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Short Communication

3D visualization of mitochondria in the conidia of *Pyricularia oryzae* using green fluorescent protein (GFP) fused with citrate synthase (*CitA*)

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Abstract

The mitochondrion is the target organelle for QoI (quinone outside inhibitor) antifungal drugs, but mitochondrial behavior in *Pyricularia oryzae* remains unclear. Conidial mitochondria in fungal isolate transformed with citrate synthase (CitA)-GFP fusion gene, were observed in situ using laser-scanning confocal microscopy and computer analysis. By harvesting at 36 h after eliminating the aerial mycelia, conidia with 1, 2, or 3 cells were observed. The 3D visualization of mitochondria in these conidia showed that mitochondria were initially shaped like dots and developed into a network as the conidia aged.

Keywords: *Pyricularia oryzae*, mitochondria, citrate synthase (CitA)-GFP, 3D visualization

Pyricularia oryzae is a phytopathogenic fungus, which is composed of multi-host pathotypes, that causes rice blast disease, one of the most serious threats to our global food security. Each year, yield losses reaches about $66 billion, amounting to enough staple food for 60 million people (Pennisi 2010). The infection process starts with conidia adhering to the rice leaf surface. With sufficient water on the leaf, conidia germinate and form a unique structure called the appressorium within 8 h (Howard et al. 1991). The appressorium then penetrates the leaf surface, and invasive hyphae then form inside the leaf to further colonize the plant (Talbot 1995). Conidia that eventually form on the plants can be rapidly dispersed by wind and heavy rain throughout the rice planting area and cause severe losses.

The mitochondria in conidia have a critical role in the survival of this fungus and provide the energy for germination and growth before the host is parasitized (Li and Calderone 2017). Therefore, mitochondria are a promising target of anti-fungal agents, such as strobilurin (QoI: quinone outside inhibitor), which binds to cytochrome *b* (CYTB) in the quinone outside (Qo) site, thereby disrupting respiration. Moreover, even more unique is that mitochondria fuse, divide and are inherited by the next generation (Mishra and Chan 2016). Recently, the emergence of resistance to QoI agents has been reported in some rice-growing areas including Japan (Ishii 2015). This resistance is caused by a base substitution in the *Cytb* gene in the mitochondrial genome (Fernández-Ortuño et al. 2008). To understand the distribution of QoI resistance and disease, we need to understand mitochondrial behavior, especially during conidial formation of *P. oryzae*, but little has been known.

The present study focused on visualizing mitochondria and generating a 3-dimensional image to analyze mitochondrial dynamics using citrate synthase (CitA)-

green fluorescent protein (GFP) fusion protein as described by Suelmann and Fischer (2000) for *Aspergillus nidulans*. Briefly, the DNA sequence encoding the N-terminal of CitA (accession MGG_07202) was PCR-amplified with two primers (5′-ATGGCCTCTGCTATGCGC-3′ and 5′-CTGGTCAAGAGTGACCTTGTC-3′) and cloned into the N-terminal of the *EGFP* gene in pBLASTR-TEF1::eGFP::Mop53BP1 (Ohara et al. 2018) using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Japan, Tokyo, Japan). The vector was then inverse-PCR amplified with two primers (5′-GCGACGGCCCCGTG-3′ and TAGGGGATATCAGCTGGATGG-3′) and re-circularized to get rid of the *Mop53BP1* gene and introduce a stop codon for *EGFP*. The resulted vector, pBLASTR-TEF1::CitA::eGFP was used to transform *P. oryzae* Ina86-137 strain according to the method of Miki et al. (2009). The transformation was confirmed with PCR and Southern hybridization of *CitA-GFP* fusion gene.

For localizing the mitochondria, conidia from transformants were stained with 300 nM aqueous solution of MitoTracker Red CMXRos (Molecular Probes, Eugene, OR, USA) in 37°C for 30 min. The results showed that the green fluorescence from the fusion protein CitA-GFP and red from MitoTracker colocalized inside the conidia (Fig. 1). MitoTracker specifically stains mitochondria and was used previously for *P. oryzae* (Zhang et al. 2015); thus, CitA-GFP is reliable for visualizing mitochondria in *P. oryzae.*

For observing mitochondria during conidial formation, various timepoint for the beginning of conidiation was investigated periodically for the transformant, because some mutants of *P. oryzae* are known to differ in the timing and ability to conidiate (Goh et al. 2017; Han et al. 2018). A portion of 2-week-old colony of the transformant with oatmeal agar attached was removed with a plastic cell scraper, then washed with sterilized distilled water to remove aerial hyphae and conidia. This piece of colonized

oatmeal agar was trimmed to 1 cm^2 and incubated in a plastic petri dish under fluorescence light at 25˚C. After 30, 36, and 72 h, conidia were harvested and counted using a hemocytometer. No conidia were found until after 33 h, when conidia with one, two, or three cells were harvested. Interestingly, fewer conidia with one cell and two cells were produced over time, but remained at 5.3% and 16.4%, respectively, even after 72 h (Fig. 2a). These results indicated that conidiation by this transformant starts after 30 h of incubation, and at least some of the single-celled conidia at 33 to 36 h will develop into three-celled conidia by 72 h. In addition, microscopic observations of conidiophores showed conidia after 36 h (Fig. 2b, c). Based on these results, 36 h was confirmed as appropriate for observing a variety of conidia (Fig. 3).

Conidia harvested after 36 h were fixed in 5% (v/v) aqueous solution of formaldehyde . Conidia were observed with a Leica TCS SP8 STED 3X laser scanning confocal microscope, equipped with HC PL APO CS2 100×/1.40 objective lens and a white light (WLL) laser (70%) (Leica Microsystems GmbH, Wetzlar, Germany); 3D mitochondrial images were generated by Las X software (Leica),. *Z*-stack images were set up with 1024 × 1024 resolution, with *z* step size of 0.2 μm. The excitation wavelength was 488 nm, and emission filter was set to 498–530 nm.

The 3D visualization allowed us to observe the mitochondria from many angles and understand mitochondrial structure much better than with 2D visualization. In conidia, mitochondria were categorized by length as dots, tubules and networks (Table 1 and Fig 4a–c). Relationship between number of cells per conidium and length category of conidial mitochondria was analyzed by counting the conidia in each category. In addition, 75 conidia each with 1, 2, or 3 cells were selected to build 3-D images with mitochondria. Conidia with each number of cells was then categorized by the most

complex type of mitochondrial morphology present., e.g., dot when only dot mitochondria were present (Fig. 4d), and network when at least one network mitochondrion was present (Fig 4e, f) . Conidia of all cell numbers were dominated by network mitochondria (Fig. 5). Few one-celled conidia were found without mitochondria or with only dot mitochondria, and none had only tubule mitochondria.

In addition, mitochondria in hyphae were observed with the same method (Fig. 4g–l). Mitochondria in hyphal tips were observed mainly as dots, and those in the basal part of hyphae (i. e. older hyphae farther from the growing tip) were mainly as tubules (Fig. 4g–i). In Fig. 4j–l morphology of mitochondria was compared in the branch (young) and main hyphae (mature). These results showed that mitochondria in the primary hyphae formed a network, but were present primariy as dots and tubules in the branch hyphae. The volume of each single mitochondrion in hyphal tips and basal part of hypha was quantified using MitoGraph software (Viana et al. 2015). The results showed that the mean volume of mitochondria in hyphal tips was always smaller (0.9 $\mu m^3 \pm 0.34$, $n = 6$) than that in the basal part (5.43 $\mu m^3 \pm 3.0$, $n = 6$).

These results might suggest that mitochondria are distributed in conidia as dots, because hyphal tips with dot mitochondria are considered to be conidiating part as observed in Fig. 3d. In addition, only a few one-celled conidia had only dot-type mitochondria. However, one-celled conidia with network-type mitochondria were also found at 36 h. Based on the fact that one-celled conidia could be harvested after 72 h (Fig. 2), some conidia could have matured without further cell division. The network-type mitochondria in one-celled conidia found at 36 h might have already matured. The form of mitochondria distributed into delopping conidia is remaining unclear and should be verified by further direct observation of conidial development, but the fact that

mitochondrial fission protein MoFis1 mediates conidiation (Zhong et al. 2016) indicates a possibility that dot-type is the form of distributed mitochondria, because MoFis1 is considered to regulate mitophagy, which is essential for the formation of dot mitochondria. In *Saccharomyces cerevisiae*, however, mitochondria that are initially distributed in daughter cells are tubule mitochondria (Osman et al. 2015).

This report is the first to visualize mitochondria of *P. oryzae* in 3-dimensions using GFP fused with citrate synthase (CitA). Recently, Khan et al. (2015) visualized mitochondria using the (MoFis1)-GFP fusion protein, and Li et al. (2016) used electron-transferring flavoprotein B (ETFB)-GFP, but they did not visualize them in 3D. The 3D visualization here enabled the observation of the mitochondrial structure in conidia and hyphae without any blind spots that occur with 2D visualization. In addition, we found that the initial mitochondrial morphology in conidia is dot-like. This technique will help elucidate the mechanism of mitochondrial inheritance in *P. oryzae* and understand the distribution of QoI-resistance in future studies.

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Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Conidia with three cells and appearance of mitochondria of *Pyricularia oryzae* viewed with bright field optics (a) and fluorescence microscopy with CitA-GFP (b), and MitoTracker (c). (d) Merged images for CitA-GFP (green) and MitoTracker (red). Contrast and brightness were adjusted with ImageJ Fiji (https://imagej.net/Fiji) .

Fig. 2 Determination of appropriate timing to observe conidiation by *Pyricularia oryzae*. (a) Conidial concentration after 30, 33, 36 and 72 h. Bars indicate standard deviation (n = 3). Conidiophores in slide culture after 30 h (b) and 36 h (c).

Fig. 3 Conidia of *Pyricularia oryzae* with 3 (a), 2 (b), and 1 cell (c) and three conidia still attached to the conidiophore (d), observed at 36h of incubation.

Fig. 4 Mitochondrial morphology in conidia and hyphae of *Pyricularia oryzae* viewed in 3D as dots (a), tubules (b), and network (c). Mitochondria viewed in 3D in 1- (d), 2- (e) and 3-celled conidia (f). Bright field image of hyphae (g, j). Confocal image and 3D visualization of mitochondria in hyphal tips (g, h, i, j, k, l). D, dot; T, tubule; N, network mitochondria; one arrowhead, hyphal branch; two arrowheads, main mycelia
.

Fig. 5 Mitochondrial morphology based on 3D visualization of 75 1-, 2- or 3-celled conidia of *Pyricularia oryzae*. Conidia were counted based on the most complex type of mitochondrion found. For example, a conidium with all types of mitochondria was

categorized as network. No conidia were categorized as tubule, which contains only tubules or a mixture of dots and tubules.

Table 1. Category of mitochondrial morphology of *Pyricularia oryzae*

Category	**Description**	**Length (μm)**
Dot	Circle and ball-like	0.23–0.75
Tubule	More oval, not branched and formed like a tube	>1.6
Network	Tubules branched and connected	>1.6

3D visualization of mitochondria in the conidia of *Pyricularia oryzae* using Green Fluorescent Protein (GFP) fused with citrate synthase (*CitA*)

Muhammad Akhid Syib'li

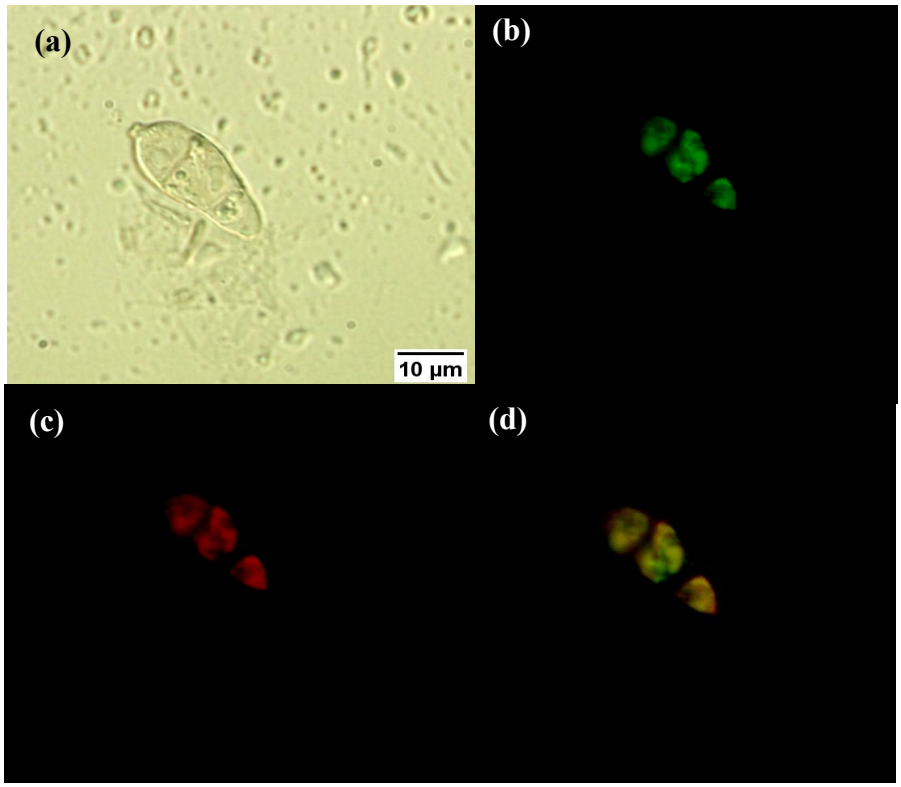
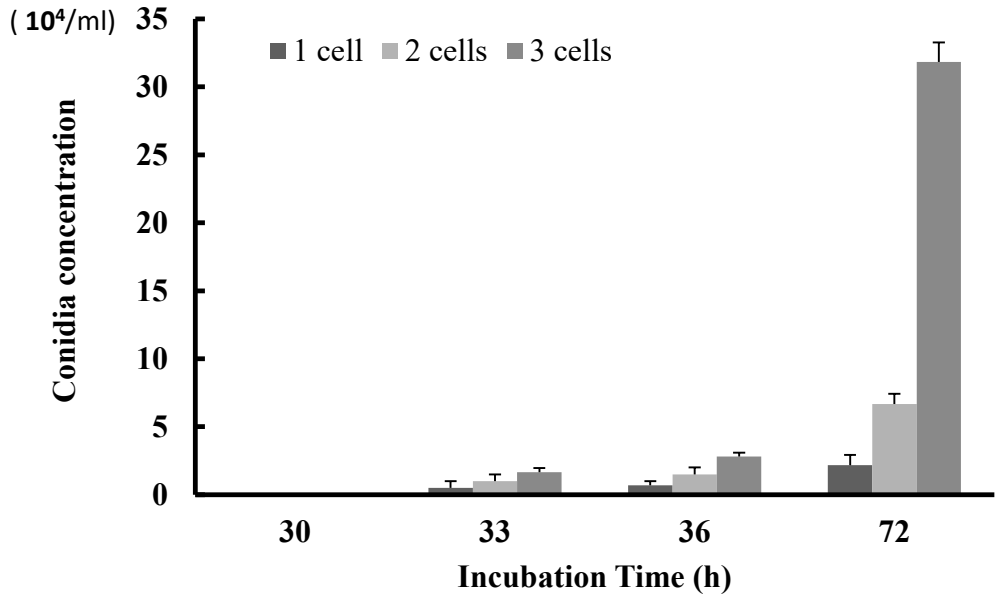


Fig. 1 Conidia with 3 cells and mitochondrial appearance. Mitochondria appearance under fluorescence microscopy with bright field (a), CitA-GFP (b), MitoTracker (c) the merged image between the result from CitA-GFP (green) and MitoTracker (red) (d). The observation was conducted by using fluorescence microscopy BX 50 (Olympus, Tokyo, Japan) and the pictures were adjusted with ImageJ Fiji (https://imagej.net/Fiji) for the contrast and brightness.

(a)



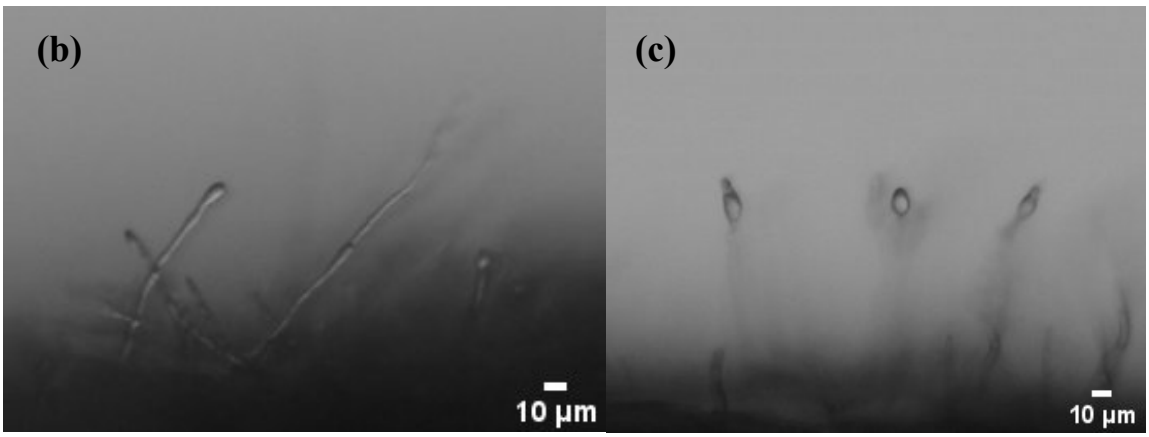


Fig. 2 Identification of the appropriate timing to observe conidiation. Conidia concentration after 30, 33, 36 and 72 h (a). The bar scale in the graphic indicate standard deviation with 3 replicates. The appearance of conidiophores after 30 h (b) and 36h (c) on slide culture.

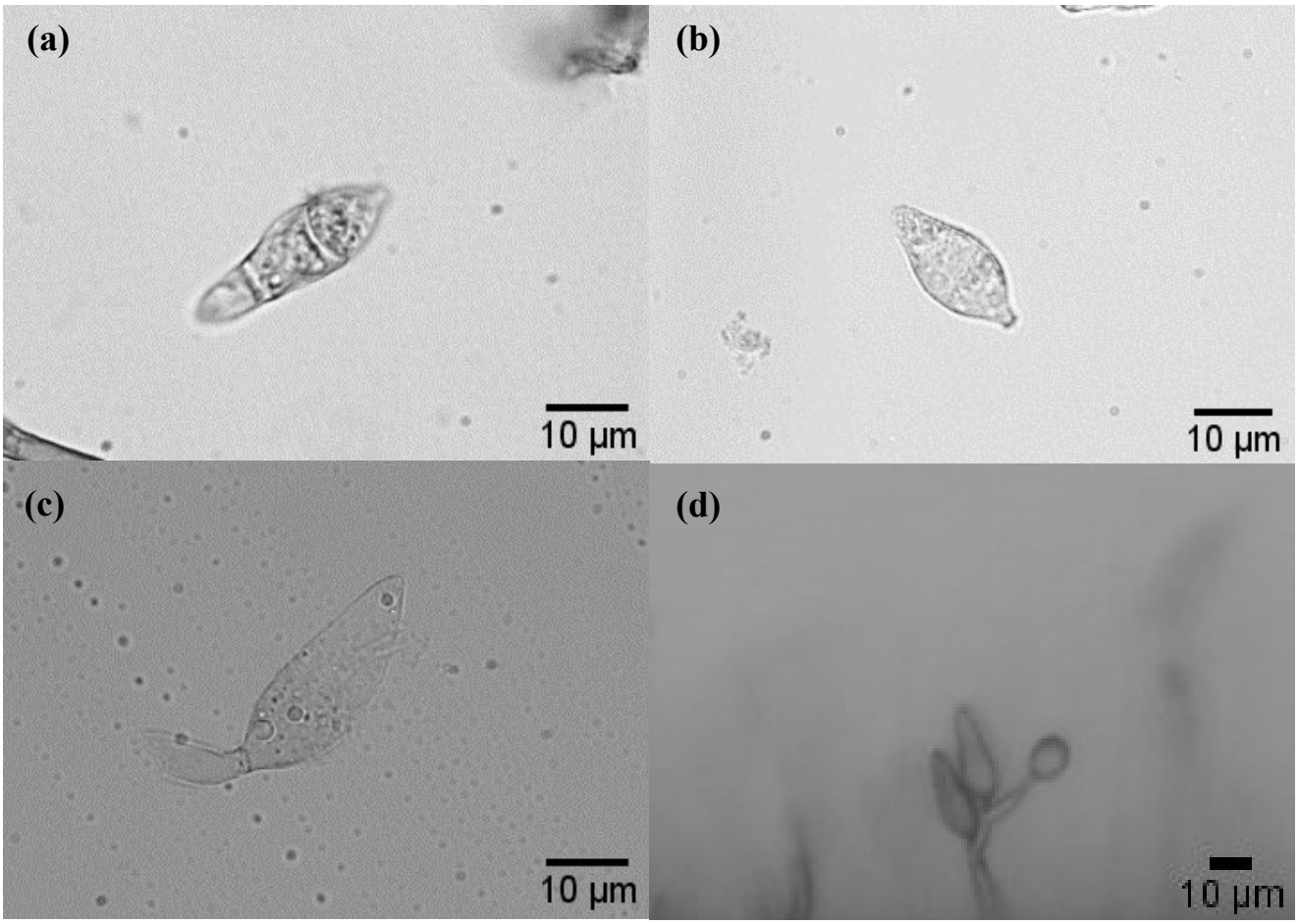


Fig. 3 Conidia with different number of cells. Microscopic image of conidia with 3 cells (a), 2 cells (b), 1 cell (c) and intact conidia with conidiophore (d).

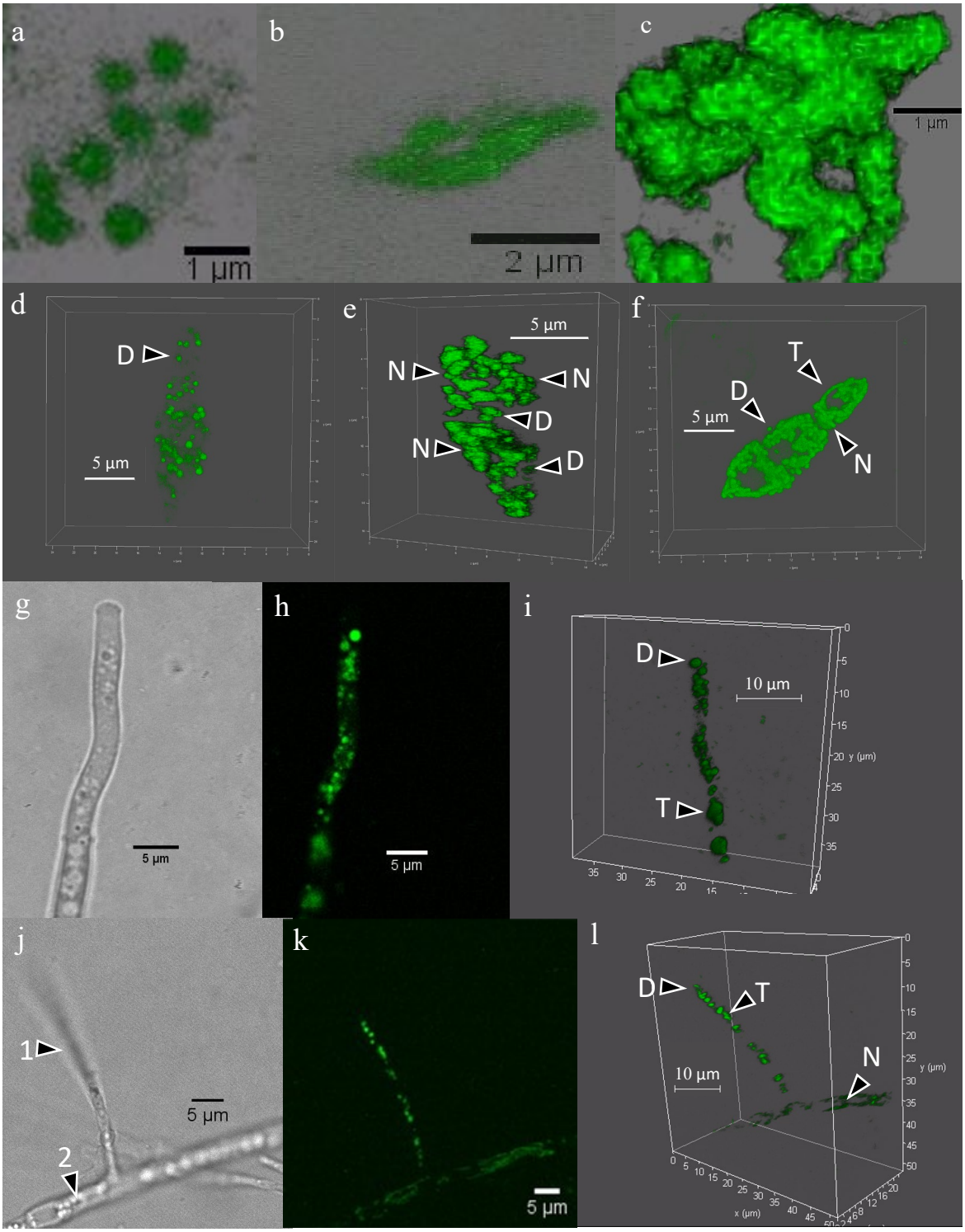


Fig. 4 Mitochondria morphology in the conidia and hyphae. 3D image of mitochondria as dots (a), tubules (b), network (c). 3D image of mitochondria in the single-cellular (d), two-cellular (e) and three-cellular conidia (f). Bright field image of hyphae (g, j). Confocal image and 3D image of mitochondria in the hyphal tips (g, h, i, j, k, l). D, N and T arrowheads indicate dot, tubule and network mitochondria, respectively. 1 and 2 arrowheads indicate the hyphal branch and main mycelia, respectively.

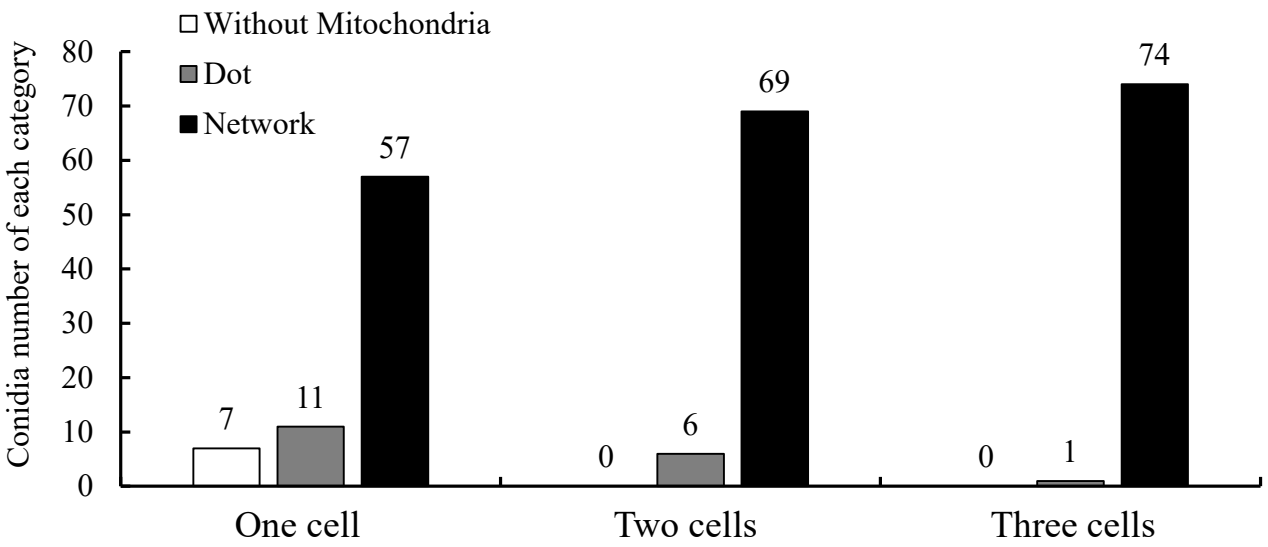


Fig. 5 Mitochondria morphology in each type of conidia.

Conidia were categorized based on their cell number. 75 conidia were selected to build 3 dimensional-object of mitochondria. Within categories, conidia were grouped into based on mitochondrial morphology appearance, such as dot when mitochondria appearance was only dotted and as tubule or network when we could find tubule or network form in the conidia cell. For example, a conidia with all types of mitochondria was categorized as network. No conidia was categorized as tubule, which contains only tubule or mixture of dot and tubule.

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Category	**Description**	**Length (µm)**
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