Biochimica et Biophysica Acta 1853 (2015) 348–360

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Quantitative analysis of mitochondrial morphology and membrane potential in living cells using high-content imaging, machine learning, and morphological binning

Anthony P. Leonard [a,b,*], Robert B. Cameron [b], Jaime L. Speiser [c], Bethany J. Wolf [c], Yuri K. Peterson [b], Rick G. Schnellmann [b], Craig C. Beeson [b], Bärbel Rohrer [a,d]

[a] Dept. of Ophthalmology, Medical University of South Carolina, USA
[b] Dept. of Drug Discovery and Biomedical Sciences, Medical University of South Carolina, USA
[c] Dept. of Public Health Sciences, Medical University of South Carolina, USA
[d] Ralph H. Johnson Memorial Veteran's Administration Hospital, USA

ARTICLE INFO

Article history:
Received 27 February 2014
Received in revised form 2 November 2014
Accepted 4 November 2014
Available online 13 November 2014

Keywords:
High content microscopy
Mitochondrial dynamics
Mitochondrial function
Morphometry
Image cytometry
Mitochondrial toxicology

ABSTRACT

Understanding the processes of mitochondrial dynamics (fission, fusion, biogenesis, and mitophagy) has been hampered by the lack of automated, deterministic methods to measure mitochondrial morphology from microscopic images. A method to quantify mitochondrial morphology and function is presented here using a commercially available automated high-content wide-field fluorescent microscopy platform and R programming-language-based semi-automated data analysis to achieve high throughput morphological categorization (puncta, rod, network, and large & round) and quantification of mitochondrial membrane potential. In conjunction with cellular respirometry to measure mitochondrial respiratory capacity, this method detected that increasing concentrations of toxicants known to directly or indirectly affect mitochondria (*t*-butyl hydroperoxide [TBHP], rotenone, antimycin A, oligomycin, ouabain, and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone [FCCP]), decreased mitochondrial networked areas in cultured 661w cells to 0.60–0.80 at concentrations that inhibited respiratory capacity to 0.20–0.70 (fold change compared to vehicle). Concomitantly, mitochondrial swelling was increased from 1.4- to 2.3-fold of vehicle as indicated by changes in large & round areas in response to TBHP, oligomycin, or ouabain. Finally, the automated identification of mitochondrial location enabled accurate quantification of mitochondrial membrane potential by measuring intramitochondrial tetramethylrhodamine methyl ester (TMRM) fluorescence intensity. Administration of FCCP depolarized and administration of oligomycin hyperpolarized mitochondria, as evidenced by changes in intramitochondrial TMRM fluorescence intensities to 0.33- or 5.25-fold of vehicle control values, respectively. In summary, this high-content imaging method accurately quantified mitochondrial morphology and membrane potential in hundreds of thousands of cells on a per-cell basis, with sufficient throughput for pharmacological or toxicological evaluation.

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1. Introduction

Mitochondrial morphology is an actively regulated and dynamic feature altered via mitochondrial dynamics (MD) — the combination of mitochondrial fission, fusion, biogenesis, and mitochondrial autophagy (mitophagy). Changes in MD have been associated with regulation of oxidative metabolism, calcium homeostasis, and apoptotic or necrotic cell death [1,2].

The evaluation of mitochondrial morphology using microscopy was originally limited to manual classification. The development of multiphoton microscopy and fluorescent dyes, coupled with high-definition videography (enabling the capture of high-resolution images in living cells over time), greatly improved the qualitative observation of MD. However, the large number of mitochondria within cells makes manual classification and quantification prohibitive, as the ~100 individual mitochondria observed within each cell translates to $>10^4$ mitochondria per high power (40×) microscopic field. Furthermore, quantification of mitochondrial morphology across a series of experiments greatly increases the volume of data and complexity of the analysis. Due to these limitations, many researchers report mitochondrial morphology via display of representative fields per treatment group and without robust quantification. Consequently, throughput is insufficient and the resultantly small subsets of mitochondria evaluated may be affected by sampling bias.

Previous studies using microscopy and computational image analysis, commonly referred to as high-content microscopy or image cytometry, have demonstrated the strength of this approach with regard to

* Corresponding author at: MUSC Room QF305, 280 Calhoun St., Charleston, SC 29425, USA. Tel.: +1 843 817 2074.

http://dx.doi.org/10.1016/j.bbamcr.2014.11.002
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limiting observer and selection bias in morphological evaluations while increasing throughput [3–8]. While several groups have made efforts to apply high-content fluorescence microscopy to the task of mitochondrial morphological evaluation [4–8], only the approach of Koopman et al. has demonstrated the throughput necessary for pharmacological/toxicological evaluation [8]. However, by looking at morphometric measures across the diverse range of mitochondrial morphological subtypes in aggregate, that approach loses power to detect subtler changes in interconnectedness, and is not well suited to quantify the abundance of morphological shapes that occur much less frequently than others. This is of particular note as in the present study, large & round (presumably swollen) mitochondria were observed at frequencies less than 10% of the abundance of other morphological shapes (Section 3.3.4). Similar to published machine learning methods characterizing mitochondrial shape, our method aims to describe interconnectedness. However, by measuring the relative frequency of four morphological phenotypes (shapes) comprised of networks, rod-like, puncta, and large & round, the method developed here additionally enables the quantification of mitochondrial swelling.

Our approach for analyzing individual mitochondrial morphologies is capable of large-scale mitochondrial measurement; that is to say millions to billions of mitochondria across thousands of cells within a single experiment can be classified according to interconnectedness and complexity. This was achieved through automated live-cell wide-field fluorescence imaging coupled with image-based computational high content analysis and the use of a machine learning-derived classifier to segregate mitochondria into four shape-based categories [9]. The method is further extended to observe changes in mitochondrial membrane potential ($\Delta\Psi_m$) by examining fluorescence intensity of a potentiometric dye within mitochondria. The development of a deterministic fully-automated algorithm improves throughput, allowing for evaluation of entire cellular populations within multiple replicate microscopic fields across repeated experiments, which in turn allows for decreased observer and sampling bias.

2. Materials and methods

2.1. Cell culture

661w photoreceptor cells were generously provided by Dr. M. Al-Ubaidi (University of Oklahoma) [10] and maintained under standard conditions using DMEM-HG media (Sigma-Aldrich #D-7777) supplemented with 10% fetal calf serum (FCS, Atlanta Biologicals #S11550) and alanylglutamine (GlutaMAX, Life Technologies #35050-061). Cells of passages 15–25 were cultured to 80% confluency before trypsinization and seeded at a cell density of 8000 cells per well in 96 well plates (Nunc Edge Plate, Thermo Scientific #167314) supplemented with 5% FCS (fetal calf serum) and edge reservoirs filled with phosphate-buffered saline (to prevent hydration-dependent microplate edge effects) (PBS; Life Technologies #14080-055). After cells reached 80% confluency (24 h), cells were washed in PBS, and media was changed to DMEM with 5.5 mM glucose without phenol red (Sigma-Aldrich #D-5030) and supplemented with 1% FCS to induce cell cycle arrest. Cells were used for experiments 24 h after this media switch. Treatments were for either 24 h (morphological analysis) or 1 h (membrane potential analysis). All compounds used for treatment were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified, and were prepared at 1000× concentration in DMSO before administration to cells (final [DMSO] = 0.1% v/v).

2.2. Live cell staining

Cells were stained with Hoechst 33342 (Anaspec #83218), and MitoTracker Deep Red FM (MTDR, Life Technologies #M22426). After determining the lowest concentration of dye necessary to acquire high signal-to-noise ratio (>3) images while maintaining exposure times under 1 s, cells were stained for 30 min at 37 °C with the above dyes in phenol-red-free DMEM supplemented with 1% v/v FCS (Hoechst at 10 μM and MTDR at 50 nM), after which the media was replaced for imaging.

2.3. Image acquisition and analysis: overview

Briefly, 661w photoreceptor cells cultured on 96-well plates were stained with Hoechst 33342 and MTDR, and imaged using wide-field fluorescence microscopy (see Fig. 1a and Section 2.4). A z stack of seven images was collected of MTDR-stained mitochondria and 2-D deconvolution applied to the stack to output a single in-focus field with out-of-focus information removed (a process that simulates confocal microscopy). Mitochondrial objects were identified from deconvolved and preprocessed images using the “object” segmentation algorithm in GE INCell Developer Toolbox 1.9.1, a variation of the “top hat” approach to segmentation (Fig. 1b–c). Developer Toolbox is available within the GE INCell Investigator 1.6.1 software package (GE Healthcare Bio-Sciences, Pittsburgh, PA). As mitochondria display a variety of shapes which indicate interconnectedness and health, four categories were established: puncta, rod, networked, large & round. Rods are an intermediate phenotype between puncta and networks. The large & round group likely represents a combination of pathologically swollen mitochondria as well as normal mitochondria undergoing fission or fusion (see Section 4). To automate classification of mitochondrial objects into these bins, 1386 mitochondria were manually classified, and a subset of 897 with 35 morphometric measures calculated on each were then used to train a classifier using conditional inference recursive partitioning [9]. The remaining 489 mitochondria (test data, not used to train classifier) were used to test its performance. The R code as well as the training and test data sets are included in Supplementary Files; the decision tree output is shown in Supplementary Fig. 2.

2.4. Automated microscopy—step 1: image acquisition

Wide-field fluorescence imaging of live cells was coupled with off-line deconvolution of the MTDR-stained mitochondria to increase acquisition speed (Fig. 1a). Lateral spatial resolution was 717 nm by Rayleigh criteria and 555 nm (3 pixels) by Nyquist criteria, whereas the average width (narrowest dimension) of an individual mitochondrion is ~500 to 1000 nm [11]. Stained cells were imaged using filters corresponding to each dye and polychroic mirror (“X”, QUAD1) on the GE INCell 2000 Analyzer automated wide-field fluorescence microscope. The objective used was a 40× Nikon ELWD NA 0.6 matched with the large-format 2048 × 2048 pixel 12-bit Coolsnap K4 camera (z/sampling height 1.55 μm, xy/lateral pixel dimensions of 0.185 μm). Resolving small objects near resolution limits like mitochondria requires high signal-to-noise ratio images. To achieve this, seven images were acquired in a set of z-stacks 1.55 μm apart on the Cy5 filter set (MTDR-stained mitochondria) to enable 2-D deconvolution (see Section 2.5.1). A single z section was obtained for Hoechst-stained nuclei on the DAPI filter set. Two series of images (fields or locations) for each set of wavelengths were acquired in each well, and all conditions were run in duplicate wells. Thus, each treatment condition was represented by 4 to 8 fields per plate, leading to 12 to 72 fields overall acquired for each condition. Each plate was treated as an individual experiment, n = 3–9 per condition.

2.5. Computational analysis

2.5.1. Step 2: image pre-processing

Preprocessing alters the rate of detection of mitochondrial objects, namely improving the ability of the segmentation algorithm to identify mitochondrial objects from background (Section 2.5.2). Image stacks were opened in the GE INCell Developer Toolbox 1.9.1 program. Using

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