Supporting Information for “Simultaneous Measurement of Individual Mitochondrial Membrane Potential and Electrophoretic Mobility by Capillary Electrophoresis”

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S-1. Experimental Section.

S-1-1. Buffers and Solutions.

Reagents and Materials used in Supporting Information. Sodium hydroxide (NaOH), methanol, EGTA, and tetraphenylphosphonium chloride (TPP⁺) were from Sigma-Aldrich (Saint Louis, MO). 3-(N-morpholino)propanesulfonic acid (MOPS) was from Acros (Geel, Belgium). EDTA was from Avocado (Heysham, Lancashire, UK). D-mannitol was from Riedel de-Haën (Seelze, Germany). Tris was from Fisher (Fair Lawn, NJ). Bovine serum albumin (BSA) was from Roche (Indianapolis, IN). Hydrochloric acid (HCl) was from Mallinckrodt (Saint Louis, MO). The TPP⁺ ion-selective electrode and Dri-Ref Ag/AgCl reference electrode were purchased from World Precision Instruments (Sarasota, FL).

Buffers and Solutions: Additional Detail. Mitochondrial isolation buffers were prepared for cells (C), liver (L), and muscle tissue (M₁ and M₂). See Table S-1-1 below for the composition of these buffers. JC-1, fluorescein, and valinomycin were dissolved in methanol, aliquotted, dried in a vacuum evaporator (LabConco, Kansas City, MO), and stored at -20 °C. Stock solutions were prepared from a new aliquot for each experiment; JC-1 and valinomycin were reconstituted and diluted in DMSO and fluorescein was reconstituted in methanol and diluted with SH buffer. JC-1 stock solutions were sonicated for 60 minutes before further dilution. A stock succinate solution was prepared by titrating succinic acid to pH 7.4 with KOH in the presence of 10 mM HEPES. The succinate solution was filtered to 0.2 µm and stored at 4 °C. A stock rotenone solution was prepared in DMSO and stored at -20 °C. The succinate concentration of 2.5 mM was chosen to limit Joule heating in the CE-LIF experiments; no detectable Joule heating was observed with this concentration of succinate in buffer SH, while higher concentrations resulted in Joule heating. A standard solution of 10 mM TPP⁺ was prepared in water and diluted with buffer Rₛ or buffer Rval for analysis of depolarized controls.

Table S-1-1. Buffer compositions for mitochondrial isolation.a

| Buffer | Description       | Composition                                      |
|--------|-------------------|-------------------------------------------------|
| C      | Isolation buffer, cells | 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 5.0 mM EDTA |
| L      | Isolation buffer, liver | 200 mM sucrose, 10 mM MOPS, 10 mM tris, 1.0 mM EGTA |
| M₁     | Isolation buffer 1, muscle | 67 mM sucrose, 50 mM KCl, 50 mM tris, 10 mM EDTA, 0.2% (w/v) BSA |
| M₂     | Isolation buffer 2, muscle | 250 mM sucrose, 10 mM tris, 3.0 mM EGTA |

a. All buffers made up in deionized water and adjusted to pH 7.4 with KOH.
Figure S-1-1. Photobleaching of SH buffer with 2.5 mM succinate to reduce background. A device containing 120 blue LEDs (467 nm, $4.2 \times 10^5$ mcd intensity) was used to reduce the fluorescence background in the SH buffer containing 2.5 mM succinate. Samples were taken at different time points. Samples of buffer were flowed through the sheath flow cuvette, and the fluorescence and standard deviation of the signal was measured. The photobleaching device reduced the average signal intensity and the standard deviation of the average signal intensity in the red channel, $593 \pm 20$ nm (A-B), and in the green channel, $520 \pm 17.5$ nm (C-D).
Mitochondrial Preparation and JC-1 Labeling.

**Mitochondrial Preparation.** Mitochondria from cell culture were isolated by differential centrifugation and mechanical homogenization.\(^1\) Cells were lifted with 0.25% trypsin in PBS and added to an equal volume of DMEM. All subsequent procedures were performed on ice or at 4 °C unless otherwise noted. Cells were washed three times with ice-cold buffer C and resuspended in 2 mL buffer C. Cells were counted with a Fuchs-Rosenthal counting chamber (Hauser Scientific, Horsham, PA) after staining with trypan blue. A typical cell concentration was \(1 \times 10^6\) cells/mL. Cells were disrupted in a 2 mL Kontes glass dounce tissue grinder (Kimble Chase, Vineland, NJ) by applying strokes with loose (0.12 mm) and narrow (0.06 mm) glass pestles by hand until cell breakage from 80-100% was achieved, as monitored by trypan blue staining. After disruption, intact cells, nuclei, and other cellular debris were eliminated from the preparation by centrifugation at 600g for 10 min. Mitochondria in the supernatant were pelleted by centrifugation at 1000g for 10 min. Mitochondria were resuspended in buffer C and kept on ice. Protein content in the mitochondrial fraction was quantified using the Pierce BCA protein assay kit according to the manufacturer’s instructions (Thermo, Rockford, IL). Typical mitochondrial protein concentration from this preparation was 1.60 ± 0.09 mg/mL.

Mitochondria were isolated from mouse liver and muscle (hamstring) tissue from C57BL6 mice using published protocols for mechanical homogenization and differential centrifugation.\(^2\) C57BL6 mice were housed in a central specific pathogen free facility and were treated in an optimally ethical and humane fashion using protocols approved by the Institutional Animal Care and Use Committee for all procedures. Briefly, a female mouse was anesthetized on the day of the experiment and the liver and hamstring muscle were excised. The liver was immersed in ice-cold buffer L and the muscle was immersed in ice-cold PBS containing 10 mM EDTA. All subsequent procedures were performed on ice or at 4 °C unless otherwise noted. For preparation of liver mitochondria, the liver was rinsed with buffer L and minced into small pieces. The pieces were rinsed and transferred to a glass 15 mL Potter-Elvehjem homogenizer (Wheaton, Millville, NJ). The liver was homogenized with 3-5 strokes of a motor-driven Teflon pestle operated at 1600 rpm (Wheaton). The homogenate was centrifuged at 600g for 10 min to remove nuclei, intact cells, and other debris. The supernatant was centrifuged at 7000g for 10 min, then the pellet was washed once with buffer L and centrifuged at 7000g for 10 min. The liver mitochondria were resuspended in a minimal amount of buffer L and kept on ice. For preparation of muscle mitochondria, the muscle was minced into small pieces which were then washed with PBS containing 10 mM EDTA. The minced muscle pieces were incubated with 0.05% trypsin in PBS with 10 mM EDTA for 30 min. The pieces were transferred to buffer M\(_1\) and homogenized with 10-15 strokes of a motor-driven Teflon pestle operated at 1600 rpm. The homogenate was centrifuged at 7000g for 10 min to remove nuclei, intact cells, and other debris. The supernatant was centrifuged at 8000g for 10 min, then the pellet was washed once with buffer M\(_2\) and centrifuged at 8000g for 10 min. The muscle mitochondria were resuspended in a minimal amount of buffer M\(_2\) and kept on ice. Protein content in each mitochondrial fraction was quantified using the Pierce BCA protein assay kit. Protein concentration from this preparation was 23 ± 5 mg/mL for liver mitochondria and 3.2 ± 0.6 mg/mL for muscle mitochondria.
Figure S-1-2 JC-1 labeling strategy for isolated mitochondria. A) Mitochondria from C2C12 cells were isolated and kept on ice in buffer R with no succinate/rotenone or valinomycin. At different time points, aliquots were taken and succinate/rotenone was added (buffer Rs). Valinomycin was added to depolarized controls (buffer Rval). Aliquots were labeled with JC-1, incubated, then added to a 96-well plate. (continued on next page)
Time points shown on the plot represent the time elapsed after mitochondrial isolation before the data was collected. Red (B) and green (C) fluorescence was measured in a plate reader (red channel: $\lambda_{ex} = 530 \pm 12.5$ nm, $\lambda_{em} = 590 \pm 17.5$ nm; green channel: $\lambda_{ex} = 485 \pm 10$ nm, $\lambda_{em} = 528 \pm 10$ nm). Red fluorescence and red/green ratio (D) decreases over time for samples containing polarized mitochondria. Additionally, aliquots labeled later in the experiment show higher red/green ratios than aliquots labeled earlier at the same point in time (e.g., the polarized sample labeled and measured at 112 minutes has higher red/green ratio than the polarized sample labeled and measured at 36 min, the polarized sample labeled and measured 200 min after isolation shows the highest red/green ratio at the final measurement, 256 min). Each data point is corrected for photobleaching caused by exposure to the excitation source in the plate reader according to the formula

$$fluor_{corrected} = n_{reads}(\Delta fluor_{photobleaching}) + fluor_{uncorrected}$$

where $n_{reads}$ is the number of times the plate was exposed to the excitation light. Photobleaching ($\Delta fluor_{photobleaching}$) was determined by reading the plate two additional times at the end of the experiment (data not shown). Plate reader settings were as described in Figure 4 in the main text, JC-1 labeling was as described in the Experimental section in the main text.
S-1-3. Bulk Measurement of JC-1 Fluorescence.

**Figure S-1-3.** Bulk fluorescence from isolated mitochondria (L6 rat myoblasts). The mitochondrial sample containing substrate is labeled “polarized” and the depolarized control containing substrate and valinomycin is labeled “depolarized.” Buffer R and 100 nM JC-1 in buffer R are included as additional controls. (A) Red fluorescence ($\lambda_{\text{ex}} = 530 \pm 12.5 \text{ nm}, \lambda_{\text{em}} = 590 \pm 17.5 \text{ nm}$). (B) Green fluorescence ($\lambda_{\text{ex}} = 485 \pm 10 \text{ nm}, \lambda_{\text{em}} = 528 \pm 10 \text{ nm}$). The mitochondrial sample and depolarized control have higher red and green fluorescence compared to buffer R and 100 nM JC-1 in buffer R, showing an uptake of JC-1 into mitochondria. (C) Red/green ratio. (D) Normalized data from mitochondrial sample and depolarized control. The sample has higher red fluorescence than the depolarized control (2 µM valinomycin), showing JC-1 aggregate formation from the higher concentration. Error bars represent the standard deviation of measurements from three different wells containing the same sample.
Validation of JC-1 as a Ratiometric Probe for Membrane Potential.

**Fluorescence Microscopy.** L6 Cells were seeded onto Lab-Tek chambered cover glass slides (ThermoFisher Scientific, Waltham, MA) overnight. Cells were depolarized with 2 µM valinomycin for 2 hours before JC-1 labeling, 0.12% DMSO was added to the media of polarized cells for 2 hours as a control. Cells were labeled by adding 500 nM JC-1 to the media. After 30 minutes, the media was replaced with PBS containing 2 µM valinomycin (depolarized cells) or 0.12% DMSO (polarized cells) and confocal fluorescence images were acquired using an Olympus IX-81 inverted fluorescence microscope (Melville, NY) equipped with a DS-IX100 disk spinning unit. A C9100-01 EM CCD camera (Hamamatsu, Bridgewater, NJ) was used to acquire images. Images were acquired in the red channel with a 2.0 s exposure time, a gain of 100, $\lambda_{ex} = 545 \pm 10$ nm, $\lambda_{em} = 597.5 \pm 27.5$ nm, and a 565 nm dichroic, (Olympus M-RFPHQ filter cube) and in the green channel with a 10.0 s exposure time, a gain of 100, $\lambda_{ex} = 470 \pm 10$ nm, $\lambda_{em} = 517.5 \pm 22.5$ nm, and a 485 nm dichroic (Olympus U-MGFPHQ filter cube). Simple PCI software (Hamamatsu) was used to adjust contrast and apply a median smooth function to the images.

![Figure S-1-4](image)

**Figure S-1-4.** Confocal fluorescence microscopy. (A-D) Confocal fluorescence microscopy images of polarized (A and B) and depolarized (C and D) L6 rat myoblast cells labeled with 500 nM JC-1 for 30 minutes. Polarized cells exhibit intense red fluorescence (A) from JC-1 aggregates and less intense green fluorescence (B); depolarized cells (2 µM valinomycin) exhibit very little red fluorescence (C) and more intense green fluorescence (D) from JC-1 monomers. Contrast is adjusted and median smooth function used. Scale bars are 10 µm. (E-H) Unmodified images. Red channel (A,C,E, and G): 2.0 s exposure time, gain of 100, $\lambda_{ex} = 545 \pm 10$ nm, $\lambda_{em} = 597.5 \pm 27.5$ nm, 565 nm dichroic, Olympus M-RFPHQ filter cube; green channel (B,D,F, and H): 10.0 s exposure time, gain of 100, $\lambda_{ex} = 470 \pm 10$ nm, $\lambda_{em} = 517.5 \pm 22.5$ nm, 485 nm dichroic, Olympus U-MGFPHQ filter cube.
Membrane Potential Measurement with TPP⁺ Ion-Selective Electrode. Measurement of the average membrane potential of isolated mitochondria was performed by a method which has been described previously. Briefly, mitochondria were isolated from L6 rat myoblasts, then the sample and depolarized control were prepared as described in the main text (but without JC-1 labeling). Total mitochondrial protein content was measured using the BCA assay as described in the main text and TPP⁺ uptake was measured by an ion-selective electrode. The response of the electrode to TPP⁺ was calibrated by 7 µL additions of 0.1 mM TPP⁺ to 700 µL of buffer Rₐ (or Rᵥ for the depolarized control) with stirring. Directly after addition of TPP⁺ to a final concentration of 3.85 µM, 75 µL mitochondrial suspensions containing 0.30 mg of total mitochondrial protein in buffer Rₐ or 0.32 mg in buffer Rᵥ (depolarized control) were added. Electrode response was recorded using an Orion 420 A+ pH meter (Thermo) and collected using a program written in LabView (National Instruments, Austin, TX). Membrane potential was calculated according to the previously described method.

![Figure S-1-5](image-url)

Figure S-1-5. Measurement of membrane potential by TPP⁺ ion-selective electrode in mitochondrial sample (A) and depolarized control (B) from L6 rat myoblasts. Before measurement of TPP⁺ uptake, mitochondria were incubated in buffer Rₐ (or Rᵥ for the depolarized control) for 10 min. The * symbols represent additions of 7 µL of 0.1 mM TPP⁺ to 700 µL of buffer Rₐ (or buffer Rᵥ for the depolarized control), # and $ represent additions of 75 µL suspensions of mitochondria in buffer Rₐ (or buffer Rᵥ for the depolarized control), respectively. Total mitochondrial protein added was 0.30 and 0.32 mg (depolarized control). Membrane potential was calculated as -125 mV and -80 mV for the sample and depolarized control, respectively.
Figure S-1-6. Fluorescence emission of JC-1 and transmittance of filter set in CE-LIF detector. The fluorescence emission spectrum of a 1 μM solution of JC-1 in buffer SH₃ was acquired using a Cary Eclipse fluorimeter (Varian, Mulgrave, Victoria, Australia) with a xenon lamp, 488 nm excitation, 5 nm excitation and emission slit widths, 30 nm/min scanning speed, and high sensitivity (800 V PMT voltage). Apparent emission below ~505 nm is spillover from the excitation light. The fluorescence intensity values were normalized to the local maxima at 595 nm (emission from the JC-1 aggregates). The absorbance spectra of the bandpass filters, dual notch filter, and dichroic mirror from the CE-LIF detector were collected using the Biotek Synergy 2 plate reader (xenon lamp, 2.4 nm monochromator bandpass, 1 nm increments) and were converted to percent transmittance. The spectrum of the dichroic mirror was collected by tilting the mirror 45 degrees to the light path (as it is positioned in the CE-LIF detector). The laser lines at 488 nm and 543.5 nm are shown for reference (not real data). See the Experimental section in the main text for a description of the CE-LIF detector filters.
Capillary Preparation.

Capillary Coating with PVA. Fused-silica capillaries were permanently coated with adsorbed PVA by a method adapted from Shen et al.\textsuperscript{4} PVA coatings reduce mitochondrial adsorption to the capillary surface.\textsuperscript{5} Briefly, three capillaries were rinsed with methanol, 1.0 M NaOH, and water for 10 min each by flushing under 10 psi nitrogen pressure. The capillaries were purged and flushed with a 5\% (w/v) PVA solution for 1 h. The PVA solution was heated to 80 °C prior to flushing through the capillaries. The solution was then purged from the capillaries. After purging, the capillaries were heated between two aluminum plates with heating tape applied to one side; temperature was controlled by a variable-voltage power source at 140 °C for 1 h. The PVA flush, N\textsubscript{2} purge, and heating steps were repeated once with the capillaries in the opposite orientation for a more robust coating. Capillaries were stored dry until use. Before use, the inlet and outlet were trimmed and the polyimide coating was burned off at the tips to produce capillaries with a total length of ~40 cm.

Figure S-1-7. Alignment of capillary using Alignflow flow cytometry beads. (A) Signal in red and green channels. Red channel y offset = 1, n = 719 coincident events. Fluorescence intensity was much higher in the red channel than in the green channel (green channel is pictured but difficult to see as shown in the plot). (B) Detail from 0 to 50 s, red channel. Y-axis shown with different scale than in A. Average event fluorescence intensity %RSD = 18\%. (C) Detail from 0 to 50 s, green channel. Y-axis shown with different scale than in A. Average event fluorescence intensity %RSD = 35\%. Average event red/green %RSD = 14\%. See experimental section in the main text for CE-LIF conditions.
S-1-7. Data Analysis.

False Positives. In addition to mitochondrial events, some of the detected events may correspond to false positives. A particular concern is the detection of JC-1 aggregates which may be present outside mitochondria. Although these aggregates should migrate in the opposite direction of the detector due to their positive charge (so no JC-1 aggregates should be detected), a blank injection was performed to measure the false positive rate. A 200 nM solution of JC-1 was injected and the number of detected coincident events was compared to the average number of events detected in four injections of mitochondria from liver tissue (see Figure S-1-8). This concentration of JC-1 was chosen to match the concentration that is present in the mitochondrial samples after dilution with buffer SH₅ for injection. The number of false positives detected was calculated as less than 5% of the total number of detected coincident mitochondrial events. An additional way to evaluate false positives is to count the number of events detected in the pre-migration window in each electropherogram (0 to 200 s). This analysis results in an average false-positive rate of 0.1% (see Table S-1-2 below).

**Figure S-1-8.** Control for false positives in CE-LIF. (A) Blank injection of 200 nM JC-1 with 0.5 nM fluorescein (peak at 423 s). The concentration of JC-1 was chosen to match the concentration that is present in the mitochondrial samples after dilution with buffer SH₅ for injection. After peak picks and coincidence analysis (see “Data Analysis” in the Experimental section in the main text), 6 events were detected in this run. (B) In four runs containing liver mitochondria (including two depolarized controls), there were 701 coincident events, for an average of 175 events per run. Mitochondrial protein content of injected samples was 3.9 ng, as determined by the BCA assay. Since the CE-LIF run of the blank injection contained 6 events, this results in a false positive rate of 3.4%. All plots: green channel y-offset = 5. CE-LIF conditions as in Figure 1 in the main text.

| Sample | Total events | Cells | Muscle | Liver |
|--------|--------------|-------|--------|-------|
|        |              | Muscle | Liver  |       |
| Mitochondria | 385 | 287 | 278 | 318 | 275 | 155 | 347 | 196 | 204 | 190 | 130 | 171 |
| Pre-migration events | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| False positive rate (%) | 0.2 | 0 | 0 | 0 | 0.3 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0 | 0.6 |

**Table S-1-2.** False positive rate based on number of events detected in pre-migration window.
**Peak Overlap.** An issue in individual organelle analysis by CE is whether peak overlap is a problem, i.e., whether most observed peaks represent individual or multiple comigrating components. Statistical overlap theory has been applied to this problem to determine a threshold for peak saturation, below which the number of observed events is a good estimate for the number of actual events, and so the effect of peak overlap on the observed distribution is minimal. This statistical test has been applied to each mitochondrial CE-LIF run and it has been determined that peak overlap is not a significant problem (see Table S-1-3).

**Table S-1-3.** Peak overlap results for a CE-LIF experiment of mitochondria from cultured cells.

| CE-LIF run | 1   | 2   | 3   | 4   | 5   | 6   |
|------------|-----|-----|-----|-----|-----|-----|
| $p_{\text{tot}}$ (total events) | 385 | 287 | 278 | 318 | 287 | 301 |
| N (number of bins) | 10 | 9 | 9 | 10 | 9 | 9 |
| $\sigma$ (event st dev, s) | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 | 0.009 |
| X (bin width, s) | 23.9 | 19.4 | 20.8 | 23.6 | 24.1 | 21.6 |
| $s = \log(\sigma/X)_t$ | -3.42 | -3.33 | -3.36 | -3.41 | -3.42 | -3.37 |
| n (events in most crowded bin) | 90 | 61 | 76 | 91 | 83 | 74 |

| $\beta_t = 0.85$ |  |
| m (threshold number of events) | 105 | 92 | 96 | 104 | 105 | 99 |
| is overlap a problem at $\beta_t = 0.85$? | No. | No. | No. | No. | No. | |

| $\beta_t = 0.90$ |  |
| m (threshold number of events) | 87 | 77 | 80 | 86 | 88 | 82 |
| is overlap a problem at $\beta_t = 0.90$? | Yes. | No. | No. | Yes. | No. | No. |

For all runs shown in Table S-1-3 above (except for two), the number of events in the most crowded bin is smaller than $m$ at both $\beta_t = 0.85$ and $\beta_t = 0.90$, indicating that peak overlap is not a problem in these runs. For the two runs in which $n$ is larger than $m$, this is only true for $\beta_t = 0.90$, and $n$ exceeds $m$ by only 3 and 5 events in each case. Results from other mitochondrial CE-LIF experiments are not shown, but peak overlap was determined not to be a significant problem in these experiments (i.e. $p_{\text{tot}} = 275, 155, 347,$ and 196 for runs of mitochondria from muscle and 204, 190, 130, and 171 for runs of mitochondria from liver). In the run containing 347 events, $n$ was larger than $m$ at $\beta_t = 0.90$, but only exceeded $m$ by 12 events and was not larger at $\beta_t = 0.85$. 
**Table S-1-4.** Fits to red vs. green data using different models.

| Fit                      | $A$          | $B$          | $C$          | red. $\chi^2$ | ss$_{res}$ | adj. $R^2$ |
|--------------------------|--------------|--------------|--------------|---------------|------------|------------|
| linear                   | c$^c$        | 0.01 ± 0.02 (200%)$^b$ | 1.31 ± 0.04 (3%) | 0.52$^c$ | 970        | 0.37       |
| $y = A + Bx$             | m            | 0.08 ± 0.01 (10%) | 1.08 ± 0.01 (1%) | 0.15 | 149        | 0.89       |
|                          | l            | 0.03 ± 0.02 (70%) | 1.03 ± 0.01 (1%) | 0.22 | 154        | 0.89       |
| polynomial, 2nd order    | c            | 0.09 ± 0.02 (20%) | 0.9 ± 0.07 (8%) | 0.16 ± 0.02 (10%) | 0.51 | 948        | 0.38       |
| $y = A + Bx + Cx^2$      | m            | 0.04 ± 0.01 (30%) | 1.28 ± 0.03 (2%) | -0.03 ± 0.01 (30%) | 0.15 | 142        | 0.90       |
|                          | l            | -0.05 ± 0.02 (40%) | 1.25 ± 0.03 (2%) | -0.04 ± 0.01 (30%) | 0.21 | 144        | 0.90       |
| exponential              | c            | -4.8 ± 1.0 (20%) | 4.9 ± 1.0 (20%) | 0.20 ± 0.03 (20%) | 0.51 | 949        | 0.38       |
| $y = A + Be^{cx}$        | m$^c$        | -9.9 ± 1.7 (20%) | 10.0 ± 1.6 (20%) | 0.08 ± 0.01 (10%) | 0.18 | 177        | 0.88       |
|                          | l            | 16 ± 2 (10%)    | -16 ± 2 (10%)   | -0.08 ± 0.01 (10%) | 0.21 | 145        | 0.90       |
| power                    | c            | 1.27 ± 0.02 (2%) |              |                | 0.53 | 988        | 0.36       |
| $y = x^{A}$              | m            | 1.04 ± 0.01 (1%) |              |                | 0.17 | 164        | 0.89       |
|                          | l            | 1.01 ± 0.01 (1%) |              |                | 0.23 | 157        | 0.89       |
| logarithm                | c            | -1000 ± 5000 (500%)$^b$ | -200 ± 900 (500%)$^b$ | 100 ± 700 (700%)$^b$ | 0.52 | 971        | 0.37       |
| $y = A - B \ln(x + C)$   | m            | -40 ± 10 (30%)  | -17 ± 3 (20%)  | 13 ± 2 (20%)   | 0.15 | 143        | 0.90       |
|                          | l            | -32 ± 9 (30%)   | -14 ± 2 (20%)  | 11 ± 2 (20%)   | 0.21 | 145        | 0.90       |
| sigmoidal                | c            | 7.0 ± 0.4 (6%)  | 1.47 ± 0.06 (4%) | 2.27 ± 0.09 (4%) | 0.53 | 976        | 0.36       |
| $y = \frac{A}{1 + e^{-B(x-C)}}$ | m            | 6.38 ± 0.09 (1%) | 1.27 ± 0.03 (2%) | 2.52 ± 0.05 (2%) | 0.18 | 175        | 0.88       |
|                          | l            | 6.3 ± 0.1 (2%)  | 1.07 ± 0.03 (3%) | 2.81 ± 0.06 (2%) | 0.26 | 177        | 0.87       |

Reduced chi-square (red. $\chi^2$), sum of squares of residuals (ss$_{res}$), and adjusted coefficient of variance (adj. $R^2$) are shown to evaluate goodness of fit.

Uncertainty values shown for each coefficient are standard error of the mean with relative standard deviation shown in parenthesis. The relative standard deviation was lowest for the linear model ($B$ coefficient) and for the power model ($A$ coefficient).

- “c” = cells, “m” = muscle, “l” = liver
- Could not produce a fit in which error was smaller than the coefficient.
- To produce a fit which resulted in error smaller than the coefficients, lower and upper bounds of -10 and 10 were used for coefficients $A$ and $B$. 
Figure S-1-9. Optimization of ROIs. The slope of a line defining the ROIs was varied and the difference in the percentage of events in the polarized ROI of samples compared to the depolarized control was maximized. (A) Cells; optimal slope was 0.96. This sample had 8% more events in the polarized ROI compared to the number of events in the polarized ROI from the depolarized control. (B) Muscle; optimal slope was 1.48. This sample had 38% more events in the polarized ROI compared to the number of events in the polarized ROI from the depolarized control. (C) Liver; optimal slope was 1.47. This sample had 9% more events in the polarized ROI compared to the number of events in the polarized ROI from the depolarized control. For direct comparison between liver and muscle samples, an average value of 1.475 was used to define ROIs in both of these samples. CE-LIF conditions as described in Figure 1 in the main text.
Table S-1-5. Number of events in all runs and ROIs of each sample type.

| Sample       | n  | n, pol. ROI | %, pol. ROI | n, depol. ROI | %, depol. ROI | % difference in n in polarized ROI |
|--------------|----|-------------|-------------|--------------|---------------|-----------------------------------|
| cells        | 950| 501         | 53%         | 449          | 47%           | 8%                                |
| cells, d.c.  | 906| 403         | 44%         | 503          | 56%           |                                   |
| muscle       | 622| 306         | 49%         | 316          | 51%           | 38%                               |
| muscle, d.c. | 351| 41          | 12%         | 310          | 88%           |                                   |
| liver        | 334| 61          | 18%         | 273          | 82%           | 8%                                |
| liver, d.c.  | 361| 61          | 10%         | 325          | 90%           |                                   |

a. The percent difference in the number of events in the polarized ROI between samples and depolarized controls (i.e. percent in polarized ROI from sample minus percent in polarized ROI from depolarized control sample) was maximized to define the ROIs.
b. “d.c.” is “depolarized control”

Figure S-1-10. Definition of ROIs. (A) Mitochondrial events from muscle sample with ROIs shown. (B) Detail of A. (C) Mitochondrial events from depolarized control with ROIs shown. D) Detail of C. CE-LIF conditions as described in Figure 1 in the main text.
S-2. Results and Discussion.

S-2-1. Validation of JC-1 as Membrane Potential Indicator.

Figure S-2-1. Retention of membrane potential-sensitive dyes in isolated mitochondria. Mitochondria were isolated from C2C12 cells (as described in section S.2 in the Supporting Information) and energized in buffer Rs or Rval (depolarized control) for 10 min at 37 °C. Aliquots were labeled with 1 µM JC-1, 1 nM rhodamine 123 (R123, Life Technologies), or 5 nM tetramethylrhodamine, methyl ester (TMRM, Life Technologies) for 10 min at 37 °C. Half of each aliquot was loaded into a well plate immediately after labeling with the dye present in solution and fluorescence intensity was acquired using the plate reader using different sensitivity settings for each dye. The other half of each aliquot was centrifuged at 10000 g for 10 min, the supernatant was discarded (to remove the free dye in solution), and the mitochondrial pellet was resuspended in buffer Rs or Rval (depolarized controls). These samples were then loaded into a well plate and fluorescence intensity was recorded. Data from these samples was acquired 28.5 min after the initial data was acquired, a time scale comparable to the amount of time required for a CE-LIF run. For JC-1, red/green ratio was measured (red channel: λex = 530 ± 12.5 nm, λem = 590 ± 17.5 nm; green channel: λex = 485 ± 10 nm, λem = 528 ± 10 nm). R123 was detected in the green channel and TMRM was detected in the red channel. All fluorescence intensities and red/green ratios were normalized to the highest value among data of a given dye type.
Figure S-2-2. Effect of depolarizers on the fluorescent properties of JC-1 in solution. Solutions of JC-1 at the indicated concentrations containing either 2 μM valinomycin or 50 μM CCCP were compared to controls (i.e. solutions without the depolarizer present). The effect on red/green ratio was much more pronounced with CCCP; consequently, valinomycin was used to depolarize mitochondria in all subsequent experiments to minimize this effect. Solutions containing valinomycin were prepared in buffer R_s, solutions containing CCCP were prepared in buffer C (plus an equivalent amount of DMSO). Plate reader settings as described in Figure 4 in the main text.
Figure S-2-3. Distributions of red/green ratios (A) and corrected electrophoretic mobility (B) from individual runs of mitochondria isolated from C2C12 cells, n = 385, 287, and 278 detected events for runs 1, 2, and 3, respectively. See Figure 2 in the main text for combined distributions and Q-Q plots. CE-LIF conditions as described in Figure 1 in the main text.
Figure S-2-4. Reproducibility of red/green ratios in multiple CE-LIF runs of depolarized control mitochondria isolated from C2C12 cells. (A) Distributions of red/green ratios in three replicate runs of depolarized control mitochondria. n = 318, 287, and 301 detected events for runs 1, 2, and 3, respectively. (B) Distribution of red/green ratios from the combined runs. (C) Q-Q plot of individual runs vs. combined data. This plot is a qualitative indication that the distributions of red/green ratios are reproducible (i.e. data points closely follow the $y = x$ line shown on the plot). There is much more run-to-run variation in the 90-95th percentiles (inset in Q-Q plot in C, normalized $ss_{res} = 509\%, 291\%,$ and $605\%$ for runs 1, 2, and 3, respectively), but the 5-85th percentiles are reproducible (normalized $ss_{res} = 23\%, 6\%,$ and $20\%$ for runs 1, 2, and 3, respectively). CE-LIF conditions as described in Figure 1 in the main text.
Figure S-2-5. Reproducibility of corrected electrophoretic mobility distributions in multiple CE-LIF runs of depolarized control mitochondria isolated from C2C12 cells. (A) Distributions of corrected mobility in three replicate runs of depolarized control mitochondria. n = 318, 287, and 301 detected events for runs 1, 2, and 3, respectively. (B) Distribution of corrected mobility from the combined runs. (C) Q-Q plot of individual runs vs. combined data (x-axis: “corr. mob., depol., combined” is the corrected mobility quantiles of all runs of the depolarized control sample combined; y-axis: “corr. mob., depol., ind. runs” is the corrected mobility quantiles of individual runs of the depolarized control sample). This plot is a qualitative indication that the distributions of corrected mobility are reproducible (i.e. data points closely follow the y = x line shown on the plot, normalized ssres = 4%, 5%, and 5% for runs 1, 2, and 3, respectively). CE-LIF conditions as described in Figure 1 in the main text.
Figure S-2-6. Reproducibility in ROIs of multiple CE-LIF runs using mitochondria isolated from C2C12 cells. Shaded markers (■, ▲, ♦) denote runs of individual samples and ×, −, + denote runs of depolarized controls. (A) Red/green ratio quantiles of polarized ROIs from individual runs of samples and depolarized ROIs from individual runs of depolarized controls vs. combined data from polarized ROIs of samples. Distributions of red/green ratios from depolarized ROIs from individual runs (data points close to x-axis) appear reproducible. (B) Corrected mobility quantiles of polarized ROIs from individual runs of samples and depolarized ROIs from individual runs of depolarized controls vs. combined data from polarized ROIs of samples. Axis labels: “corr. mob., all pol. ROIs” is “corrected mobility, all polarized ROIs combined” and “corr. mob., ind. ROIs” is “corrected mobility from individual ROIs.” The y = x line is shown on each plot for comparison purposes. See Table S-2-1 for normalized \( s_{res} \) for each run. CE-LIF conditions as described in Figure 1 in the main text.
Table S-2-1. Sum of squares of residuals (ss\textsubscript{res}) and normalized ss\textsubscript{res} from all Q-Q plots shown in the main text and supporting information.

| Figure | Sample | Data | Comparison | Quantiles | ss\textsubscript{res} | Norm. ss\textsubscript{res} |
|--------|--------|------|------------|-----------|-----------------|---------------------|
| 2 B    | cells  | red/green | run 1 vs combined runs | all       | 12.2            | 353%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.1            | 26%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 12.1           | 352%                 |
| 2 B    | cells  | red/green | run 2 vs combined runs | all       | 4.8             | 221%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.03           | 18%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 4.8            | 221%                 |
| 2 B    | cells  | red/green | run 3 vs combined runs | all       | 13.5            | 371%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.02           | 13%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 13.4           | 370%                 |
| 2 D    | cells  | mobility | run 1 vs combined runs | all       | 7.28×10\textsuperscript{-10} | 7%       |
|        |        |        | run 2 vs combined runs | all       | 7.65×10\textsuperscript{-10} | 7%       |
|        |        |        | run 3 vs combined runs | all       | 3.50×10\textsuperscript{-10} | 5%       |
| S-2-4 C| cells  | red/green | run 1 vs combined runs, d.c. | all       | 21.0            | 510%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.04           | 23%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 21.0           | 509%                 |
| S-2-4 C| cells  | red/green | run 2 vs combined runs, d.c. | all       | 6.9             | 291%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.003          | 6%                   |
|        |        |        |            | 90-95\textsuperscript{th} | 6.8            | 291%                 |
| S-2-4 C| cells  | red/green | run 3 vs combined runs, d.c. | all       | 29.7            | 606%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.03           | 20%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 29.6           | 605%                 |
| S-2-5 C| cells  | mobility | run 1 vs combined runs, d.c. | all       | 2.16×10\textsuperscript{-10} | 4%       |
|        |        |        | run 2 vs combined runs, d.c. | all       | 3.33×10\textsuperscript{-10} | 5%       |
|        |        |        | run 3 vs combined runs, d.c. | all       | 4.29×10\textsuperscript{-10} | 5%       |
| 5 B    | cells  | red/green | d.c. vs overall sample | all       | 4.3             | 210%                |
|        |        |        |            | 5-85\textsuperscript{th} | 0.1            | 24%                  |
|        |        |        |            | 90-95\textsuperscript{th} | 4.3            | 208%                 |
| 5 B    | cells  | red/green | depol. ROI vs pol. ROI | all       | 314.2           | 1444%               |
|        |        |        |            | 5-85\textsuperscript{th} | 13.9           | 304%                 |
|        |        |        |            | 90-95\textsuperscript{th} | 300.2          | 1412%                |
| 5 D    | cells  | mobility | d.c. vs overall sample | all       | 5.30×10\textsuperscript{-10} | 6%       |
| 5 D    | cells  | mobility | depol. ROI vs pol. ROI | all       | 1.83×10\textsuperscript{-8} | 37%      |

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| Sample | Type | Region | Condition | ROI Comparison | All | p-value |
|--------|------|--------|-----------|----------------|-----|---------|
| S-2-6 A | cells | red/green | pol. ROI from run 1 vs combined pol. ROIs | all | 3.7 | 156% |
|        |      |         | pol. ROI from run 2 vs combined pol. ROIs | all | 3.3 | 148% |
|        |      |         | pol. ROI from run 3 vs combined pol. ROIs | all | 12.4 | 287% |
|        |      |         | depol. ROI from d.c. run 1 vs combined pol. ROIs | all | 314.3 | 1444% |
|        |      |         | depol. ROI from d.c. run 2 vs combined pol. ROIs | all | 313.6 | 1443% |
|        |      |         | depol. ROI from d.c. run 3 vs combined pol. ROIs | all | 314.7 | 1446% |
| S-2-6 B | cells | mobility | pol. ROI from run 1 vs combined pol. ROIs | all | $1.29 \times 10^{-9}$ | 10% |
|        |      |         | pol. ROI from run 2 vs combined pol. ROIs | all | $1.29 \times 10^{-9}$ | 10% |
|        |      |         | pol. ROI from run 3 vs combined pol. ROIs | all | $1.50 \times 10^{-9}$ | 10% |
|        |      |         | depol. ROI from d.c. run 1 vs combined pol. ROIs | all | $2.48 \times 10^{-8}$ | 43% |
|        |      |         | depol. ROI from d.c. run 2 vs combined pol. ROIs | all | $1.30 \times 10^{-8}$ | 31% |
|        |      |         | depol. ROI from d.c. run 3 vs combined pol. ROIs | all | $1.80 \times 10^{-8}$ | 36% |
| S-2-10 B | muscle | red/green | d.c. vs overall sample | all | 58.4 | 529% |
|        |      |         | depol. ROI vs pol. ROI | all | 203.5 | 501% |
| S-2-10 B | liver | red/green | d.c. vs overall sample | all | 1.7 | 139% |
|        |      |         | depol. ROI vs pol. ROI | all | 70.1 | 447% |
| 6 C | muscle | mobility | d.c. vs overall sample | all | $1.07 \times 10^{-8}$ | 34% |
|        |      |         | depol. ROI vs pol. ROI | all | $2.14 \times 10^{-8}$ | 49% |
| 6 C | liver | mobility | d.c. vs overall sample | all | $1.50 \times 10^{-8}$ | 37% |
|        |      |         | depol. ROI vs pol. ROI | all | $1.78 \times 10^{-8}$ | 47% |

a. The normalized $ss_{res}$ is the square root of the $ss_{res}$ divided by the median of the data from the x-axis, reported as a percentage: **normalized** $ss_{res} = \sqrt{ss_{res}/median} \times 100%$

b. Abbreviations: “d.c.” is “depolarized control,” “pol.” is “polarized” and “depol.” is “depolarized.”
**Figure S-2-7.** Response of plate reader to changes in JC-1 concentration and amount of mitochondria. Red/green ratio, green, and red fluorescence of solutions of JC-1 in buffer Rs (A-C) and mitochondria at different dilutions (D-F). Some precipitation of red aggregates was observed in the 2 µM JC-1 solution. In (G-I), red/green ratio and fluorescence is normalized to the undiluted, original mitochondrial sample. Mitochondria were isolated from L6 cells (as described in section S.2 in the Supporting Information) and energized in buffer Rs (with 10 mM succinate and 10 µM rotenone) for 30 min at 37 °C. Samples were labeled with 1 µM JC-1 for 5 min at 37 °C and then diluted (percentages shown are relative to the amount of mitochondria in the original sample) with buffer Rs. Red channel: λ<sub>ex</sub> = 485 ± 10 nm, λ<sub>em</sub> = 590 ± 17.5 nm; green channel: λ<sub>ex</sub> = 485 ± 10 nm, λ<sub>em</sub> = 528 ± 10 nm.
S-2-4. Region of Interest (ROI) Analysis.

**Figure S-2-8.** Mirror comparison of red/green ratio distributions between sample and depolarized control (overall distributions) using mitochondria isolated from C2C12 cells. White bars represent data from depolarized controls. \( n = 950 \) events, 3 runs. Depolarized control: \( n = 906 \) events, 3 runs. See Figure 5 in the main text for Q-Q plot comparisons of these distributions. CE-LIF conditions as in Figure 1 in the main text.

S-2-5. Dependence of Electrophoretic Mobility on Membrane Potential.

**Figure S-2-9.** Mirror comparison of corrected electrophoretic mobility distributions between sample and depolarized control (overall distributions) using mitochondria isolated from C2C12 cells. White bars represent data from depolarized controls. \( n = 950 \) events, 3 runs. Depolarized control: \( n = 906 \) events, 3 runs. See Figure 5 in the main text for Q-Q plot comparisons of these distributions. CE-LIF conditions as in Figure 1 in the main text.
**S-2-6. Application to Liver and Muscle Tissue Mitochondria.**

**Figure S-2-10.** Red/green ratios determined by CE-LIF of mitochondria from muscle and liver tissue. White bars represent data from depolarized controls. (A) Distributions. (B) Q-Q plots. Comparisons are made between samples and depolarized controls (all events) and between events in polarized ROIs and events in depolarized ROIs from depolarized controls. The different ranges used for $x$ and $y$ axes in B causes the slope of the $y = x$ line to appear not equal to $45^\circ$. See Table S-2-1 for the normalized $ss_{res}$ for the Q-Q plot. See Table S-1-5 for the number of events. CE-LIF conditions as in Figure 1 in the main text.

**Figure S-2-11.** Mirror distributions of corrected electrophoretic mobility determined by CE-LIF of mitochondria from muscle and liver tissue. White bars represent data from depolarized controls. Comparisons are made between samples and depolarized controls (all events) and between events in polarized ROIs and events in depolarized ROIs from depolarized controls. See Figure 6 in the main text for Q-Q plot comparisons of these distributions. See Table S-1-5 for number of events. CE-LIF conditions as in Figure 1 in the main text.
References

(1) Kostal, V.; Fonslow, B. R.; Arriaga, E. A.; Bowser, M. T. *Anal Chem* **2009**, *81*, 9267-9273.
(2) Frezza, C.; Cipolat, S.; Scorrano, L. *Nat Protoc* **2007**, *2*, 287-295.
(3) Labajova, A.; Vojtiskova, A.; Krivakova, P.; Kofranek, J.; Drahota, Z.; Houstek, J. *Anal Biochem* **2006**, *353*, 37-42.
(4) Shen, Y. F.; Smith, R. D. *J Microcolumn Sep* **2000**, *12*, 135-141.
(5) Whiting, C. E.; Arriaga, E. A. *Electrophoresis* **2006**, *27*, 4523-4531.