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Protein Labeling With FlAsH and ReAsH

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Summary

The ability to image biochemical and phenotypical changes in living cells has become crucial for the investigation and understanding of the molecular mechanisms that govern all physiological cellular functions in health and disease. Genetically encoded reporters derived from fluorescent proteins (FPs) have proved to be extremely useful for localization and interaction studies in living cells. However, the large size and spectral properties of FP impose certain limitations for their use. The recently developed Fluorescein Arsenical Hairpin (FlAsH/tetracysteine) binder technology emerged as a promising alternative to FP for protein labeling and cellular localization studies. The combination of a small genetically encoded peptide tag with a small molecule detection reagent makes this technology particularly suitable for the investigation of biochemical changes in living cells that are difficult to approach with fluorescent proteins as molecular tags. We describe the practical application of this technology to image protein dynamics in living cells.

Key Words: Biarsenical; FlAsH; fluorescein; fluorescent proteins; Gateway; high content screening; live cell imaging; Lumio; protein kinase C; protein labeling; ReAsH; resorufin; tag; tetracysteine.

1. Introduction

Current high content analysis is mostly performed as end-point analysis using immunofluorescence as the primary labeling/detection method. This method although valuable because of its flexibility and ease of use is by default limited to end-point analysis. The ability to perform real-time analysis of protein dynamics in living cells is critical for the in-depth understanding of the complex biochemical and phenotypical changes associated with cell behavior and function *(1)*. Traditionally, detection and analysis of proteins in living cells relied primarily on the use of fluorescent proteins (FPs) as genetically encoded tags *(2)*. Despite its proven versatility and utility for the analysis of protein dynamics, there are certain limitations for the use of FPs. Because of their size (28 kD) FPs have the potential to interfere with the activity, localization, or conformation of its fusion partner and can be usually fused only to the N- or C-terminus of a protein *(3)*. In addition, FPs offer only limited spectral variety with a relative paucity of useful red FP versions, although the development of improved red FP variants has been reported recently *(4)*.

In recent years, a number of alternative live cell labeling methods have been introduced, solving some of the problems of FPs (such as the spectral limitations) *(5,6)*. However, all published methods rely on the fusion of rather sizable polypeptides to the protein of interest, and, therefore, share with FP, the drawback of potential steric interference of the tag with the function of the target protein.

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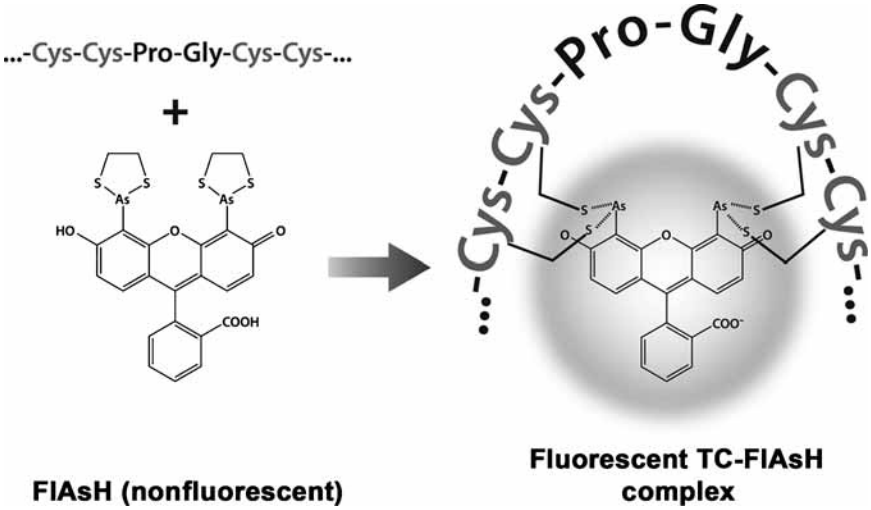

Fig. 1. Molecular principle of FlAsH binding to the TC motif.

Recently Roger Tsien's group at UCSD developed a method of specifically labeling proteins in living cells using a high-affinity pair consisting of a small peptide tag and a small molecule label *(7)*. This technology exploits the high affinity of the tetracysteine (TC) motif, consisting of two cysteine pairs separated by a two amino acid spacer (CCXXCC), for organo-arsenical compound under reducing conditions (**Fig. 1**). This principle was successfully utilized by generating fluorescein arsenical hairpin binder (FlAsH), a derivative of the membrane permeable dye fluorescein that contains thioarsolan substitutions in the 4′ and 5′ positions. The two cysteine pairs bind with high affinity (an effective dissociation constant of 10^{-11} *M* has been reported in **ref.** *8* through the formation of four reversible covalent bonds to the thioarsolan groups of FlAsH (**Fig. 1**). The genetic fusion of the TC tag to a target protein allows the specific labeling (and detection) of the fusion protein in living cells on incubation with a biarsenic-conjugated fluorescent dye *(7)*. The earliest TC tags were designed as short putative α-helical motifs following the assumption that a α-helix provides an optimal binding environment *(7)*. However, further systematic investigation of numerous TC motifs with different spacers determined that the insertion of the helix-breaking amino acids glycine and proline resulted in substantially enhanced affinity of FlAsH for the TC motif *(8)*. Another fortuitous aspect of this technology is that the unbound ligand shows little fluorescence until it binds to the TC motif, resulting in a massive increase in fluorescence on binding *(7)*. The Tsien laboratory expanded the range of colors available for TC labeling by the development of two additional biarsenical dyes, the resorufin-derived red fluorescing dye ReAsH and the blue fluorescing biarsenical CHoXAsH, a 3,6-dihydroxy xanthone derivative *(8)*. This technology has also been applied to the investigation of protein conformation (*see* **Note 1**). For example, two environmentally sensitive biarsenical dyes based on fluorescein and nile red were developed by Yoshio Umezawa's lab to monitor calcium-induced conformation changes in calmodulin *(12,13)*.

Despite its relative limited use so far, there is a remarkable variety of published FlAsH applications (*see* **Table 1**) *(3,6–20)* underlining both its versatility and potential for different applications that reach beyond imaging. The TC/FlAsH™ technology has been further developed and commercialized by Invitrogen (Molecular Probes; Carlsbad, CA) under the brand name TC-FlAsH (formerly Lumio™ green) and TC-ReAsH (formerly Lumio red) in a convenient kit format for labeling of TC-tagged proteins in living cells. Invitrogen offers an additional kit (Lumio Green detection kit) designed for the specific detection of proteins in polyacrylamide gels.

Table 1
Published FlAsH Applications

Method	Protein of interest	Cell system	Reference
Fluorescence assisted light inactivation	Synaptotagmin connexin 43	Drosophila, third instar larvae	***9,11***
FRET	A_{2A} adenosine receptor	CHO	***10***
Conformational analysis – environment sensitive probe	Calmodulin	HEK293	***12,13,26***
Electron microscopy	Connexin43	HeLa	***14,15***
Single molecule Detection/analysis	Calmodulin	n.a.	***16***
Affinity purification	Kinesin	n.a.	***17***
Protein translocation/tracking	Connexin43	HeLa	***14,15***
	HIV	HeLa, Jurkat	***18***
	Glucocorticoid receptor	Hela, CHO	**Fig. 3A**
	PKC-α	HeLa, CHO	**Fig. 3B**
	β-tubulin	HeLa, CHO	**Fig. 3C**
	β-tubulin	*Saccharomyces cerevisae*	***2***
	Cytochrome-*c*		***19***
Multicolor pulse chase analysis	Connexin43		***14***
Oligomerization/aggregation analysis	Ebola virus protein 40	293T	***20***
	CRABP I	*Escherichia coli*	***21–23***
	Phospholamban	In vitro	***24***
Electrophoresis		n.a.	***25***

CRABP, cellular retinoic acid-binding protein.

There are currently no examples for the use of FlAsH in high content screening (HCS) applications published in the scientific literature. Existing HCS analysis in living cells relies exclusively on the use of FP as the tagging/detection method, with the previously mentioned limitations inherent to this approach. The TC/FlAsH labeling technology should prove valuable in complementing FP in live cell imaging. The combination of small tag size and the high cell permeability and affinity of FlAsH/ReAsH provide the ideal means of pursuing challenging applications such as multiplex imaging, FRET-based analysis of protein conformation/interaction ***(10,20,21)*** and pulse chase experiments ***(14)*** in living cells.

In this report we attempt to provide a systematic and practical introduction to the use of TC/FlAsH technology for the labeling of proteins in living cells. Although staining of TC-tagged proteins in living cells with FlAsH is a relatively simple and straightforward procedure, the reader should keep in mind that new applications usually require a certain degree of optimization and empirical testing of some of the variables we describe to obtain the desired results. As previously mentioned, TC/FlAsH labeling has been successfully used for a variety of specialized imaging and biochemical applications (**Table 1**). A detailed description of these applications would exceed the scope of this report. The reader is instead referred to the literature for further information.

2. Materials

2.1. Generation and Expression of TC-Tagged Fusion Proteins

1. TC-tagging expression vector (e.g., pCDNA6.2™/Lumio-Dest, Invitrogen, www.invitrogen.com).
2. Reagents and equipment for standard molecular biology techniques.
3. *Escherichia coli* TOP10™ (Invitrogen).
4. CHO-K1 cell line (or any other cell line suitable for live cell analysis by microscopy).
5. Standard cell culture reagents and equipment required for the cultivation of mammalian cells (plastic ware: Corning, www.corning.com\lifescienes\, all media/supplements: Invitrogen-GIBCO).

6. Lipofectamine™ 2000 (Invitrogen).
7. Opti-MEM® I-reduced serum medium (Invitrogen).

2.2. Staining With FlAsH Reagent

1. FlAsH/ReAsH: TC-FlAsH/TC-ReAsH In-Cell Tetracysteine Detection Kits (Invitrogen-Molecular Probes, formerly: Lumio In-Cell Labeling Reagent).
2. Labtek II Chambered cover slips (Nunc, www.nalgenunc.com).
3. Glass bottom 35 mm cell culture dishes (MatTek Corp., Ashland, MA; www.mattek.com).
4. EDT_2 (1,2-ethanedithiol, purum >98%, FLUKA [St. Louis, MO]; www.sigmaaldrich.com).
5. 2,3 Dimercapto-1 propanol (also known as BAL = British antilewisite, ACROS, www.acros.com).
6. Patent blue V, sodium salt (Fluka).
7. Inverted Microscope (we used for all imaging examples shown in this report either a Zeiss Axiovert 25 or a Deltavision Image Restoration system from Applied Precision [Issaquah, WA]; www.zeiss.com, www.api.com).

3. Methods

The methods described herein outline all steps required to express, label, and detect TC-fusion proteins in mammalian cells. Special attention is given to the labeling procedure. We show a number of examples, such as labeling of glucocorticoid receptor, tubulin-β and protein kinase C-α (PKC-α), and to demonstrate successful detection of TC-tagged proteins in living cells, to emphasize the versatility of this live cell labeling methodology (**Fig. 2A–C**).

3.1. Generation and Expression of TC-Tagged Fusion Proteins

The detection of proteins with FlAsH requires the generation of an expression construct containing the gene of interest and a fused or integrated TC-tag. The basic core sequence for the TC tag is CCXXCC. The most common amino acids used for the XX spacer are proline and glycine (as used also in Invitrogen's expression vectors), but the use of a number of different spacers has been reported *(8)*.

The TC tag can easily be added to either the N- or C-terminus of the target protein. In addition, the small size of the TC tag often allows its insertion into specific domains of the target protein without disturbing protein structure or function, as demonstrated for the A_{2A} adenosine receptor *(10)* and the mammalian cellular retinoic acid-binding protein I *(20,21,23)*. These studies suggest that short α-helices or loops are the most suitable sites for integration/substitution with the TC tag. Overall, the TC tag provides excellent flexibility with respect to tag placement combined with minimal potential for interference with overall structure and function of the target protein. The small size of the TC tag also allows for simple addition through PCR and cloning of the PCR product into any desired expression vector using standard recombinant DNA methods. A spacer sequence between protein and TC tag is normally not required. An additional option is the use of Invitrogen's Gateway® system that includes mammalian expression vectors already containing N- or C-terminal TC tags. The Gateway system allows for the fast and convenient generation of N- or C-terminal TC-fusion proteins using lambda phage-based site-specific recombination. Additional information about the Gateway cloning system and Gateway-Lumio vectors can be found on Invitrogen's website (www.invitrogen.com). As with any other fusion construct, proper expression, localization, and function of the fusion protein needs to be validated against the wild-type protein before using the construct in experiments (*see* **Note 2**).

TC-fusion proteins are suitable for both stable and transient expression in a wide variety of organisms and cell lines (*see* **Table 1**). We transiently expressed a number of different proteins (structural proteins, kinases, GPCRs, and nuclear hormone receptors, and so on) in a range of different cell lines (HEK 293, HeLa, CHO, A549, and NIH3T3) using standard transfection methods (Lipofectamine™ and Lipofectamine 2000) without experiencing any problems regarding protein expression or cell viability. The following protocol provides guidance for the generation and use of TC-tagged fusion proteins.

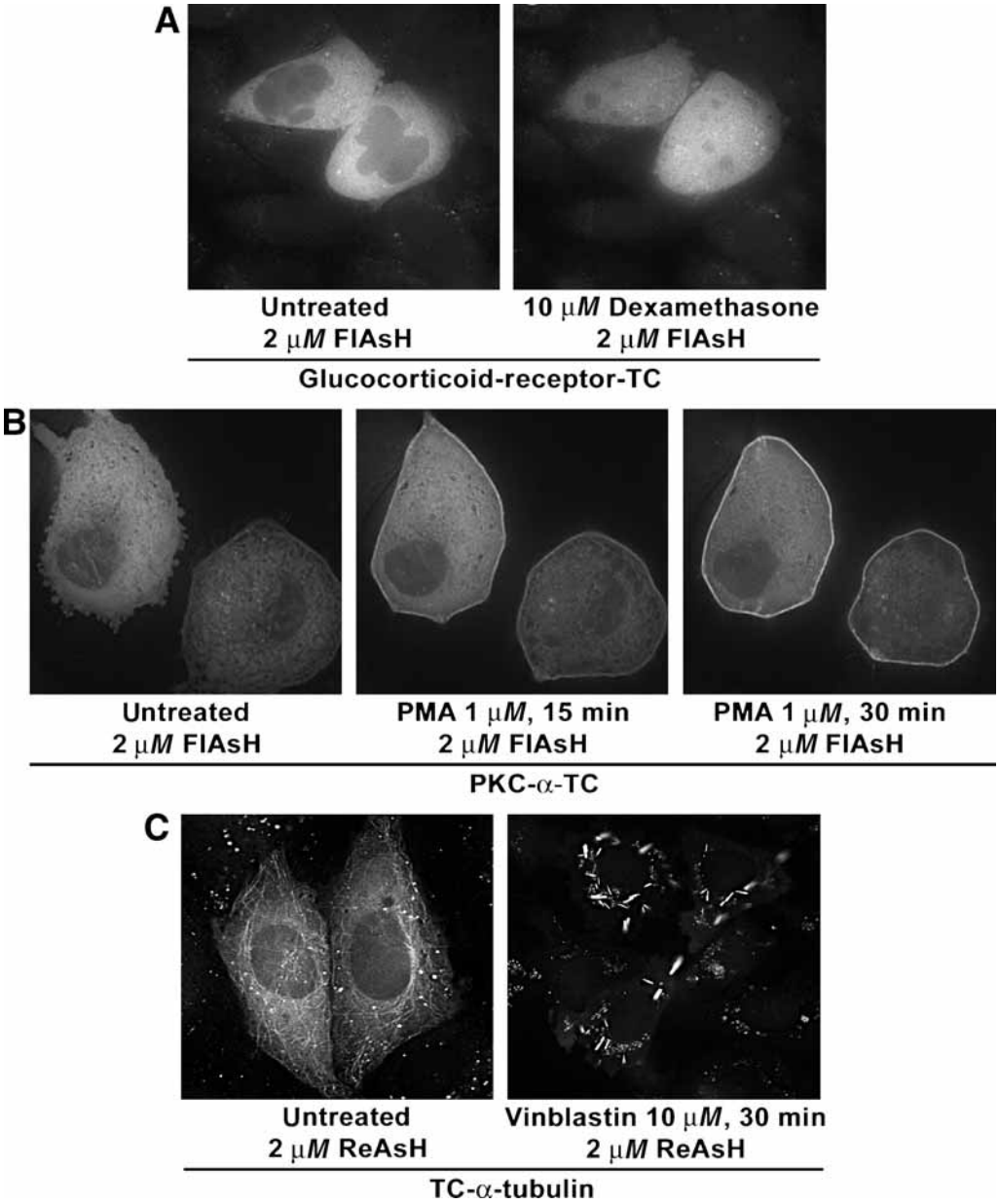

Fig. 2. Examples for imaging protein dynamics in living cells using FlAsH/ReAsH staining. CHO cells were transiently transfected with **(A)** Glucocorticoid receptor-TC (GR-TC), **(B)** PKC-α-TC or **(C)** TC-tubulin-α. After transferring the cells onto chambered cover slips the cells were labeled with 2 μ*M* FlAsH or ReAsH (as indicated) for 60 min at 37°C and subsequently washed three times with Opti-MEM containing 200 μ*M* EDT2. **(A)** The cells transfected with Glucocorticoid receptor were left untreated or treated for 20 min with either 10 μ*M* Dexamethasone. Dexamethasone binds and activates GR resulting in the translocation of GR-TC from cytoplasm to the nucleus. **(B)** The cells transfected with PKC-a were left untreated or treated for 20 min with 1 μ*M*. PMA treatment leads to activation of PKC-a followed by translocation from the cytoplasm to the plasma membrane. **(C)** The cells transfected with TC-β-tubulin were left untreated or treated for 60 min with 10 m*M* vinblastine which induces microtubule depolymerization and the appearance of short tubulin aggregates. The two images show different fields within the sample.

Images were aquired on a Deltavision Image Restoration System (Olympus IX-71 inverted microscope equipped with a Plan-Apo ×60, NA 1.4 objective). All images were subjected to deconvolution processing.

3.2. Generation of TC-Tagged PKC-α Expression Construct

We are using PKC-α as an example to illustrate the practical steps required for generating, expressing, and labeling TC-tagged proteins. For this example, the DNA of PKC-α was cloned through PCR directly from cDNA generated from HeLa cell mRNA using standard molecular biology techniques.

3.2.1. Cloning

1. For the generation of C-terminal TC-fusion constructs we modified Invitrogen's mammalian expression vector pcDNA6™/V5-His by replacing the His tag with the TC tag.
2. The full-length PKC-α fragment was cloned into the *Hind*III and *Xho*I sites of the modified pcDNA6™/V5(-TC) vector. The inclusion of the V5 tag did not interfere with the labeling or function of the target protein in cells and was additionally used to independently verify expression and correct localization of the PKC-α-fusion protein through indirect immunofluorescence.
3. The plasmid DNA was transformed into the TOP10™ *E. coli* strain and plated on LB plates containing ampicillin (50 mg/mL).
4. Individual colonies were selected and grown in LB (+ ampicillin).
5. The plasmid DNA was purified and checked for proper insert size and verified by sequencing before further use.
6. The modification of the expression vector and subsequent insertion of the PKC-α DNA was carried out following standard molecular biology protocols. A detailed description would exceed the scope of this article.

We also had success using the previously mentioned Gateway cloning system to generate N-terminal TC-tagged expression constructs for numerous different proteins (e.g., TC-tubulin-α, **Fig. 2C**). For these constructs we utilized Invitrogen's Ultimate™ ORF collection, which offers more than 4000 sequence validated open reading frames in Gateway entry vectors. The desired entry clones were transferred by recombination into the Gateway N-terminal Lumio destination vector (pCDNA™6.2/Lumio-DEST).

3.2.2. Expression of TC-Fusion Proteins in CHO-K1 Cells

We have expressed many distinct TC-tagged fusion proteins (*see* **Subheading 3.1.**) in different cellular backgrounds without encountering any problems with insufficient expression or toxicity that could be attributed to the addition of the TC tag. The transfection protocol provided serves as a working example. It is recommended to optimize transfection protocols for each novel experimental setup (e.g., different expression systems, cell lines, and transfection methods), before performing large-scale experiments (*see* **Note 3**).

We used Lipofectamine 2000 as transfection reagent for our transient and stable transfections. Other transfection reagents were used with similar overall results regarding transfection efficiency and cell viability. For detailed information regarding principles of transfection and application protocols we refer the reader to the manufacturer's protocol for Lipofectamine 2000. Later we describe a standard protocol routinely employed in our laboratory for the transfection of CHO-K1 cells.

3.2.2.1. Day 1

1. Plate 0.5×10^6 CHO-K1 cells in 3 mL Dulbecco's modified Eagle's medium (DMEM) + 10% FCS in a 6-well plate and incubate for 16 h under cell culture conditions. The CHO-K1 cells used in this experiment were cultivated in DMEM supplemented with nonessential amino acids, penicillin, streptomycin, 10 m*M* HEPES and 10% FCS. The cells were kept at 37°C in a humidified incubator and an atmosphere containing 5% CO_2.

3.2.2.2. Day 2

1. Remove medium from cells and replace with 2.5 mL of fresh complete DMEM medium + 10% FCS.
2. Prepare two microtubes with 250 µL OPTI-MEM I (tubes A and B). Add 4 µg of the plasmid DNA to tube A and 10 µL of Lipofectamine 2000 to tube B and mix gently. Mix content of both tubes by pipeting, and incubate the transfection mixture for at least 15 min at room temperature.

3. Add 500 µL transfection mixture to the cells and incubate for 24 h under cell culture conditions. We generally achieve between 30 and 60% transfection efficiency using this particular cell line and transfection protocol.
4. Plate the cells according to imaging application. For applications requiring the use of oil immersion objectives (and limited working distance) we used either chambered-cover slips (Nunc Labtek II, cover slips made of German glass 1.5 thickness [0.16–0.19 mm]), which can be purchased with 1, 2, 4, or 8 individual chambers. For applications that require better accessibility (e.g., for subsequent microinjection, and so on), 35 mm glass bottom dishes are more suitable. The glass surface normally provides an excellent substrate for most adherent cells. If necessary, the cover slips/dishes can be coated with Matrigel (or any other adherence promoting factors, such as collagen, and poly-L-lysine) to improve adherence. A coating of poly-L-lysine will allow the attachment of suspension cells to glass or plastic surfaces. We also used 96-well plates for experiments, which allow the handling and observation of large number of samples and the use HCS/HTS instrumentation. However, the use of plastic materials for imaging purposes requires objective with long working distance and inherently limits resolution and image quality and is therefore not suitable for applications that require the acquisition of high-quality images of subcellular structures. For general imaging, the cell density should not exceed 70–80% confluency in order to achieve optimal cell morphology. After plating, the cells should be incubated for additional 24 h under cell culture conditions to allow for cell adherence.

3.3. Staining With FlAsH Reagent

The staining protocol provided here should be used as general guidance for the staining of living cells and should be modified as required to achieve optimal and consistent results. In general, staining cells with FlAsH is a fast and uncomplicated process and will provide in most cases highly specific and sensitive staining of TC-tagged proteins. However, a couple of issues need to be addressed in order to obtain the best results with this labeling technology.

The single most important problem using TC/FlAsH is unspecific background staining *(27,28)*, which might lead to poor signal-to-noise ratio especially for low-expressing targets (*see* **Note 4**). Although the FlAsH reagent has considerable specificity for the TC motif over individual cysteine residues *(7,8)*, the sheer number of available cysteines within a cell will result in some background labeling. It is therefore important to adjust incubation and wash conditions to optimize signal-to-noise ratio. This might require some additional experimentation, especially for low-expressing proteins. The most commonly used method for background reduction is the inclusion of small dithiols, such as EDT_2 and BAL, during labeling and washing procedures. These dithiols effectively compete with single cysteines for FlAsH and, therefore, prevent most nonspecific intracellular labeling. However, it should be kept in mind that at high concentrations (low millimolar) EDT_2 and BAL will eventually displace most of the TC-bound FlAsH/ReAsH. It is therefore recommended to carefully assess wash conditions. An additional source of background staining is the nonspecific association of FlAsH with proteins independent of dithiols *(28)*, presumably owing to interaction with exposed hydrophobic sites. This type of background is particularly pronounced in damaged and dead cells, which often appear as extremely bright rounded up cells in the sample. The inclusion of the uncharged dye Disperse Blue 3 (provided with Invitrogen TC-FlAsH/ReAsH Kit) will suppress most of this type of dithiol-independent background. Further background suppression can be achieved by the addition of high concentrations of a quenching dye such as Patent blue *(28)*.

The second important issue to be considered when using TC/FlAsH for labeling experiments is the requirement for a strongly reducing environment. FlAsH will not bind to oxidized cysteines. The use of TC/FlAsH should be therefore limited to proteins/protein domains that reside in the cytoplasm and nucleus (*see* **Note 5**). Membrane spanning proteins should be fused to the TC tag at their cytoplasmic portion. The use of TC/FlAsH for extracellular proteins (or domains) or for proteins that reside in the lumen of intracellular compartments, such as ER, Golgi, or endosomes should be avoided. The use of FlAsH for in vitro application requires complete reduction of the

FlAsH-binding cysteines. A helpful and informative discussion of in vitro FlAsH applications can be found in Griffin et al. *(28)*. The reader is also referred to the Invitrogen website (www.invitrogen.com) for further information about FlAsH as in vitro protein labeling reagent (Lumio green).

3.3.1. Procedure for Loading Cells With FlAsH

1. Prepare 2 µ*M* FlAsH staining solution by diluting the FlAsH stock solution 1/1000 (Invitrogen TC-FlAsH In-Cell labeling reagent is provided as 2 m*M* stock solution in DMSO) in Opti-MEM I. This staining solution yielded satisfactory results in all our experiments. The use of Opti-MEM I or any other low/no serum medium or salt solution (e.g., Hank's balanced salt solution) is highly recommended because FlAsH reagent is known to bind serum proteins *(28)*. We did not observe significantly improved staining results (for imaging applications) after using higher concentrations of FlAsH reagent, which might also lead to increased background. As previously discussed, the inclusion of dithiols greatly reduced background staining. We found it generally beneficial for the signal-to-noise ratio to include some EDT_2 in the staining solution. As shown in **Fig. 2**, the inclusion of increasing concentrations of EDT_2 led to considerable reduction in nonspecific staining. Nevertheless, higher concentrations resulted in a reduction of specific signal. We recommend the addition of a 5- to 50-fold excess of EDT_2 in the staining solution to suppress nonspecific staining. The exact concentration of EDT_2 should be determined empirically in a pilot experiment for each construct and cell line. The required amount of staining (and subsequently washing) solution is dependent on the used cultivation vessel (35 mm dish: 2 mL/8-well LabtekII: 0.4 mL/96-well plate: 100 µL/well).
2. Remove DMEM medium from cells and wash once with Opti-MEM I to remove detached (=dead/damaged) cells and cell debris, which are typically present at slightly elevated levels after transfection. Dead cells and debris can be a considerable source for background because of the high level of nonspecific FlAsH uptake.
3. Add FlAsH staining solution and incubate for 30 min at 37°C under cell culture conditions. The incubation time can be varied according to need and labeling result. We incubated the samples between 30 and 60 min with the staining solution and found little difference in staining intensity and quality. Longer periods of incubations might result in increased background.
4. Remove staining solution and wash once with Opti-MEM I to remove residual dye.

3.4. Reduction and Suppression of Background Staining in Cells

The inclusion of EDT_2 (or BAL) in the staining solution will substantially but not completely prevent background staining (the Invitrogen TC-FlAsH In-Cell labeling kit includes BAL wash buffer). For further reduction of background, it is recommended that the samples be washed once or more times with Opti-MEM I containing EDT_2 (or BAL) to remove most nonspecifically bound FlAsH. The concentration of dithiols and the number of washes required for optimal background reduction needs to be established empirically in pilot experiments for each construct, cell line, and application. **Figure 3** provides an example for the application of multiple rounds of washes with different concentrations of EDT_2. We found in our experiments that two or three wash steps with EDT_2 concentrations between 100 and 500 µ*M* yield the best results (with respect to signal-to-noise ratio). The application of higher concentrations of EDT_2 will result in a gradual loss of specific signal. It should also be mentioned that BAL is a more effective competitor for FlAsH than EDT_2. The use of BAL needs therefore to be adjusted accordingly in order to prevent the loss of specific signal. However, the intense odor of EDT_2 makes the use of BAL more convenient for the end-user.

3.4.1. Standard Procedure for Background Reduction/Suppression

The following protocol yielded satisfactory results for most of our experiments and is routinely applied to new experimental settings (e.g., new target protein or cell line).

1. Preparation of wash medium. We routinely use Opti-MEM to prepare wash medium, but most other cell culture media and salt solutions (including supplements) will be also suitable for this purpose. Our standard wash medium is prepared by adding EDT_2 to a final concentration of 200 µ*M*. The wash medium

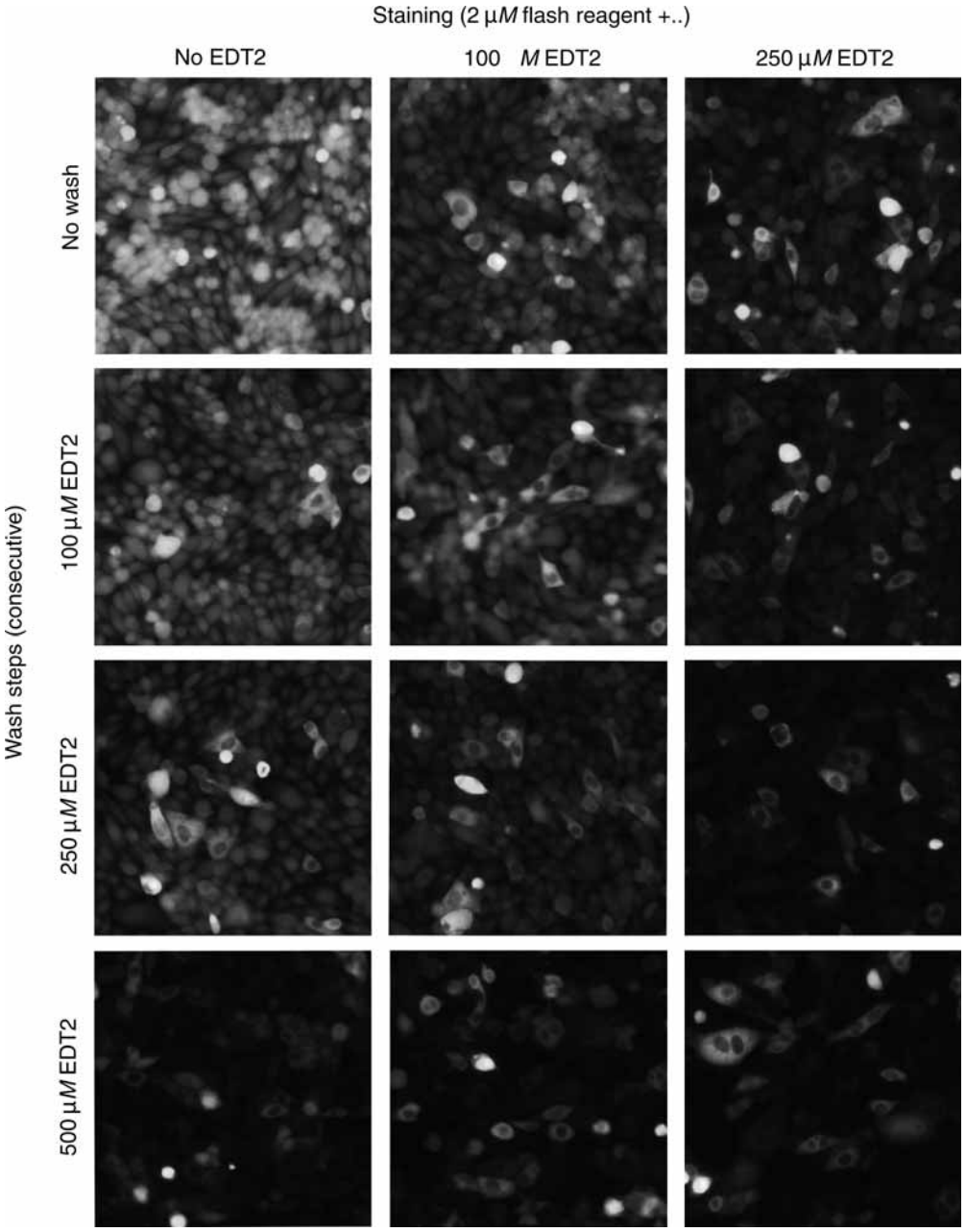

Fig. 3. Use of EDT_2 for background reduction during FlAsH loading and washing. CHO cells were transiently transfected with PKC-α-TC. The cells were labeled with 2 μ*M* FlAsH in Opti-MEM in absence or presence of EDT_2 (100 or 250 μ*M*, as indicated in the top row) for 60 min at 37 min. The samples were then subjected to consecutive wash steps with increasing concentrations of EDT_2 (100, 250, and 500 μ*M* in Opti-MEM) to remove background staining (as indicated on the left side). Images were taken with a Zeiss Axiovert 25 using a ×10 objective, using identical exposure times.

is always prepared fresh (because of the instability of EDT_2/BAL). As previously mentioned EDT_2 has a strong odor and should be handled in the fume hood. We usually prepare a small amount (100 μL) of a 100 m*M* EDT_2 stock solution in DMSO to carry out an experiment. We discard all plastic ware that comes in contact with EDT_2 in separate plastic bags to avoid unnecessary odor development.

2. Add wash solution to the sample and incubate for 5–10 min at 37°C. The required amount of wash and assay medium is dependent on the used cultivation vessel (35 mm dish: 2 mL/8-well LabtekII: 0.4 mL/96-well plate: 100 µL/well).
3. Repeat wash three times.
4. Wash once with Opti-MEM to remove residual traces of EDT_2.
5. Add assay medium to the sample. The assay medium can be any phenol red-free cell culture medium (including supplements and FCS). To reduce dithiol-independent background we include in the final assay medium 20 µ*M* disperse blue 3 and 0.5 m*M* patent blue V.

3.5. Microscopy

The images shown in this report were taken either with a Zeiss Axiovert 25 microscope (Fluar ×10, NA 0.5 objective) equipped with a Pixera Penguin 600CL CCD camera or with a Deltavision Image Restauration System (Olympus IX 71 inverted microscope, ×60 Planapo, NA 1.4 objective) equipped with a Photometrix Coolsnap HQ camera. The excitation and emission filters were chosen according to the spectral properties of FlAsH (ex_{max} 508 nm/em_{max} 528 nm) and ReAsH (em_{max} 593 nm/em_{max} 608 nm) (*see* **Note 6**). High-resolution images (**Figs. 1** and **2**) were subjected to deconvolution processing.

3.6. Application Examples for FlAsH Staining in Living Cells

As mentioned before, there are currently no published examples for the use of FlAsH/Lumio labeling in HCS. We tested a number of well-known biological models used in our laboratory as practical examples for the use of FlAsH to investigate protein dynamics in living cells and its potential use for the development of high content assays. These examples also demonstrate the functionally inert nature of the TC tag (*see* **Note 7**). The proteins were tagged either on the N- (TC-β-tubulin) or C-terminus (PKC-α-TC, glucocorticoid receptor-TC) with the TC motif. Both PKC-α and glucocorticoid receptor (**Fig. 2A,B**), show a stimulus (PMA and dexamethasone, respectively) dependent translocation of the target proteins between different compartments. The stimulation of PKC-α with phorbol ester PMA induces conformational changes followed by the translocation of PKC-α from the cytoplasm to the plasma membrane, whereas treatment with dexamethasone results in the activation of glucocorticoid receptor and its movement from the cytoplasm to the nucleus. The third example (**Fig. 2C**) shows vinblastine (a well-known antimicrotubule compound) dependent dissolution of microtubules to small crystal like tubulin aggregates as an example for drug-induced phenotypic changes. The staining of β-tubulin was conducted using ReAsH instead of FlAsH, demonstrating that both dyes are suitable for monitoring protein dynamics in living cells (*see also* **refs. *13*** and ***14***).

4. Notes

1. In a recent publication from Roger Tsien's laboratory two new TC sequences ***(29)*** with higher affinity for biarsenical dyes and better quantum yield have been reported (sequence 1: FLNCCPGCCMEP, sequence 2: HWRCCPGCCKTF). The enhanced properties of these TC tags allow for more stringent washing conditions and therefore lower background staining, resulting in improved signal-to-noise ratio. The authors claim an at least 20-fold increase in contrast compared with the standard CCPGCC motif. These claims have not yet been evaluated in our laboratory.
2. Toxicity: In general we did not observe any obvious signs of compromised-cell health during the labeling procedures with FlAsH and ReAsH. However, it has been reported that the use of ReAsH might cause temporary changes (24–48 h post-labeling) in cell morphology. These phenotypical changes are presumably caused by the generation of singlet oxygen when ReAsH is illuminated by a high-intensity light source ***(11)***. The generation of reactive oxygen species makes ReAsH an excellent choice for fluorophore-assisted light inactivation experiments ***(11)***, but could potentially lead to altered behavior of the target protein in imaging applications that require high-intensity illumination. We did not observe any signs of toxicity caused by the application of dithiols (EDT_2 and BAL) for background reduction up to concentrations of 500 µ*M*.

3. Sensitivity: According to R. Tsien, FlAsH labeling is at least one magnitude less sensitive than GFP (*1,8*). FlAsH might not the best method to label weakly expressing or unstable proteins. It is a good idea to validate expression levels by an independent method (e.g., Western blot or immunofluorescence using a protein-specific antibody).
4. Specificity: Background is a frequent issue with FlAsH labeling. The need for background reduction was stressed before. In addition, the choice of cell type/line needs to be considered. It has been frequently reported that some cell lines (e.g., 293T and CHO) show relatively high residual background, which might interfere with the detection of weakly expressing proteins. It is therefore recommended to test expression and labeling in multiple cell lines and to select the best suited cell line.
5. Reducing environment: Staining with FlAsH requires a reducing environment (*28*). It is therefore not recommended to use FlAsH for labeling of extracellular proteins (or extracellular domains of proteins) or intracellular proteins that reside or translocate into the lumen of the ER, Golgi, and vesicular compartments.
6. Selection of labeling reagent: For imaging applications it is in general preferable to use FlAsH as labeling reagent. One reason is that the earlier-mentioned potential for phenotypic changes induced by ReAsH under certain conditions, the other reason is the substantially higher photostability of FlAsH as compared to ReAsH.
7. The use of genetically encoded tags always includes the possibility of interference of the tag with the function and localization of the tagged protein. This is rarely reported, but we have occasionally encountered this problem, especially while working with proteins that are part of larger assemblies (e.g., FP-tagged tubulin). Although the TC tag provides a unique size-advantage over every other live-cell labeling technology, commercially available or described in literature, there is still the possibility of interference, especially if used as integrated tag or in combination with linkers. A recent report by L. Rudner et al. shows that HIV-1 Gag would localize differently depending on the use of either TC alone or a TC-linker combination (*18*). It is therefore extremely important to validate proper function and localization of the tagged-protein against wild-type protein (e.g., by immunohistochemistry/fluorescence).

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