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Fluorescent probes for imaging formaldehyde in biological systems

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Formaldehyde (FA) is a common environmental toxin but is also endogenously produced through a diverse array of essential biological processes, including mitochondrial one-carbon metabolism, metabolite oxidation, and nuclear epigenetic modifications. Its high electrophilicity enables reactivity with a wide variety of biological nucleophiles, which can be beneficial or detrimental to cellular function depending on the context. New methods that enable detection of FA in living systems can help disentangle the signal/stress dichotomy of this simplest reactive carbonyl species (RCS), and fluorescent probes for FA with high selectivity and sensitivity have emerged as promising chemical tools in this regard.

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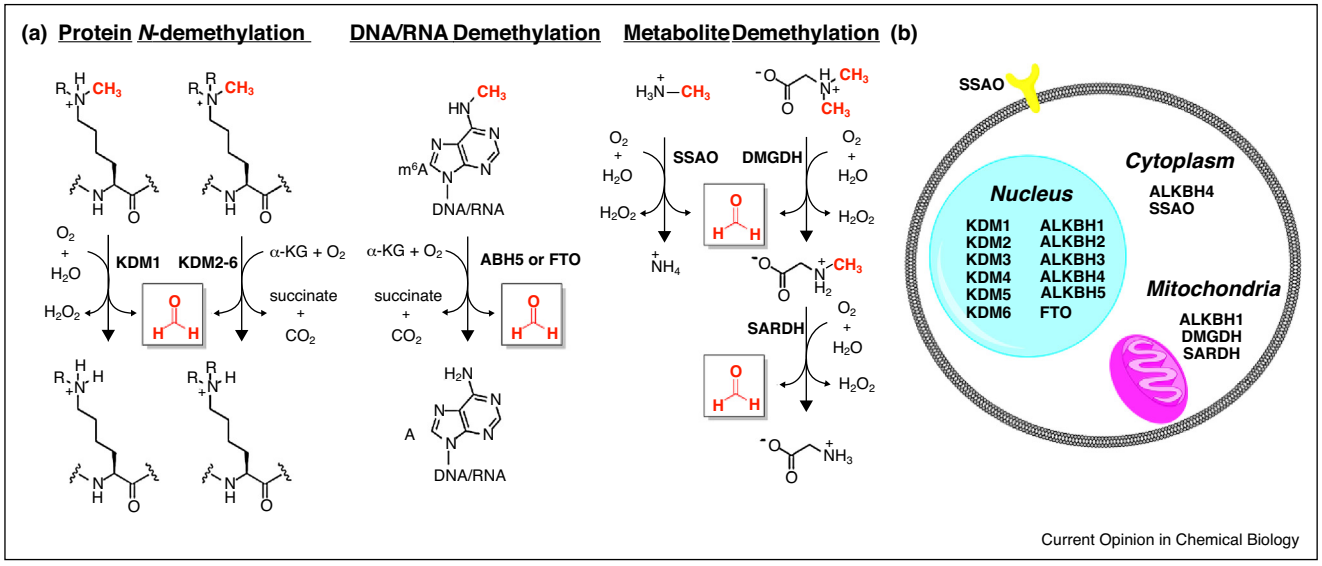
Introduction

Formaldehyde (FA) is an endogenously-produced reactive carbonyl species (RCS) released in biological processes ranging from epigenetics to one carbon metabolism (Figure 1) [1,2]. Although more widely known as an environmental toxin and carcinogen [3], FA has been reported to exist at relatively high concentrations intracellularly under normal physiological conditions, reaching up to 500 μM in certain organelles [4,5••]. FA is a one carbon fuel involved in maintenance of cell homeostasis, and its abundance and high reactivity suggest a potential role as a physiological signaling molecule [6,7•,8]. FA is the product of demethylation events of *N*-methylated amino acid residues (*e.g.*, lysine, arginine, histidine) mediated by demethylase enzymes [9], such as lysine specific demethylase 1 (KDM1) [10,11] and Jumonji domain-containing proteins (Figure 1) [12–14]. FA is also produced through *N*-demethylation of DNA and RNA bases, such as m^6A, by AlkB homologues (ALKBH) [15]. Methylation and demethylation events signal transcription factors to promote or repress transcription, and disruption of normal methylation markers could have wide ranging effects in disease states, particularly cancer progression [16,17]. Similarly, metabolism of methylated amines, including the abundant endogenous metabolite methylamine, by semicarbazide-sensitive amine oxidases (SSAO) releases FA [18•,19]. In addition, several demethylase enzymes utilize tetrahydrofolate (THF) as a cofactor to bind FA, yielding 5,10-methylene-THF, known as 'active formaldehyde' [20,21]. Folate derivatives are essential for mitochondrial one-carbon metabolism, in which demethylation events release FA which is further incorporated into important cellular building blocks such as amino acids, purines, and phospholipids [22]. A canonical example is the sarcosine pathway, where dimethylglycine dehydrogenase (DMGDH) and sarcosine dehydrogenase (SARDH) demethylate dimethylglycine sequentially, yielding two equivalents of FA and glycine [23•]. Owing to the toxic nature of FA, it is efficiently metabolized as its glutathione adduct, *S*-(hydroxymethyl)glutathione, by the enzyme alcohol dehydrogenase 5 (ADH5, also known as formaldehyde dehydrogenase, *S*-nitrosogluthathione reductase, and alcohol dehydrogenase 3) [24–27].

The aforementioned examples provide motivation for developing new FA detection technologies for biological study. Owing to the prevalence of FA in industrial settings as well as in household items, several sensitive FA detection methods have been devised [28], including methods utilizing high performance liquid chromatography (HPLC) [29,30], gas chromatography (GC) [31], mass spectrometry (MS) [32], as well as preconcentration/chemical ionization MS [33]. However, the study of FA and its roles in physiology and pathology is still limited by a lack of detection methods that can give spatiotemporal resolution in living cells and more complex biological specimens. One emerging approach utilizes fluorescence-based probes, which offer high selectivity and sensitivity and can be used *in situ*. Reactivity-based fluorescent probes have been successfully employed to image a variety of biological analytes [34–36], including the carbonyl species carbon monoxide [37–41] and methylglyoxal [42], and recent efforts have focused on the development of reactivity-based probes for FA. This review aims to summarize the current progress toward creating responsive and selective fluorescent reporters for FA, along with an outlook toward improvements in the field.

Figure 1



(a) Selected enzymatic pathways that generate FA in biological systems. **(b)** Subcellular localization of FA-generating enzymes. Abbreviations: α-KG, α-ketoglutarate; KDM, lysine demethylase; ALKBH, AlkB homologues; SSAO, semicarbazide-sensitive amine oxidases; DMGDH, dimethylglycine dehydrogenase; SARDH, sarcosine dehydrogenase; FTO, fat mass and obesity-associated protein.

Design considerations for fluorescent formaldehyde probes

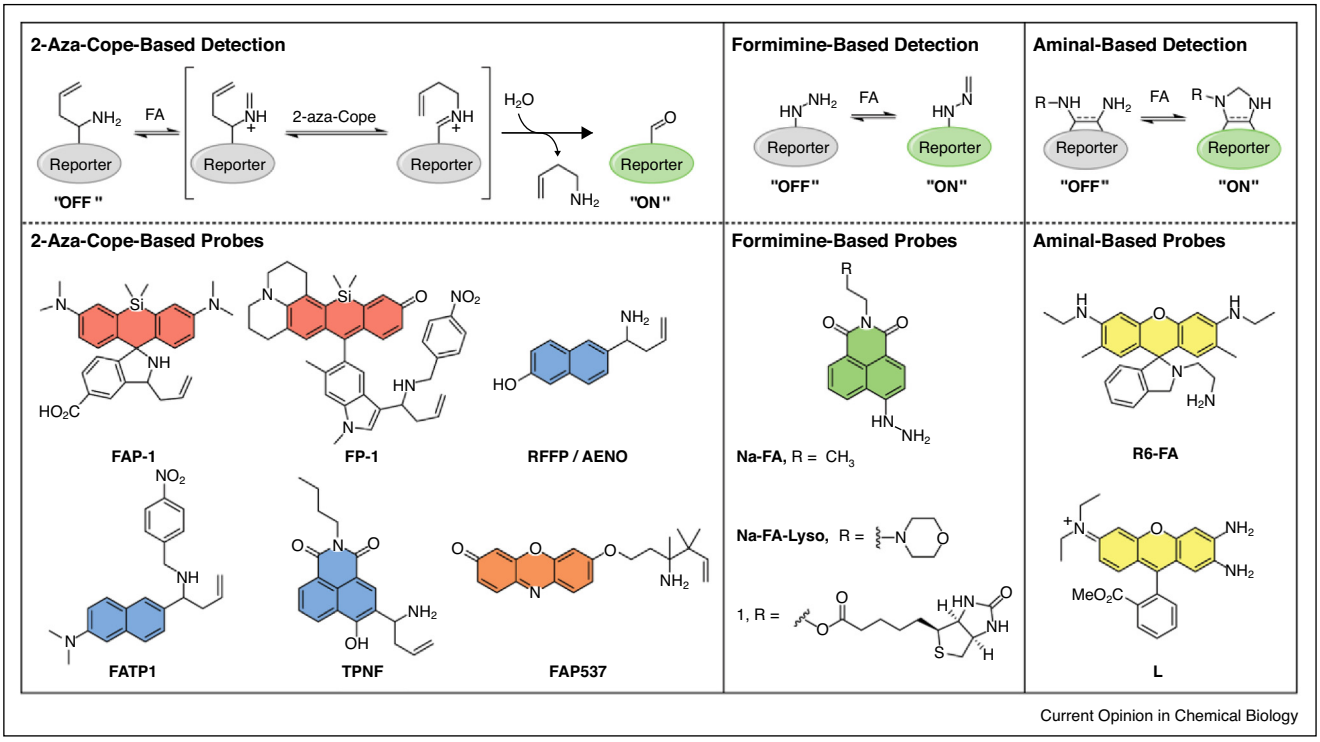
Effective fluorescent probes for imaging FA in living cells and higher specimens must meet several criteria. Key considerations include fast reactivity with FA and selectivity of the reaction against other biological analytes, especially other biologically-relevant RCS which possess similar electrophilic carbonyl groups. These competing RCS, including acrolein, 4-hydroxynonenal, and methylglyoxal, as well as other aldehyde-containing metabolites such as acetaldehyde, are generated primarily during metabolism and oxidative stress conditions, such as ethanol metabolism and lipid peroxidation [43,44]. Compared to the high micromolar endogenous concentrations of FA, steady-state levels of other RCS range from high nanomolar to low micromolar [45] and are estimated to be higher in various disease states [46]. Thus, the large variety of continually-generated RCS and their similar electrophilic reactivity makes selectivity perhaps the most important hurdle to overcome for developing reliable FA detection methods.

Several different but complementary mechanisms of FA detection have been reported, including 2-aza-Cope-, formimine-, and aminal-based detection methods (Figure 2), all offering unique advantages and disadvantages that are summarized below and in Table 1. In addition, such probes rely on a number of fluorescent scaffolds suitable for biological imaging with excitation wavelengths that span the visible to near-infrared spectrum and/or possess reasonable two-photon cross-sections [47]. Ultraviolet excitation is less desirable owing to interference from native autofluorescence from chromophores in the cell such as NADH and flavins as well as the potential for photodamage and oxidative stress induced by irradiation with high-energy light. FA detection can be realized by attaching various reactivity switches to fluorophore platforms, utilizing well-developed fluorescent response mechanisms ranging from spirocyclization, photoinduced electron transfer (PET), internal charge transfer (ICT), fluorophore uncaging, and ratiometric detection [48].

FA probes utilizing the 2-aza-Cope reaction

Our laboratory [49••] and Chan's laboratory [50••] independently and simultaneously developed the 2-aza-Cope-based strategy utilizing a homoallylic amine as a selective trigger for FA reactivity. In this scheme, the homoallylamine condenses with FA, undergoes the 2-aza-Cope rearrangement, and hydrolyzes to yield an aldehyde or ketone product (Figure 2). The 2-aza-Cope reaction exhibits high selectivity for FA over other RCS and carbonyl species, such as acetaldehyde, methylglyoxal, and 4-hydroxynonenal. The change in nucleophilicity and electronic properties on conversion of a homoallylamine to a carbonyl can be harnessed to provide a fluorescence turn-on or ratio change. Initial probes utilized near-infrared-emitting silicon rhodamine/rhodol scaffolds as the fluorophore with different sensing mechanisms to elicit a fluorogenic response. Our laboratory reported Formaldehyde Probe 1 (FAP-1), whose homoallylamine favors an initial spirocyclized, weakly emissive state [49••]. After FA reaction, the product aldehyde is incapable of spirocyclization, leading to an open, fluorescent product

Figure 2



Molecular probes for the detection of FA. Three general detection strategies have been taken: 2-aza-Cope-based, formimine-based, and aminal-based. Coloring reflects excitation wavelengths.

with an eight–fold turn-on response to 100 μM FA after 1 hour. High sensitivity and selectivity allowed FAP-1 to respond to exogenously-added FA in living cells within 30 min, and was also able to detect a decrease in endogenous FA levels in cells upon LSD1 inhibition. Concurrent work by the Chan group reported FP-1, which utilizes a PET mechanism to elicit a fluorescent response [50••]. Specifically, a nitrobenzene moiety was appended

Table 1

Summary of fluorescent FA probes

Probe	λ_{ex} (nm)	λ_{em} (nm)	Response to FA *in vitro*	Biological application
aza-Cope-based probes				
FAP-1 [49••]	645	662	Eight–fold turn-on to 0.1 mM FA (1 hour, 20 mM PBS)	Cells (HEK293T, MCF7)
FP1 [50••]	620	649	Seven–fold turn-on to 0.5 mM FA (3 hours, PBS)	Cells (HEK293T, NS1)
RFFP [51]	318	359 (451 post-FA)	53-fold ratio change to 3 mM FA (4 hours, 25 mM PBS/1% acetone)	Cells (HeLa)
AENO [52]	319	513	ca. 110-fold turn-on to 5 mM FA (3 hours, 10 mM PBS/20% DMF)	Cells (HeLa)
FATP1 [53]	390	526	25-fold turn-on to 0.2 mM FA (3 hours, 20 mM PBS/5% DMSO)	Cells (HEK293T, MCF7), Tissue (liver)
TPNF [54]	350	510	20-fold turn-on to 5 mM FA (3 hours, 10 mM PBS/0.5% DMSO)	Cells (HeLa), Zebrafish
FAP573 [55•]	573	585	4.2-fold turn-on to 0.1 mM FA (2 hours, 20 mM PBS/0.1% DMSO)	Cells (HEK293T, HAP1, MEF)
Formimine-based probes				
Na-FA [58••]	440	543	900-fold turn-on to 0.1 mM FA (30 min, 10 mM PBS/1% DMSO)	Cells (HeLa), Tissue (liver)
Na-FA-Lyso [60]	440	541	350-fold turn-on to 0.2 mM FA (30 min, 10 mM PBS/1% DMSO)	Cells (HeLa)
1 [59]	428	541	140-fold turn-on to 0.5 mM FA (8 min, 10 mM PBS/1% DMSO)	Cells (4T-1, 3T3), Tissue (tumor)
Aminal-based probes				
R6-FA [61••]	530	560	7.4-fold turn-on to 0.01 mM FA (10 sec, 25 mM PBS/50% DMF)	Cells (HeLa)
L [62]	520	620	ca. Seven–fold turn-on to 10 mM FA (10 mM Tris/30% EtOH)	Cells (L929)

to the homoallylamine, which quenches the rhodamine through donor-excited PET. After the 2-aza-Cope reaction, hydrolysis liberates the fluorophore from the nitrobenzene moiety, resulting in seven–fold fluorescence turn-on response to 250 μM FA after 3 hours. In cells, FP-1 displayed a 1.2-fold turn-on to 1 mM FA after 3 hours. These reports demonstrated the feasibility of utilizing fluorescence-based probes to detect FA in living cells, and specifically the utility of the 2-aza-Cope reaction as a detection platform.

The 2-aza-Cope strategy has subsequently been extended to other fluorophore scaffolds, establishing the generality of this reactive trigger for FA detection. Indeed, several groups have reported 2-aza-Cope probes for FA based on an ICT mechanism using an unfunctionalized homoallylamine switch. A homoallylamine appended to a naphthalene fluorophore furnished a UV-excitable ratiometric probe (reported by different groups as RFFP and AENO) [51,52]. These first-generation probes are highly selective for FA and exhibit good ratiometric responses, but are limited by relatively slow reaction kinetics, taking hours to display a statistically significant turn-on signal to millimolar levels of exogenously added FA and thus limiting their applicability to image endogenous FA pathways. The first examples of two-photon FA probes FATP1 [53] and TPNF [54] were applied to image FA in tissue and zebrafish, respectively. While FATP1 and TPNF require a 3–hour incubation in cells to show a statistically significant turn-on response, both probes illustrate the compatibility of the 2-aza-Cope reaction with tissue and whole organism settings.

Although highly selective for FA, the use of a parent homoallylamine for the 2-aza-Cope reaction leaves room for improvement owing to its relatively slow reaction kinetics. Indeed, in organic synthesis, 2-aza-Cope reactions are often performed at higher temperatures to promote reactivity; however, biological constraints preclude altering temperature above physiological levels, and as such improving reactivity must be addressed through structural modifications of the homoallylamine trigger. Recent efforts from our lab led to the development of a 2-aza-Cope-based trigger for FA with improved kinetics through structural modification of the homoallylamine moiety [55•]. In particular, appending *gem*-dimethyl substituents accelerated the 2-aza-Cope reaction about 10-fold through the Thorpe–Ingold effect [56], allowing for a responsive and selective series of FA probes. One such probe, FAP573, was capable of visualizing increased endogenous FA levels in ADH5 knockout cells compared to the wildtype counterpart. We have also utilized this motif to develop a first-generation ratiometric FA probe with visible excitation and emission profiles [57•]. Overall, this aforementioned collection of fluorescent probes demonstrates the broad applicability of the 2-aza-Cope reaction for detection of biological FA, and further extensions of this strategy will allow for tuning of excitation/emission wavelengths and subcellular localization, as well as expansion to other imaging modalities.

Formimine and aminal-based FA probes

Alternative reactivity-based approaches for FA detection exploit the formimine- or aminal-forming properties between FA and amines. Both approaches display fast reaction kinetics but are more challenging to tune for FA selectivity over other aldehydes, thus offering distinct advantages and disadvantages over the 2-aza-Cope strategy. Lin and colleagues have elegantly employed a hydrazine moiety to create a series of probes using the 1,8-naphthalimide fluorophore (Na-FA) [58••]. The free amine on the hydrazine initially quenches fluorescence through a PET mechanism, which is blocked after formimine formation. By modifying the fluorescent scaffold, biotin-guided (1) [59] and lysosome-targeting (Na-FA-Lyso) [60] FA probes were created. Na-FA displays an *in vitro* 900-fold increase in fluorescence to 100 μM FA in 30 min, but *in cellulo* reactivity appears to be more sluggish and comparable to 2-aza-Cope congeners. A key set of tissue imaging experiments include endogenous changes in FA from the addition of sodium bisulfite, a FA scavenger which lowers levels of free FA by sequestration as the bisulfite adduct. Bisulfite addition shows a decrease in fluorescence compared to basal conditions, suggesting the probes are capable of imaging changes in endogenous FA *in cellulo*. An important consideration in performing such FA sequestration experiments is to verify the *in vitro* response of FA probes toward bisulfite alone does not display fluorescence quenching. Formimine-based probes can also respond to simple aldehydes such as acetaldehyde, albeit with much less of a response compared to FA, and this promiscuity provides a promising starting point to create probes for acetaldehyde and other RCS in addition to FA.

Aminal formation-based probes exploit slightly different reactivity to detect FA. The Lin group reported R6-FA, which utilizes a spirocyclization method, similar to FAP-1, to induce a fluorescence turn-on [61••]. The spirocyclized amine moiety is linked to a free, FA-reactive amine. The Schiff base resulting from reaction with FA is more electrophilic than the xanthone, resulting in aminal heterocycle formation and concurrent fluorescent turn-on. While highly reactive (FA-induced turn-on occurs within 10 s), this method displays relatively poor selectivity for FA over other carbonyl-containing species. Along similar lines, the Zeng group utilized aminal heterocycle formation to create a dual methylglyoxal/FA probe (L) [62]. However, fluorescence turn-on by FA and turn-off by methylglyoxal makes *in cellulo* experiments difficult to interpret, as both analytes are present in the micromolar concentration range, limiting its application.

Conclusions and outlook

Development of a variety of fluorescent probes for FA has greatly expanded the capabilities to image FA in living biological systems. All three strategies discussed in this review show promise for further interrogation of the roles FA plays in biology. However, probe sensitivity remains hindered by either reaction kinetics for the 2-aza-Cope strategy or selectivity for the formimine- and aminal-based detection methods. Improving reaction kinetics is particularly important owing to the rapid elimination of FA, which has a measured half-life of approximately 90 s in organisms [63]. The presence of competing endogenously produced RCS also makes selectivity a key design consideration for confidence in interpreting the fluorescence response as a consequence of FA reactivity alone. Further investigation into the mechanisms of previously unstudied FA biological pathways will benefit from a method able to combine the advantages of the two approaches to create a responsive and selective modality. The tunability of the homoallylamine moiety for the 2-aza-Cope strategy, as demonstrated in FAP573, presages the potential for increased reactivity while conserving selectivity. Because of its generality, as showcased by the various probes discussed above, the 2-aza-Cope strategy shows promise for extension into alternate imaging modalities. Indeed, we recently reported a positron emission tomography-based probe, [^{18}F]FAC-FDG, for *in vivo* FA imaging [64]. Likewise, the fast kinetics of formimine formation offer a large advantage which could be harnessed through further tuning to inhibit reactivity toward competing carbonyl species. In particular, increased steric encumbrance could afford enhanced selectivity over larger aldehydes. Future experiments with improved FA probes will help elucidate the roles FA may play in physiology and disease related to demethylation events, one-carbon metabolism, and downstream effects of FA metabolism.

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