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Fully automated software for quantitative measurements of mitochondrial morphology

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Abstract

Mitochondria undergo dynamic changes in morphology in order to adapt to changes in nutrient and oxygen availability, communicate with the nucleus, and modulate intracellular calcium dynamics. Many recent papers have been published assessing mitochondrial morphology endpoints. Although these studies have yielded valuable insights, contemporary assessment of mitochondrial morphology is typically subjective and qualitative, precluding direct comparison of outcomes between different studies and likely missing many subtle effects. In this paper, we describe a novel software technique for measuring the average length, average width, spatial density, and intracellular localization of mitochondria from a fluorescent microscope image. This method was applied to distinguish baseline characteristics of Human Umbilical Vein Endothelial Cells (HUVECs), primary Goto-Kakizaki rat aortic smooth muscle cells (GK SMCs), primary Wistar rat aortic smooth muscle cells (Wistar SMCs), and SH-SY5Ys (human neuroblastoma cell line). Consistent with direct observation, our algorithms found SH-SY5Ys to have the greatest mitochondrial density, while HUVECs were found to have the longest mitochondria.

Mitochondrial morphology responses to temperature, nutrient, and oxidative stressors were characterized to test algorithm performance. Large morphology changes recorded by the software agreed with direct observation, and subtle but consistent morphology changes were found that would not otherwise have been detected. Endpoints were consistent between experimental repetitions ($R = 0.93$ for length, $R = 0.93$ for width, $R = 0.89$ for spatial density, and $R = 0.74$ for localization), and maintained reasonable agreement even when compared to images taken with compromised microscope resolution or in an alternate imaging plane. These results indicate that the automated software described herein allows quantitative and objective characterization of mitochondrial morphology from fluorescent microscope images.

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Disclosures

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Keywords

Mitochondria; Morphology; Microscopy; Image processing

1. Introduction

Mitochondrial morphology has become a topic of intensive investigation in recent years. Whereas a PubMed search for “mitochondrial morphology” returns only 222 results from the year 2000 or before, the same search returned 1331 results as of 4/14/2015, and many of these papers are published in high profile journals. This vast body of research is unsurprising in light of recent reports indicating that mitochondrial dynamics are involved in regulating cellular respiration (Chen et al., 2005), calcium dynamics (Rizzuto et al., 2012), free radical production (Yu et al., 2006), substrate catabolism (Liesa and Shirihai, 2013), cell survival (Karbowski and Youle, 2003), cell activation (Alano et al., 2002), and perhaps epigenetics (Picard et al., 2013). Alterations in mitochondrial dynamics have been reported in a wide variety of disease states including diabetes (Yoon et al., 2011), obesity (Dietrich et al., 2013), aging (Seo et al., 2010), vascular proliferative disorders (Ong and Hausenloy, 2010), and several genetic conditions that directly influence regulation of mitochondrial morphology (Liesa et al., 2009). Clearly, mitochondrial dynamics are critically involved in human and cellular physiology and their study is thus warranted.

A great deal has been learned about mitochondrial morphology and dynamics in recent years, yet the current state of the art for assessing mitochondrial morphology leaves much to be desired. Of the first 50 results on a PubMed search for “mitochondrial morphology” sorted by relevance (as of 4/14/2015), 31 are primary research articles assessing mitochondrial networking. Of these, 3 used molecular or histologic markers as surrogates for mitochondrial morphology measurements, 18 reported subjective assessments of morphology, 4 performed morphological measurements by hand, and 6 used some manner of software measurement. It is troubling that most common methods for assessing mitochondrial morphology involve no objective measurements of mitochondrial morphology. Subjective assessment is ill-suited to comparison of findings between studies or between cell types, and molecular markers do not necessarily relate to actual morphology in an intuitive manner. Because these methods are relatively insensitive, the interventions employed to perturb mitochondrial morphology are often extreme relative to what a cell might encounter in-vivo. Taking measurements by hand avoids these pitfalls, but it is slow, thus limiting sample sizes and increasing time per experimental iteration. A quantitative, objective, and consistent software method of measuring mitochondrial morphology is attractive in that it allows comparison between studies or cell types, might detect more subtle changes in morphology than subjective assessment, and allows for efficient analysis of a large sample size.

Some laboratories use software tools, such as the Mitochondrial Morphology plugin in ImageJ (Aboud et al., 2015; Li et al., 2013; Xie and Chung, 2012). This software can allow introduction of investigator bias since the user selects the thresholding parameters, and the endpoints reported to the user are rather limited. More sophisticated methods of identifying

mitochondria have been developed, including the Fourier-based algorithm of Koopman et al. (Koopman et al., 2006), the statistical normalization-based algorithm of Peng et al. (Peng et al., 2010), and the hierarchical model-based segmentation algorithm of Lin et al. (Lin et al., 2005) that was originally developed for detecting nuclei and has been applied to mitochondrial measurements (Mitra et al., 2009), among others. These detection methods have enabled more sophisticated structural analyses such as the automated morphological subtyping of Peng et al. (Peng et al., 2011) and the categorization scheme of Farrand et al. (Farrand et al., 2013), further increasing the potential value of these computational tools. To date, the adoption of these algorithms has been quite limited as discussed in the previous paragraph, and it is noteworthy that the use of computational methods for quantification of mitochondrial morphology is largely restricted to laboratories with quantitative expertise. This suggests that usability and comprehensibility of existing software solutions to their target audience in life science research may be limiting the adoption of these technologies.

We propose that a practical software solution for the measurement of mitochondrial morphology should meet five basic criteria: algorithm performance should not require a specific microscope or sample preparation, software expertise should not be required to operate the software, endpoints should be consistent between experimental repetitions, no specific portion of the mitochondrial network should be excluded from analysis or incorrectly analyzed due to algorithm performance, and the endpoints reported to the user should hold intuitive and tangible meanings. The final point may seem “soft” on first glance, but we contend that if the output does not align with visual impressions or cannot readily be understood by the user, it will be difficult to trust the results. These criteria were used to guide our experimental design. Our software shows considerable consistency between experimental repetitions and thus allows for sensitive detection of morphological consistency. By using an established algorithm for mitochondrial image binarization that has already been tested with multiple sample preparations and microscopes (Peng et al., 2010), we ensure that our technique is usable for as broad an audience as possible. Automation of the analysis precludes the need for software expertise on the part of the end user, and the methods developed for removing aberrantly detected mitochondria successfully removed mitochondrial bodies which were obviously detected incorrectly. A variety of experimental perturbations were used to test the ability of this software to track dynamic changes in network morphology, and dynamic changes were in fact detected with each perturbation. Finally, investigation of the effects of optical slice thickness and plane selection indicate that the measurements obtained by this software are largely consistent even in the face of subtle changes in imaging procedures.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagles Medium (DMEM) 5 mM and 25 mM glucose and non-essential amino acids and Laughlin's F12 Medium were obtained from Thermo Scientific Hyclone, and trypsin and trypsin inhibitor were purchased from Fisher Scientific. Fetal bovine serum (FBS) was procured from Gemini Bioproducts. Hank's Balanced Salt Solution (HBSS) was purchased from Corning Life Sciences. Secondary detection antibodies Alex Fluor 488 and

546 as well as primary antibodies to α -actin were purchased from Life Technologies. Antibodies to TOM20 (rabbit) and nitrotyrosine (mouse) were procured from Santa Cruz Biotechnology. Rhodamine phalloidin was obtained from Invitrogen.

2.2. Cell culture

Primary rat vascular smooth muscle cells (SMCs) were cultured in low glucose (5 mM) DMEM with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acid blend, and 1% Pen/Strep, all expressed as % by volume. For SMC starvation media, the same proportions were maintained, except that FBS was reduced to 0.1%. Human umbilical vein endothelial cells (HUVECs) were cultured in F-12 k medium (Hyclone #SH30526.01) with 10% FBS, 1% Pen/Strep, 0.05 mg/mL endothelial cell growth supplement, and 0.1 mg/mL heparin. SH-SY5Ys (ATTC neuronal cell line) were cultured in 45% F-12 k medium, 45% low glucose DMEM, and 10% FBS. All cells were cultured at 37 °C at 5% CO₂. Media was changed at least every three days, and cells were split 2:1 at 80% confluence. Experiments were performed on cells at 50%–70% confluence. Unless otherwise specified, cell culture materials were obtained from Santa Cruz Biotechnology.

2.3. Fixation and staining

Prior to fixation, phosphate buffer solution (PBS) rinse and paraformaldehyde solutions were warmed to 37 °C (except as otherwise indicated). Samples were washed 3× in warm PBS prior to incubation in 4% paraformaldehyde at 37 °C for 15 min. Following fixation, samples were washed 3× in warm PBS and quenched in 50 mM NH₄Cl for 15 min at room temperature. Samples were then rinsed 3× with room temperature PBS and stored immersed in PBS at 4 °C until staining. All samples were stained within one week of fixation. Prior to staining, cells were permeabilized by incubation in 0.1% TX-1000 at 37 °C for 15 min. Following permeabilization, samples were rinsed 3× with room temperature PBS and then blocked by incubation in 10% fetal bovine serum (FBS) in PBS for 25 min and 37 °C. Samples were subsequently incubated in a primary antibody mix in 5% FBS in PBS at 37 °C for one hour. Following treatment with primary antibody, samples were washed 3× with 5% FBS in PBS and incubated in a secondary antibody mix in 5% FBS in PBS at room temperature for one hour. Samples were then washed 3× in PBS, incubated in DAPI diluted 1:10,000 in PBS at room temperature for 10 min, washed 3× in PBS again, then washed once in distilled water. Coverslips were then mounted to microscope slides for imaging. The primary antibody mix consisted of a Tom20 antibody (Santa Cruz Biotechnology sc-11415) diluted 400× to stain mitochondria and a nitrotyrosine antibody (Santa Cruz Biotechnology sc-32757) diluted 100× to stain the cytoplasm. The secondary antibody mix consisted of Alexafluor 488 (Life Technologies A-11008) diluted 1000× to react with the Tom20 antibody and Alexafluor 546 (Life Technologies A-11030) diluted 500× to react with the nitrotyrosine antibody. In order to verify that the binarization scheme developed in this study is not stain-specific, some cells were stained with a primary antibody for α -actin (Life Technologies A-2547) rather than Nitrotyrosine. Others were stained using Phalloidin (Invitrogen R415) rather than using antibodies to detect the cytoplasm. Following staining, slides were stored protected from light at 4 °C.

2.4. Microscopy and imaging techniques

The primary imaging system used in these experiments was an Olympus FV1000 confocal microscope provided by the UC Denver Advanced Light Microscopy Core. A 488 nm laser was used to excite AlexaFluor 488 and a 543 nm laser was used to excite AlexaFluor 546. Built-in filter settings for these fluorophores were used to detect fluorescence. Slides were imaged using a 60× oil immersion objective at a resolution of 206.8 nm/pixel and an image size of 1024 pixels by 1024 pixels. An exposure time of 15 ms per pixel was used and the average of three frame acquisitions was used as the raw microscope image. In addition to the Olympus FV1000, further imaging was performed on a Leica SP8 confocal microscope at the Denver VA Medical Center. A white light laser was tuned to excitation wavelengths of 488 nm and 546 nm for AlexaFluor 488 and 546, respectively. Similar to images acquired on the Olympus system, a 60× oil immersion objective was used on the Leica system. The total image size for images acquired using the Leica SP8 was 1024 pixels by 1024 pixels with a pixel size of 180.4 nm. A scanning frequency of 600 Hz was used for an exposure time of 1.6 μ s per pixel. 6 line averages and 3 frame averages were used to reduce the influence of instrument noise.

2.5. Image binarization for computational measurements

Images were imported into Matlab in 16-bit TIF format and binarized using custom-built software. Prior to binarization, 2×2 binning was applied to raw mitochondrial images to reduce the influence of noise. The noise-reduced images were then normalized so that the minimum pixel intensity value was 0 and the maximum pixel intensity value was 1. The adaptive image enhancement algorithms of Peng et al. (Peng et al., 2010) with a parameter value of $\frac{T_{std}}{Std(I)} = 0.55$ were combined with the thresholding algorithm of Otsu (Otsu, 1975) in order to binarize the Tom20 (mitochondrial) channel. The DAPI (nucleus) channel was binarized directly by Otsu thresholding.

The algorithms used to binarize the cytoplasm (nitrotyrosine or α -actin) channel are outlined in Fig. 1. Nitrotyrosine (cytoplasm) channel appears as punctate dots of fluorescence throughout the cytoplasm, whereas α -actin appears as striated fibers throughout the cytoplasm. In both cases, signal heterogeneity within the cytoplasm is the primary challenge of image binarization. The process for binarizing the cytoplasm channel (regardless of staining technique used) is outlined in Fig. 1. Prior to processing, images were normalized such that the minimum pixel intensity was 0 and the maximum pixel intensity was 1 (Fig. 1A). A Gaussian blur was used to transform the punctate and/or striated distribution of fluorescence into a more uniform distribution of fluorescence within the cytoplasm (Fig. 1B). It was observed that more peripheral regions of the cytoplasm are often more similar in pixel intensity to background than to pixel intensity in perinuclear regions of the cytoplasm. A second Gaussian blur was therefore used to obtain an approximation of background pixel intensity, similar to the method employed in binarization of the mitochondrial channel (Fig. 1C). The difference in pixel intensities between the once-smoothed and twice-smoothed images was then used to construct an intensity prominence image (Fig. 1D). Candidate threshold values between 0 and 1 were tested with a resolution of 0.02. The threshold value which minimized the ratio of edge length to background area was then selected to binarize

the image (Fig. 1E). All Matlab scripts employed in this study are included in the associated Data in Brief article (McClatchey et al., submitted for publication).

2.6. Removal of aberrantly detected mitochondrial bodies

Raw mitochondrial and cytoplasm images were binarized as described in the previous section. Bodies comprising less than 15 pixels were excluded from further analysis as too small (subject to error due to discrete pixel size), and those for which the projected area to perimeter ratio was greater than $0.3 \mu\text{m}$ were excluded from further analysis as too wide (likely out of focus). These criteria were determined empirically to remove bodies which were obviously detected aberrantly and were used to prevent error in algorithm measurements due to faulty image binarization. The results of this scheme are illustrated in Fig. 2. These criteria removed only those mitochondrial bodies for which the total number of pixels was so small as to cause discretization of mitochondrial measurements along with the very widest mitochondrial bodies (Fig. 2A). Note that the image in Fig. 2A was selected for demonstration purposes because an unusually large number of mitochondrial bodies were rejected. For all experimental groups tested, the overall average was less than 20% rejected. When compared to the raw mitochondrial image, the bodies removed were reliably those which were either out of focus or spuriously detected due to noise (Fig. 2B). A histogram of mitochondrion widths (discussion of length and width calculations in the following paragraphs) prior to removal of aberrantly detected bodies shows a discontinuous distribution with physiologically implausible values (Fig. 2C), whereas the same histogram following removal of aberrantly detected bodies shows a continuous distribution with physiologically plausible values (Fig. 2D). Similar results were obtained for histograms of mitochondrion length (data not shown). The chosen limits of 15 pixels minimum and maximum projected area to perimeter ratio of $0.3 \mu\text{m}$ might need to be adjusted for different imaging systems. It is unlikely that the issue of measurement discretization for bodies comprising less than 15 pixels can be avoided, and so image resolution must be great enough that genuine mitochondrial bodies are generally larger than 15 pixels. Similarly, the projected area to perimeter ratio serves as a proxy for mitochondrial width and will thus be sensitive to changes in pixel size. Specifically, the projected area to perimeter ratio can never be less than pixel size, and so pixel sizes greater than or equal to $0.3 \mu\text{m}$ will result in all mitochondrial bodies being excluded from analysis with the exclusion criteria given. All Matlab scripts employed in this study are included in the associated Data in Brief article (McClatchey et al., submitted for publication).

2.7. Computation of raw mitochondrial morphology parameters

The total number of illuminated pixels in each binary mitochondrial image was multiplied by the pixel size in μm^2 to obtain an estimate of mitochondrial network size. The number of illuminated pixels bordering non-illuminated pixels was multiplied by the pixel edge length in μm to obtain an estimate of projected mitochondrial network perimeter. The number of mitochondrial bodies was detected using a built-in function in Matlab (see *bwconncomp*) in Matlab documentation). Using mitochondrial bodies that were not rejected according to the criteria discussed in the previous section, average mitochondrion size was then taken to be the total mitochondrial network size divided by the number of mitochondrial bodies. This calculation is equivalent but computationally faster than summing the individual

mitochondrial bodies and taking a numerical mean. The average mitochondrion perimeter was likewise taken to be the total mitochondrial network perimeter divided by the number of mitochondrial bodies. Next, average mitochondrion size and perimeter were used to calculate average mitochondrion length and width. Observing that most of the mitochondria in the network are shaped like long circular tubes with rounded ends, the geometric relationships depicted in Fig. 3A and Eqs. (1)–(2) below were used to solve for average length and width. In these equations, P is perimeter, A is area, W is width, and L is length.

$$P_{mean} = \pi * W_{mean} + 2 * L_{mean} \quad (1)$$

$$A_{mean} = \frac{\pi}{4} * W_{mean}^2 + W_{mean} * L_{mean} \quad (2)$$

The raw parameters computed using the algorithms described in the previous section were subsequently corrected for total system error in measurement and for more general field-of-view parameters, such as total cytoplasm area and number of cells. Total system error comprises the microscope point spread function (PSF), algorithm error in binarization of raw mitochondrial images, and discretization error in computational measurement of these binarized mitochondrial images. Rather than attempting to measure or estimate each of these sources of error independently, the magnitude of the total system error was directly measured as the difference between the known 400 nm diameter of fluorescent beads (CytoDiagnostics FBC-620-400) and their measured diameter using the same image processing scripts as were used to measure mitochondrial network parameters. For both microscopes used, 400 nm beads were measured at concentrations spanning four orders of magnitude. Bead images were binarized using identical methods to those used to binarize mitochondrial images, and measured bead diameter was inferred from total projected area and perimeter using similar methods to those outlined in Eqs. (1)–(2), except that a spherical rather than tubular shape was assumed. Raw bead diameter measurements were corrected for signal–noise ratio (SNR), as described in the following section. No significant differences were found between calculated bead diameters when measured at different concentrations for either microscope system, indicating that clustering of beads did not compromise this analysis (Fig. 3B). It was found that the Olympus FV1000 microscope overestimated bead diameter by 185 nm, while the Leica SP8 overestimated bead diameter by 101 nm. Raw length and width values were then calibrated by subtracting the bead diameter overestimation obtained using the imaging system and setup corresponding to each mitochondrial image. This calibration serves to correct for system bias resulting from imperfect optics and image processing.

Total cytoplasm area was estimated from the total number of illuminated pixels in the binarized cytoplasm image multiplied by pixel area in μm^2 . The total number of cells in the image was estimated from number of nuclei. The total projected area of the mitochondrial network was divided by the total cytoplasm area to obtain an estimate of the fraction of the cytoplasm filled by mitochondria as a normalized metric of mitochondrial mass. Likewise,

the total projected area of the mitochondrial network and the total number of mitochondria were divided by the number of cells in the image to obtain an estimate of total mitochondrial mass per cell and total number of mitochondrial bodies per cell, respectively. Noting that localization of the mitochondria relative to the nucleus varied by treatment condition and cell type, we sought to quantify this localization. To assess localization of mitochondria within the cytoplasm, we introduced a parameter termed “perinuclear preference” defined as the natural log of the ratio of the mitochondrial mass in the 30% of the cytoplasm closest to the nearest nucleus to the mitochondrial mass in the 30% of the cytoplasm furthest from the nearest nucleus. A perinuclear preference value of 0 indicates equivalent mitochondrial mass in the perinuclear and peripheral regions, a positive value indicates higher mitochondrial mass in the perinuclear space, and a negative value indicates higher mitochondrial mass in the periphery. For most inter-group comparisons, normalized parameters were used rather than raw parameters in order to reduce the influence of machine error, random differences in cell size or confluence between fields of view, etc. on reported end-points. All Matlab scripts employed in this study are included in the associated Data in Brief article (McClatchey et al., submitted for publication).

2.8. Correction for signal–noise ratio

Observing that images with apparently similar mitochondrial morphology sometimes yielded noticeably different measurements, especially of mitochondrial width, we investigated the influence of signal–noise ratio (SNR) on measures of mitochondrial morphology. We defined SNR as the ratio of mean pixel intensity in foreground pixels (as determined by binarization) to mean signal intensity in background pixels in raw mitochondrial images. As illustrated in Fig. 4, it was found that raw measurements of bead diameter (Fig. 4A), mitochondrion width (Fig. 4B), and mitochondrion length (Fig. 4C) vary inversely with SNR. The relationship between SNR and mitochondrion width was found to be consistent across cell types and similar to that between SNR and apparent bead diameter. Simple linear regression was used to correct for the effects of SNR on these measurements. The relationship between SNR and mitochondrion length, although statistically significant ($p < 0.05$), was found to be different between different cell types and considerably less pronounced than for mitochondrion width or apparent bead diameter. Measurements of mitochondrion length were therefore not corrected for SNR in order to avoid undue data manipulation. Finally, measures of mitochondrial mass and fraction of cytoplasm filled by mitochondria were scaled with corrections in mitochondrion width. All Matlab scripts employed in this study are included in the associated Data in Brief article (McClatchey et al., submitted for publication).

2.9. Post-processing of data

Following initial measurements and corrections, outliers in the data set were automatically detected and removed by the software. Any image for which any parameter measured was greater than three standard deviations from the treatment group mean was removed from the data set entirely. Remaining images were then used to calculate treatment group means and SEMs for each experimental repetition and across all experimental repetitions. In order to verify that this mode of data grooming was not limiting sample size, the total number of images included and rejected in each analysis was monitored. Less than 10% of images were

removed from analysis in all image sets analyzed. All Matlab scripts employed in this study are included in the associated Data in Brief article (McClatchey et al., submitted for publication).

2.10. Statistics and software packages

For comparisons involving three or more groups, a one-way ANOVA was used to assess statistical significance. For comparisons involving two groups only, an unpaired t-test was used to assess statistical significance. To assess correlations and their statistical significance, Pearson's R and the corresponding t-value were computed. Effects for which $p < 0.1$ were considered trending and those for which $p < 0.05$ were considered significant. For comparisons in which one-way ANOVA was employed, Tukey's post-test was used to correct for the effects of multiple comparisons. All post-hoc statistical analyses were performed in Prism GraphPad version 5.0. The mitochondrial measurement algorithms and automated post-processing were coded into Matlab 2013. Where available, built-in functions in these software packages were used for statistical computations.

3. Results

3.1. Consistency of measurements between experimental repetitions

In order for a mitochondrial morphology measurement software to be maximally impactful, it must be demonstrated that it yields consistent results and the minimum detectable effect size must be known. For all groups imaged on the Olympus FV100 microscope, each experimental repetition mean for every metric reported was compared to matched experimental repetition means. Each experimental repetition included 3 images each from 3 separate slides for a total of $n = 9$ images per experimental repetition. Baseline and perturbed (perturbations discussed in subsequent sections) mitochondrial networks for HUVECs, GK SMCs, Wistar SMCs, and SH-SY5Ys were employed in this analysis. With 3 experimental repetitions per treatment group, 3 permutations of matched experimental repetitions can be achieved without including the same data twice or drawing self-self comparisons: repetition 1 vs repetition 2, repetition 2 vs repetition 3, and repetition 1 vs repetition 3. Nitrotyrosine was used to detect the cytoplasm in these experiments.

Fig. 5 summarizes assessments of consistency between experimental repetitions within treatment groups. Treatment group differences were observed to be qualitatively consistent between experimental repetitions (Fig. 5A). Strong consistency was observed between subsequent experimental repetitions in mitochondrial length (Fig. 5B, $R = 0.93$) and mitochondrial width (Fig. 5C, $R = 0.93$). Likewise, strong agreement was observed between subsequent experimental repetitions in fraction of cytoplasm filled by mitochondria (Fig. 5E, $R = 0.89$), and modest consistency was observed for perinuclear preference (Fig. 5F, $R = 0.74$). Fig. 5D lists the mean and standard deviation of discrepancies between successive experimental repetitions for each mitochondrial morphology metric. Since $n = 9$ images per experimental repetition, the mean values reported in Fig. 5D reflect a minimum detectable effect size with $n = 9$ for each metric. These minimum detectable effect sizes were 330 nm for mean mitochondrion length, 12 nm for mean mitochondrion width, 0.54% for fraction of cytoplasm filled by mitochondria, and 0.2 for perinuclear preference. Collectively, these

results indicate that very small changes in mitochondrial morphology are detectable using this system.

3.2. Baseline cell line characteristics

This section documents baseline mitochondrial network characteristics of GK SMCs, Wistar SMCs, HUVECs, and SH-SY5Ys. Nitrotyrosine was used to detect the cytoplasm in this experiment. Fig. 6 summarizes differences between these four cell lines. Representative microscope images for each cell line are shown in Fig. 6A. Consistent with visual inspection, HUVECs were found to have the greatest mean mitochondrion length (Fig. 6B) at 7.5 μm while Wistar SMCs had the shortest at 4.6 μm . Fig. 6C shows cell line differences in mean mitochondrion width; SH-SY5Ys had the widest mitochondria at 243 nm while HUVECs had the thinnest at 112 nm. Cell line differences in fraction of cytoplasm filled by mitochondria are shown in Fig. 6D; SH-SY5Ys had the greatest fraction of the cytoplasm filled by mitochondria at 7.5% while HUVECs had the least at 2.5%. Fig. 6E shows cell line differences in perinuclear preference; HUVEC mitochondria showed the greatest degree of perinuclear localization with a perinuclear preference of 3.06, while SH-SY5Ys showed the least with a perinuclear preference of 1.46. Cell line differences reported by the software fully matched with visual assessment for fraction of cytoplasm filled by mitochondria and perinuclear preference, and cell line differences in mean mitochondrion length and width agreed with visual assessment for all cell lines except for the SH-SY5Ys. Comparison of raw and binarized SH-SY5Y images revealed that the accuracy of binarization was impaired for these cells (please refer to processed images in the corresponding Data in Brief article (McClatchey et al., submitted for publication)). Mitochondrial morphology studies reporting direct measurement of mitochondrial length typically report values in the range of 1–15 μm (Rintoul et al., 2003; Nozaki et al., 2001; Wang et al., 2013; Korobova et al., 2013; Choi et al., 2015; Cagalinec et al., 2013; Korobova et al., 2014; Gan et al., 2014), consistent with the values obtained in this analysis. Literature values for mitochondrial width/diameter generally fall in the range of tens to hundreds of nanometers (Moran et al., 2010; Finley et al., 2012; Karam et al., 2003; Santamaría et al., 2006; Holloway et al., 2010), also consistent with those obtained in this analysis.

3.3. Effects of fixation temperature in HUVEC cells

To assess the impact of the fixation conditions on mitochondrial network parameters, we tested the impacts initial reagent temperatures and incubation temperature during fixation on mitochondrial morphology. HUVEC cells were cultured without experimental perturbation for 6–10 passages. Cells were divided into one of four experimental groups and fixed according to the protocol described above, using nitrotyrosine to detect the cytoplasm. Treatment group-specific adjustments to temperature were assigned as outlined below. Group 1 was fixed using reagents pre-heated to 37 °C and all incubation steps during fixation were performed at 37 °C; Group 2 was fixed using room-temperature reagents and all incubation steps during fixation were performed at 37 °C; Group 3 was fixed using room-temperature reagents and all incubation steps during fixation were performed at room temperature; Group 4 was fixed using reagents pre-heated to 37 °C and all incubation steps during fixation were performed at room temperature.

Fig. 7 summarizes the effects of fixation temperature on mitochondrial morphology in HUVEC cells. Insets of representative microscope images are shown in Fig. 7A. Visual observation of the samples indicated a more fragmented mitochondrial network in the group treated with room temperature formaldehyde and incubated at room temperature during fixation, and possibly a more peripheral distribution of mitochondrial mass in the group treated with pre-warmed formaldehyde and incubated at 37 °C during fixation compared to other groups. It was found that the average mitochondrion length was significantly ($p < 0.0001$) reduced in the group treated with room temperature formaldehyde and incubated at room temperature during fixation relative to every other group, and that no other treatment group differences in mitochondrial length were statistically significant (Fig. 7B). No group differences were found in average mitochondrion width ($p > 0.05$). Although visual analysis suggested perinuclear localization of mitochondria in all groups treated with room temperature reagents or incubated at room temperature at any stage during fixation, perinuclear preference did not vary significantly between groups ($p > 0.05$). Finally, no group differences were found in total fraction of the cytoplasm filled by mitochondria, as would be expected given that the time required for fixation is considerably less than the time required for substantial mitochondrial turnover.

3.4. High glucose exposure time course with primary rat SMCs

As part of a broader investigation into long-term phenotypic changes to vascular smooth muscle cells in diabetes (Keller et al., in revision), we sought to characterize the responses of mitochondrial network morphology to acute glucose stress in smooth muscle cells from diabetic and non-diabetic hosts. Primary smooth muscle cells from the aorta of GK or Wistar rats were cultured in low glucose (5 mM) smooth muscle growth media until passages 8–10. Prior to treatment, cells were switched to low glucose, serum free starvation media for 48 h. Serum starved cells were exposed to high glucose (25 mM), serum free media. For each cell type (GK and Wistar) in each repetition of each experiment, 15 slides were prepared. Of these 15 slides, three were merely rinsed with high glucose media before fixation and staining (0 min exposure), three were fixed and stained after 10 min in high glucose media, three were fixed and stained after 30 min in high glucose media, three were fixed and stained after 60 min in high glucose media, and the remaining three were fixed and stained after 240 min in high glucose media. These time points were selected based on empirical observation that mitochondrial morphology was highly dynamic in the first hour of high glucose exposure and then underwent more gradual changes in the subsequent several hours. Nitrotyrosine was used to detect the cytoplasm in this experiment.

Fig. 8 summarizes the response of GK and Wistar SMCs to exposure to 25 mM glucose exposure. Statistically significant ($p < 0.05$) changes in mean mitochondrion length (Fig. 8A) were observed for both GK and Wistar SMCs, including fragmentation between 10 and 30 min in 25 mM glucose, and networking between 60 and 240 min in 25 mM glucose. These changes were not visible to the naked eye, as illustrated by representative microscope images at 10 and 30 min in 25 mM glucose (Fig. 8B). Although these changes were too subtle for direct visual assessment, dynamic changes detected using our algorithm were consistent between experimental repetitions (e.g. Fig. 5A). In addition to these differences in

dynamic changes in length, GK SMCs had significantly ($p < 0.0001$) longer mitochondria than did Wistar SMCs at all time-points recorded. Similarly, although average mitochondrion width did not change significantly over time ($p > 0.05$) for either cell type, Wistar SMCs had significantly ($p < 0.0001$) wider mitochondria than did GK SMCs at all time-points recorded (data not shown). A number of studies in a variety of cell types have reported mitochondrial fragmentation upon acute exposure to high concentrations of glucose (Yu et al., 2011; Trudeau et al., 2010; Yu et al., 2006). Yu et al. report results of particular interest, showing a similar two-phase mitochondrial morphology response in neuronal cells to that which we observed in primary SMCs, consisting of rapid mitochondrial fission followed by gradual mitochondrial fusion (Yu et al., 2011). To our knowledge, no previous studies have reported a quantitative time course of mitochondrial morphology response to incubation in 25 mM glucose in primary vascular SMCs.

3.5. Hydrogen peroxide exposure with SH-SY5Y neurons

Oxidative stress has been reported to perturb mitochondrial morphology. These experiments examined the impact of H_2O_2 in a neuronal cell line. SH-SY5Y neuronal cells were cultured without experimental perturbation for a minimum of 5 passages. Cells were then changed to media containing either 200 nM or 0 nM H_2O_2 for 2 h at least 24 h after the previous media change. Cells were then fixed and stained as described above, using nitrotyrosine to detect the cytoplasm.

Fig. 9 summarizes the effects of hydrogen peroxide exposure on mitochondrial morphology in SH-SY5Y cells. Mitochondria from cells exposed to hydrogen peroxide were found to be visibly more fragmented than controls and more peripherally located, although the visible difference was subtle (Fig. 9A). This difference was reflected in algorithmic measurements, which indicated a significant ($p < 0.0001$) reduction in average mitochondrion length with hydrogen peroxide exposure (Fig. 9B) and a significant ($p < 0.01$) reduction in perinuclear preference with hydrogen peroxide exposure (Fig. 9C). Studies in a variety of cell types report mitochondrial fragmentation upon acute exposure to hydrogen peroxide (Fan et al., 2010, Jahani-Asl et al., 2007, Jendrach et al., 2008), although the specific effects of hydrogen peroxide on mitochondrial localization has not previously been reported to our knowledge.

3.6. Influences of optical slice thickness on mitochondrial morphology measurements

Because of the 3-dimensional nature of real mitochondrial networks and the 2-dimensional nature of the microscope images analyzed in this study, the thickness of the optical slice (ΔZ) would be expected to change measures of mitochondrial morphology. To assess this possibility, 3 repetitions of 3 slides each of HUVECs and primary rat carotid SMCs were cultured for at least 3 passages, and then fixed and stained as described above, using either α -actin (for carotid SMCs) or rhodamine phalloidin (for HUVECs) to detect the cytoplasm. Optical slice thickness was modulated by changing the microscope pinhole diameter and repeating acquisition of the same images with different pinhole settings in order to facilitate single-cell level analysis.

Fig. 10 summarizes the results of this analysis. With the exception of perinuclear preference, all metrics show a statistically significant ($p < 0.05$) bias at all pinhole diameters relative to the default pinhole diameter of 1 Airy Unit (AU) (Fig. 10A). Unexpectedly, measured values mean mitochondrion length were found to be significantly reduced ($p < 0.05$) at 2 AU ($Z = 1.614 \mu\text{m}$) relative to 1 AU ($Z = 0.896 \mu\text{m}$). Biases observed in mitochondrion length (at all pinhole diameters), mitochondrion width (at 0.5 AU, $Z = 0.593 \mu\text{m}$ and 1.5 AU, $Z = 1.246 \mu\text{m}$), fraction of cytoplasm filled by mitochondria (at 0.5 AU, $Z = 0.593 \mu\text{m}$ and 1.5 AU, $Z = 1.246 \mu\text{m}$), and perinuclear preference (at all pinhole diameters) were less than the detectable effect size determined from analysis of experimental repetition consistency (Fig. 5D), while those for mitochondrion width and fraction of cytoplasm filled at 2.0 AU ($Z = 1.614 \mu\text{m}$) were greater than the detectable effect size. Apparent bead diameters (Fig. 10B) were found to increase with increasing pinhole diameter, likely due to changes in signal-noise ratio (SNR) given that all beads imaged were in a single plane. For pinhole diameters of 1.5 AU ($Z = 1.246 \mu\text{m}$) and 2 AU ($Z = 1.614 \mu\text{m}$), the increases in apparent bead diameter were statistically significant ($p < 0.05$), and so pinhole diameter-specific values of measured bead diameter were used for calibration of these image sets. Multiple linear regression analysis (Fig. 10C) was used to assess the relative contributions of slice thickness and SNR to single-cell discrepancies in each metric from matched values at 1 AU ($Z = 0.896 \mu\text{m}$). Log change in SNR and linear change in Z were used as predictor variables, while linear changes in mitochondrion length, mitochondrion width, and fraction of cytoplasm filled by mitochondria along with log changes in perinuclear preference were used as dependent variables for regression analysis. This analysis indicates that SNR alone influenced changes in measured mitochondrion length with changing pinhole diameter, while both SNR and Z were statistically significant ($p < 0.05$) predictors of biases in mean mitochondrion width and fraction of cytoplasm filled by mitochondria. The independent contributions of Z to discrepancies in width and fraction of cytoplasm filled likely stem from increased detection of out-of-focus mitochondria with increasing slice thickness. SNR (Fig. 10D) was found to be significantly reduced ($p < 0.05$) in 1.5 AU ($Z = 1.246 \mu\text{m}$) and 2 AU ($Z = 1.614 \mu\text{m}$) images relative to 1 AU ($Z = 0.896 \mu\text{m}$) images, further supporting the notion that changes signal quality rather than slice thickness per-se substantially contributed to the observed changes in mitochondrial morphology metrics with changing pinhole diameter. These observations indicate that subtle changes in optical slice thickness or microscope resolution are unlikely to compromise mitochondrial network measurements, but that optimal software performance will be achieved with optimal microscope settings (i.e. pinhole diameter of 1 AU).

3.7. Influences of plane selection on mitochondrial morphology measurements

Mitochondrial morphology has been observed to vary with location in the cell. The specific plane selected for imaging thus might be expected to influence the metrics of mitochondrial morphology reported in this study. To assess this possibility, the same samples that were used for analysis of optical slice thickness effects were re-imaged with consistent optical slice properties but at different image planes. First, a primary plane was selected to include the largest possible portion of the complete mitochondrial network. Images from an overlapping plane $0.3 \mu\text{m}$ above the representative plane and a non-overlapping plane $1.2 \mu\text{m}$ above the representative plane ($Z = 0.896 \mu\text{m}$) were compared to those from the

representative plane. A z-stack ranging from 2 μm to 2 μm above the representative plane with an axial resolution of 0.3 μm was used to create a maximum intensity projection of the entire mitochondrial network for each image.

Fig. 11 summarizes the results of this analysis, including a sample image in the representative plane (Fig. 11A), overlapping plane (Fig. 11B), non-overlapping plane (Fig. 11C), and maximum intensity projection (Fig. 11D). Correlation analysis (Fig. 11E) indicates strong agreement ($p < 0.0001$) between the representative plane and overlapping plane for all metrics, as well as strong agreement ($p < 0.0001$) between the representative plane and maximum intensity projection. Weak but statistically significant agreement ($p < 0.05$) was observed between the representative plane and non-overlapping plane. Statistically significant ($p < 0.05$) biases were observed between chosen planes for most metrics with most combinations of planes (Fig. 11D), but the only biases larger than the minimum detectable effect size (Fig. 5D) were those for comparison of the representative and non-overlapping planes. Compared to the representative plane, the non-overlapping plane displayed a mean mitochondrion length reduced by 520 nm, a mean mitochondrion width increased by 39 nm, and a perinuclear preference increased by 0.42. These changes are consistent with visual assessment in that the non-overlapping plane typically sectioned a portion of the cell that was thicker than the rest of the cell due to the nucleus, with only portions of mitochondrial bodies detected, somewhat thicker mitochondria than for the cell as a whole, and perinuclear localization of mitochondria. The strong agreement between representative and overlapping planes indicates that this software is relatively robust to minor changes in plane selection, and the strong agreement between representative planes and maximum intensity projections indicates that a single plane is in fact sufficient for mitochondrial measurements in cell types with squamous morphology such as those used in this study. For cell types with more 3-dimensional morphology such as lymphocytes or pancreatic β cells, use of a maximum intensity projection may be preferable to a single plane.

4. Discussion

In light of the tremendous quantity of research in recent years assessing mitochondrial morphology endpoints, it is important that quantitative, objective, practical, and consistent methods of obtaining mitochondrial morphology measurements are developed. The algorithms documented in this manuscript show excellent consistency in measured endpoints between experimental repetitions and robustness in the face of alternate microscopes, sample preparations, or microscope settings. Our results indicate slightly better performance in measurements of length, width, and mitochondrial mass than in measurements of mitochondrial localization. Our observation of consistent results from one experimental repetition to the next along with the documented general functionality of the binarization scheme employed here (Peng et al., 2010, 2011) hold promise for enhancing consistency of results between different laboratories. In addition, a collaborator was successfully able to apply this software for quantification of mitochondrial morphology in Human Fetal Lung cells infected with VZV virus (Badani et al., in revision). Compared to previous software techniques for assessment of mitochondrial morphology, the software developed in this study requires fewer user inputs and reports endpoints that are more readily measurable by

alternate techniques (e.g. mean length and width rather than categorical subtyping). Finally, the differences between cell types and treatment groups detected by this software agree with subjective visual assessment of mitochondrial morphology from raw microscope images. Collectively, our results indicate that these methods enable quantitative, objective, and consistent measurements of mitochondrial morphology using an automated software approach.

Further evidence for this method's utility can be found in comparisons of our experimental results to published observations. Studies in several cell types have reported mitochondrial fragmentation upon acute exposure to high concentrations of glucose (Yu et al., 2011; Trudeau et al., 2010; Yu et al., 2006). Yu et al. reported a two phase, fission-fusion mitochondrial morphology response consistent with the time course observed in this study (Yu et al., 2011). Finally, studies in several cell types report mitochondrial fragmentation upon acute exposure to hydrogen peroxide (Fan et al., 2010, Jahani-Asl et al., 2007, Jendrach et al., 2008), echoing our own results in SH-SY5Y neurons. Our findings that an unsupervised algorithm was able to recreate these results indicates that subjective or user-guided assessment of mitochondrial morphology is no longer necessary.

Moreover, the quantitative values for mitochondrial morphological parameters obtained in this study are realistic. Mitochondrial morphology studies reporting direct measurement of mitochondrial length typically report values in the range of 1–15 μm (Rintoul et al., 2003; Nozaki et al., 2001; Wang et al., 2013; Korobova et al., 2013; Choi et al., 2015; Cagalinec et al., 2013; Korobova et al., 2014; Gan et al., 2014), consistent with the values obtained in this study. Literature values for mitochondrial width/diameter generally fall in the range of tens to hundreds of nanometers (Moran et al., 2010; Finley et al., 2012; Karam et al., 2003; Santamaría et al., 2006; Holloway et al., 2010), also consistent with those obtained in this study. Moreover, in studies that measure dynamic changes in both length and width, width has been reported to be far more stable than length (Moran et al., 2010; Santamaría et al., 2006), agreeing with our findings of many statistically significant changes of average mitochondrion length but none in average mitochondrion width.

An interesting methodological finding in this study is that fixation at room temperature resulted in significantly and substantially different mitochondrial morphology than did fixation at 37 °C. We tested this effect after observing markedly different mitochondrial morphologies between samples fixed with cold formaldehyde vs warm formaldehyde during preliminary methods development. This study did not attempt to determine the mechanism underlying these differences, but it is reasonable to propose that studies of mitochondrial morphology in fixed cells should use 37 °C as a physiologically relevant temperature during fixation. Given that many studies in the literature use cooler or unspecified fixation temperatures, it is possible that mitochondrial morphology endpoints in reports such as these may have been compromised.

One advantage of the algorithms developed in this study is their ability to reliably detect differences in mitochondrial morphology that are too subtle to see with the naked eye, as was the case for both Wistar and GK SMCs exposed to 25 mM glucose. With this advantage comes the potential for aberrant detection of artificially significant results. We found that a

total of 3 slides per treatment group or time point per experimental repetition with 3 experimental repetitions and 3 images per slide for a total of $n = 27$ images and 9 slides per treatment group was adequate to achieve statistical significance in these cases. This large sample size was necessary in part because of the great heterogeneity of mitochondrial network morphologies observed in a single slide and in part due to the subtlety of some of the effects recorded. It would not be feasible to use sample sizes this large if measurements had been performed by hand. Using our algorithms, little additional time is dedicated to analysis with increasing sample size. In the event of a subtle effect which is detected by the software but not by the naked eye, we highly recommend comparing the results from individual experimental repetitions as in Fig. 6A. For purposes of this study, if an endpoint difference was not visible to the naked eye, it was included only if successive experimental repetitions gave qualitatively similar results.

In the introduction of this paper we proposed that software measurements of mitochondrial morphology should not require a specific microscope, software expertise should not be required to apply the method, endpoints should be consistent between experimental repetitions, no specific portion of the mitochondrial network should be excluded from analysis or incorrectly analyzed due to algorithm performance, and the endpoints reported to the user should hold intuitive and tangible meanings. Because our software is fully automated, no computational expertise is required to operate it. Endpoints were found to be quantitatively similar between successive experimental repetitions, and the method of binarization employed in this study has been proven superior to simple thresholding in detecting the entire mitochondrial network and it has also been successfully applied across multiple imaging systems and sample preparations (Peng et al., 2010). The specific software technique described in this paper has been successfully applied not only for methodological validation, but also for cell biology research both within our group (Keller et al., in revision), and in a collaborating laboratory (Badani et al., in revision). Finally, the primary endpoints reported to the user are average length and width of a single mitochondrial body, which can easily be replicated with by-hand measurements.

The measurements yielded by this software proved to be not only realistic but also exquisitely sensitive to subtle changes in mitochondrial morphology, potentially enabling measurement of previously undetectable effects. Moreover, the quantitative nature of this method allows separate assessment of effect existence and effect size. In order to elicit detectable morphometric changes, non-physiological experimental perturbations have been reported. Beyond simple quantification of mitochondrial length and width, this software allows assessment of differential effects in perinuclear and peripheral mitochondria, and could very well be used to measure experimental endpoints and the single-mitochondrion level in experiments that call for this analysis. Although fixed cells were used in this study, the method employed for measurement is equally applicable to live cells and perhaps even to videomicroscopy if applied on a frame-by-frame basis. In principle, these algorithms could also be applied to morphologic measurements of other intracellular structures such as the endoplasmic reticulum or Golgi apparatus. The investigators will gladly provide this software to other research groups upon request and assist in adoption of this technology.

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Fig. 1. Method used to binarize cytoplasm images. Cytoplasm is stained using a monoclonal antibody for nitrotyrosine or α -actin. The raw microscope image (A) is then blurred twice using a Gaussian blur, and the difference between the once-blurred (B) and twice-blurred (C) images is used to estimate the local intensity prominence. This intensity prominence image (D) is then thresholded to create a binary image (E) of the cell's cytoplasm.

Fig. 2.

Removal of poorly detected mitochondrial bodies. Individual mitochondrial bodies with a total size of less than 15 pixels or a calculated width greater than 500 nm were excluded from analysis in order to limit the influences of noise and out of focus light, respectively. (A) Mitochondria excluded from analysis in a representative HUVEC cell fall into categories of either so small that discrete pixel size compromises measurement (small bodies) or unrealistically wide (large bodies). (B) These exclusion criteria were empirically determined to effectively remove out of focus mitochondrial bodies (red, many in bottom left) and image noise aberrantly detected (red, many in bottom right). Although nearly 50% of detected mitochondrial mass was discarded in this sample image, over 80% of detected mitochondrial mass was used for analysis in all experimental groups tested. (C) Histogram of mitochondrial width in a representative HUVEC cell prior to removal of poorly detected bodies. The distribution is discontinuous and includes physiologically implausible values (e.g. width <0 or width >500 nm). (D) Histogram of mitochondrial width in a representative HUVEC cell after removal of poorly detected bodies. The distribution is continuous and includes physiologically plausible values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Calculation of mitochondrial dimensions and calibration of software measurements using fluorescent beads of known size. (A) Deterministic relationship between edge length, cross-sectional area, and length and width. Assuming the typical mitochondrion to be shaped like an elongated tube, the average length and width in the network can be calculated from the total network area, edge length, and number of mitochondria. (B) The same technique was used to calculate bead diameter as was used for mitochondrial dimensions, but assuming spherical rather than tubular morphology. Raw bead diameter measurements were corrected for signal-noise ratio (SNR) as previously discussed (see Fig. 2A). At bead concentrations spanning four orders of magnitude, calculated bead diameter remained constant at 585 nm using the Olympus microscope and 501 nm using the Leica microscope, compared to a true mean diameter of 400 nm. The total system bias of 185 nm and 101 nm for the Olympus and Leica images, respectively, was then subtracted from average mitochondrion length and width values to correct for the microscope PSF and errors in image processing.

Fig. 4.

Effects of signal-noise ratio on measured mitochondrial dimensions. Signal-noise ratio (SNR) was determined by dividing the mean pixel intensity in foreground regions (as determined by binarization) by the mean pixel intensity in background regions. SNR was found to contribute to variance in dimensional measurements of both fluorescent beads and mitochondrial networks. (A) SNR is the dominant source of variance in measurement of fluorescent beads of known, fixed size. Decreasing SNR is associated with over-estimation of bead diameter. (B) SNR is the dominant source of variance in measurements of mitochondrial width. The data shown is from HUVEC cells with no perturbation. The slope of the regression line relating inverse signal strength $((\text{SNR}-1)^{-1})$ to mitochondrial width did not vary significantly between cell types ($p > 0.05$) and did not vary significantly ($p > 0.05$) from the slope of the regression line for fluorescent beads. Measurements of mitochondrion width were therefore corrected for SNR using a linear regression as shown above and extrapolating to an SNR of infinity. (C) SNR contributes significantly to variance in measurements of mitochondrial length. This effect varies greatly in magnitude and was therefore not used for correction of raw mitochondrial measurements in order to avoid spurious detection of differences in mitochondrial length between experimental groups.

Fig. 5. Consistency of experimental repetitions. (A) Time course of mean mitochondrion length in GK SMCs exposed to 25 mM glucose. Individual experimental repetitions (n = 9 images) are shown in red, while the overall average (n = 27 images) is shown in black. (B) Agreement between repetitions in mitochondrion length. Very strong agreement is observed between successive experimental repetitions (R = 0.93). (C) Agreement between repetitions in mitochondrion width. Strong agreement is observed between successive experimental repetitions (R = 0.93). (D) Magnitude of discrepancy between experimental repetitions for each of the reported measures. Mitochondrion length differs by an average of 330 nm between experimental repetitions, as opposed to 12 nm for mitochondrion width, 0.54% for fraction of cytoplasm filled by mitochondria, and 0.2 for perinuclear preference (log of ratio of mitochondrial mass in perinuclear space to mitochondrial mass in peripheral space). (E) Agreement between repetitions in mitochondrial mass. Strong agreement is observed between successive experimental repetitions (R = 0.89). (F) Agreement between repetitions in perinuclear preference. Modest agreement is observed between successive experimental repetitions (R = 0.74). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Cell line characteristics. (A) Representative microscope images of cell types used in this study. Mitochondria were stained using a Tom20 antibody, cytoplasm using a nitrotyrosine antibody, and nuclei using DAPI. All panels are shown at 4× zoom to improve clarity; the original images are 4× this size. (B) Average mitochondrion length by cell type. HUVECs had the longest mitochondria at 7.5 μm while Wistar SMCs had the shortest at 4.6 μm. Each cell type displayed a significantly different ($p < 0.0001$) average mitochondrion length from every other cell type, with the exception of GK SMCs and SH-SY5Ys, which did not differ significantly ($p > 0.05$). (C) Average mitochondrion width by cell type. SH-SY5Ys had the widest mitochondria at 243 nm while HUVECs had the thinnest at 112 nm. All cell type differences were statistically significant ($p < 0.0001$), except for HUVECs and GK SMCs, which did not differ significantly ($p > 0.05$). (D) Mitochondrial mass by cell type. SH-SY5Ys had the greatest fraction of the cytoplasm filled by mitochondria at 7.5% while HUVECs had the least at 2.5%. All cell type differences were highly significant ($p < 0.0001$). (E) Perinuclear preference (log of ratio of mitochondrial mass in perinuclear space to mitochondrial mass in peripheral space) by cell type. HUVEC mitochondria showed the greatest degree of perinuclear localization with a perinuclear preference of 3.06, while SH-SY5Ys showed the least with a perinuclear preference of 1.46. All cell type differences were statistically significant ($p < 0.0001$), except for SH-SY5Ys and GK SMCs, which did not differ significantly ($p > 0.05$).

Fig. 7.

Effects of temperature during fixation on mitochondrial morphology. HUVEC cells were fixed using either body-temperature or room-temperature reagents and subsequently incubated at either body-temperature or room-temperature for fifteen minutes during fixation. (A) Representative images of mitochondrial networks following each treatment. All panels are shown at 4× zoom to improve clarity; the original images are 4× this size. Cells fixed with room-temperature reagents and incubated at room temperature show visible mitochondrial fragmentation. (B) Mean mitochondrion length by treatment. Cells fixed with room-temperature reagents and incubated at room temperature show a highly significant ($p < 0.0001$) reduction in mean mitochondrion length compared to other treatments, which do not differ significantly ($p > 0.05$).

Fig. 8.

Dynamic mitochondrial network responses. GK (red) and Wistar (black) SMCs were incubated in 25 mM glucose for the times indicated in the figures. (A) Mean mitochondrion length vs time in 25 mM glucose. Mean mitochondrion length was greater in GK SMCs than in Wistar SMCs at all time-points measured ($p < 0.0001$). Wistar SMCs displayed a highly dynamic response to glucose exposure including significant fragmentation between 10 and 30 min ($p < 0.01$) and a significant increase in networking between one hour and four hours ($p < 0.0001$). Heterogeneity was somewhat greater within groups among GK SMCs, but fragmentation and subsequent networking were statistically significant ($p < 0.05$) at the same time points as for the Wistar SMCs. (B) Representative images of the two cell types at baseline (top) and following 30 mins incubation in 25 mM glucose (bottom). Baseline cell type differences were more readily visible than were dynamic changes in mitochondrial morphology. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9.

Effects of 200 nM H_2O_2 on mitochondrial morphology in SH-SY5Ys. (A) Representative images before and after peroxide treatment. All panels are shown at 4 $\times$ zoom to improve clarity; the original images are 4 $\times$ this size. Mitochondria were visibly fragmented and seemed to have redistributed towards the peripheral space following treatment, but changes were difficult to observe due to the very small size of the cells. (B) Mean mitochondrion length before and after peroxide treatment. Hydrogen peroxide induced a significant decrease in mean mitochondrion length ($p < 0.0001$). (C) Perinuclear preference (log of ratio of mitochondrial mass in perinuclear space to mitochondrial mass in peripheral space) before and after peroxide treatment. A significant decrease ($p < 0.01$) in perinuclear preference was observed, indicated redistribution of mitochondria from perinuclear to peripheral spaces.

Fig. 10.

Influences of optical slice thickness on mitochondrial morphology measurements. Slice thickness was modulated by changing pinhole diameter. This technique has the additional effect of changing signal-noise ratio (SNR). (A) Table of measurement biases introduced by modulation of slice thickness by changing pinhole diameter. All measurements except for perinuclear preference were significantly biased ($p < 0.05$) at all slice thicknesses relative to a pinhole diameter of 1.0 AU. (B) Measured bead diameter at various pinhole settings. Apparent bead diameter was significantly greater at pinhole diameters of 1.5 AU ($p < 0.05$) and 2.0 AU ($p < 0.0001$) relative to 1.0 AU. Apparent bead diameter did not change from baseline at 0.5 AU ($p > 0.05$). Because all beads imaged were in the same plane, differences in apparent bead diameter reflect changes in SNR. (C) Statistical significance of SNR and slice thickness influences on mitochondrial morphology measures in multiple linear regression. Both slice thickness and SNR were found to influence measurements of mitochondrial width and fraction of cytoplasm filled by mitochondria. After correcting for the influences of SNR, slice thickness was found to have no independent effect ($p > 0.05$) on measurements of mitochondrion length. (D) SNR by pinhole diameter. Consistent with the effects of pinhole diameter on apparent bead diameter, SNR is significantly reduced at 1.5 AU ($p < 0.05$) and 2.0 AU ($p < 0.01$) relative to 1.0 AU, while SNR at 1.0 AU and 0.5 AU do not differ significantly ($p > 0.05$).

Fig. 11.

Influences of image plane selection on measurements of mitochondrial morphology. (A) A representative plane was chosen for each image to best illustrate the complete mitochondrial network. (B) The representative plane was compared to an overlapping plane 0.3 μm above the representative plane. (C) The representative plane was also compared to a non-overlapping plane 1.2 μm above the representative plane. (D) Finally, the representative plane was compared to a maximum intensity projection spanning the entire mitochondrial network. (E) Comparison of mitochondrial morphology measurements in alternate planes or projections to measurements in a representative plane. Strong agreement is observed for all metrics between overlapping planes. Weak but statistically significant ($p < 0.05$) agreement is observed for metrics compared between non-overlapping planes. Strong agreement is observed between a representative plane and a maximum intensity projection. Although many statistically significant measurement biases are observed, the only one for which effect size is great enough to be meaningful for experimental design is in perinuclear preference in the non-overlapping plane. For most cells imaged, the non-overlapping plane did not include peripheral portions of the cytoplasm, thus yielding an inappropriate assessment of mitochondrial network localization.