

## 4

### “FIAsh” 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  $\sim 28$  kDa ( $\sim 230$  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.

## 4.1.1

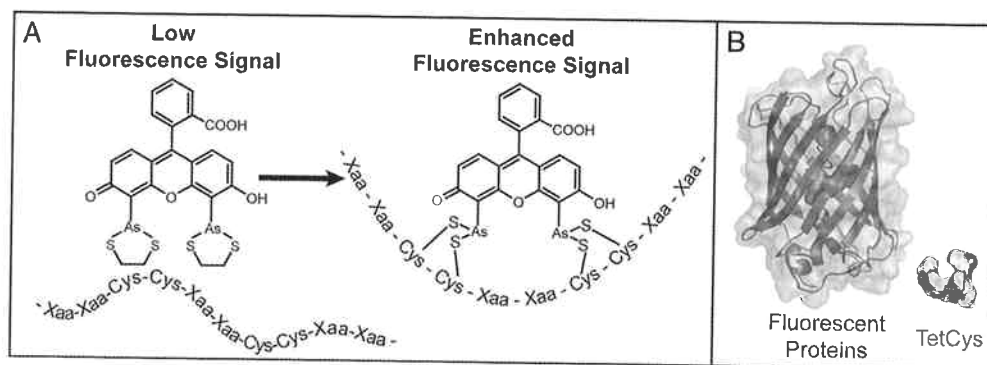
**The Biarsenical-Tetracysteine System**

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 FAsH (Fluorescein Arsenical Hairpin Binder), a derivative of the well-known fluorophore fluorescein [4]. For FAsH, dissociation constants in the sub-nanomolar range (Table 4.1) for a peptide containing the TetCys motif have been reported. In solution, FAsH 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  $\beta$ -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 FAsH-EDT<sub>2</sub> exhibits low fluorescence, whereas bound FAsH 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 <i>et al.</i> [4]
WDCCPGCCK	0.67	310 000	4	Adams <i>et al.</i> [11]
WDCCGPCCK	0.44	50 000	72	Adams <i>et al.</i> [11]
WDCCPCCK	0.60	100 000	150	Adams <i>et al.</i> [11]
WDCCGCCK	0.55	35 000	100	Adams <i>et al.</i> [11]
WDCCDEACCK	0.23	65 000	92 000	Adams <i>et al.</i> [11]
FLNCCPGCCMEP	0.78	n.d.	n.d.	Martin <i>et al.</i> [12]
HRWCCPGCCKTF	0.65	n.d.	n.d.	Martin <i>et al.</i> [12]

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

#### 4.1.2

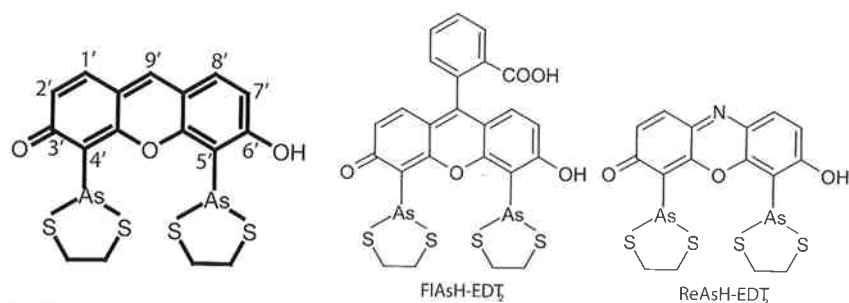
##### Improved TetCys Motifs

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 (Resorufin-based Arsenical Hairpin Binder) 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 <sub>2</sub> ; 9'-OH	380 nm	430 nm	Adams et al. 2002
F <sub>2</sub> FlAsH	2'-7'-F <sub>2</sub> ; 9'-(2-C <sub>6</sub> H <sub>4</sub> COOH)	500 nm	522 nm	Spagnuolo et al. 2006
CrAsH	9'-(2,5-C <sub>6</sub> H <sub>4</sub> [COOH] <sub>2</sub> )	515 nm	534 nm	Cao et al. 2006
FlAsH	9'-(2-C <sub>6</sub> H <sub>4</sub> COOH)	511 nm	527 nm	Griffin et al. 1998
F <sub>4</sub> FlAsH	9'-(2-C <sub>6</sub> F <sub>4</sub> COOH)	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<sub>2</sub> and ReAsH-EDT<sub>2</sub> 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.

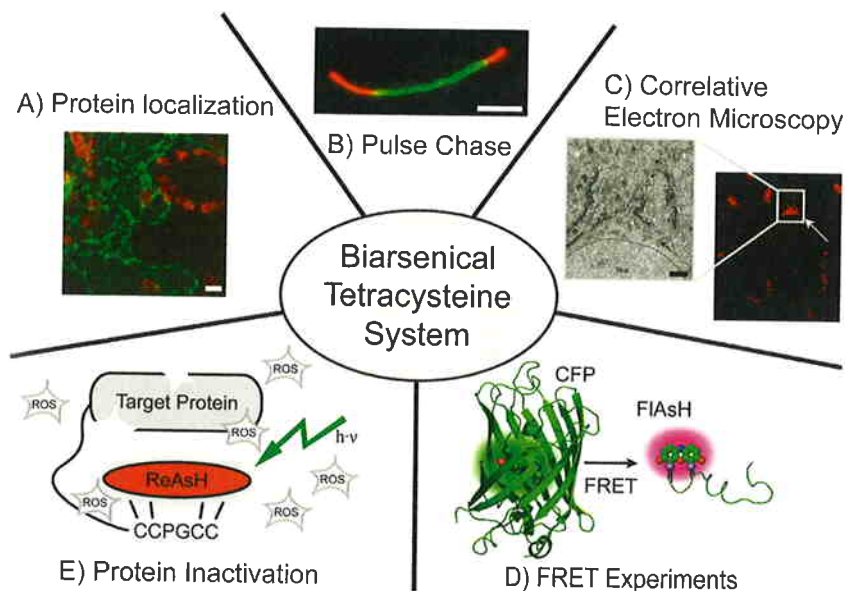
#### 4.1.4

##### 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 FIAsh-labelled *Arabidopsis* cells expressing TetCys-labelled synthetic glycomodule peptides [22]. (B) Sequential labeling with FIAsh 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 FIAsh has been utilized to study conformational changes or to create environment-sensitive probes. (E) Intense irradiation of FIAsh 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  $\mu$ m.

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 FIAsh 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.

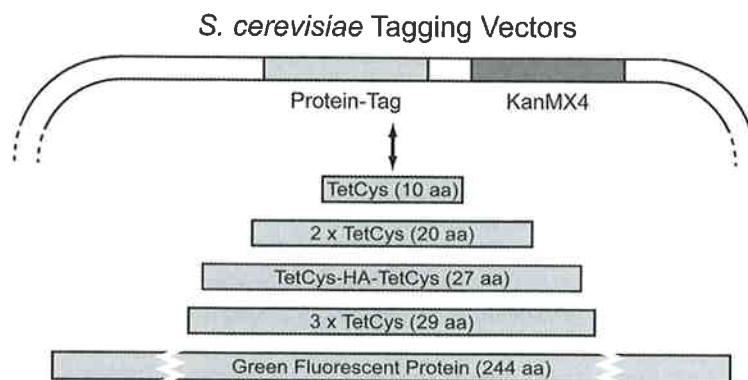
Organism	CCXCC	CCXXCC	CCPGCC
<i>Arabidopsis thaliana</i>	41	52	—
<i>Candida albicans</i>	3	3	—
<i>Caenorhabditis elegans</i>	38	30	—
<i>Drosophila melanogaster</i>	122	149	—
<i>Homo sapiens</i>	64	68	—
<i>Mus musculus</i>	58	64	—
<i>Rattus norvegicus</i>	27	22	—
<i>Saccharomyces cerevisiae</i>	5	7	1)
<i>Saccharomyces pombe</i>	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 ( $\beta$ -tubulin; Tub2) were investigated. Tub2 tagged with either 1 $\times$  TetCys (10 amino acids [aa]) or 2 $\times$  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.

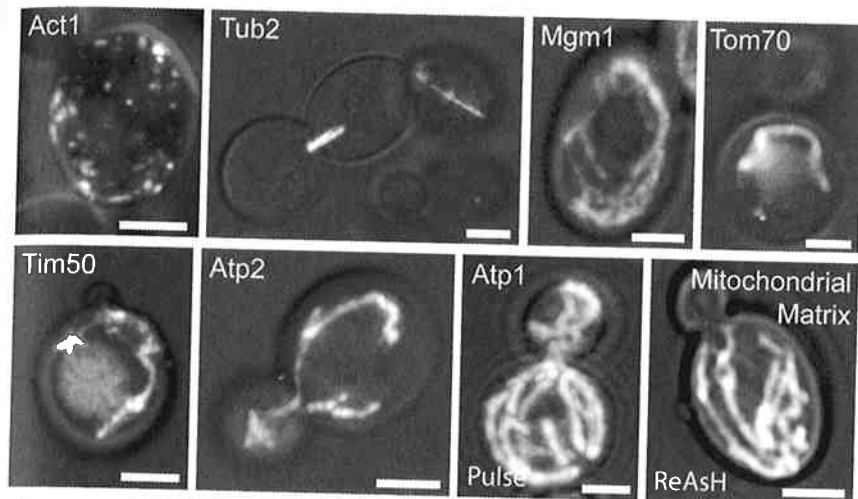
Protein-tag	Bleaching rate, $t_{1/2}$ (s)
1× TetCys	1.8
2× TetCys	3.5
3× TetCys	9.5
TetCys-HA-TetCys	4.5
GFP	16.0

3× TetCys (29 aa) or with GFP (244 aa) resulted in nonviable haploid cells [9]. Hence, for  $\beta$ -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 FAsH 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). FAsH 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 FAsH 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 FAsH-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 FAsH-EDT<sub>2</sub>. Since FAsH-EDT<sub>2</sub> 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  $2\times$  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 FAsH. Scale bars:  $2\mu\text{m}$ .

We have used both methods to FAsH-label various yeast strains expressing  $2\times$  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 FAsH labeled proteins that could be tagged with the  $2\times$  TetCys or the  $3\times$  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 <sup>-1</sup>	ammonium sulfate	5 g l <sup>-1</sup>
complete supplement mixture (see Ref. [31])	0.6 g l <sup>-1</sup>	glucose	20 g l <sup>-1</sup>

## Mitochondria complete medium:

yeast nitrogen base	1.7 g l <sup>-1</sup>	ammonium sulfate	5 g l <sup>-1</sup>
		galactose	10 g l <sup>-1</sup>
		ethanol	20 ml l <sup>-1</sup>
complete supplement mixture (see Ref. [31])	0.6 g l <sup>-1</sup>	glycerol	15 g l <sup>-1</sup>

## 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<sub>2</sub> and ReAsH-EDT<sub>2</sub> 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<sub>2</sub> or 200 μM ReAsH-EDT<sub>2</sub> 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.