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Combining Morphological and Textural Features to Characterize Mitochondrial Structure

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Abstract— **Understanding the dynamics of cellular responses is crucial when evaluating treatments and/or pathological conditions. Changes in sub-cellular structures can be assessed by imaging and used to evaluate cellular responses to external factors. One of the key sub-cellular structures which can provide us with information about cellular metabolic states are mitochondria. They are the cell 'power- houses' responsible for energy production. They also produce bioactive molecules that regulate a wide range of cellular functions. Thus, investigating changes in mitochondria organization/structure and their localization within the cell may provide new opportunities to identify functional phenotypes and activation states within tissues. The mitochondrial structure changes dynamically and the main formed structures can be classified as network-like arrangements, puncti, or rods. In this study, super-resolution confocal microscopy was used to assess mitochondrial structure and a combination of image- based morphological and textural features are used to extractive objective information about the main mitochondrial structure's shapes such as fiber orientation, length, concentration based on pixel intensities, degree of structural organization and other related features. Our preliminary results show that morphological feature-based metrics were not successful in differentiating the mitochondrial arrangements. However, textural features provided the potential to allow the quantification of differences between puncta and fiber-network structures. Rods- like structures are still under investigation. By combining morphological and textural features we aim to create an image- based score using mitochondrial structures commonly present in cells to perform a systematic image-based differentiation of cellular metabolic states and activation *in vitro* and *ex vivo*.**

Keywords— **Mitochondria, Confocal microscopy, morphology, MiNA, GLCM**

I. Introduction:

When studying cell biology, the investigation of sub-cellular components such as mitochondria becomes a relevant tool to assess metabolic activity and cellular states in responsiveness to external stimuli.

Not only are mitochondria crucial in Adenosine Triphosphate (ATP) production, but also produces bioactive molecules that regulate cell function, such as Nicotinamide adenine dinucleotide (NADH). Mitochondria changes dynamically [2]. Its morphology typically comprises of different types of structures, such as small spheres, short tubules to elongated tubules as well as networks, which can be often associated to cell, tissue type and functional states [3]. In addition, fusion and fission leads to mitochondria structures that range from fragmented to dense networks of large fiber-like connected structures [4]. To visualize and distinguish sub-cellular organelles, it is crucial to use high resolution microscopy, like confocal imaging. Confocal microscopy is a powerful tool to visualize sub-cellular structures with a great level of detail [5]. By utilizing laser scanning technology and a pinhole aperture, the technique's advantage lies in its ability to reduce background noise and out-of-focus light, resulting in sharper, clearer images. Through fluorescent labeling or dyes targeting specific cellular components, we can selectively visualize mitochondria in fixed and living cells, observing their movement, distribution, and interactions with other cellular structures in real-time. This work explores the integration of high-resolution imaging and image analysis approaches to obtain quantitative metrics associated to mitochondrial structure. By differentiating between some of the most common arrangements found intracellularly (puncta, finer, and rods), we aim to provide a strategy to characterize and classify different cellular metabolic states and map potential responses to treatments, infections, and changes within the cellular microenvironment.

II. Methods:

A. *Cells source and cell culture procedure*

All animal procedures were approved by the Carleton University Animal Care committee and were conducted in accordance with the guidelines provided by the Canadian Council for Animal Care. Bone marrow cells were isolated from the tibias and femurs of 3-5-month-old C57BL/6J mice (The Jackson Laboratory). Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) media with 10% (v/v) Fetal Bovine Serum (FBS), 1% (v/v/) penicillin/streptomycin (Life Technologies), and 15% L929 fibroblast cell-conditioned medium on a 100 mm Petri dish as previously described [6]. After 10 days of differentiation, the resulting primary mouse bone marrow derived macrophages (BMDM) were detached, counted, and plated into µ-Slide 8 Well (ibidi) at 5 x 10^4 cells/cm^2 under 5mM glucose conditions (DMEM medium without glucose/glutamine/pyruvate/phenol red supplemented with 5 mM glucose, 1 mM Pyruvate, 4 mM

Glutamine, 10% FBS [Life technologies], and 15% L929-conditioned media). Cells were allowed to rest overnight before staining in a humidified incubator at 37°C, at atmospheric pressure, 5% (v/v) carbon dioxide/air, and 95% humidity.

B. Cellular staining

Mitochondria were stained using MitoTracker™ Deep Red FM (MTDR) (ThermoFisher Scientific, Canada), cell nuclei were stained using DAPI (ThermoFisher Scientific, Canada), filamentous actin (F-actin) was labelled using Alexa Fluor™ 488 Phalloidin (Phalloidin, ThermoFisher Scientific, Canada). Reagents used for staining include 4% paraformaldehyde (PFA, Sigma-Aldrich, Canada) in Phosphate-buffered saline (PBS, ThermoFisher Scientific, Canada), 0.2% (v/v) Triton X-100 (Fisher Scientific, Canada) in PBS, and 2% (wt/v) bovine serum albumin (Tocris Bioscience) in PBS (2% BSA-PBS). Live cells were stained with 300 nM MTDR in 5 mM glucose DMEM media without FBS and L929-conditioned media and were placed in a humidified incubator at 37°C, atmospheric pressure, 5% (v/v) carbon dioxide/air, and 95% humidity for 40 minutes. Subsequently, cells were fixed in 4% PFA in PBS (20 minutes at room temperature (RT)) and permeabilized with 0.2% Triton X-100 in PBS (10 minutes at RT). The cells were then incubated with 2% BSA-PBS for blocking non-specific binding (30 minutes at 4°C) and stained with 165 nM Phalloidin in 2% BSA-PBS for 1 hour at RT in the dark. The nuclei were stained with 300 nM DAPI in 2% BSA-PBS for 5 minutes at RT in the dark. The stained cells were stored in PBS at 4°C in the dark until imaging.

C. Microscopy Imaging

The stained BMDM cells were imaged using the Zeiss LSM 980 confocal microscope part of the Tissue Engineering and Applied Materials (TEAM) Hub imaging facility at Carleton University. The microscope was set up to acquire super resolution images using Airyscan. This microscope utilizes an Airyscan 2 detector (Carl Zeiss AG, Germany), setup with beam splitters in 405 nm and 514/639 nm, used to image DAPI and MTDR/633 dyes, respectively. A Plan-Apochromat x63/1.4 oil immersion objective (Carl Zeiss AG, Ger- many) was used. The image parameters were set as follows: a digital gain of 700V, laser power of 2% and 0.5% were set for MTDR/633 and DAPI respectively, and LSM scan speed was set to 5.

D. Image Analysis

A total of 77 images were evaluated in this preliminary phase. Image pre-processing was performed using MATLAB (version R2023b, MathWorks), and included image contrast enhancement, Wiener, and Gaussian filter to address speckle noise and smooth the image, respectively. Morphological information about the mitochondrial structures were obtained by using the MiNA plugin designed for Fiji [7]. The features were obtained following the steps outlined by A.J. Valente, et al [7]. The morphological features obtained associated to fiber-like, rods and puncta structures of mitochondria were footprint/fiber area, branch, and network length. OrientationJ (Fiji plugin) was also used to characterize the orientation and isotropy of structures (mostly fibers) [8]. The OrientationJ plugin is based on gradient structure tensor of a local neighborhood of pixels. Finally, using MATLAB, we obtained first order statistics from each image (mean, variance, skewness, and kurtosis) [9]. Textural features, which can be associated to fiber organization, homogeneity, and smoothness for example, were also obtained using our MATLAB custom script. The textural features explored are obtained from a Gray Level Co-Occurrence Matrix (GLCM), which quantifies how often pixel values occur, which quantifies how often pixel values occur, which then provides information about a region of interest (ROI) [10]. Textures were obtained for 4 different angles (0, 45, 90, 135) and window size was 8 pixels. Additional tests were performed considering the cell- body as the ROI. All the extracted features, both morphological and textural, will be combined to create a score that will be used to characterize the different mitochondrial structures (i.e., fiber-network, rods, puncta) and be correlated with cellular functional states, responses to treatments and/or external stimuli.

E. Statistical Analysis

The Shapiro-Wilks test was used to define data normality. Non-parametric features were mitochondrial footprint, average branch length, correlation, and entropy; parametric features were average branch length, energy, contrast, and homogeneity. The Wilcoxon signed-rank test and paired t-test were selected for the non-parametric and parametric analysis respectively. The Wilcoxon signed-rank test and paired t-test was done using MATLAB's statistical and machine learning toolbox. As a result, the null hypothesis (H_0) is that there is no difference between puncta and fiber-network. Outliers were removed using Grubbs test from MATLAB's statistical and machine learning toolbox.

III. Preliminary Results:

A. Mitochondrial Network Analysis (MiNA)

This analysis was used to quantify the mitochondrial footprint, and the length of the fiber-like mitochondrial network. Mitochondrial footprint refers to the area or volume consumed by the mitochondrial signal [7]. Average branch length refers to the accumulation of all the lines that makeup the mitochondria, and the average network branch length refers to the accumulation of all the attached lines that make up the mitochondria (fiber-network structure) [7].

Figure 1 shows the structures that are being characterized in this study, but also how the MiNA considers the structure.

The fiber-network structure is an accumulation of branches and connecting points. The puncta are usually small high intensity clusters of pixels. Rods are branches that do not have connections and are considered individuals.

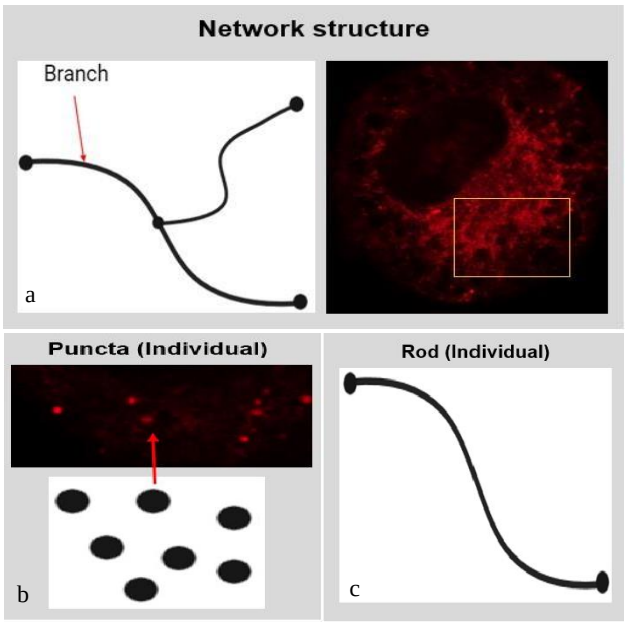

Fig. 1: Representative mitochondrial structure. a) Example of network structure. b) Example of puncta structure. c) Example of Rod structure. Created with BioRender.com.

Figure 2 shows that MiNA works when there is a clear network (Figure 2d). However, it does not work as well when the images have more puncti. MiNA skeletonizes the image and creates network structures when there are no fiber-like structures in the image.

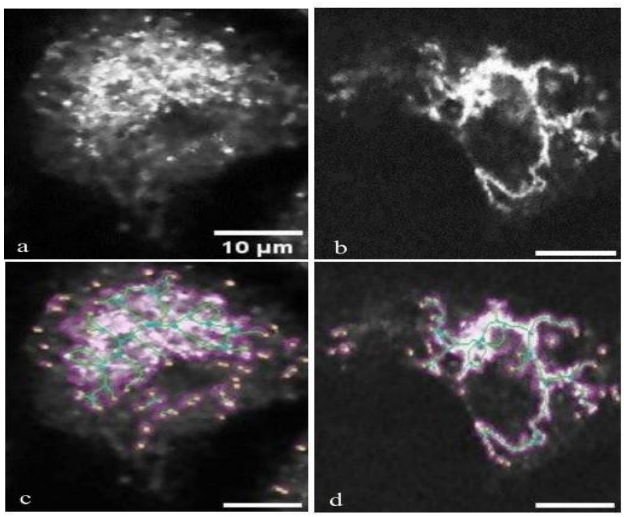

Fig. 2: Representative confocal microscopy images (a, b) before and after MiNA processing (c, d).

When comparing the results for the mitochondrial footprint, average branch length and network branch length no difference was statistically significant, as per values displayed in Table 1. The heterogeneity of the mitochondrial distribution on each individual cell contributes to the challenge of successfully distinguishing which structural feature will be the most prominent. We anticipate that increasing the number of images containing these specific structures might allow us to successfully distinguish them in the near future, expanding the analysis to characterize responses due to treatments or disease states.

Table 1 Features obtained from MiNA

Structure	Mitochondrial footprint [µm]	Average Branch Length [µm]	Average-Network Branch Length [µm]
Fiber-Network	357.2 (158.9)	3.01 (0.64)	6.01 (3.17)
Puncta	314.1 (118.4)	2.96 (0.72)	5.34 (3.03)

B. Gray Level Co-Occurrence matrix (GLCM)

Texture analysis was used to characterize mitochondrial structure considering the relationships between pixels, and therefore, assessing its structure regarding level of organization, fragmentation, smoothness, for example.

When comparing the textural features obtained from each structural group, fiber-networks and puncti structures, entropy, gauging the disorder level of structures within an image, and energy, assessing the uniformity or homogeneity of structures within the image, were significantly distinct statistically. The next steps will involve collecting additional images containing rod-like structures and investigate if textures will be able to capture significant differences, as rods and networks tend to have more similar structures than when compared to puncti.

Table 2 GLCM textural features

Structure	E	CN	CR	H	ET
Fiber-Network	**0.27 (0.12)**	0.20 (0.07)	0.97 (0.02)	0.92 (0.12)	**0.94 (0.07)**
Puncta	**0.24 (0.08)**	0.20 (0.06)	0.97 (0.01)	0.92 (0.02)	**0.97 (0.03)**

E: Energy; CN: Contrast; CR: Correlation; H: Homogeneity; ET: Entropy.

IV. Conclusion:

Mitochondria along with the other cellular organelles can provide information about the cell state and indicate specific responses associated to changes in the cellular microenvironments due to external stimuli (e.g., pathological alterations, therapeutic interventions). Characterizing the distinct types of mitochondrial structures provides us with information about the cellular metabolic state.

This preliminary study shows textural analysis can reliably capture differences between puncta and fiber-network mitochondrial structures, without the need of extensive processing. In the next stages, different image features will be further explored and combined to distinguish all 3 main types of mitochondrial structures commonly observed. Various gray level texture analysis matrices, like the Gray Level Run Length Matrix (GLRLM), enable the extraction of diverse features such as fragmentation level, fiber lengths, and coarseness. Finally, a score will be created based on the extracted image features in unstimulated cells and cells stimulated with diverse inflammatory and microbial ligands to characterize the cell activation/metabolic state, providing an objective metric to support functional data. The images will be categorized into the different groups of structures by visual inspection and then compared against the characterization done by the image-based score. These scores will then be tested to see if they can identify activation states across diverse healthy and diseased tissues.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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