

Supplementary information 1.

Selective Uncoupling of Individual Mitochondria within a Cell using a Mitochondria-Targeted Photoactivated Protonophore

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Complete references for references 2a and 20b

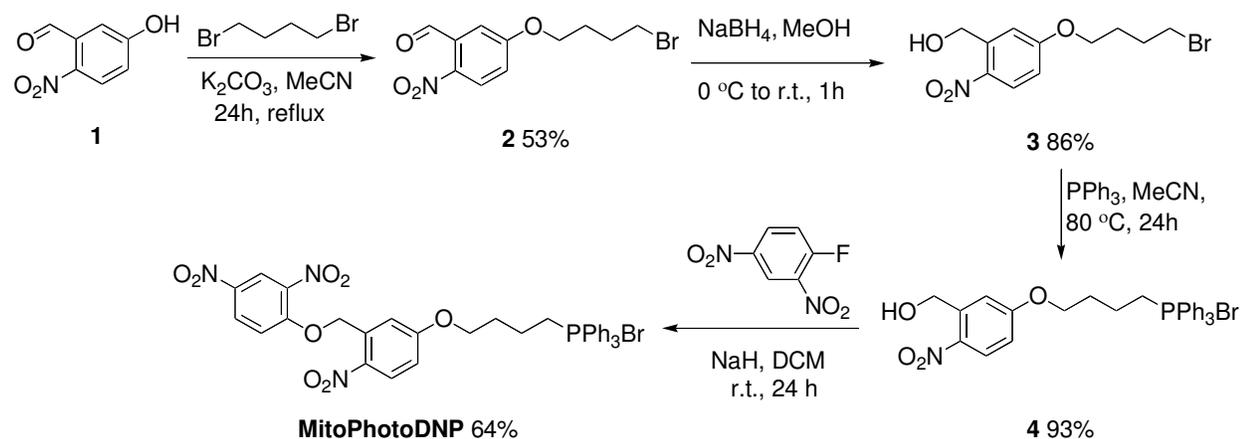
2(a) Cochemé, H. M.; Quin, C.; McQuaker, S. J.; Cabreiro, F.; Logan, A.; Prime, T. A.; Abakumova, I.; Patel, J. V.; Fearnley, I. M.; James, A. M.; Porteous, C. M.; Smith, R. A. J.; Saeed, S.; Carré, J. E.; Singer, M.; Gems, D.; Hartley, R. C.; Partridge, L.; Murphy, M. P. *Cell Metab.* **2011**, *13*, 340-350.

20(b) Rodriguez-Cuenca, S.; Cochemé, H. M.; Logan, A.; Abakumova, I.; Prime, T. A.; Rose, C.; Vidal-Puig, A.; Smith, A. C.; Rubinsztein, D. C.; Fearnley, I. M.; Jones, B. A.; Pope, S.; Heales, S. J. R.; Lam, B. Y. H.; Neogi, S. G.; McFarlane, I.; James, A. M.; Smith, R. A. J.; Murphy, M. P. *Free Radic. Biol. Med.* **2010**, *48*, 161-172

Synthesis: general comments

All reactions under an inert atmosphere were carried out using oven-dried or flame-dried glassware. Solutions were added via syringe. Dichloromethane and acetonitrile were dried where necessary using a solvent drying system, PuresolvTM, in which solvent is pushed from its storage container under low nitrogen pressure through two stainless steel columns containing activated alumina and copper. Reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. ¹H, ³¹P and ¹³C NMR spectra were obtained on a Bruker AVIII/500 spectrometer operating at 500, 202 and 125 MHz respectively or a Bruker AVIII/400 spectrometer operating at 400, 162 and 100 MHz respectively. All coupling constants are measured in Hz. DEPT was used to assign the signals in the ¹³C NMR spectra as C, CH, CH₂ or CH₃. ESI-MS were carried out on a Thermofisher LTQ Orbitrap XL at the University of Swansea, other mass spectra (MS) were recorded on a Jeol JMS700 (Mstation) spectrometer. Infra-red (IR) spectra were obtained on a Shimadzu FTIR-8400S spectrometer using attenuated total reflectance (ATR) so that the IR spectrum of the compound (solid or liquid) could be directly detected (thin layer) without any sample preparation.

Synthesis of MitoPhotoDNP



Scheme S1

Synthesis of 5-(4'-bromobutoxy)-2-nitrobenzaldehyde 2

1,4-Dibromobutane (10.6 ml, 89.8 mmol, 3.0 eq) was added to a mixture of aldehyde **1** (5.00 g, 29.9 mmol, 1.0 eq) and potassium carbonate (10.3 g, 74.8 mmol, 2.5 eq) in dry acetonitrile (150 ml). The resulting mixture was heated at 80 °C under an atmosphere of argon for 24 h.

After cooling the potassium carbonate was filtered off and washed with EtOAc (50 ml). The combined organics were concentrated under vacuum and the residue purified by column chromatography on silica eluting Et₂O-petrol (40-60) (1:1). The product-containing fractions were recrystallised from Et₂O-petrol (40-60) to give the *alkyl bromide 2* as pale green fine needles (4.81 g, 53%).

M.p. 48-49 °C.

δ_{H} (400 MHz: CDCl₃): 1.97-2.11 (4H, m, 2 × CH₂), 3.49 (2H, t, J = 6.3 Hz, CH₂Br), 4.14 (2H, t, J = 5.9 Hz, CH₂O), 7.13 (1H, dd, J = 2.9 and 9.1 Hz, H-4), 7.29 (1H, d, J = 2.9 Hz, H-6), 8.15 (1H, d, J = 9.1 Hz, H-3), 10.46 (1H, s, CHO).

δ_{C} (100 MHz: CDCl₃): 27.46 (CH₂), 29.05 (CH₂), 30.98 (CH₂), 68.02 (CH₂), 113.46 (CH), 118.85 (CH), 127.26 (CH), 134.28 (C), 142.16 (C), 163.25 (C), 188.50 (CHO).

LRMS (CI) m/z: 304 [(M+H)⁺, (⁸¹Br), 100%], 302 [(M+H)⁺, (⁷⁹Br), 100%], 274 [(M+H)⁺ (⁸¹Br) – CH₂O, 40%], 272 [(M+H)⁺ (⁷⁹Br) – CH₂O, 40%].

HRMS (CI) m/z: 304.0003 and 302.0025. C₁₁H₁₃NO₄⁸¹Br requires (M+H)⁺, 304.0008 and C₁₁H₁₃NO₄⁷⁹Br requires (M+H)⁺, 302.0028.

Found: C, 43.83; H, 3.98; N, 4.62%. C₁₁H₁₂NO₄Br requires C, 43.73; H 4.00; N 4.64%,

ν_{max} (ATR)/cm⁻¹: 2995 (Ar-H), 1689 (C=O), 1577 (C-NO₂)

Synthesis of 5-(4'-bromobutoxy)-2-nitrobenzyl alcohol 3

Sodium borohydride (713 mg, 18.9 mmol, 1.9 eq) was added slowly to a solution of *aldehyde 2* (3.00 g, 9.93 mmol, 1.0 eq) in methanol (60 ml) cooled to 0 °C. After stirring for 1 h at 0 °C the solution was poured in water (300 ml), acidified to pH 1 then extracted in EtOAc (150 ml). The aqueous layer was re-extracted with EtOAc (75 ml) and the combined organic layer washed with water (2 × 300 ml) then dried over magnesium sulfate and concentrated under vacuum to give the *alcohol 3* as a pale green solid (2.61 g, 86%).

M.p. 56-58 °C.

δ_{H} (500 MHz: CDCl₃): 1.96-2.01 (2H, m, CH₂), 2.04-2.09 (2H, m, CH₂), 2.79 (1H, s, OH), 3.48 (2H, t, J = 6.5 Hz, CH₂Br), 4.10 (2H, t, J = 6.0 Hz, CH₂CH₂O), 4.97 (2H, 2., CH₂OH), 6.85 (1H, dd, J = 2.8 and 9.1 Hz, H-4), 7.20 (1H, d, J = 2.8 Hz, H-6), 8.14 (1H, d, J = 9.1 Hz, H-3).

δ_{C} (125 MHz: CDCl₃): 27.54 (CH₂), 29.13 (CH₂), 33.10 (CH₂), 62.79 (CH₂), 67.67 (CH₂), 113.40 (CH), 114.39 (CH), 127.93 (CH), 140.11 (C), 140.33 (C), 163.46 (C).

LRMS (CI) m/z: 306 [(M+H)⁺, (⁸¹Br), 100%], 304 [(M+H)⁺, (⁷⁹Br), 100%], 288 [(M+H)⁺ (⁸¹Br) – H₂O, 30%], 286 [(M+H)⁺ (⁷⁹Br) – H₂O, 30%].

HRMS (CI) m/z : 306.0171 and 304.0183. $C_{11}H_{15}NO_4^{81}Br$ requires $(M+H)^+$, 306.0164 and $C_{11}H_{15}NO_4^{79}Br$ requires $(M+H)^+$, 304.0185.

Found: C, 43.57; H, 4.61; N, 4.63%. $C_{11}H_{14}NO_4Br$ requires C, 43.44; H 4.64; N 4.61%,
 $\nu_{max}(ATR)/cm^{-1}$: 3315 (OH), 2958 (Ar-H), 1614 (Ar-H), 1576 (C-NO₂)

Synthesis of {4-[3'-(hydroxymethyl)-4'-nitrophenoxy]butyl}triphenylphosphonium bromide **4**

Triphenylphosphine (2.56 g, 9.74 mmol, 3.0 eq) was added to a solution of alkylbromide **3** in dry acetonitrile (15 ml). The resulting solution was heated to 80 °C under an atmosphere overnight. After cooling, the solution was added slowly to rapidly stirred diethyl ether (~200 ml). The resulting precipitate was filtered off then dissolved in the minimum of dichloromethane and precipitated from ~200 ml of rapidly stirred diethyl ether. This was repeated once more to give the *phosphonium salt 4* as an off-white solid (1.75 g, 93%).

NOTE: The phosphonium salt is slightly light sensitive when in solution.

M.p. decomp >115 °C.

δ_H (500 MHz: CDCl₃): 1.77-1.85 (2H, m, PCH₂CH₂), 2.11-2.16 (2H, m, OCH₂CH₂), 3.69-3.75 (2H, m, PCH₂), 4.24 (2H, t, J = 6.2 Hz, CH₂O), 4.91 (2H, s, CH₂OH), 4.95 (1H, broad s, OH), 6.61 (1H, dd, J = 2.6 and 9.1 Hz, H-6'), 7.53 (1H, d, J = 2.6 Hz, H-2'), 7.63-7.67 (6H, m, Ar-H), 7.74-7.80 (9H, m, Ar-H), 7.94 (1H, d, J = 9.1 Hz, H-5').

δ_C (125 MHz: CDCl₃): 18.84 (d, J = 3.7 Hz, CH₂), 22.02 (d, J = 51.2 Hz, CH₂), 28.87 (d, J = 16.7 Hz, CH₂), 61.53 (CH₂), 67.04 (CH₂), 113.38 (CH), 113.51 (CH), 117.87 (d, J = 86.1 Hz, C), 127.10 (CH), 130.48 (d, J = 12.5 Hz, CH), 133.55 (d, J = 10.0 Hz, CH), 135.11 (d, J = 2.9 Hz, CH), 139.33 (C), 143.09 (C), 163.04 (C).

δ_P (202 MHz: CDCl₃): 24.26

LRMS (ESI) m/z : 486 [M⁺ (phosphonium cation), 100%].

HRMS (ESI) m/z : 486.1818. $C_{29}H_{29}NO_4P$ requires M^+ , 486.1829.

$\nu_{max}(ATR)/cm^{-1}$: 3273 (OH), 1612 (Ar-H), 1573 (C-NO₂)

Synthesis of {4-[3'-(2'',4''- dinitrophenoxy)methyl]-4'-nitrophenoxy]butyl}triphenylphosphonium bromide (MitoPhotoDNP)

Sodium hydride (112 mg, 2.82 mmol, 1.3 eq) was added to a solution of 2,4-dinitrofluorobenzene (0.37 ml, 3.0 mmol 1.4 eq) and alcohol **4** (1.23 g, 2.17 mmol) in dry dichloromethane (15 ml) at r.t.. The solution was shielded from the light then stirred under an atmosphere of argon overnight. The solution

was diluted with dichloromethane (30 ml) and then poured into ~10% NaBr solution (100 ml). The layers were separated and the aqueous layer was re-extracted with dichloromethane (20 ml) and the combined organic layers washed with NaBr solution (2 × 100 ml). The organic layer was dried over magnesium sulfate and concentrated under vacuum. The residue was then purified by column chromatography on silica eluting CH₂Cl₂ to CH₂Cl₂-MeOH (9:1). After concentration the impure product was dissolved in the minimum of dichloromethane and precipitated from ~200 ml of rapidly stirred diethyl ether to give the *MitoPhotoDNP* as an orange solid (1.02 g, 64%).

M.p. decomp >75 °C.

δ_{H} (500 MHz: CDCl₃): 1.84-1.94 (2H, m, PCH₂CH₂), 2.24-2.30 (2H, m, OCH₂CH₂), 3.94-4.01 (2H, m, PCH₂), 4.32 (2H, t, J = 6.2 Hz, CH₂O), 5.77 (2H, s, ArCH₂O), 7.03 (1H, dd, J = 2.8 and 9.2 Hz, H-6'), 7.50 (1H, d, J = 9.2 Hz, H-6''), 7.56 (1H, d, J = 2.8 Hz, H-2'), 7.66-7.71 (6H, m, Ar-H), 7.76-7.79 (3H, m, Ar-H), 7.84-7.89 (6H, m, Ar-H), 8.23 (1H, d, J = 9.2 Hz, H-5'), 8.51 (1H, dd, J = 2.8 and 9.2 Hz, H-5''), 8.84 (1H, d, J = 2.8 Hz, H-3'').

δ_{C} (125 MHz: CDCl₃): 19.18 (d, J = 3.7 Hz, CH₂), 22.27 (d, J = 50.8 Hz, CH₂), 29.06 (d, J = 17.0 Hz, CH₂), 68.05 (CH₂), 69.38 (CH₂), 114.05 (CH), 114.54 (CH), 115.35 (CH), 118.15 (d, J = 86.0 Hz, C), 122.34 (CH), 128.29 (CH), 129.82 (CH), 130.47 (d, J = 12.6 Hz, CH), 133.70 (d, J = 10.0 Hz, CH), 135.02 (d, J = 3.7 Hz, CH), 138.42 (C), 139.10 (C), 140.53 (C), 155.97 (C), 163.81 (C).

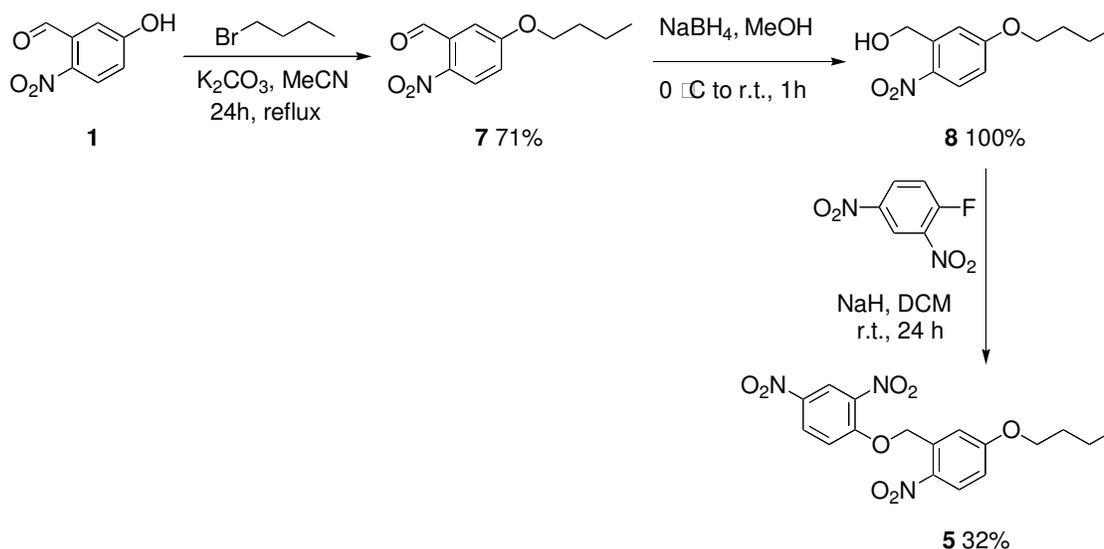
δ_{P} (202 MHz: CDCl₃): 24.29

LRMS (FAB) m/z: 652 [M⁺ (phosphonium cation), 100%], 636 (15%), 262 (30%).

HRMS (FAB) m/z: 652.1843. C₃₅H₃₁N₃O₈P requires M⁺, 652.1849.

ν_{max} (ATR)/cm⁻¹: 1603 (Ar-H), 1580 (C-NO₂), 1509 (Ar-H).

Synthesis of control compound 5



Scheme S2

Synthesis of 4-butoxy-2-(2',4'-dinitrophenoxymethyl)-1-nitrobenzene **5**

Sodium hydride (71 mg, 1.78 mmol, 1.3 eq.) (60% dispersion in mineral oil) was added portion-wise to a solution of alcohol **8** and 2,4-dinitro-1-fluorobenzene (0.23 ml, 1.91 mmol, 1.4 eq.). The solution was stirred overnight under argon at r.t.. The resulting yellow solid was extracted into EtOAc (50 ml) and then washed with 1M HCl. The organic layer was dried over magnesium sulfate and concentrated under vacuum. The resulting solid was washed with diethyl ether (3 × 50 ml) to give the *ether* **5** as a white fluffy solid. (173 mg, 32%).

M.p. 175-177 °C

δ_{H} (500 MHz: D-6 DMSO): 0.94 (3H, t, $J = 7.4$ Hz, CH_3), 1.39-1.50 (2H, m, CH_2CH_3), 1.69-1.79 (2H, m, OCH_2CH_2), 4.15 (2H, t, $J = 6.6$ Hz, CH_2O), 5.86 (2H, s, ArCH_2), 7.16 (1H, dd, $J = 2.8$ and 9.2 Hz, H-5), 7.38 (1H, d, $J = 2.7$ Hz, H-3), 7.72 (1H, d, $J = 9.4$ Hz, H-6'), 8.25 (1H, d, $J = 9.1$ Hz, H-6), 8.56 (1H, dd, $J = 2.9$ and 9.3 Hz, H-5'), 8.83 (1H, d, $J = 2.8$ Hz, H-3').

δ_{C} (125 MHz: CDCl_3): 13.54 (CH_3), 18.54 (CH_2), 30.28 (CH_2), 68.33 (CH_2), 68.93 (CH_2), 113.68 (CH), 114.19 (CH), 116.22 (CH), 121.56 (CH), 128.10 (CH), 129.71 (CH), 134.41 (C), 138.83 (C), 139.29 (C), 140.19 (C), 155.09 (C), 163.17 (C).

Found: C, 52.14; H, 4.38; N, 10.67%. $\text{C}_{17}\text{H}_{17}\text{N}_3\text{O}_8$ requires C, 52.18; H 4.38; N 10.74%.

LRMS (CI) m/z 409 [28%, $(\text{M}+\text{NH}_4)^+$], 208 (100).

HRMS (CI) m/z : 409.1355. $\text{C}_{17}\text{H}_{21}\text{N}_4\text{O}_8$ requires $(\text{M}+\text{NH}_4)^+$, 409.1354.

$\nu_{\max}(\text{ATR})/\text{cm}^{-1}$: 1602 (Ar-H), 1587 (C-NO₂), 1496 (Ar-H), 1330 (C-NO₂).

Synthesis of 5-butoxy-2-nitrobenzaldehyde 7

1-Bromobutane (2.9 ml, 27 mmol, 1.5 eq) was added to a mixture of aldehyde **1** (3.00 g, 18.0 mmol, 1.0 eq) and potassium carbonate (3.70 g, 26.9 mmol, 1.5 eq) in dry acetonitrile (90 ml). The resulting mixture was heated at 80 °C under an atmosphere of argon for 24 h. After cooling, the potassium carbonate was filtered off and washed with EtOAc (50 ml). The combined organics were concentrated under vacuum and the residue purified by kugelrohr distillation to give the *aldehyde 7* as a yellow oil which slowly solidified. (2.84 g, 71%).

M.p. 39-41 °C

δ_{H} (500 MHz: CDCl₃): 1.00 (3H, t, J = 7.4 Hz, CH₃), 1.46-1.57 (2H, m, CH₂CH₃), 1.79-1.87 (2H, m, OCH₂CH₂), 4.12 (2H, t, J = 6.5 Hz, CH₂O), 7.14 (1H, dd, J = 2.8 and 9.1 Hz, H-4), 7.30 (1H, d, J = 2.8 Hz, H-6), 8.14 (1H, d, J = 9.1 Hz, H-3), 10.47 (1H, s, CHO).

δ_{C} (125 MHz: CDCl₃): 13.63 (CH₃), 18.98 (CH₂), 30.77 (CH₂), 69.01 (CH₂), 113.67 (CH), 118.73 (CH), 127.16 (CH), 134.30 (C), 141.88 (C), 163.61 (C), 188.54 (CH).

Found: C, 59.18; H, 5.86; N, 6.32%. C₁₁H₁₃NO₄ requires C, 59.19; H 5.87; N 6.27%.

LRMS (CI) m/z: 224 [(M+H)⁺, 100%], 167 (95%).

HRMS (CI) m/z: 224.0927. C₁₁H₁₄NO₄ requires (M+H)⁺, 224.0923.

$\nu_{\max}(\text{ATR})/\text{cm}^{-1}$: 1692 (C=O), 1577 (C-NO₂), 1511 (Ar-H).

Synthesis of 5-butoxy-2-nitrobenzyl alcohol 8

Sodium borohydride (644 mg, 17.04 mmol, 1.9 eq.) was added portion wise to a solution of aldehyde **7** (2.00 g, 8.97 mmol) in MeOH (60 ml) at 0 °C. After complete addition the solution was stirred at 0 °C for 1 h. The reaction mixture was quenched with 1M HCl, then extracted into EtOAc (150 ml). The aqueous layer was re-extracted with EtOAc (50 ml) and the combined organics washed with water (2 × 200 ml). The organic layer was dried over magnesium sulfate and concentrated under vacuum to give the *alcohol 8* as a yellow oil which slowly solidified. (2.01 g, 100%).

M.p. 52-56 °C

δ_{H} (500 MHz: CDCl₃): 0.98 (3H, t, J = 7.4 Hz, CH₃), 1.44-1.57 (2H, m, CH₂CH₃), 1.77-1.83 (2H, m, OCH₂CH₂), 2.65 (1H, t, J = 6.4 Hz, OH), 4.05 (2H, t, J = 6.5 Hz, CH₂O), 4.98 (2H, d, J = 6.0 Hz, CH₂OH), 6.87 (1H, dd, J = 2.8 and 9.1 Hz, H-4), 7.19 (1H, d, J = 2.8 Hz, H-6), 8.16 (1H, d, J = 9.1 Hz, H-3).

δ_C (125 MHz: $CDCl_3$): 13.73 (CH_3), 19.09 (CH_2), 30.96 (CH_2), 63.06 (CH_2), 68.55 (CH_2), 113.53 (CH), 114.71 (CH), 128.02 (CH), 140.09 (C), 140.19 (C), 163.88 (C).

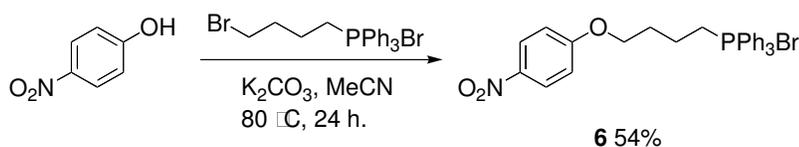
Found: C, 58.64; H, 6.76; N, 6.23%. $C_{11}H_{15}NO_4$ requires C, 58.66; H 6.71; N 6.22%.

LRMS (CI) m/z: 226 [$(M+H)^+$, 100%], 208 (30), 194 (30).

LRMS (CI) m/z: 226.1077. $C_{11}H_{14}NO_4$ requires $(M+H)^+$, 226.1080.

$\nu_{max}(ATR)/cm^{-1}$: 3290 (OH), 1606 (Ar-H), 1586 (C-NO₂), 1505 (Ar-H), 1323 (C-NO₂).

Synthesis of control compound 6



Scheme S3

Synthesis of [4-(4-nitrophenoxy)butyl]triphenylphosphonium bromide 6

Potassium carbonate (144 mg, 1.04 mmol, 1.0 eq.) was added to a solution of 4-nitrophenol (145 mg, 2.08 mmol, 2.0 eq.) and 4-bromobutyltriphenylphosphonium bromide (500 mg, 1.04 mmol, 1.0 eq.) in dry acetonitrile (10 ml). The suspension was then heated to 80 °C under an atmosphere of argon overnight. After cooling the solid was filtered off and washed with dichloromethane. The filtrate was then concentrated under vacuum and redissolved in dichloromethane (30 ml) and washed with 1.0 M HBr solution. The organic layer was dried over magnesium sulfate and concentrated under vacuum. After concentration the impure product was dissolved in the minimum of dichloromethane and precipitated from ~200 ml of rapidly stirred diethyl ether. The resulting precipitate was filtered off then redissolved in the minimum of dichloromethane and precipitated from ~200 ml of rapidly stirred diethyl ether. This was repeated once more to give the *phosphonium salt* **6** as an orange solid (367 mg, 54%).

M.p. 48-50 °C.

δ_{H} (500 MHz: CDCl₃): 1.80-1.88 (2H, m, PCH₂CH₂), 2.22-2.25 (2H, m, OCH₂CH₂), 3.89-3.95 (2H, m, PCH₂), 4.24 (2H, t, J = 6.0 Hz, CH₂O), 6.92 (2H, d, J = 7.2 Hz, H-2' and H-6'), 7.64-7.67 (6H, m, Ar-H), 7.74-7.78 (3H, m, Ar-H), 7.80-7.85 (6H, m, Ar-H), 8.10 (2H, d, J = 7.2 Hz, H-3' and H-5').

δ_{C} (125 MHz: CDCl₃): 19.04 (d, J = 3.9 Hz, CH₂), 22.1 (d, J = 50.8 Hz, CH₂), 29.07 (d, J = 16.9 Hz, CH₂), 67.58 (CH₂), 114.60 (CH), 118.07 (d, J = 86.0 Hz, C), 125.78 (CH), 130.41 (d, J = 12.6 Hz, CH), 133.60 (d, J = 10.0 Hz, CH), 134.99 (d, J = 2.9 Hz, CH), 141.23 (C), 163.82 (C).

δ_{P} (202 MHz: CDCl₃): 24.47

LRMS (FAB) m/z: 456 [M⁺ (phosphonium cation), 100%].

HRMS (FAB) m/z: 456.1734. C₂₈H₂₇NO₃P requires M⁺, 456.1729.

ν_{max} (ATR)/cm⁻¹: 1604 (Ar-H), 1589 (C-NO₂), 1497 (Ar-H), 1330 (C-NO₂).

UV spectra of control compounds 4-6 and MitoPhotoDNP

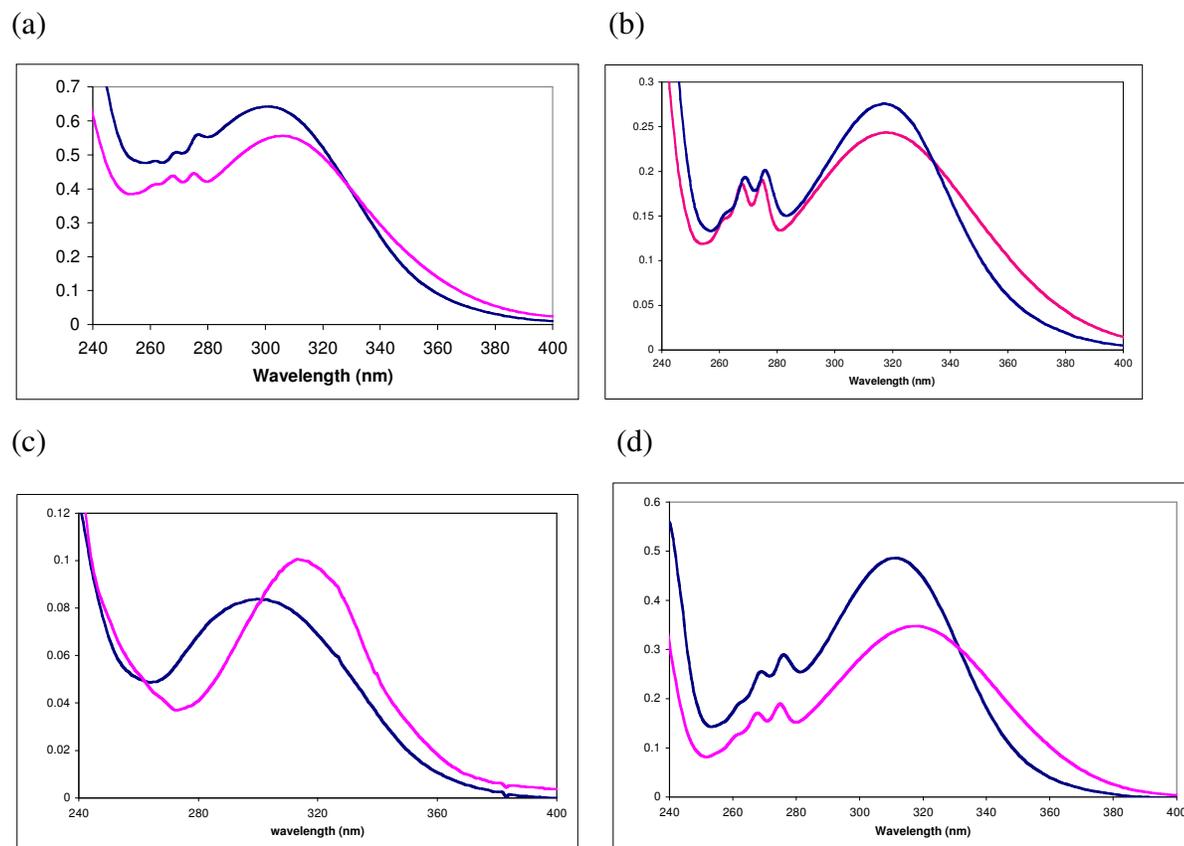


Figure S1 UV spectra of (a) MitoPhotoDNP (b) MitoPhotoWater **4** (c) PhotoDNP **5** and (d) MitoNonPhoto **6** at 30 μM in chloroform (blue) and water [pink, 0.1% DMSO for (a), (b) and (d), 1% DMSO for (c) due to lower water solubility in the absence of the TPP group].

NMR experiment showing UV-induced DNP release from MitoPhotoDNP

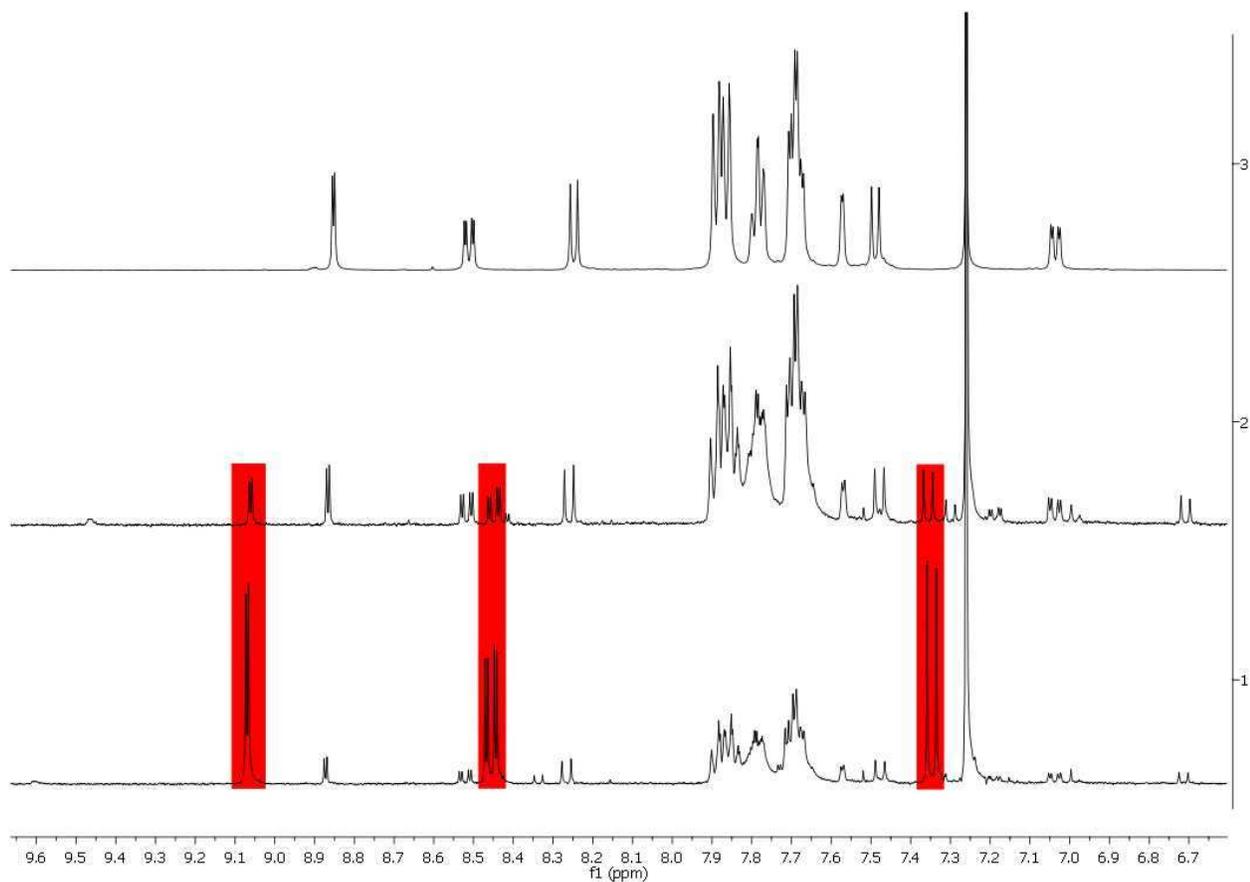


Figure S2 A sample of MitoPhotoDNP was irradiated in CDCl_3 (2.6 mg in ~ 0.7 ml in a standard NMR tube) in a Luzchem light chamber using UVA (316-400 nm) with a power of 80 W (10 bulbs at 8W each) and release of DNP was observed. The top spectrum is before irradiation, the second after, and the third after spiking the NMR sample with DNP.

Electrode responses for uptake of MitoPhotoDNP into isolated mitochondria

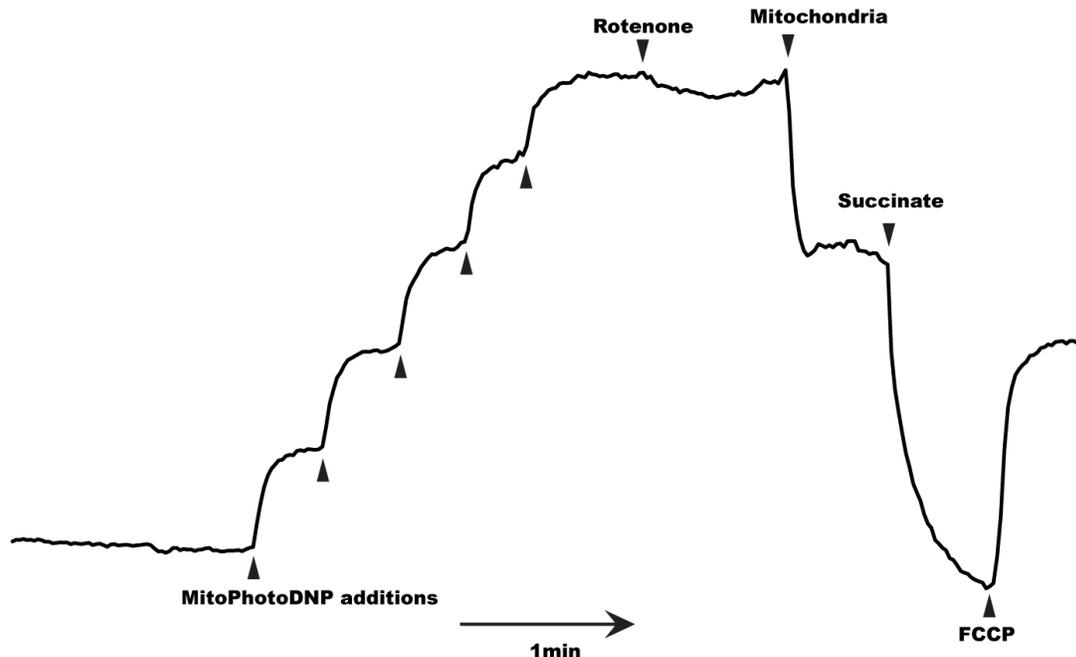


Figure S3 An electrode selective for the TPP cation was inserted into a stirred chamber thermostated at 37 °C containing 3 ml KCl medium (120 mM KCl, 1 mM EGTA (glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid), 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.2). The electrode response was calibrated by 5 sequential additions of 1 μ M MitoPhotoDNP. Rotenone (4 μ g/ml) was added, followed by rat liver mitochondria (2 mg protein mL⁻¹), succinate (10 mM) and the uncoupler FCCP (500 nM) where indicated.

Intact cell analysis: general methods

Preparation of freshly dissociated cells

Male guinea pigs (~500 g) were humanely killed by overdose with sodium pentobarbital in accordance with the guidelines of the Animal (Scientific Procedures) Act UK 1986. A segment of intact distal colon (~5 cm) was transferred to oxygenated (95% O₂, 5% CO₂) physiological saline solution composed of (in mM): 118.4 NaCl, 25 NaHCO₃, 4.7 KCl, 1.13 NaH₂PO₄, 1.3 MgCl₂, 2.7 CaCl₂, and 11 glucose, pH 7.4. After removal of the mucosa from the tissue, single smooth muscle cells were enzymatically dissociated.¹ All experiments and loading of cells with fluorescent dyes were carried out at room temperature (20 ± 2°C).

Imaging

Cells were loaded with the $\Delta\Psi_m$ -sensitive dye tetramethylrhodamine ethyl ester perchlorate (TMRE, 10 nM) for 30 min in the presence of wortmannin (10 μ M; to prevent contraction) and were stained in combination with either the $\Delta\Psi_m$ -insensitive dye MitoTracker Green-FM (1 μ M for the latter 15 min of the loading period) or the calcium-sensitive fluorophore fluo4-AM (10 μ M for 30 min). After this cells were allowed to settle in a custom-made 2 ml chamber with a No. 0 coverslip on the bottom. They were then washed with extracellular solution containing (in mM): 80 Na glutamate, 40 NaCl, 20 tetraethylammonium chloride, 1.1 MgCl₂, 3 CaCl₂, 10 HEPES, and 30 glucose, pH 7.4 with NaOH and additionally TMRE (10 nM).

Two-dimensional images were obtained on an inverted epifluorescence microscope (Nikon TE2000U, Nikon, Surrey, UK) with a 40 \times 1.3 NA S-Fluor oil objective. Single cells were illuminated with the output of a 75 W xenon arc lamp (LPS-220B, Photon Technology International (PTI) Inc., Ford, UK) with selected wavelengths controlled via a monochromator (DeltaRAM X, PTI Inc.; 475 and 560 ± 5 nm) guided via an optical light guide, through a field stop diaphragm and a ND4 filter before being reflected off a custom-made long-pass dichroic mirror (Chroma, Rockingham, VT, USA) reflective in the ranges 470-495 and 550-570 nm as well as <400 nm. Emitted light was collected through the objective and dichroic and transmitted to a cooled, back-illuminated frame transfer CCD camera (Phometrics Cascade 512B, Roper Scientific, Trenton, NJ, USA) controlled by EasyRatio pro software (1.2.1.87, PTI Inc.). Full frame images (512 pixels \times 512 pixels), with a pixel size of 397 nm at the cell, were acquired sequentially with 10 ms exposure for each dye. All test compounds were allowed to equilibrate with the cells for 30 min prior to UV photolysis. Initially the cells' TMRE and

MitoTracker fluorescence was imaged (with a 10 s timelapse) during this loading period to ensure that no change in mitochondrial membrane potential occurred to the test compound alone (without UV light). Subsequently, the test compounds were allowed to equilibrate with the cells during the period of dye loading.

UV Photolysis

The output of a 355 nm laser (DPSL 50 mW Nd:YAG, Rapp OptoElectronic, Hamburg, Germany) was guided via a fibre-optic cable through the side-port of the microscope and attenuated with neutral density filters such that the resultant 2 μm -diameter beam had a power of 100 μW at the coverslip on the microscope stage. A preselected circular region of the cell of ~ 10 μm diameter was scanned three times with this beam (taking 150 ms for a 10 μm diameter circle or 85 ms for a 7.6 μm diameter circle as in Figure 2). Region selection and scanning were achieved with a computer-controlled galvanometer-driven positioning system (UG40 laser micromanipulator, Rapp OptoElectronic).

Electrophysiology

Freshly-isolated smooth muscle cells were patch-clamped using conventional tight-seal whole cell recording methods as described previously.¹ The extracellular solution was as above. The pipette solution contained (in mM): 85 $(\text{Cs})_2\text{SO}_4$, 20 CsCl, 1 MgCl_2 , 30 HEPES, 3 MgATP, 2.5 pyruvic acid, 2.5 malic acid, 1 NaH_2PO_4 , 5 creatine phosphate, and 0.5 guanosine phosphate. Whole cell currents were measured using an Axopatch 200B (MDS Analytical Technologies), low-pass filtered at 500 Hz, digitally sampled at 1.5 kHz using a Digidata interface and pClamp (version 10; MDS Analytical Technologies), and stored for analysis.

Data analysis

Images were analysed using MetaMorph 7.5.0.0 (MDS Analytical Technologies from PTI Inc.). Regions of interest were drawn around the area of the cell exposed to UV light and the intensity of TMRE and MitoTracker fluorescence each measured over a 20-frame average 15 s before and 30 s after UV exposure. The difference between the ratio of TMRE to MitoTracker fluorescence after UV and the TMRE/MitoTracker ratio prior to UV was then calculated for each cell. Results are expressed as the mean \pm s.e.m. for n cells, significance was calculated using independent Student's t-tests with $p < 0.05$ considered significant.

The ratio of TMRE to MitoTracker Green fluorescence was used in preference to TMRE fluorescence alone as it compensates for any artefacts that might arise due to even very slight movement or focus changes during the course of the experiment. Both fluorophores localise to mitochondria, however only one (TMRE) is sensitive to changes in the mitochondrial membrane potential.

Alternatively fluo4 fluorescence was measured in a region drawn to encompass the entire cell. Values were expressed as a ratio of fluorescence normalised to basal values prior to addition of MitoPhotoDNP.

MTT assay of cell toxicity

To evaluate cell viability, mouse C2C12 myoblast cells were plated on 96-microwell cell-culture plate (1×10^4 cells/well in 100 μ L of Dulbecco's modified medium containing 10% heat-inactivated fetal calf serum plus 100 U/ml penicillin and 100 μ g/ml streptomycin) and allowed to settle for 8 hrs. After this time the medium was replaced with fresh medium containing varying concentrations of MitoPhotoDNP (10nM - 100uM) and incubated overnight at 37°C in a humidified incubator with 5% CO₂. Cell death was measured using an MTT assay (CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay, Promega): 20 μ L of MTT solution (1 mg/ml) was added to each well and incubated at 37°C for 2 hr. The absorbance of formazan was measured at 490 nm in a microtiter plate reader. The background absorbance of the reaction solution alone (without cells) was subtracted from the sample absorption values before data analysis.

Drugs and Chemicals

TMRE, MitoTracker Green-FM and fluo4-AM were purchased from Life Technologies (Paisley, UK). All other drugs and reagents were purchased from Sigma (Poole, UK).

Dose response of mitochondrial depolarization by 2,4-dinitrophenol

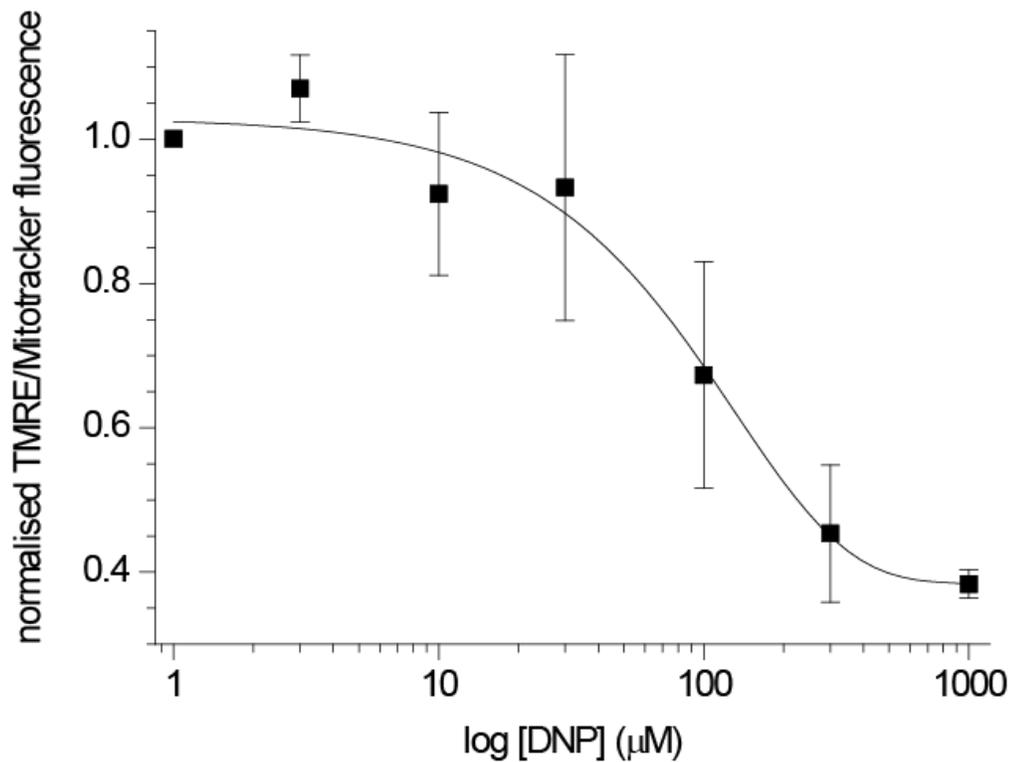


Figure S4: Dose response curve of mitochondrial depolarization with increasing extra-cellular concentration of dinitrophenol. Freshly isolated smooth muscle cells were loaded with the $\Delta\Psi_m$ -sensitive fluorophore TMRE (10 nM) and the $\Delta\Psi_m$ -insensitive fluorophore MitoTracker Green-FM (1 μM) plus wortmannin (10 μM) to prevent contraction. Each concentration of dinitrophenol (DNP) was washed into the cells' bathing solution and allowed to equilibrate for 10 min prior to imaging. The dyes were excited at 560 and 475 nm respectively and fluorescence images captured sequentially with 10 ms exposure for each. Whole cell TMRE and MitoTracker fluorescence was normalised to values prior to DNP addition. Values are mean fluorescence ratio \pm SEM, $n=6$.

Expanded version of Figure 2

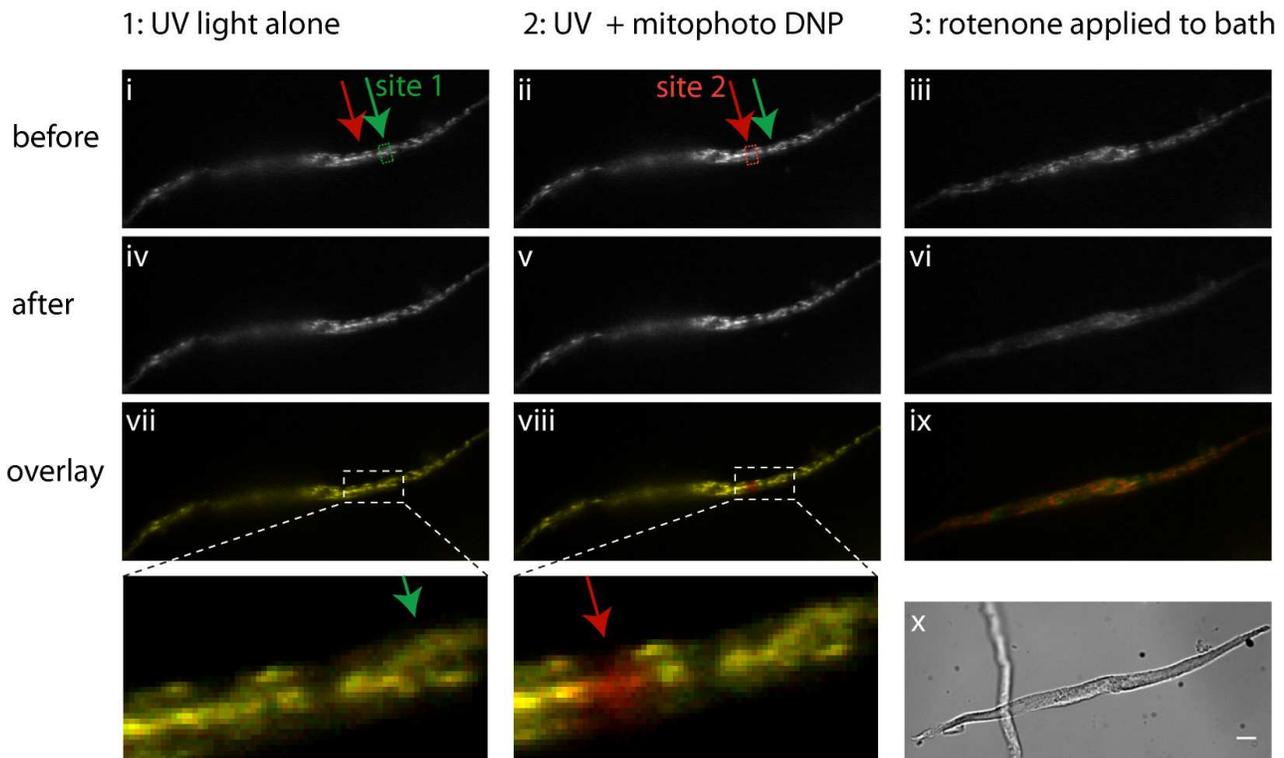


Figure S5: Localised mitochondrial depolarization following localised photolysis of MitoPhotoDNP, expanded version of Figure 2. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) display a punctate fluorescent staining (i). The cell displayed underwent: (1) a brief, localised exposure to UV laser light (355 nm for 85 ms in region shown in green, panel i) in the absence of MitoPhotoDNP; (2) MitoPhotoDNP (200 nM) was washed into the cell's bathing medium, allowed to equilibrate for 15 min and then a second region of the cell was exposed to UV light (85 ms, in region shown in red, ii); (3) finally the mitochondrial inhibitors rotenone (2 μ M) plus oligomycin (3 μ M) were washed into the bathing medium. TMRE fluorescence was detected before (i, ii & iii) and after (iv, v & vi) each of the three treatments and any differences highlighted by overlaying artificially coloured images in which "before" is red and "after" is green, such that no change results in yellow regions, loss of TMRE staining (hence mitochondrial depolarization) results in red regions and any gain of fluorescence results in green (panels vii, viii & ix; close-up regions indicated below). A brightfield image of the cell plus 10 μ m scale bar is shown in panel x.

Summarised data: mitochondrial depolarization evoked by MitoPhoto DNP and control compounds 4-6

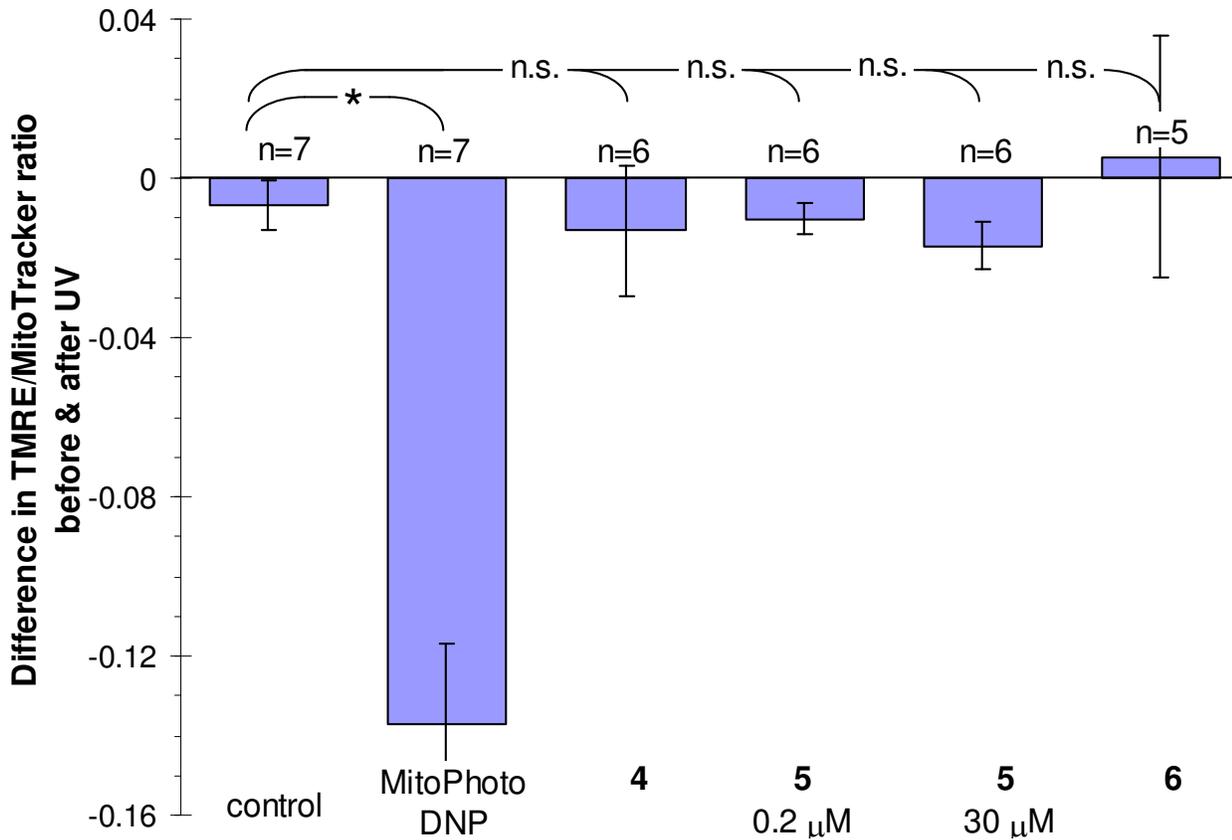


Figure S6: MitoPhoto DNP alone significantly depolarised mitochondria within a ~10 μm -diameter sub-region of cells exposed to UV light. Freshly isolated smooth muscle cells loaded with TMRE (10 nM) and MitoTracker Green (1 μM) were imaged during a brief exposure (~150 ms) to UV light in the presence of each of the test compounds shown. The ratio of TMRE:MitoTracker fluorescence within the regions exposed to UV light was measured offline with image analysis software and the difference in ratio before and after is shown. Values are the mean difference in ratio \pm s.e.m. for n cells, with $p < 0.05$ considered significant (*), n.s. = not significant.

Mitochondrial depolarization evoked by MitoPhoto DNP

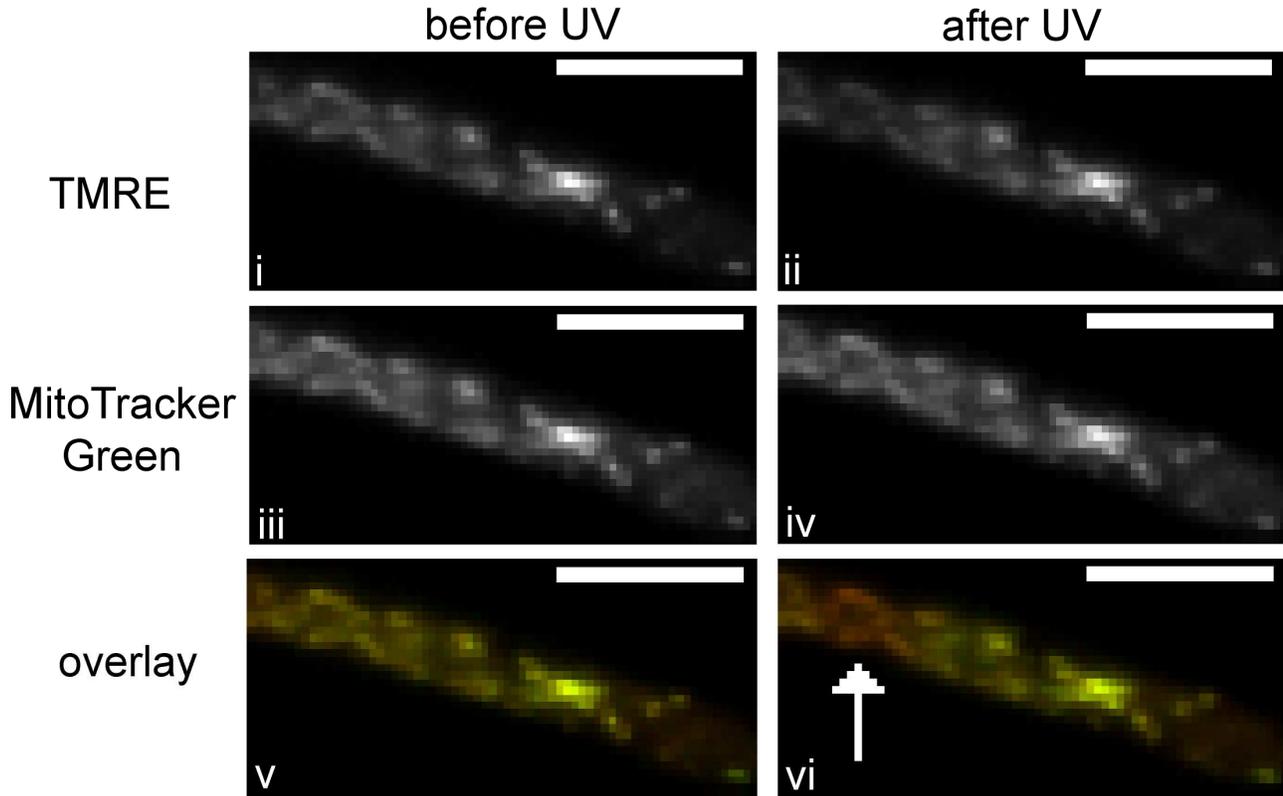


Figure S7: Localised mitochondrial depolarization following localised photolysis of MitoPhotoDNP. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) and MitoTracker Green (1 μ M) display a punctate fluorescent staining of each dye (i & iii). Cells were imaged during a brief exposure (\sim 150 ms, in region indicated by arrow in panel vi) to UV light. TMRE and MitoTracker fluorescence was detected before (i & iii) and after (ii & iv) UV exposure. Overlaid images (v & vi) show MitoTracker fluorescence coloured red and TMRE fluorescence green, such that regions of overlap are yellow (as in panel v, before UV exposure) and any decrease in TMRE fluorescence, hence mitochondrial depolarization, is red (as in the region exposed to UV light, \uparrow , in panel vi). Scale bar = 10 μ m in all panels.

Lack of mitochondrial depolarization evoked by control compounds

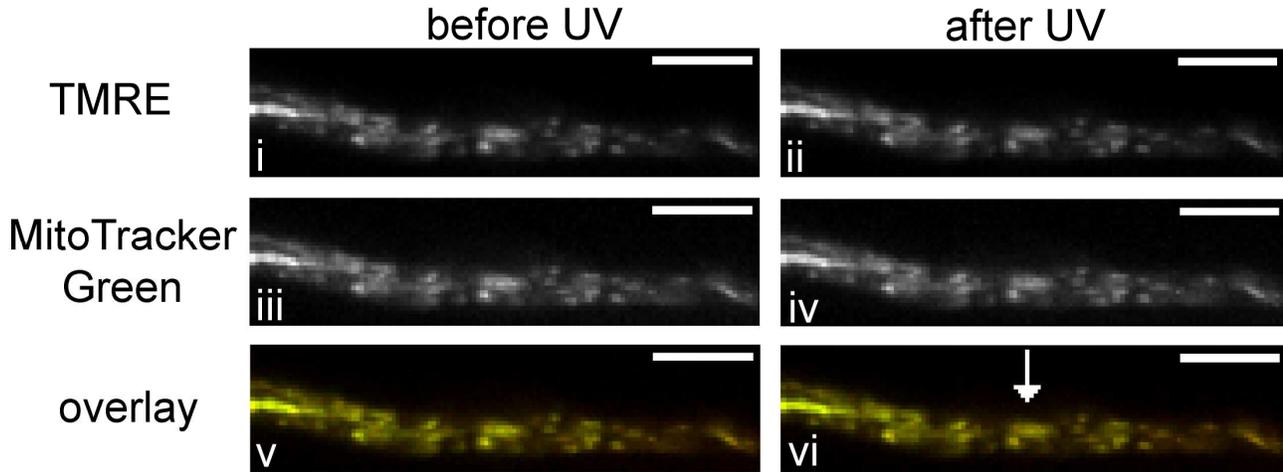


Figure S8: No mitochondrial depolarization occurred following localised photolysis of control compound **4**. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) and MitoTracker Green (1 μ M) display a punctate fluorescent staining of each dye (i & iii). Cells were imaged during a brief exposure (\sim 150 ms, in region indicated by arrow in panel vi) to UV light. TMRE and MitoTracker fluorescence was detected before (i & iii) and after (ii & iv) UV exposure. Overlaid images (v & vi) show MitoTracker fluorescence coloured red and TMRE fluorescence green, such that regions of overlap are yellow (as in panels v & vi, before and after UV exposure) and any decrease in TMRE fluorescence, hence mitochondrial depolarization, is red (not seen). Scale bar = 10 μ m in all panels.

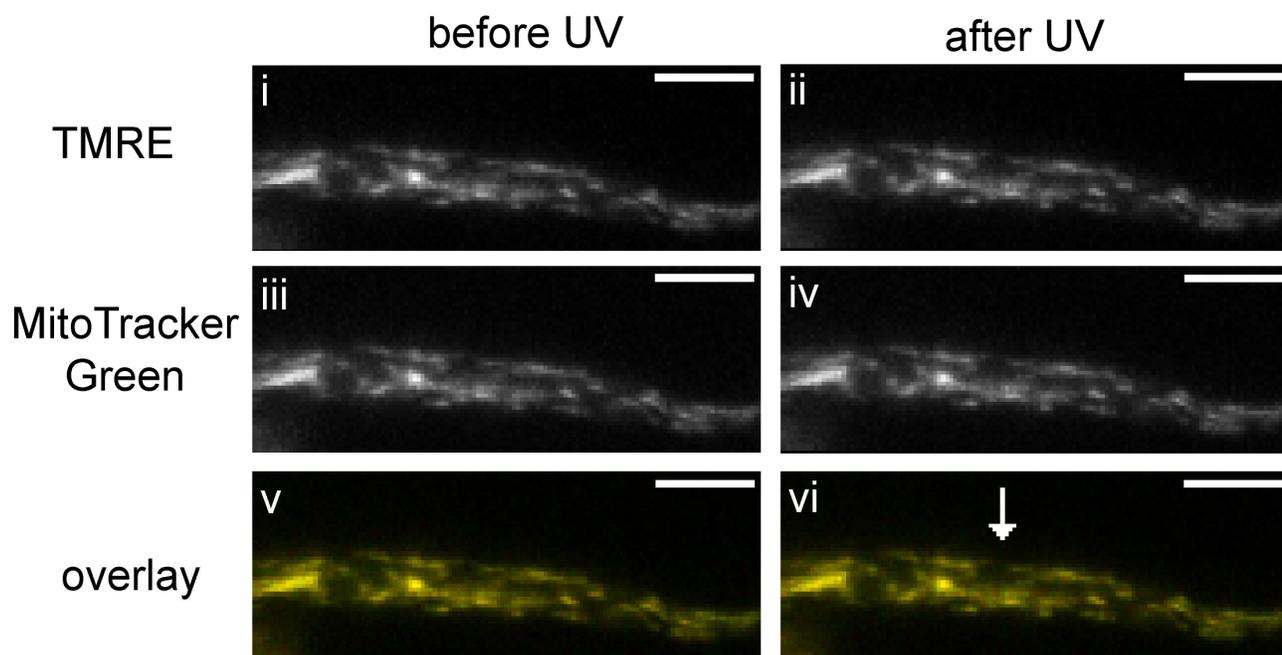


Figure S9: No mitochondrial depolarization occurred following localised photolysis of control compound **5**. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) and MitoTracker Green (1 μ M) display a punctate fluorescent staining of each dye (i & iii). Cells were imaged during a brief exposure (\sim 150 ms, in region indicated by arrow in panel vi) to UV light. TMRE and MitoTracker fluorescence was detected before (i & iii) and after (ii & iv) UV exposure. Overlaid images (v & vi) show MitoTracker fluorescence coloured red and TMRE fluorescence green, such that regions of overlap are yellow (as in panels v & vi, before and after UV exposure) and any decrease in TMRE fluorescence, hence mitochondrial depolarization, is red (not seen). Scale bar = 10 μ m in all panels.

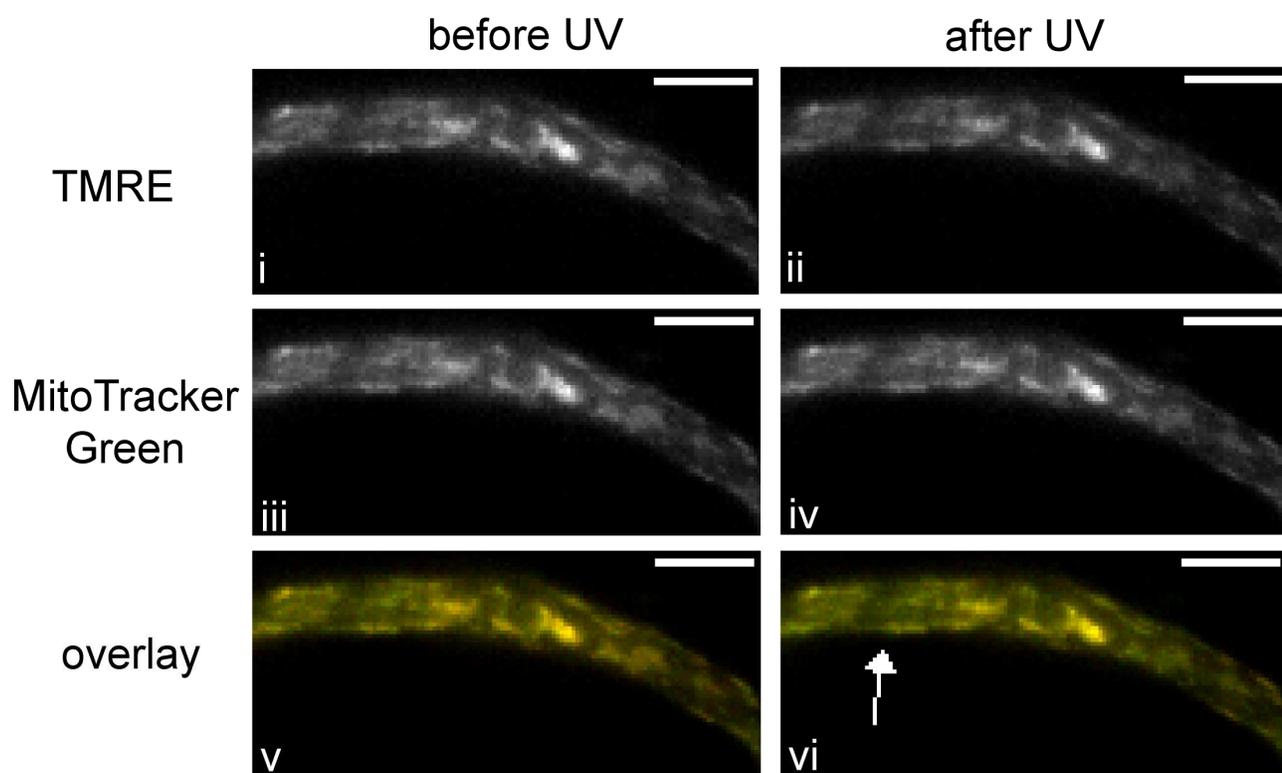


Figure S10: No mitochondrial depolarization occurred following localised photolysis of control compound **6**. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) and MitoTracker Green (1 μ M) display a punctate fluorescent staining of each dye (i & iii). Cells were imaged during a brief exposure (\sim 150 ms, in region indicated by arrow in panel vi) to UV light. TMRE and MitoTracker fluorescence was detected before (i & iii) and after (ii & iv) UV exposure. Overlaid images (v & vi) show MitoTracker fluorescence coloured red and TMRE fluorescence green, such that regions of overlap are yellow (as in panels v & vi, before and after UV exposure) and any decrease in TMRE fluorescence, hence mitochondrial depolarization, is red (not seen). Scale bar = 10 μ m in all panels.

HPLC of irradiated MitoPhotoDNP and MitoPhotoWater 4

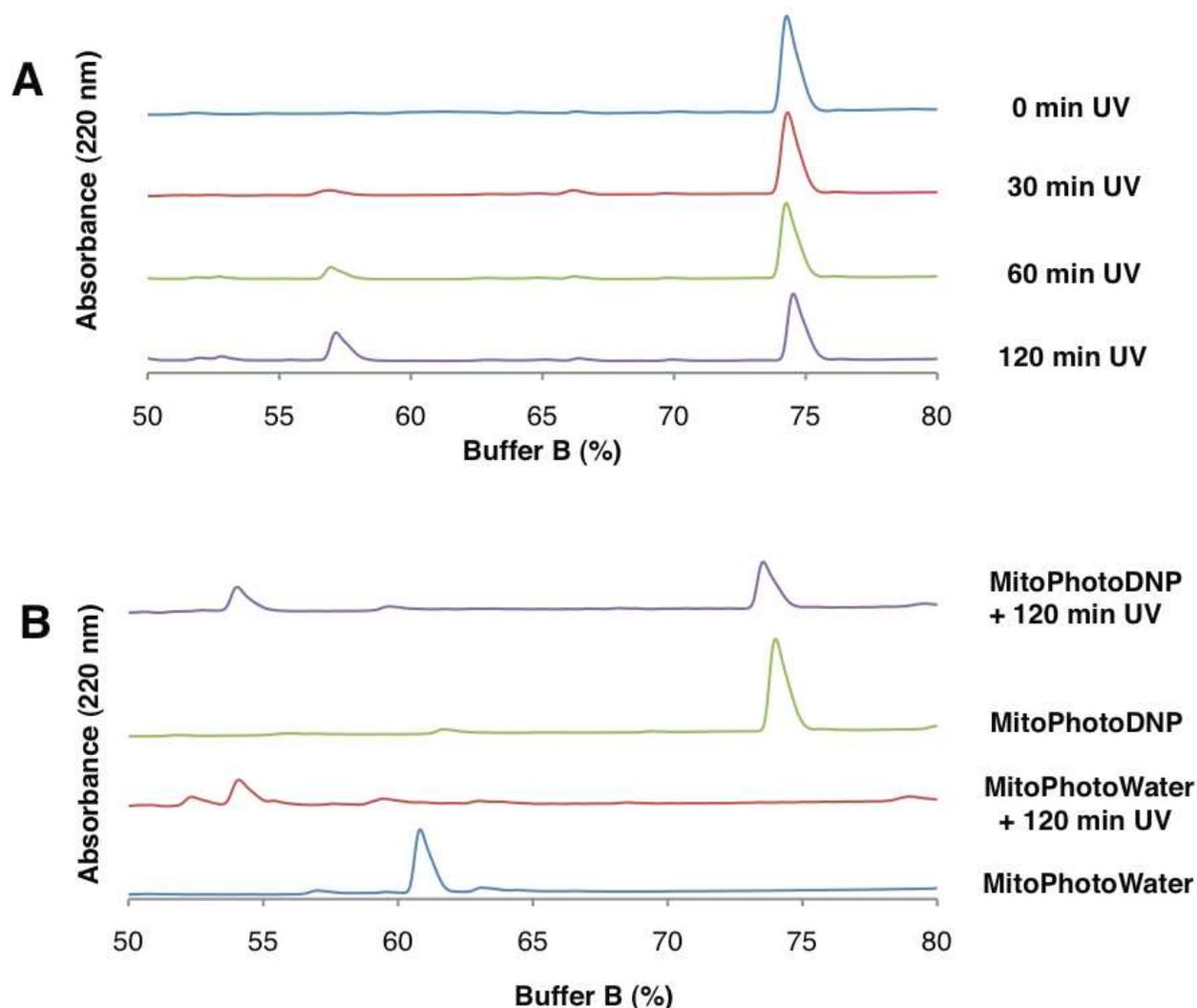


Figure S11. **A** MitoPhotoDNP breaks down in a time dependent manner when exposed to UV light. **B** MitoPhotoDNP and MitoPhotoWater **4** appear to breakdown to the same product on exposure to UV light. 30 μ L samples of 100 μ M MitoPhotoDNP or MitoPhotoWater **4** in DMSO were incubated for up to 2 hours in uncapped eppendorfs approximately 3 cm below a 365 nm UV light source (UVGL-58 Mineralight Lamp, UVP, Upland, CA, USA). Subsequently 20 μ L of these samples was injected into an HPLC (Gilson, model 321) with output detected at 220 nm (Gilson, model 151). MitoPhotoDNP, MitoPhotoWater **4** and their breakdown products were separated on a C18 column using a linear gradient from 100% Buffer A (0.1% TFA) to 100% Buffer B (acetonitrile containing 0.1% TFA).

Measures of cellular toxicity of MitoPhotoDNP

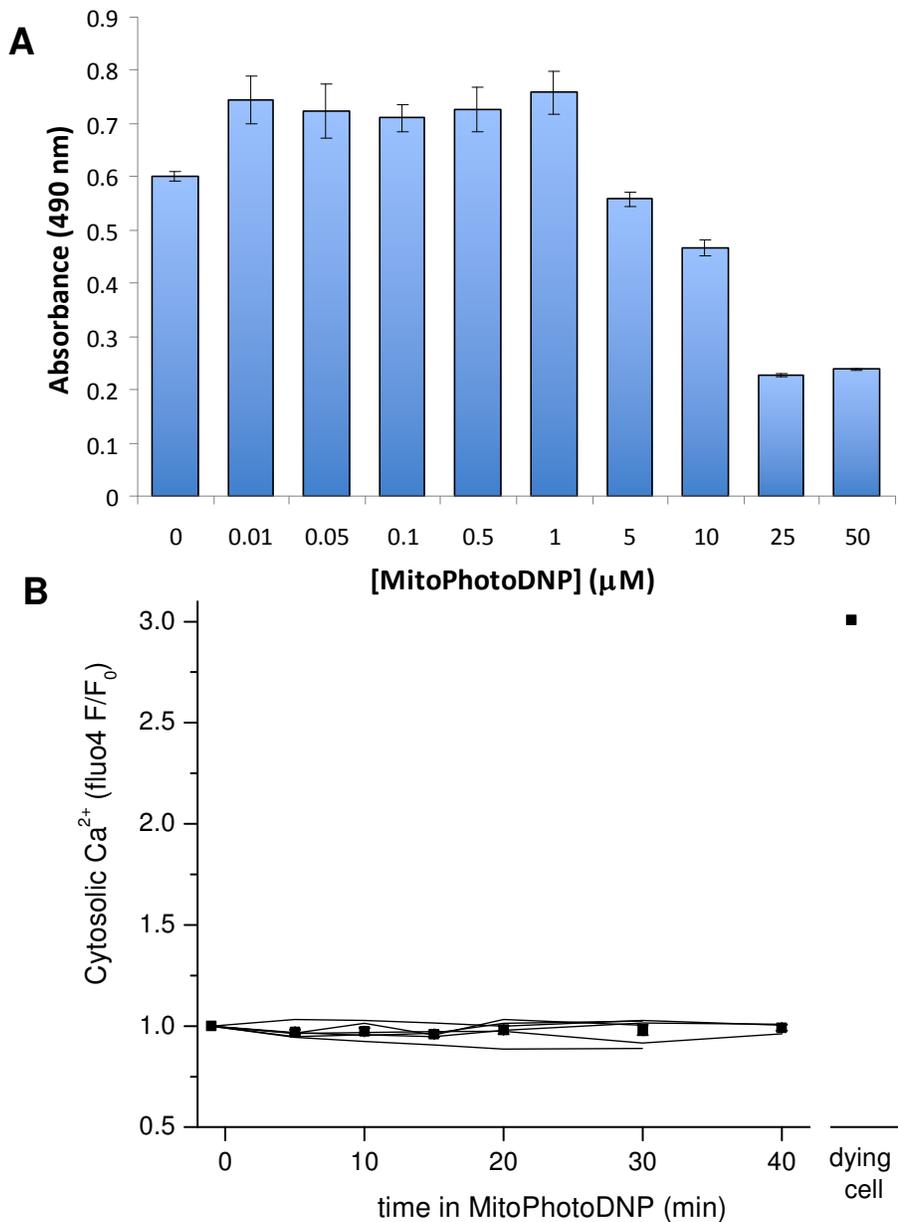


Figure S12. MitoPhotoDNP does not evoke cellular toxicity. **A** MitoPhotoDNP does not evoke toxicity at concentrations below 5 μM in a MTT viability assay of C2C12 myoblasts (1×10^4 cells/well in a 96-microwell culture plate). Background corrected absorbance at 490 nm of MTT product (formazan) is shown for $n=8$ wells for each, mean \pm s.e.m. **B** Freshly-isolated native colonic smooth muscle cells do not show an increase in cytosolic calcium concentration (as measured by fluo4 fluorescence) during loading with MitoPhotoDNP (200 nM) as would be expected if MitoPhotoDNP were toxic ($n=6$ cells from separate isolations, individual timecourses shown by lines, mean \pm s.e.m). As a point of comparison, fluo4 fluorescence is also shown of a damaged smooth muscle cell which has just fallen off an electrophysiology patch pipette. The increase in fluorescence is characteristic of a stressed cell and inability to control cytoplasmic calcium concentration.

Sustained localized mitochondrial depolarization in patch clamped cells

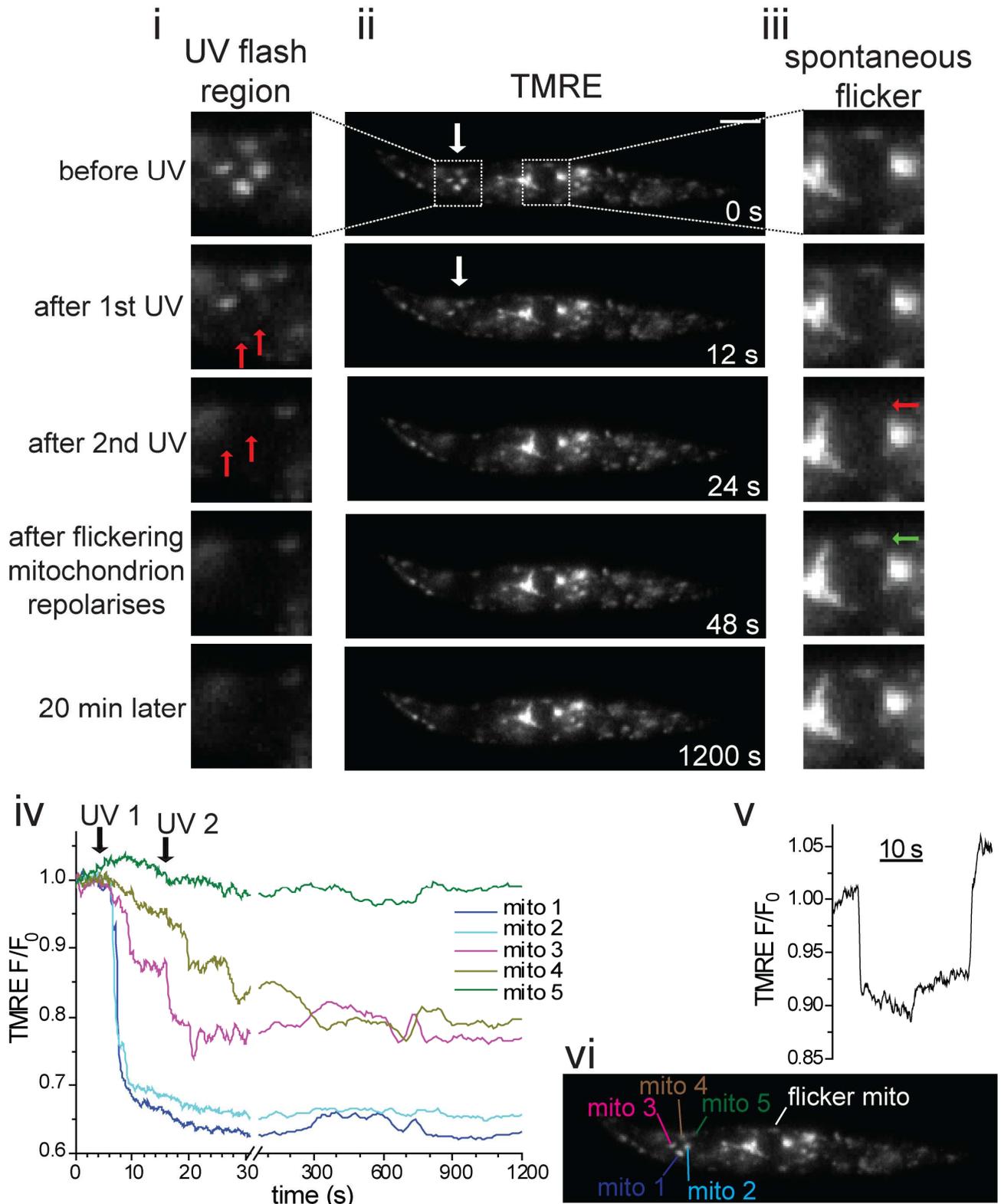


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Figure S13. MitoPhotoDNP-evoked mitochondrial depolarization does not reverse within the timescale of biological experiments. Freshly-isolated colonic smooth muscle cells loaded with TMRE (10 nM) and MitoPhotoDNP (200 nM) were patch-clamped in the whole-cell configuration to provide the metabolites pyruvate and malate plus ATP and phosphocreatine directly to the cytosol. Punctate mitochondrial fluorescence disappeared as mitochondria depolarized (red arrows, i) specifically in the region of UV exposure (2 exposures of 100 ms each, white arrow, ii) and did not re-appear over the subsequent 20 min. On the other hand, a spontaneous mitochondrial “flicker” was observed (iii) in which a mitochondrion depolarized (red arrow) and then recovered (green arrow), demonstrating that mitochondrial repolarization can be observed in these cells. The line traces in panel iv show TMRE fluorescence in regions of interest drawn around four mitochondria that depolarise following UV-photolytic release of DNP (2 UV exposures indicated by arrows) plus one proximal mitochondrion that was not subject to UV exposure so did not depolarize (positions shown in vi). Panel v shows TMRE fluorescence in a region of interest drawn around the mitochondrion displaying a spontaneous “flicker” of membrane potential. Scale bar = 10 μ m.

Supporting Information References

(1) McCarron, J.G.; Muir, T.C. *J. Physiol.* **1999**, *516*,149-161.