4
"FlAsH" Protein Labeling

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4.1
Introduction

Fluorescence microscopy has developed into a tremendously powerful tool for the study of protein dynamics within cells in time and space. A milestone for live-cell imaging has been the discovery, cloning and heterologous expression of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (reviewed in [1]). Since then, the discovery of further fluorescent proteins in other species, as well as various mutagenesis approaches, has diversified the spectra and the properties of these probes [2, 3]. In the meantime fluorescent proteins have been utilized for an overwhelming number of applications in cell biology. Despite the numerous benefits of the fluorescent proteins, there are limitations for their use, the most dominant being their relatively large molecular weight of ~28 kDa (~230 amino acids). Due to this size, fluorescent proteins may interfere with the correct localization, stability or functionality of their respective fusion partners.

In 1998 Roger Tsien's group developed an alternative approach for the specific labeling of proteins in living cells using the high-affinity interaction between a 6-residue peptide that includes four cysteines and membrane-permeable biarsenical molecules [4]. Since its invention, the biarsenical-tetracysteine system has undergone some notable improvements and has been employed in a number of studies.

In this chapter we describe briefly the principle of this labeling system, its major current applications and limitations, and provide information for its practical use. Several comprehensive articles describing the application of this system in mammalian cells are available [5, 6]. Here we focus on the use of the biarsenical-tetracysteine system in the budding yeast *Saccharomyces cerevisiae*, although we note that many practical aspects are transferable to other organisms as well.

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4.1.1 The Biarsenical-Tetracysteine System

This labeling system makes use of the high affinity of fluorescent compounds containing two arsenic atoms to four appropriately spaced cysteines (TetCys motif: Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa is not cysteine) (Figure 4.1A). The first utilized biarsenical dye was FlAsH (*F*luorescein *A*rsenical *H*airpin *B*inder), a derivative of the well-known fluorophore fluorescein [4]. For FlAsH, dissociation constants in the sub-nanomolar range (Table 4.1) for a peptide containing the TetCys motif have been reported. In solution, FlAsH is complexed with two 1,2-ethanedithiol (EDT) molecules. It is membrane permeable and in solution only weakly fluorescent, yet becomes brightly fluorescent upon binding to a target peptide. This improves the signal to noise ratio since, predominantly, the peptide-bound fluorophore is detected.

The DNA sequence coding for the TetCys motif may be fused to a target gene by standard techniques, resulting in fusion proteins with the TetCys motif at the N- or C-terminus or incorporated into the protein. Upon association of the biarsenical fluorophore to the binding sequence, the fluorescence develops immediately, whereas the proper folding of a fluorescent protein requires tens of minutes to hours.

Even more important, the TetCys motif is up to 25 times smaller than a fluorescent protein (~230 amino acids) (Figure 4.1B). Several examples where the protein function is less perturbed by a TetCys motif than by a fluorescent protein have been reported, including G protein-coupled receptors [7], cAMP-dependent protein kinase [8] and β-tubulin [9].

Despite its obvious merits, the biarsenical-tetracysteine system has its disadvantages, the most pressing being poor signal to noise ratios due to unwanted background staining and pronounced photobleaching. To alleviate these obstacles,

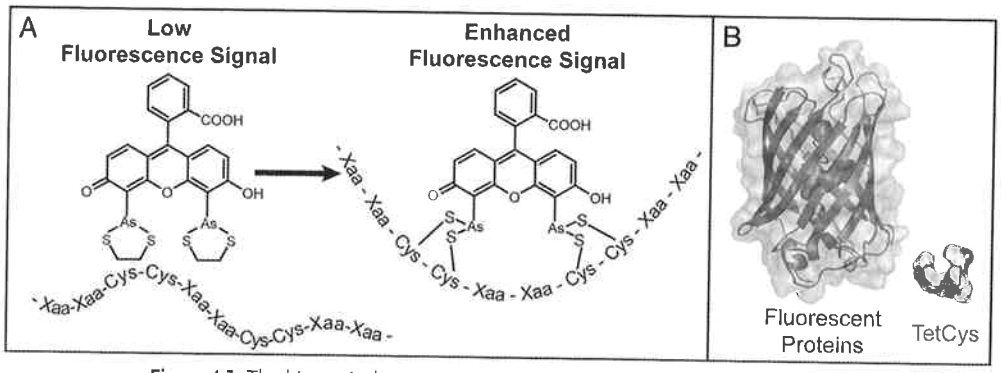


Figure 4.1 The biarsenical-tetracysteine system. (A) Binding of the biarsenical dye to the TetCys motif. Unbound FlAsH-EDT_2 exhibits low fluorescence, whereas bound FlAsH is brightly fluorescent. (B) Size comparison of a fluorescent protein with the TetCys motif. GFP comprises ~230 amino acid residues, whereas the TetCys motif may be as short as 6 amino acids.

Table 4.1 Binding motifs for the Biarsenical-Tetracysteine System.

Motif	Quantum efficiency	K_{on} ($M^{-1} s^{-1}$)	K_d (pM)	Source
WEAAAREACCRECCARA	0.50	65 000	70	Griffin *et al.* [4]
WDCCPGCCK	0.67	310 000	4	Adams *et al.* [11]
WDCCGPCCK	0.44	50 000	72	Adams *et al.* [11]
WDCCPCCK	0.60	100 000	150	Adams *et al.* [11]
WDCCGCCK	0.55	35 000	100	Adams *et al.* [11]
WDCCDEACCK	0.23	65 000	92 000	Adams *et al.* [11]
FLNCCPGCCMEP	0.78	n.d.	n.d.	Martin *et al.* [12]
HRWCCPGCCKTF	0.65	n.d.	n.d.	Martin *et al.* [12]

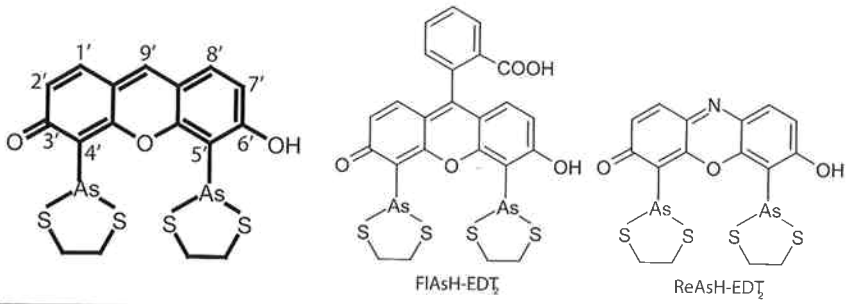
substantial efforts have been undertaken to improve the TetCys motif, the available fluorophores and the staining procedures.

4.1.2
Improved TetCys Motifs

Utilization of the original TetCys motif (Cys-Cys-Arg-Glu-Cys-Cys) [4] generally results in comparatively strong background staining in mammalian cells. The sensitivity and the detection limit of the method were estimated to be an order of magnitude worse than those of GFP [10]. To alleviate this problem, two studies led by the Tsien lab identified several improved TetCys motifs (Table 4.1) [11, 12]. They found that the CCPGCC-FlAsH complex was five-fold more stable than the previously utilized CCRECC-FlAsH complex [11]. Subsequently, the residues flanking the core motif were also systematically optimized [12]. FLNCCPGCCMEP and HRWCCPGCCKTF were shown to tolerate washes with high concentrations of the antidote EDT while retaining the bound fluorophore. Since both sequences also resulted in higher fluorescence quantum yields, a ~20-fold increase in contrast was reported when using these improved TetCys motifs instead of the previous one. Because FLNCCPGCCMEP gives somewhat higher quantum yields than HRWCCPGCCKTF, the former is currently the TetCys motif of choice for most applications. We note, however, that for labeling applications in *S. cerevisiae*, we did not observe advantages of these extended TetCys motifs over CCPGCC.

4.1.3
Fluorescent Biarsenical Ligands

Although the optimization of the TetCys motif has led to important improvements in affinity and brightness, the limited photostability and pH sensitivity of FlAsH precludes many potential applications. Hence there is a large demand for improved biarsenical dyes. In 2002 a red-emitting resorufin-based analog called ReAsH (*R*esorufin-based *A*rsenical *H*airpin *B*inder) was introduced [11]. More recently, several other novel biarsenical ligands have been developed, some of which are listed



Ligand	Modification	Excitation	Emission	Source
CHoXAsH	2'-7'- Cl_2; 9'- OH	380 nm	430 nm	Adams et al. 2002
F_2FlAsH	2'-7'-F_2; 9'-(2-C_6H_4COOH)	500 nm	522 nm	Spagnuolo et al. 2006
CrAsH	9'-(2,5-C_6H_4[COOH]$_2$)	515 nm	534 nm	Cao et al. 2006
FlAsH	9'-(2-C_6H_4COOH)	511 nm	527 nm	Griffin et al. 1998
F_4FlAsH	9'-(2-C_6F_4COOH)	528 nm	544 nm	Spagnuolo et al. 2006
ReAsH	9'-Aza	593 nm	608 nm	Adams et al. 2002

Figure 4.2 Fluorescent biarsenical ligands. An overview of the chemical structures of some important TetCys motif binding probes is given. Currently only FlAsH-EDT_2 and ReAsH-EDT_2 are commercially available.

in Figure 4.2. For detailed information on the properties of these new probes, we refer the reader to [4, 11, 13, 14]. Despite promising properties, none of these novel probes have been widely used so far. Therefore it remains to be seen whether these novel ligands, or others which are still to come, will prove to be superior to FlAsH and ReAsH. Currently only these two biarsenical dyes are commercially available from Invitrogen under the brand names TC-FlAsH/Lumio green and TC-ReAsH/Lumio red.

4.1.4 Applications of the Biarsenical-Tetracysteine System

There is a remarkable variety of reported applications for this labeling system (Figure 4.3). The usability of this approach is reflected by the increasing number of studies using biarsenical dyes [7, 15–27].

Arguably, most studies exploited the small size of the TetCys motif to visualize tagged proteins within cells (Figure 4.3A). The majority of such localization applications relied on the more photostable FlAsH rather than ReAsH.

In combination the two biarsenical dyes have been used to visualize the age of TetCys tagged proteins [16, 19]. To this end, all TetCys motifs of a tagged protein in a cell are initially saturated with FlAsH. Any free dye is then washed out and the cell is allowed to synthesize new copies of the tagged protein. Subsequent staining with a biarsenical dye of different color (i.e., ReAsH) results in exclusive labeling of the newly synthesized tagged protein. Gaietta and colleagues employed this approach in an elegant experiment for the analysis of the trafficking of connexin43 [16]. They demonstrated that the newly synthesized connexin43 was predominantly incorpo-

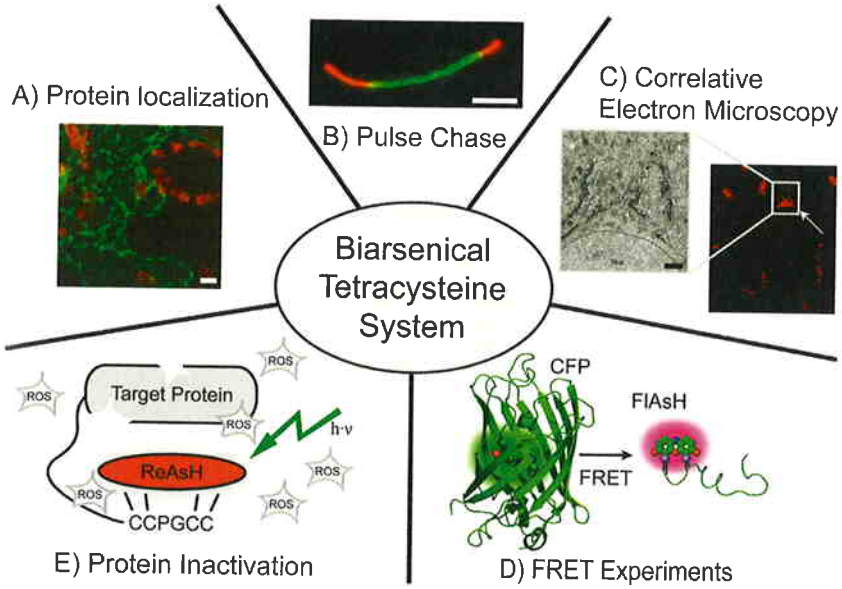


Figure 4.3 Applications of the biarsenical-tetracysteine system. Arguably, the current major application areas for the biarsenical-tetracysteine system are the following. (A) Visualization of intracellular protein localizations. Shown are FlAsH-labelled *Arabidopsis* cells expressing TetCys–labelled synthetic glycomodule peptides [22]. (B) Sequential labeling with FlAsH and ReAsH can indicate the age of proteins. This approach was used to study the life cycle of connexin43 as it was trafficked into and out of gap junctions [16]. (C) ReAsH labeled structures can be visualised in both fluorescence and electron microscopy. This correlative approach has been utilized to study Golgi vesicles throughout mitosis [24]. (D) FRET of, for example, CFP and FlAsH has been utilized to study conformational changes or to create environment-sensitive probes. (E) Intense irradiation of FlAsH or ReAsH can generate ROS, which inactivate the tagged protein, providing a genetically targeted strategy for the light driven knockout of specific proteins. All microscopic images used with permission. Scale bars (A–C): 1 μm.

rated at the periphery of existing gap junctions, whereas older connexins were removed from the center of the plaques (Figure 4.3B).

In a different application scheme, ReAsH has been demonstrated to be useful for correlative protein localization in light and electron microscopy. Upon illumination, ReAsH generates singlet oxygen species which locally oxidize diaminobenzidine (DAB) into an osmiophilic polymer which can be identified by electron microscopy. Such a photoconversion protocol has been utilized to study ReAsH labeled Golgi vesicles throughout mitosis (Figure 4.3C) [24] as well as connexin trafficking [16].

Fluorescence resonance energy transfer (FRET) between a fluorescent protein and a nearby biarsenical dye has been exploited in a few studies (Figure 4.3D). For example, the energy transfer from CFP to FlAsH was used to monitor G protein-coupled receptor activation in living cells [7]. In this study the CFP/TetCys construct showed a 3–5 times higher FRET signal than the corresponding CFP/YFP construct. In a different FRET application, a combinatorial tag consisting of GFP

and a TetCys motif proved to be beneficial for photoconversion [24]. In this case, only the ReAsH bound to the TetCys motif accepts the energy from the adjacent GFP and produces singlet oxygen, thereby increasing the specificity of photoconversion.

A further field of application of the biarsenical-tetracysteine system is fluorophore assisted light inactivation of genetically targeted proteins (Figure 4.3E) [17, 18]. Here, the reactive oxygen species (ROS) that are generated by the intense illumination of FlAsH or ReAsH inactivate the tagged protein. Because of their high reactivity, the ROS act in close vicinity to the site of their generation. Thus this technique allows inactivation of tagged proteins in living cells with high spatial and temporal precision. So far only a few reports utilizing this promising technique have been published.

4.1.5 Staining in Various Model Organisms

Since its first application in cultured mammalian cells [4], the biarsenical-tetracysteine system has been successfully adapted for use in several other organisms including plants [22], budding yeast [9] and bacteria [28].

In principle most, if not all, organisms should be amenable to this labeling system, albeit various challenges may need to be faced. Physical barriers like cell walls may impede the staining procedure. Further, the system works only in a reducing environment, although this problem may be overcome by adding the membrane-permeant reducing agent tributylphosphine to the cells [24]. Also, different cell types are likely to exhibit distinct abilities to export the biarsenical dyes from the cytoplasm which will require an adaptation of the specific labeling procedure.

A major challenge in the application of the biarsenical-tetracysteine system in most cell types is nonspecific staining. One part of the unwanted background labeling appears to result from a low-affinity interaction of the biarsenical dyes with various cellular components and is frequently resolved by extensive washing. Another source of background staining, which is more difficult to address, is due to endogenous proteins containing biarsenical dye binding motifs. Although TetCys motifs of the CCXXCC type bind FlAsH with the highest affinity, motifs of the CCXCC type also display considerable binding affinities (Table 4.1). Hence endogenous proteins exhibiting CCXCC or CCXXCC sequences may represent binding partners for the biarsenical compounds. To estimate the frequency of the natural occurrence of these sequences, we performed data base searches on the sequenced genomes of some important model organisms (Table 4.2). We find that budding yeast, which displays only little unspecific background upon staining, contains relatively few endogenous potential binding partners. In this organism, there is one predicted protein which contains the CCPGCC motif; however this protein is apparently not, or only weakly expressed and thus does not contribute to an unwanted background signal. Human cells, in contrast, exhibit a higher background and also the number of potential binding partners is 10 times larger. Hence a substantial amount of the observed background staining in mammalian cells might indeed be due to endogenous proteins with a TetCys motif.

Table 4.2 Number of possible binding motifs for biarsenical dyes.

Organism	CCXCC	CCXXCC	CCPGCC
Arabidopsis thaliana	41	52	—
Candida albicans	3	3	—
Caenorhabditis elegans	38	30	—
Drosophila melanogaster	122	149	—
Homo sapiens	64	68	—
Mus musculus	58	64	—
Rattus norvegicus	27	22	—
Saccharomyces cerevisiae	5	7	1)
Saccharomyces pombe	1	1	—

4.1.6 FlAsH Labeling in *S. cerevisiae*

Functional analysis of the budding yeast *S. cerevisiae* has been greatly aided by the ease with which genes can be modified within the genome by site-specific homologous recombination. This so-called epitope-tagging allows the modification of practically any ORF without perturbing its transcription [29].

In order to utilize this method for the tagging of yeast proteins with the TetCys motif, we have previously constructed a small series of tagging vectors containing between one and three TetCys motifs (Figure 4.4) [9]. In that study the effects of the different tag lengths on the functionality of a host protein (β-tubulin; Tub2) were investigated. Tub2 tagged with either 1× TetCys (10 amino acids [aa]) or 2× TetCys (20 aa) was able to substitute Tub2. In contrast, C-terminal tagging of Tub2 with

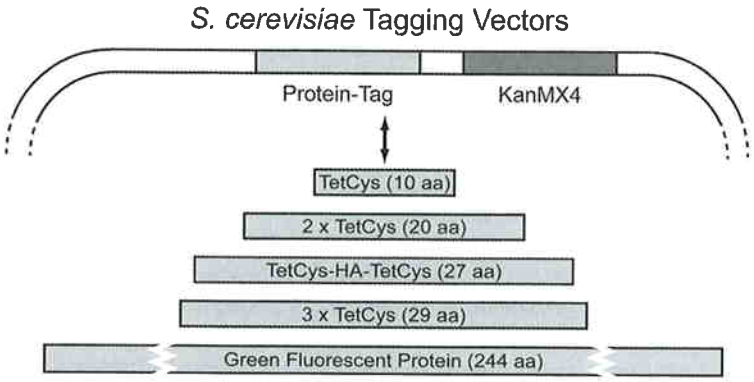


Figure 4.4 Vectors for epitope tagging in budding yeast. Schematic representation of the tagging modules (for details see [9]). The miscellaneous tagging plasmids are identical except for the sequences of the epitopes. Therefore, the same set of primers can be used to tag an ORF with any of these epitopes.

Table 4.3 Comparison of photostability.

Protein-tag	Bleaching rate, $t_{1/2}$ (s)
1× TetCys	1.8
2× TetCys	3.5
3× TetCys	9.5
TetCys-HA-TetCys	4.5
GFP	16.0

3× TetCys (29 aa) or with GFP (244 aa) resulted in nonviable haploid cells [9]. Hence, for β-tubulin, the size of the tag is crucial. Similar effects have also been reported for other proteins [7, 8], although it can be anticipated that for many host-proteins the size of the tag is only of minor relevance [30].

We compared the influence of concatenated TetCys motifs on the bleaching rate of the fluorescence signal in FlAsH labeled cells (Table 4.3). To this end we tagged the alpha subunit of the mitochondrial ATP synthase (Atp1) with the various motifs. The photobleaching rates were measured with an epi-fluorescence microscope equipped with a mercury lamp (100 W) and a CCD camera. We found the resistance against photobleaching to increase with the number of TetCys motifs attached to the host protein (Table 4.3). FlAsH labeled proteins tagged with one TetCys motif were so prone to photobleaching that we do not recommend the 1× TetCys motif for use in budding yeast. Tagging with the 2× TetCys or 3× TetCys motifs significantly reduced the bleaching rates. This reduced bleaching is likely due to FRET between the two or three FlAsH molecules binding to these motifs. Separation of two TetCys motifs by the hemagglutinin (HA) epitope (11 aa) (Figure 4.4) increases the overall brightness, as well as the resistance against photobleaching, slightly compared to the 2× TetCys motif, probably because the binding sites are better accessible for the biarsenical dyes. However, since the TetCys-HA-TetCys tag is of similar size to the 3× TetCys tag, but the latter gives rise to brighter labeled specimens (not shown), we do not recommend the TetCys-HA-TetCys tag, either. Instead, we recommend either the 2× TetCys or the 3× TetCys for most applications in budding yeast. Whenever the larger 3× TetCys motif is tolerable, it is likely to outperform the 2× TetCys motif, albeit at the expense of a slightly larger size.

Two approaches for the FlAsH-labeling of yeast cells expressing TetCys tagged proteins are currently routinely used in our laboratory (detailed protocols are given in the last section of this chapter). The first method relies on the overnight-cultivation of a liquid yeast culture in a growth medium containing FlAsH-EDT_2. Since FlAsH-EDT_2 is relatively expensive, it is advisable to use very small culture volumes which require careful adjustments of the growth conditions. Once established, this labeling protocol generally results in a homogenous labeling of the whole culture. The second approach requires a brief electric discharge provided by a standard electroporator to bring the biarsenical dyes into the cells. This approach is quick and reliable, however, generally only a small fraction (2–10%) of the cells will be labeled.

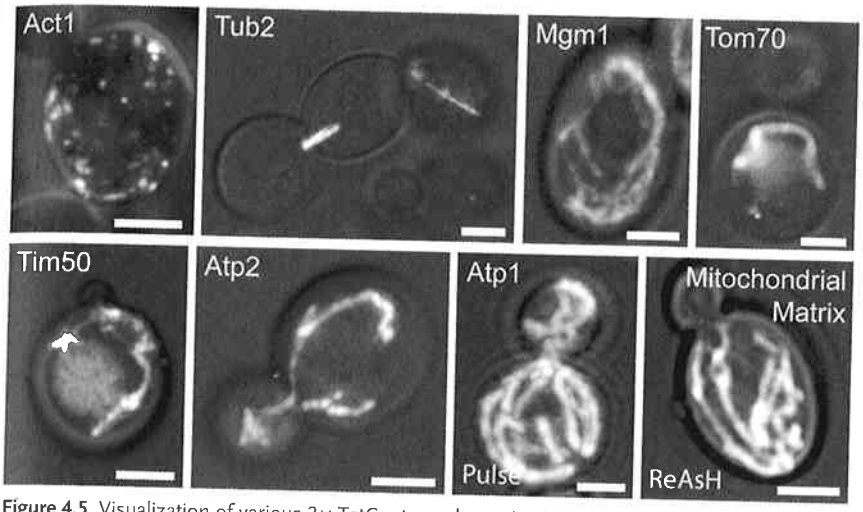


Figure 4.5 Visualization of various 2× TetCys tagged proteins in *S. cerevisiae*. Examples include the actin (Act1) and tubulin (Tub2) cytoskeleton, and different compartments of mitochondria. The tagged alpha subunit of the mitochondrial F_1F_0 ATP synthase (Atp1) was labeled using the pulse staining protocol. The mitochondrial matrix was labeled with ReAsH. All other proteins were labeled with FlAsH. Scale bars: 2 µm.

We have used both methods to FlAsH-label various yeast strains expressing 2× TetCys-tagged proteins localizing to different cellular structures (amongst others tubulin, actin, mitochondrial inner and outer membrane), demonstrating the general applicability of the method (Figure 4.5). We succeeded in visualizing most FlAsH labeled proteins that could be tagged with the 2× TetCys or the 3× TetCys motif. Nevertheless, image acquisition of less abundant proteins may be challenging, even with sophisticated low-light sensitive microscopes. In our experience, cells labeled with ReAsH generally exhibited an unsatisfactory fluorescence signal so that we abstained from using this compound to visualize proteins expressed at endogenous levels. ReAsH was however used to visualize an overexpressed protein targeted to the mitochondrial matrix (Figure 4.5).

4.1.7 Outlook

The biarsenical-tetracysteine system is an important asset in the toolbox for live cell imaging. Its major advantages are the small size of the tagging motif and the varied (fluorescent) labels that can be utilized for different applications. Nevertheless, at present, fluorescent proteins outperform the biarsenical-tetracysteine system with respect to fluorescence intensity and photostability, albeit, of course, at the cost of a larger tag. Recent progress in the development of novel biarsenical dyes promises to alleviate many of the current limitations, and should make this labeling system even more significant in the future.

4.2
Use of the Biarsenical-Tetracysteine System in *S. cerevisiae*

In this section we focus on two different protocols to label proteins tagged with the TetCys motif expressed in the budding yeast *S. cerevisiae*. Many details of the methods described in the following may be adapted to other organisms.

4.2.1
Materials

4.2.1.1 Growth Media

Growth media exhibiting low autofluorescence are advantageous. Since yeast extract is a major source for autofluorescence in many common growth media, we recommend the use of a synthetic growth medium. The following growth media work well.

Synthetic complete medium (SC-Medium):			
yeast nitrogen base	$1.7\,g\,l^{-1}$	ammonium sulfate	$5\,g\,l^{-1}$
complete supplement mixture (see Ref. [31])	$0.6\,g\,l^{-1}$	glucose	$20\,g\,l^{-1}$
Mitochondria complete medium:			
yeast nitrogen base	$1.7\,g\,l^{-1}$	ammonium sulfate	$5\,g\,l^{-1}$
		galactose	$10\,g\,l^{-1}$
		ethanol	$20\,ml\,l^{-1}$
complete supplement mixture (see Ref. [31])	$0.6\,g\,l^{-1}$	glycerol	$15\,g\,l^{-1}$

4.2.1.2 Buffers (Required Stock Solutions)

HBS-buffer (pH 7.0):	150 mM NaCl; 20 mM HEPES
Tris solution (pH 7.5):	1 M Tris/HCl

Dye Solution FlAsH-EDT_2 and ReAsH-EDT_2 are available from Invitrogen and are delivered in a concentration of 2 mM. Both dyes are sensitive against oxidation and should be aliquoted before use. Freeze and thaw cycles as well as extended light exposures must be avoided.

Biarsenical Dye Working Solution

200 µM FlAsH-EDT_2 or 200 µM ReAsH-EDT_2 in 1 M Tris-HCl (pH 7.5).

This working solution is needed for the overnight staining protocol only (see below). It should be prepared immediately before use. Storage is not recommended.

Washing Buffer The antidote 1,2-ethanedithiol (Sigma-Aldrich, St.Louis, MO, USA) exhibits a strong offensive smell. A fume hood must be used. We recommend using an extra set of pipettes for EDT because this volatile compound can easily contaminate the pipettes, which may be obstructive for other molecular biology applications.

washing buffer:	1 mM EDT in HBS-buffer (vortex thoroughly, prepare freshly)

4.2.2 Labeling Protocols

For the staining of budding yeast cells two different protocols (overnight staining and pulse staining) were established in our laboratory.

For overnight staining, the cells are propagated in a growth medium containing the dye (FlAsH or ReAsH). This approach requires adjustment of the growth parameters and generally results in a uniform labeling of the cell culture. In contrast, pulse staining, which utilizes an electric discharge to trigger the internalization of the dye, is comparatively simple but tends to be of lower efficiency. A detailed description of both methods is given below, followed by a short version of the protocols and a troubleshooting section.

4.2.2.1 Overnight Staining

Here, the cells are grown for 12 h in the dye-containing medium. The long incubation time in the staining medium is necessary to enable the biarsenical dyes to reach the interior of the cells at the concentrations required for sufficient labeling. For this protocol we found the haploid yeast strains of the genetic background BY4741/BY4742 to be more suitable than the diploid strain BY4743.

Preparation of Yeast Cells During overnight staining the cells should be kept continuously in the logarithmic growth phase and therefore the starting cell density has to be carefully adjusted. To this end the growth rate of the yeast strain of interest should be determined in the absence of the biarsenical dye but otherwise under staining conditions. (Since EDT has a slight influence on the doubling time it should be added at this step.) Once the growth rate is determined a logarithmically growing pre-culture is diluted accordingly to proceed with the staining.

FlAsH-Staining The staining of the yeast cells is typically performed in a volume of ~200 µl. To this end, we recommend the use of a 48-well microtiter plate. To avoid excessive evaporation of the growth medium during staining, adjacent wells are filled with media.

For staining 2–6 µl biarsenical dye working solution (final dye concentration: 2–6 µM) and 1 µl washing buffer (final EDT concentration: 5 µM) are added to 200 µl growth medium. Generally, 2–4 µM FlAsH-EDT_2 and 4–6 µM ReAsH-EDT_2 are sufficient. The EDT is required to lessen unspecific staining. Finally, an adequate amount of cells from a logarithmically growing preculture is added.

The cells are grown overnight at 30 °C in the 48-well microtiter plate in a rotating shaker at ~120 rpm. To keep them in the dark, the plates are covered with aluminum foil. To maximize the yield, the culture should be grown until the late logarithmic growth phase.

Washing After overnight staining the cells are transferred to an Eppendorf-cup and harvested by centrifugation (2 min, 1500 g). The pellet is resuspended in 1 ml growth medium containing 30–50 µM EDT and incubated for 10–15 min. After this destaining step the cells are peleted and resuspended in growth medium (without EDT). Further washing steps may be beneficial before the cells are mounted for microscopy (see below).

4.2.2.2 Pulse Staining

This approach relies on the electroporation of the cells in the presence of the dye, which is accomplished with a standard electroporator. This method reduces the required time for the whole staining procedure to less than 1 h. Cells grown in liquid media or on agar plates may be used. The unspecific background tends to be considerably lower than in the case of the overnight staining approach. The major disadvantage of this method is the low portion of stained cells. Generally 2–10% of all cells are labeled efficiently.

Preparation of Yeast Cells The cells need not necessarily be in the logarithmic growth phase. However, a large number of dead cells interferes with the staining efficiency because the dead cells tend to accumulate biarsenical dyes.

Prior to electroporation, the cells are washed twice with distilled water (room temperature) to remove ions. The cells are then harvested by centrifugation in an Eppendorf-cup. The amount of cells is not critical. The cell pellet is resuspended in 40 µl distilled water and 0.2–0.4 µl biarsenical dye is added from the undiluted 2 mM stock solution. Finally the cells are transferred in a standard electroporation cuvette with a width of 2 mm.

Staining The uptake of the dye is induced by an electric discharge, delivered by an electroporator. The best staining was achieved with the following parameters: 600 Ω, 0.5 kV, 25 µF. Immediately after electroporation, 160 µl growth medium containing 1 µl fresh washing buffer (final EDT concentration: 5 µM) is added. Before washing the cells are incubated for ~30 min at 30 °C.

Washing The cells are harvested and destained as described for the overnight procedure (see above). The time needed for destaining may be reduced to ~5 min.

4.2.2.3 Mounting and Microscopy

Live cells are mounted in growth-medium or buffer solution. To this end the cells are harvested by centrifugation, resuspended in a small volume of growth medium or buffer and mounted below a cover slip. Alternatively, and for longer observation times, the cells may be taken up in 1% low melting agarose (Type VII, Sigma, St Louis,

MO, USA) in growth-medium kept at ~40 °C. However, the low melting agarose may exhibit slight autofluorescence that may interfere with the weak FlAsH signal.

Generally, FlAsH and ReAsH are prone to photobleaching, rendering the imaging potentially challenging. We typically use epifluorescence microscopy to determine the staining efficiency. Subsequently confocal microscopy or other imaging techniques may be used. Imaging with high light intensities should be avoided and sensitive detection systems are advisable.

4.3 Short Protocols

4.3.1 Overnight Staining

Labeling:

- harvest an adequate amount of cells from a logarithmically growing culture (2 min at 1500 g)
- prepare: 200 µl growth-medium with 1 µl fresh washing buffer
- dilute the biarsenical dye 1 : 10 in Tris-buffer
- add 2–6 µl of diluted biarsenical dye to the culture
- inoculate the medium with cells (so that a logarithmic growth for at least 12 h is ensured)
- grow cells for at least 12 h in a rotating shaker at 30 °C (in the dark).

Washing:

- harvest cells by centrifugation in an Eppendorf-cup (2 min at 1500 g)
- resuspend in 1 ml of growth-medium
- add 30–50 µl of fresh washing buffer
- incubate for 10–15 min, pellet the cells by centrifugation (4 min at 1500 g)
- wash the cells for ~1 min in growth-medium to remove EDT
- harvest the cells for microscopy.

Required time: ~13–18 h

4.3.2 Pulse Staining

Labeling:

- prepare: 160 µl growth-medium with 1 µl fresh washing buffer
- wash cells (from liquid culture or agar plate) twice in 1 ml H_2O
- pellet the cells and resuspend them in 40 µl H_2O
- add 0.2–0.4 µl undiluted biarsenical dye to the cells
- transfer the sample into an electroporation cuvette

- Pulse label at 600 Ω, 25 µF, 0.5 kV and immediately add the 160 µl growth-medium with EDT
- transfer the cells to an Eppendorf cup
- incubate in the dark for ~30 min in a rotating shaker at 30 °C.

Washing:

- harvest cells by centrifugation in an Eppendorf-cup (2 min at 1500 g)
- resuspend in 1 ml growth-medium
- add 30–50 µl of fresh washing buffer
- incubate for ~5 min, pellet the cells by centrifugation (4 min at 1500 g)
- wash the cells for ~1 min in growth-medium to remove EDT
- harvest the cells for microscopy.

Required time: ~1 h

4.4 Troubleshooting

Problem: Weak or no fluorescence signal	
Insufficient labeling	We recommend establishing the labeling protocol with an abundant tagged protein.
	Try the alternative protocol (electroporation or overnight staining).
	EDT may be omitted from the staining solution.
	In rare cases increasing the dye concentration (up to 10 µM) and incubation time (in case of overnight staining) is useful.
Too extensive washing	Reduce the length of the de-staining step and/or reduce the concentration of EDT in the de-staining solution.
The biarsenical dye is not working	Both, FlAsH and ReAsH, are very sensitive to multiple freeze–thaw cycles and oxidation.
	ReAsH changes its color upon oxidation from red to blue/purple. ReAsH with a purple color does not work properly.
	FlAsH and ReAsH aliquots (5–10 µl) are best stored at −80 °C.
Signal is too low	Fusion proteins labeled with the biarsenical-tetracysteine system generally display a weaker fluorescence signal than GFP-fusion proteins.

	The TetCys motif may not be accessible by the dye. The motif might be buried within the folded protein or a membrane. The target cysteines must be in their reduced form. Cysteines that are in the lumen of the secretory pathway or outside cells tend to oxidize spontaneously. Use of tributylphosphine to reduce the cysteines [24] may alleviate the problem.
Problem: Background is too high	
Insufficient destaining/ bright vacuoles	Frequently, unbound biarsenical dyes are accumulated in the vacuoles during the staining procedure and a slightly stained cytosol may be observed. This problem is normally alleviated by increasing the duration of the destaining steps and/or the concentration of EDT in the de-staining solution.
Problem: Very bright individual cells	
Dead yeast cells accumulate biarsenical dyes	If too many dead cells are in the solution, this may reduce the amount of free dye and reduce the staining efficiency of the healthy cells. Care must be taken to minimize the number of dead cells.
Problem: Fast loss of fluorescence signal	
Photobleaching	FlAsH and ReAsH are prone to photobleaching. Reduce irradiation intensities. At low oxygen concentrations, the overall FlAsH fluorescence signal may decrease. (This happens, for example, when live yeast cells consume the oxygen under the coverslip.) Sometimes a higher FlAsH fluorescence signal is observed in close vicinity to air bubbles.

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