Current Protocols

In vivo Determination of Mitochondrial Function using Luciferase-Expressing *Caenorhabditis elegans*: Contribution of Oxidative Phosphorylation, Glycolysis, and Fatty Acid Oxidation to Toxicant-Induced dysfunction

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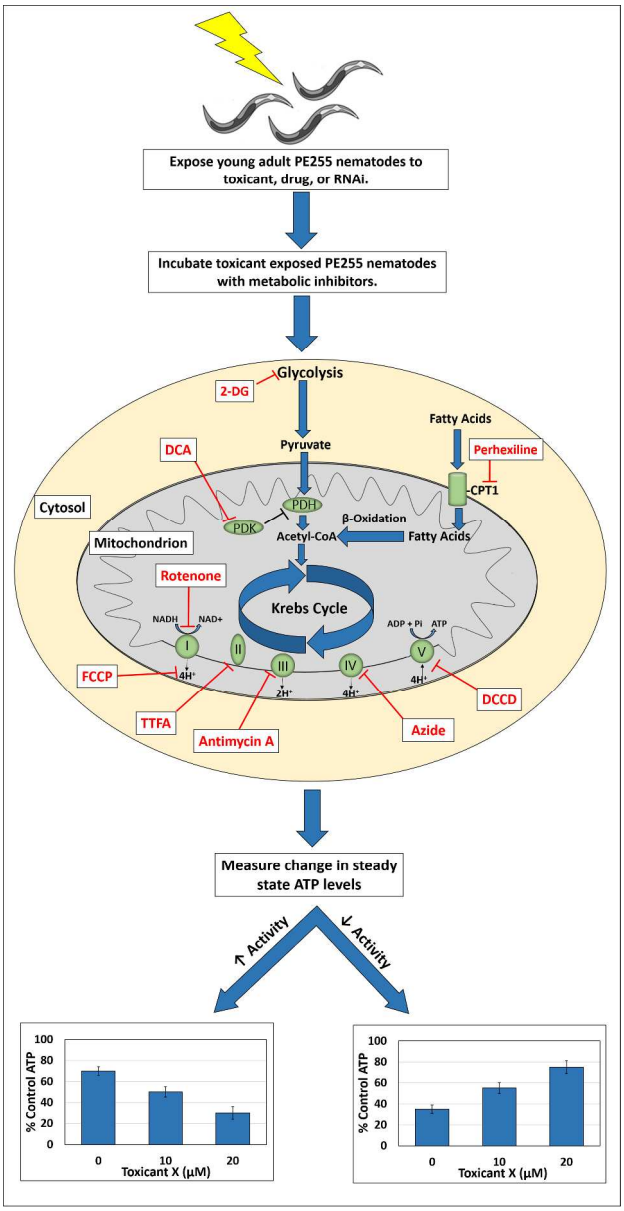
Complete List of Authors:	Luz, Anthony; Duke University, Nicholas School of the Environment Lagido, Cristina; Institute of Medical Sciences, School of Medical Sciences Hirschey, Matthew; Duke University School of Medicine, Duke Molecular Physiology Institute; Duke University School of Medicine, Sarah W. Stedman Nutrition and Metabolism Center; Duke University School of Medicine, Departments of Medicine and Pharmacology & Cancer Biology Meyer, Joel; Duke University, Nicholas School of the Environment
Keywords:	Caenorhabditis elegans, mitochondrial toxicity, PE327, PE255, glycolysis, oxidative phosphorylation, fatty acid oxidation
Abstract:	Mitochondria are a target of many drugs and environmental toxicants; however, how toxicant-induced mitochondrial dysfunction contributes to the progression of human disease remains poorly understood. To address this issue, *in vivo* assays capable of rapidly assessing mitochondrial function need to be developed. Here, using the model organism *Caenorhabditis elegans*, we describe how to rapidly assess the *in vivo* role of the electron transport chain, glycolysis or fatty acid oxidation, in energy metabolism following toxicant exposure, using a luciferase-expressing ATP-reporter strain. Alterations in mitochondrial function subsequent to toxicant exposure are detected by depleting steady-state ATP levels with inhibitors of the mitochondrial electron transport chain, glycolysis, or fatty acid oxidation. Differential changes in ATP following short-term inhibitor exposure indicate toxicant-induced alterations at the site of inhibition. Because a microplate reader is the only major piece of equipment required, this is a highly accessible protocol for studying toxicant-induced mitochondrial dysfunction *in vivo*.



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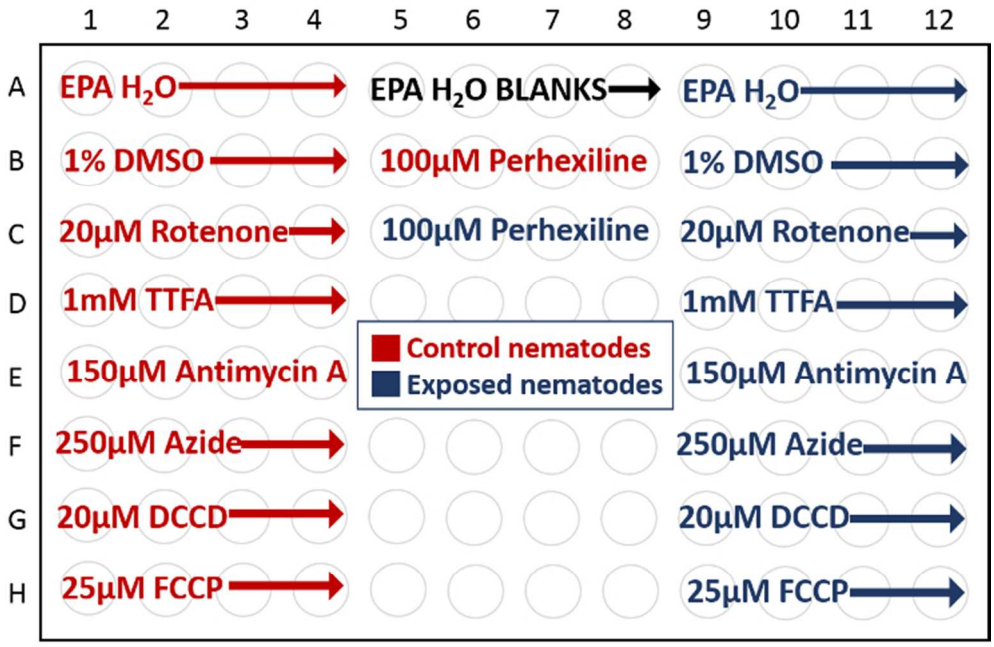

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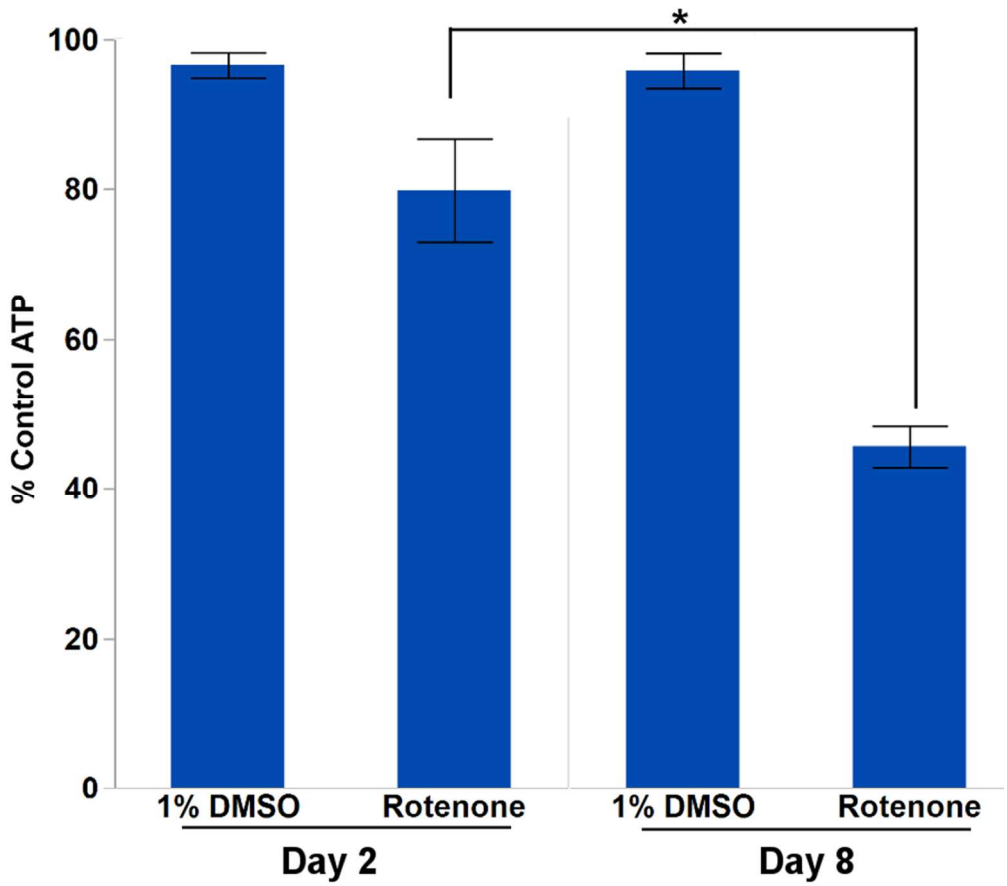

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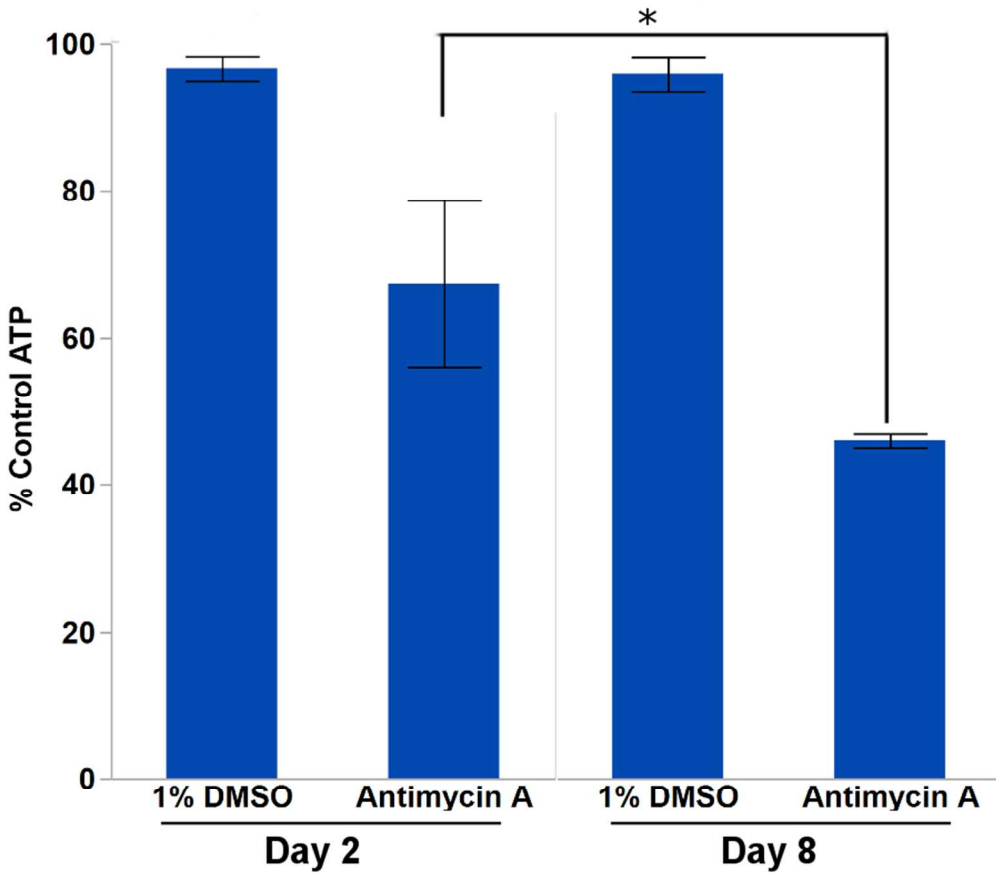

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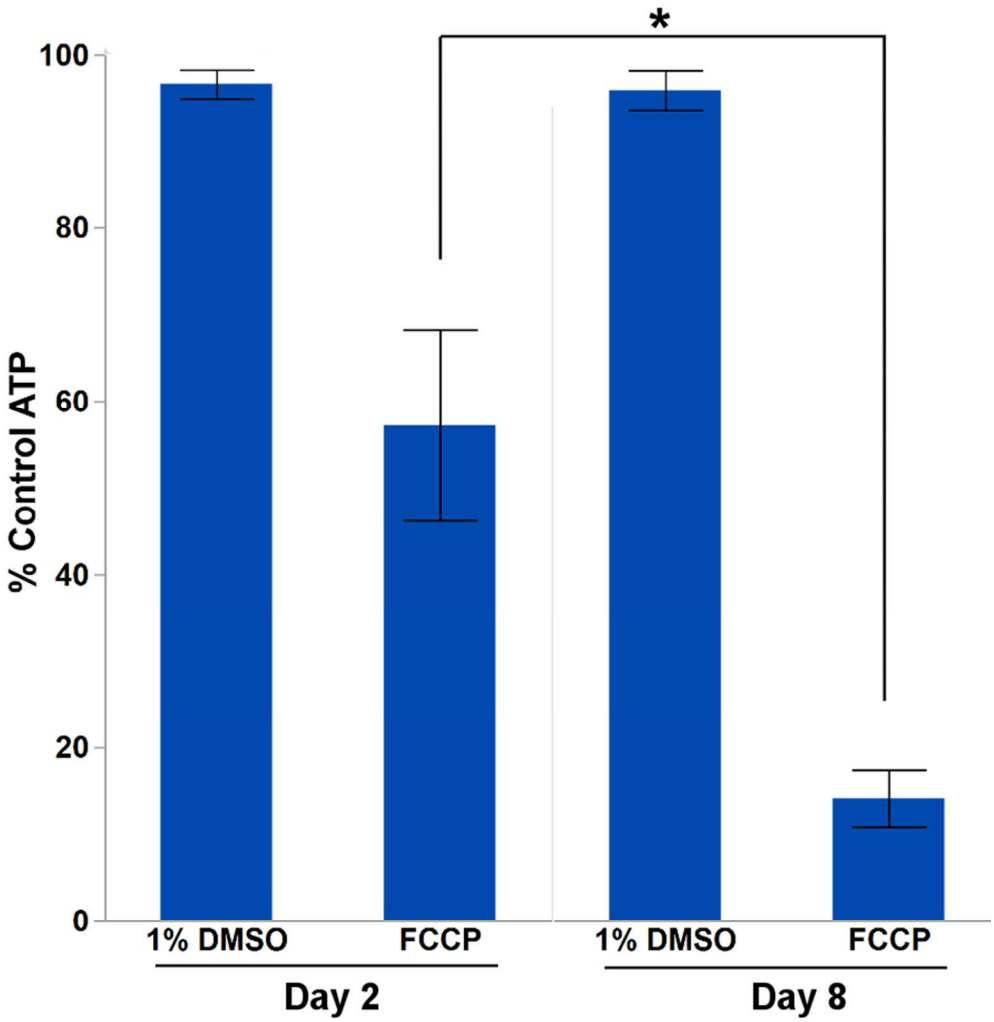

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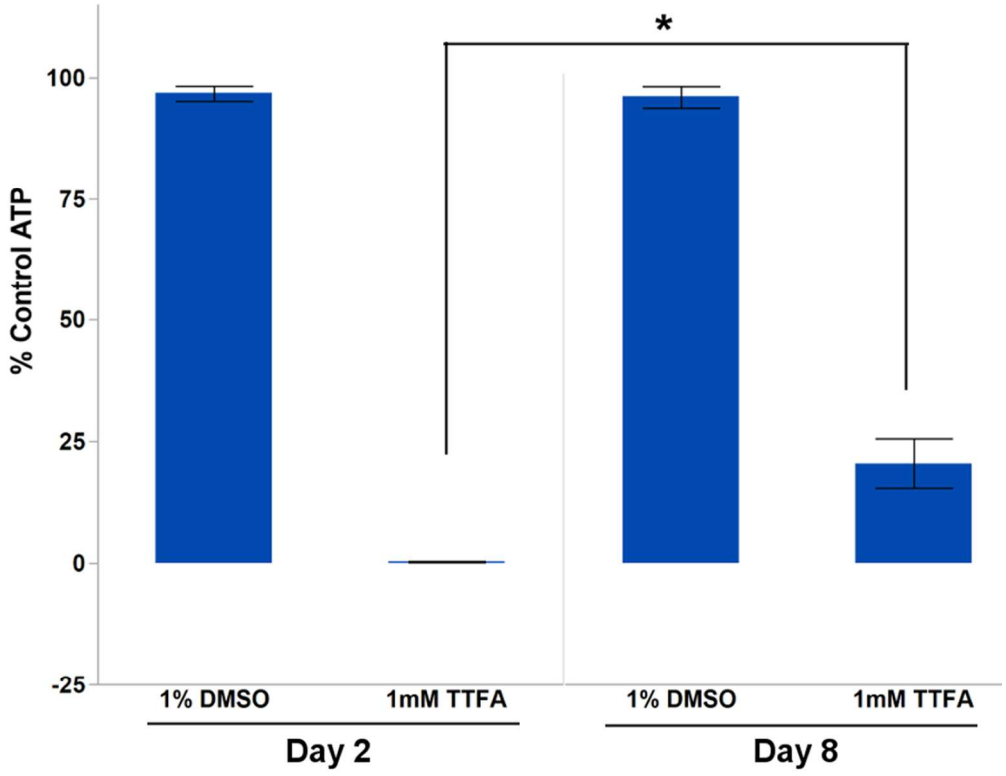

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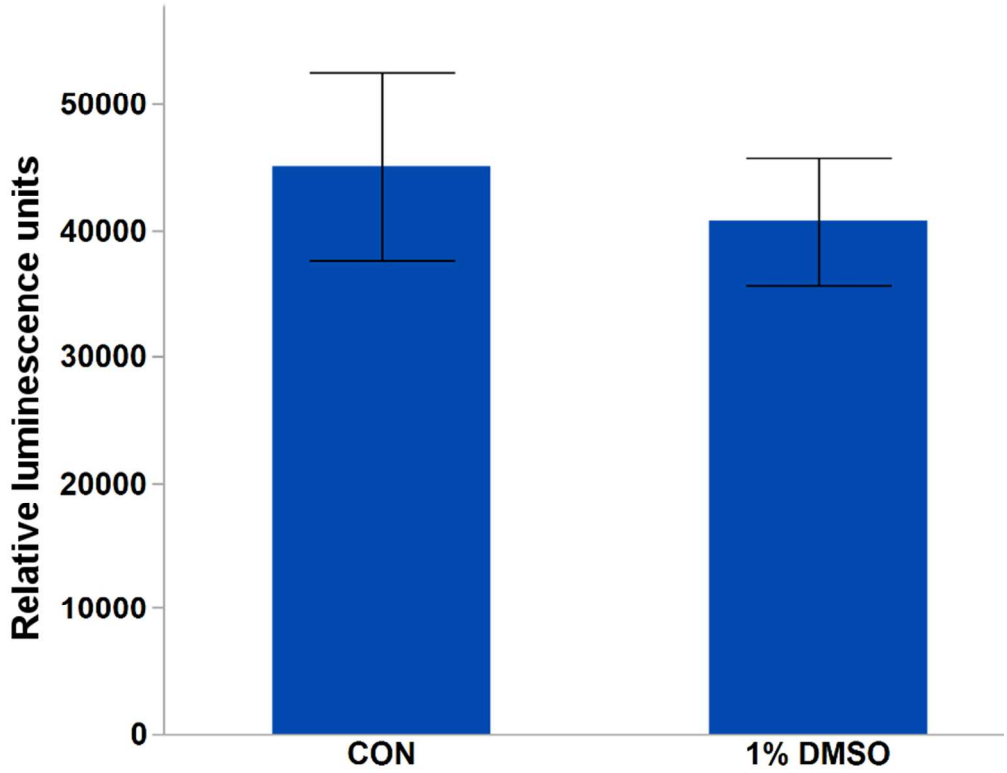

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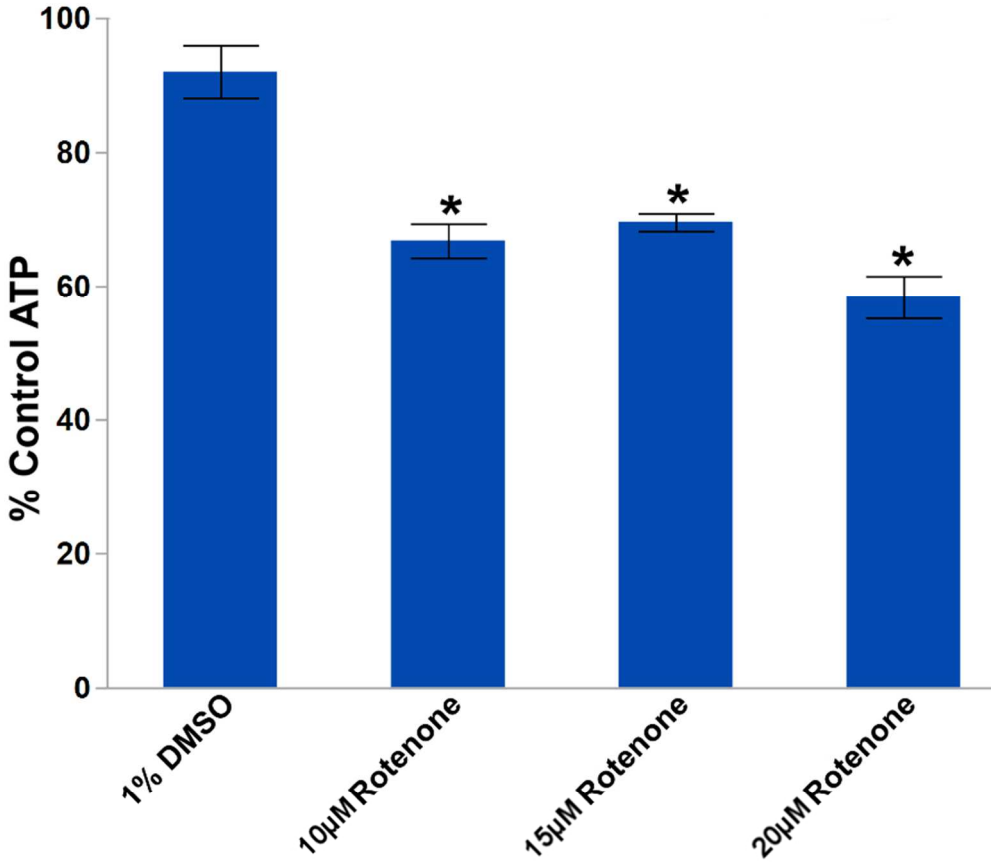

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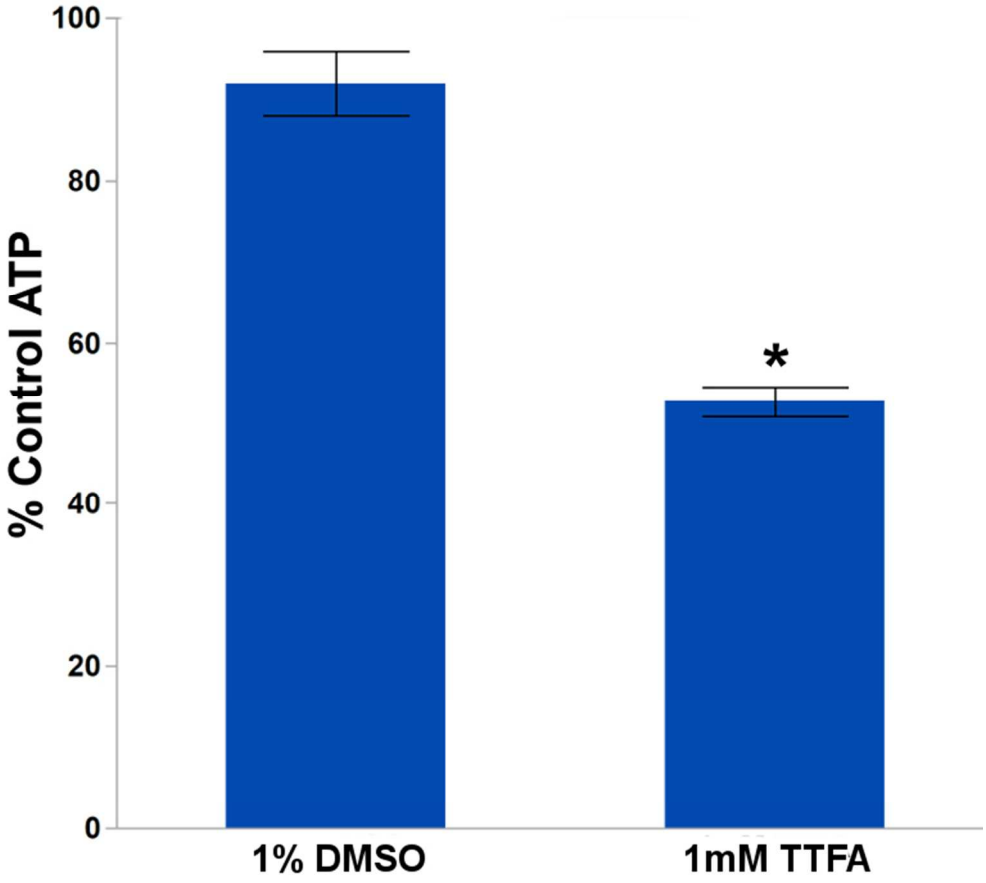

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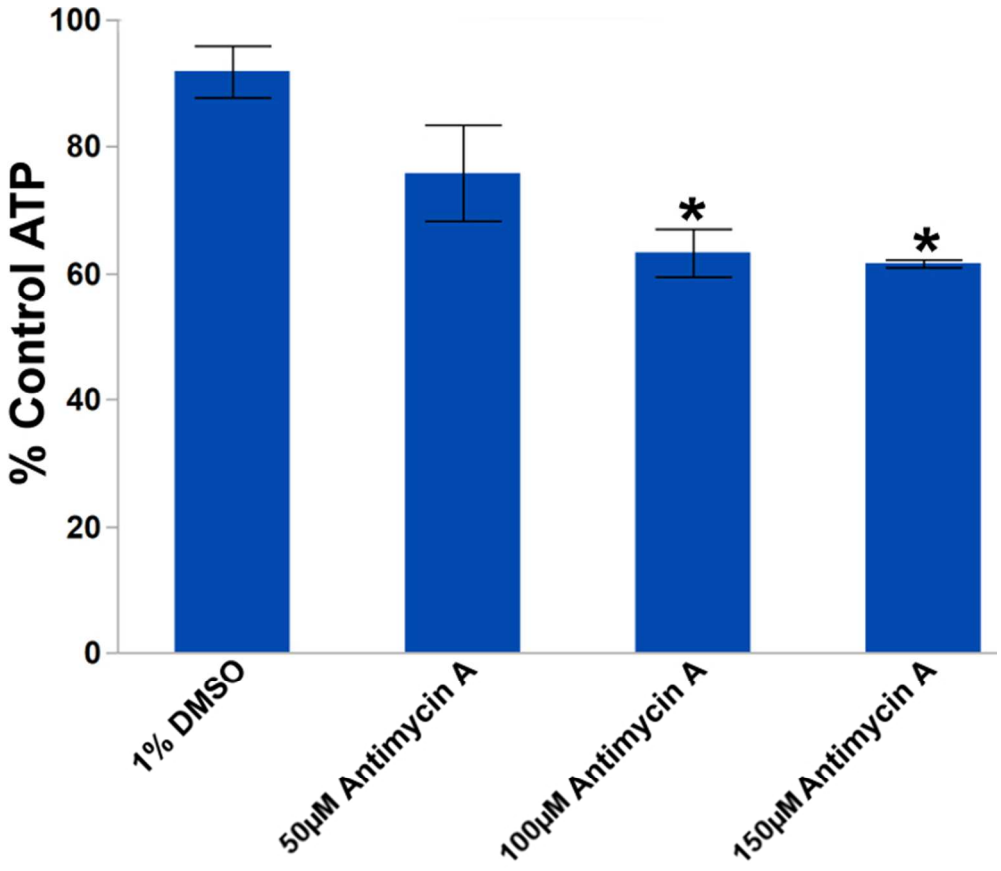

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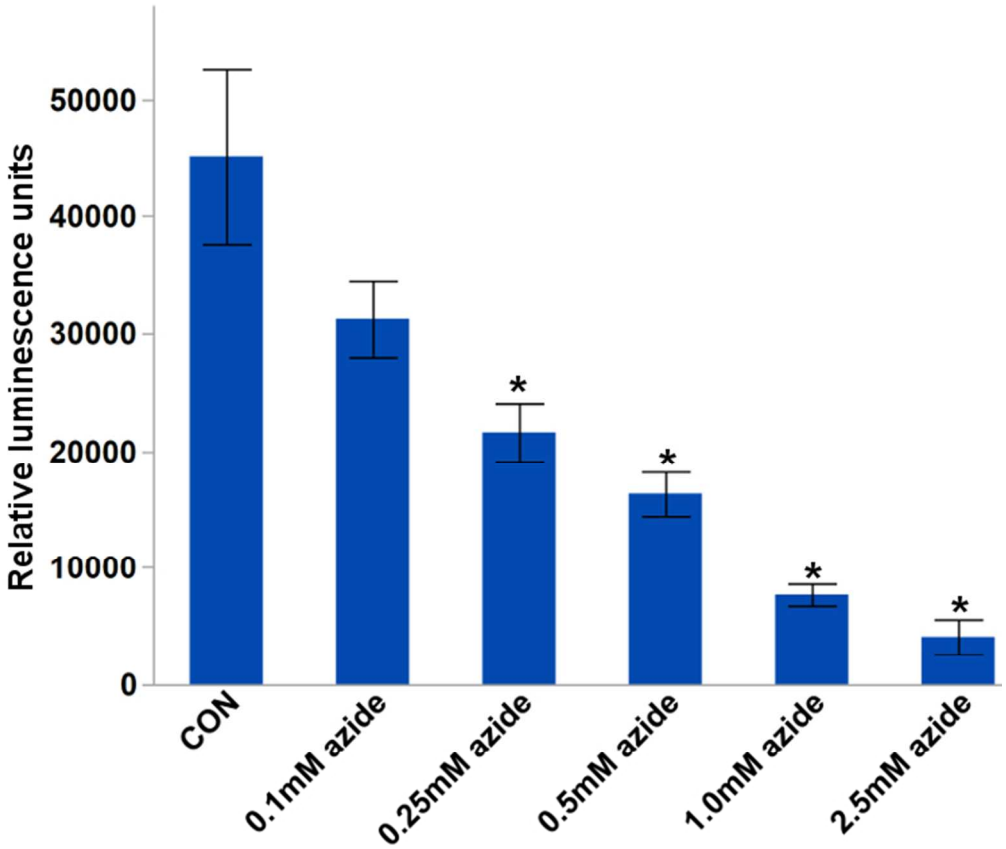

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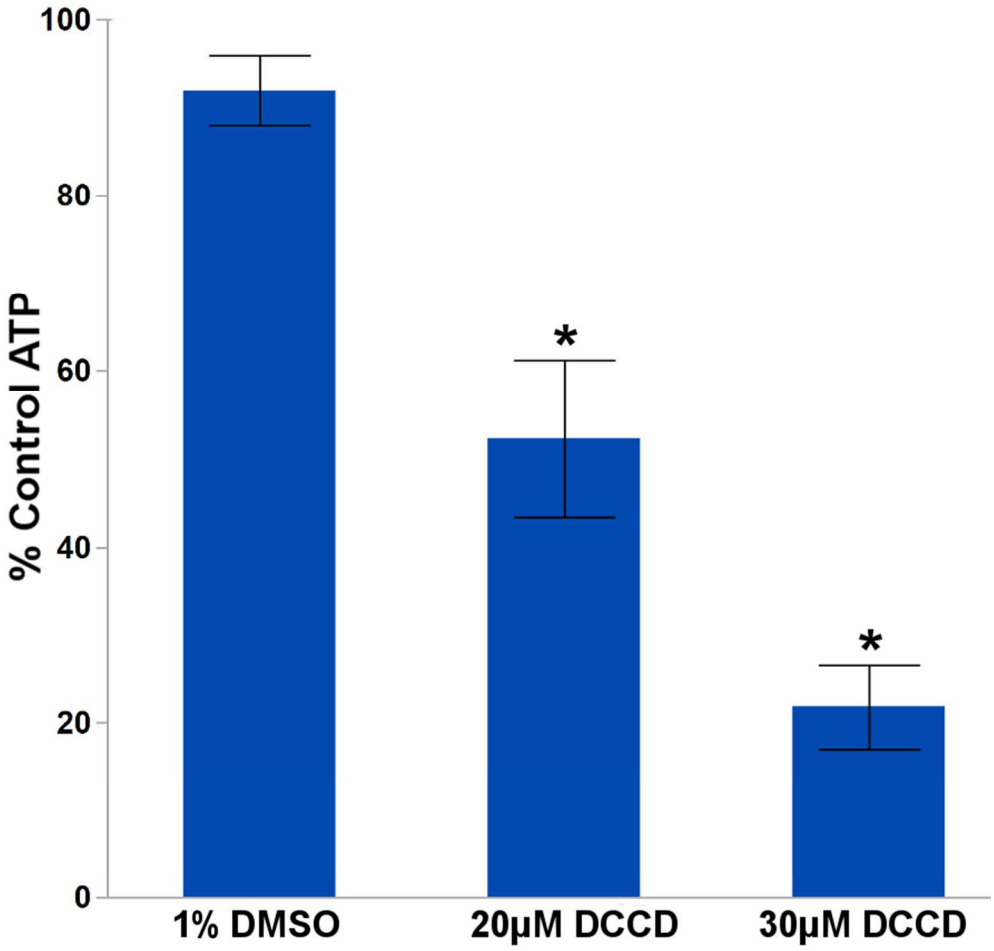

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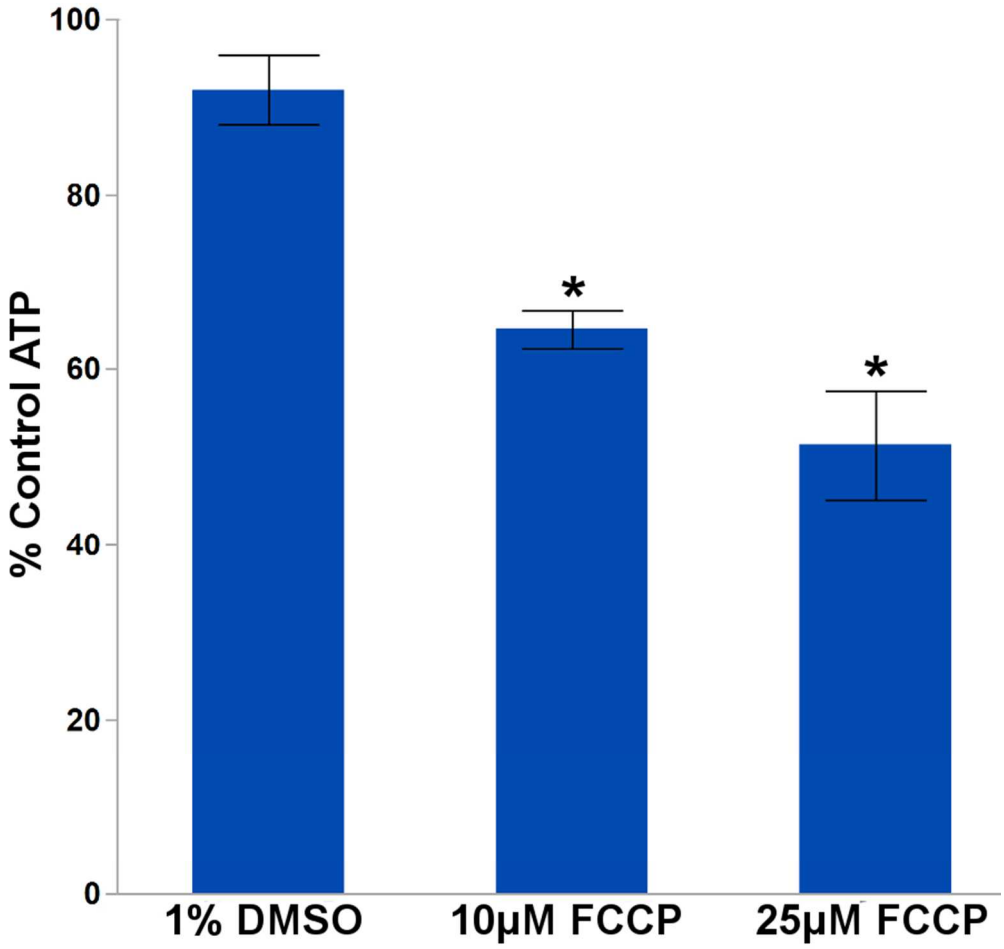

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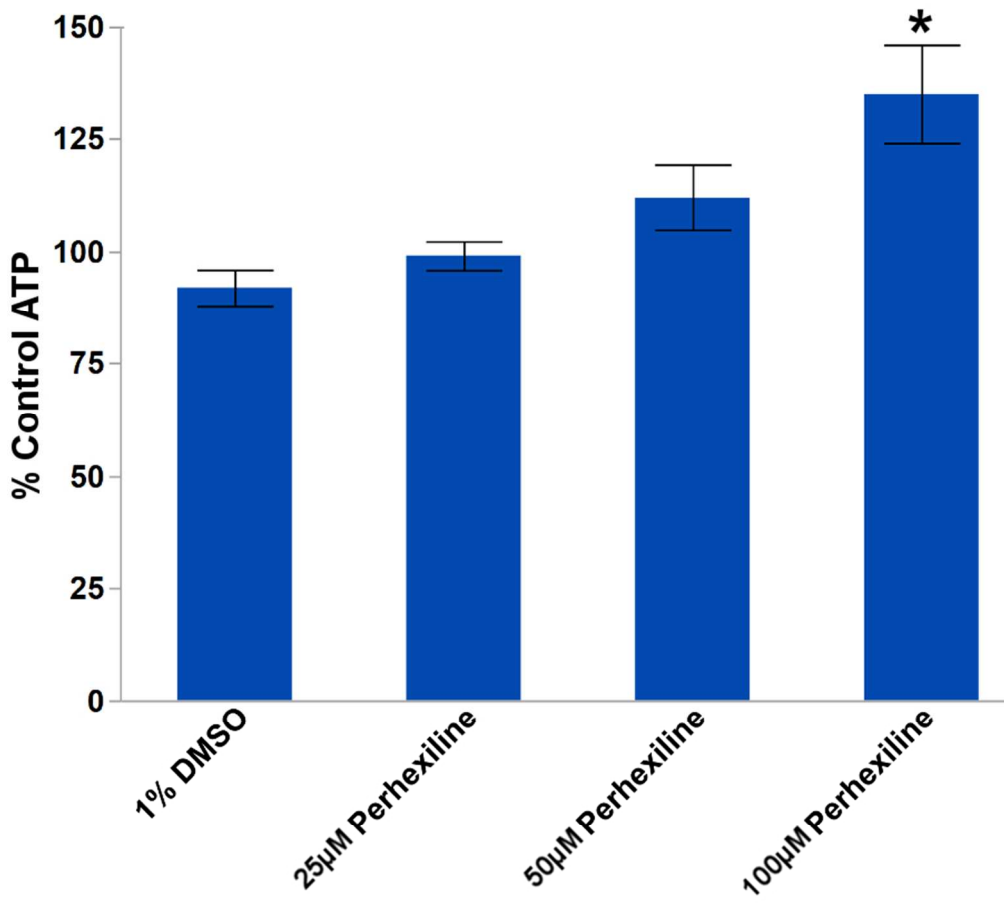

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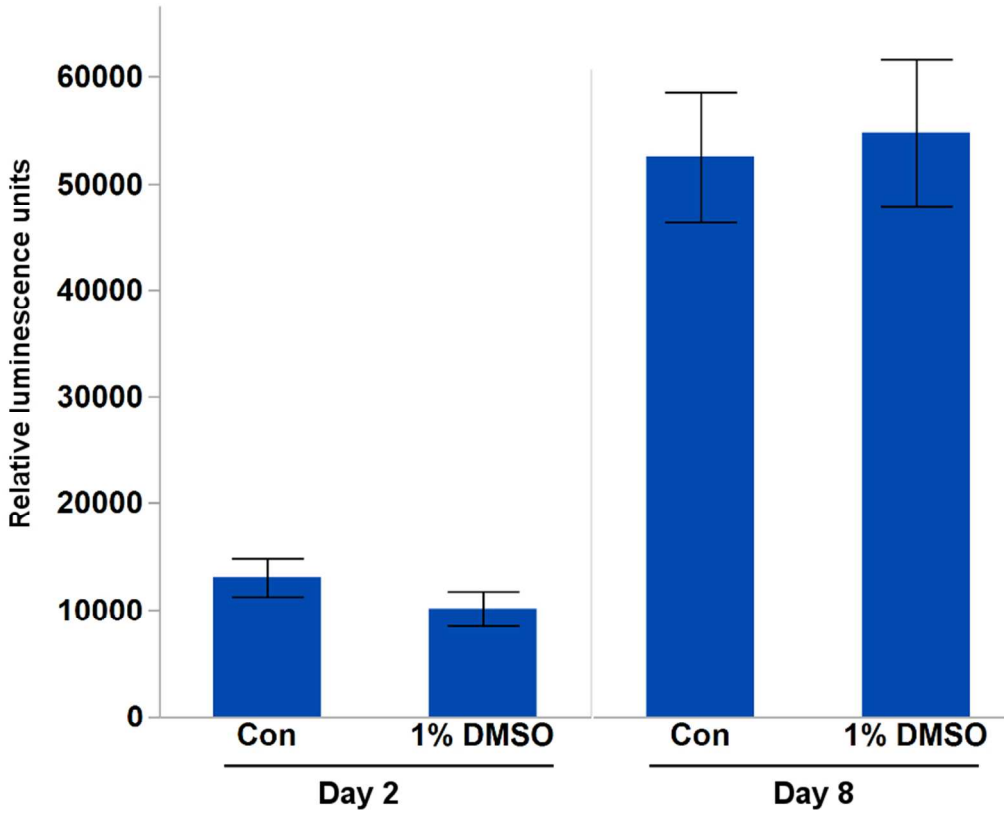

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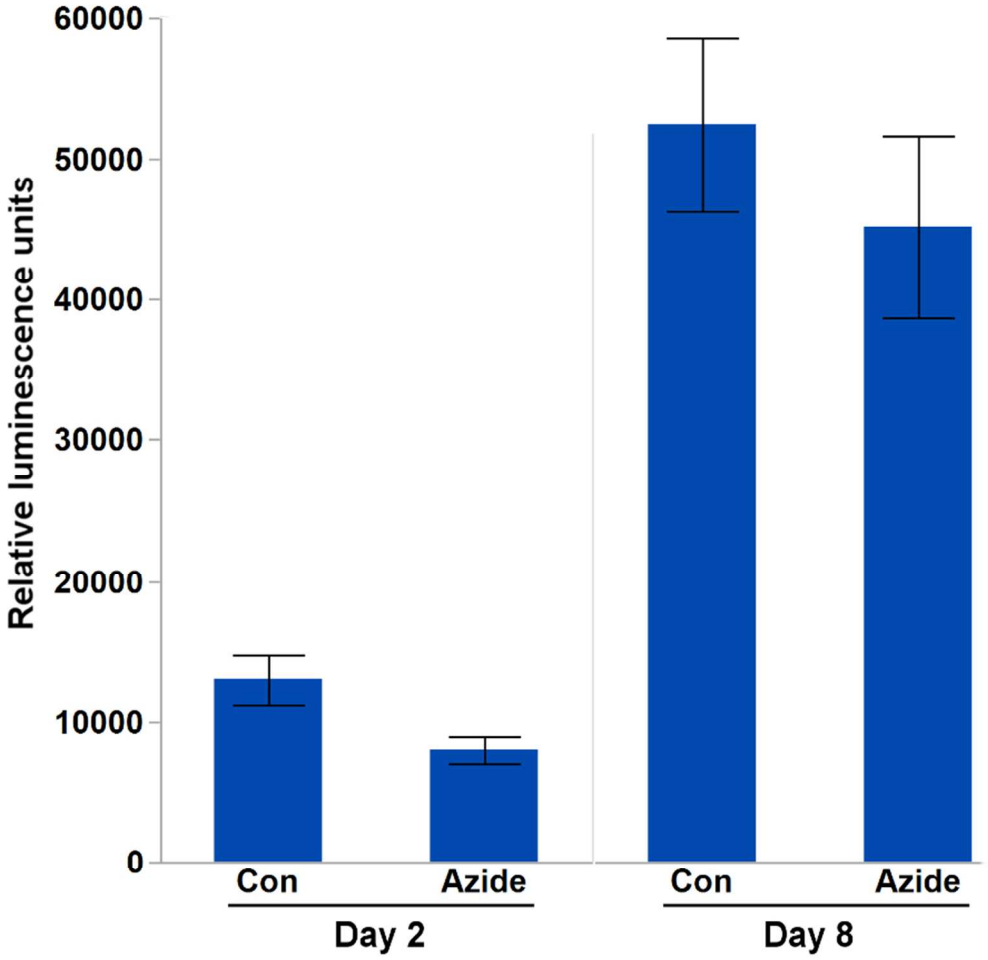

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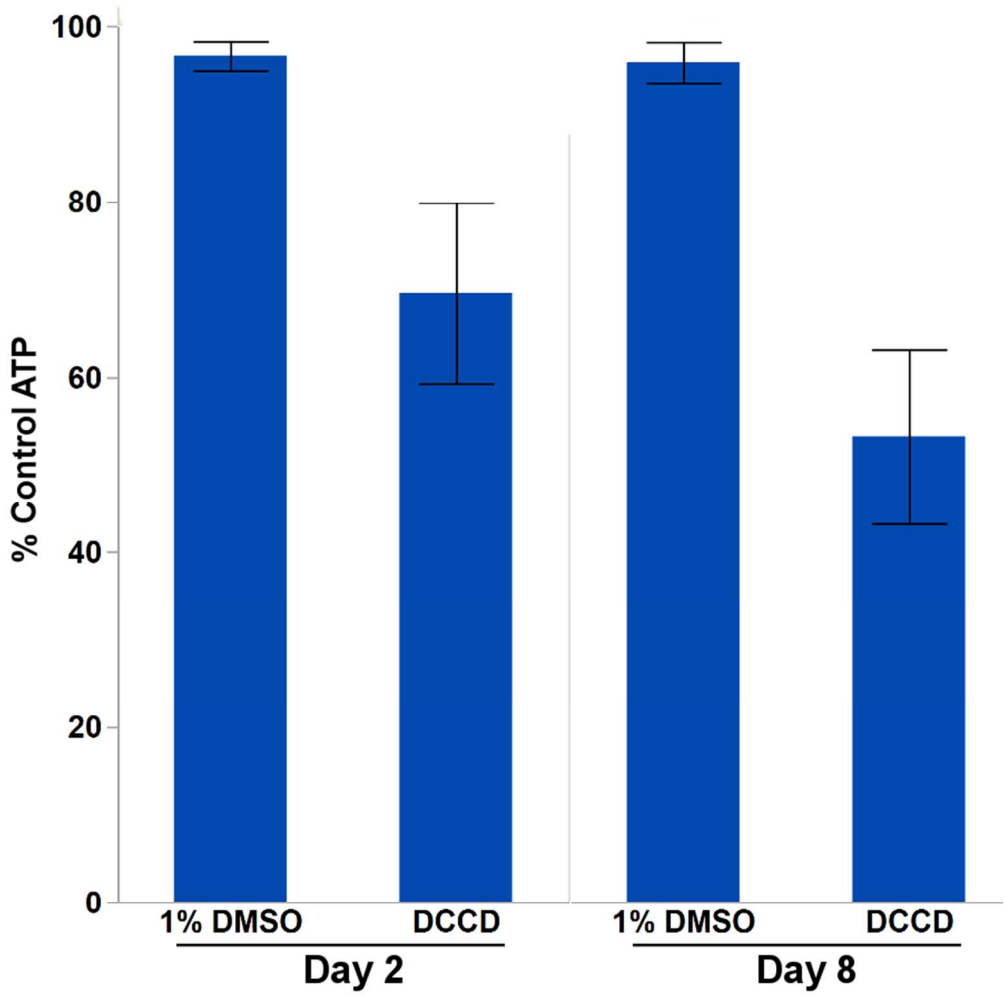

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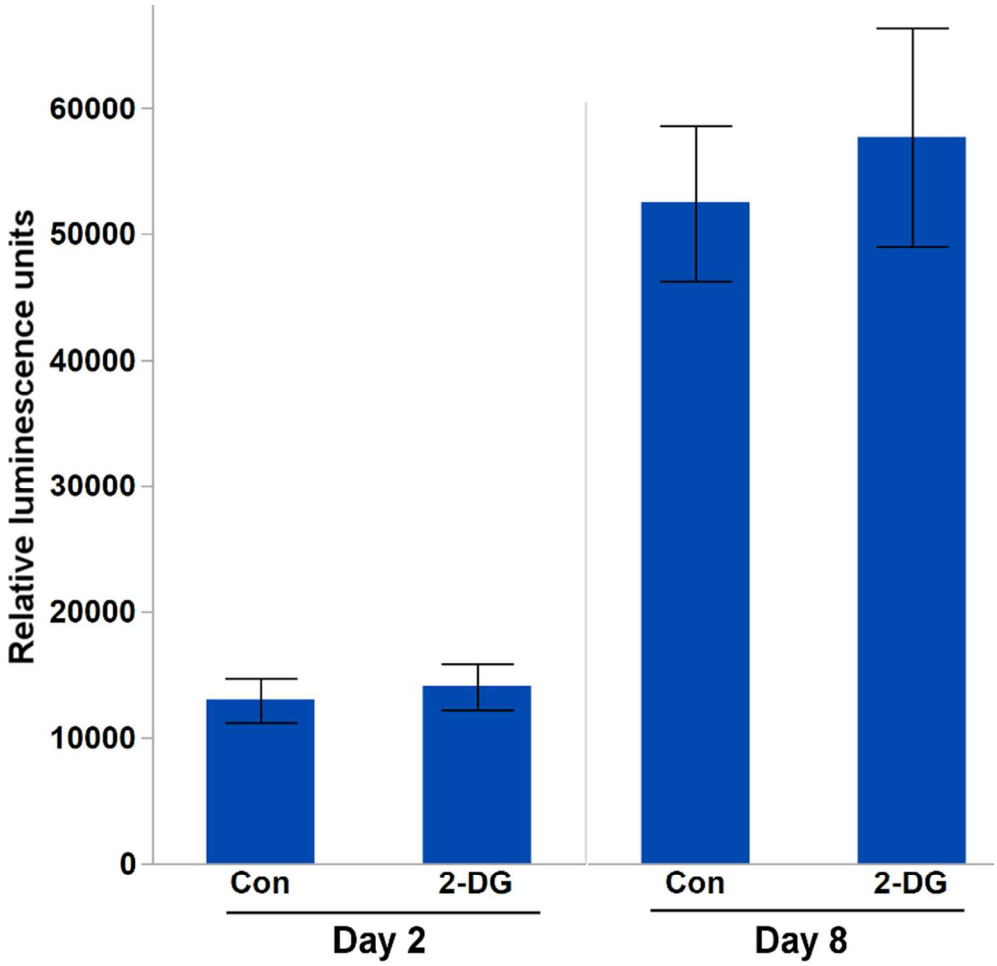

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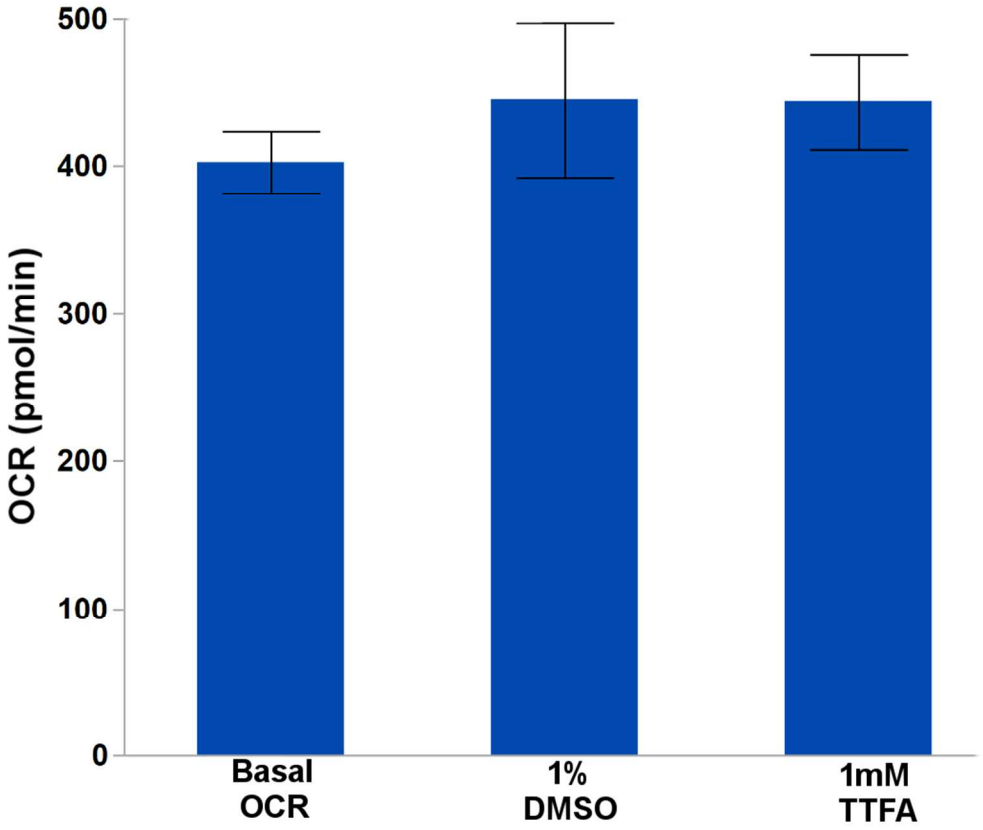

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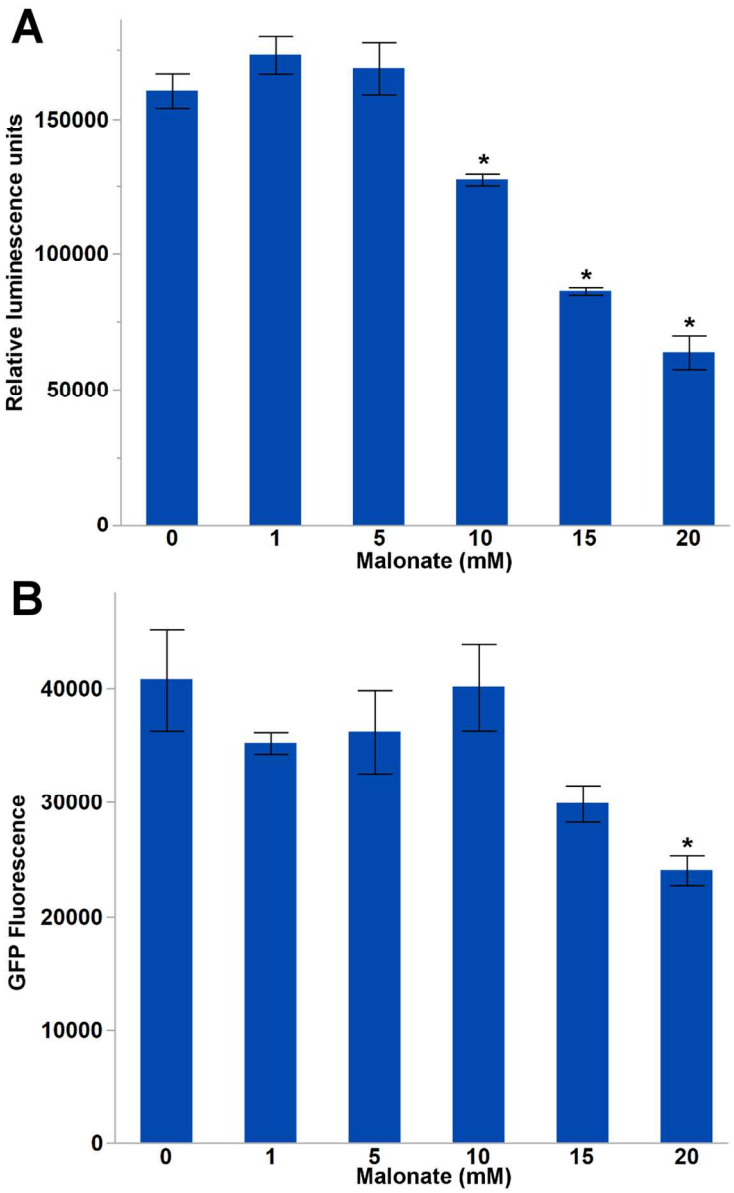

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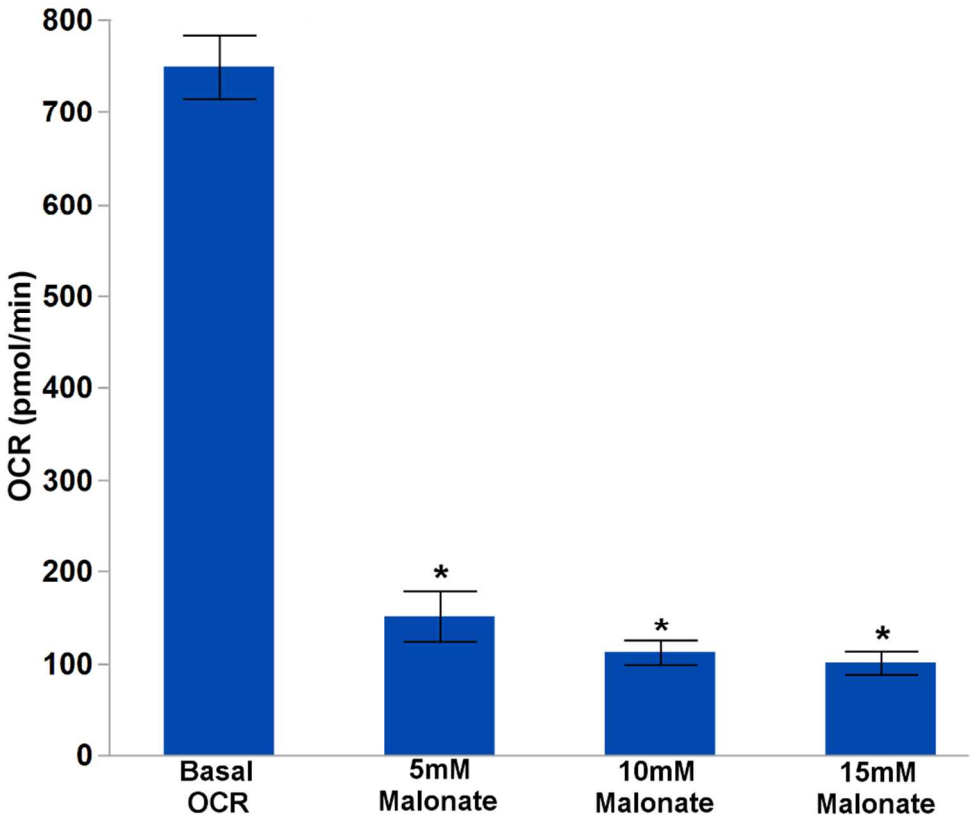

88x73mm (300 x 300 DPI)

96-well plate Layout **Numerical GFP values (lower outline) in A1 correspond to the labels

	1	2	3
A	EPA H2O	EPA H2O	EPA H2O
B	20uM Rotenone	20uM Rotenone	20uM Rotenone
C	100uM Antimycin A	100uM Antimycin A	100uM Antimycin A
D	0.5mM azide	0.5mM azide	0.5mM azide
E	20uM DCCD	20uM DCCD	20uM DCCD
F	10uM FCCP	10uM FCCP	10uM FCCP
G	100uM Perhexiline	100uM Perhexiline	100uM Perhexiline
H	BLANK	BLANK	BLANK

96-well plate Layout

	1	2	3
A	53144	54622	46656
B	36688	41814	46441
C	35725	27948	34129
D	41386	35435	40984
E	43275	40468	36857
F	39080	41755	50130
G	37506	41891	42920
H	8470	8769	8895

TREATMENT	GFP	Blank Corrected GFP
EPA H2O	53144	44383.25
EPA H2O	54622	45861.25
EPA H2O	46656	37895.25
EPA H2O	47006	38245.25
1% DMSO	50321	41560.25
1% DMSO	55464	46703.25
1% DMSO	58244	49483.25
1% DMSO	50957	42196.25
10uM Rotenone	33379	24618.25
10uM Rotenone	43788	35027.25
10uM Rotenone	39449	30688.25
10uM Rotenone	36304	27543.25
20uM Rotenone	36688	27927.25
20uM Rotenone	41814	33053.25
20uM Rotenone	46441	37680.25
20uM Rotenone	39808	31047.25
1mM TTFA	41014	32253.25
1mM TTFA	49201	40440.25
1mM TTFA	48271	39510.25
1mM TTFA	58729	49968.25
50uM Antimycin A	48014	39253.25
50uM Antimycin A	52760	43999.25
50uM Antimycin A	52199	43438.25

50uM Antimycin A	36526	27765.25
100uM Antimycin A	35725	26964.25
100uM Antimycin A	27948	19187.25
100uM Antimycin A	34129	25368.25
100uM Antimycin A	45799	37038.25
0.1mM azide	44863	36102.25
0.1mM azide	33847	25086.25
0.1mM azide	55980	47219.25
0.1mM azide	50725	41964.25
0.25mM azide	45507	36746.25
0.25mM azide	41603	32842.25
0.25mM azide	35321	26560.25
0.25mM azide	37448	28687.25
0.5mM azide	41386	32625.25
0.5mM azide	35435	26674.25
0.5mM azide	40984	32223.25
0.5mM azide	42634	33873.25
1.0mM azide	35703	26942.25
1.0mM azide	40804	32043.25
1.0mM azide	38885	30124.25
1.0mM azide	46117	37356.25
2.5mM azide	35354	26593.25
2.5mM azide	29877	21116.25
2.5mM azide	33552	24791.25
2.5mM azide	39901	31140.25
20uM DCCD	43275	34514.25
20uM DCCD	40468	31707.25
20uM DCCD	36857	28096.25
20uM DCCD	38683	29922.25
30uM DCCD	44007	35246.25
30uM DCCD	43659	34898.25
30uM DCCD	43804	35043.25
30uM DCCD	45695	36934.25
25uM FCCP	41545	32784.25
25uM FCCP	34741	25980.25
25uM FCCP	38030	29269.25
25uM FCCP	40877	32116.25
10uM FCCP	39080	30319.25
10uM FCCP	41755	32994.25
10uM FCCP	50130	41369.25
10uM FCCP	34880	26119.25
25uM Perhexiline	40236	31475.25
25uM Perhexiline	38514	29753.25
25uM Perhexiline	56140	47379.25
25uM Perhexiline	55708	46947.25
50uM Perhexiline	45828	37067.25
50uM Perhexiline	56348	47587.25

50uM Perhexiline	54484	45723.25
50uM Perhexiline	49267	40506.25
100uM Perhexiline	37506	28745.25
100uM Perhexiline	41891	33130.25
100uM Perhexiline	42920	34159.25
100uM Perhexiline	40708	31947.25
BLANK	8470	
BLANK	8769	
BLANK	8895	
BLANK	8909	
Avg. Blank	8760.75	

; in A1 (i.e. A1=EPA H2O=53144).

4	5	6	7	8
EPA H2O	1% DMSO	1% DMSO	1% DMSO	1% DMSO
20uM Rotenone	1mM TTFA	1mM TTFA	1mM TTFA	1mM TTFA
100uM Antimycin A	0.1mM azide	0.1mM azide	0.1mM azide	0.1mM azide
0.5mM azide	1.0mM azide	1.0mM azide	1.0mM azide	1.0mM azide
20uM DCCD	30uM DCCD	30uM DCCD	30uM DCCD	30uM DCCD
10uM FCCP	25uM Perhexiline	25uM Perhexiline	25uM Perhexiline	25uM Perhexiline
100uM Perhexiline				
BLANK				

4	5	6	7	8
47006	50321	55464	58244	50957
39808	41014	49201	48271	58729
45799	44863	33847	55980	50725
42634	35703	40804	38885	46117
38683	44007	43659	43804	45695
34880	40236	38514	56140	55708
40708				
8909				

9	10	11	12
10uM Rotenone	10uM Rotenone	10uM Rotenone	10uM Rotenone
50uM Antimycin A	50uM Antimycin A	50uM Antimycin A	50uM Antimycin A
0.25mM azide	0.25mM azide	0.25mM azide	0.25mM azide
2.5mM azide	2.5mM azide	2.5mM azide	2.5mM azide
25uM FCCP	25uM FCCP	25uM FCCP	25uM FCCP
50uM Perhexiline	50uM Perhexiline	50uM Perhexiline	50uM Perhexiline

9	10	11	12
33379	43788	39449	36304
48014	52760	52199	36526
45507	41603	35321	37448
35354	29877	33552	39901
41545	34741	38030	40877
45828	56348	54484	49267

Well	Treatment	Luminescence	Blank Corrected Luminescence
A01	EPA H2O	95879	95693
A02	EPA H2O	87098	86912
A03	EPA H2O	65358	65172
A04	EPA H2O	75069	74883
A05	1% DMSO	75934	75748
A06	1% DMSO	89019	88833
A07	1% DMSO	84544	84358
A08	1% DMSO	83279	83093
A09	10uM Rotenone	25677	25491
A10	10uM Rotenone	36228	36042
A11	10uM Rotenone	28115	27929
A12	10uM Rotenone	27079	26893
B01	20uM Rotenone	28325	28139
B02	20uM Rotenone	23191	23005
B03	20uM Rotenone	34175	33989
B04	20uM Rotenone	26808	26622
B05	1mM TTFA	25938	25752
B06	1mM TTFA	32949	32763
B07	1mM TTFA	33612	33426
B08	1mM TTFA	45100	44914
B09	50uM Antimycin A	38080	37894
B10	50uM Antimycin A	42191	42005
B11	50uM Antimycin A	44775	44589
B12	50uM Antimycin A	28906	28720
C01	100uM Antimycin A	22706	22520
C02	100uM Antimycin A	19517	19331
C03	100uM Antimycin A	22945	22759
C04	100uM Antimycin A	27754	27568
C05	0.1mM azide	39487	39301
C06	0.1mM azide	30461	30275
C07	0.1mM azide	46714	46528
C08	0.1mM azide	43911	43725
C09	0.25mM azide	25582	25396
C10	0.25mM azide	26019	25833
C11	0.25mM azide	19749	19563
C12	0.25mM azide	24233	24047
D01	0.5mM azide	16638	16452
D02	0.5mM azide	18501	18315
D03	0.5mM azide	15294	15108
D04	0.5mM azide	22737	22551
D05	1.0mM azide	8364	8178
D06	1.0mM azide	7135	6949
D07	1.0mM azide	7555	7369
D08	1.0mM azide	8973	8787
D09	2.5mM azide	5003	4817
D10	2.5mM azide	2994	2808

D11	2.5mM azide	5229	5043
D12	2.5mM azide	4194	4008
E01	20uM DCCD	26207	26021
E02	20uM DCCD	21607	21421
E03	20uM DCCD	20212	20026
E04	20uM DCCD	17001	16815
E05	30uM DCCD	8813	8627
E06	30uM DCCD	12410	12224
E07	30uM DCCD	7001	6815
E08	30uM DCCD	9204	9018
E09	25uM FCCP	27633	27447
E10	25uM FCCP	27080	26894
E11	25uM FCCP	30338	30152
E12	25uM FCCP	31714	31528
F01	10uM FCCP	26352	26166
F02	10uM FCCP	40644	40458
F03	10uM FCCP	47692	47506
F04	10uM FCCP	30568	30382
F05	25uM Perhexiline	64496	64310
F06	25uM Perhexiline	54201	54015
F07	25uM Perhexiline	84419	84233
F08	25uM Perhexiline	76679	76493
F09	50uM Perhexiline	65417	65231
F10	50uM Perhexiline	85760	85574
F11	50uM Perhexiline	80121	79935
F12	50uM Perhexiline	78934	78748
G01	100uM Perhexiline	69763	69577
G02	100uM Perhexiline	68426	68240
G03	100uM Perhexiline	78677	78491
G04	100uM Perhexiline	73089	72903
H01	BLANK	170	
H02	BLANK	179	
H03	BLANK	196	
H04	BLANK	198	
	Avg. Blank	186	

Treatment	GFP	Luminescence	Avg. GFP	Normalization Factor	ATP
EPA H2O	44383.25	95693	34494.01	1.286694297	74371.2
EPA H2O	45861.25	86912		1.329542312	65369.9
EPA H2O	37895.25	65172		1.098603686	59322.6
EPA H2O	38245.25	74883		1.108750374	67538.2
1% DMSO	41560.25	75748		1.204854008	62869
1% DMSO	46703.25	88833		1.353952345	65610.1
1% DMSO	49483.25	84358		1.434546041	58804.7
1% DMSO	42196.25	83093		1.22329199	67925.7
10uM Rotenone	24618.25	25491		0.713696313	35716.9
10uM Rotenone	35027.25	36042		1.015458823	35493.3
10uM Rotenone	30688.25	27929		0.889668878	31392.6
10uM Rotenone	27543.25	26893		0.798493636	33679.7
20uM Rotenone	27927.25	28139		0.809626003	34755.6
20uM Rotenone	33053.25	23005		0.958231501	24007.8
20uM Rotenone	37680.25	33989		1.09237072	31114.9
20uM Rotenone	31047.25	26622		0.900076482	29577.5
1mM TTFA	32253.25	25752		0.93503907	27541.1
1mM TTFA	40440.25	32763		1.172384605	27945.6
1mM TTFA	39510.25	33426		1.145423405	29182.2
1mM TTFA	49968.25	44914		1.448606452	31005
50uM Antimycin A	39253.25	37894		1.137972837	33299.6
50uM Antimycin A	43999.25	42005		1.27556193	32930.6
50uM Antimycin A	43438.25	44589		1.259298238	35407.8
50uM Antimycin A	27765.25	28720		0.804929536	35680.1
100uM Antimycin A	26964.25	22520		0.781708115	28808.7
100uM Antimycin A	19187.25	19331		0.556248701	34752.4
100uM Antimycin A	25368.25	22759		0.735439216	30946.1
100uM Antimycin A	37038.25	27568		1.073758795	25674.3
0.1mM azide	36102.25	39301		1.046623651	37550.3
0.1mM azide	25086.25	30275		0.727263884	41628.6
0.1mM azide	47219.25	46528		1.368911463	33989
0.1mM azide	41964.25	43725		1.216566185	35941.3
0.25mM azide	36746.25	25396		1.065293558	23839.4
0.25mM azide	32842.25	25833		0.952114497	27132.2
0.25mM azide	26560.25	19563		0.769995938	25406.6
0.25mM azide	28687.25	24047		0.831658812	28914.5
0.5mM azide	32625.25	16452		0.945823551	17394.4
0.5mM azide	26674.25	18315		0.773300859	23684.2
0.5mM azide	32223.25	15108		0.934169354	16172.7
0.5mM azide	33873.25	22551		0.982003742	22964.3
1.0mM azide	26942.25	8178		0.781070323	10470.2
1.0mM azide	32043.25	6949		0.928951057	7480.48
1.0mM azide	30124.25	7369		0.873318215	8437.93
1.0mM azide	37356.25	8787		1.082977786	8113.74
2.5mM azide	26593.25	4817		0.770952625	6248.11
2.5mM azide	21116.25	2808		0.612171449	4586.95

2.5mM azide	24791.25	5043	0.718711676	7016.72
2.5mM azide	31140.25	4008	0.902772602	4439.66
20uM DCCD	34514.25	26021	1.000586677	26005.7
20uM DCCD	31707.25	21421	0.919210237	23303.7
20uM DCCD	28096.25	20026	0.814525404	24586.1
20uM DCCD	29922.25	16815	0.867462126	19384.1
30uM DCCD	35246.25	8627	1.021807751	8442.88
30uM DCCD	34898.25	12224	1.011719044	12082.4
30uM DCCD	35043.25	6815	1.015922672	6708.19
30uM DCCD	36934.25	9018	1.070743779	8422.18
25uM FCCP	32784.25	27447	0.950433046	28878.4
25uM FCCP	25980.25	26894	0.753181425	35707.2
25uM FCCP	29269.25	30152	0.848531305	35534.3
25uM FCCP	32116.25	31528	0.931067367	33862.2
10uM FCCP	30319.25	26166	0.87897137	29768.9
10uM FCCP	32994.25	40458	0.956521059	42297
10uM FCCP	41369.25	47506	1.199316815	39610.9
10uM FCCP	26119.25	30382	0.75721111	40123.6
25uM Perhexiline	31475.25	64310	0.912484432	70477.9
25uM Perhexiline	29753.25	54015	0.862562725	62621.5
25uM Perhexiline	47379.25	84233	1.373549949	61325
25uM Perhexiline	46947.25	76493	1.361026036	56202.5
50uM Perhexiline	37067.25	65231	1.074599521	60702.6
50uM Perhexiline	47587.25	85574	1.379579981	62029
50uM Perhexiline	45723.25	79935	1.325541618	60303.7
50uM Perhexiline	40506.25	78748	1.174297981	67059.6
100uM Perhexiline	28745.25	69577	0.833340263	83491.7
100uM Perhexiline	33130.25	68240	0.960463772	71049
100uM Perhexiline	34159.25	78491	0.990295036	79260.2
100uM Perhexiline	31947.25	72903	0.926167966	78714.7

Treatment	Avg. ATP	% Control ATP
EPA H2O	66650.46	
1% DMSO	63802.39	95.73
10uM Rotenone	34070.61	53.40
20uM Rotenone	29863.93	46.81
1mM TTFA	28918.47	45.33
50uM Antimycin A	34329.53	53.81
100uM Antimycin A	30045.39	47.09
0.1mM azide	37277.32	55.93
0.25mM azide	26323.20	39.49
0.5mM azide	20053.87	30.09
1.0mM azide	8625.60	12.94
2.5mM azide	5572.86	8.36
20uM DCCD	23319.92	36.55
30uM DCCD	8913.91	13.97
25uM FCCP	33495.54	52.50
10uM FCCP	37950.09	59.48
25uM Perhexiline	62656.73	98.20
50uM Perhexiline	62523.73	98.00
100uM Perhexiline	78128.90	122.45

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Contact Author	Joel Meyer joel.meyer@duke.edu, 919-613-8109		
Author Addresses	Nicholas School of the Environment, PO Box 90328, Duke University, Durham, North Carolina, 27708 School of Medical Sciences, Institute of Medical Sciences, Aberdeen AB25 2 ZD, UK Department of Medicine, 500 N. Duke St., 50-201, Duke University, Durham, North Carolina, 27708		

	anthony.luz@duke.edu, 919-613-8022 c.lagido@abdn.ac.uk, +44 (0)1224-437300 matthew.hirschey@duke.edu, 919-479-2315 joel.meyer@duke.edu, 919-613-8109
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In vivo Determination of Mitochondrial Function using Luciferase-Expressing *Caenorhabditis elegans*: Contribution of Oxidative Phosphorylation, Glycolysis, and Fatty Acid Oxidation to Toxicant-Induced dysfunction.

Anthony L. Luz[1,4], Cristina Lagido[2,5], Matthew D. Hirschey[3,6], and Joel N. Meyer[*1,7]
[1]Nicholas School of the Environment, PO Box 90328, Duke University, Durham, North Carolina, 27708
[2]School of Medical Sciences, Institute of Medical Sciences, Aberdeen AB25 2 ZD, UK
[3]Department of Medicine, 500 N. Duke St., 50-201, Duke University, Durham, North Carolina, 27708
[4]anthony.luz@duke.edu, 919-613-8022
[5]c.lagido@abdn.ac.uk, +44 (0)1224-437300
[6]matthew.hirschey@duke.edu, 919-479-2315
[7]joel.meyer@duke.edu, 919-613-8109
*Address correspondence to: Joel N. Meyer

[*Au: CP template requires that the protocol be described in stepwise guidelines. Please number the steps in each protocol.]

ABSTRACT

Mitochondria are a target of many drugs and environmental toxicants; however, how toxicant-induced mitochondrial dysfunction contributes to the progression of human disease remains poorly understood. To address this issue, *in vivo* assays capable of rapidly assessing mitochondrial function need to be developed. Here, using the model organism *Caenorhabditis elegans*, we describe how to rapidly assess the *in vivo* role of the electron transport chain, glycolysis or fatty acid oxidation, in energy metabolism following toxicant exposure, using a luciferase-expressing ATP-reporter strain. Alterations in mitochondrial function subsequent to toxicant exposure are detected by depleting steady-state ATP levels with inhibitors of the mitochondrial electron transport chain, glycolysis, or fatty acid oxidation. Differential changes in ATP following short-term inhibitor exposure indicate toxicant-induced alterations at the site of inhibition. Because a microplate reader is the only major piece of equipment required, this is a highly accessible protocol for studying toxicant-induced mitochondrial dysfunction *in vivo*.

Keywords: *Caenorhabditis elegans*, mitochondrial toxicity, PE255, PE327, glycolysis, oxidative phosphorylation, fatty acid oxidation

INTRODUCTION

Mitochondria are best known for the role they play in ATP production via oxidative phosphorylation; however, mitochondria also play crucial roles in apoptosis (Susin et al., 1999), calcium homeostasis (Duchen, 2000) and retrograde signaling (Liu and Butow, 2006), thus playing diverse roles in cellular and organismal health. Mitochondrial dysfunction is causative and/or associated with numerous human diseases, including cancer (Baysal et al., 2000; Wallace, 2012; Yan et al., 2009), metabolic syndrome (Bugger, 2008), and various neurological disorders (Beal, 2005; Lin and Beal, 2006). Furthermore, growing evidence has demonstrated that mitochondria are an important target of many drugs (e.g. antibiotics and nucleoside reverse transcriptase inhibitors) (Dykens and Will, 2007; Guan, 2011; Poirier et al., 2015) and environmental toxicants (e.g. polycyclic aromatic hydrocarbons and pesticides) (Backer and Weinstein, 1980; Meyer et al., 2013; Tanner et al., 2011), and toxicant-induced mitochondrial dysfunction has been implicated in many diseases, including cancer and neurodegeneration (Robey et al., 2015; Tanner et al., 2011; Zhao et al., 2014).

Because mitochondrial function is dependent upon cellular context and environmental cues (Chan, 2012; McBride et al., 2006), it is critical to develop assays capable of rapidly assessing mitochondrial function, *in vivo*, following toxicant exposure. A short lifecycle (2-3 weeks), high reproductive rate (~300 offspring per gravid adult), and highly conserved mitochondrial biology (Tsang and Lemire, 2003) and biochemical pathways (Braeckman et al., 2009) contribute to the utility of the model organism *Caenorhabditis elegans* for studying toxicant-induced mitochondrial dysfunction. Furthermore, significant overlap between the activities of Toxcast phase I and II libraries have recently been described between nematodes and zebrafish, further validating *C. elegans* as an important non-mammalian model (Boyd et al., 2015). Currently, mitochondrial respiration in *C. elegans* can be measured via low-throughput Clark type electrodes (Braeckman et al., 2002), or with the higher-throughput, but more expensive Seahorse XF^{e} Bioanalyzer (Luz et al., 2015a; Luz et al., 2015b). Additionally, small metabolites, such as ATP, pyruvate, or NADH can be extracted from nematodes and used to assess mitochondrial health (Brys et al., 2010; Krijgsveld et al., 2003); however, this is a time consuming process. Alternatively, transgenic, firefly luciferase-expressing PE255 nematodes can be used to rapidly assess steady-state ATP levels *in vivo* (Lagido et al., 2009; Lagido et al., 2008). This transgenic model has proven valuable to environmental toxicologists, and has been used to study the effects of heavy metals and 3,5-dichlorophenol (Lagido et al., 2009; Lagido et al., 2001), 5-fluoro-2-deoxyuridine (Rooney et al., 2014), sewage sludge extract (McLaggan et al., 2012), the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Bodhicharla et al., 2014), and ultraviolet C radiation (Bess et al., 2012; Bess et al., 2013; Leung et al., 2013) on steady-state ATP-levels, and has more recently been used to track nematode development (Olmedo et al., 2015), and screen drug-libraries (Lagido et al., 2015).

Here, using PE255 luciferase-expressing nematodes and well-established inhibitors of the mitochondrial electron transport chain (ETC), glycolysis, and fatty acid oxidation (FAO) we describe a novel method that can be used to rapidly screen for alterations in mitochondrial energy metabolism following drug or toxicant exposure. Short-term incubation with these inhibitors depletes steady-state ATP levels. Thus, differential depletion of ATP in toxicant exposed nematodes in response to inhibitors indicates the relative contribution of the targeted cellular process to energy metabolism. Using this approach we recently demonstrated induction

of a Warburg-like effect in arsenite exposed PE255 *glp-4* nematodes (Luz et al., Submitted), which was confirmed via Seahorse XF^e and small metabolite analysis, thus further demonstrating this protocol's utility in detecting toxicant-induced mitochondrial dysfunction.

BASIC PROTOCOL 1

Luciferase-based *in vivo* assessment of mitochondrial energy metabolism in *C. elegans*

Here, using the PE255 ATP reporter strain (Lagido et al., 2015; Lagido et al., 2008), and the well-known pharmacological inhibitors rotenone (complex I), thenoyltrifluoroacetone (TTFA, complex II), antimycin A (complex III), sodium azide (complex IV), dicyclohexylcarbodiimide (DCCD, ATP synthase), carbonyl cyanide-p-trifluoromethoxyphenylhydrazon (FCCP, mitochondrial uncoupler), perhexiline (fatty acid oxidation (FAO)), and 2-deoxy-D-glucose (2-DG, glycolysis)) we outline how to rapidly assess mitochondrial energy metabolism following toxicant exposure. All of these inhibitors have previously been demonstrated to work in *C. elegans* (Luz et al., 2015b; Schulz et al., 2007; Taylor et al., 2013; Zubovych et al., 2010). Short-term (1.0 or 4.5 hour) incubation with inhibitors results in changes in steady-state ATP levels. Thus, altered function at the site of inhibition is detected through differential depletion of steady-state ATP levels. For example, the magnitude of ATP depletion following inhibition of ETC complex I with rotenone will be less than in toxicant exposed relative to unexposed nematodes if toxicant exposure has the effect of reducing complex I activity. This is because complex I is already contributing less to maintenance of steady-state ATP levels. Alternatively, the magnitude of ATP depletion will be greater in toxicant exposed nematodes if toxicant exposure is increasing activity of complex I. Figure 1 details the main principles of this assay.

Materials:

REAGENTS:

OP50 seeded K-agar plates (see Support Protocol 1)
Synchronous populations of L1 PE255 nematodes (see Support Protocol 2; Transgenic (PE255) N2 (wild type) and PE327 *glp-4* (*bn2*) nematodes available through the *Caenorhabditis* Genetics Center, University of Minnesota)
K-medium (see recipe)
Inhibitor stocks (Table 1 outlines all required inhibitors, as well as storage conditions)
Dimethylsulfoxide (DMSO)
Unbuffered EPA H_2O (see recipe)
0.1% (v/v) Triton X-100 (diluted in ddH_2O; store at room temperature indefinitely)
Glass microscope slides
Disposable reagent reservoirs
Multi-channel pipette (capable of pipetting 20-200µl)
White 96-well plates without lids
Luminescence buffer (see recipe)

EQUIPMENT:

Incubator (capable of maintaining temperatures in the range of 15-25°C)
Centrifuge (e.g. Beckman Coulter equipped for 15mL tubes)
Dissecting light microscope
Horizontal vortexer (e.g. Eppendorf MixMate PCR 96)
Orbital shaker
Microplate reader (FLUOstar OPTIMA, BMG Labtech) equipped with luminescence optic, 502nm emissions filter, and 485 nm excitation filter

Nematode culturing [*Au: This seems like a supporting protocol]
Nematodes are cultured on k-agar plates seeded with *E. coli* strain OP50 as previously described (Stiernagle, 1999).
Using a sterile Pasteur pipet transfer age-synchronous L1 PE255 nematodes, obtained from sodium hydroxide bleach treatment (see Support Protocol 2), to an OP50 seeded k-agar plate (see Support Protocol 1). Culture the nematodes until the appropriate life stage for toxicant or drug exposure is reached.

Both PE255 glp4 (strain PE327) *and PE255 N2 (wild type) nematodes are available for purchase for a nominal fee, through the National Institutes of Health-supported Caenorhabditis Genetics Center (CGC, University of Minnesota).*

This assay was originally developed using germline-deficient, PE255 glp-4 (bn2) nematodes, which are maintained at the permissive temperature of 15°C. Shifting glp-4 nematodes to the restrictive temperature, 25°C, results in sterile, germ cell free nematodes (Beanan and Strome, 1992). However, we have successfully used most of the concentrations of inhibitors outlined in this assay (see Table 1) with both L4 and 8 day old adult PE255 N2 nematodes, which are maintained at 20°C.
Toxicant or Drug Exposure
This assay can be used to assess mitochondrial function following toxicant or drug exposure. The precise length of exposure is at the discretion of the experimenter. However, if using PE255 N2 nematodes we recommend assaying prior to, or after the reproduction period, as reproduction will add variability to experiments. Nematodes can be exposed in liquid or on agar; however, liquid exposures can facilitate drug uptake in nematodes (Zheng et al., 2013). Finally, be sure to thoroughly rinse toxicant exposed nematodes 3-4 times with 15ml k-medium to remove excess toxicant prior performing this assay.
Preparation of Inhibitors [*Au: This seems like it belongs either in a Reagents & Solutions section or as supporting protocol. Isn't this described in the Table? If this is the same as what is described in table 1 then this section is redundant and can only be referenced as Table 1.]

Prepare stocks of 2mM rotenone, 100mM TTFA, 15mM antimycin A, 2mM sodium azide, 2mM DCCD, 2.5mM FCCP, 10mM perhexiline, and 400mM 2-DG in either 100% unbuffered EPA water or DMSO as outlined in Table 1. To minimize freeze/thawing, stocks can be stored in 30µl aliquots at either 4°C or -20°C (see Table 1).

Titrations of each drug were performed in sterile young adult (cultured on agar for 72h at 25°C) PE255 glp-4 nematodes (Supplementary Figures 1-8). Concentrations of each ETC inhibitor that result in a 40-60% depletion of ATP after a one hour exposure were then chosen.

A 4.5 hour exposure to 50mM 2-DG gave the most consistent reduction in luminescence in the context of arsenite exposure (Luz et al., Submitted), thus was chosen for all future experiments.

A 1 hour exposure to 100µM perhexiline increased nematode luminescence (~25%) in PE255 glp-4-deficient nematodes (Supplemental Figure 8), and thus was chosen for all future experiments. Our rationale for this result is detailed in the Anticipated Results, perhexiline section.
Dilute inhibitor stocks with unbuffered EPA H_2O to the appropriate 8X working concentrations as outlined in Table 1.

All inhibitors are dissolved in either DMSO or unbuffered EPA water. 8X Working stocks contain zero or 8% DMSO, such that when 12.5µl of the 8X working stock is pipetted into a well of a white 96-well plate (containing 50 nematodes in 87.5µl unbuffered EPA H_2O) the inhibitor is diluted to its final, 1X, working concentration in 1% DMSO. A one hour exposure to 1% DMSO does not significantly affect ATP levels in young adult PE255 glp-4, or L4 or 8 day old PE255 N2 nematodes (Supplemental Figure 1, 9).

Preparation of nematodes for inhibitor exposure

Remove PE255 nematodes from the incubator. If nematodes are being exposed to a drug or toxicant (either on agar or in liquid), be sure to rinse the nematodes thoroughly to remove excess toxicant.

Excess toxicant can be removed by transferring toxicant exposed nematodes to a new 15ml centrifuge tube, and resuspending them in 15ml unbuffered EPA H_2O. Nematodes can then be pelleted by centrifuging at 2200 RCF for 2 minutes at room temperature. The supernatant can then be discarded in accord with your university's guidelines. This process should then be repeated an additional 2-3 times to ensure toxicant is completely removed through dilution.

Resuspend the nematodes to a final concentration of 1.0±0.2 nematodes per microliter in unbuffered EPA H_2O.

The minimal acceptable concentration is 0.6 nematodes per microliter, as this concentration results in approximately 50 nematodes per 87.5µl; however, we recommend diluting nematodes to a concentration of 0.8-1.2 nematodes per microliter for all samples to minimize variation. To estimate the number of nematodes per microliter, trim the tip of a 200µl pipette tip, and pipette 20µl of 0.1% Triton X-100 up and down. The triton prevents worm loss due to sticking. Pipet four 20µl drops of nematodes onto a glass slide and count the number of nematodes per 20µl on a dissecting light microscope. Be sure to use a new, triton rinsed tip for each drop, and invert the centrifuge tube several times between counts to resuspend the nematodes.

Calculate the volume required to obtain 50 nematodes.

For example, if your concentration of nematodes is 1.0 nematode per microliter, you will pipette 50µl into each well of the 96-well plate to achieve 50 nematodes per well.

Pour 5ml 0.1% Triton X-100, 5ml unbuffered EPA H_2O, and the nematode suspension into three separate, new, 25ml disposable reagent reservoirs.

Using a 200µl multi-channel pipette, pipet 50 nematodes into the appropriate wells of a white 96-well plate.

Prior to pipetting nematodes into a white 96-well plate trim the pipet tips with scissors to increase each tips circumference, which allows large adult nematodes to be pipetted without injury. Then rinse the pipette tips with 0.1% Triton X-100 by pipetting up and down, which prevents nematode loss due to sticking. Nematodes can then be re-suspended in the reagent reservoir prior to their addition to the 96-well plate by pipetting up and down 3-4 times with the multi-channel pipette. Use new, trimmed, triton-rinsed tips each time you resuspend and transfer nematodes.

Figure 2 illustrates how a 96-well plate may be set up for an experiment containing two experimental groups. For example, each group (i.e. control and toxicant exposed) is pipetted into 4 wells of a 96-well plate for each inhibitor or control (i.e. EPA H_2O or 1% DMSO) used. We recommend setting up 2 plates, one for the one hour inhibitor exposure (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline), and one for the 4.5 hour inhibitor exposure (2-DG; not shown, but can be setup in a manner similar to Figure 2. Note that 2-DG does not require a 1% DMSO control, as it is dissolved in EPA H_2O).

Using a multi-channel pipette bring the volume in each well to 87.5µl with unbuffered EPA H_2O.

EPA H_2O controls (i.e. nematodes unexposed to inhibitors or DMSO) and blank wells can be brought to a final volume of 100µl with unbuffered EPA H_2O.

Using a 20µl pipette, add 12.5µl of each 8X inhibitor (prepared in steps 2-3 and outlined in Table 1) to the appropriate wells. Figure 2 outlines how nematodes can be loaded into a 96 well plate; however, this will vary depending upon the number of exposure groups, and inhibitors chosen for each experiment (outlined in Figure 2).

It should take no longer than 3-4 minutes to load all of the inhibitors for each plate. This is important, because the inhibitor incubation period is only 60 minutes (for ETC, and FAO inhibitors); thus, a longer loading period will introduce variability into the assay. If necessary, samples can be divided onto multiple 96-well plates to limit the amount of time it takes to load all of the inhibitors. However, be sure to include the appropriate EPA H_2O and DMSO controls, as well as blanks for each plate. The addition of inhibitors should be staggered 15-20 minutes for each plate to avoid overlap on the plate reader.

At minimum, two plates will be run. The first plate is designated for one hour inhibitor incubations (rotenone, TTFA, antimycin A, azide, DCCD, FCCP, perhexiline, EPA H_2O and 1% DMSO controls), while the second plate is for the 4.5 hour inhibitor incubations (i.e. 2-DG, EPA H_2O control).

Vortex the white 96-well plate for 10 seconds at 1000 rpm using a horizontal vortexer after the final inhibitor has been added.

Vortexing will help ensure that inhibitors are mixed and completely in solution.

Place the 96-well plate on an orbital shaker at 20°C for 60 minutes or 4.5 hours depending upon which inhibitors are being tested.

Measuring steady-state ATP levels

In the presence of ATP, firefly luciferase catalyzes the oxidation of luciferin to generate light. Thus, steady-state ATP levels can be determined *in vivo* by measuring light output, which is proportional to steady-state ATP levels in PE255 nematodes (Lagido et al., 2008). Nematode luminescence can be measured using a microplate reader equipped with a luminescence optic and filters capable of measuring GFP fluorescence (502nm emissions, 485nm excitation). Below we detail how to measure ATP in PE255 nematodes using a FLUOstar OPTIMA (BMG LABTECH) plate reader; however, precise instruction will vary depending upon the microplate reader model being used. A recent visual presentation of this assay is also available (Lagido et al., 2015).

Prepare the luminescence buffer (see recipe) 15 minutes prior to the end of the incubation period. **[*Au: This seems like a reagents & solutions section]**

Luminescence buffer can be prepared in 15ml centrifuge tube covered in foil to protect light-sensitive luciferin.
Turn on the plate reader and open the OPTIMA software 15 minutes before the incubation period has ended. Prepare the plate reader for measuring GFP fluorescence.
PE255 nematodes express a firefly luciferase – GFP fusion protein. Thus by normalizing each wells luminescence reading to GFP, you can account for overall enzyme content, which will help to normalize each well for slight discrepancies in nematode size and overall nematode counts.
Under reader configuration, select the fluorescence optic.
Select test setup. Click on fluorescence intensity, and make a new program for measuring GFP. Name (i.e. PE255::GFP) and save the program for future use. Guidelines for preparing the program are outlined below.
Plate type: (fill in with the appropriate plate brand)
Optic used: Top
Excitation filter: 485nm
Emission filter: 502nm
Position delay (s): 0.2
Kinetic window: 1
Number of cycles: 1
Measurement start time (s): 0.0
Number of flashes: 10

Open the newly designed program. Under the Layout tab select the appropriate sample containing wells. Click Okay.
Approximately 10 minutes before the incubation period has ended, insert the 96-well plate into the microplate reader. Click on measure and select the appropriate protocol for measuring GFP. Name the current run in the pop-up menu.
Click on the gain tab and highlight the entire plate. Click gain adjust.
Gain adjusting the entire plate will identify the well with the highest GFP fluorescence, which will be used to normalize the entire plate. The raw gain value should be somewhere around 58,000, although this may vary for other microplate reader models.

Click start measurement. When the measurement has finished exit the OPTIMA software. Turn off the plate reader.

GFP measurements are automatically saved in the OPTIMA software.

Carefully remove the fluorescence optic from the microplate reader and replace it with the luminescence optic.

For more details see the plate reader's user manual.

Turn on the plate reader and open the OPTIMA software. Click on Reader Configuration and select luminescence optic.

Prime the injector (if applicable) for luminescence buffer injection. First, insert the injector needle into a waste container (50ml centrifuge tube covered with foil) and the tubing into a 50ml centrifuge tube containing 70% ethanol. Next, select the priming function and flush the injector needle with 2ml 70% ethanol, following by 2ml ddH_2O, and finally prime the injector needle with 1.5ml of luminescence buffer.

If your plate reader is not equipped with an injecting apparatus you can manually pipette the luminescence buffer into your 96-well plate using a multi-channel pipette and then read luminescence 3 minutes later.

Remove the injector needle from the waste container and place it into the machine's injection port.

Select test setup. Click on luminescence intensity, and make a new program for measuring luminescence. Name (i.e. PE255-Luminescence) and save the program for future use.

Guidelines for measuring luminescence are outlined below.

Plate type: (fill in with the appropriate plate brand)
Optic used: Top
Gain: 3600
Emission filter: lens
Position delay (s): 0.2
Shaking width (mm): 7
Shaking mode: orbital
Number of cycles: 2
Cycle time (s): 180
Measurement start time (s): 0.0
Measurement interval time (s): 1.0

Injector Setup

Volume (µl): 50
Pump used: 1
Pump speed (µl/s): 420
Pump syringe volume (ml): 0.5
Injection cycle: 1
Injection start time (s): 0.0

Open the newly designed program. Under the layout tab select the appropriate sample containing wells.

Click on the injection tab. Make sure all sample containing wells are set to have 50µl of luminescence buffer injected. Click Okay.
Click on Measure and select the appropriate protocol for measuring luminescence. Name the current run in the pop-up menu. Click start.
When the luminescence measurement finishes the results automatically save in the OPTMA software.
When finished with the instrument, wash the injector tubing. First, remove the injector needle from the plate reader and place it into the waste container. Place the injector tubing into ddH_2O, and rinse the tubing with 3ml ddH_2O, followed by 3ml 70% ethanol. Finally, dry the injector tubing by back flushing 3ml into the waste container.
Back flushing the injector pushes air through the line to dry it out; any remaining ethanol in the line should then evaporate.
Open results in the OPTIMA Software. Blank correct each well's GFP and luminescence values by subtracting the EPA H_2O blank GFP and luminescence values, respectively. An example of how to normalize data is provided in Supplemental File 1.
Each well's blank-corrected luminescence can be divided by the corresponding blank-corrected GFP value. Alternatively, we generate normalization factors for each well by dividing each wells blank-subtracted GFP value by the average GFP value for the entire plate. Blank-corrected luminescence values can then be divided by the corresponding normalization factor as detailed in Supplemental File 1.

Statistical Analysis

Assuming normally distributed data (which has been our experience), assess the effects of each drug initially with a one-, two-, or three-way ANOVA, depending upon how many factors you have (i.e. time, strain, dose, etc.). If different developmental stages are compared, ATP levels may vary enough that logarithmic transformation of the data is necessary to permit comparison of exposure-or strain-related differences across ages. When warranted, post-hoc analysis can be performed.

SUPPORT PROTOCOL 1

Preparing OP50 seeded k-agar plates

Nematodes are cultured on *Escherichia coli* OP50 seeded k-agar plates. The preparation of OP50 seeded K-agar plates has previously been described (Lewis and Fleming, 1995; Stiernagle, 1999), and is briefly outlined below.

Materials:

Potassium chloride (KCl),
Sodium chloride (NaCl)
Bacto-peptone
Bacto-agar
1M Calcium chloride ($CaCl_2$, dissolved in ddH_2O and autoclaved)
1M Magnesium sulfate ($MgSO_4$, dissolved in ddH_2O and autoclaved)
10 mg/ml Cholesterol (dissolved in ethanol and filter sterilized)
5mL 1.25 mg/ml Nystatin (dissolved in ethanol)
LB broth (see recipe)

E. coli OP50 (which can be purchased from the *Caenorhabditis* Genetics Center, University of Minnesota)

Erlenmeyer flask (2L or larger)
Magnetic spin bar
Magnetic hot plate
Autoclave tape
Autoclave
Serological pipette (25 or 50 mL)
Motorized pipette aid (e.g. Drummond)
Petri dishes (100 x 50mm)
Inoculating loop
37°C Shaking incubator
Repeating pipette (e.g. Eppendorf) & 10 mL displacement tips
Glass hockey stick spreaders
Rotating pedestal
Bunsen burner

Pouring k-agar plates

Weigh and add 2.36g potassium chloride, 3.0g sodium chloride, 2.5g bacto-peptone, and 20g bacto-agar to a 2L Erlenmeyer flask containing 1L ddH_2O and a magnetic spin bar. Cover the flask with foil and add autoclave tape.

Prior to autoclaving the agar can be mixed gently on a magnetic spin plate; however, this is not required, as autoclaving will dissolve all ingredients.

Autoclave to sterilize.

A 30 minute liquid cycle at 121°C and 17psi is sufficient for sterilization.

Place sterilized k-agar on a magnetic spin plate with the spin bar turned on and let the agar cool to ≈55°C.

Once cooled, add 1ml 1M $CaCl_2$, 1ml 1M $MgSO_4$, 1 ml 10 mg/ml cholesterol, and 5 ml 1.25 mg/ml nystatin (dissolve in 100% EtOH) to the k-agar.

The nystatin is an anti-fungal used to help prevent contamination.

Using a 50 mL serological pipet, carefully pipette 17mL k-agar into a 100x50mm sterile petri dish. Gently swirl the agar to ensure the entire surface of the plate is covered.

Caution! K-agar plates are easily contaminated. To minimize contamination risk use sterile technique. One liter of k-agar fills approximately 60 petri dishes.

Let the k-agar plates cool and solidify overnight (12-18 hours).

Growing *E. coli* OP50

Using sterile technique, inoculate 50mL sterile LB broth (see recipe) with *E. coli* OP50 using a sterile inoculating loop.

Incubate the LB broth at 37°C, while shaking at 250 rpm, overnight (16 hours).

Seeding K-agar plates with OP50

Using sterile technique pipette 300µl of OP50 culture onto the center of each k-agar plate.

We use a 10mL Eppendorf repeating pipettor, which minimizes the number of times that you will have to pipette directly from the OP50 culture, thus minimizing the risk of contamination.

Using a sterile glass hockey stick and a rotating pedestal spread the OP50 on the surface of the k-agar.

To sterilize the glass hockey stick dip it in 70% ethanol, and then pass it through the flame of a bunsen burner and allow the ethanol to burn off. Re-sterilize the hockey stick every five plates.

Let the OP50 seeded k-agar plates dry at room temperature (~48 hours). Dry OP50 plates can then be stored at 4°C for up to 3 months.

SUPPORT PROTOCOL 2

Age-synchronizing nematodes via sodium hypochlorite treatment

Synchronous populations of L1 nematodes can be generated by treating gravid adult nematodes with sodium hypochlorite solution. Nematode eggs are resistant to sodium hypochlorite, while gravid adults are not, which allows for the isolation of eggs. Eggs are then allowed to hatch overnight (12-18 hours) in the absence of food, resulting in synchronous populations of L1 nematodes (Lewis and Fleming, 1995). Note that the timing may need to be reduced if using RNAi or crossing luciferase reporter strains with strains carrying mutations, because some genetic manipulations sensitize eggs to this treatment.

Materials

OP50 seeded k-agar plates (see Support Protocol 1) containing gravid adult nematodes
K-medium (see recipe)
70% ethanol
15 mL Centrifuge tubes (e.g. Corning Falcon)
Centrifuge (e.g. Beckman Coulter equipped for 15mL tubes)
Glass hockey stick spreader
Bunsen burner

Orbital shaker
Pasteur pipette
50ml cell culture flask
Dissecting light microscope

Using sterile k-medium wash gravid adult nematodes from the k-agar plate into a new 15 mL centrifuge tube. Pellet nematodes by centrifuging at 2200 RCF for 2 minutes at room temperature. Discard the supernatant.
Pipette 2-3ml k-medium onto k-agar plate and gently loosen eggs from the surface of the agar using a sterile glass hockey stick spreader. Carefully pour the loosened eggs into the 15 ml centrifuge tube containing the gravid adult nematodes, and spin at 2200 RCF for 2 minutes. Discard the supernatant.
Glass hockey stick spreaders can be sterilized by dipping them in 70% ethanol, and then passing them through the flame of a Bunsen burner, allowing the ethanol to burn off.
Carefully add 10ml of sodium hydroxide bleach solution (see recipe) to the nematode pellet. Place the centrifuge tube on an orbital shaker in a 20°C incubator for 8 minutes.
8 minutes is sufficient time for the sodium hydroxide bleach solution to disintegrate adult nematodes, but not eggs, allowing for the isolation of a large quantity of nematode eggs. After 8 minutes, if adult nematodes remain, place the tube back on the orbital shaker for up to 2 additional minutes.
After 8 minutes, spin the centrifuge tube at 2200 RCF for 2 minutes, discard the supernatant, and resuspend the pelleted eggs in 15 ml k-medium.
Take care not to disturb the egg pellet when removing the bleach solution; however, no more than 50-100µl of bleach solution should remain prior to resuspension in k-medium.
Spin the resuspended eggs for an additional 2 minutes at 2200 RCF. Discard all but 0.5-1.0ml of the supernatant.
Using a sterile glass Pasteur pipette, resuspend the pelleted eggs, and transfer them to a sterile 50ml cell culture flask containing 8ml complete k-medium (see recipe).
Incubate the flask overnight (12-18 hours) on an orbital shaker at 20°C to obtain a synchronous population of L1 nematodes.
Under a dissecting light microscope check to make sure the majority of the eggs have hatched. Pour the L1 nematodes into a sterile 15 mL centrifuge tube, and centrifuge at 2200 RCF for 2 minutes. Discard the supernatant.
Transfer the nematodes to an OP50 seeded k-agar plate (see support protocol 1) using a Pasteur pipette and incubate the nematodes at the appropriate temperature until the desired life stage is reached.

REAGENTS AND SOLUTIONS

Complete k-medium

150µl 1M calcium chloride ($CaCl_2$; sterilized via autoclave)
150µl 1M magnesium sulfate ($MgSO_4$; sterilized via autoclave)
25µl 10mg/ml cholesterol (dissolve in 100% ethanol and filter sterilize)
50mL sterile k-medium
Store at room temperature for up to one week

K-medium

2.36g Potassium chloride (KCl)
3g Sodium chloride (NaCl)
1L ddH_2O
Autoclave to sterilize

Store at room temperature, indefinitely, under sterile conditions

LB broth

0.5g Tryptone
0.25g Yeast extract
0.5g Sodium chloride (NaCl)
50mL ddH_2O
Autoclave to sterilize
Store at room temperature, indefinitely, under sterile conditions

Luminescence Buffer

6.925ml 0.2M Na_2PO_4 (store at room temperature)
3.075ml 0.1M Citric acid (store at room temperature)
100µl DMSO
100µl 5% Triton X-100 (diluted in ddH_2O)
100µl 10mM Luciferin salt (dissolved in ddH_2O; store in 100µl aliquots at -20°C; protect from light)
Make fresh prior to ATP measurements

Sodium hydroxide bleach solution

6mL Clorox bleach (non-germicidal, regular bleach*, 8.25% sodium hypochlorite)
5 Sodium hydroxide pellets (NaOH; Avantor Performance Materials)
44mL ddH20
Shake until NaOH pellets are completely dissolved
Each pellet weighs ~89mg.

Store at room temperature for up to 3 days.
*It is important to use non-germicidal bleach, as some bleaches contain germicides that are toxic to nematodes.

Unbuffered EPA water (Weber, 1991)

60mg Magnesium sulfate ($MgSO_4 \cdot 7\ H_2O$)
60mg Calcium sulfate ($CaSO_4 \cdot 2\ H_2O$)
4mg Potassium chloride (KCl)
1L ddH_2O
Store at room temperature, indefinitely, under sterile conditions

COMMENTARY

Background Information

The protocol described in this unit details how to rapidly assess the contribution of different pathways to steady-state ATP levels following drug or toxicant exposure in the model organism *C. elegans*. As mitochondrial function is dependent upon cellular context (Chan, 2012; McBride et al., 2006), this approach offers the advantage of an *in vivo* model, as well as all of the other benefits associated with working with nematodes. For example, simple RNAi gene knockdown technology (Kamath et al., 2003), and/or outbreeding the PE255 transgene into any of thousands of genetically deficient strains (Thompson et al., 2013) could extend this protocol's utility to include genetic and gene-environment interaction studies.

Although this protocol offers many advantages over other assays capable of assessing mitochondrial respiratory chain health, it also has limitations. This protocol cannot distinguish between direct enzyme inhibition, substrate limitation, or changes in overall enzyme content as causative of observed changes in inhibitor based ATP-depletion. Instead, this protocol offers an economical way to thoroughly assess mitochondrial health that can then be followed up with more targeted assays, such as metabolomics, Seahorse Analysis, and gene or protein expression studies. Another drawback of this protocol is that it does not directly measure changes in steady-state ATP levels, but instead measures changes bioluminescence generated by the ATP-powered firefly luciferase enzyme. However, targeting of mitochondrial respiratory chain genes by RNAi gave a bioluminescence response that correlated with steady-state ATP levels in PE255 nematodes (Lagido et al., 2015; Lagido et al., 2008). Finally, differences in ATP depletion between control and exposed nematodes may be due to compensatory increases in ATP production via other routes, such as glycolysis. However, if this is the case then toxicant-exposed nematodes would be expected to be less sensitive to ATP depletion induced by all ETC inhibitors; thus, toxicant-induced changes at only one or two of the ETC complexes is highly suggestive of altered function at the site of inhibition rather than a compensatory increase in ATP production via an alternative route.

In addition to the inhibitors optimized for this protocol, inhibitors of other metabolic pathways could also be used to further assess toxicant-induced mitochondrial dysfunction. For example, dichloroacetate (DCA), a pyruvate dehydrogenase kinase (negative regulator of the Krebs cycle) inhibitor could be used to assess changes in Krebs cycle activity. We have previously tested this inhibitor in the context of arsenite exposure, but observed no significant changes in bioluminescence under any conditions tested (1-6 hour exposure to either 0.5 or 1.0mM DCA) (Luz et al., Submitted). Likewise, Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES), a glutaminase (GLS1) inhibitor, could be used to test for toxicant-induced changes in glutamine metabolism (Shukla et al., 2012). However, not all metabolic inhibitors will inhibit their intended targets in nematodes. For example, the mitochondrial uncoupler 2,4-dinitrophenol and the ATP synthase inhibitor oligomycin A do not inhibit mitochondrial respiration in nematodes (Luz et al., 2015a). It is probable that the nematode cuticle, a thick collagenous barrier, limits the uptake of these inhibitors, as oligomycin can inhibit mitochondrial respiration in cuticle-deficient (*bus-8*) nematodes (Luz et al., 2015a); thus, we use the less-specific ATP synthase inhibitor, DCCD, in the current protocol.

Finally, under certain conditions the ETC complex II inhibitor TTFA can also function as a mitochondrial uncoupler. However, 1mM TTFA did not increase mitochondrial respiration

(Supplemental Figure 13), suggesting that TTFA-induced depletion of ATP (Supplemental Figure 3) is due to complex II inhibition. Nonetheless, we have also optimized malonate, a competitive inhibitor of complex II for the current protocol (Thorn, 1953). Exposure to 15mM malonate for one hour reduces ATP by approximately 50% (Supplemental Figure 14A), and reduces mitochondrial respiration (Supplemental Figure 15); however, higher concentrations of malonate were inexplicably found to reduce GFP fluorescence (Supplemental Figure 14B), which under certain conditions may confound results. Thus, we recommend researchers consider the caveats to working with TTFA and malonate prior to performing experiments.

Critical Parameters

Number of nematode per well

This protocol has been optimized to work with 50 nematodes per well of a 96-well plate, and has successfully been used with both L4 and 8 day old adult nematodes. Fewer nematodes (25) have been tested, but tend to give variable luminescence values (data not shown). Alternatively, if this assay is to be used with L2 or L3 nematodes, the experimenter will have to load >50 nematodes per well, but must be careful not to overload the wells as this could result in anoxic conditions.

Age and genetic background of nematodes

Early and later life stages are widely considered more sensitive to certain exposures. Thus, concentrations of inhibitors may need to be adjusted depending upon the life stage being investigated. In agreement with this, we observed increased sensitivity to ATP depletion with rotenone, antimycin A, and FCCP in 8 day old PE255 N2 nematodes (Figures 3-5). In contrast, reduced sensitivity to TTFA was observed in 8 day old PE255 N2 nematodes (Figure 6), while no age related sensitivities were observed for azide, DCCD, or 2-DG (Supplemental Figures 10-12).

Genetic deficiencies can also sensitize (or protect) an organism from toxicity following toxicant exposure. Therefore, if this protocol is adapted to RNAi studies or if the PE255 transgene is crossbred into other genetically-deficient strains, inhibitor concentrations may need to be adjusted. However, we have crossbred the PE255 transgene into mitochondrial fission-deficient nematodes (*drp-1*), and observed similar responses to inhibitors as with PE255 N2 nematodes (data not shown). Interestingly, young adult PE255 *glp-4* nematodes appear less sensitive to TTFA than either L4 or 8 day old PE255 N2 nematodes, as 1mM TTFA reduced luminescence ~50% in *glp-4* (Supplemental Figure 3) and 80-99% in N2 nematodes (Figure 6). On the other hand, young adult PE255 *glp-4* nematodes appear more sensitive to sodium azide than either L4 or young adult PE255 N2, as 0.25mM azide reduced luminescence ~50% in *glp-4*-deficient nematodes (Supplemental Figure 5), but did not have a statistically significant effect on luminescence in PE255 N2 (Supplemental Figure 10). Thus, concentrations of TTFA (500µM is effective for N2 versus 1000µM for *glp-4*) and sodium azide (500µM is effective for N2 versus 250µM for *glp-4*) will need to be adjusted depending upon the genetic background.

Troubleshooting

Table 2 highlights some of the common problems encountered with this protocol, indicates potential causes, and outlines potential solutions to these problems.

Anticipated Results

ETC Inhibitors:

Incubation with inhibitors of the mitochondrial ETC (i.e. rotenone, TTFA, antimycin A, sodium azide, DCCD, FCCP) should dramatically (40-60%) reduce nematode luminescence, as these inhibitors directly interfere with ATP production via oxidative phosphorylation. Therefore, if prior toxicant exposure alters the activity of one or more ETC complexes the magnitude of ATP depletion will be significantly altered compared to unexposed nematodes. For example, if toxicant exposure reduces complex I activity the magnitude of ATP depletion following a one-hour incubation with rotenone will be reduced compared to unexposed nematodes.

2-Deoxy-D-Glucose:

If drug or toxicant exposure increases glycolysis, then incubation with the glycolysis inhibitor 2-DG should reduce nematode luminescence, whereas 2-DG should have little to no effect on untreated nematode bioluminescence because of the relatively small baseline contribution of glycolysis to ATP.

Perhexiline:

Perhexiline, a prophylactic anti-anginal medication, prevents mitochondrial fatty acid oxidation (FAO) by inhibiting mitochondrial carnitine palmitoyltransferase-1 (CPT-1), thus preventing the transport of long chain fatty acids into mitochondria (Kennedy et al., 1996). Inhibition of FAO with perhexiline results in a shift in cardiac metabolism from the utilization of fatty acids to glucose, which is beneficial because glucose oxidation requires less oxygen per unit of ATP generated. Thus, perhexiline increases cardiac efficiency (Kjekshus and Mjøs, 1972; Mjos and Kjekshus, 1971). Like cardiac myocytes (Stanley et al., 1997), germline-deficient nematodes elevate fatty acid oxidation (Ratnappan et al., 2014). Thus, we hypothesized that treatment of PE255 *glp-4*-deficient nematodes with perhexiline would increase nematode luminescence by increasing the efficiency of ATP production, which our findings support (Supplemental Figure 8). In the context of toxicant exposure the effect of perhexiline on luminescence may prove more difficult to interpret. We postulate that toxicant exposures that disrupt glucose catabolism may result in increased FAO, in which case perhexiline would be expected to decrease nematode luminescence by inhibiting FAO. This would provide initial evidence for toxicant-induced changes in FAO that can be confirmed with gene expression or metabolomics studies.

Time Considerations

It will take approximately 48 hours to culture a synchronous population of L4 PE255 nematodes; however, the overall duration of nematode culturing will depend upon the desired larval stage when toxicant exposure is to be initiated. Likewise, the length of toxicant exposure will vary from experiment to experiment. However, following toxicant exposure the entire assay can be performed in approximately six hours (1.5 hours to prepare inhibitors, load nematodes into 96-well plates and start inhibitor exposures, and 1 - 4.5 hours of inhibitor exposure).

When performing assays inhibitors must be pipetted into the appropriate wells of the 96-well plate in a timely manner. This is especially important for the 60 minute inhibitor incubation, as delays will introduce variability into the experiment. The addition of all inhibitors should take no more than 3-4 minutes when using a single channel pipette; however, this can

be further reduced by using a multi-channel pipette or by splitting samples onto multiple 96-well plates. However, the addition of inhibitors should be staggered by 15-20 minutes if multiple 96-well plates are being run to ensure no overlap on the microplate reader.

Approximately 10-15 minutes prior to the end of the inhibitor exposure the plate reader can be prepped for the pending GFP and luminescence measurements. Depending upon the plate reader, it will take 5-10 minutes to gain adjust and measure GFP per 96-well plate. Thus to avoid extended incubation periods we typically begin GFP measurements 5-10 minutes before the end of the incubation period. This allows luminescence to be measured immediately following the end of the 1 or 4.5 hour incubation period, which will help limit variability between experiments.

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FIGURE LEGENDS

Figure 1. Workflow for assessing toxicant-induced mitochondrial dysfunction in luciferase expressing PE255 *C. elegans*.

Figure 2. Example of how toxicant exposed nematodes can be loaded into a 96-well plate for inhibitor exposure.

Figure 3. Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 20µM rotenone than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.006$), treatment ($p<0.0001$), and their interaction ($p=0.008$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). N=3-4. Bars±SEM.

Figure 4. Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 150µM antimycin A than 2 day old nematodes (2 way ANOVA, main effect of treatment ($p<0.0001$), and time*treatment interaction ($p=0.002$), but not time ($p=0.19$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). N=3-4. Bars±SEM.

Figure 5. Eight day old PE255 N2 nematodes are more sensitive to ATP depletion following a one hour exposure to 25µM FCCP than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.02$), treatment ($p<0.0001$), and their interaction ($p=0.02$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). N=3-4. Bars±SEM.

Figure 6. Eight day old PE255 N2 nematodes are less sensitive to ATP depletion following a one hour exposure to 1mM TTFA than 2 day old nematodes (2 way ANOVA, main effects of time ($p=0.01$), treatment ($p<0.0001$), and their interaction ($p=0.008$)). Asterisk denotes statistical significance ($p<0.05$) for post-hoc comparison (Tukey's HSD). N=3. Bars±SEM.

Supplemental Figure 1. One hour exposure to 1% DMSO does not affect luminescence in young adult PE255 *glp-4* nematodes compared to nematodes in EPA water control (One way ANOVA, $p=0.65$). N=4. Bars±SEM.

Supplemental Figure 2. One hour exposure to 10, 15, and 20µM rotenone significantly reduced ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way

ANOVA, p=0.0004). 20µM Rotenone was chosen for future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars±SEM.

Supplemental Figure 3. One hour exposure to 1mM TTFA significantly reduced ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.0005). 1mM TTFA was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars±SEM.

Supplemental Figure 4. One hour exposure to 100 and 150µM antimycin A significantly reduced ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.005). 150µM Antimycin A was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=2-4. Bars±SEM.

Supplemental Figure 5. One hour exposure to 0.25, 0.5, 1.0, 2.5mM sodium azide significantly reduced ATP compared to the EPA water control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.001). 0.25mM Sodium azide was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=2-3. Bars±SEM.

Supplemental Figure 6. One hour exposure to 20 and 30µM DCCD significantly reduced ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.0002). 20µM DCCD was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars±SEM.

Supplemental Figure 7. One hour exposure to 10 and 25µM FCCP significantly reduced ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.0008). 25µM FCCP was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars±SEM.

Supplemental Figure 8. One hour exposure to 100µM perhexiline significantly increased ATP compared to the 1% DMSO control in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.0061). 100µM Perhexiline was used in all future experiments. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars±SEM.

Supplemental Figure 9. One hour exposure to 1% DMSO does not affect luminescence in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (two way ANOVA, main effect of time (p=0.0003), but not treatment (p=0.96) or their interaction (p=0.83)). N=3-7. Bars±SEM.

Supplemental Figure 10. One hour exposure to 0.25mM sodium azide did not significantly affect ATP levels in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (2 way ANOVA, main effect of time (p<0.0001), but not treatment (p=0.20) or their interaction(p=0.81)). N=3-4. Bars ± SEM.

Supplemental Figure 11. One hour exposure to 20µM DCCD reduced ATP levels in 2 and 8 day old N2 nematodes (two way ANOVA, main effect of treatment (p=0.003), but not time (p=0.33) or their interaction (p=0.38)). N=3-7. Bars±SEM.

Supplemental Figure 12. Four and a half hours exposure to 50mM 2-DG did not significantly affect ATP levels in 2 or 8 day old PE255 N2 nematodes compared to EPA water control (2 way ANOVA, main effect of time (p<0.0001), but not treatment (p=0.56) or their interaction (p=0.70)). N=3. Bars ± SEM.

Supplemental Figure 13. Exposure to 1mM TTFA did not affect mitochondrial respiration in young adult PE255 *glp-4* nematodes (one way ANOVA, p=0.53). N=7-22. Bars ± SEM.
Supplemental Figure 14. One hour exposure to malonate significantly reduced (A) ATP levels (one way ANOVA, p<0.0001) and (B) GFP fluorescence (one way ANOVA, p=0.014) in young adult PE255 *glp-4* nematodes. Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=3. Bars ± SEM.
Supplemental Figure 15. Exposure to malonate reduced mitochondrial respiration in young adult PE255 *glp-4* nematodes (one way ANOVA, p<0.0001). Asterisk denotes statistical significance (p<0.05) for post-hoc comparison (Tukey's HSD) to control. N=4-12. Bars ± SEM.

TABLES

Table 1. Preparation of Inhibitors

Inhibitor (Target)	**Stock Concentration**	**Working Concentration (8x)**	**Final Concentration (1x)**	**Incubation period (Hours)**
Rotenone (ETC Complex I)	2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	160µM Dissolved in 8% DMSO **<u>To make</u>**: Add 24µl 2mM Rotenone (100% DMSO) to 276µl unbuffered EPA H_2O	20µM in 1% DMSO	1
*TTFA (ETC Complex II)	100mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)	8mM Dissolved in 8% DMSO **<u>To make</u>**: Add 24µl 100mM TTFA to 276µl unbuffered EPA H_2O	1mM in 1% DMSO	1
¥Malonate (ETC Complex II)	120mM dissolved in 100% unbuffered EPA H_2O (store at 4°C in 1mL aliquots)	120mM dissolved in 100% unbuffered EPA H_2O	15mM in 100% unbuffered EPA H_2O	1
Antimycin A (ETC Complex III)	15mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	1.2mM Dissolved in 8% DMSO **<u>To make</u>**: Add 24µl 15mM Antimycin A (100% DMSO) to 276µl unbuffered EPA H_2O	150µM in 1% DMSO	1

**Sodium Azide (ETC Complex IV)	2mM dissolved in 100% unbuffered EPA H_2O (store at 4°C in 1mL aliquots)	2mM dissolved in 100% unbuffered EPA H_2O	250µM in 100% unbuffered EPA H_2O	1
DCCD (ATP synthase)	2mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	160µM Dissolved in 8% DMSO **To make**: Add 24µl 2mM DCCD (100% DMSO) to 276µl unbuffered EPA H_2O	20µM in 1% DMSO	1
FCCP (Mitochondrial uncoupler)	2.5mM dissolved in 100% DMSO (store at -20°C in 30µl aliquots)	200µM Dissolved in 8% DMSO **To make**: Add 24µl 2.5mM FCCP (100% DMSO) to 276µl unbuffered EPA H_2O	25µM in 1% DMSO	1
Perhexiline (Fatty acid oxidation)	10mM dissolved in 100% DMSO (store at 4°C in 30µl aliquots)	800µM Dissolved in 8% DMSO **To make**: Add 24µl 10mM Perhexiline (100% DMSO) to 276µl unbuffered EPA H_2O	100µM in 1% DMSO	1
2-DG (Glycolysis)	400mM dissolved in unbuffered EPA H_2O (store at 4°C in 30 µl aliquots)	400mM dissolved in unbuffered EPA H_2O	50mM in 100% unbuffered EPA H_2O	4.5

Concentrations of ETC inhibitors listed in Table 1 caused roughly a 40-60% reduction in bioluminescence in young adult PE255 *glp-4* deficient nematodes (Supplemental Figures 2-7), and with the exceptions of TTFA and sodium azide, cause similar reductions in both L4 and 8 day old PE255 N2 nematodes.

*500µM TTFA reduces luminescence approximately 50% in PE255 N2 nematodes (data not shown), while 1000µM causes an 80-99% reduction in PE255 N2 bioluminescence (Figure 6).

**250µM Sodium azide has no significant effect on PE255 N2 bioluminescence, while 500µM azide reduces bioluminescence ~50%.

¥ Malonate, a competitive inhibitors of ETC complex II, can be used in place of TTFA at the discretion of the experimenter. Pros and cons of this are discussed in the Background Information section.

TABLE 2. Trouble Shooting		
Problem	**Possible cause**	**Solution**
Inhibitor caused no, or only a minor decrease in luminescence	Concentration of inhibitor is too low.	Increase inhibitor concentration.
	Inhibitor precipitated out of solution	It is imperative that inhibitor stocks equilibrate to room temperature prior to their addition to the white 96-well plates, as the rapid temperature change may cause the inhibitors to precipitate out of solution.
	Luminescence optic not installed properly	Check that the luminescence optic has been installed properly.
	Luciferin was not added to the luminescence buffer.	Re-prepare the luminescence buffer taking care to add luciferin to the buffer.
	Luminescence buffer failed to inject	Ensure that your plate reader's injector needle has been properly installed and/or that luminescence buffer was properly added prior to measuring luminescence.
Inhibitor resulted in greater than a 90% loss of luminescence.	Inhibitor concentration is too high.	Decrease the concentration of inhibitor used.

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