

A ratiometric fluorescent sensor for the mitochondrial copper pool

Clara Shen, Jacek L. Kolanowski, Carmen Tran, Amandeep Kaur, Mia C. Akerfeldt, Matthew S. Rahme, Trevor W. Hambley, Elizabeth J. New

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7. References

1. GENERAL EXPERIMENTAL METHODS

All solvents used were laboratory grade and were dried over appropriate drying agents when required. MilliQ water was used to prepare all aqueous solutions. Merck 230-400 mesh Kieselgel 60 was used for column chromatography and Merck Kieselgel 60 0.25 mm F254 precoated sheets were used for analytical thin layer chromatography. Chemicals were obtained from Sigma-Aldrich (St. Louis, MO), Alfa Aesar and Combi Blocks and used as received.

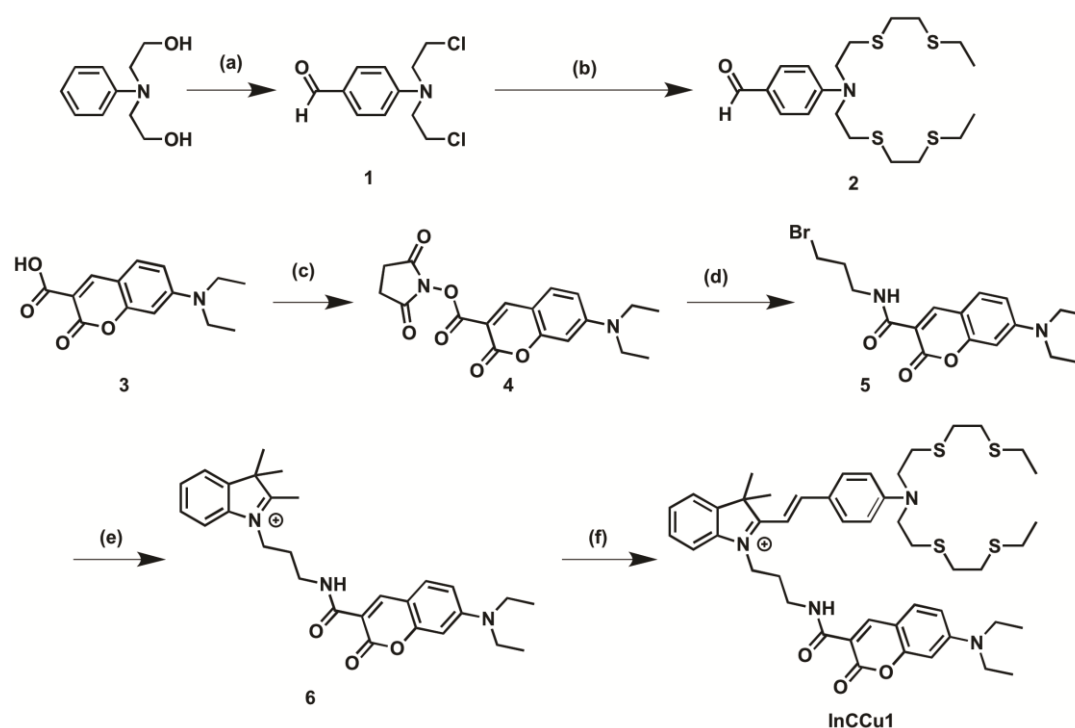
2. CHARACTERISATION INSTRUMENTATION

^1H -NMR spectra were collected in commercially available deuterated solvents (Cambridge Isotope Laboratories, Cambridge, MA) on a Bruker DRX 300 or Bruker Ascend 400 and 500 spectrometer. ^{13}C -NMR spectra were recorded on Bruker Ascend 400 and 500 spectrometers at frequencies of 100 MHz and 125 MHz. All chemical shifts are reported in the standard δ notation of parts per million using the peak of the residual solvent proton signals as an internal reference. J values are given in Hertz.

Fluorescence spectroscopic measurements were performed in 20 mM HEPES adjusted to pH 7.4. Fluorescence measurements were collected using a bench top Perkin Elmer Enspire Multimode Plate Reader at 10 nm slit widths.

Low resolution ESI-MS was recorded on a Bruker Amazon SL mass spectrometer operating on Electrospray Ionisation (ESI). High-resolution mass spectrometry was performed on a Thermo LTQ Orbitrap XL Mass spectrometer. Samples were diluted in methanol and added to a nano-spray tip coated with silver before running by positive ion nano-spray. The instrument was configured with a mass range 150-2000 m/z , ionspray voltage 1.4 kV and running with a resolving power of 100,000 at m/z 400.

3. SYNTHESIS



Scheme S1 Synthesis of **InCCu1**. (a) POCl_3 , DMF (b) 3-thiapentane-1-thiol, Na, EtOH (c) *N*-hydroxysuccinimide, EDC.HCl, DMF (d) 3-bromopropan-1-amine hydrobromide, DCM, triethylamine (e) 2,3,3-trimethylindolenine (f) 2, EtOH.

3.1. Synthesis of InCCu1

1¹ and **3**² were prepared according to literature procedures.

4-(bis(2-((2-(ethylthio)ethyl)thio)ethyl)amino)benzaldehyde (**2**)

3-Thiapentane-1-thiol was prepared according to a reported procedure and used without purification.³ To prepare **2**, sodium metal (230 mg, 10.0 mmol) was cut up into small 1-2 mm pieces and dissolved in ethanol (20 mL). 3-thiapentane-1-thiol (1.22 g, 10.0 mmol) was added to this solution and was stirred for 1 h under nitrogen atmosphere at 40 °C. Then, a solution of **1** in DMF (5 mL) was added and the reaction mixture stirred for 3 h at 40 °C. Water (50 mL) was then added, and the mixture was extracted with dichloromethane (3 x 50 mL). The organic fractions were combined and washed with water (50 mL) and brine (50 mL) sequentially. The combined organic extract was dried over MgSO_4 and the solvent reduced by rotary evaporation to give viscous orange oil. The crude oil product was immediately purified by flash column chromatography (1:20 MeOH:DCM) to yield **2** as a pale orange oil (2.28 g, 55%). ¹H-NMR (500 MHz, CDCl_3): δ 9.73 (s, 1H), 7.73 (d, 2H, *J* 9.0 Hz), 6.68 (d, 2H, *J* 9.0 Hz), 3.64 (t, 4H, *J* 7.5 Hz), 2.74 (m, 12H), 2.55 (q, 4H, *J* 7.5 Hz), 1.24 (t, 6H, *J* 7.5 Hz) ppm. ¹³C-NMR (125 MHz, CDCl_3): δ 190.1, 151.4, 132.3, 126.0, 111.0, 51.5, 32.6, 31.9, 30.9, 29.4, 26.2 ppm.

2,5-dioxopyrrolidin-1-yl 7-(diethylamino)-2-oxo-2H-chromene-3-carboxylate (4)

A solution of EDC.HCl (880 mg, 4.60 mmol) and *N*-hydroxysuccinimide (530 mg, 4.60 mmol) was dissolved in anhydrous DMF (15 mL). A solution of **3** (800 mg, 3 mmol) in DMF (5 mL) was added dropwise. The reaction mixture was stirred in darkness for 48 h at room temperature. The resulting solution was then poured onto ice water (150 mL) and the resulting yellow precipitate was filtered off by vacuum filtration to give **4** as a pale yellow solid which was dried in air overnight (390 mg, 35%) and used in the next step without further purification. ¹H-NMR (200 MHz, CDCl₃): δ 8.58 (s, 1H), 7.38 (d, 1H, *J* 9.0 Hz), 6.64 (dd, 1H, *J* 9.0, 2.5 Hz), 6.46 (s, 1H), 3.48 (q, 4H, *J* 7.0 Hz), 2.89 (s, 4H), 1.26 (t, 6H, *J* 7.0 Hz) ppm.

***N*-(3-bromopropyl)-7-(diethylamino)-2-oxo-2H-chromene-3-carboxamide (5)**

4 (300 mg, 0.60 mmol) was dissolved in DCM (20 mL) with triethylamine (128 µL, 0.90 mmol). To this solution was added 3-bromopropan-1-amine hydrobromide (201 mg, 0.90 mmol) and the reaction mixture was stirred overnight at room temperature in the dark. The solution was subsequently diluted with 50 mL of DCM and washed sequentially with NaHCO₃ (0.2 M, 50 mL), HCl (0.2 M, 50 mL) and brine. The organic layer was dried over MgSO₄ and the solvent was reduced by rotary evaporation to give **5** as a yellow solid (190 mg, 59%). ¹H-NMR (400 MHz, CDCl₃): δ 8.90 (s, 1H), 8.70 (s, 1H), 7.43 (d, 1H, *J* 9.0 Hz), 6.65 (dd, 1H, *J* 9.0, 2.0 Hz), 6.50 (d, 1H, *J* 2.0 Hz), 3.59 (q, 2H, *J* 6.5 Hz), 3.47 (m, 6H), 2.20 (quin, 2H, *J* 6.5 Hz), 1.25 (t, 6H, *J* 7.0 Hz) ppm. ¹³C-NMR (100 MHz, CDCl₃): δ 163.5, 162.8, 157.7, 152.6, 148.1, 131.1, 110.1, 110.0, 108.4, 96.6, 45.1, 38.1, 32.6, 30.8, 12.4 ppm.

1-(3-(7-(diethylamino)-2-oxo-2H-chromene-3-carboxamido)propyl)-2,3,3-trimethyl-3H-indol-1-ium (6)

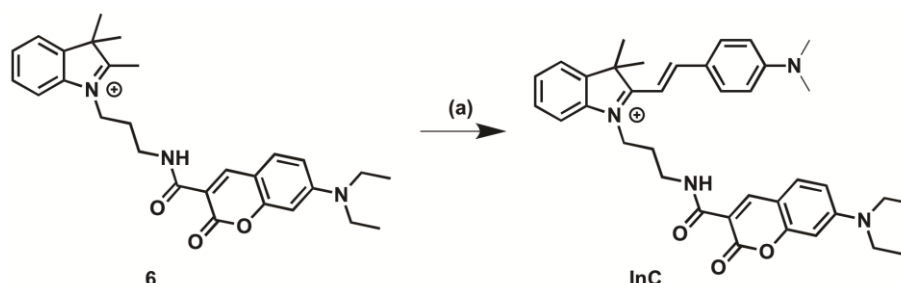
6 was prepared by heating a neat mixture of **5** (140 mg, 0.36 mmol) and 2,3,3-trimethylindolenine (59 µL, 0.36 mmol) at 100 °C overnight. This produced a dark pink solid onto which was poured ether (20 mL) and vigorously scraped to retrieve the solid. The resulting suspension was filtered and rinsed with ether and dried in air to give **6** as a dark purple solid that was used in the next step immediately without further characterisation or purification (90 mg, 53%). ESI-MS calculated 460.26 for [M⁺] found 460.21.

InCCu1

Compounds **2** (79 mg, 190 µmol) and **6** (88 mg, 190 µmol) were heated to reflux in a solution of ethanol (10 mL) overnight with MgSO₄ (catalytic) under nitrogen atmosphere. The resulting dark purple solution was reduced by rotary evaporation and immediately purified by flash column chromatography (silica, 1% MeOH/DCM to 5% MeOH/DCM) to yield **InCCu1** as a dark purple solid (61 mg, 41%). HR-MS found: M⁺, 859.3780. C₄₇H₆₃N₄O₃S₄⁺ requires 859.3778. ¹H-NMR (500 MHz, CDCl₃): δ 8.97 (m, 1H), 8.59 (s, 1H), 8.27 (s, 1H), 8.12 (d, 1H, *J* 15.0 Hz), 7.78 (d, 1H, *J* 15.0 Hz), 7.42 (m, 5H), 6.81 (d, 2H, *J* 8.5 Hz), 6.64 (m, 1H), 6.49 (s, 1H), 4.99 (m, 2H), 3.73 (m, 6H), 3.45 (q, 4H, *J* 7.0 Hz), 2.85-2.75 (m, 12H), 2.58 (q, 4H, *J* 7.5 Hz), 2.31 (m, 2H), 1.80 (s, 6H), 1.25 (m, 14H) ppm. ¹³C-NMR (125 MHz, CDCl₃): δ 179.8, 163.6,

162.5, 157.7, 155.4, 152.6, 152.6, 147.9, 142.5, 141.1, 135.9, 131.1, 129.4, 127.9, 123.6, 122.4, 113.3, 112.6, 110.0, 108.3, 106.8, 96.6, 51.8, 51.2, 45.1, 44.8, 37.1, 32.8, 31.9, 29.8, 28.6, 27.7, 26.2, 14.8, 12.4 ppm.

3.2. Synthesis of InC



Scheme S2. Synthesis of control probe **InC** from **6** (Scheme 1). (a) Dimethylaminobenzaldehyde, EtOH.

Compound **6** was prepared as for **InCCu1**, but in the final step was reacted with dimethylaminobenzaldehyde instead of **2**. **6** (30 mg, 65 μ mol) and *N,N*-dimethylaminobenzaldehyde (9.72 mg, 65 μ mol) were heated to reflux in a solution of ethanol (5 mL) overnight with MgSO_4 (catalytic) under nitrogen atmosphere. The resulting dark purple solution was reduced by rotary evaporation and immediately purified by flash column chromatography (silica, DCM to 3% MeOH/DCM) to yield **InC** as a dark purple solid (22 mg, 57%). HR-MS found: M^+ , 591.3323. $\text{C}_{37}\text{H}_{43}\text{N}_4\text{O}_3^+$ requires 591.3330. ^1H -NMR (500 MHz, CDCl_3): δ 9.00 (m, 1H), 8.59 (s, 1H), 8.14 (s, 1H), 8.09 (d, 2H, J 15.0 Hz), 7.58 (d, 2H, J 15.0 Hz), 7.47-7.37 (m, 5H), 6.77 (d, 2H, J 8.5 Hz), 6.64 (d, 1H, J 8.5 Hz), 6.48 (s, 1H), 4.90 (t, 2H, J 7.0 Hz), 3.73 (m, 2H), 3.46 (q, 4H, J 7.0 Hz), 3.16 (s, 6H), 2.32 (m, 2H), 1.80 (s, 6H), 1.24 (m, 6H) ppm. ^{13}C -NMR (75 MHz, CDCl_3): δ 179.0, 162.5, 157.7, 155.4, 155.2, 152.6, 148.0, 142.2, 141.1, 131.2, 129.3, 127.6, 122.7, 122.3, 113.1, 112.6, 112.6, 110.1, 110.1, 108.2, 105.3, 96.5 ppm.

4. FLUORESCENCE DATA COLLETION

MilliQ water was used to prepare all aqueous buffers and solutions. All spectra were collected in HEPES buffer (20 mM, pH 7.4). Excitation of **InCCu1** was provided at 430 nm for *in vitro* studies with collection from 450-750 nm. In the titration experiments, copper was delivered in the form of $[\text{Cu}(\text{MeCN})_4][\text{PF}_6]$ in acetonitrile stock solution (10 mM), which was diluted into HEPES buffer (20 mM, pH 7.4).

The binding affinity of **InCCu1** to Cu(I) was measured using thiourea as a competitive ligand to provide buffered and extremely low concentrations of Cu(I) in solution as previously reported.⁴ The stability constants used for thiourea binding were taken from reported literature.

5. CELLULAR EXPERIMENTS

5.1. Cell culture

All cellular studies were performed on DLD-1 cells (passage number below 20) cultured as a monolayer at 37 °C in 5% CO₂ humidified incubator in advanced Dulbecco's modified eagle medium (Adv. DMEM) supplemented with 2.5 mM glutamine and 2% foetal bovine serum (FBS) without antibiotics. Cells were regularly mycoplasma tested.

5.2. Viability assay

Viability was determined using the standard AlamarBlue assay. In a 96-well plate, approximately 1×10^4 DLD-1 cells in 100 µL complete medium (Adv. DMEM supplemented with FCS and glutamine) were seeded into each well and allowed to adhere overnight in an incubator. 2 mM stock solution of CisPt in PBS was used to prepare serial dilutions ranging between 0 – 0.8 mM (40% PBS in media). Then 10 µL of the appropriate solution was added to the wells containing cells to final concentrations ranging from 0 - 80 µM. Control wells were treated with corresponding volumes of the vehicle (40% PBS in media). Each treatment was performed in triplicates. After a 16 h incubation period, 10 µL of AlamarBlue solution was added to each well and the cells were incubated for additional 3 h and the plates were shaken for 1 min. The fluorescence of each well was recorded at 590 nm using a plate reader (λ_{ex} = 570 nm).

5.3. Microscopes and software

Single-photon confocal images were acquired using an Olympus FluoView FV1000 inverted light confocal microscope (single-photon images) with Olympus UPLanSApo 60X water-immersion objective lens (NA = 1.20). Excitation light at 405 nm (1% laser power) was provided by a FV5-LDPSU 405 nm laser and Melles Griot laser was used as a source of 633 nm excitation light (1% laser power). Multi-photon images were acquired using a Leica SP5 II confocal multi-photon microscope with HC PLApoCS2 63X water-immersion objective lens (NA = 1.20). Excitation light of 820 nm was provided by the Mai Tai DeepSee™ Ti:Sapphire femtosecond pulsed laser. Cells were imaged directly after treatment with the probe, in a temperature-controlled incubator at 37 °C (on Olympus FV1000 microscope) or directly on the stage (on Leica SP5 II microscope). Images were collected using FV10-ASW viewer software v1.7 (Olympus) and Leica Application Suite Advanced Fluorescence Version: 2.8.0 build 7266 viewer software. Image analysis was performed using FIJI (National Institutes of Health).

5.4. Cell preparation and treatments

DLD-1 cells were cultured as described above. Before each experiment, a stock suspension of cells in logarithmic growth phase was prepared in media described above and 2 mL of this suspension of cells (30 000 – 35 000 cells per dish) were added onto 35 mm glass-bottom dishes (MatTek Corporation)

48 h before imaging to ensure the adherence of cells and restoration of their normal growth before any treatments.

Cells were treated with 2 mL of supplemented media (glutamine and FBS) containing vehicle control (phosphate-buffered saline, PBS) or copper sulfate (20 μ L of 10 mM stock solution of copper sulfate in PBS to the final concentration of 100 μ M) and then washed 3 times with PBS. After the given incubation time, cells were washed 3 times with PBS and incubated in 1 mL solution of a desired concentration of **InCCu1** (0.1 or 0.2 μ M) in Adv. DMEM media (supplemented only with 2.5 mM glutamine) and prepared immediately before the addition to cells by diluting a 1000x concentrated stock solution of the probe in DMSO with the media (final concentration of DMSO in cell media was less than 0.1%). After 15 minutes cells were washed 3 times with PBS and imaged in 10 % FBS in PBS immediately after treatment.

For colocalisation experiments, cells were treated firstly for 15 min with **InCCu1** (50 nM, from a 50 μ M DMSO stock solution). After washing thrice with PBS, cells were then treated for 15 min with MitoTracker Deep Red (100 nM, from a 100 μ M DMSO stock solution) before imaging in 10% fetal bovine serum (FBS) in PBS immediately after the treatment.

5.5. Imaging experiments.

Images were typically collected at two channels: blue channel collecting 425 – 525 nm light and red channel collecting 570 nm – 670 nm light.

Images on Olympus FV1000 microscope were collected upon excitation with a 405 nm laser at 1% laser power at 1024x1024 resolution applying 4x line averaging. Images on Leica SP5 II microscope were collected upon excitation with 820 nm pulsing multi-photon laser at 512x512 resolution applying 4x line averaging.

In co-localisation experiments, images were collected on Olympus FV1000 microscope similarly to the method described above but at three channels: blue channel (425 nm – 525 nm), red channel (570 nm – 620 nm) and deep red channel (655 nm – 755 nm), upon simultaneous excitation with 405 laser and 633 nm laser. At least five fields of view per sample were photographed and analysed on ImageJ.

6. Flow cytometry

Flow cytometric analyses were performed using BD biosciences LSRFortessa equipped with a 56 mW 405 nm coherent laser. Cells were cultured as detailed above for at least 24 h before an appropriate treatment was added in fresh AdvDMEM media. Treatment included vehicle (PBS) or 10 μ M cisplatin solution prepared immediately before treatment by diluting 10 μ L of 2 mM stock solution in PBS in 2 mL of supplemented media. After 16 h cells were treated additionally with vehicle (PBS) or copper sulfate (as above) for 2 h. Then they were washed 3 times with PBS before the addition of **InCCu1** (0.2

μ M, 15 min) solution in AdvDMEM media supplemented with glutamine (2.5 mM) but without FBS. After 15 minutes cells were washed 3 times with PBS prior to the addition of trypsin (5 mins) to cause detachment from the dish, followed by addition of cold 10 % (v/v) FBS in PBS. After centrifugation cells were resuspended in cold 10% (v/v) of FBS in PBS to the concentration of approximately 100 000 cells / mL and stored on ice prior to analysis with flow cytometer. Emission upon excitation at 405 nm was collected in the blue channel (450/50 nm) and red channel (585/15 nm). Approximately 40 000 events were collected for each run with appropriate gating applied to isolate healthy and single cells according to the FSC/SSC plot. Dead cells were the population in the bottom-left quadrant, and constituted less than 4% of total cells for all treatments. Data obtained were extracted using FlowJo software (Tree Star) and FlowPy (v5.2), and analysed using Microsoft Excel.

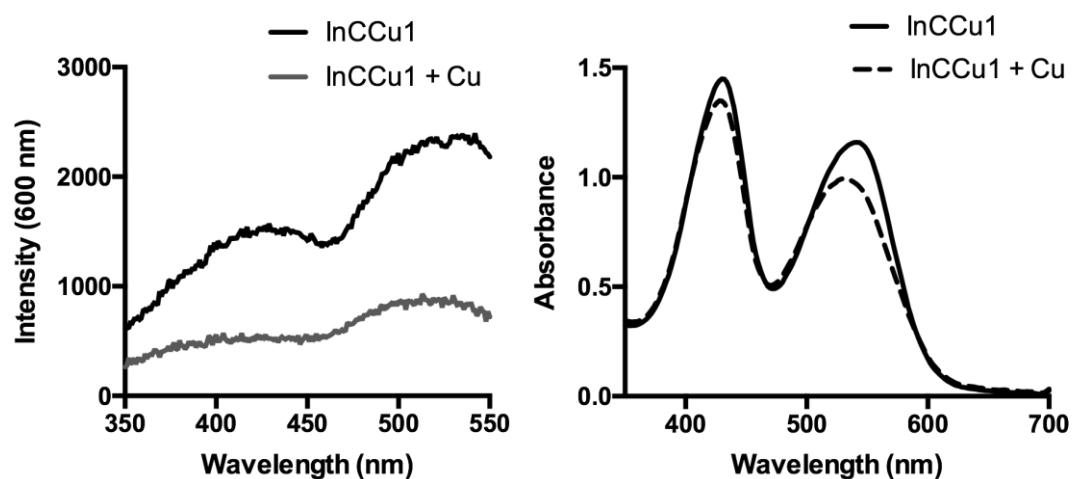


Figure S1. The excitation and absorption spectra of **InCCu1** alone and in the presence of Cu(I). (Left): Excitation spectra of **InCCu1** (100 μ M in HEPES pH 7.4) and **InCCu1** + Cu(I) (1.0 eq.) with emission at 600 nm. (Right): Absorbance spectra of **InCCu1** (100 μ M in HEPES pH 7.4) and **InCCu1** + Cu(I) (1.0 eq.).

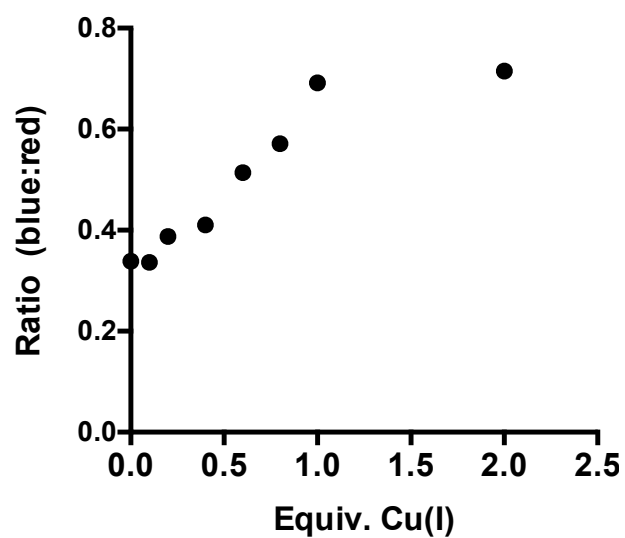


Figure S2. Fluorescence response of **InCCu1** (100 μ M in HEPES 20 mM, pH 7.4) to increasing amounts of Cu(I), with 430 nm excitation and emission collected from 450-650 nm. Ratio was determined by $I_{450-525} / I_{550-650}$.

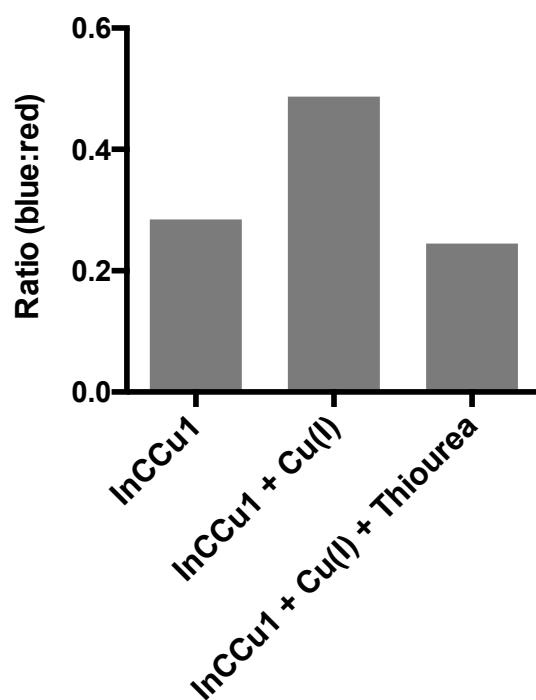


Figure S3. Reversibility of response of **InCCu1** to Cu(I). The fluorescence of **InCCu1** (100 μ M in HEPES 20 mM, pH 7.4) was measured upon addition of Cu(I) (1.0 equivalent), and then with subsequent addition of thiourea.

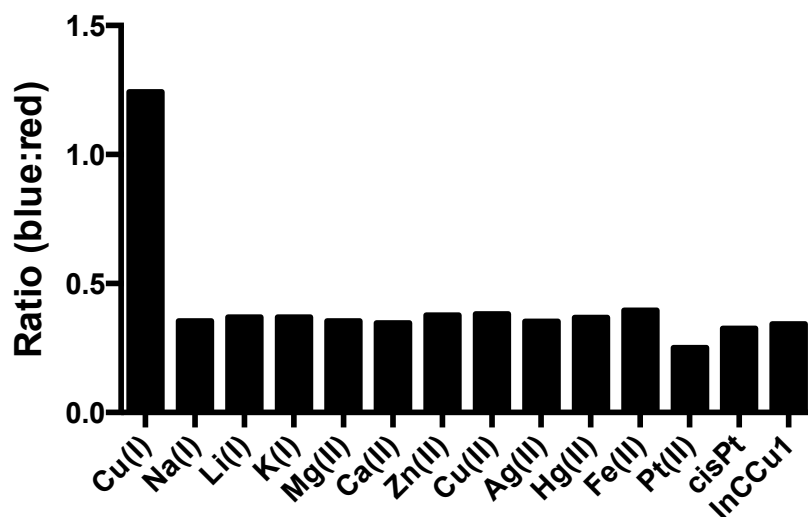


Figure S4. Selectivity of **InCCu1** for copper over metal ions. Integrated ratio of fluorescence response of 100 μ M **InCCu1** to various metals (1.0 equivalents of each) delivered as nitrate salts (Pt was delivered in the form of KPtCl_4 and cisplatin). Excitation provided at 430 nm, and ratio was determined by dividing integrated emission from 450-515 nm with 550-650 nm.

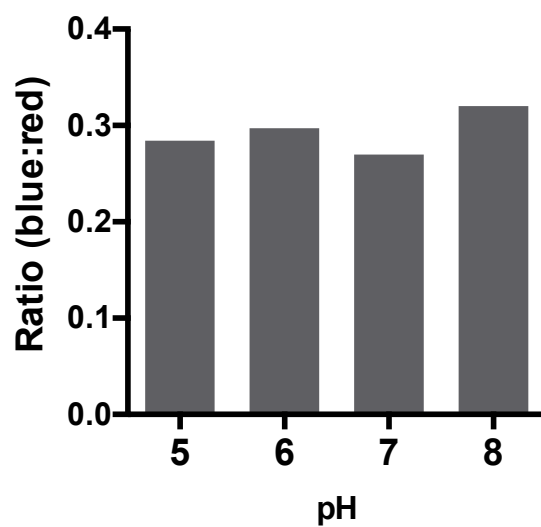


Figure S5. The ratio of blue to red intensity of **InCCu1** with varying pH. Spectra were collected in HEPES buffer with **InCCu1** (100 μ M). Excitation provided at 430 nm, and ratio was determined by dividing integrated emission from 450-520 nm with 550-700 nm.

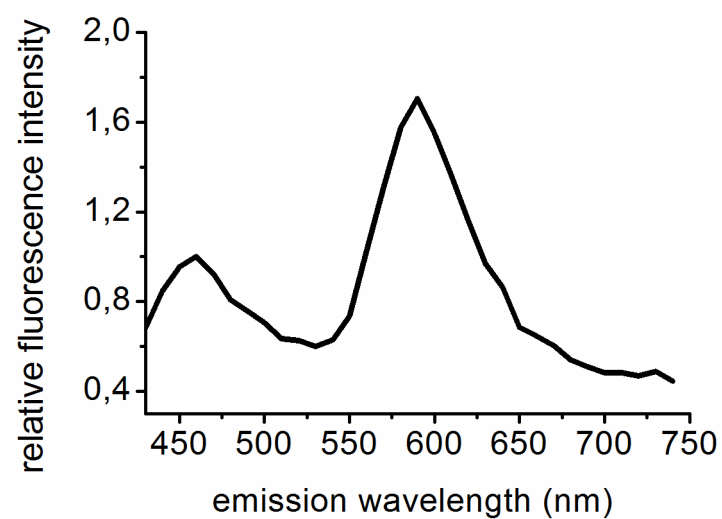


Figure S6. Intracellular emission spectrum of **InCCu1**. Spectral scan of DLD-1 cells treated with **InCCu1** (0.1 μ M, 15 min), taken at 10 nm steps (with 10 nm bandwidth) from 430-650 nm, with 405 nm excitation.

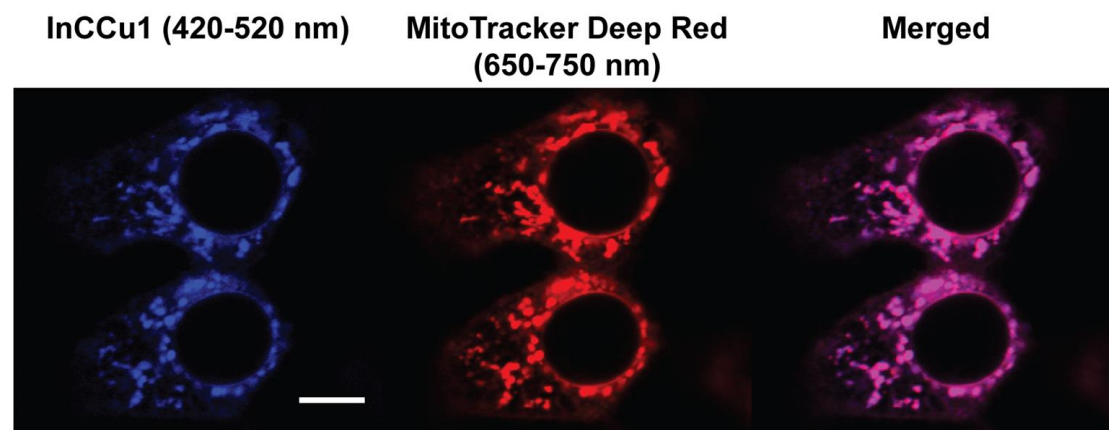


Figure S7. InCCu1 localises in mitochondria. Confocal microscopy images of DLD-1 cells upon simultaneous excitation with 405 nm and 633 nm laser. Images of blue channel ($\lambda_{em} = 420 - 520$ nm) and deep red channel ($\lambda_{em} = 650 - 750$ nm) were obtained from cells treated with MitoTracker Deep Red FM (100 nM, 15 min), followed by the treatment with **InCCu1** (100 nM, 15 min) Pearson's correlation coefficient between **InCCu1** and MitoTracker Deep Red FM is 0.96, suggesting high co-localisation. Scale bar represents 10 μ m.

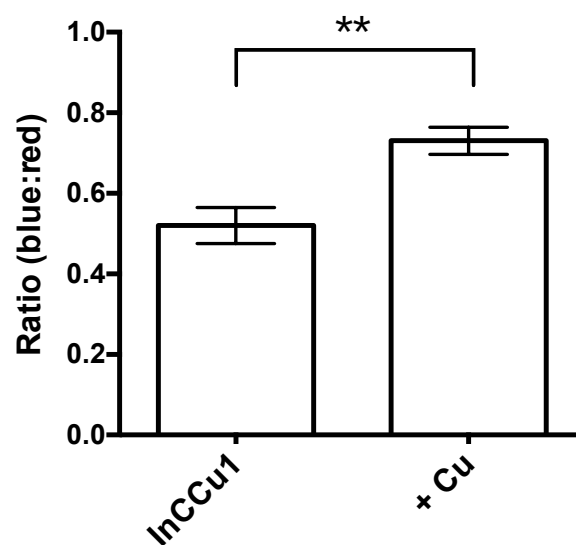


Figure S8. InCCu1 response to changes in mitochondrial copper measured upon multiphoton excitation ($\lambda_{\text{ex}} = 820 \text{ nm}$). Ratio of mean intensities of two channels (channel 1 – 425 – 475 nm, channel 2 - 570 – 670 nm) from DLD-1 cells treated with InCCu1 (200 nM, 15 min), following 16 h treatment with vehicle control or copper sulfate (100 μM). Error bars represent standard error of mean of quintuplicate measurements.

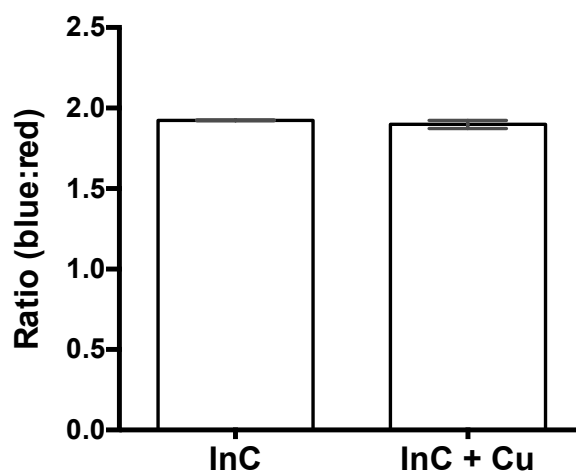


Figure S9. The ratio of **InC** emission does not respond to copper. **InC** (50 μ M in HEPES 20 mM, pH 7.4) fluorescence (λ_{ex} 425 nm; λ_{em} 445 – 675 nm) was measured alone or in the presence of copper ($[\text{Cu}(\text{MeCN})_4][\text{PF}_6]$, 5 equivalents). Data represents ratio of $I_{445-525}/I_{550-675}$. Error bars represent standard error of mean of triplicate measurements.

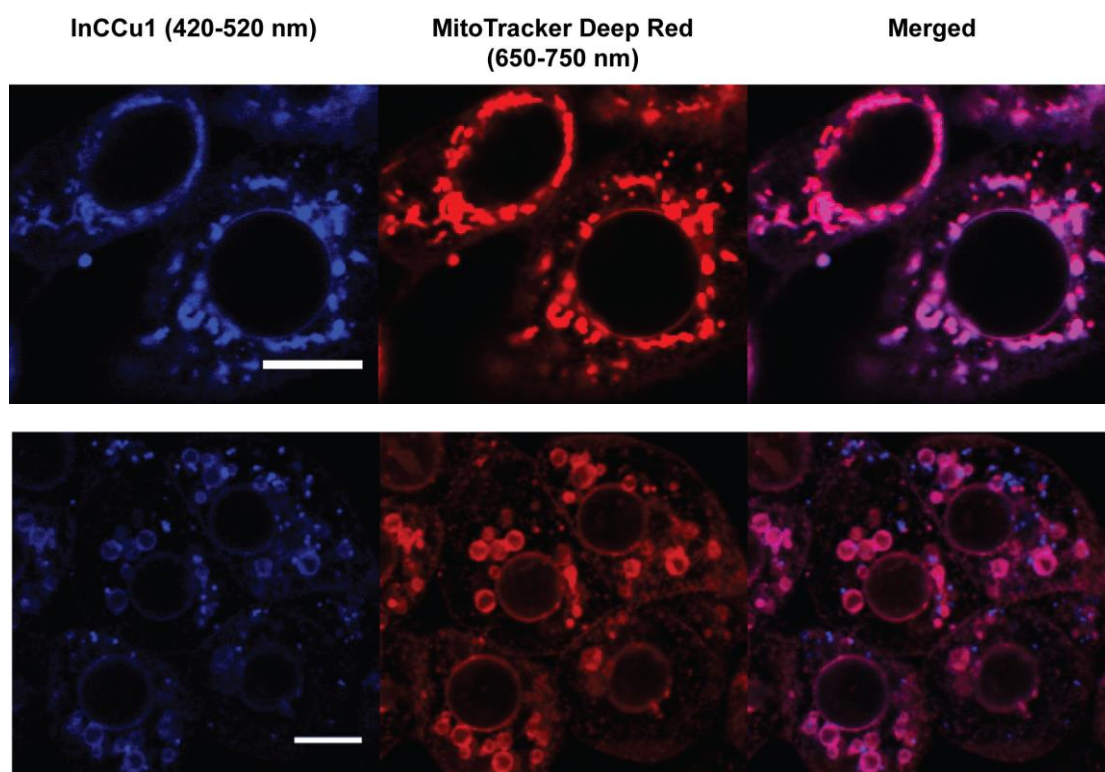


Figure S10. Cisplatin does not influence the localisation of **InCCu1**, but uncoupling the mitochondrial membrane potential with FCCP results in delocalisation of both probe and MitoTracker Deep Red. (Top): Confocal microscopy images of DLD-1 cells upon simultaneous excitation with 405 nm and 633 nm laser. Images of the blue channel (420-520 nm) and deep red channel (650-750) were obtained from cells treated with cisplatin (10 μ M , 16 h) followed by MitoTracker Deep Red (100 nM, 15 min) and then treatment with **InCCu1** (100 nM, 15 min). (Bottom): The same cells were then treated with FCCP to uncouple mitochondrial oxidative phosphorylation and imaged again with the same lasers. Scale bars represents 10 μ m.

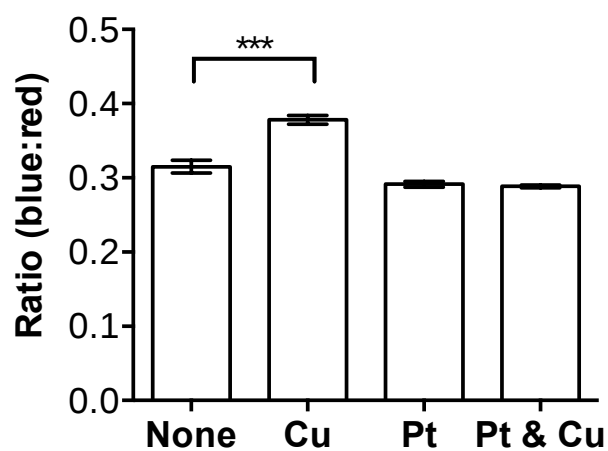


Figure S11. Flow cytometry reveals that cisplatin treatment compromises mitochondrial copper uptake. Ratio of intensities from DLD1 cells (red emission 585/15 / blue emission 450/50, excitation at 405 nm) for flow cytometric analysis of DLD-1 cells treated with **InCCu1** (0.2 μ M, 15 min) following treatment. Mean blue/red ratio for each treatment averaged for single and live cells. Error bars represent standard error of mean for quintuplicate measurements.

7. REFERENCES

- 1 M. T. Morgan, P. Bagchi and C. J. Fahrni, *J. Am. Chem. Soc.*, 2011, **133**, 15906–15909.
- 2 A. Kaur, M. A. Haghighatbin, C. F. Hogan and E. J. New, *Chem. Commun.*, 2015, **51**, 10510–10513.
- 3 L. Zeng, E. W. Miller, A. Pralle, E. Y. Isacoff and C. J. Chang, *J. Am. Chem. Soc.*, 2006, **128**, 10–11.
- 4 D. W. Domaille, L. Zeng and C. J. Chang, *J. Am. Chem. Soc.*, 2010, **132**, 1194–1195.