Published in final edited form as:

Anal Methods. 2017 ; 9(23): 3418–3421. doi:10.1039/C7AY01238A.

Imaging Acetaldehyde Formation During Ethanol Metabolism in Living Cells using a Hydrazinyl Naphthalimide Fluorescent Probe

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Abstract

The metabolism of ethanol to acetaldehyde has been visualized in living lung epithelial cells using a hydrazinyl naphthalimide fluorescent probe. Utilizing a condensation reaction between carbonyls and a hydrazine moiety, we demonstrate that the fluorescent probe (Aldehydefluor-1) AF1 reacts with a range of reactive carbonyl species including formaldehyde, acetaldehyde, glyoxylic acid, and methyl glyoxal. With AF1, it is possible to directly visualize endogenous carbonyl metabolites. Here, we have applied it towards the visualization of acetaldehyde generated from alcohol dehydrogenase mediated ethanol metabolism, validating it as a useful tool to study the roles of alcohol in respiratory disease and other pathological mechanisms.

Acetaldehyde plays a notorious role as a toxic intermediate in the enzymatic metabolism of ethanol.^{1,2,3} The undesired side effects of this metabolism – headache, nausea, dry mouth, vertigo, and sensitivity to light and sound – mark the well-known symptoms of a hangover. The lungs are particularly vulnerable to ethanol-mediated tissue damage due to repeated exposure to ethanol after ingestion.⁴ Mammalian lungs express class I alcohol dehydrogenase^{5,6} and ethanol metabolism can potentially proceed via oxidative or non-oxidative mechanisms.⁷ Heavy ethanol consumption has been implicated as a risk factor in chronic obstructive pulmonary disease (COPD) and the term "whiskey bronchitis" has been used to describe this ethanol-mediated etiology.⁸ Interestingly, ethanol can both relieve⁹ and exacerbate¹⁰ acute and chronic airway obstruction, depending on factors such as the mode of delivery and genetics. Studies have demonstrated that administration of alcohol to patients with asthma results in significant bronchodilation.¹¹ However, it has also been demonstrated that acetaldehyde, the first product of ethanol metabolism, initiates bronchoconstriction.¹⁰ There exists a clear, but complicated, relationship between ethanol metabolism and respiratory ailments such as asthma.

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[†]Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here].
See DOI: 10.1039/x0xx00000x

Despite progress, studying lung epithelial ethanol metabolism remains challenging due to a lack of methods for the direct detection of metabolic products in living models. Measurements in mammalian lung tissue homogenates show a steep dependence on experimental conditions, particularly pH.^{12,13} As a result, the precise biochemical events that lead to these pathologies remain unclear. This is mainly due to the furtive nature of acetaldehyde, necessitating careful sample processing and detection methods such as spectrophotometry, nuclear magnetic resonance imaging (NMR), or gas chromatography mass-spectrometry (GC-MS), which typically result in processing and destruction of the sample.¹⁴ The use of carbonyl-responsive fluorescent probes^{15–21} could potentially circumvent these problems by allowing for the real-time, unobtrusive visualization of acetaldehyde as it is produced in living cells.

Inspired by a previous study,²² we anticipated that a hydrazinyl naphthalimide-based fluorescent probe would react with aldehydes to yield hydrazone products, providing a fluorescence response that would increase with higher levels of aldehydes. If combined with the proper controls, this probe could be useful for tracking enzymatically generated acetaldehyde in living cells. With this goal in mind, the fluorescent probe **AF1** was synthesized from the commercially available starting material 4-bromo-1,8-naphthalimide, a fluorescent naphthalic scaffold (Scheme 1). Introduction of a hydrazine moiety using a nucleophilic aromatic substitution reaction provides **AF1**, which displays quenched fluorescence likely due to photoinduced electron transfer (PET) quenching. Reaction of **AF1** with acetaldehyde results in the formation of the hydrazone structure, which was confirmed via ¹H NMR and high resolution mass spectrometry (Fig. S5).

After synthesizing **AF1**, the purified crystalline product was aliquoted into individual Eppendorf tubes and kept at –20 °C until use. For initial experimentation, an aliquot of **AF1** was removed from storage at –20 °C and allowed to thaw before addition of enough dimethyl sulphoxide (DMSO) to make a 2.5 mM solution. This dissolved aliquot was then refrozen until the next experiment, and this freeze-thaw cycle repeated until no aliquot remained. However, a significant decrease in fluorescent turn-on was observed after a single freeze-thaw cycle of a given dissolved **AF1** aliquot (Fig. S6), indicating the importance of using a fresh aliquot for experiments. This reduction in signal over time may help explain differences in selectivity of fluorescent probes with similar chemical structures.¹⁹ All further experiments were run with **AF1** aliquots dissolved in DMSO the same day of experimentation.

Upon reacting 10 μM **AF1** with 200 μM acetaldehyde in a buffered aqueous system, a 13-fold increase in fluorescence emission at 551 nm was observed after 60 minutes (Fig. 1). Selectivity studies were performed with 10 μM **AF1** and 100 μM of biologically relevant reactive carbonyls, reducing sugars, and selected sulphur, oxygen, and nitrogen species (Fig. 2). In addition to the fluorescence response towards acetaldehyde, we note that a significant turn-on was observed for formaldehyde, glyoxylic acid, and methyl glyoxal, indicating that this class of hydrazinyl-based aldehyde probes is not completely selective for a single reactive carbonyl species. The increased response for formaldehyde agrees with previous studies showing that formaldehyde is more effective at trapping hydrazine groups than more

substituted carbonyls like acetone²³ and follows known reactivity trends of aldehydes and ketones with aryl hydrazine molecules.²⁴

Having characterized the response and selectivity, the fluorescent probe **AF1** was then characterized in living human lung epithelial cells (A549) using an EVOS-fl fluorescence microscope equipped with a GFP filter cube. First, the ability of **AF1** to image exogenous acetaldehyde was examined. Only low fluorescent intensity was observed upon addition of **AF1** to A549 cells (Fig. 3a), with a 2-fold increase in fluorescent response upon addition of 100 μ M acetaldehyde (Fig. 3b) and a 5-fold increase upon addition of 1 mM acetaldehyde (Fig. 3c). This trend clearly indicates the ability of **AF1** to report on exogenous acetaldehyde in a cellular environment.

Next, we analysed the efficacy of **AF1** as a tool in the measurement of endogenous acetaldehyde, specifically as a result of alcohol dehydrogenase mediated metabolism of ethanol.⁷ Primary lung epithelial cells display significant biological responses to ethanol upon incubation between 2 and 6 hours.^{25–27} Therefore, we decided to utilize **AF1** to monitor dose-dependent acetaldehyde formation with 2-hour incubations with increasing concentrations of ethanol. We observed an increase in fluorescence emission with increasing ethanol concentration, which reached a maximum and statistically significant ($p < 0.01$) value at 50 mM ethanol (Fig. 4). In order to confirm that the observed signal was due to the activity of alcohol dehydrogenase, the selective class I alcohol dehydrogenase inhibitor fomepizole (FPZ) was employed (Fig. 5).^{28,29} A comparison of cells incubated with solely ethanol (Fig. 5b) to cells incubated with both ethanol and FPZ (Fig. 5c) showed a visible and significant ($p < 0.05$) decrease in overall fluorescent intensity in the cells allowed to co-incubate with FPZ. This key experiment reveals a decrease in the metabolism of ethanol to acetaldehyde, indicating that signal from **AF1** under these conditions is predominately due to cellular acetaldehyde formation.

Based on these results, the following conclusions can be made: (1) The hydrazinyl naphthalimide scaffold provides a fluorescence response to a range of aldehydes including acetaldehyde, formaldehyde, glyoxylic acid, and methyl glyoxal, (2) aliquots must be used shortly after preparation to maintain a reliable response, and (3) **AF1** can be used to detect cellular generated acetaldehyde from ethanol metabolism in A549 cells. Similar hydrazinyl naphthalimide scaffolds have been used for detecting other reactive carbonyl species,^{19,22} highlighting the need to carefully interpret results in light of appropriate controls. In this study, fomepizole was employed as an inhibitor for alcohol dehydrogenase, indicating that the metabolism of ethanol to acetaldehyde is responsible for the probe's response. While a fluorescent probe with higher selectivity for acetaldehyde would be preferred, this study provides a singular example of acetaldehyde imaging in living cells and should serve as a useful method for researchers in the field.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Institute Of General Medical Sciences of the National Institutes of Health under Award Number R15GM114792. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional support was provided by Southern Methodist University (start-up funds to A.R.L. and Engaged Learning funds to A.G.R.) We acknowledge Sara Lange for assistance with preliminary experiments. A.R.L. discloses a financial stake in Biolum Sciences, LLC, a company developing asthma monitoring technology.

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Figure 1. Fluorescence response after treating 10 μM **AF1** with 200 μM CH_3CHO in 20 mM HEPES buffer (pH 7.4) containing 0.2% DMSO for 0, 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes. $\lambda_{\text{ex}} = 440$ nm.

Figure 2.

Fluorescence response of 10 μM **AF1** to biologically relevant reactive carbonyl species and selected reactive sulphur, oxygen, and nitrogen species in 20 mM HEPES buffer (pH 7.4) containing 0.2% DMSO. Bars represent fold turn-on at 550 nm acquired 1, 5, 10, 15, 20, 25, and 30 minutes after addition of the analyte. Data were acquired using $\lambda_{\text{ex}} = 440$ nm. Error bars are $\pm$ S.D. Legend (all 100 μM except glutathione at 5 mM): (1) blank; (2) acetaldehyde; (3) formaldehyde; (4) 4-hydroxynonenal; (5) glyoxal; (6) glyoxylic acid; (7) methyl glyoxal; (8) sodium pyruvate; (9) α -ketoglutaric acid; (10) glutathione, 5 mM; (11) DEA NONOate; (12) Angeli's salt; (13) fructose; (14) glucose.

Figure 3.

Fluorescence microscopy images of exogenous acetaldehyde detection in live A549 cells using **AF1**. (a) A549 cells incubated with 10 μM **AF1** for 60 minutes at 37 $^{\circ}\text{C}$. (b) A549 cells incubated with 10 μM **AF1** for 60 minutes at 37 $^{\circ}\text{C}$ with 100 μM acetaldehyde added for the final 30 minutes. (c) A549 cells incubated with 10 μM **AF1** for 60 minutes at 37 $^{\circ}\text{C}$ with 1 mM acetaldehyde added for the final 30 minutes. (d)–(f) Brightfield images of the fields in (a)–(c). (g) Quantification of cellular imaging experiments. Error bars are S.E. Values are the average of $n = 16$ fields of cells from 5 biological replicates.

Figure 4.

Fluorescence microscopy images of acetaldehyde generated during ethanol metabolism in live A549 cells using **AF1**. (a) A549 cells incubated with 10 μ M **AF1** for 180 minutes at 37 $^{\circ}$ C. (b) A549 cells incubated with 10 μ M **AF1** for 180 minutes at 37 $^{\circ}$ C with 25 mM ethanol added for the final 150 minutes. (c) A549 cells incubated with 10 μ M **AF1** for 180 minutes at 37 $^{\circ}$ C with 50 mM ethanol added for the final 150 minutes. (d)–(f) Brightfield images of the fields in (a)–(c). (g) Quantification of cellular imaging experiments. Error bars are S.E. Control values are $n = 34$ fields of cells from 8 biological replicates, 50 mM ethanol

values are averages from $n = 34$ fields of cells from 8 biological replicates, and other values are averages of $n = 9$ – 10 fields of cells from 3 biological replicates. Statistical significance was assessed using a two-tailed student's t-test. ** $p < 0.01$.

Figure 5.

Fluorescence microscopy images of the inhibition of acetaldehyde generated during ethanol metabolism in live A549 cells using AF1. (a) A549 cells incubated with 10 μ M AF1 for 180 minutes at 37 °C. (b) A549 cells incubated with 10 μ M AF1 for 180 minutes at 37 °C with 50 mM ethanol added for the final 150 minutes. (c) A549 cells incubated with 10 μ M AF1 for 180 minutes at 37 °C with 50 mM ethanol and 1 mM fomepizole added for the final 150 minutes. (d)–(f) Brightfield images of the fields in (a)–(c). (g) Quantification of cellular imaging experiments. Error bars are S.E. Control values are n = 34 fields of cells from 8

biological replicates, 50 mM ethanol values are averages from $n = 34$ fields of cells from 8 biological replicates, 50 mM EtOH + FPZ values are the average of $n = 21$ fields of cells from 4 biological replicates. Statistical significance was assessed using a two-tailed student's t-test. ** $p < 0.01$, * $p < 0.05$.

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Scheme 1. Synthesis and reaction-based acetaldehyde detection of **AF1**.