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# INTRODUCING TETRACYS MOTIFS AT TWO DIFFERENT SITES RESULTS IN A FUNCTIONAL DOPAMINE TRANSPORTER

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We have introduced tetracysteine motifs into different positions of the dopamine transporter (DAT) for specific FlAsH labeling. Two of the constructs expressed at the cell surface and were functional as determined by [$^3$H] dopamine uptake experiments. The N-terminally modified transporter showed uptake levels comparable to the wild-type DAT, while the construct with tetracysteine motif at position 511 displayed an uptake level about 1/3 of its wild-type counterpart. In addition, these two transporter constructs were visualized on the cell surface following labeling with a fluorescent cocaine analog. YFP introduced into the same N-terminal position was also shown to have surface staining in agreement with activity tests. We propose that these two sites are suitable targets for tetracysteine labeling to be used in FlAsH staining studies, while p134, p342, p427, p433 and p517 sites are not.

*Keywords:* Dopamine transporter – FlAsH – tetracysteine – fluorescent labeling – confocal

## INTRODUCTION

The monoamine transporters are transmembrane proteins that are responsible for removing the monoamines dopamine, norepinephrine and serotonin from the synaptic cleft. All three transporters, the dopamine transporter (DAT), the serotonin (SERT) and the norepinephrine transporter (NET) belong to the family of Neurotransmitter: Sodium Symporters (NSS), also referred to as the $Na^+/Cl^-$-dependent transporters, that include the transporters for other neurotransmitters such as γ-amino butyric acid (GABA) and glycine [6, 17, 20].

The DAT is a presynaptic membrane protein in dopaminergic neurons that tightly regulates dopamine neurotransmission by the rapid uptake of released dopamine

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back into the presynaptic nerve terminal and thereby plays a key role in the regulation of dopaminergic neurotransmission. DAT is also a target for commonly abused psychostimulants, such as cocaine and amphetamine, which makes it focus of interest in the field of neuropharmacology. The dopamine uptake mechanism is believed to involve a series of conformational changes that gate the substrate's passage through the transporter. Similar to GPCRs, the twelve transmembrane domain of transporters hampers high-resolution structural studies and studies that aim to elucidate activation mechanisms mostly depend on indirect biochemical and biophysical methods such as mutagenesis or spectral analyses. By fluorescently labeling the transporter, we aim to clarify intramolecular conformational movements, interactions among the different parts of the protein, ligand induced changes and changes through the translocation process.

Fluorescence resonance energy transfer (FRET) is a powerful tool to study conformational changes in proteins such as G-protein coupled receptors [8, 15] and neurotransmitter transporters [10]. The cell-based fluorescent probes in current use are mostly derivatives of the green fluorescent protein (GFP) from *Aequorea Victoria*. A commonly used pair suitable for this purpose is the cyan/yellow pair (CFP/YFP). However, due to their large sizes (~30 kDa) these fluorescent proteins are generally not favorable as reporters of functional conformational changes within proteins [18]. Recently two short (6–8 residue) organic probes have been introduced as an alternative labeling system in intact cells, the green fluorescent probe, FlAsH, and the red fluorescent probe, ReAsH. These probes have a very high affinity for four appropriately placed cysteines in the CCXXCC motif, where X is any amino acid other than cysteine. A proline and glycine in these positions have been shown to result in the highest binding affinity to date [1, 14].

Several studies in the monoamine transporters have indicated that the first and third intracellular loops are conformationally active during substrate or inhibitor binding for the members of the monoamine transporter family [2, 5, 16].

In this study, we have aimed to create FlAsH labeling sites on the intracellular face of the DAT at locations presumed to be both accessible for labeling and conformationally active. Importantly, the constructs were generated before the LeuT structure was available [21]. Modeling the DAT on the LeuT structure resulted in a shorter fourth intracellular loop than we previously predicted, thus moving two of our FlAsH labeling sites into transmembrane domain 8 (TM8).

We report here the functional expression of two tetraCys tagged DAT constructs (N-terminus position 1 and position 511). Moreover, we show that YFP introduced into the N-terminal position also yields a functional protein. The ability to insert a foreign six residue peptide sequence in the N-terminus and fifth intracellular loop without losing surface expression and basic functionality should serve as a basis for future modifications of the tetraCys sequence and provide a useful tool for FRET studies.

# METHODS

## *Site-directed mutagenesis*

A synthetic hDAT gene with artificially introduced restriction enzyme sites (synDAT) was used as a template in site-directed mutagenesis studies [13]. This gene was inserted into the bicistronic mammalian expression vector pCIHygro [13]. The constructs containing tetraCys motif were synthesized by two-step PCR using Phusion polymerase (Finnzymes, Espoo, Finland) and SynDAT as a template. All constructs were confirmed by restriction enzyme digestion and by automated DNA sequence analysis (MWG Biotech, Ebersberg, Germany). All restriction enzymes were from New England Biolabs (Beverly, USA).

## *Cell culture and transfection*

Human embryonic kidney (HEK) 293 cells were grown in DMEM medium with 10% fetal calf serum and 0.01 mg/ml gentamicin (Invitrogen) at 37 °C in a humidified 5% $CO_2$ atmosphere. The cells were seeded at a density of $1–1.5 \times 10^6$ for stable, $3–3.5 \times 10^6$ for transient cultures per 75 $cm^2$ flask. Transfection was performed using either Lipofectamine or Lipofectamine 2000 reagent (Invitrogen). Briefly, 2 μg of DNA was combined with 8 μl of lipofectamine for 30 min at room temperature and then this mixture was added to the previously seeded cells. Transfection was allowed to occur during a 5 hour incubation at 37 °C in a $CO_2$ incubator. For stable cell lines, the transfected pool was selected using 350 μg/ml hygromycine (Invitrogen).

## *Uptake experiments*

Uptake assays were performed using 2,5,6-[$^3$H]-dopamine (7–21 Ci/mmol) (Amersham Biosciences) [21]. Cells were seeded at a density of $1 \times 10^5$ cells/well in 24 well plates coated with 20 mg/ml poly-D-lysine (Sigma) in PBS buffer two days before the assay. The uptake buffer contained 10 μM catechol-O-methyltransferase inhibitor Ro 41-0960 (COMT) to prevent dopamine degradation. Nonlabeled dopamine ligands (Research Biochemicals) were added in concentration 10 nM to 1 mM. After addition of radioactive dopamine (90 nM [$^3$H] dopamine), the uptake reaction was performed in 5 min at 37 °C. All samples were analyzed in triplicate. Statistically analyzed assays are the result of at least three independent experiments, since this is found to be sufficient for ligand binding and activity tests [13]. The samples were counted in a Wallac Tri-Lux β-scintillation counter (NEN). Data was analyzed by nonlinear regression analysis using Prism 3.02 fitting and plotting Software (GraphPad San Diego, CA).

## *Fluorescent labeling*

FlAsH/Lumio Green or FlAsh/Lumio Red labeling was performed according to the instructions of the manufacturer (Lumio In-Cell Labeling Kits, Cat. No: 12589-040, Invitrogen). 1,2-Ethandithiol (EDT) was from Sigma (W 348406). Rhodamine labeled cocaine analogue JHC 1-64 was provided by Amy H. Newman (NIDA-IRP, Baltimore, MD). JHC 1-64 was prepared as a 10 mM stock in DMSO. Staining was performed using 5 nM JHC 1-64 for 20 minutes at room temperature.

## *Confocal imaging*

HEK-293 cells stably expressing tetraCys mutants were grown in phenol red-free medium for 48 h in 8-well Lab-Tek II glass chamber slides (Nalge) before analysis. Stained cells were visualized using a Zeiss (Oberkochen, Germany) LSM 510 confocal laser scanning microscope with an oil immersion 63× water objective. The AlexaFluor 568 was excited at 543 nm with a HeNe laser and the emitted light was detected using a 585 nm long-pass filter [3]. A Zeiss LSM 510 confocal laser scanning microscope equipped with a 150 mW Ar-Kr laser was used for excitation of Lumio at 488 nm, and the emitted light passed through a 505-nm long-pass filter. YFP labeled constructs were also observed under similar conditions. To visualize JHC 1-64 labeled cells, excitation wavelength was adjusted to 543 nm and an LP 585 filter was used in detection.

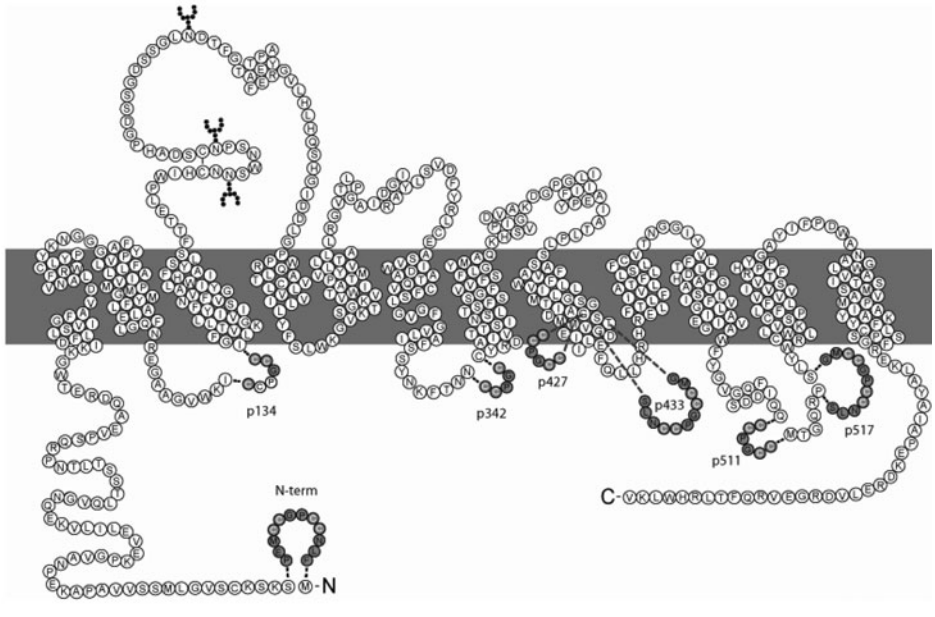



*Fig. 1.* Schematic representation of different tetraCys tag positions inserted into SynDAT

# RESULTS

## *Determining functionality of tetraCys mutants*

A tetraCys motif was introduced into different positions of the human DAT (Fig. 1) as described in Methods. The N-terminal tetraCys construct has FLNCCPGCCMEP motif in the first position. In addition, we introduced CCPGCC motif at p134 (IL1), p342 (IL3), p427 and p433 (TM8), p511 and p517 (IL5). To investigate functionality of those constructs we have performed uptake assays in either stable (N-terminal,

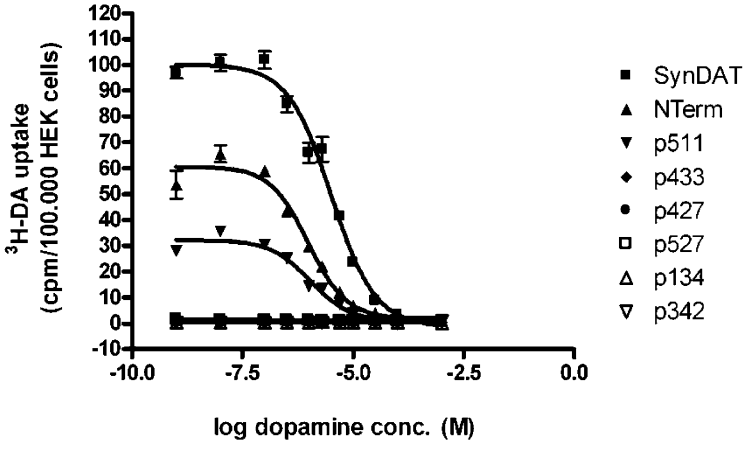



*Fig. 2.* [$^3$H]dopamine uptake of tetraCys labeled constructs. Data are means of triplicate series for each concentration and values are percent of control [$^3$H]dopamine uptake for wild type DAT (SynDAT)

*Table 1*
Kinetic constants for dopamine uptake in HEK 293 cells expressing dopamine transporter and tetraCys labeled constructs

| | $EC_{50}$ (S.E. interval) μM | $K_m$ (DA) (S.E. interval) μM | $V_{max}$ (means ± S.E. interval) fmol/min/$10^5$ cells |
|---|---|---|---|
| Control (SynDAT) | 3.2 (2.4–4.1) | 1.63 [1.35–1.96] | 21237 ± 2064 |
| N-term TC | 0.95 (0.69–1.3) | 0.8 [0.72–0.89] | 4302 ± 1333 |
| p511 | 1 (0.7–1.4) | 1.97 [1.07–3.65] | 2130 ± 1579 |
| p433 | 5.2 (n. d.)* | 5.13 (n. d.) | 2371 (n. d.) |
| p427 | 1.16 (n. d.) | 11.5 (n. d.) | 4339 (n. d.) |
| p517 | 2.1 (n. d) | 5.3 (n. d) | 3200 (n. d.) |
| p134 | 42.11 (0.56–316) | 42.0 (n. d) | 1232 (n. d.) |
| p342 | 996.8 (n. d.) | 124 [15.4–996] | – |

$V_{max}$ values are means of five independent experiments ($n = 5$).
* n. d.: not determined.

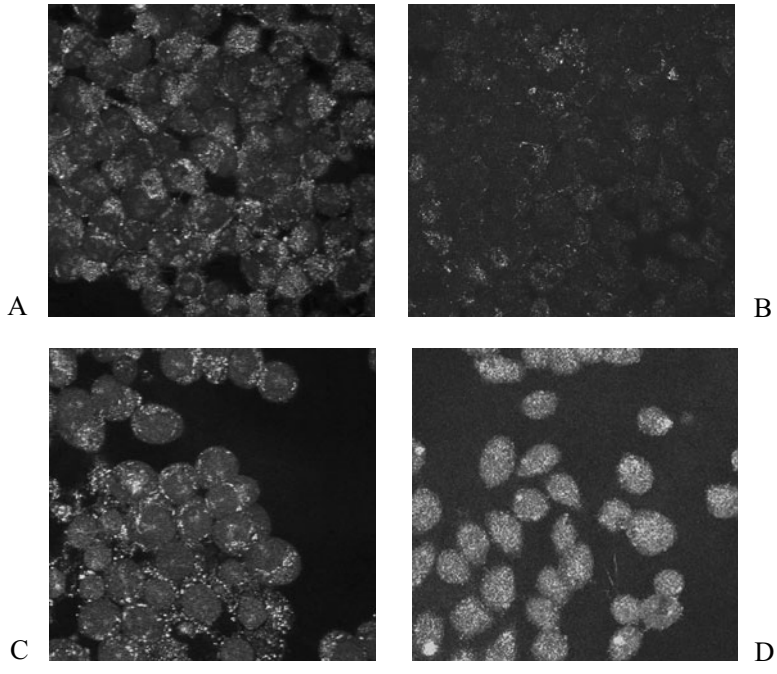



*Fig. 3.* Effect of different EDT concentrations on FlAsH labeling. Cells were incubated for 30 min in staining buffer. A – WT SynDAT, 0.5 μM FlAsH/5 μM EDT labeling, wash with 500 μM EDT; B – N-term tetraCys, 0.5 μM FlAsH/5 μM EDT labeling, wash with 500 μM EDT; C – N-term tetraCys, 2.5 μM FlAsH/25 μM EDT labeling, wash with 250 μM EDT; D – N-term tetraCys, 2.5 μM FlAsH/25 μM EDT labeling, wash with 250 μM EDT

p427, p433, p511 and p517) or transient (p134, p342) cell lines (Fig. 2). Only the N-terminal and p511 tetraCys constructs were func tional. The N-terminal tetraCys and p511 tetraCys constructs transported dopamine with a $K_m$ that was comparable to the wild type DAT, although $V_{max}$ values were reduced to approximately 20% and 10% of wild type, respectively (Table 1). We were not able to determine affinities for the rest of the constructs.

## *No specific FlAsH labeling was detected*

A wide range of conditions were tested with different concentrations of EDT, different incubation times, temperatures and washing conditions. Application of the FlAsH protocol without EDT as suggested by manufacturer resulted in green staining homogeneously distributed over the cell. To exclude the possibility that this pattern is a result of high background or low expression, we repeated experiments adding various concentrations of EDT (Fig. 3). The EDT/FlAsH ratio was kept at 10 or 20 with an approximately 15–20 minute preincubation to ensure complete binding of EDT.

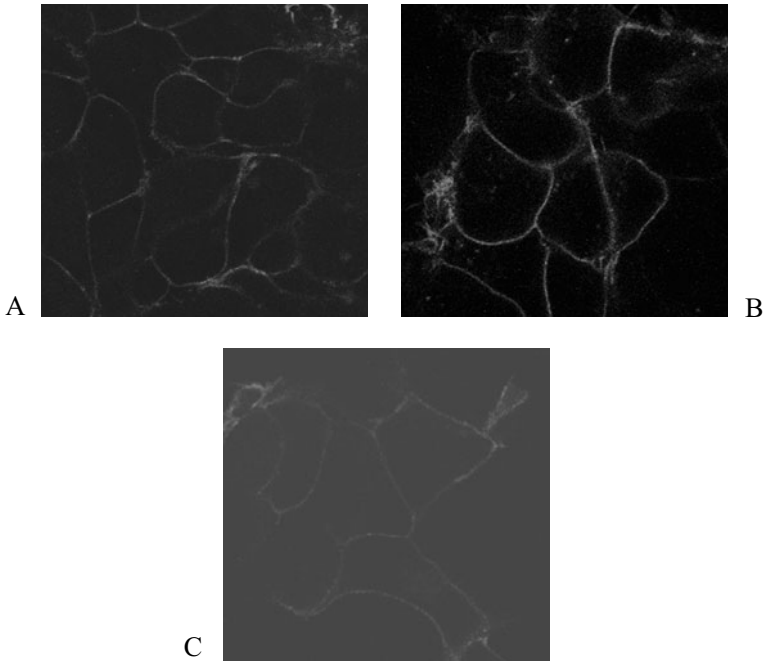



*Fig. 4.* JHC 1-64 labeling of the same constructs. Rhodamine labeled cocaine analogue JHC 1-64 is used to show surface expression in the wild type (A) and functional tetraCys labeled N-term (B) and p511 (C) constructs

After staining, 100/200-fold EDT was used in washes (250/500 mM). We also analyzed cells after washing with lower concentrations of EDT or omitting the wash step. All visualizations were done under Disperse Blue to prevent nonspecific hydrophobic interactions [7]. No differences in staining were observed between wild-type and the mutants. Bright spots that have been observed inside the cell were also observed in the stable SynDAT control cell lines, excluding the possibility of a perturbation in transporter trafficking due to the toxic effects of the system. This has been verified by surface staining studies using JHC 1-64 as described below.

## *Expression of tetraCys labeled constructs*

Surface expression of SynDAT, N-terminal and p511 tetraCys constructs was determined using the 5 nM rhodamine labeled fluorescent cocaine analog JHC 1-64 [4]. In both mutant constructs we observed surface staining as expected for the wild type (Fig. 4). Specificity of surface staining was confirmed with 10 μM nonfluorescent cocaine analogue RTI-55 (data not shown) as previously reported [4].

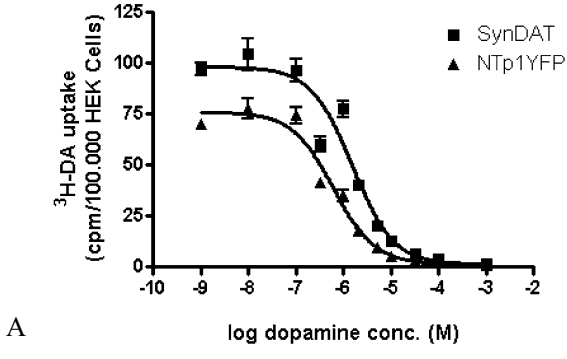
To further verify expression of the transporter, we established another construct that contained YFP at the same location where the N-terminal tetraCys motif is

inserted. This YFP construct was functional (Table 2, Fig. 5A) and showed clear surface expression under examination with confocal microscopy (Fig. 5B).

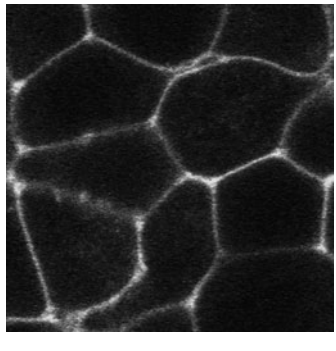
*Table 2*
Kinetic constants for dopamine uptake in HEK 293 cells expressing N-terminally YFP labeled dopamine transporter

| | $EC_{50}$ (S.E. interval) μM | $K_m$ (DA) (S.E. interval) μM | $V_{max}$ (means ± S.E. interval) fmol/min/$10^5$ cells |
|---|---|---|---|
| Control (SynDAT) | 1.5 (n. d.)* | 1.47 (n. d.) | 12388 (n. d.) |
| N-term YFP | 0.61 (n. d.) | 0.56 (n. d.) | 3842 (n. d.) |

* n. d.: not determined.




A



B

*Fig. 5.* YFP positioned at the N-terminal of transporter have been analyzed for uptake (A) and visualized using confocal as described in Methods (B)

## DISCUSSION

It has been shown that phosphorylation of certain serine residues at N-terminal has an important effect on amphetamine induced efflux of DAT [9], however the previously suggested role of N-terminal phosphorylation on internalization has been ruled out by recent studies [3]. To further investigate this role and ligand induced changes *in vitro,* our study has aimed to detect conformational changes using fluorescent labeling probes which were inserted into different locations of DAT. We introduced tetraCys motifs in the N-terminal and IL1, IL3, IL4 and IL5 regions of DAT. Interaction of the N-terminal tag with probes located in other positions of the protein (e.g. IL4 or IL5) can impose important constraints on the structure and the effect of ligand binding can be clarified using FRET analysis.

A recent study has shown that selected tetraCys sequences FLNCCPGCCMEP and HRWCCPGCCKTF have higher affinities for biarsenical dyes, giving higher fluorescence quantum yields and higher resistance to dithiols, thereby increasing signal-to-noise ratio [14]. Based on these observations, we introduced an FLNCCPGCCMEP motif as a fusion to the N-terminal. We also tested a corresponding construct where YFP was fused to the N-terminus for functionality and surface expression. The N-terminal-tetraCys construct had an uptake activity ~60% of the wild type protein. A minimized tetraCys motif (CCPGCC) inserted into intracellular loop 5 (p511) was also functional, though at a lower level (~30%) in comparison with the wild type DAT. Apparent dopamine affinities were similar for both constructs. YFP inserted into p511 position, however, yielded a nonfunctional protein. Among the seven constructs tested, only insertions of the tetraCys motif at these two positions were tolerated without compromising surface expression.

The fluorescent cocaine analogue JHC 1-64 was used to determine surface expression of the N-terminal-tetraCys and p511 constructs. We found that both constructs have the surface expression similar to that of the wild type. The specificity of the signal was confirmed using nonfluorescent cocaine analog RTI-55.

Since DAT binds one cocaine molecule per transporter, there should be one JHC 1-64 bound per DAT. Likewise, the tetraCys constructs should only be labeled with one FlAsH molecule per transporter. The FlAsH molecule consists of a fluorescein fluorophore to which two arsenic ions are conjugated. The fluorescein molecule itself is a very bright fluorophore with a quantum yield of 0.95 [11]. The fluorescein in the FlAsH molecule is likewise very bright when bound to the tetraCys motif and reaches quantum yield values between 0.5 and 0.7 [1]. The Rhodamine Red fluorophore of JHC 1-64 is also very bright and has a quantum yield comparable to that of fluorescein. Thus, one would expect that the tetraCys constructs that are clearly visible on the cell surface following fluorescent JHC 1-64 binding would also be clearly visible upon labeling with FlAsH. In addition, expression of a DAT construct with YFP tagged in the position of the N-term tetraCys is also clearly visible on the cell surface. However, following rigorous experimentation, we were not able to obtain specific FlAsH labeling of the tetraCys containing DAT constructs on the cell surface.

The FlAsH (Lumio Green) compound was supplied as precomplexed to the arsenoxide antidote 1,2-ethandithiol $(EDT)_2$ which has been shown to stabilize the biarsenic reagents and used to minimize nonspecific labeling and toxicity. The FlAsH molecule is membrane permeable and becomes highly fluorescent on binding the CCXXCC motif. FlAsH was indicated to bind specifically to this sequence which is very rare in cell proteins [1]. In spite of this, there are some recent reports indicating to the nonspecific labeling of endogenous proteins, especially in certain cell types including HEK293 that result in lower staining efficiencies [19]. Langhorst et al. showed that FlAsH accumulates in active mitochondria as an indication of toxic side effects of arsenic [12]. EDT is also very toxic, thereby limitations on incubation times and concentrations should be well considered. In agreement with these observations, we have detected homogeneous and bright green staining all over the cells with local brighter spots, mostly in the cytoplasm, both in the wild type and in tetraCys fusions. Prolonged incubation times resulted in rounded cell morphology, an indication of toxic effects.

The FlAsH labeling procedure was applied in both stable and transient cell lines according to the guidelines of the manufacturer. In addition, we modified the labeling conditions to include longer incubation times, different concentrations and ratios of the reagents FlAsH and EDT from high nM to low mM range, and screened the labeling conditions in different media and various buffers containing glucose as energy source. Further optimization is obviously necessary to observe specific FlAsH signal for transporters. The use of monothiol β-mercaptoethanol (2-ME) or BAL instead of EDT to increase efficiency and reduce toxicity of the process or to introduce a second tetracysteine tag sequence in tandem can be an alternative approach to optimize the procedure for use in live cell imaging [12].

In conclusion, we successfully introduced tetraCys tag into seven different positions of DAT and determined two acceptable positions, N-term and p511 which preserve activity of the transporter. The activities of these constructs were verified by uptake assays and surface staining. These two positions were also tested with YFP tag. N-term YFP DAT was found to have biochemical characteristics similar to wild type DAT, whereas p511 was found to be nonfunctional. As a result, we propose that these two sites (N-term and p511) are suitable targets for tetraCys labeling to be used in FlAsH staining studies, while p134, p342, p427, p433 and p517 are not.

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