The ability to visualize biomolecules within living specimen by engineered fluorescence tags has become a major tool in modern biotechnology and cell biology. Encoding fusion proteins with comparatively large fluorescent proteins (FPs) as originally developed by the Chalfie and Tsien groups is currently the most widely applied technique. As synthetic dyes typically offer better photophysical properties than FPs, alternative strategies have been developed based on genetically encoding unique tags such as Halo and SNAP tags, which offer high specificity but are still fairly large. Small tags like multi-histidine or multi-cysteine motifs may be used to recognize smaller fluorophores, but within the cellular environment they frequently suffer from poor specificity as their basic recognition element is built from native amino acid side chains. Such drawbacks may be overcome by utilizing bioorthogonal chemistry that relies on coupling exogenous moieties of non-biological origin under mild physiological conditions. A powerful chemistry that fulfills these requirements is the Huisgen type (3+2) cycloaddition between azides and alkynes (a form of click chemistry). By utilizing supplementation-based incorporation techniques and click reactions Beatty et al. coupled azide derivatized dyes to Escherichia coli expressing proteins bearing linear alkynes. However, this azide–alkyne cycloaddition required copper(I) as a catalyst (CuAAC), which strongly reduces biocompatibility (but see Ref. [7]). This limitation has been overcome by Bertozzi and co-workers, who showed that the “click” reaction readily proceeds when utilizing ring-strained alkynes. Since then this strain-promoted azide–alkyne cycloaddition (SPAAC) has found increasing applications in labeling, for example, carbohydrates, nucleotides, and lipids. Further expanding the potential of this approach, Ting and co-workers engineered a lipolic acid ligase which ligates a small genetically encoded recognition peptide to a cyclooctyne-containing substrate. In a second step the incorporated cyclooctyne moiety then functioned as a specific site for labeling in cells.

The direct genetic encoding of fluorescent unnatural amino acids (UAAs) has overcome many drawbacks of previous approaches by offering exquisite specificity, freedom of placement within the target protein, and minimal, if any, structural change. This approach was first achieved by Summerer et al. who evolved a leucyl tRNA synthetase (tRNA/RS) pair from E. coli to genetically encode the UAA dansylalanine into Saccharomyces cerevisiae. In response to the amber stop codon TAG, dansylalanine was readily incorporated into proteins by the host translational machinery. This approach has since been used to genetically encode several small dyes but owing to the need to evolve new tRNA/RS pairs and potential size limitations imposed by the translational machinery, larger dyes with enhanced photophysical properties have not yet been encoded.

Targeted incorporation of UAAs should in principle make it possible to genetically incorporate strained alkylene and azide functional groups. In fact, a variety of azides have been genetically encoded by the use of, for example, engineered tyrosyl Methanococcus jannaschii tRNA/RS, leucyl E. coli tRNA/RS, and pyrrolysine Methanosarcina bakeri/mazei tRNA/RS pairs (Tyr = tyrosine, Leu = leucyl, pyl = pyrrolyl). However, genetically encoding the functionality necessary for metal-free click ligations, that is, the strained alkylene, has not been achieved to date, owing to the large size of the cyclic side-chain moiety. Direct encoding of strained alkynes offers numerous advantages over encoding the azide functionality. Many commercial compounds, such as fluorescent dyes or fatty acids, are only available as the azide derivatives and thus not compatible with a copper-free click reaction with the available genetically encoded UAAs. Another major advantage of directly encoding strained alkynes is that they can react with fluorogenic azides, which are dyes whose fluorescence is dramatically quenched in the azide form and increases strongly after a successful click reaction (Scheme 1). This fluorogenic approach was applied, for example, to specifically label lipids within living mammalian cells with a coumarin azide derivative, which offered high contrast due to a lack of background fluorescence. The benefit of fluorogenic labels has also been highlighted in recent work of the Weisleder group who utilized the ability of tetrazines to quench fluorescence and to react with strained dienophiles in a Diels–Alder cycloaddition.

To overcome the current limitations of engineering copper-free click chemistry into living cells, we aimed to genetically encode strained alkynes into E. coli. We reasoned that the natural amber suppressor pyrrolysine tRNA/RS pair from M. mazei might be a suitable starting point because the amino acid substrate binding pocket is partially exposed.
offering space to harbor a larger side chain. Furthermore, the carbamate bond at the lysine side chain was recently shown to be an important discriminator for successful incorporation of UAAAs\cite{16,19,20}. Accordingly, we synthesized lysine derivative 1 in six steps, starting from a commercially available cycloheptene precursor as outlined in Scheme S1 of the Supporting Information. In the last step, the tert-butoxycarbonyl (Boc) protected 1 required particularly mild deprotection with 70\% formic acid, as other standard conditions resulted in cleavage of the side-chain cyclooctyne moiety. The average yield per step was above 80\% and allowed convenient synthesis of gram quantities. We then tested the amber suppression efficiency of the wildtype (WT) system tRNA\textsuperscript{pyl/pylRS}\textsuperscript{WT} by co-expressing a green fluorescent protein (GFP) reporter construct. This construct contains an amber TAG mutation at the amino acid position 39 (GFP TAG). Hence, full-length and fluorescent GFP\textsuperscript{TAG} was efficiently expressed only in the presence of either 1 or 2 for this mutant tRNA\textsuperscript{pyl/pylRS}\textsuperscript{WT} pair and mass spectrometry confirmed the site-specific incorporation of the UAA into the protein (Supporting Information, Table S1). As scaling up the synthesis of 1 was easier than of 2, we focused on compound 1 for further experiments and determined that typically more than 10 mg GFP\textsuperscript{TAG} were obtained from a 1 L culture in the presence of 1 mM 1, demonstrating high suppression efficiency and fidelity of our tRNA\textsuperscript{pyl/pylRS}\textsuperscript{WT} construct.

To investigate the utility of 1, we aimed to perform single-molecule (sm) studies of labeled proteins. In particular, single-molecule observation of fluorescence resonance energy transfer (FRET) is a widely applied tool to study protein structure and dynamics. smFRET typically requires covalent, high-yielding, site-specific labeling of proteins with photostable dyes because of the low concentrations used for single-molecule studies and the high demand for overcoming the fundamental noise limit. To this end, we purified GFP\textsuperscript{TAG}–1 and ligated the commercially available azide derivative of Atto647N under mild conditions. Owing to the spectral properties of this fluorescent species, the natural GFP chromophore served as a donor (D) while Atto647N served as the acceptor dye (A). We then observed freely diffusing GFP\textsuperscript{TAG}–1,Atto647N molecules were analyzed based on their labeling stoichiometry (S) and for the occurrence of energy transfer ($E_{\text{FRET}}$). As shown in Figure 2a, we clearly observed a species that contains both D and A molecules ($S_0=0.5$, $E_{\text{FRET}}=1$). This observed high FRET efficiency is in good agreement with the crystal structure of GFP,\cite{22} indicating that the dye attached at position 39 is within 30 Å of the GFP chromophore.

As mentioned above, the cyclooctyne moiety is ideally suited to achieve site-specific labeling within the living cell. To verify the feasibility of such experiments with our approach, we expressed the red fluorescent protein mCherry\textsuperscript{TAG} in E. coli in the presence of 1 mM 1 and tRNA\textsuperscript{pyl/pylRS}\textsuperscript{WT}. Cells were then incubated in a 50 µM solution of the fluorescent coumarin azide 3.\textsuperscript{20} Figure 2 shows that only cells expressing mCherry\textsuperscript{TAG}–1,3 exhibit a strong fluorescence when excited at $\lambda = 405$ nm. The fluorescent in vivo labeling was also confirmed by fluorescence spectrometry (Figure 2c) and by analyzing the fluorescence of cell lysate using SDS PAGE (see Supporting Information Figure S3–S5, also for additional imaging experiments with other proteins).

In summary, we have genetically encoded one of the most potent functional groups for in vivo chemistry into E. coli and...
demonstrated its basic utility for in vivo labeling as well as high-resolution single-molecule measurements. SPAAC chemistry is now available to site-specifically and non-invasively modify proteins in living cells. As the tRNA\(^{pyl}\)/pylRSAF showed no obvious dependence on linker length (1 versus 2), it is conceivable that slightly altered derivatives, such as dibenzocyclooctynes, \[24\] could be directly used in this system.

Keywords: amino acids · click chemistry · gene expression · proteins · strained molecules

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