and a subsequent hold for further 20 min. The flow rate was kept constant at 1 ml min^{-1} . Bimane derivatives were detected fluorimetrically (RF2000, Dionex, Germering, Germany) with excitation at 395 nm and emission at 477 nm. For comparison of the *in vivo* labelling with labelling of low molecular-weight thiols in cell extracts, cells were ground in 200 mM methanesulfonic acid. After addition of an equal volume of 4 M sodium methanesulfonate – 0.2 N methanesulfonic acid thiols were labelled with bimane by adding 200 mM EPPS – methanesulfonate with 5 mM diethylenetriaminepenta-acetic acid (DTPA) and 3 mM MBB, pH 8.2 for 10 min in the dark (Fahey and Newton, 1987). The reaction was stopped by addition of methanesulfonic acid to 100 mM. To test for completeness of GSH *in vivo* labelling with MCB, cell extracts were prepared after *in vivo* labelling and additionally labelled with MBB *in vitro* as described above. For the determination of oxidized glutathione (GSSG), the reduced GSH was bound by addition of *N*-ethylmaleimide (NEM) first. After removal of excess NEM by extracting five times with toluene, GSSG was reduced by the addition of 2 mM DTT and subsequently labelled with MBB as described above. For a recovery analysis, bimane-thiol standard solutions of known concentration were subjected to the extraction method in 200 mM methanesulfonic acid, and free thiol solutions of known concentration were subjected to the *in vitro* labelling procedure. Recovery was always >98% of the starting concentration.

Laser scanning microscopy

A shallow chamber was made by sticking adhesive tape (150 μm thick) to a slide and cutting out the centre to a size of $\approx 1 \times 1$ cm. A small number of cells in culture medium supplemented with MCB and PI were transferred to this chamber and covered by a glass cover slip (No. 1.5, Agar Scientific, Stansted, UK). Cells were imaged immediately afterwards either by CLSM or TPLSM. Confocal microscopy used an MRC600 CLSM (Bio-Rad Microscience, Hemel Hempstead, UK) and a 442 nm HeCd laser (Liconix, Santa Clara, CA, USA) attached to an inverted microscope (Nikon Diaphot) equipped with a Nikon 60 \times 1.4 NA Plan Apochromat oil-immersion lens. Images were collected at zoom 1 with a pixel spacing of 0.275 μm . The pinhole was set to 5.0 and images were Kalman averaged over three frames. For TPLSM, a MRC-1024MP (Bio-Rad Microscience) attached to an upright microscope (Olympus BX50WI, Olympus, Southall, UK) was used. Fluorescence of GSB was excited at 770 nm by a femtosecond-pulsed tuneable mode-locked Ti:sapphire laser (Mira 900, Coherent, Cambridge, UK) equipped with a 5 W solid state pump laser (Verdi, Coherent) using an Olympus 60 \times UPlanApo1.2-NA water-immersion lens. Emitted light from the focal point was collected with external detectors equipped with a green/red filter block (550DLCP) and red (D606/90 nm) and green (D535/50 nm) emission filters to separate the blue-green GSB signal from the red fluorescence of PI. Blocking filters (HQ575/150 nm) were used on both channels to remove any reflected infrared light. For measurement of the cytoplasm to vacuole volume ratio stacks of serial optical sections along the z-axis were collected at 1 μm intervals throughout the complete depth of the sample chamber.

For quantitative measurements of GSB by TPLSM, single optical sections were collected over 2 h at intervals between 1 and 10 min. Images were collected with a pixel spacing of 0.2 μm and Kalman averaged over three or four frames. A four-step protocol was used to quantify the total amount of GSB formed after 2 h labelling. First, the average fluorescence intensity was measured for both cytoplasm and vacuole from manually delimited regions of interest in a number of cells. Second, the average background signal, measured from a nearby region of the medium, was subtracted from the average fluorescence value. Third, the remaining fluorescence intensity was converted to concentration by calibration against a series of solutions with known amounts of GSB that were imaged under identical instrument settings. Fourth, the GSB concentrations in the cytoplasm and vacuole were summed, taking into account the volume ratio between the cytoplasm and vacuole as measured either by segmentation of confocal images or by the Cavalieri estimator for the TPLSM images (see below).

Measurement of cytoplasmic volume

Cells were separated from the suspension medium by spinning an aliquot of the cell suspension through a 0.45 μm Nylon membrane (AnaSpin Filter System, Anachem, Luton, UK). Subsequently, the weight of the cells was determined and converted into cell volume. For this conversion the density of the cells was determined to be 1.02 g ml^{-1} by sedimentation in sucrose solutions of known density. The relative volume of cytoplasm in the cells was determined either by a segmentation approach applied to stacks of serial optical sections taken by CLSM, or by a stereological approach applied to stacks of serial optical sections collected by TPLSM. Area measurements on optical sections were done by manually drawing lines around the

different compartments using MPL software (Bio-Rad Microscience). Segmented areas were multiplied by the distance between two slices (1 μm) to give volumes. For the stereological approach, the Cavalieri method (Gundersen and Jensen, 1987) was used. The Cavalieri estimator of volume was applied using Digital Stereology V4.5 (Kinetic Imaging, Liverpool, UK). Starting with a uniform random section from a stack, the Cavalieri estimator was repeatedly applied to different sections at defined intervals until the coefficient of error was smaller than 10%. Both volume measurement by segmentation and volume estimation by the Cavalieri method were performed on cells in which the GSB conjugate had been transported into the vacuole (negative contrast of the cytoplasm), as well as on cells in which the GSB, and therefore the fluorescence, remained in the cytoplasm (negative contrast of the vacuole). In order to prevent ATP-dependent vacuolar transfer of conjugates and exclusively label the cytoplasm, cells were either pre-incubated in 0.1 mM NaN_3 and then labelled with 100 μM MCB, or were labelled with 100 μM MCB on ice.

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