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Capillary electrophoretic analysis reveals subcellular binding between individual mitochondria and cytoskeleton

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Abstract

Interactions between the cytoskeleton and mitochondria are essential for normal cellular function. An assessment of such interactions is commonly based on bulk analysis of mitochondrial and cytoskeletal markers present in a given sample, which assumes complete binding between these two organelle types. Such measurements are biased because they rarely account for non-bound 'free' subcellular species. Here we report on the use of capillary electrophoresis with dual laser induced fluorescence detection (CE-LIF) to identify, classify, count and quantify properties of individual binding events of mitochondria and cytoskeleton. Mitochondria were fluorescently labeled with DsRed2 while F-actin, a major cytoskeletal component, was fluorescently labeled with Alexa488-phalloidin. In a typical subcellular fraction of L6 myoblasts, 79% of mitochondrial events did not have detectable levels of F-actin, while the rest had on average ~2 zeptomole F-actin, which theoretically represents a ~ 2.5- μ m long network of actin filaments per event. Trypsin treatment of L6 subcellular fractions prior to analysis decreased the fraction of mitochondrial events with detectable levels of F-actin, which is expected from digestion of cytoskeletal proteins on the surface of mitochondria. The electrophoretic mobility distributions of the individual events were also used to further distinguish between cytoskeleton-bound from cytoskeleton-free mitochondrial events. The CE-LIF approach described here could be further developed to explore cytoskeleton interactions with other subcellular structures, the effects of cytoskeleton destabilizing drugs, and the progression of viral infections.

Keywords

mitochondria; cytoskeleton; phalloidin; DsRed; capillary electrophoresis; fluorescence; subcellular

INTRODUCTION

Interactions between the cytoskeletal network and other organelles (i.e., nuclei,¹ Golgi,² lysosomes³, mitochondria⁴) are essential to cell function. In the case of mitochondria, these interactions appear to play critical roles in mitochondrial biogenesis,⁵ localization and morphology.⁶ These interactions are also responsible for keeping mitochondria and cytoskeleton tightly bound after isolation of subcellular fractions, which is beneficial when investigating subcellular interactions, but detrimental when preparing a subcellular fraction devoid from cytoskeletal impurities.⁷

The abundances of cytoskeleton and mitochondria in a highly purified bulk subcellular fraction are a surrogate of the interactions between these two subcellular structures. Unfortunately, the methods used (e.g., Western blots and ELISAs)⁸ are not adequate to

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distinguish between 'free' and bound subcellular structures that are co-isolated during the subcellular fractionation procedure. Fluorescence⁹ and electron¹⁰ microscopies are commonly used to observe mitochondria-cytoskeleton interactions. Fluorescence microscopy is limited by its spatial resolution. Electron microscopy is tedious and impractical for the analysis of a large number of binding events.

We previously demonstrated that capillary electrophoresis with post-column laser induced fluorescence detection (CE-LIF) is an excellent tool for assessing the properties of individual subcellular particles,¹¹ such as mitochondria¹² or cytoskeletal aggregates.¹³ The potential of CE-LIF to describe binding between two subcellular structures has not been explored. Here we expand on the use of individual organelle CE-LIF to describe subcellular binding between cytoskeleton and mitochondria. We fluorescently labeled mitochondria with DsRed2 targeting the mitochondria matrix¹⁴ and F-actin (a cytoskeletal protein) with Alexa488-phalloidin (A488-PHD).¹⁵ Individual mitochondria from L6 myoblasts were easily classified as cytoskeleton-bound or cytoskeleton-free. The CE-LIF method measured changes F-actin levels of individual mitochondria before and after treatment with trypsin, an enzyme known to remove cytoskeletal proteins from the surface of mitochondria.¹⁶ This method also provides electrophoretic mobility distributions of individual organelles, which support that cytoskeleton-bound and cytoskeleton-free mitochondria have distinct surface properties. Future applications of this CE-LIF method could include evaluation of cytoskeletal destabilizing drug treatments, quantitation of subcellular interactions in chemotaxis, and monitoring of the progression of viral infections.

EXPERIMENTAL SECTION

Chemicals and reagents

Sucrose was purchased from Roche (Indianapolis, IN). Digitonin was obtained from Calbiochem (San Diego, CA). Ethanol and DMSO were purchased from Fisher Scientific (Pittsburgh, PA). D-mannitol was purchased from Riedel de-Haën (Seelze, Germany). Trypsin solution (10×, 5 g/L trypsin, 2 g/L EDTA·4 Na, 8.5 g/L NaCl), N-(2-hydroxyethyl) (piperazine)-N-(ethanesulfonic acid) (HEPES), phosphate-buffered saline (10× PBS, containing 100 mM KH₂PO₄/Na₂HPO₄ solution, pH 7.4, 27 mM KCl, 1370 mM NaCl), potassium hydroxide (KOH), hydrochloric acid (HCl), formaldehyde, poly-L-lysine, Triton X-100 and Trypan Blue were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM), OPTI-MEM reduced-serum medium, Geneticin, fetal calf serum, lipofection reagent DMRIE-C, ProLong® anti-fade reagent conjugated with DAPI, fluorescein and Alexa488 Phalloidin were purchased from Invitrogen (Carlsbad, CA). Stock solutions of 1 mM fluorescein were made in ethanol. The stock solution of 100 g/L digitonin was prepared in DMSO and diluted to 10 mg/mL in mitochondria isolation buffer immediately before using.

Buffers

Mitochondria isolation buffer (buffer M) contained 70 mM sucrose, 5 mM HEPES, 5 mM EDTA and 210 mM mannitol. Capillary electrophoresis buffer (CE buffer) contained 250 mM sucrose and 10 mM HEPES. All buffers were prepared in Milli-Q water, filtered with a 0.22 µm membrane filter and adjusted to pH 7.4 with 1M KOH.

Cell culture

Adherent L6 rat myoblasts were grown in DMEM medium supplemented with 10% (v/v) fetal calf serum (FCS), 50 µg/mL gentamicin at 37 °C and 5% CO₂ in 75 cm² vented flasks. Cells were split every 3–4 days before they reached confluence. For splitting, cells were

rinsed with PBS, lifted with 5 mL of 0.25 g/L trypsin for 5 min and diluted in fresh medium in 1:20–1:40 ratio.

Stable transfection of cells with DsRed2-mito

Cells were transfected with a plasmid harboring genes responsible for neomycin/kanamycin resistance and expression of mito-DsRed2, a fusion protein that targets mitochondria to release the red fluorescent protein DsRed2 into the mitochondrial matrix (Clontech, Mountain View, CA). Transfections were performed using the lipofection reagent DMRIE-C according to the manufacturer's instructions. Briefly, DNA plasmid and DMRIE-C were resuspended separately in OPTI-MEM medium to form a DNA-lipid complex. After a 5-minute incubation both solutions were mixed together and incubated for 30 minutes. The cells were washed with the OPTI-MEM and the DNA-lipid complex was layered on the cells. After 6 hours, serum-enriched medium (DMEM+20% FBS) was added. After 24 hours, cells were lifted, seeded into a T25 culture flask and cultured in the presence of 600 µg/mL geneticin for ~14 days. Cells producing DsRed2 were further enriched using fluorescence-activated cell sorting (FACS Aria, BD BioSciences, San Jose, CA). Only 35% of the most fluorescent cells were gated and collected for further culturing. Approximately 85% of cells retained their DsRed2 marker after 15 days of culturing in DMEM medium containing 200 µg/mL geneticin.

Confocal microscopy

Cells were cultured overnight in DMEM in a 4-well LabTek #1.5 chambered slide (Nunc, Rochester, NY) coated with poly-L-lysine. Cells were fixed with 3.7% formaldehyde in DMEM for 15 min, washed three times with PBS and permeabilized with 0.1% Triton X-100. After another wash with PBS, cells were stained with 165 nM A488-PHD (1:200 dilution of the original solution) for 25 min at room temperature in the dark. Lastly, cells were washed three times with PBS and mounted using the ProLong® Anti-Fade reagent with DAPI (Invitrogen) and imaged.

Confocal fluorescence images were acquired using an Olympus IX-81 inverted microscope equipped with a 120-W mercury lamp, a DS-IX100 disk spinning unit and a C9100-01 EM CCD camera (Hamamatsu corp., Bridgewater, NJ). All images were captured with a 60× oil immersion objective (N.A.=1.45). Alexa488-PHD, DsRed2 and DAPI were imaged using FITC (ex. 460 – 500 nm, 505 nm dichroic, em. 510 – 560 nm), TRITC (ex. 510 – 560 nm, 565 nm dichroic, em. 570 – 650 nm) and DAPI (ex. 325 – 375 nm, 460 nm dichroic, em. 470 – 750 nm) filter cubes (Chroma, Rockingham, VT), respectively. Images were collected and analyzed using Simple PCI 5.3 software (Hamamatsu Corp., Sewickley, PA). Z-stacks of confocal images were deconvoluted with the Huygens software (Scientific Volume Imaging, Hilversum, The Netherlands) and displayed as their maximum fluorescence projection.

Mitochondria preparation and A488-PHD labeling

All operations during mitochondria sample preparations were performed on ice. Cells were harvested using 5 mL of 0.25 g/L trypsin and washed three times with cold buffer M, counted using a Fuchs-Rosenthal hemocytometer (Hausser Scientific, Horsham, PA) and diluted to 5.0×10^6 cells/mL with buffer M. A 1-mL aliquot of cell suspension was mixed with 10 µL of 10 mg/L digitonin and incubated for 5 min. After permeabilization, cells were gently disrupted in a Dounce homogenizer with 0.0005–0.0025 in. clearance (Kontes, Vinland, NJ) until Trypan Blue staining revealed that approximately 90% of cells were disrupted (~20 – 30 strokes). Subcellular fractions were prepared by differential centrifugation. Briefly, cell lysate was centrifuged at $600 \times g$ for 10 min to eliminate intact cells, cell debris and the nuclear fraction. A mitochondria-enriched pellet was obtained by

centrifugation at $12,000 \times g$ for 10 min and then resuspended in buffer M. Aliquots of this mitochondrial-enriched preparation was pipetted into three separate vials and used (i) as control, (ii) for direct staining with A488-PHD or (iii) for staining with A488-PHD after trypsin treatment (0.75 mg/mL for 1h at 37 °C). For A488-PHD staining, the treatment was at 165 nM (1:200 dilution of the A488-PHD original reagent solution) for 20 minutes at 4 °C in the dark. After staining, mitochondria were washed twice with buffer M using differential centrifugation and then resuspended in the CE buffer.

Bulk assay

An aliquot of 50 μ L of mitochondrial preparation was pipetted into one of the wells of a 96-well plate and the fluorescence corresponding to A488-PHD (exc. 490 nm, em, 520 nm) and DsRed2 (ex. 530 nm, em. 580 nm) resp. were measured using a 96-well plate reader (Biotek, Winooski, VT). The DsRed2 fluorescence was used for quantitation of mitochondrial content in each well. The ratio of the fluorescence intensities of A488-PHD over DsRed2 was used as the metric for comparison among samples. Each sample was read three times in three separate wells (nine readings total).

CE-LIF setup

Capillary electrophoresis experiments were performed using a custom-built CE instrument with post-column dual LIF detection, which has been described previously.¹² The 488-nm line from an argon-ion laser (12 mW, Melles Griot, Irvine, CA) was used as an excitation source. Fluorescence from Alexa488 and DsRed2 were spectrally resolved by the 560-nm long-pass dichroic mirror (DCLP560, Omega filters, Brattleboro, VT, USA), passed through interference filters transmitting in the range 505–535 nm (520DF30, Omega Optical, Brattleboro, VT, USA) and 607.5–662.5 nm (635DF55, Omega Optical), respectively, and detected by two separate photomultiplier tubes (R1477, Hamamatsu Corp., Bridgewater, NJ). The output signal from each photomultiplier tube was digitized at 200 Hz using a NiDAQ I/O board (PCI-MIO-16XE-50, National Instruments, Austin, TX) and stored as separate binary files. The detector was aligned using a continuous injection of 5×10^{-10} M fluorescein in CE buffer at -400 V/cm into the capillary. The limit of detection for fluorescein and A488-PHD for the CE-LIF instrument was ~ 4 and ~ 2 zM, respectively.

All separations were carried out in 42-cm long, 50- μ m i.d., 150- μ m o.d. fused silica capillaries (Polymicro Technologies, Phoenix, AZ) permanently coated with poly-acryloyl-amino propanol. The coating procedure has been published previously.¹⁷ All mitochondrial samples were injected into the capillary using a 2s hydrodynamic injection at 10.1 kPa (corresponding to 3 nL) and separated at -400 V/cm.

Data analysis

Binary data files were analyzed using Igor Pro software (Wavemetrics, Lake Oswego, OR). Dual-trace electropherograms were filtered using a median filter to eliminate broad bands and peaks with $S/N > 5$ were selected for further processing. Intense peaks saturating the detector were excluded ($< 4\%$ of total number of peaks). Identification of coincident peaks in dual trace electropherograms was done using a previously reported procedure in Igor Pro Software.¹⁸ The intensities of peaks in each trace were corrected for spectral cross-talking. Cross-talking from the A488-PHD signal to the DsRed2 signal and vice versa were 3% and 9%, respectively. The peaks in the pre-migration window (0 – 200 s) of both traces were analyzed using the same procedure in order to estimate the carryover from the previous run. The carryover was $4 \pm 2\%$ and was considered as a bias in the measurement of events in the migration window.

The number of F-actin molecules per event (n_a) was calculated from the peak height (h) using the expression:

$$n_a = (s \times R \times e) \times h \quad (\text{Eq. 1})$$

where s is the sensitivity of the detector determined from the injection of the A488-PHD in the same separation buffer ($s = 5.02 \times 10^{-20}$, mole/area-unit), R is the average area to peak height ratio ($R = 0.0192$, area-unit/V) of individually detected mitochondria and e is the detector efficiency ($e = 0.88$).¹⁹ This calculation assumes quantitative and stoichiometric (1:1) binding between A488-PHD and F-actin.²⁰

The electrophoretic mobility of each detected (μ_i) event was calculated using the following equation:

$$\mu_i = L^2 / (V \times t_m) + \mu_{\text{EOF}} \quad (\text{Eq. 2})$$

where t_m is the migration time of the detected event, V is the applied voltage, L is the capillary length and μ_{EOF} is the electroosmotic flow. The μ_{EOF} was $(0.85 \pm 0.01) \times 10^{-4} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$ as determined by current monitoring method.²¹

Histograms of the CE mobility were generated by binning individual electrophoretic mobility values into intervals of equal size ($2.5 \times 10^{-5} \text{ cm}^2 \text{V}^{-1} \text{s}^{-1}$) and normalizing the peak number of each bin to the total number of detected peaks.

A potential issue during the CE analysis of particles is the co-migration of multiple particles in the single time domain. To address this issue, we calculated the probability of detecting multiple peaks corresponding to co-migrating mitochondria using the statistical overlap theory (SOT), which was previously modified to be applied to the organelle CE.²² We calculated the critical saturation threshold for the most crowded region (330 – 430 s) of the A488-PHD trace shown in Figure 3A and compared it to the observed number of peaks. The number of detected peaks ($n = 72$) was lower than the peak saturation threshold value ($m = 102$) suggesting the peak overlap was not a significant issue ($\alpha = 0.05$, $\beta_1 = 0.9$).

Quantile-quantile plots (q-q plot)²³ were used to compare distributions of individual electrophoretic mobilities (e.g. cytoskeleton-bound versus cytoskeleton-free mitochondria). Quantiles increasing by 5% (i.e., 5%, 10%, 15%, etc.) of the test and reference distributions were plotted as 'y' and 'x' coordinates, respectively.

For both, CE and bulk data, a two-tailed, unpaired t-test was used to test for statistical differences between the means.

RESULTS AND DISCUSSION

Fluorescence labeling of cytoskeleton and mitochondria

Fluorescence microscopy has been used to investigate mitochondria-cytoskeleton interactions within the cell.⁹ Direct observation of such interactions by fluorescence microscopy is limited by the spatial resolution defined by the diffraction limits at the wavelengths of observation. On the other hand, this technique is suitable to evaluate and select subcellular fluorescent probes. Phalloidins have become an elegant tool for visualizing actin microfilaments. They bind to filamentous F-actin with high affinity ($K_d < 30 \text{ nM}$), and do not bind to other cytoskeletal components such as the monomeric G-actin.²⁴ As an example, labeling with A488-PHD clearly displays the actin cytoskeleton without any delocalized fluorescence (Figure 1a). The DsRed2 label used in these studies is a fusion protein that has a mitochondrial targeting sequence. Upon translocation of this protein into

mitochondria, DsRed2 accumulates in the mitochondrial matrix, rendering these organelles fluorescent (Figure 1B). Matrix localization makes DsRed2 a good indicator of mitochondrial intactness after mitochondrial isolation, because mitochondria with a compromised inner membrane leak out their DsRed2 cargo, becoming undetectable. Furthermore, both A488-PHD and DsRed2 are detected using different excitation/emission settings (Figure 1C) providing the basis for selective detections of cytoskeleton and mitochondria, respectively.

Bulk analysis of cytoskeleton binding to mitochondria

Spectrofluorometry is commonly used to measure subcellular properties in bulk. As such, simultaneous measurements of mitochondrial and cytoskeletal contents could provide an 'index' of interaction between these two subcellular structures. We determined both the A488-PHD and DsRed2 fluorescence intensities of bulk mitochondrial preparations and then obtained the ratio of these fluorescence intensities (Figure 2). Using this procedure, the fluorescence of DsRed2 was used to normalize the A488-PHD fluorescence intensity for the mitochondria content in the sample. As expected, A488-PHD was not detected in control samples (i.e., mitochondrial samples not labeled with A488-PHD), confirming that autofluorescence of mitochondrial samples is not a limiting factor in bulk measurements (Figure 2). On the other hand, the A488-PHD stained sample (PHD in Figure 2) showed a dramatic increase in the overall response.

A test to confirm that cytoskeleton is bound to mitochondria consists of treating the mitochondria sample with trypsin prior to A488-PHD labeling. The overall A488-PHD normalized fluorescence decreased to $8 \pm 2\%$ ($N = 3$) of the fluorescence of mitochondrial preparation treated only with A488-PHD, suggesting that trypsin removed cytoskeletal remnants (i.e., F-actin) bound to mitochondria. Treatment of mitochondria with high concentrations of trypsin or long incubation times may also disrupt mitochondria,²⁵ biasing the results of the test used here. Nevertheless, we did not observe significant differences in DsRed2 fluorescence (i.e., leakage from mitochondria) between samples treated and not treated with trypsin (Supplementary material, Figure S1). Thus, the trypsin treatments used here are adequate to remove cytoskeleton from mitochondria and do not significantly disrupt these organelles.

Detecting binding between cytoskeleton and individual mitochondria using CE-LIF

The spectrofluorometric bulk assay described above is rapid, but cannot distinguish among free A488-PHD, cytoskeleton bound to mitochondria or to other organelles, and cytoskeleton that is co-isolated in the fraction but is not bound to any organelle. Individual organelle CE-LIF can overcome these limitations.

CE with dual LIF detection is suitable to identify and quantify fluorescent compounds within individual mitochondria.¹⁹ Here we demonstrate that this technology is also adequate to characterize binding of mitochondria to other subcellular structures such as the cytoskeleton. Individual mitochondria were identified and detected by virtue of their DsRed2 contents while cytoskeleton (F-actin) was detected and quantified due to the labeling with A488-PHD (Figure 3). Narrow peaks, (43 ± 8 ms wide at the baseline, $n = 27$) correspond to either (i) mitochondria bound by cytoskeleton, i.e. peaks seen by both detectors (* in Figure 3B); (ii) mitochondria without detectable amounts of cytoskeletal F-actin, i.e. peaks that appear only in the DsRed2 trace ($\emptyset$ in Figure 3B); (iii) cytoskeletal aggregates or cytoskeleton bound to organelles other than mitochondria; i.e. peaks that appear only in the A488-PHD trace ($\times$ in Figure 3B). Another advantage of CE-LIF analysis is that excess of free A488-PHD dye appears as a broad peak in the electropherogram (i.e. broad peak at migration time = 872 s, upper trace, Figure 3A). While this peak is completely

electrophoretically separated from the majority of the mitochondria, the remaining overlaps can be resolved using a previously reported algorithm that utilizes a median filter to dissect 'broad' and 'narrow' features in the electropherogram.²⁶ Lastly, the CE-LIF electropherograms show true positive peaks only after ~ 250 s, which correspond to the time required for electromigration of mitochondria from the injection end of the capillary to the dual LIF detector. The features of CE-LIF analysis of particles make it possible to identify, classify, count, and quantify properties of individually detected events.

On average, the analysis of 3 nL of sample, equivalent to the lysate taken from 2 cells, resulted in detection of 260 ± 66 mitochondrial events (Average $\pm$ Std. Dev.; $N = 3$). These mitochondrial events (i.e., events detected due to their DsRed2 contents) were further classified as cytoskeleton-bound (i.e., events showing detection of A488-PHD) and cytoskeleton-free mitochondria. In a control, (i.e., mitochondrial sample not stained with A488-PHD), we found that 11% of mitochondria showed a corresponding green signal in the A488-PHD trace, which likely results from mitochondrial autofluorescence (Table 1). Since spectral cross talking between the detectors is taken into account, these events do not result from cross talking from the DsRed2 to the A488-PHD signal. Since autofluorescence is intrinsic to mitochondria and is influenced by cell status and cell origin, untreated sample controls were needed to assess the rate of false positives in the CE-LIF results. A total of 32% of the mitochondrial events had detectable levels of A488-PHD (Table 1). Thus, taking into account the false positive counts due to autofluorescence, CE-LIF reveals that 21% of mitochondria are binding to cytoskeleton. The remaining mitochondrial events correspond to cytoskeleton-free mitochondria. Cytoskeleton-free mitochondrial events may result from (i) undetectable levels of A488-PHD, (ii) removal of the cytoskeleton during the sample preparation, which is ultimately desired in the preparation of subcellular fractions, or (iii) organelles that were not bound to cytoskeleton in the original intracellular milieu. Particularly interesting would be to extend the CE-LIF analysis to monitor the level of cytoskeleton as cell or tissue homogenization progresses (e.g., sampling from the cell homogenate while increasing the number strokes used in the mechanical homogenization process) or when comparing different homogenization protocols (e.g., nitrogen cavitation versus mechanical homogenization). Another interesting application would be to induce biochemically changes in the binding of mitochondria to the cytoskeleton, (e.g., by depolarizing mitochondria which causes their redistribution from subcellular regions in which they closely associated with actin microfilaments to other regions),²⁷ and then use CE-LIF analysis to monitor binding events between mitochondria and cytoskeleton. These are future applications and are outside the scope of this report.

A dramatic finding of the CE-LIF analysis is the high abundance of events detected only by the A488-PHD detector (i.e., 'Other organelles' in Table 1). These events would contribute to the total A488-PHD fluorescence detected in bulk assays (c.f., Figure 2) dramatically biasing the measurement of interactions between mitochondria and cytoskeleton. While we cannot rule out the possibility that these events represent mitochondria with undetectable levels of DsRed2 or mitochondria that were damaged and leaked DsRed2 during the sample preparation procedure, it is likely that these events are caused by other subcellular structures bound to cytoskeleton or cytoskeleton aggregates. Other organelles, such as lysosomes, nuclei and peroxisomes,¹⁻³ and fragments of outer cell membrane²⁸ may remain bound to cytoskeletal remnants and appear as A488-PHD fluorescent events. In the future, combining A488-PHD labeling with selective labeling of these other subcellular structures with either fluorescent fusion proteins (e.g., DsRed2 targeted to peroxisomes) or chemical probes (e.g., LysoTracker Red) may further reveal the origin of these cytoskeleton binding events.

CE-LIF analysis of individual mitochondria-cytoskeleton binding events in samples treated with trypsin showed a decrease in the number of these events and increase in the number of

cytoskeleton-free mitochondria ('Free mitochondria' in Table 1). In fact, the number of binding events ($16 \pm 10\%$) is close to the one we observed in the control sample ($11 \pm 5\%$). These results are consistent with the trends observed by the spectrofluorometric analysis of bulk preparations (c.f. Figure 2). Furthermore, the similar total number of mitochondrial (DsRed2) peaks in both trypsin-treated and non-treated sample (291 and 311, respectively) confirms that mitochondrial disruption is not significant during the trypsin treatment. Furthermore, F-actin has 34 potential trypsin cleavage sites²⁹ making it highly susceptible to this enzyme's action. Thus, the CE-LIF analysis further confirms that the gentle trypsin treatment used here is capable of 'shaving off' proteins, including actin, from the mitochondrial surface.³⁰

Amounts of F-actin bound to individual mitochondria

We used the A488-PHD fluorescence intensities of the CE-LIF results to calculate to the amount of the cytoskeleton bound to each particle (c.f. Equation 1). The 25%, 50% (median) and 75% quartiles were 0.18×10^{-21} , 0.28×10^{-21} and 1.41×10^{-21} mol F-actin, respectively (PHD box plot in Figure 4). As expected, both control (not PHD labeled) and trypsin treated samples prior to PHD labeling displayed decreased fluorescence. The level of autofluorescence in the control was equivalent to $(0.3 \pm 0.2) \times 10^{-21}$ mol of F-actin per event (average $\pm$ Std. Dev., $n=3$), while the level of fluorescence in trypsin treated sample was $(0.3 \pm 0.3) \times 10^{-21}$ mol of F-actin per event (Figure 4). These average levels were not significantly different ($p = 0.3$). On the other hand, the average for cytoskeleton-bound mitochondrial events was $(1.5 \pm 2.8) \times 10^{-21}$ mol of F-actin, which was significantly different ($p < 0.002$). Thus, the trypsin treatment is effective at decreasing the amounts of cytoskeleton bound to the surface of the mitochondria.

The A488-PHD fluorescence intensities could also be used to estimate the length of the cytoskeletal filaments bound to mitochondria. Within the cell, the actin network is a dense array of short-branched filaments.²⁸ An actin filament is 9 nm in diameter and 1 μm in length and has 370 F-actin molecules.³¹ Since the average amount of F-actin per mitochondrial event reported here is 1.54 zmoles (732 F-actin molecules), the average actin network length is 2.1 μm per event. Using similar calculations, the actin network length per event spans from 191 nm (10% percentile) to 10 μm (90% percentile) with a median of 462 nm.

The comparison of the fluorescence intensities of A488-PHD and DsRed2 of the individual CE-LIF mitochondrial events helps further explore the nature of these events. Besides false positives (autofluorescence) individual cytoskeleton-mitochondria binding events may include both individual mitochondria bound to cytoskeleton and cytoskeleton-tethered mitochondria. Plotting of individual A488-PHD versus DsRed2 fluorescence intensities gives a glimpse into this complex behavior (Supplementary material, Figure S2). While autofluorescence is correlated to DsRed2 levels (Supplementary material, Figure S2, Panel A), those events that deviate positively from this trend (Figure S2, Panel B) are more likely to represent cytoskeleton binding with individual mitochondria (i.e. at low DsRed2 levels) or with several mitochondria (i.e. at high DsRed2 levels). Together, these observations could be very useful in future assessment of cytoskeleton disrupting drugs or quantitative measurement of F-actin in mitochondrial fractions.

Electrophoretic assessment of cytoskeleton-free and cytoskeleton-bound mitochondria

One of the parameters affecting the electrophoretic mobility of individual biological particles (e.g. mitochondria) is their surface charge. The previously reported CE-LIF determination of the electrophoretic mobility profiles of individual mitochondria¹² could be extended to characterize differences between cytoskeleton-bound and -free mitochondria.

Using the migration times of individual events detected in the DsRed2 trace, we calculated the electrophoretic mobilities of individual events. Although all reported mobilities are negative (c.f. Equation 2), in Figure 5 we report them as positive values for clarity. When comparing the cytoskeleton-free mitochondria with and without A488-PHD treatment ('Free PHD' and 'Free Control') we found out that the electrophoretic mobilities were the same (both medians were $-3.3 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$, $p = 0.53$); Figure 5A). Furthermore, we compared the respective mobility distributions using q-q plots.²³ The q-q plot of the cytoskeleton-free mitochondria comparing A488-PHD labeled versus unlabeled samples (i.e., diamonds representing μ_{Free} (PHD) versus μ_{Free} (Control) in Figure 5B) shows that majority of the plot is close to the 45-degree identity distribution line thereby indicating that these two mobility distributions are similar. Thus, both the median values and q-q plot suggest that A488-PHD labeling does not have a significant influence on the mobility of cytoskeleton-free mitochondria.

In contrast, labeling of a sample with A488-PHD showed that the median electrophoretic mobility distribution of cytoskeleton-bound mitochondria ('Cyto-bound PHD' in Figure 5) is slightly less negative with respect to that of cytoskeleton-free mitochondria ('Free PHD' in Figure 5A). The respective values were -3.1 and $-3.3 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$. Furthermore, the q-q plot for this comparison (diamonds representing $\mu_{\text{Cyto-bound}}$ (PHD) versus μ_{Free} (PHD) in Figure 5C) shows that A488-PHD labeling decreases the magnitude of the electrophoretic mobility in 70% of the distribution of cytoskeleton-bound mitochondria relative to the distribution of cytoskeleton-free mitochondria (i.e., there are 14 markers below the 45-degree identity distribution line in Figure 5C). This difference in mobility could result from the combined effects of charge contributions of cytoskeleton and A488-PHD, bound to F-actin upon labeling, to the electrical charge on the mitochondrial surface.

Next, we evaluated the effect of trypsin treatment on the electrophoretic mobility of cytoskeleton-bound and cytoskeleton-free mitochondria. The mobility distributions of both types of mitochondria were more negative when treated with trypsin, relative to samples not treated with trypsin, ('Cyto-bound Trypsin+PHD' versus 'Cyto-bound PHD' and 'Free Trypsin+PHD' versus 'Free PHD', Figure 5). The medians of cytoskeleton-free mitochondria in the trypsin-treated and untreated samples were -4.0×10^{-4} and $-3.3 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$, respectively; the medians of cytoskeleton-bound mitochondria in the trypsin-treated and untreated samples were -4.0 and $-3.1 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$, respectively. The upward shift from the 45-degree line in the q-q plot (triangles representing μ_{Free} (Trypsin+PHD) versus μ_{Free} (Control) in Figure 5B) further confirms that the electrophoretic mobilities of mitochondria become more negative when the samples are treated with trypsin. These results are expected as trypsin may increase the number of negative charges on the surface of mitochondria as it exposes the c-terminus of arginine and lysine residues upon hydrolysis of F-actin and other proteins at the mitochondrial surface.

Trypsin treatment uncovered an interesting behavior in the mobility distributions of the cytoskeleton-bound mitochondria when compared to cytoskeleton-free mitochondria detected in the same run. Despite that their medians are the same (i.e., $-4.0 \times 10^{-4} \text{ cm}^2\text{V}^{-1}\text{s}^{-1}$; marked as 'Cyto-bound Trypsin+PHD' and 'Free Trypsin+PHD' in Figure 5A), the corresponding q-q plot reveals that these two distributions are different as shown by shift of some quantiles from the 45-degree identity line (triangles representing $\mu_{\text{Cyto-bound}}$ (Trypsin+PHD) versus μ_{Free} (Trypsin+PHD) in Figure 5C). These results suggest that overall trypsin treatments make the average surface of cytoskeleton-free and cytoskeleton-bound mitochondria more similar, but that each distribution is characterized by other fundamental differences likely associated with the surface of mitochondria. This detailed information would have been lost in bulk or fluorescence microscopy assays.

CONCLUSIONS

The CE-LIF analysis of binding events between mitochondria and cytoskeleton revealed that isolated mitochondria are found as cytoskeleton-bound and -free organelles. This approach is superior to bulk analysis in regards to the ability to discriminate against co-existing cytoskeleton not bound to mitochondria. In addition, it suggests the size of cytoskeletal network bound to isolated mitochondria. Trypsin treatments ‘shaved off’ cytoskeleton, which was consistent with the decrease in the number of cytoskeleton-bound mitochondria and amounts of F-actin detected in mitochondrial events after such treatments. The CE-LIF methodology described here has exquisite sensitivity and could be used to monitor the presence of cytoskeleton and other organelles during the purification of subcellular fractions in metabolic or proteomic studies. This methodology could also be applied to monitor the effects of cytoskeleton destabilizing drugs (e.g., Taxol), cellular reorganization in chemotaxis³² and progression of viral infections.³³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Fluorescence imaging of mitochondria and cytoskeleton. (A) F-actin cytoskeleton labeled with A488-PHD. (B) Mitochondria labeled with DsRed2. (C) Overlay of (A) and (B); the nucleus is counterstained with DAPI. The images are deconvoluted Z-stacks of confocal images. Exposure time was 10 s for each channel. Bar = 10 μm .

Figure 2.

Bulk determination of F-actin in mitochondrial fractions using A488-PHD staining. Control: sample not labeled with A488-PHD; PHD: sample labeled with A488-PHD; Trypsin+PHD: sample treated with trypsin prior to labeling with A488-PHD. Mitochondria fractions prepared from L6 cells expressing DsRed2 targeted to mitochondria. Both DsRed2 and A488-PHD fluorescence signals were measured three times in three wells (N=3) of a 96-well plate. The average DsRed2 fluorescence in each well was used to normalize the average A488-PHD fluorescence for the content of mitochondria in the respective well. The y-axis represents the normalized A488-PHD fluorescence relative to maximum response.

Figure 3.

Electropherogram of individual mitochondria-cytoskeleton binding events. (A) Top and bottom traces show A488-PHD and DsRed2 detection, respectively. (B) Migration time window 538–546 s from (A) showing representative individually detected events. The detected events are classified as: mitochondria interacting with cytoskeleton (*), mitochondria without cytoskeleton ($\emptyset$) and other organelles interacting with cytoskeleton (X). Separations were performed in 50 μm i.d. poly-AAP-coated fused silica capillary at -400 V/cm in SH buffer. Samples were hydrodynamically injected into the capillary using an 11 kPa negative pressure for 2 s. A488-PHD and DsRed2 fluorescence were collected using filters 520DF35 and 635DF55, respectively. Top traces in both panels were offset for clarity.

Figure 4.

F-actin bound to mitochondria. Control: Sample not labeled with A488-PHD ($n = 42$); PHD: sample labeled with A488-PHD ($n = 82$); Trypsin+PHD: sample treated with trypsin prior to labeling with A488-PHD ($n = 47$). Boxes represent 0.25, 0.5 and 0.75 percentiles; whiskers represent 0.1 and 0.9 percentiles; markers show values outside these ranges. Other experimental conditions were as described in Figure 2.

Figure 5.

Comparisons of electrophoretic mobilities of individual mitochondria. **(A)** Comparison of medians and quartiles. *Control*: mitochondria, without any labeling ($n = 267$); *Free PHD*: cytoskeleton-free mitochondria after A488-PHD labeling ($n = 110$); *Free Trypsin+PHD*: cytoskeleton-free mitochondria in samples treated with trypsin and labeled with A488-PHD ($n = 243$); *Cyto-bound PHD*: cytoskeleton-bound mitochondria after A488-PHD labeling, ($n = 82$); *Cyto-bound Trypsin+PHD*: cytoskeleton-bound mitochondria in samples treated with trypsin and labeled with A488-PHD ($n = 47$). Boxes represent 0.25, 0.5 and 0.75 percentiles; whiskers represent 0.1 and 0.9 percentiles. **(B)** q-q plot of electrophoretic mobility distributions of cytoskeleton-free mitochondria versus mitochondria in the control. *PHD*: sample labeled with A488-PHD (diamonds); *Trypsin+PHD*: sample treated with trypsin and labeled with A488-PHD (triangles). **(C)** q-q plot of electrophoretic mobility distributions comparing cytoskeleton-bound (*Cyto-bound*) versus cytoskeleton-free (*Free*) mitochondria. *PHD*: sample labeled with A488-PHD (diamonds); *Trypsin+PHD*: sample treated with trypsin and labeled with A488-PHD (triangles). All mobilities are in ($\times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$). The distance between markers is 0.05 percentile. The electrophoretic mobilities in parts (A)–(C) were calculated using Equation 2 and then expressed as positive numbers for clarity. Other experimental conditions were as described in Figure 2.

Table 1

Abundance of cytoskeleton-bound and -free mitochondrial events detected by CE-LIF in mitochondrial samples

Sample ¹	Cytoskeleton-bound mitochondria	'Free' mitochondria	Other organelles
Control ²	11±5%	89±5%	7±7%
A488-PHD	32±17%	68±17%	166±123%
Trypsin + A448-PHD	16±10%	84±10%	60±11%

¹In a given sample, the total number of mitochondrial events is 100%. A total of 647, 795 and 291 mitochondrial peaks were analyzed in the Control, A488-PHD and Trypsin+A488-PHD treated samples, respectively.

²Control: sample not labeled with A488-PHD.