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BOOK OF PROCEEDINGS

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Slight cooling during growth induced changes in filamentous fungi hypha mitochondrial morphology

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Abstract: Adaptive changes in mitochondrial morphology are associated with changes in the mitochondrial function and metabolic fitness of eukaryotic cells. We previously described in young hyphae of the filamentous fungus *Phycomyces blakesleeanus* a dramatic effect of an increase in ambient temperature during growth: a 3°C warmer environment compared with a control temperature of 22°C resulted in the appearance of long elongated ("tubular") mitochondria accompanied by an increase in lipid droplet density. Here, we examined how cooler ambient temperature (18°C) during growth affects mitochondrial morphology in *P. blakesleeanus* compared with the control grown at 22°C. We used two-photon fluorescence imaging (TPEF) of live hyphae stained with the vital mitochondrial dye rhodamine 123. Extraction of relevant parameters (number, size, and shape of mitochondria) from TPEF images was performed using the Ilastik machine learning-based software. The suitability of the Ilastik analysis was compared with the Particle Analysis (ImageJ). Cold treatment resulted in the appearance of tubular mitochondrial morphology that was absent in the control group. Tubular mitochondrial morphology appears to be an adaptive feature that occurs in both warmer and colder conditions and is likely part of the stress response.

Keywords: *Phycomyces*, Imaging, TPEF, Machine-learning, Ilastik

1. Introduction

Mitochondria are very plastic organelles capable of morphologically adapting in order to meet cellular needs. The conditions that require more efficient quality control or greater respiratory capacity lead to fragmentation or fusion of mitochondria,

respectively [1]. We have previously shown that a 3°C warmer environment induces the appearance of tubular mitochondria in the filamentous fungus *Phycomyces blakesleeanus* [2]. Here we investigate the effects of a 3°C cooler environment on mitochondrial morphology.

2. Methods

Hyphal cells of the unicellular filamentous fungus *Phycomyces blakesleeanus* (Burgeff), wt (NRRL 1555(−)), were grown in illuminated stationary plates at 22°C for 16-21 h, as described previously [3], or at 18°C for cold treatment. Mitochondria stained with 5 μM rhodamine123 (Rhd123) were imaged *in vivo* by two-photon fluorescence microscopy (TPEF) of hyphae. Images were obtained from five separate cultures. For two-photon excitation of Rhd123, we used a Ti:Sa laser tuned to 800 nm (160 fs pulse duration, 76 MHz repetition rate) focused with the Zeiss Plan Neofluar 40x1.3 objective. The signal was detected through a MF530/43 bandpass interference filter (ThorLabs, USA). Details of the experimental setup for TPEF were described in [4]. Raw images were analyzed in both Ilastik and ImageJ Particle Analysis software packages using individually thresholded images to achieve high-quality segmentation of the bright structures representing mitochondria. Only images with a sufficiently high signal-to-noise ratio (to clearly distinguish the structures from the background) were analyzed. Statistics: ANOVA with multiple comparisons and Holm-Sidac correction, and an unpaired two-tailed t test with Welch's correction for unequal variances. Confidence levels for statistical significance were: 0.05 (*), 0.01 (**), 0.005 (***).

3. Results and Discussion

TPEF images showed that Rhd123-stained mitochondria of different shapes and sizes were distributed throughout the control hyphae (n = 6) and that there was no conspicuous region of high density in the growing hyphal tip (Fig. 1a, left). In cold-treated hyphae (n = 12), there was a similar range of mitochondrial shapes and sizes as in the control group, except for some longer mitochondria (Fig. 1a, right). To quantify mitochondrial morphology in an efficient and accurate manner, we trained a machine learning-based software routine in Ilastik to sort them into groups: round, ellipsoid, and elongated. The classifications obtained, such as the example shown in Fig. 1b (a colored mask superimposed on the image), matched well with the initial images. The same images were subjected to ImageJ Particle Analysis (Fig. 1c), and the obtained morphological measures were compared with the Ilastik-derived data for each hypha (Fig. 1d,e). The number of mitochondria determined by ImageJ was significantly lower than the number counted by Ilastik ($p < 0.0001$) (Fig. 1d left), while the total area occupied by mitochondria was similar in ImageJ and Ilastik ($p=0.46$) (Fig. 1d right). Visual inspection of the ImageJ-generated masks confirmed that many individual mitochondria were not well separated. Therefore, we analyzed the Ilastik -generated dataset in more detail. The results are shown in Fig. 2.

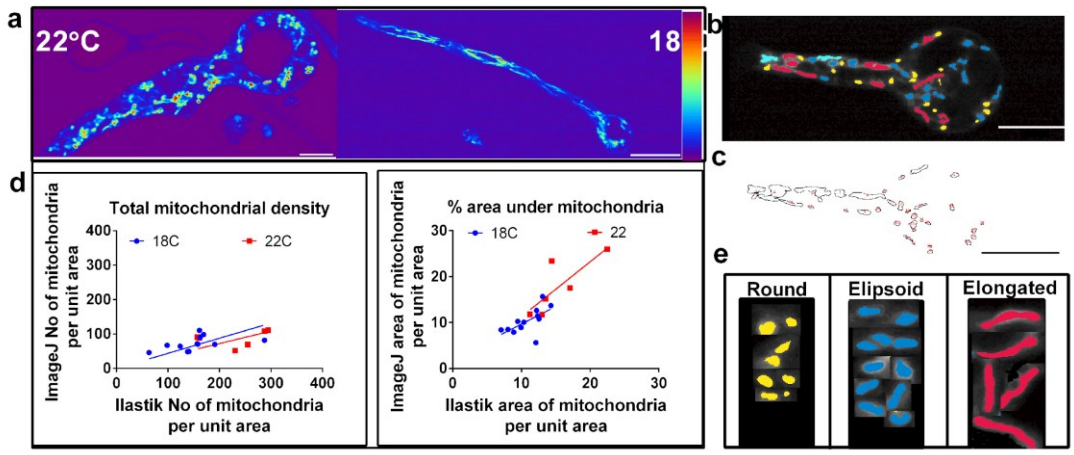

Figure 1. Mitochondrial morphology analysis from *P. blakesleeanus* hypha TPEF images. a.) Representative TPEF images of Rhd123-stained hypha grown in control (22°C) and cool (18°C) conditions. The color bar on the right, blue indicates lowest signal values. b-c.) Results of segmentation during image analysis using Ilastik (b) and Particle Analysis in ImageJ (c). d.) Comparison of values obtained by Ilastik and ImageJ analysis: Mitochondrial density (left) and percent of hyphal area occupied by mitochondria (right). All points are shown, with obtained linear fit coefficients: for mitochondrial density, k = 0.44 ± 0.04 (18°C) and k = 0.36 ± 0.05 (22°C); for % area under mitochondria, k = 0.76 ± 0.3 (18°C) and k=1.2±0.4 (22°C). e.) Representative examples of shapes of mitochondria for each of the main morphology groups: round, ellipsoid and elongated. Scales are shown in the bottom of images: a.)10 μm (left) and 20 μm (right); b.) 10 μm; c.) 10 μm.

We found that the number of mitochondria per unit area of hyphae (Fig. 2a) was lower in the 18°C group, which is consistent with the expected changes in mitochondrial morphology toward greater connectivity during adaptation to stress. On the other hand, the finding that the total area under the mitochondria (Fig. 2b) decreased with cooling suggests an increase in mitophagy, or alternatively, that increased fragmentation of mitochondria resulted in a portion of very small structures below the detection limit (submicron size). Overall, these results suggest that the dynamic restructuring of the mitochondrial network occurs in cooled hyphae. We examined a subset of representative hyphae from both groups in more detail. The density of each morphological type was similar in both groups, so the data were pooled (Fig. 2c). The length of the mitochondria in each morphological group is shown in Fig. 2d for the 22°C group and in Fig. 2e for the 18°C group. Significantly longer elongated (tubular) mitochondria were found in the 18°C group compared with the 22°C group (Fig. 2f).

3. Conclusions

The tubular morphology of mitochondria is an adaptive feature that occurs under colder conditions, similar to the changes previously observed in warmer environments, and is likely part of the stress response. Slight changes in ambient temperature can elicit a mitochondrial response, that is, dynamic changes in morphology.

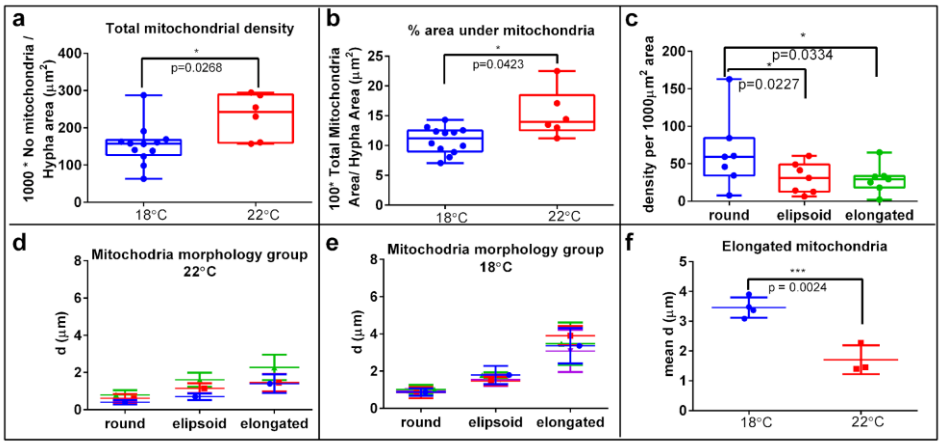

Figure 2. Parameters of mitochondrial morphology obtained from Ilastik image analysis, shown as box and whiskers plots enclosing the 25th and 75th percentile range with the line representing the median and the whiskers extending to the minimal and maximal value, all points shown. (a.,b.,f.) Comparison of hypha grown at 18°C (n=12) and at 22°C (n=6). T-test with Welch's correction: a.) Mitochondrial density (defined as the number of mitochondria per 1000 µm² of hypha area). b.) Mitochondrial area in a hypha normalized to the area of that hypha (% area under mitochondria). c.) The density of mitochondria belonging to each morphological type (round, ellipsoid, elongated) from pooled subsets of Control (n =3) and Cold-treated (n=4) hypha images. Anova with Holm-Sidac correction. d-e.) The length (d) of mitochondria belonging to each morphological type in hypha grown at 22°C (graph in 2d.) and 18°C (graph in 2e.) shown as average value and SD in each hypha (18°C group, n=4 and 22°C group, n=3). f.) Average length of elongated mitochondria. T-test with Welch's correction.

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