Fluorescent Labeling

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The Power of Fluorogenic Probes**

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In fluorescence microscopy, as in many analytical methods, the signal-to-noise ratio is crucial for the success of the experiment. Ideally, there should be no fluorescence at the resting level and a large increase in the emission should be observed after manipulation of the sample. This scenario is, to a certain extent, achieved when bioluminescence is used, since the background is limited to autofluorescence. The use of bioluminescence in single-cell experiments, however, is almost impossible, simply because the number of photons emitted does not suffice to produce a useful image. Fluorogenic dyes have the ability to fill the gap, and numerous fluorescence probes that increase their fluorescence intensity upon a cellular event have been developed.

One of the prominent early examples is the heavily used calcium indicator Fluo-3, which is quenched through photo-induced electron transfer (PET) in the calcium-free state and brightly fluorescent when calcium is bound.[1] As will later be seen, this principle was used recently by the Bertozzi research group, whereby a chemical labeling reaction successfully eliminated the PET and thus led to a massive increase in the fluorescence.[2] Some of the early genetically encoded calcium sensors are also fluorogenic.[3] Another approach to generate fluorogenic fluorescent proteins is to use so-called split fluorescent proteins, which form the intact fluorophore only after a missing part of the β-barrel has been added to complete the structure of the fluorescent protein. Such split proteins are often used to study protein–protein interactions, and the onset of fluorescence is an indicator of the successful assembly of the protein complex.[4] Fluorogeneity is particularly desirable for protein labeling in a complex biological environment. Thus, several techniques have been developed very recently in which a specific artificial amino acid is treated with a fluorogenic dye.[5] When it comes to fluorescence labeling inside cells, it is generally desirable to have the bioorthogonally reactive group directly involved in the fluorophore quenching (Figure 1a). A successful reaction then produces the fully fluorescent biomolecule. [3+2] Cycloaddition reactions with and without copper catalysis and reverse electron demand Diels–Alder reactions make use of fluorogenic dyes in which the reactive group on the dye (azide/alkyne or tetrazine) also quenches the fluorophore (Figure 2a–c).[6] Similar results were achieved with FlAsH labels, where the reaction and concomitant steric fixation of two bisarsenite groups resulted in effective fluorescence of the previously quenched xanthene dye (Figure 2d).[7] An alternative method for in vivo protein labeling requires the fusion of a self-alkylating or self-acylating unit to the protein of interest.[8] The small proteins SNAP-tag or CLIP-tag react spontaneously with benzylguanine–fluorophore conjugates. While benzylguanine is a modest quencher, the addition of a suitable quenching unit (Figure 1b) provides full fluorogeneity (Figure 2e).[9] Similarly, the β-lactamase-based TEM-tag self-acylates from a β-lactam substrate on β-elimination of a suitable quencher (Figure 2f).[10]

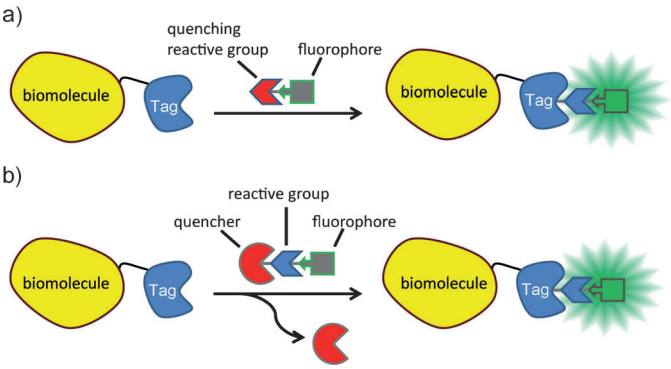

Figure 1. The two most popular approaches employed in fluorogenic labeling of biomolecules. a) The reactive moiety itself acts as quencher, and fluorescence is enhanced when its photophysical properties are altered during the labeling reaction. b) A fluorescence quenching molecule is covalently attached to the reactive group of the fluorophore conjugate and released in the course of the labeling reaction.

Functional groups with largely delocalized electrons are most suitable for PET quenching of the fluorophores. Simple but very successful examples are the azido groups used in fluorogenic coumarins and Lucifer yellow (Scheme 1a).[6b,11] This principle is much more difficult to achieve with xanthene dyes.

So far, only one example of a fluorogenic Oregon Green tetrazine conjugate has been reported that can be used for in vivo labeling of a biomolecule by a Diels–Alder reaction with *trans*-cyclooctene residues.[6c] The Bertozzi research group addressed the intrinsic difficulty of generating fluorogenic xanthene dyes by employing a rational approach that

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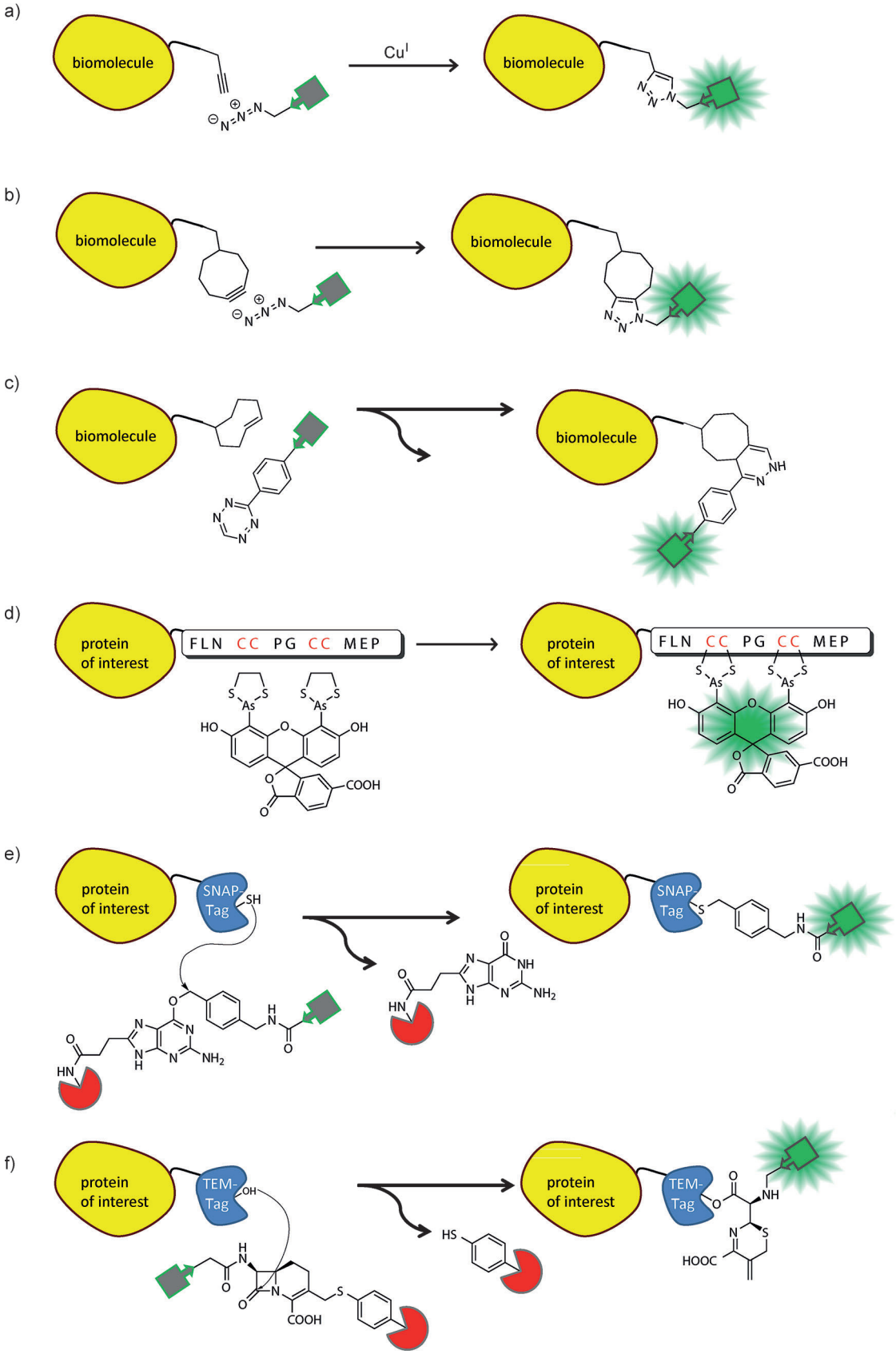
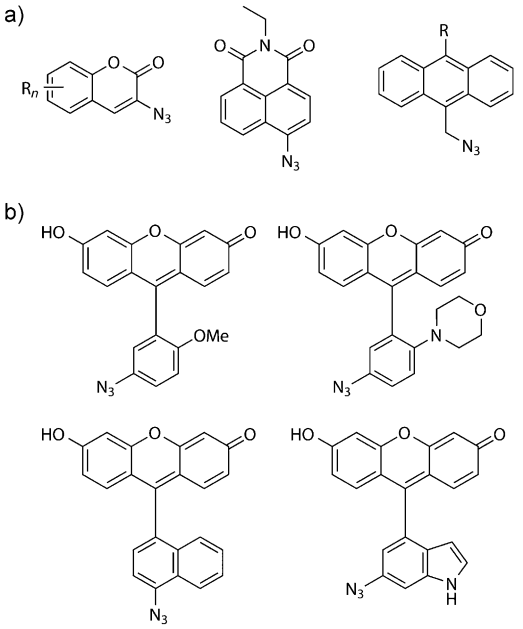


Figure 2. Examples of biomolecule labeling with fluorogenic dyes. a) Copper-catalyzed alkyne/azide cycloaddition. b) Strain-promoted alkyne/azide cycloaddition. c) Strain-promoted alkene/tetrazine cycloaddition of the Diels–Alder type. d) FlAsH labeling of a tetracysteine motif. e) SNAP-tag labeling with a quenched fluorophore–benzylguanine conjugate. f) TEM-tag labeling with a quenched fluorophore-β-lactam conjugate.



Scheme 1. Azide-quenched fluorogenic dyes. a) Typical examples. b) Examples of the newly introduced, rationally designed xanthene derivatives.

was introduced by Nagano and co-workers.[12] Briefly, by using experimental approaches in combination with density functional theory (DFT) calculations they established a correlation between the E_{HOMO} energy (HOMO = highest occupied molecular orbital) of the pendant aryl ring of the fluorescein derivatives and the respective quantum yield. Pendant aryl rings with low E_{HOMO} values quench the fluorescence by PET, whereas those with high E_{HOMO} values do not. Following this line of thought, Bertozzi and co-workers postulated that azidoarylxanthene derivatives should be almost completely nonfluorescent because of their low E_{HOMO} values, whereas the corresponding triazolyl products formed from a [2+3] cycloaddition with an alkyne should be highly fluorescent.

They synthesized a set of azidoarylxanthene derivatives (Scheme 1 b) and analyzed the changes in the photophysical properties after a click reaction. An up to 34-fold enhancement in the fluorescence was detected upon triazole formation. This increase is sufficient for the system to be used as high-contrast fluorogenic probes for in vivo experiments. Moreover, reduction of the azide to the corresponding amine did not generate fluorescent species. Thus, the detrimental effects of this common side reaction are eliminated, which at times results in high background fluorescence when other fluorogenic azides are used. It has to be emphasized, that this rational approach yields fluorogenic compounds in approximately 50 % of the cases, which is far superior to undirected screens.[2]

The experimental usefulness of the azidoarylxanthenes was demonstrated by fluorogenic protein labeling, as well as no-wash labeling of alkynyl-modified cell-surface glycoproteins in fixed cells. In conclusion, the new set of fluorogenic fluorescein dyes expands the color palette available for in vivo labeling. It would be very useful in the future to use the dyes inside intact cells. This should be feasible, when the intrinsic cell-permeability of the newly introduced azidoarylxanthene is considered. The negative charge on these dyes is especially useful, since it ensures that the compounds stay in the cytosol rather than being absorbed into membranes, as is the case with most other fluorogenic dyes. In intact cells, fluorogeneity will solve the problem of dye entry in cases of ineffective labeling, which frequently produces unwanted background fluorescence.

Despite these improvements, the wish-list of microscopists in terms of dyes is still quite long. Better photostability, lack of blinking, and membrane-permeability are the most desired features. The ultimate goal in this field would be the development of highly fluorogenic dyes suitable for high-resolution imaging.

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