Supporting Information:

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

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1. Synthetic Procedures.

General Methods. All reactions were performed in oven-dried glassware under an atmosphere of N2. Reagents were used without further purification. Reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Alfa Aesar (Ward Hill, MA). Analytical thin layer chromatography was performed using Silicycle 60 F254 silica gel (pre-coated sheets, 0.25 mm thick). Plates were visualized by fluorescence quenching under UV light. 1H and 13C NMR spectroscopy was performed on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz). Splitting patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; br, broad. Chemical abbreviations are indicated as follows: EtOH, ethanol; EtOAc, ethyl acetate; H2O, water; N2, nitrogen. High-resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) at the Shimadzu Center for Advanced Analytical Chemistry at the University of Texas, Arlington.
6-bromo-2-(2-methoxyethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (1). 4-bromo-1,8-naphthalic anhydride (570 mg, 2.06 mmol, 1.0 equiv) was dissolved in 30 mL of EtOH followed directly by the addition of 2-methoxyethylamine (0.18 mL, 2.1 mmol, 1.0 equiv). The reaction was allowed to stir for 12 h at reflux. The reaction mixture was concentrated to yield compound 1 (67.9 mg, 98% yield) as a brown solid and was used in the next step without further purification. \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 8.66 (d, 1H, \(J = 7.5\) Hz), 8.57 (d, 1H, \(J = 7.5\) Hz), 8.42 (d, 1H, \(J = 8.0\) Hz), 8.04 (d, 1H, \(J = 8.0\) Hz), 7.85 (t, 1H, \(J = 8.0\) Hz), 4.43 (t, 2H, \(J = 6.0\) Hz), 3.73 (t, 2H, \(J = 6.0\) Hz), 3.37 (s, 3H); \(^{13}\)C NMR (500 MHz, CDCl\(_3\)) \(\delta\) 163.75, 133.36, 132.23, 131.41, 131.14, 130.64, 130.38, 129.08, 128.13, 123.04, 122.19, 69.65, 58.90, 39.47; HRMS calcd for C\(_{15}\)H\(_{12}\)BrNO\(_3\)Na [M+Na]\(^+\) 355.9893, found 355.9904.

Figure S1. \(^1\)H NMR (500 MHz) spectrum of 1 in CDCl\(_3\).
Figure S2. $^{13}$C NMR (125 MHz) spectrum of 1 in CDCl$_3$.

6-hydrazineyl-2-(2-methoxyethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione, Aldehydefluor 1 (AF1). Compound 1 (213 mg, 0.638 mmol, 1.0 equiv) was dissolved in 10 mL of 2-methoxyethanol followed directly by the addition of hydrazine monohydrate (1.25 mL, 25.5 mmol, 40 equiv). The reaction was allowed to stir for 12 h at 110 °C. The mixture was then concentrated. The resulting product was purified by recrystallization in hot EtOH to afford AF1 as an orange crystalline solid (50.4 mg, 26% yield). $^1$H NMR (500 MHz, DMSO-d$_6$) δ 9.12 (s, 1H), 8.57 (d, 1H, $J$ = 6.3 Hz), 8.38 (d, 1H, $J$ = 6.3 Hz), 8.25 (d, 1H, $J$ = 8.6 Hz), 7.60 (t, 1H, $J$ = 7.5), 7.21 (d, 1H, $J$ = 8.6 Hz), 4.18 (t, 2H, $J$ = 6.3 Hz), 3.52 (t, 2H, $J$ = 6.3 Hz), 3.20 (s, 3H); $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ 164.40, 163.44, 134.84, 131.23, 128.90, 124.70, 118.98, 114.40, 109.95, 104.60, 69.35, 58.44, 40.04, 38.65; HRMS calcd for C$_{15}$H$_{14}$N$_3$O$_3$ [M–H]$^+$ 284.1041, found 284.1032.
Figure S3. $^1$H NMR (500 MHz) spectrum of AF1 in DMSO-$d_6$.

Figure S4. $^{13}$C NMR (125 MHz) spectrum of AF1 in DMSO-$d_6$. 
6-(2-ethylidenehydrazineyl)-2-(2-methoxyethyl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (2). AF1 (1.6 mg, 0.0054 mmol) was dissolved in 1 mL of DMSO-d₆ followed directly by the addition of two drops of acetaldehyde, upon which the solution brilliantly fluoresced. The reaction was allowed to sit for 8 h before NMR spectra were collected. 

¹H NMR (500 MHz, CDCl₃) δ 11.02 (s, 1H), 8.69 (d, 1H, J = 8.6 Hz), 8.43 (d, 1H, J = 8.0 Hz), 8.29 (d, 1H, J = 8.6 Hz), 7.76 (q, 1H, J = 5.2 Hz), 7.72 (dd, 1H, J = 8.6 Hz, 7.4 Hz), 7.47 (d, 1H, J = 8.6 Hz), 4.19 (t, 2H, J = 6.3 Hz), 3.53 (t, 2H, J = 6.4 Hz), 1.19 (d, 3H, J = 5.2 Hz); HRMS calcd for C₁₇H₁₆N₃O₃ [M-H] - 310.1197, found 310.1207.

Figure S5. ¹H NMR (500 MHz) spectrum of AF1 and acetaldehyde in DMSO-d₆.

2. Spectroscopic Materials and Methods. All spectroscopic measurements were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Samples were measured in quartz cuvettes (Starna, Atascadero, CA) with dimensions of 1 cm x
0.1 cm. Acetaldehyde (CH\textsubscript{3}CHO) and other stock solutions were prepared in DI water the day of each experiment and kept on ice.

**Fluorescence Response.** Wavelength scans of fluorescent emission of 10 µM AF1 before and after the addition of 200 µM acetaldehyde were acquired in 20 mM HEPES buffer (pH 7.4). Solutions were prepared in a total of 500 µL HEPES including 2 µL of a 2.5 mM AF1 stock solution in DMSO (10 µM final concentration). Fluorescent emission of the solution was collected at 1, 5, 10, 15, 20, 25, and 30 minutes after the addition of acetaldehyde (Figure 1) or 30 minutes after the addition of acetaldehyde (Figure S6). Relative turn-on refers to fluorescent turn-on at the time indicated divided by fluorescent emission of the blank.

![Figure S6](image)

**Figure S6.** Decrease in fluorescence response of 10 µM AF1 to 200 µM acetaldehyde over time. 400 µL DMSO was added to probe aliquot to make 2.5 mM and stored in the freezer between uses. Data were acquired in 20 mM HEPES buffered to pH 7.4 containing 0.2% DMSO with excitation at λ\textsubscript{ex} = 440 nm. Emission was collected between 460 and 650 nm. Points represent relative turn on of 10 µM AF1 in response to 200 µM acetaldehyde after 30 minutes of incubation on various days.

3. **Selectivity Studies.** Selectivity studies for AF1 were performed in 20 mM HEPES (pH 7.4) containing 0.2% DMSO and monitored using an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) every 5 min over 30 min. Reactions were performed as triplicates. All probe aliquots were prepared as 2.5 mM stock solutions in DMSO and were utilized immediately after dissolving.

Blank: Into an Eppendorf tube was added 498 µL 20 mM HEPES followed by 2 µL of 2.5 mM AF1 (10 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of AF1.
Acetaldehyde (100 µM): A 50 mM stock solution of acetaldehyde was made by adding 1.4 µL acetaldehyde to 499 µL chilled DI water. This solution was further diluted to 5 mM (100 µL of 50 mM stock into 900 µL chilled DI water). Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM acetaldehyde stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of acetaldehyde.

Formaldehyde (100 µM): A 5 mM stock solution of formaldehyde was prepared by adding 14.9 µL of 37% w/v formaldehyde to 40 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM formaldehyde stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of formaldehyde.

4-Hydroxynonenal (100 µM): A 5 mM stock solution of 4-hydroxynonenal was prepared by adding 3.1 µL 4-hydroxynonenal (1 mg per 100 µL EtOH) to 37 µL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM 4-hydroxynonenal stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of 4-hydroxynonenal.

Glyoxal (100 µM): A 5 mM stock solution of glyoxal was prepared by adding 11.4 µL of 40% w/v glyoxal to 20 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM glyoxal stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of glyoxal.

Glyoxylic Acid (100 µM): A 5 mM stock solution of glyoxylic acid was prepared by adding 9.6 mg glyoxylic acid to 20.8 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM glyoxylic acid stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of glyoxylic acid.

Methylglyoxal (100 µM): A 5 mM stock solution of methylglyoxal was prepared by adding 5 µL methylglyoxal to 16.3 mL chilled DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM methylglyoxal stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of methylglyoxal.

Acetone (100 µM): A 5 mM stock solution of acetone was prepared by adding 7.3 µL acetone to 20 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM acetone stock (100 µM). This solution
was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of acetone.

**Sodium Pyruvate (100 µM):** A 5 mM stock solution of sodium pyruvate was prepared by adding 7.5 mg sodium pyruvate to 13.6 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM sodium pyruvate stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of sodium pyruvate.

**α-Ketoglutaric Acid (100 µM):** A 5 mM stock solution of α-ketoglutaric acid was prepared by adding 3.6 mg α-ketoglutaric acid to 3.18 mL DI water. Into an Eppendorf tube was added 488 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 5 mM α-ketoglutaric acid stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of α-ketoglutaric acid.

**Glutathione (5 mM):** A 250 mM stock solution of glutathione was prepared by dissolving 23.3 mg glutathione in 303 µL DI water. Into an Eppendorf tube was added 488 µL of 20 mM Hepes, 2 µL of 2.5 mM AF1 (10 µM), and 10 µL of 250 mM glutathione stock (5 mM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of glutathione.

**H₂O₂ (100 µM):** A 11.6 mM stock solution of H₂O₂ was prepared by adding 100 µL 11.63 M H₂O₂ to 900 µL DI water. Into an Eppendorf tube was added 494 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 4.3 µL of 11.6 mM H₂O₂ stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of H₂O₂.

**DEANONOate (100 µM):** A 6.03 mM stock solution of DEANONOate was prepared by dissolving DEANONOate in 0.01 M NaOH (verified by UV-Vis, ε = 6500 M⁻¹ cm⁻¹). Into an Eppendorf tube was added 490 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 8 µL of 6.03 mM DEANONOate stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of DEANONOate.

**Angeli’s Salt (AS) (100 µM):** A 32.7 mM stock solution of AS was prepared by dissolving AS in 0.01 M NaOH (verified by UV-Vis, ε = 6100 M⁻¹ cm⁻¹). Into an Eppendorf tube was added 497 µL of 20 mM HEPES, 2 µL of 2.5 mM AF1 (10 µM), and 1.5 µL of 32.7 mM AS stock (100 µM). This solution was vortexed and transferred to a quartz cuvette, where its fluorescent intensity was measured 1, 5, 10, 15, 20, 25, and 30 min after the addition of AS.

### 4. Cell Culture and Fluorescence Microscopy

Adenocarcinoma human alveolar basal epithelial cells (A549) were purchased from ATCC. Complete cell culture media was
prepared by adding 10% (v/v) FBS (HyClone) to F12K (ATCC), and 1% (v/v) pen/strep (10,000 units/ml penicillin and 10,000 µg/ml streptomycin purchased from HyClone) and used for cell culture. Cells were passaged every 3 to 4 days in T75 flasks until healthy enough for experimentation. All experiments were performed in sterile 6-well plates (Falcon), which were seeded with cells 24 hours prior to use. 6-well plates were incubated in 5% CO₂ at 37 °C overnight until roughly 75% confluent. During treatment of the cells for experimentation, F12K was replaced with Fluorobrite™ DMEM (Thermo Fischer Scientific) for clearer fluorescent imaging. A final concentration of 10 µM AF1 was used in all experiments. Fluorescent imaging was done using an EVOS-fl fluorescent microscope (Advanced Microscopy Group) equipped with a GFP filter cube.

**Image Quantification.** To reduce human bias, images were analyzed in ImageJ via the method outlined in Figure S7. The raw images were converted to 8-bit images. A threshold was determined using the Huang algorithm¹ and proper selection of cells was confirmed by visual inspection as shown in Figure S7. A selection was created based on this threshold and the average pixel intensity was measured. Typically, three fields per well were measured and at least 3 biological replicates were performed for each set of conditions. All images were analyzed identically. Figure S8–S10 show two additional biological replicates for images in Figures 3–5, respectively. Table S1 shows a summary of all quantified cellular imaging data acquired in this study.

![Figure S7](image-url)

**Figure S7.** Turn-on of A549 cells upon addition of 10 µM AF1 and 100 µM acetaldehyde (b) along with corresponding brightfield image (a). Quantification of cells was performed using ImageJ software. Image was opened in ImageJ and converted to an 8-bit image. The threshold was then adjusted via the Huang algorithm with a dark background. Finally, the cells were selected as shown in (c) and the mean pixel intensity was measured.

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**Figure S8.** Turn-on of A549 cells upon addition of 10 μM AF1 and (a, h) 0 μM acetaldehyde (b, i) 100 μM acetaldehyde (c, j) 1 mM acetaldehyde and their corresponding brightfield images (d, e, f, k, l, m). A549 cells were first incubated with AF1 for 30 minutes before the addition of acetaldehyde. After a second 30-minute incubation, they were imaged at 40% light intensity and a 500 ms exposure on an EVOS-fl microscope.

**Figure S9.** Turn-on of A549 cells upon addition of 10 μM AF1 and (a, b) 0 mM EtOH (c, d) 25 mM EtOH (e, f) 50 mM EtOH A549 cells were first incubated with 10 μM AF1 for 30 min before the addition of EtOH. After a second 120 min incubation, they were imaged at 50% light intensity and a 250 ms exposure on an EVOS fluorescent microscope.

**Figure S10.** Turn-on of A549 cells upon addition of 10 μM AF1 and (a, b) 0 mM EtOH (c, d) 50 mM EtOH (e, f) 50 mM EtOH and 1 mM fomepizole. A549 cells were first incubated with 10 μM AF1 for 30 min before the addition of EtOH and fomepizole. After a second 120 min incubation, they were imaged at 50% light intensity and a 250 ms exposure on an EVOS fluorescent microscope.
Table S1. Mean pixel intensities of all images acquired in this study.

| Acetaldehyde / mM | 0 | 100 | 1000 | 0 | 0 | 0 | 0 | 0 | EIDH / mM | 0 | 0 | 0 | 0 | 0 | 25 | 50 | 100 | 200 | 500 |
|-------------------|---|-----|------|---|---|---|---|---|----------------|---|---|---|---|---|----|---|----|----|----|----|
| Date              | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 | 8/17/16 | 8/19/16 |
| Settings          | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity | 50% light intensity |
| Plate #           | 1 | 2 | 3 | 4 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| Field #           | 1 | 2 | 3 | 4 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |

The data for the variable 50% light intensity vs 50 mM EtOH was found to be a significant outlier (P < 0.05) by the Grubbs' test and was removed from the analysis.

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