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Current Opinion in Chemical Biology

Extending fluorescence microscopy into anaerobic environments

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Fluorescence microscopy is a powerful tool for investigating living cells. While widely used fluorescent proteins, such as green fluorescent protein (GFP), have had huge impact in biological imaging because they provide genetically encoded, highly specific labeling, these probes require oxygen to generate fluorescence. This crucial oxidative step has limited the use of GFP-like proteins in anaerobic bacterial systems and restricted live-cell studies of obligate anaerobes and their biology. This review discusses alternative approaches to labeling proteins in anaerobic bacteria that are compatible with live-cell fluorescence microscopy in strict oxygen-free environments. The advantages, disadvantages, and likelihood of successful implementation for each approach are considered to provide context and guide further advances in anaerobic fluorescence labeling.

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Current Opinion in Chemical Biology 2019, **51**:98–104

This review comes from a themed issue on **Molecular imaging**

Edited by **Philipp Kukura** and **Sua Myong**

For a complete overview see the Issue and the Editorial

Available online 26th June 2019

https://doi.org/10.1016/j.cbpa.2019.05.008

Introduction

Fluorescent proteins (FPs) are among the most widely used tools to investigate a range of *in vitro* and *in vivo* biological phenomena. These probes remain one of the most prominent and robust tools in biology due to the simplicity of genetically encoding FPs, which is accomplished by appending an FP gene to a target gene. Both the discoveries of new FPs and protein engineering efforts have generated extensive libraries of FPs with wide ranges of excitation and emission spectra, enhanced stokes shifts and brightness; other FPs have been developed to have useful properties such as photo-conversion [1] and photo-activation [2,3]. FPs have been reviewed extensively in the literature [4–7].

However, the use of fluorescence microscopy is limited in anaerobic conditions; conventional FPs such as the blue-green GFP [8], the red DsRed [9], and the far-red mKate [10] families of enzymes require oxygen to generate a mature fluorescent chromophore [11]. This essential step precludes the use of a wide range of established FPs in obligate anaerobes. Developing robust fluorescent probes suited for anaerobic imaging would allow biological exploration of anaerobic systems and extend live-cell fluorescence imaging to medically important organisms and microbial communities, including members of gut and soil microbiomes.

This review provides an overview of alternative strategies that have been, or could be, employed to label bacterial cells: these tools include oxygen-independent fluorescent proteins, bioconjugation techniques, and target-based approaches (Figure 1). We discuss the contexts in which these strategies are likely to succeed, and we describe future efforts that could make an approach more robust and easier to employ.

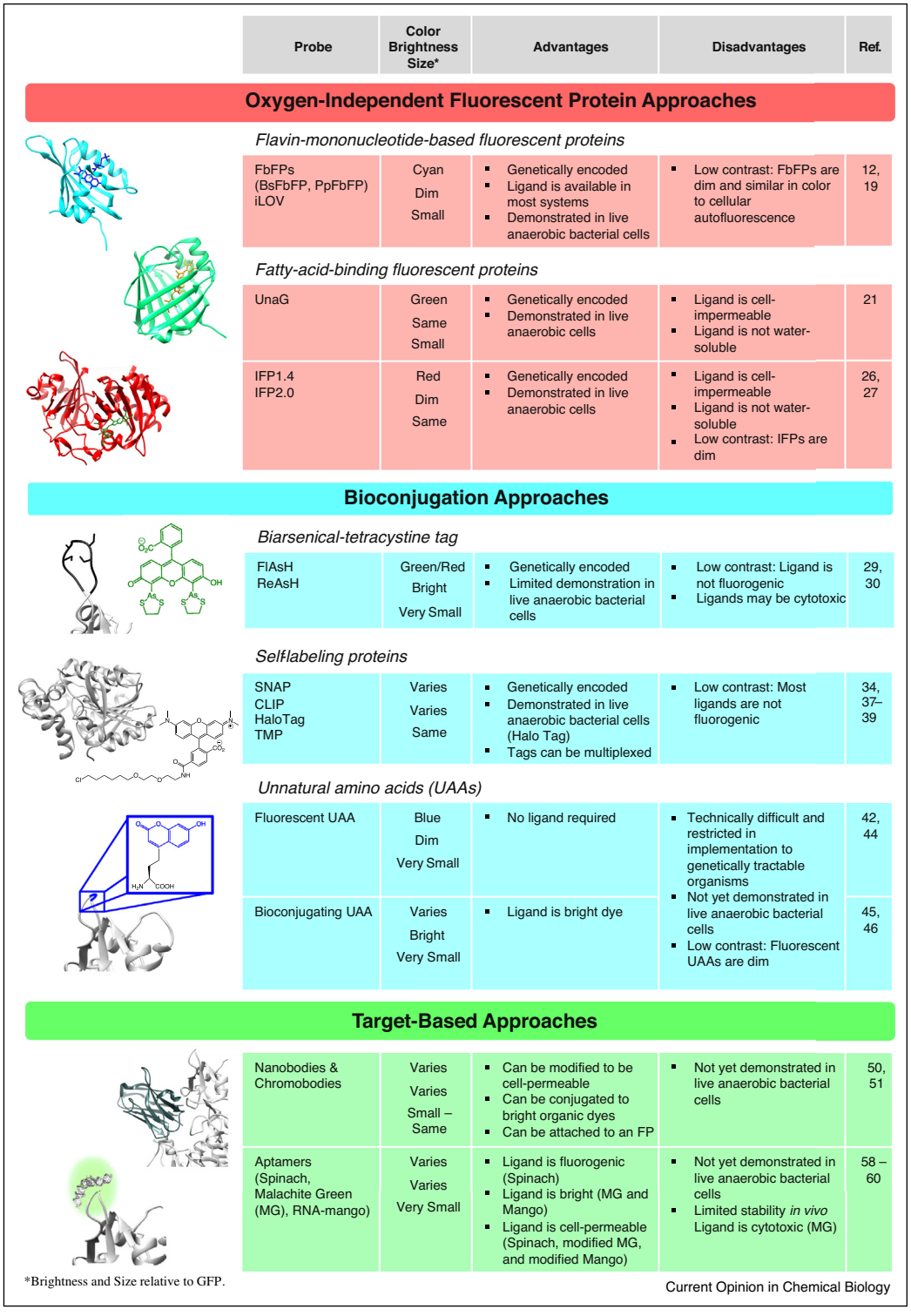
Oxygen-independent fluorescent protein approaches

Flavin-mononucleotide-based fluorescent proteins

Flavin mononucleotide (FMN)-based fluorescent proteins (FbFPs) rely on the photoactive light-oxygen-voltage (LOV) domain to produce blue fluorescence. Native LOV proteins covalently bind the FMN cofactor and are found in bacterial and plant photosensors. While native LOV proteins are typically non-fluorescent, FbFPs have been engineered to fluoresce [12]. FbFPs have been used in many biological conditions, including as fluorescent markers for labeling anaerobic gut bacteria [13] and hypoxically cultured mammalian cells [14], and as reporters for gene expression in anaerobically cultured bacteria [15,16]. The development of FbFPs has been summarized recently [17,18].

FbFPs provide distinct advantages over GFP-like FPs and other oxygen-independent FPs. FbFPs are smaller than GFP-like FPs (10–15 kDa versus 27 kDa), and may therefore be less disruptive to cellular signaling or protein–protein interactions. FbFPs like iLOV are also monomeric [17,19] (whereas GFP-like FPs may oligomerize), which further decreases the risk that these tags will introduce artifacts into the biological system under investigation. An advantage of FbFPs over other oxygen-independent FPs is that FMN, and its precursor molecule riboflavin, are essential molecules in metabolism and do

Figure 1



Summary of probes for anaerobic live-cell imaging.

not typically need to be externally supplied in live cell imaging.

Despite these advantages, the weak fluorescence signal of FbFPs has prevented wide adoption of these tools. While engineering efforts have made FbFPs brighter and more photostable [20•], the relatively weak fluorescence signal is difficult to distinguish from the intrinsic cellular autofluorescence background. Further directed evolution and protein engineering targeted at the FMN-binding pocket may increase FbFP brightness and might red-shift the excitation and emission peaks away from the blue intrinsic fluorescence signal to provide the requisite sensitivity.

Fatty-acid-binding fluorescent proteins

Fatty-acid-binding proteins (FABPs) reversibly bind and transport fatty acids between intra-cellular and extracellular membranes. Fluorescent FABPs also bind to the porphyrin-derived chromophores bilirubin and biliverdin, which are breakdown products of heme metabolism, to generate fluorescence. As such, these proteins are promising candidates for anaerobic fluorescence microscopy. Since these proteins bind exogenously added bilirubin and biliverdin, there is no oxygen requirement for fluorescence production.

One native fluorescent FABP, UnaG, is isolated from Japanese unagi eels and only becomes fluorescent when constituted with bilirubin [21•,22]. UnaG binds bilirubin with high affinity and high specificity, and has similar excitation and emission wavelengths to GFP. UnaG has previously been used to label mammalian HeLa cells [21•] and has been developed into a protein–protein interaction sensor [23] and a calmodulin sensor [24]. Similar to FbFPs, UnaG is smaller in size (15 kDa) than oxygen-dependent FPs [21•].

Infrared and far-red fluorescent FABPs have also been developed and are comparable in spectral characteristics to the far-red mKate FP family. The first FABP, IFP1.4, was developed from a bacterial phytochrome [25] and binds biliverdin [26], although it suffers from dim fluorescence due to low quantum yield. The cellular brightness of IFP1.4 was improved by directed evolution protein engineering to generate IFP2.0, which has been successfully utilized for imaging neurons in *Drosophila* [27].

FABPs have high promise for anaerobic imaging, but have not yet been demonstrated in bacterial systems. However, bacterial cell walls are largely impermeable to the bilirubin and biliverdin cofactors, which may limit UnaG or IFP1.4/2.0 labeling in bacteria to outer membrane targets. Furthermore, the two cofactors are highly insoluble in aqueous solution and may not be compatible with live-cell systems where bilirubin or biliverdin cannot first be reconstituted with the enzyme. Other commonly used strategies, such as electroporation or osmotic shock, may in the future be employed to mitigate these limitations.

Bioconjugation approaches

Biarsenical–tetracysteine tags

Whereas fusions to traditional FPs may disturb a biological system due to steric bulk, a smaller peptide tag that reacts with a substrate can be used for targeted labeling [28]. For instance, the tetracysteine peptide tag binds to biarsenical substrates [29,30]. This system uses a short sequence of 6–20 amino acid residues that includes a CCXXCC motif, in which four reactive cysteine residues flank two other canonical amino acids. These cysteines can covalently react with either a green fluorescein (FlAsH) or a red resorufin (ReAsH) dye that has been modified to contain two arsenical moieties. This system allows site-specific labeling of a target protein or peptide.

Although FlAsH has previously been used to label the anaerobic bacterium *Bacteroides thetaiotaomicron*, the biarsenical–tetracysteine system's primary limitation is a high incidence of non-specific ligand binding [31]. The FlAsH and ReAsH ligands are not fluorogenic, thus any unbound or nonspecifically bound ligand in the experimental sample will increase background noise. Furthermore, many other endogenous proteins contain cysteines, and free thiols may readily react with the FlAsH or ReAsH ligand to cause non-specific labeling and further increase background noise. To minimize off-target labeling by FlAsH and ReAsH ligands, the labeling and subsequent washing steps could be performed with 1,2-ethanedithiol or 2,3-dimercaptopropanol, which will react with excess ligand.

Self-labeling protein tags

Another alternative to genetically encoded FPs is genetically encoded 'self-labeling' proteins. Similar to FPs, a self-labeling protein tag is appended to the target of interest. However, self-labeling proteins are not intrinsically fluorescent and instead react with ligands containing a fluorophore.

These self-labeling proteins combine the specificity and ease of a genetically encoded tag with the functional diversity of synthetic chemistry, as swapping the reactive ligand changes the functionality of the tag. Large libraries of functionalized ligand substrates encompassing a wide range of spectral properties for fluorescence microscopy, including enhanced brightness and photo-activation, have been generated [32••]. The orthogonal HaloTag, SNAP, and CLIP tags can be used in conjunction for simultaneous multi-color fluorescence microscopy [33]. The use of fluorescent ligands is also advantageous as synthetic dyes are typically brighter and more photostable than FPs.

These self-labeling proteins do not require oxidation for ligand conjugation and fluorescence. The HaloTag, derived from a bacterial halogenase, covalently binds molecules containing a chloroalkane moiety [34]; HaloTag technology has previously been used in anaerobic single-molecule tracking experiments in the gut microbe *B. thetaiotaomicron* [35,36••]. Similarly, fusing a target protein to dihydrofolate reductase (eDHFR) allows for labeling with trimethoprim fluorophores [37]. The SNAP [38] and CLIP [39] tags, derived from the human DNA repair protein *O*6-alkylguanine-DNA alkyltransferase, react with *O*6-alkylguanine (AG) and *O*2-benzylcytosine (BC) substrates, respectively; multiple AG and BC substrates with attached fluorescein (green), rhodamine (red), and Cy5 (far-red) fluorescent probes have been generated.

As with all fluorescent probes, self-labeling proteins require some degree of optimization with regard to tag placement and ligand choice. Like traditional FPs, self-labeling protein tags may be disruptive and require strategic placement to minimize artificial interactions in *in vitro* and *in vivo* experiments. Ligands can easily be introduced to a biological sample by supplementing the growth medium, but non-specific ligand interactions may occur if excess ligand is not removed since the unbound ligands themselves may also be dimly fluorescent and result in increased background noise signal. The ligands are typically not cell permeable and may be limited to outer membrane labeling, though ligands may be introduced into permeabilized cells. Another avenue of optimization may be through ligand or fluorophore modification. The addition of elements such as carboxyl groups, which can be removed by endogenous esterases, or sugars that can be imported by cellular uptake channels would enhance the ligand cell permeability.

Unnatural amino acids

The biosynthetic incorporation of unnatural amino acids (UAAs) containing intrinsically fluorescent or chemically functional side-chains into target proteins, facilitated by the development of methods to expand the genetic code, presents a further avenue for labeling proteins under anaerobic conditions [40,41]. Genetically encoded UAAs are inserted directly into a target protein and circumvent issues of steric bulk or oligomerization that can plague GFP-like FPs.

Intrinsically fluorescent UAAs, such as the coumarin-derived amino acid (CouAA) [42,43], have been developed and incorporated into *Escherichia coli* [42] and *Saccharomyces cerevisiae* [44] proteins. Fluorescent UAAs are brighter and more red-shifted than the weakly emissive canonical amino acids (phenylalanine, tyrosine, and tryptophan), but these UAAs remain in the blue spectral region, which limits the signal-to-background ratio in *in vivo* imaging.

Rather than using intrinsically fluorescent UAAs, chemically functional UAAs provide a reactive handle to conjugate a fluorescent ligand [45,46]. This type of site-specific labeling incorporates brighter dyes and allows a multitude of different dye ligand colors to be conjugated to proteins of interest. A more comprehensive overview of bioconjugation strategies employing unnatural amino acids can be found in other reviews [47,48••].

Although both fluorescent and chemically functional UAAs are theoretically capable of working in anaerobic conditions, neither has been demonstrated to date. UAA incorporation is technically difficult to implement because expanded genetic code technologies are restricted to genetically tractable, easily manipulated organisms; experimental challenges include UAA placement on the target gene and tRNA synthetase and tRNA evolution. Furthermore, no more than two to three UAAs can be incorporated into a single target, which may limit the amount of attainable fluorescence signal.

Target-based approaches

Nanobodies

While antibodies have long been used as important diagnostic and imaging tools in research, nanobodies are an alternative to bulky full-length antibodies and can be used for the same *in vitro* applications. Nanobodies contain only a single variable domain from heavy chain antibodies (12–15 kDa) and are generated to directly target proteins or nucleic acids or to recognize generalized peptide tags [49•]. Nanobodies must be modified for live-cell imaging in two key ways: (1) they must be functionalized with a cell-penetrating tag, as nanobodies do not natively traverse cell membranes, and (2) nanobodies must be functionalized with conjugated fluorophores for imaging. Fluorescent nanobodies, also known as chromobodies, have previously been used for live-cell imaging through GFP attachment [50–53], and would need to instead be attached to an oxygen-independent FP (such as those discussed previously) or to a fluorescent dye for anaerobic applications. Previous reviews have covered developments in antibody imaging technology including chromobody applications [54,55].

Nanobodies and chromobodies possess both advantages and disadvantages for fluorescent imaging in anaerobic bacteria. An advantage of nanobodies is that they, like antibodies, label all ectopically expressed and endogenous target molecules through specific binding, whereas FP-labeled systems typically consist of overexpressed fusion proteins from plasmids that leave a background of unlabeled endogenous target molecules. Although the smaller size of nanobodies may provide an advantage over antibodies and GFP-like FPs, nanobodies possess only a

single variable domain, which may limit the specificity of a nanobody for a target.

We foresee applying nanobodies to live-cell imaging in obligate anaerobic bacteria by generating nanobodies against identifying features of species (lipopolysaccharides, outer membrane proteins, etc.) for labeling in the context of multi-species imaging. For anaerobic fluorescence, chromobodies could be attached to oxygen-independent FPs, such as the ones described previously, or conjugated to fluorescent dyes.

Aptamers

Aptamers refer to single-stranded DNA or RNA molecules that have been selected through *in vitro* evolution techniques to recognize ligands of interest. Aptamer recognition can derive from complementary base pairing of target nucleotide sequences or recognition of peptide tags [56], and aptamers have been used to visualize processes such as transcription in live HEK293T cells [57•]. Furthermore, unlike antibodies, aptamers do not require additional steps of fixing and permeabilizing cells for successful implementation; aptamers can be expressed *in vivo* by the target organism, or the probe can be added into an experiment after generation in a benchtop thermocycler.

Upon binding, a subset of aptamers can switch on ligand fluorescence [58–60]. The Spinach aptamer system is one such class of fluorogenic aptamers: Spinach switches on the fluorescence of the GFP fluorophore analog 3,5-difluoro-4-hydroxybenzylidine imidazolinone (DFHBI) [58]. DFHBI is a fluorogenic molecule, which only becomes fluorescent upon interaction with the complementary aptamer. DFHBI is cell permeable, nontoxic, and can easily be supplemented into experimental samples. The Spinach system has been diversified to produce a multitude of colored aptamer-fluorophore pairs with shifted excitation and emission spectra [57•,61].

We foresee the use of Spinach and other fluorescent aptamers in anaerobic bacterial systems, since fluorescent dyes and the GFP chromophore analogs do not require any oxygen to produce fluorescence. However, no one has yet reported the use of aptamers in anaerobic live-cell imaging. Despite engineered improvements [62], Spinach-DFHBI complexes remain dimmer than GFP, and all aptamers are susceptible to poor intracellular folding and nuclease-targeted degradation [63]. In accordance with the unique conditions of each experimental system and the relative signal per tag, increasing the copy number of aptamers *in vivo* may improve contrast during live-cell imaging for sufficient detection. Additional biochemical validation is required when increasing the aptamer copy number to ensure that no artifacts get introduced into the system.

Conclusions

When choosing fluorescence probes, one must consider the intrinsic advantages and disadvantages of each labeling strategy. An ideal probe should balance brightness (for high contrast), minimal biological disturbance (to not affect the interrogated system), specificity (to provide an accurate image) and ease of implementation (such that the highest possible proportion of target molecules are labeled).

Fluorescence microscopy has allowed us to visualize biological phenomena in living systems, and much of this exploration has hinged on the widespread adoption and simplicity of using GFP-like fluorescent proteins. Unfortunately, GFP-like FPs depends on oxygen for fluorescence, thus anaerobic systems have yet to be extensively explored by fluorescence microscopy. The anaerobic systems that have been precluded include, but are not limited to, bacteria in gut microbiomes, soil microbiomes, and the deep seas. While we have detailed approaches and implementation of different fluorescent labels in anaerobic bacteria, we foresee that many of the described approaches may also be applied to extend fluorescence imaging to measuring anaerobic biological phenomena in yeast, mammalian, and plant systems.

Conflict of interest statement

Nothing declared.

Acknowledgements

This work was supported by Army Research Office grant W911NF-18-1-0339 to JSB; Army Research Office grant W911NF-16-1-0147, Defense Threat Reduction Agency grant HDTRA1-16-1-0004, and National Institutes of Health grant GM 093088 to ENGM. HEC was also supported by the National Science Foundation Graduate Research Fellowship Program (grant DGE-1256260).

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. McKinney SA, Murphy CS, Hazelwood KL, Davidson MW, Looger LLA: **Bright and photostable photoconvertible fluorescent protein**. *Nat Methods* 2009, **6**:131-133.

2. Patterson GH, Lippincott-Schwartz J: **A photoactivatable GFP for selective photolabeling of proteins and cells**. *Science* 2002, **297**:1873-1877.

3. Subach FV, Patterson GH, Manley S, Gillette JM, Lippincott-Schwartz J, Verkhusha VV: **Photoactivatable MCherry for high-resolution two-color fluorescence microscopy**. *Nat Methods* 2009, **6**:153-159.

4. Nienhaus K, Nienhaus GU: **Fluorescent proteins for live-cell imaging with super-resolution**. *Chem Soc Rev* 2014, **43**:1088-1106.

5. Dean KM, Palmer AE: **Advances in fluorescence labeling strategies for dynamic cellular imaging**. *Nat Chem Biol* 2014, **10**:512-523.

6. Mishin AS, Belousov VV, Solntsev KM, Lukyanov KA: **Novel uses of fluorescent proteins**. *Curr Opin Chem Biol* 2015, **27**:1-9.

7. Rodriguez EA, Campbell RE, Lin JY, Lin MZ, Miyawaki A, Palmer AE, Shu X, Zhang J, Tsien RY: **The growing and glowing toolbox of fluorescent and photoactive proteins**. *Trends Biochem Sci* 2017, **42**:111-129.

8. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC: **Green fluorescent protein as a marker for gene expression**. *Science* 1994, **263**:802-805.

9. Baird GS, Zacharias DA, Tsien RY: **Biochemistry mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral**. *Proc Natl Acad Sci U S A* 2000, **97**:11984-11989.

10. Shcherbo D, Merzlyak EM, Chepurnykh TV, Fradkov AF, Ermakova GV, Solovieva EA, Lukyanov KA, Bogdanova EA, Zaraisky AG, Lukyanov S *et al.*: **Bright far-red fluorescent protein for whole-body imaging**. *Nat Methods* 2007, **4**:741-746.

11. Tsien RY: **The green fluorescent protein**. *Annu Rev Biochem* 1998, **67**:509-544.

12. Drepper T, Eggert T, Circolone F, Heck A, Krauß U, Guterl J-K, Wendorff M, Losi A, Gärtner W, Jaeger K-E: **Reporter proteins for in vivo fluorescence without oxygen**. *Nat Biotechnol* 2007, **25**:443-445.

13. Landete JM, Peirotén Á, Rodríguez E, Margolles A, Medina M, Arqués JL: **Anaerobic green fluorescent protein as a marker of bifidobacterium strains**. *Int J Food Microbiol* 2014, **175**:6-13.

14. Walter J, Hausmann S, Drepper T, Puls M, Eggert T, Dihné M: **Flavin mononucleotide-based fluorescent proteins function in mammalian cells without oxygen requirement**. *PLoS One* 2012, **7**:e43921.

15. Drepper T, Huber R, Heck A, Circolone F, Hillmer A-K, Büchs J, Jaeger K-E: **Flavin mononucleotide-based fluorescent reporter proteins outperform green fluorescent protein-like proteins as quantitative in vivo real-time reporters**. *Appl Environ Microbiol* 2010, **76**:5990-5994.

16. Lobo LA, Smith CJ, Rocha ER: **Flavin mononucleotide (FMN)-based fluorescent protein (FbFP) as reporter for gene expression in the anaerobe *Bacteroides fragilis***. *FEMS Microbiol Lett* 2011, **317**:67-74.

17. Mukherjee A, Schroeder CM: **Flavin-based fluorescent proteins: emerging paradigms in biological imaging**. *Curr Opin Biotechnol* 2015, **31**:16-23.

18. Wingen M, Potzkei J, Endres S, Casini G, Rupprecht C, Fahlke C, Krauss U, Jaeger K-E, Drepper T, Gensch T: **The photophysics of LOV-based fluorescent proteins – new tools for cell biology**. *Photochem Photobiol Sci* 2014, **13**:875-883.

19. Chapman S, Faulkner C, Kaiserli E, Garcia-Mata C, Savenkov EI, Roberts AG, Oparka KJ, Christie JM: **The photoreversible fluorescent protein iLOV outperforms GFP as a reporter of plant virus infection**. *Proc Natl Acad Sci U S A* 2008, **105**:20038-20043.

20. • Mukherjee A, Weyant KB, Agrawal U, Walker J, Cann IKO, Schroeder CM: **Engineering and characterization of new LOV-based fluorescent proteins from *Chlamydomonas reinhardtii* and *Vaucheria frigida***. *ACS Synth Biol* 2015, **4**:371-377.
This paper details the development of brighter iLOV variants that can be used in anaerobic live-cell imaging.

21. • Kumagai A, Ando R, Miyatake H, Greimel P, Kobayashi T, Hirabayashi Y, Shimogori T, Miyawaki A: **A bilirubin-inducible fluorescent protein from eel muscle**. *Cell* 2013, **153**:1602-1611.
This paper is the first characterization of the bilirubin-dependent fluorescent protein, UnaG, which should theoretically be compatible with anaerobic live-cell imaging.

22. Shitashima Y, Shimozawa T, Kumagai A, Miyawaki A, Asahi T: **Two distinct fluorescence states of the ligand-induced green fluorescent protein UnaG**. *Biophys J* 2017, **113**:2805-2814.

23. To T-L, Zhang Q, Shu X: **Structure-guided design of a reversible fluorogenic reporter of protein-protein interactions**. *Protein Sci* 2016, **25**:748-753.

24. Shitashima Y, Shimozawa T, Asahi T, Miyawaki A: **A dual-ligand-modulable fluorescent protein based on UnaG and calmodulin**. *Biochem Biophys Res Commun* 2018, **496**:872-879.

25. Wagner JR, Brunzelle JS, Forest KT, Vierstra RD: **A light-sensing knot revealed by the structure of the chromophore-binding domain of phytochrome**. *Nature* 2005, **438**:325.

26. Shu X, Royant A, Lin MZ, Aguilera TA, Lev-Ram V, Steinbach PA, Tsien RY: **Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome**. *Science* 2009, **324**:804-807.

27. Yu D, Gustafson WC, Han C, Lafaye C, Noirclerc-Savoye M, Ge W-P, Thayer DA, Huang H, Kornberg TB, Royant A *et al.*: **An improved monomeric infrared fluorescent protein for neuronal and tumour brain imaging**. *Nat Commun* 2014, **5**:3626.

28. Pomorski A, Kre?żel A: **Exploration of biarsenical chemistry—challenges in protein research**. *ChemBioChem* 2011, **12**:1152-1167.

29. Griffin BA, Adams SR, Tsien RY: **Specific covalent labeling of recombinant protein molecules inside live cells**. *Science* 1998, **281**:269-272.

30. Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH: **Multicolor and electron microscopic imaging of connexin trafficking**. *Science* 2002, **296**:503-507.

31. Karunatilaka KS, Coupland BR, Cameron EA, Martens EC, Koropatkin NK, Biteen JS: *Single-Molecule Imaging Can Be Achieved in Live Obligate Anaerobic Bacteria* 2013, **vol 8590** pp 85900K-85900K – 7.

32. •• Grimm JB, Muthusamy AK, Liang Y, Brown TA, Lemon WC, Patel R, Lu R, Macklin JJ, Keller PJ, Ji N *et al.*: **A general method to fine-tune fluorophores for live-cell and *in Vivo* imaging**. *Nat Methods* 2017, **14**:987-994.
This paper describes modifications, or as the authors describe as 'fine-tuning', that subtly change the fluorescence emission of chemical dyes used in bioconjugation techniques.

33. Stagge F, Mitronova GY, Belov VN, Wurm CA, Jakobs S: **Snap-, CLIP- and halo-tag labelling of budding yeast cells**. *PLoS One* 2013, **8**:e78745.

34. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M *et al.*: **HaloTag: a novel protein labeling technology for cell imaging and protein analysis**. *ACS Chem Biol* 2008, **3**:373-382.

35. Karunatilaka KS, Cameron EA, Martens EC, Koropatkin NM, Biteen JS: **Superresolution imaging captures carbohydrate utilization dynamics in human gut symbionts**. *mBio* 2014, **5**:e02172-14.

36. •• Tuson HH, Foley MH, Koropatkin NM, Biteen JS: **The starch utilization system assembles around stationary starch-binding proteins**. *Biophys J* 2018, **115**:242-250.
This paper uses HaloTag technology and single-molecule tracking analysis to study the assembly of the starch utilization system in the obligate anaerobe gut bacterium *Bacteroides thetaiotaomicron*.

37. Gallagher SS, Sable JE, Sheetz MP, Cornish VW: **An in vivo covalent TMP-tag based on proximity-induced reactivity**. *ACS Chem Biol* 2009, **4**:547-556.

38. Keppler A, Gendreizig S, Gronemeyer T, Pick H, Vogel H, Johnsson K: **A general method for the covalent labeling of fusion proteins with small molecules in vivo**. *Nat Biotechnol* 2003, **21**:86-89.

39. Gautier A, Juillerat A, Heinis C, Corrêa IR Jr, Kindermann M, Beaufils F, Johnsson K: **An engineered protein tag for multiprotein labeling in living cells**. *Chem Biol* 2008, **15**:128-136.

40. Noren CJ, Anthony-Cahill SJ, Griffith MC, Schultz PG: **A general method for site-specific incorporation of unnatural amino acids into proteins**. *Science* 1989, **244**:182-188.

41. Bain JD, Diala ES, Glabe CG, Dix TA, Chamberlin AR: **Biosynthetic site-specific incorporation of a non-natural amino acid into a polypeptide**. *J Am Chem Soc* 1989, **111**:8013-8014.

42. Wang J, Xie J, Schultz PG: **A genetically encoded fluorescent amino acid**. *J Am Chem Soc* 2006, **128**:8738-8739.

43. Charbon G, Brustad E, Scott KA, Wang J, Løbner-Olesen A, Schultz PG, Jacobs-Wagner C, Chapman E: **Subcellular protein localization by using a genetically encoded fluorescent amino acid**. *ChemBioChem* 2011, **12**:1818-1821.

44. Summerer D, Chen S, Wu N, Deiters A, Chin JW, Schultz PG: **A genetically encoded fluorescent amino acid**. *Proc Natl Acad Sci U S A* 2006, **103**:9785-9789.

45. Beatty KE, Liu JC, Xie F, Dieterich DC, Schuman EM, Wang Q, Tirrell DA: **Fluorescence visualization of newly synthesized proteins in mammalian cells**. *Angew Chem* 2006, **118**:7524-7527.

46. Baskin JM, Prescher JA, Laughlin ST, Agard NJ, Chang PV, Miller IA, Lo A, Codelli JA, Bertozzi CR: **Copper-free click chemistry for dynamic in vivo imaging**. *Proc Natl Acad Sci U S A* 2007, **104**:16793-16797.

47. Chin JW: **Expanding and reprogramming the genetic code**. *Nature* 2017, **550**:53-60.

48. •• Lang K, Chin JW: **Cellular incorporation of unnatural amino acids and bioorthogonal labeling of proteins**. *Chem Rev* 2014, **114**:4764-4806.

This review provides a comprehensive overview of chemoselective reactions and the incorporation of fluorescent or bioconjugating unnatural amino acids.

49. • Virant D, Traenkle B, Maier J, Kaiser PD, Bodenhöfer M, Schmees C, Vojnovic I, Pisak-Lukáts B, Endesfelder U, Rothbauer U: **A peptide tag-specific nanobody enables high-quality labeling for DSTORM imaging**. *Nat Commun* 2018, **9**:930.

This paper describes a peptide-tag and nanobody-dye recognition system that can be used for single-molecule dSTORM imaging in live-cells.

50. Rothbauer U, Zolghadr K, Tillib S, Nowak D, Schermelleh L, Gahl A, Backmann N, Conrath K, Muyldermans S, Cardoso MC *et al.*: **Targeting and tracing antigens in live cells with fluorescent nanobodies**. *Nat Methods* 2006, **3**:887-889.

51. Olichon A, Surrey T: **Selection of genetically encoded fluorescent single domain antibodies engineered for efficient expression in *Escherichia coli***. *J Biol Chem* 2007, **282**:36314-36320.

52. Ries J, Kaplan C, Platonova E, Eghlidi H, Ewers HA: **Simple versatile method for GFP-based super-resolution microscopy via nanobodies**. *Nat Methods* 2012, **9**:582.

53. Maier J, Traenkle B, Rothbauer U: **Real-time analysis of epithelial-mesenchymal transition using fluorescent single-domain antibodies**. *Sci Rep* 2015, **5**.

54. Kaiser PD, Maier J, Traenkle B, Emele F, Rothbauer U: **Recent progress in generating intracellular functional antibody fragments to target and trace cellular components in living cells**. *Biochim Biophys Acta BBA Proteins Proteom* 2014, **1844**:1933-1942.

55. Traenkle B, Rothbauer U: **Under the microscope: single-domain antibodies for live-cell imaging and super-resolution microscopy**. *Front Immunol* 2017, **8**.

56. Song W, Strack RL, Jaffrey SR: **Imaging bacterial protein expression using genetically encoded RNA sensors**. *Nat Methods* 2013, **10**:873-875.

57. • Song W, Filonov GS, Kim H, Hirsch M, Li X, Moon JD, Jaffrey SR: **Imaging RNA polymerase III transcription using a photostable RNA–fluorophore complex**. *Nat Chem Biol* 2017, **13**:1187.

This paper details the development of a red-shifted and more photostable aptamer complex based on the chromophore of DsRed and its utilization in live-cell imaging.

58. Paige JS, Wu KY, Jaffrey SR: **RNA mimics of green fluorescent protein**. *Science* 2011, **333**:642-646.

59. Babendure JR, Adams SR, Tsien RY: **Aptamers switch on fluorescence of triphenylmethane dyes**. *J Am Chem Soc* 2003, **125**:14716-14717.

60. Dolgosheina EV, Jeng SCY, Panchapakesan SSS, Cojocaru R, Chen PSK, Wilson PD, Hawkins N, Wiggins PA, Unrau PJ: **RNA mango aptamer-fluorophore: a bright, high-affinity complex for RNA labeling and tracking**. *ACS Chem Biol* 2014, **9**:2412-2420.

61. Song W, Strack RL, Svensen N, Jaffrey SR: **Plug-and-play fluorophores extend the spectral properties of spinach**. *J Am Chem Soc* 2014, **136**:1198-1201.

62. Strack RL, Disney MD, Jaffrey SR: **A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat RNA**. *Nat Methods* 2013, **10**:1219-1224.

63. Lakhin AV, Tarantul VZ, Gening LV: **Aptamers: problems, solutions and prospects**. *Acta Naturae* 2013, **5**:34-43.