Site-specific Labeling An Intracellular Protein with Unnatural Amino Acids by Genetic Code Expansion for Spectroscopy and Imaging

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Abstract

To study the intracellular function of a protein of interest (POI), the protein has to be labeled with a probe that can be spectroscopically distinguished from cellular backgrounds. Fluorescent labeling of POI is accomplished by organic dyes conjugated to antibodies specific to the protein or genetic incorporation of fluorescence protein fused at one the end of POI. However, both labeling methods results in expansion of the physical dimensions of the labeled structures due to the POI-fluorophore distance. Also, the fused fluorescent protein can perturb the endogenous structures due to the large size, the different charge or polarity states and the oligomeric nature. Here, we adapt genetic code expansion which can incorporate unnatural amino acids to the specific residue of a POI. Incorporated unnatural amino acids such as alkyne-derived amino acids enable us to probe a POI with the shortest possible linkage distance between the fluorophore and protein via click reaction between the clickable unnatural amino acid and fluorophore. We expect the incorporated unnatural amino acids to a specific protein in human cells can be utilized in site-specific imaging based on Raman, IR and fluorescence and in spectroscopic studies of proteins inside living human cells.

Backgrounds: Genetic Code Expansion

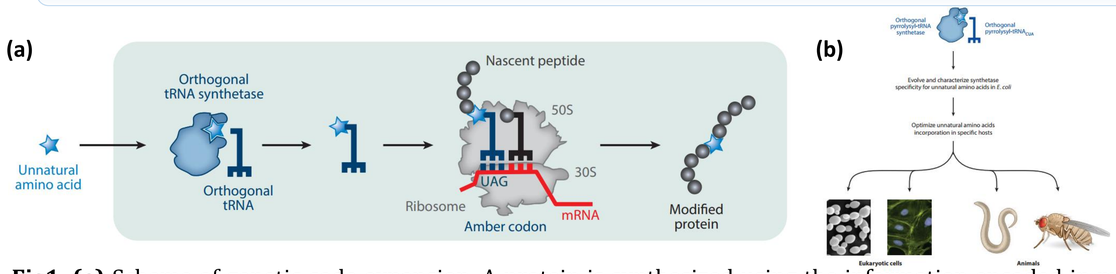


Fig1. (a) Scheme of genetic code expansion. A protein is synthesized using the information encoded in mRNA and by added amino acids transported by tRNA. When an amber stop codon is encountered during translation, orthogonal aminoacyl tRNA synthetase (PylRS) attaches unnatural amino acids to orthogonal tRNA (PylT) that brings unnatural amino acid to the peptide. Blue star: unnatural amino acid. Gray circles: natural amino acids. **(b)** Genetic code expansion can be used in various animals.

Annual Review of Biochemistry, 2014

Methods

Methanosarcina mazei pyrrolysyl-tRNA synthetase (PylRS) and tRNA$^{Pyl}_{CUA}$(PylT) from *Methanosarcina mazei* has successfully demonstrated super-resolution fluorescence imaging (Fig. 2) We transfected human cells were transfected with two DNA plasmids encoding (1) PylRS and PylT and (2) a protein of interest containing amber stop codon (TAG) using Super PiggyBac Transposase for stably expressing the genes. After transfection, unnatural amino acids were added in culture medium to be incorporated at the site of amber stop codon.

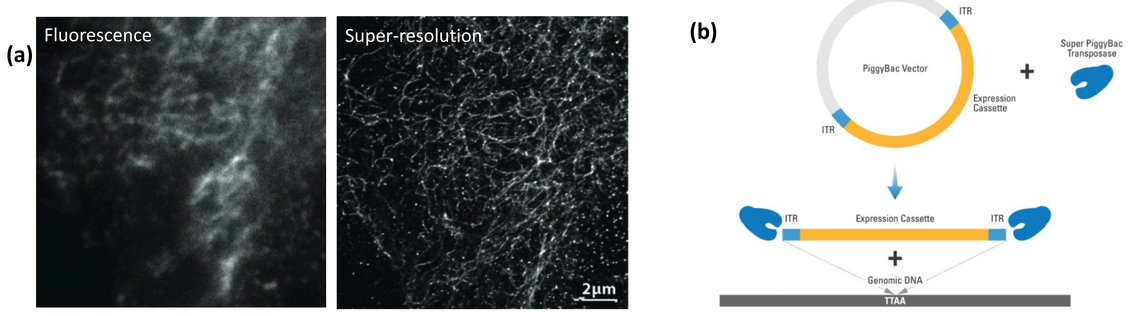
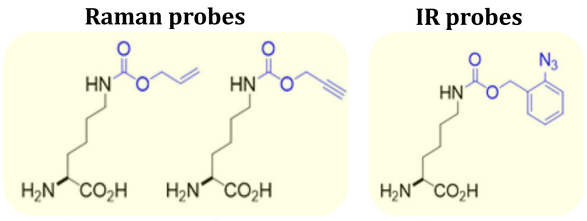


Fig2. (a) Conventional (left) and super-resolution (right) fluorescence images of vimentin^{N116}. **(b)** Scheme of stable gene incorporation using piggybac. Piggybac transposase uses cut-and-paste mechanism to transfer DNA into genome. Piggybac transposase recognizes ITR at both end of the expression cassette, cut the ITRs and then insert the resultant ITR-DNA-ITR into the genome at TTAA.

Angew.Chem.Int.Ed.2016 *Nature Methods*, 2016

Future Plan

Raman probes **IR probes**



Unnatural amino acids in **Fig 7** are all previously used for genetic code expansion with PylRS/PylT in mammalian cells. Synthesized protein which contains these amino acids can be detected using various spectroscopic and microscopic method (table1).

Fig7. Various modified unnatural amino acids modified from lysine containing Raman and IR-active chemical groups.

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Table1.

Amino acids	Microscopy Instrument To Use	Potential Applications
1,2	Stimulated Raman scattering (SRS)	Protein assemblies that cannot be labeled with fluorophores (e.g. tightly stacked fibrils such as amyloid fibrils)
3	Mid-IR photothermal microscopy	
2,3	Single-molecule localization fluorescence microscopy (e.g. STORM)	Protein assemblies with small dimensions (<10 nm)

References

[1] ElsÃ¤sser, Simon J, et al. “Genetic Code Expansion in Stable Cell Lines Enables Encoded Chromatin Modification.” Nature Methods, vol. 13, no. 2, 2016, pp. 158–164., doi:10.1038/nmeth.3701.

[2] Liu, Jihe, et al. “Genetic Code Expansion in Zebrafish Embryos and Its Application to Optical Control of Cell Signaling.” *Journal of the American Chemical Society*, vol. 139, no. 27, 2017, pp. 9100–9103., doi:10.1021/jacs.7b02145

[3] Chin, Jason W. “Expanding and Reprogramming the Genetic Code of Cells and Animals.”*Annual Review of Biochemistry*, vol. 83, no. 1, 2014, pp. 379–408., doi:10.1146/annurev-biochem-060713-035737

Results 1: Genetic Code Expansion in Human Cells

Cells were co-transfected with plasmids to incorporate noncanonical amino acdis(ncAAs) in the specific site of protein of interest(POI), and supplied with noncanonical amino acids in each figure. About 2 days after transfection, fluorescence was observed using cell culture microscope indicating that the ncAAs were inserted at the TAG of sfGFP.

Validation of Genetic Code Expansion in Human Cells

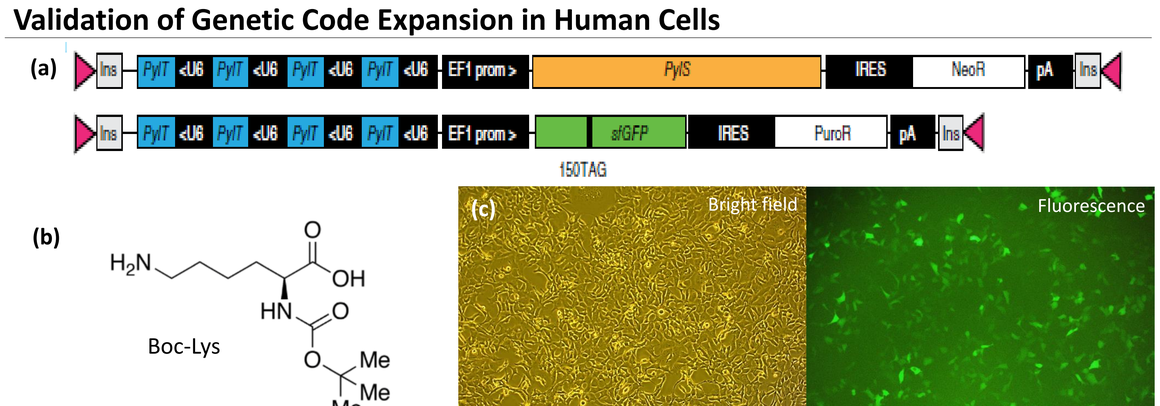

Fig3. (a) PylS and PylT containing plasmids with sfGFP(150TAG) **(b)** Boc-lys used as substrate of PylRS **(c)** HEK293AD cells were imaged 2days after transfection. Left: bright field image, Right: GFP channel fluorescence image. Cells which emit fluorescence on right shows that both PylRS/ tRNA$^{Pyl}_{CUA}$ and cytoplasm(TAG) plasmids are well incorporated into human cells. Scale bar: 10mm

Validation of PylRS-BCNKRS

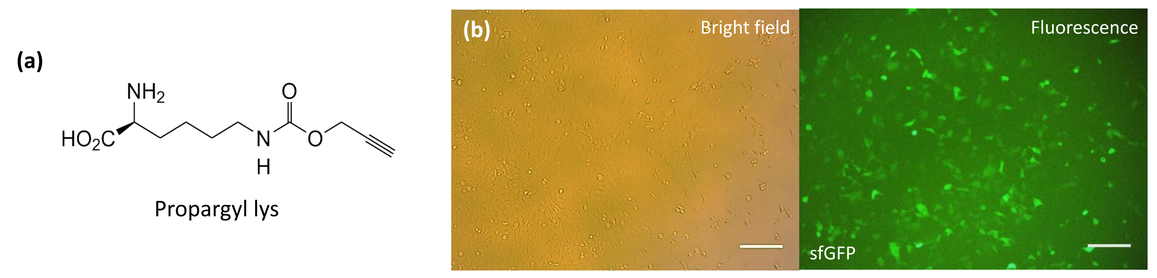

Fig4. (a) Propargyl lysine is used as substrate of PylRS-BCNKRS **(b)** HEK293AD cells were imaged 2days after transfection. Left: bright field image, Right: GFP channel fluorescence image. Scale bar: 10mm

Results 2: Labeling a Protein of Interest

Validation of POI(TAG) using avi-tag

Cells were co-transfected with plasmids which substituted PylRS to PylRS-BCNKRS, as well as a plasmid with vimentin gene with the C-terminal is followed by additional amber codon (TAG) and avi-tag, a short peptide sequence of GLNDIFEAQKIEWHE(amino acid sequence). When co-expressed with biotin ligase enzyme (BirA) and supplied with propargyl lysine(Fig 4(b)), BirA attaches biotin to the avi-tag peptide.

This result shows that transfection of three plasmids (PylRS/ tRNA$^{Pyl}_{CUA}$, vimentin(TAG) plasmid and BirA plasmid) is also working in human cells without perturbing endogenous condition. Especially, avi-tag enable us to check the synthesis of TAG protein because if the protein is broken during amber codon suppress, the avi-tag cvannot be expressed in cells.

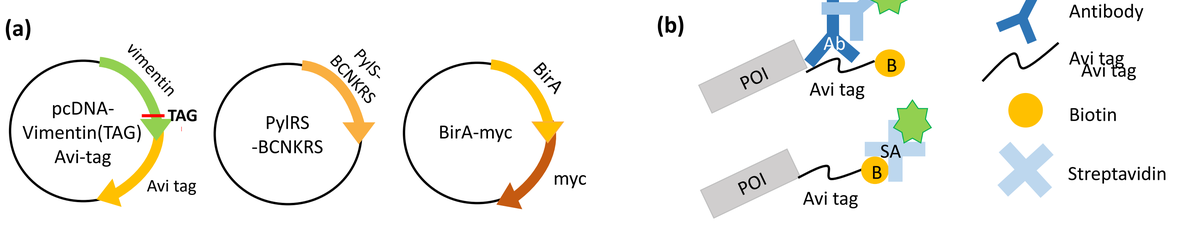

Fig5. (a) Three plasmids containing (1) PylS-BCNKRS, PylT, (2) vimentin(TAG) avi-tag and(3) birA biotin ligase **(b)** Scheme of fluorescence labeling using antibody(up) and streptavidin(bottom)

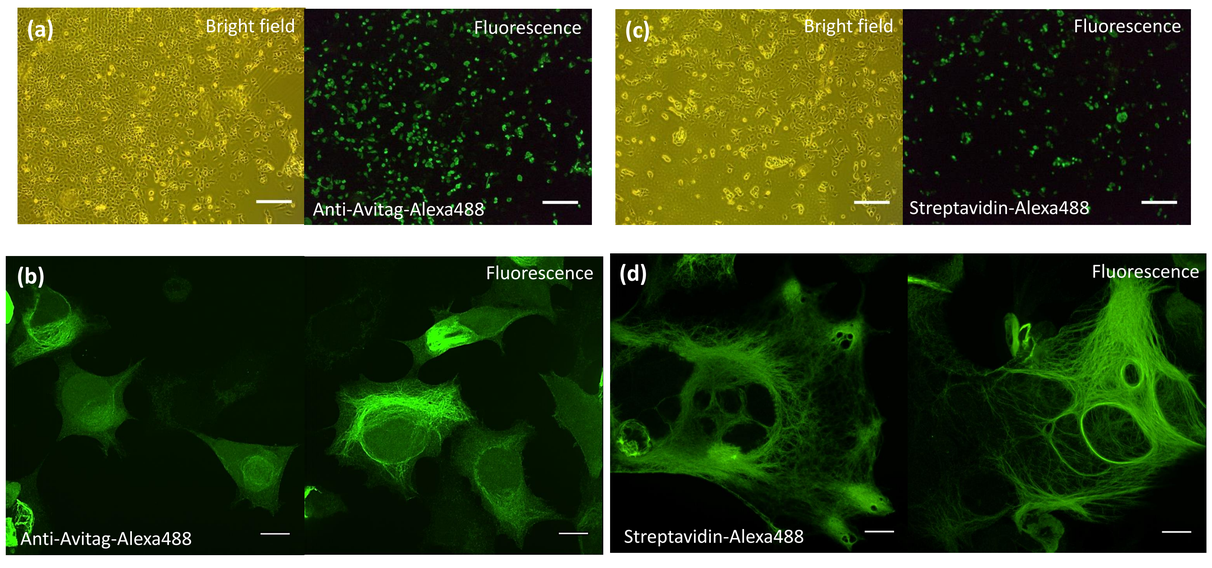

Fig6. (a) Transfected cells were imaged. Before imaging expressed avi-tag is labeled by immunofluorescence using anti-avi-tag antibody(as in (b) up). Scale bar: 10mm **(b)** Cells were imaged using spinning confocal microscopy. Scale bar: 100um **(c)**Cells were imaged 2days after transfection. Before imaging, endogenously bioinylated avi-tag by BirA ligase was labeled using streptavidin-Alexa488(as in (b) bottom). This result shows that incorporation of three plasmids is workinng. And expression of vimentin(TAG) using unnatural amino acids and endogenously biotinylation is working with POI(TAG) protein. Left: bright field image, Right: GFP channel fluorescence image. Scale bar: 10mm **(d)**Cells were imaged using spinning confocal microscope. These cells were labeled using streptavidin-Alexa488 before imaging. Scale bar: 100um