



Article

Experimental Conditions That Influence the Utility of 2'7'-Dichlorodihydrofluorescein Diacetate (DCFH₂-DA) as a Fluorogenic Biosensor for Mitochondrial Redox Status

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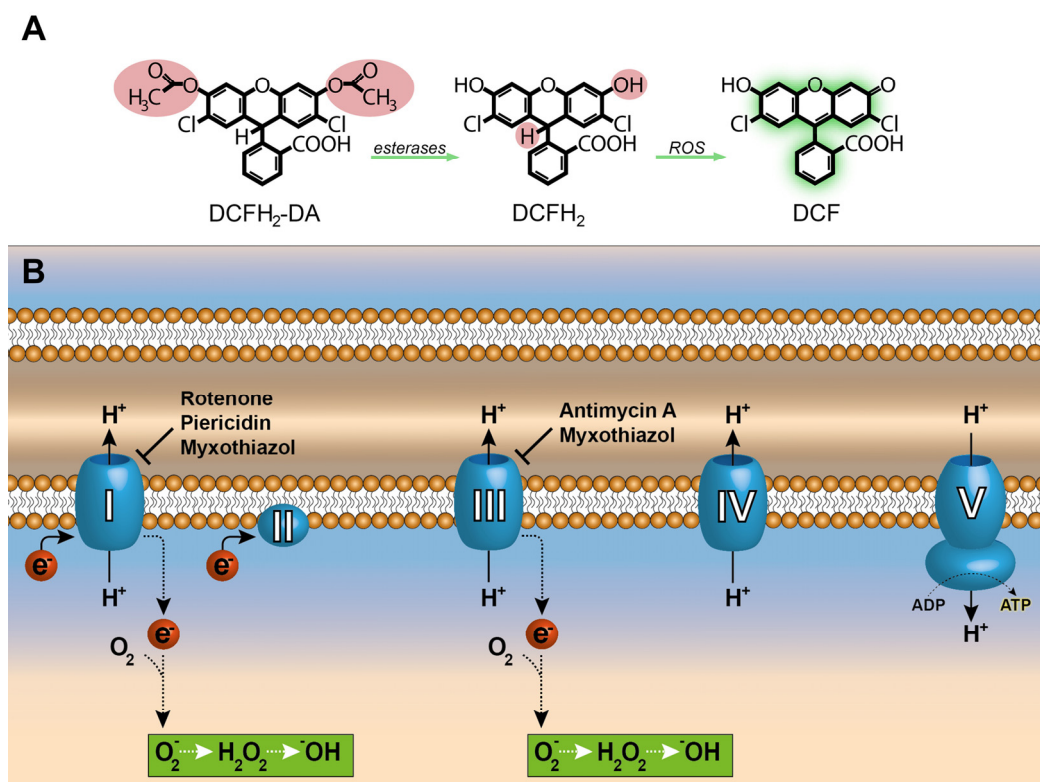


Figure S1. Reaction scheme of DCFH₂-DA to DCF (A) and a simplified overview of the electron transport chain and used inhibitors (B).

Table S1. List of chemicals and reagents.

Compound	Purity	Supplier	Additional information
Accutase	n/a	Innovative Cell Technologies [€]	
Accumax	n/a	Innovative Cell Technologies [€]	
Antimycin A	n/a	Sigma-Aldrich [#]	
bovine serum albumin (BSA),	≥ 96%	Sigma-Aldrich [#]	
2'7'-dichlorodihydrofluorescein diacetate (DCFH ₂ -DA)	n/a	Life Technologies [†]	
Dihydrorhodamine 123	n/a	Life Technologies [†]	
DMSO	≥ 99.5%	Merck Millipore [*]	
DNA stain Hoechst 33342	n/a	ImmunoChemistry Technologies [‡]	
Dulbecco's modified Eagle medium	n/a	Lonza [†]	Phenol red-free
Dulbecco's modified Eagle medium/Ham's F12	n/a	Hyclone [‡]	1:1 volume ratio
Ethanol absolute (CH ₃ CH ₂ OH)	≥ 99.5%	J.T. Baker ^{&}	

Fetal calf serum (FCS)	n/a	Bodinco [?]	
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	≥ 99%	Merck Millipore [*]	
Human recombinant insulin solution	≥ 27 U/mg	Sigma-Aldrich [#]	
Hydrocortisone 21-hemisuccinate	n/a	Sigma-Aldrich [#]	
Myxothiazol	≥ 98%	Sigma-Aldrich [#]	
L-glutamine	n/a	Lonza [†]	
Methanol (MeOH)	≥ 99%	Alfa Aesar [§]	
MitoSOX	n/a	Life Technologies [‡]	
Rotenone	≥ 95%	Sigma-Aldrich [#]	
tris(hydroxymethyl)aminomethane (TRIS),	≥ 99.8%	Sigma-Aldrich [#]	
Penicillin/streptomycin	n/a	Lonza [†]	
Sterile phosphate-buffered saline	n/a	Fresenius Kabi [§]	
Piericidin A	≥ 95%	Santa Cruz Biotechnology [†]	
Roswell Park Memorial Institute medium 1640 (RPMI-1640)	n/a	Lonza [†]	Phenol red-free
Water	Milli-Q	Merck Millipore ^{&}	18.2 MΩ · cm at 25 °C
William's E medium	n/a	Lonza [†]	Phenol red-free
William's E medium	n/a	Lonza [†]	With phenol red
William's E medium	n/a	MT-diagnostics [^]	Without pyruvate and glucose

[€] San Diego, CA

[&] Center Valley, PA

[#] St. Louis, MO

[?] Bloomington, MN

[‡] Carlsbad, CA

[§] Karlsruhe, Germany

^{*} Darmstadt, Germany

[§] Graz, Austria

⁽ Alkmaar, the Netherlands

[†] Santa Cruz, CA

[†] Basel, Switzerland

[^] Etten-Leur, the Netherlands

[‡] South Logan, UT

Table S2. Mitochondrial inhibitor concentrations and incubation times used in published protocols.

Rotenone

Ref	Concentration(s) used [μM]	Incubation time [h]	Cells cell lines
[1]	2	24	Human hepatocytes (obtained during liver transplantation)

[2]	6.3 ;25	24 ;48; 72	HepG2
[3]	0.1; 0.5; 1.0; 2.0; 5.0	24 ;48; 72	HepG2
[4]	0.001; 0.01; 0.1; 1; 10; 100	24	HepG2
[5]	0.5	24	HepG2
[6]	12.5; 25; 50; 100; 250	24	HepG2
[7]	0.1	18	Sk-Hep-1
[8]	0.5; 1; 1.5; 2.0; 2.5; 5; 7.5; 10	4; 6; 12; 24	WB-F344

Antimycin A

Ref	Concentration(s) used [μ M]	Incubation time [h]	Cells organelles
[9]	15	Not listed	Isolated rat liver mitochondria
[10]	15	0; 6; 12; 24	Rat hepatocytes
[11]	20	1	Eel hepatocytes
[12]	200	2	Rat hepatocytes
[13]	5	0–4	Rat hepatocytes
[14]	10	Continuous	Rat subsarcolemmal mitochondria
[15]	10	Continuous	Heart mitochondria of pigeons and rats
[16]	1	Continuous	Isolated rat brain mitochondria

Myxothiazol

Ref	Concentration(s) used [μ M]	Incubation time [h or min]	Cells organelles
[17]	3.0; 1.5	“Several min”	Beef and sheep heart submitochondrial particles
[18]	4	8 h	Mouse muscle cells
[11]	5	5 h	Eel hepatocytes
[19]	0.6; 1.0; 5.0; 10.0	5; 15; 30; 60 min	Rat hepatocytes
[20]	10	3 min	Submitochondrial particles of rat hepatocytes
[21]	0–3.0	20 min	Rat hepatocytes

Piericidin A

Ref	Concentration(s) used [μ M]	Incubation time [h]	Cells organelles
[22]	0.2	Continuous	Isolated mitochondria bovine heart
[23]	2	Continuous	Rat skeletal muscle mitochondria
[24]	0–0.050	9	Mouse neurons
[25]	1	Continuous	Bovine heart mitochondria

[26]	1	Continuous	Bovine heart mitochondria
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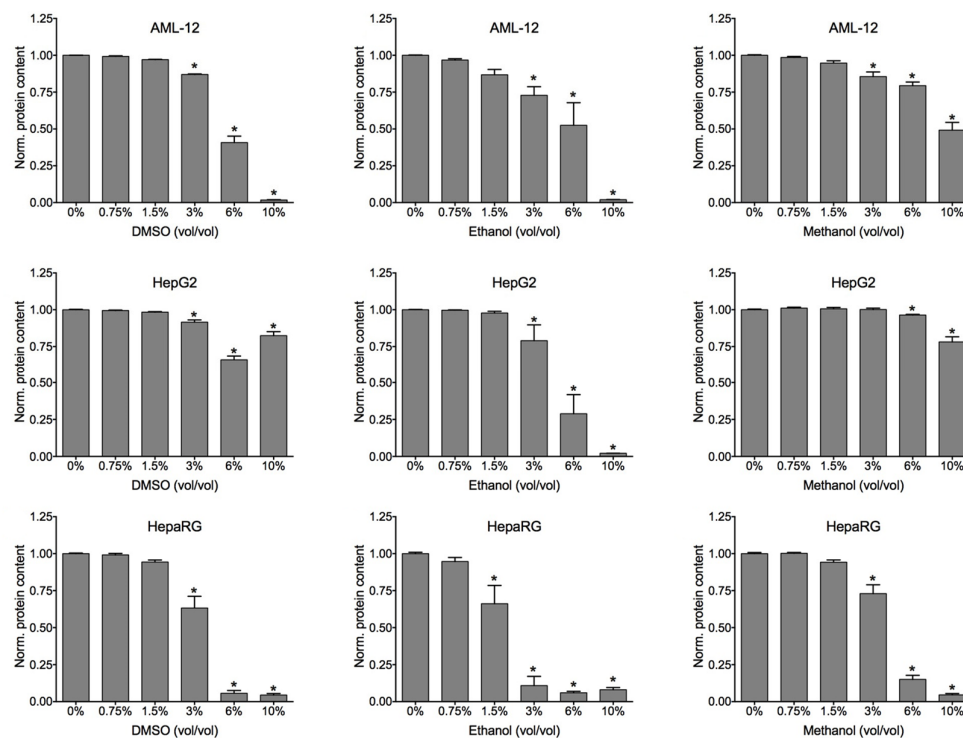


Figure S2. Solvent toxicity in hepatocyte cell lines. AML-12, HepG2, and HepaRG cells were cultured as described in the main manuscript. Directly (AML-12, HepG2) or 4 weeks (HepaRG) after reaching confluence, the monolayer was washed with 1 mL of PBS, after which the cells received fresh culture medium containing 0 – 10% (vol/vol) of ethanol, DMSO, or methanol. Following 72 h of incubation, the protein content per well was determined as a measure for solvent toxicity using the sulforhodamine B assay according to [27]. The results were normalized to the protein content of the control wells (i.e., 0% solvent) and are presented as mean \pm SD (n=4 per concentration). Intragroup differences were tested using a one-way ANOVA with Dunnett's multiple comparison test, where * indicates $p < 0.05$ versus the 0% solvent group.

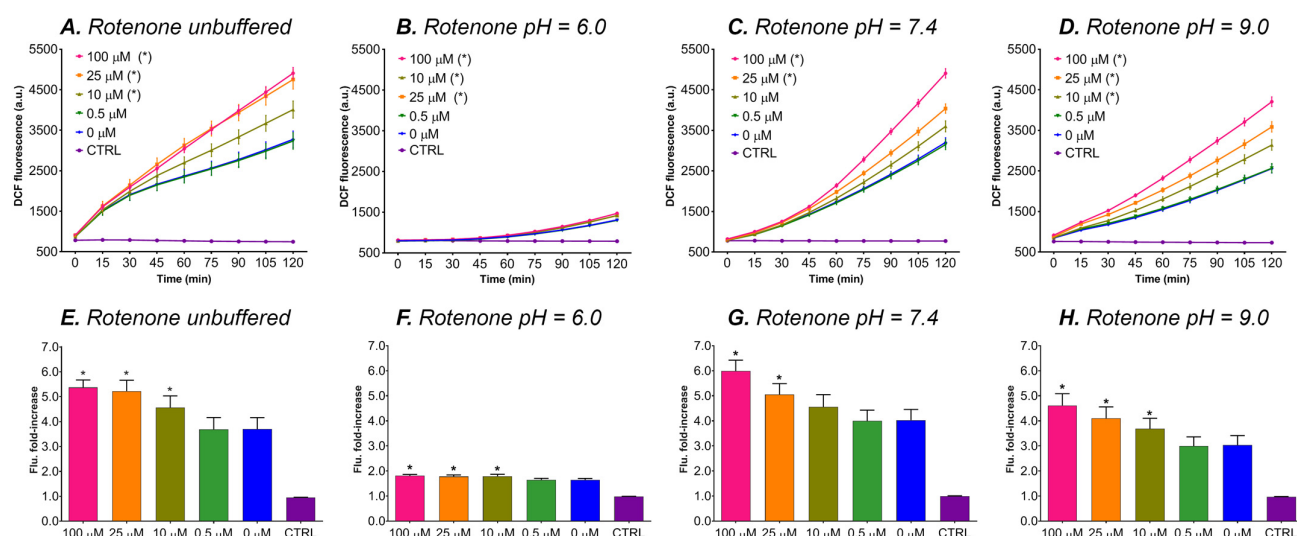


Figure S3. Influence of rotenone on DCF formation under cell-free conditions in unbuffered assay medium (**A** and **E**) or in assay medium buffered with 25 mM HEPES set to pH = 6.0 (**B** and **F**), pH = 7.4 (**C** and **G**), or pH = 9.0 (**D** and **H**) (i.e., the data summarized in Table 1 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (**E-H**) for clarity. For all shown experiments, * indicates $p < 0.05$ versus the 0 μ M (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 1 legend).

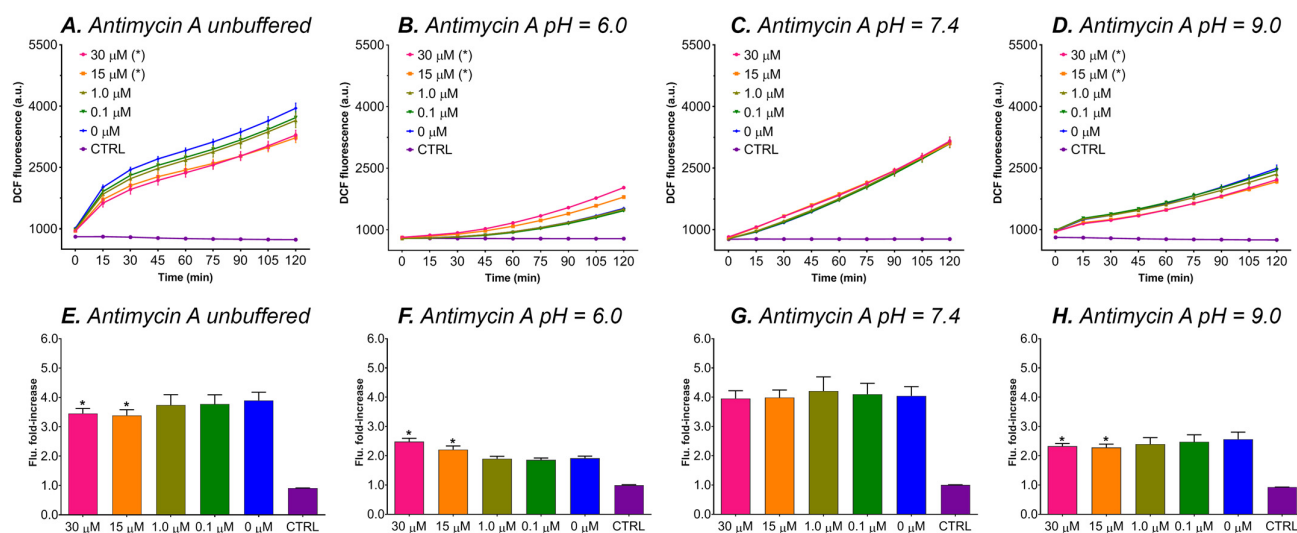


Figure S4. Influence of antimycin A on DCF formation under cell-free conditions in unbuffered assay medium (**A** and **E**) or in assay medium buffered with 25 mM HEPES set to pH = 6.0 (**B** and **F**), pH = 7.4 (**C** and **G**), or pH = 9.0 (**D** and **H**) (i.e., the data summarized in Table 1 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (**E-H**) for clarity. For all shown

experiments, * indicates $p < 0.05$ versus the 0 μM (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 1 legend).

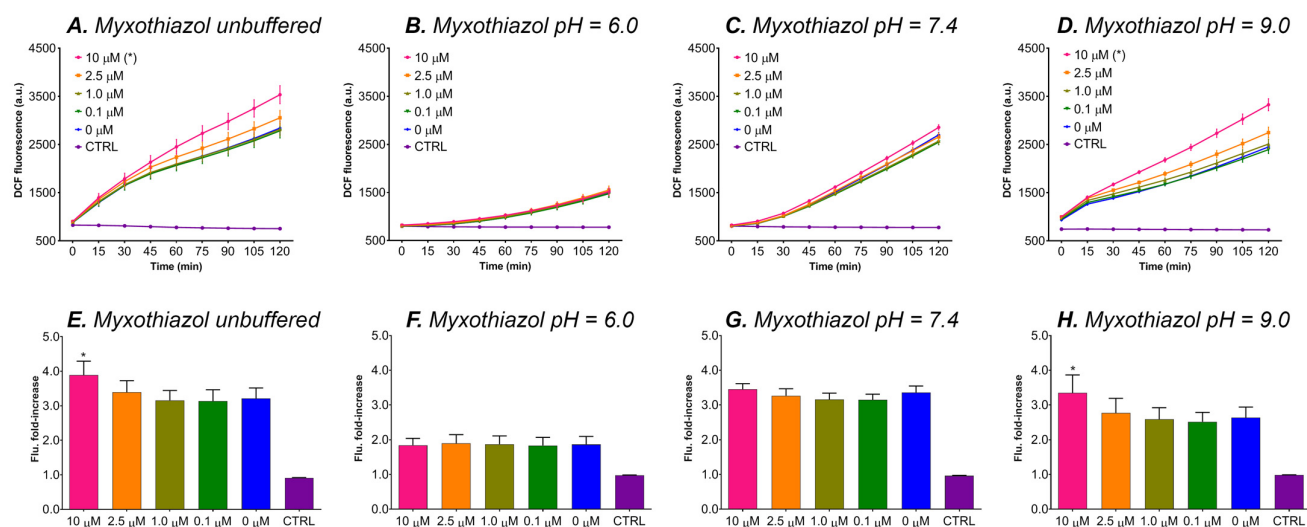


Figure S5. Influence of myxothiazol on DCF formation under cell-free conditions in unbuffered assay medium (A and E) or in assay medium buffered with 25 mM HEPES set to pH = 6.0 (B and F), pH = 7.4 (C and G), or pH = 9.0 (D and H) (i.e., the data summarized in Table 1 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (E-H) for clarity. For all shown experiments, * indicates $p < 0.05$ versus the 0 μM (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 1 legend).

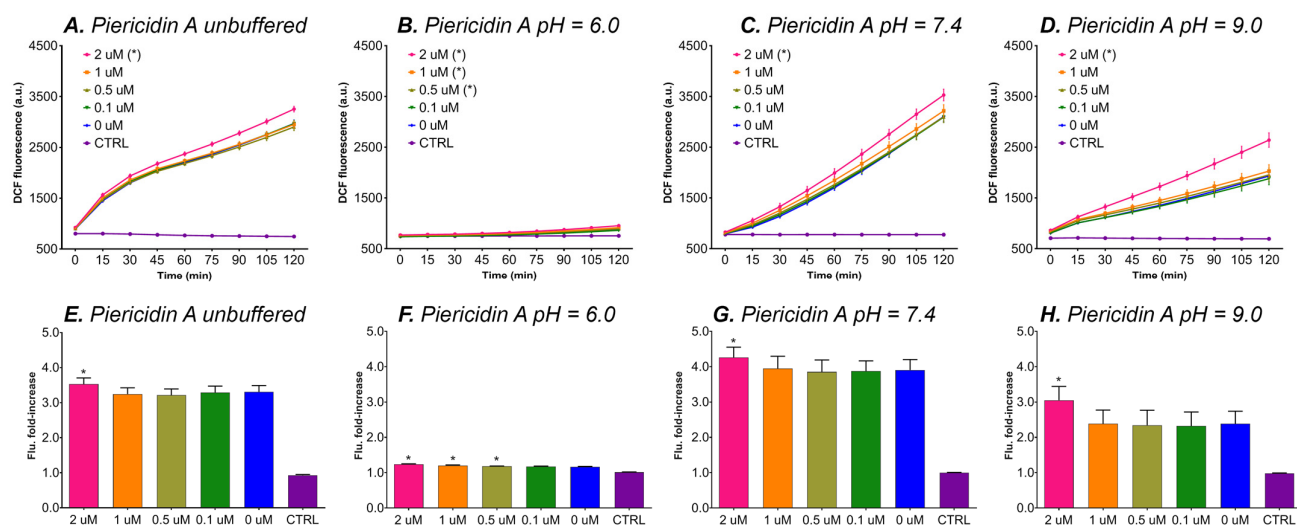


Figure S6. Influence of antimycin A on DCF formation under cell-free conditions in unbuffered assay medium (A and E) or in assay medium buffered with 25 mM HEPES set to pH = 6.0 (B and F), pH = 7.4 (C and G), or pH = 9.0 (D and H) (i.e., the data summarized in Table 1 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (E-H) for clarity. For all shown experiments, * indicates $p < 0.05$ versus the 0 μM (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 1 legend).

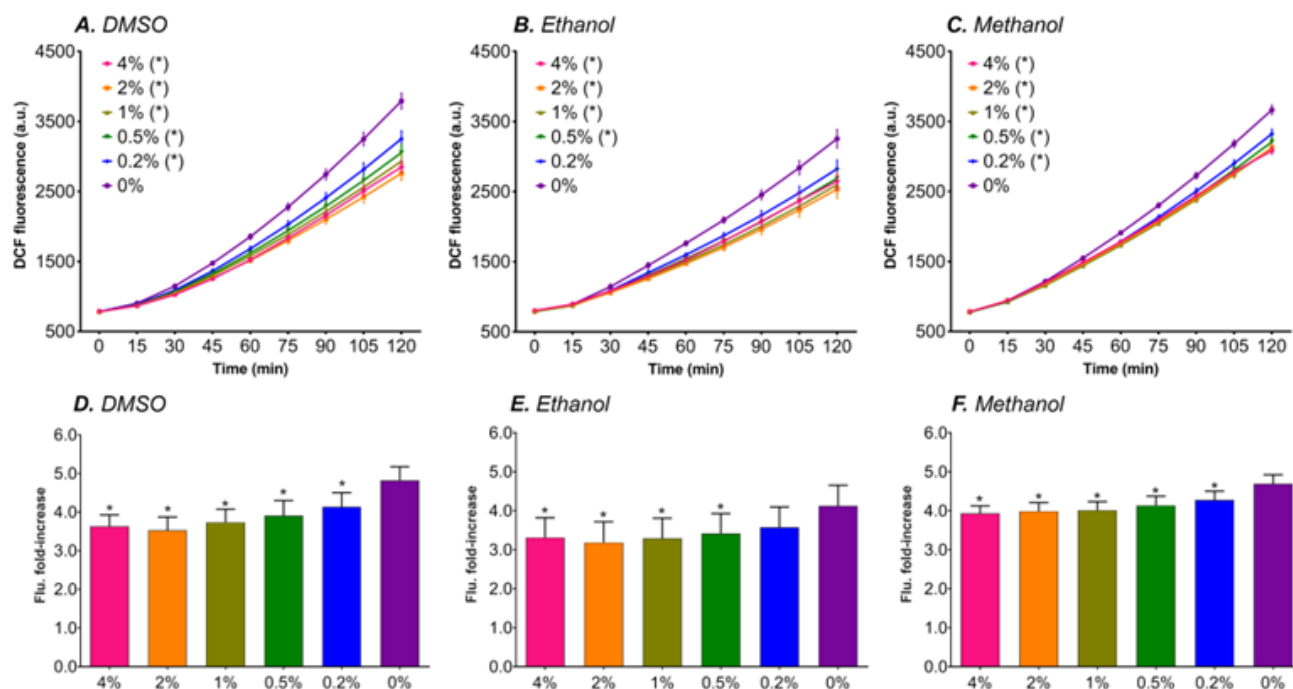


Figure S7. Influence of solvents on DCF formation under cell-free conditions in DMSO (**A** and **D**), ethanol (**B** and **E**), and methanol (**C** and **F**) in milliQ buffered with 25 mM HEPES set to pH = 7.4 (i.e., the data summarized in Table 2 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (**D-F**) for clarity. For all shown experiments, * indicates $p < 0.05$ versus the 0 μ M (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 2 legend).

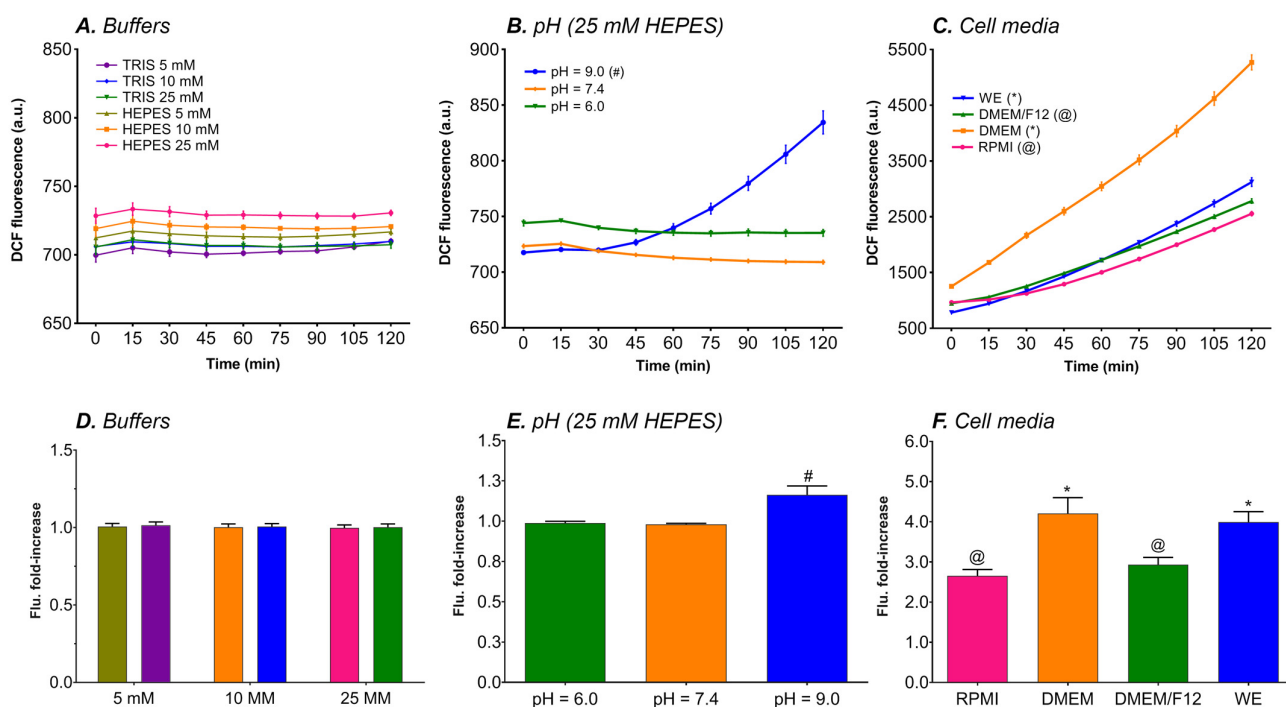


Figure S8. Influence of buffers (**A** and **D**), pH (**B** and **E**), and cell-culture media (**C** and **F**) on DCF formation under cell-free conditions (i.e., the data summarized in Table 2 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (**D-F**) for clarity. # indicates $p < 0.05$ versus both other pH values, * indicates $p < 0.05$ versus all other culture media, and @ indicates $p < 0.05$ versus DMEM and WE. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 2 legend).

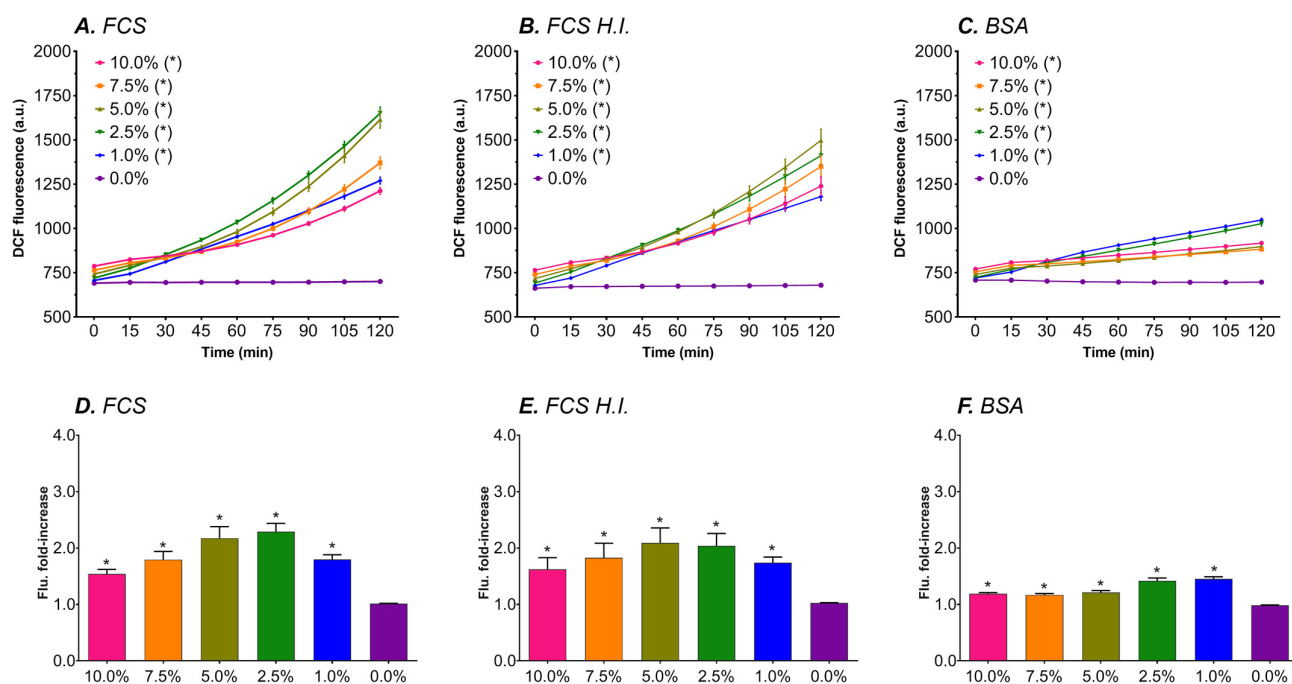


Figure S9. Influence of fetal calf serum (FCS, **A** and **D**), heat-inactivated fetal calf serum (**B** and **E**), and bovine serum albumin (**C** and **F**) on DCF formation under cell-free conditions (i.e., the data summarized in Table 2 of the main manuscript). DCF fluorescence was recorded for 2 h using a microplate reader and is presented as mean \pm SEM for 8 values per condition. Additional experimental details are provided in the main manuscript (see Section 3.1 and the Table 1 legend). The mean (\pm SD) fold-increase in DCF fluorescence over 2 h is presented in panels (**D-F**) for clarity. For all shown experiments, * indicates $p < 0.05$ versus the 0 μ M (i.e., vehicle control) group. Statistical analysis was performed as described in the main manuscript (see Section 2.6 and Table 2 legend)

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