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# A Genetically Encoded Fluorescent Probe in Mammalian Cells

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Supporting Information

**ABSTRACT:** Fluorescent reporters are useful in vitro and in vivo probes of protein structure, function, and localization. Here we report that the fluorescent amino acid, 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap), can be site-specifically incorporated into proteins in mammalian cells in response to the TAG codon with high efficiency using an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase (aaRS) pair. We further demonstrate that Anap can be used to image the subcellular localization of proteins in live mammalian cells. The small size of Anap, its environment-sensitive fluorescence, and the ability to introduce Anap at specific sites in the proteome by simple mutagenesis make it a unique and valuable tool in eukaryotic cell biology.

The ability to selectively label proteins with fluorescent probes has greatly facilitated the study of protein structure and function both *in vitro* and *in vivo*. One widely used method involves the fusion of a fluorescent protein to the target protein;[1−3] however, this strategy is generally limited to C- or N-terminal fusions. Furthermore, the large size of the fluorescent protein tag (>20 kDa) can significantly perturb the structure or activity of the target protein.[2,4] Other strategies, employing the attachment of a protein tag to the target, which can be subsequently labeled with fluorescent probes (e.g., SNAP-tag[5,6] and Halo-tag[7]), also suffer from similar limitations. Various chemical and enzymatic strategies have also been developed to selectively label short peptide tags or uniquely reactive unnatural amino acid (UAA) residues in the target protein with small fluorescent probes. Examples include the use of cell-permeable bis-arsenical dyes that bind a genetically encoded tetracysteine motif in the target protein;[1,8] bio-orthogonal conjugation to uniquely reactive non-natural side chains;[9−11] enzymatic labeling of a target peptide tag with fluorescent probes (e.g., biotin ligase,[12] sortase,[13] formylglycine-generating enzyme,[14] phosphopantetheinyl transferase,[15] lipoic acid ligase,[16] etc.). These methods, though very useful, can be limited by labeling selectivity and efficiency, compatibility with living cells, and a limited number of compatible labeling sites within a folded protein.

An alternative approach involves the co-translational incorporation of a small UAA with a fluorescent side chain directly into the target protein in a site-specific manner. To this end, we have developed orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pairs that incorporate amino acids containing 5-hydroxycoumarin and dansyl side chains in response to the amber nonsense codon in *Escherichia coli* and *Saccharomyces cerevisiae*, respectively.[17,18] Recently, a new fluorescent amino acid, 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (Anap), was genetically encoded in *S. cerevisiae*.[19] Anap is an amino acid derivative of 6-propionyl-2-(*N*,*N*-dimethyl)-aminonaphthalene (prodan), an environmentally sensitive fluorophore that is widely used in biochemistry and cell biology.[20,21] The absorption and emission maxima for Anap in water are 360 and 490 nm, respectively; the extinction coefficient is 17 500 $cm^{-1}$ $M^{-1}$, and the quantum yield is 0.48 (determined in EtOH; excitation 360 nm). Like other prodan probes, the fluorescence of Anap undergoes a substantial shift in emission maximum and intensity with changes in solvent polarity, e.g., upon transitioning from water (490 nm) to EtOAc (420 nm, Figure 1). Here we report the development of

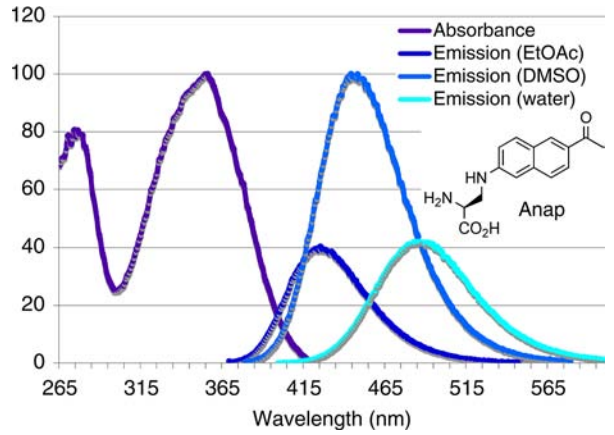


**Figure 1.** Structure and normalized absorption (purple) and fluorescence (dark blue, in EtOAc; light blue, in DMSO; cyan, in water) spectra of Anap.

an expression system for the Anap-specific tRNA/aaRS pair in mammalian cells, enabling its site-specific incorporation into proteins in these cells with excellent selectivity and efficiency by simple mutagenesis. Furthermore, we show that the subcellular localization of proteins in mammalian cells can be determined using site-specifically incorporated Anap as a fluorescent reporter.

An Anap-specific $tRNA_{CUA}^{EcLeu}$/aaRS (AnapRS) pair was evolved from *E. coli* leucyl-tRNA/LeuRS using a series of positive and negative selections in *S. cerevisiae*.[19] Since *S. cerevisiae* and mammalian cells have similar tRNA identity

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elements,[22] we anticipated that the *E. coli*-derived $tRNA_{CUA}^{EcLeu}$/AnapRS pair would also be orthogonal and functional in mammalian cells. To test this hypothesis, we constructed the suppression plasmid pAnap (Figure 2A),

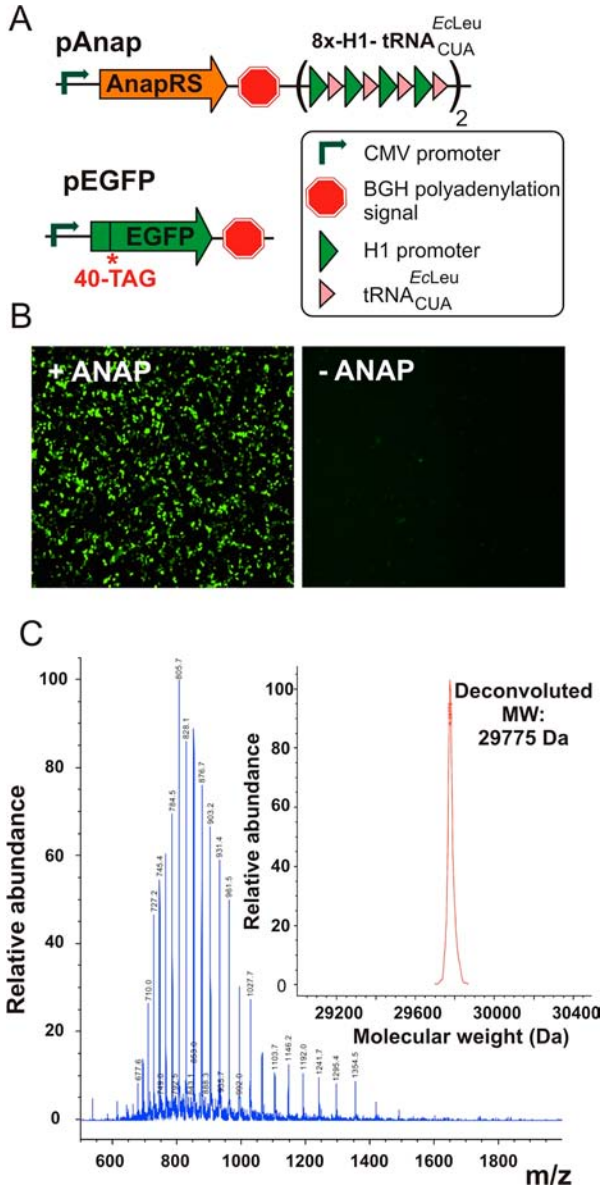


**Figure 2.** Evaluation of the activity of Anap-specific $tRNA_{CUA}^{EcLeu}$/AnapRS in mammalian cells. (A) Construction of pAnap and pEGFP plamids. Identities of different genetic elements used in these constructs are shown. (B) Expression of EGFP-40-Anap in HEK293 cells in the presence (left) and absence (right) of 0.5 mM Anap, achieved by co-transfection of pAnap and pEGFP, analyzed by fluorescence microscopy. (B) ESI-MS analysis of purified EGFP-40-Anap mutant. Expected molecular weight, 29 773 Da; observed, 29 775 Da.

encoding expression cassettes for the $tRNA_{CUA}^{EcLeu}$/AnapRS pair. The transcription of mammalian tRNAs by RNA polymerase III depends on two conserved intragenic transcriptional control elements known as the A and B box sequences.[23] The human H1 promoter, which has been previously shown to allow expression of tRNAs that lack such transcriptional elements in mammalian cells,[24] was used to the express $tRNA_{CUA}^{EcLeu}$. In addition, a poly(A) tail was added to the 3′ terminus of this tRNA to enhance its transcription and maturation in mammalian cells. In order to further increase the expression level of the tRNA, a gene cluster containing eight tandem repeats of the H1 promoter $tRNA_{CUA}^{EcLeu}$ gene was used. Finally, the Anap-specific aminoacyl-tRNA synthetase (AnapRS) was expressed from a non-regulated CMV promoter.

To test the amber (TAG) suppression efficiency of this new pair in mammalian cells, the reporter plasmid pEGFP (Figure 2A) was used, which encodes enhanced green fluorescent protein (EGFP) with a TAG mutation at a permissive site (Tyr40TAG) and a C-terminal hexa-histidine tag. When pAnap was transiently co-transfected into Human Embryonic Kidney 293 (HEK293) or Chinese Hamster Ovary (CHO) cells along with pEGFP, efficient EGFP expression was observed by fluorescence microscopy only in the presence of 0.5 mM Anap, demonstrating that the $tRNA_{CUA}^{EcLeu}$/AnapRS pair is functional in mammalian cells (Figure 2B). The lack of EGFP expression in the absence of Anap also confirms that this pair is not cross-reactive to endogenous synthetases. The mutant EGFP was isolated using Ni-NTA affinity chromatography with an isolated yield of ~1.2 mg/L (or per $1.2 \times 10^9$ adhesive CHO cells); for comparison, the yield of wild-type (wt) EGFP in a similar experiment was 6 mg/L. ESI-MS analysis of the purified mutant EGFP protein revealed a molecular weight consistent with the incorporation Anap (Figure 2C). These observations demonstrate that Anap can be selectively incorporated into proteins in mammalian cells in response to the amber nonsense codon (TAG).

Next, we explored whether this genetically encoded Anap could be used as a fluorescent reporter to probe the localization of a protein in mammalian cells. We first used Histone H3 as a target, since it reliably localizes in the nucleus, a phenotype that can be easily verified using fluorescence microscopy. A reporter plasmid was constructed in which an amber mutant of H3 (Thr59TAG) that introduces Anap in a flexible, solvent-exposed loop was expressed from a CMV promoter. A C-terminal mCherry tag was also included to provide a control signal for comparison. Only when the in-frame TAG codon is suppressed by the Anap-specific $tRNA_{CUA}^{EcLeu}$/AnapRS pair will the full-length protein be expressed with both Anap and mCherry reporters. Sixteen hours after this reporter plasmid was co-transfected into CHO cells with pAnap, the cells were washed with PBS, incubated in fresh medium for 2 h, and analyzed by fluorescence microscopy using both mCherry (540 nm excitation; 580−610 nm emission) and Anap (360 nm excitation; 470−510 nm emission). Anap fluorescence was localized in the nucleus and overlapped well with the mCherry signal, demonstrating the utility of Anap as a probe for protein localization (Supplementary Figure S1, Figure 3). Expression of the $tRNA_{CUA}^{EcLeu}$/AnapRS alone in the presence of Anap (and the absence of the amber mutant) was associated with minimal background fluorescence that did not interfere with the detection (Supplementary Figure S1).

To test whether expression of $tRNA_{CUA}^{EcLeu}$/AnapRS has an effect on cell viability, CHO cells were grown under a variety of conditions (Supplementary Table 1): (1) CHO cells were grown in the presence or absence of 0.5 mM Anap; (2) CHO cells were transfected with $tRNA_{CUA}^{EcLeu}$/AnapRS and pHistone and grown in the presence or absence of 0.5 mM Anap; (3) CHO cells were transfected with wt mCherry and grown in the absence of Anap; and (4) CHO cells were incubated with transfection reagent (Fugene HD) and grown in the absence of


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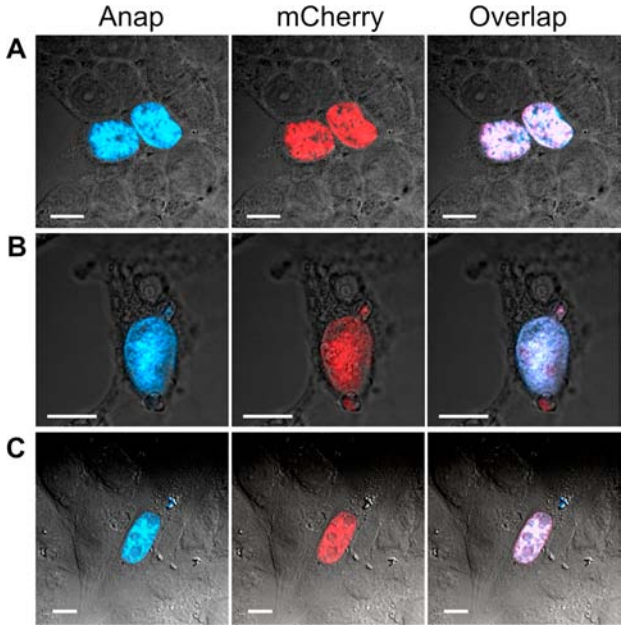


**Figure 3.** Nuclear localization of Histone-H3 (Thr59-Anap)-mCherry (C-terminal) fusion protein in HEK293 (panel A), HeLa (panel B) and CHO (panel C) cells, visualized using Anap (first column, pseudo-colored in blue, superimposed with the bright-field image) or mCherry (second column, pseudo-colored in red, superimposed with the bright-field image) as fluorescent reporters by laser scanning confocal microscopy. A composite image of these is shown in the third column. Scale bars, 10 $\mu$m.

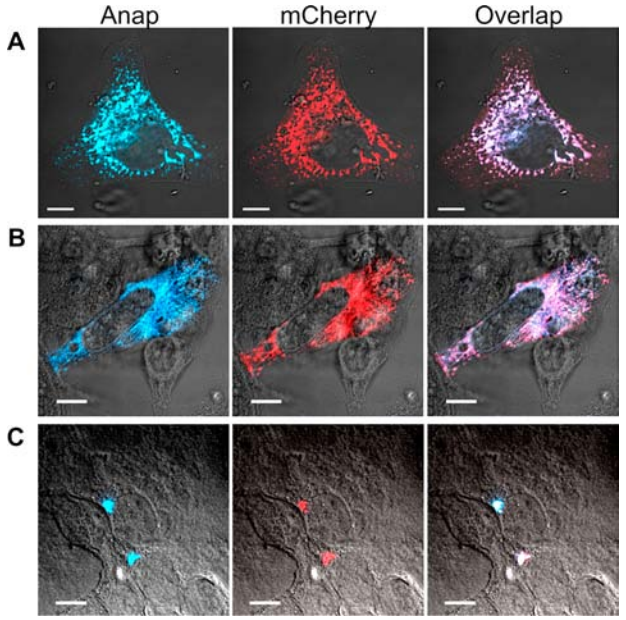


**Figure 4.** Subcellular localization of endoplasmic reticulum (ER) and Golgi-resident proteins in human cell lines visualized using Anap (first column, pseudo-colored in blue, superimposed with the bright-field image) or mCherry (second column, pseudo-colored in red, superimposed with the bright-field image) as fluorescent reporters by confocal microscopy: (A) ER-resident Grp94 (Ser127-Anap)-mCherry (C-terminal) in HeLa cells. (B) ER-resident Grp94 (Leu7-Anap)-mCherry (C-terminal) in HeLa cells. (C) Golgi resident GalT1 (Ser3-Anap)-mCherry (C-terminal) in HEK293 cells. Scale bars, 10 $\mu$m.

Anap. Cells grown under these different conditions all showed similar growth rates. Based on Trypan assay, the numbers of viable cells grown under different conditions were similar and all higher than 90% of the population after 24 h of cultivation (Supplementary Table 1).

To minimize the background from free Anap, we explored the possibility of using it at a lower concentration that does not compromise the fidelity and efficiency of its incorporation into target proteins. A 10 $\mu$M Anap concentration in the medium was found to be sufficient to sustain robust expression of EGFP (Tyr40TAG), and the protein expressed under these conditions was homogeneous and exhibited the correct mass (Supplementary Figure S2). Using this optimized condition, we repeated the experiment with different mammalian cells (HEK293, HeLa, and CHO cells) and visualized H3 localization in the nucleus using laser scanning confocal microscopy. In each case, Anap signal correlated well with the mCherry signal and localized in the nucleus (Figure 3). To further demonstrate the utility of Anap as a fluorescence probe of cellular localization, other reporter plasmids were constructed, harboring amber mutants of proteins known to specifically localize in the endoplasmic reticulum (Grp94 for Ser127TAG and Leu7TAG) or Golgi (GalT1for Ser3TAG). These proteins were again expressed from a CMV promoter with a C-terminal mCherry tag. The reporter plasmids were co-transfected with pAnap in different mammalian cells (HEK293, CHO, HeLa) in the presence of 10 $\mu$M Anap, and fluorescence images were obtained using laser scanning confocal microscopy. In each case, appropriate localization of the protein was observed using Anap fluorescence, as confirmed by a good correlation with the control mCherry signal (Figure 4).

In addition to conventional fluorescence microscopy, Anap fluorescence can also be visualized by two-photon excitation (excitation, 730 nm, two-photon; emission, 420−500 nm, Supplementary Figure S3). Two-photon excitation of the Anap-mCherry double-labeled histone H3 revealed excellent overlap between Anap (excitation, 730 nm, two-photon; emission, 420−500 nm) and mCherry (excitation, 543 nm; emission, 600−700 nm) fluorescence in the nucleus (Supplementary Figure S3). The ability to probe Anap fluorescence using conventional fluorescence microscopy as well as using two-photon excitation, which provides deeper tissue penetration, efficient light detection, and reduced phototoxicity,[25] further enhances its utility as a fluorescent probe.

By analyzing the localization of Anap-labeled proteins in various mammalian cells, we demonstrate that the fluorescence of Anap is bright enough to be useful as a cellular probe. Background fluorescence was low and did not interfere with the analyses. The small size of Anap (MW = 273 Da) is expected to minimally perturb protein structure, dynamics, function, and localization. Anap can be incorporated into virtually any site in a protein, provided that it does not impair its folding or function. Finally, the intensity and emission maxima of Anap are sensitive to its environment, making it a useful probe of biomolecular interactions and conformational changes in proteins.[19] These properties make Anap a unique and useful fluorescent probe for investigating protein structure and function directly in living mammalian cells.


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## ■ ASSOCIATED CONTENT

### Supporting Information

Materials and methods; additional images from regular and two-photon fluorescence microscopy experiments; viability data for CHO cells grown under different conditions; expression analysis of GFP-40-Anap under various Anap concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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