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Short communication

Method for the separation of mitochondria and apicoplast from the malaria parasite *Plasmodium falciparum*

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ABSTRACT

The growth and the survival of the human malaria parasite *Plasmodium falciparum* are critically dependent on the functions of the two organelles - the mitochondrion and the apicoplast. However, these two organelles have been known to be difficult to separate from each other when they are released from *Plasmodium* cell. We have been searching for the conditions with which separation of the mitochondrion and the apicoplast is achieved. In this study, we investigated how the two organelle's separation is affected when the pressure of the nitrogen gas to disrupt the *Plasmodium* cells by nitrogen cavitation method is lowered from the pressure regularly applied (1200 psi). The parasite cell was sufficiently disrupted even when nitrogen cavitation was carried out at 300 psi. The obtained mitochondrial sample was much less contaminated by DNA compared with the sample prepared using the gas at the regular pressure. After the fractionation by Percoll density gradient, the mitochondrion and the apicoplast from the 300 psi cell lysate exhibited different separation profiles. This is the first experimental evidence that indicates the mitochondrion and the apicoplast of *P. falciparum* are separable from each other.

Malaria, one of the most challenging global health problems of the modern world, is caused by infection of protozoan parasites, genus *Plasmodium* [1]. Although the life of the parasites largely depends on the host's metabolisms, *Plasmodium* keep own metabolic pathways that are critically important for their growth and survival. Such important pathways often involve the parasites' two organelles, the mitochondrion and the apicoplast. This means these organelles are potential targets of inhibitors that are useful for drug intervention of malaria. Indeed, compounds such as atovaquone [2,3] and fosmidomycin [4] that specifically interfere the functions of the parasite's mitochondrial respiratory chain and the apicoplast isoprenoid biosynthesis, respectively, are known to be effective antimalarials. Thus, it is important to understand the function and properties of each organelle in detail.

Both the mitochondrion and the apicoplast contain their own genomic DNA. However, these organellar genomes encode only a few of the enzymes or proteins that are important for the growth and survival of the parasite [5,6]. Bioinformatic analyses have predicted that many proteins and enzymes encoded in the nuclear genome are targeted to these organelles post-translationally and are involved in various functions of each organelle [7–9]. Some of those predictions have been proved by experimental evidences [10–12], but many are still being left speculative. Therefore, isolation of these organelles from the parasite will help to know the real function of them and understand their importance for the parasite's life.

Following the pioneering attempts to isolate the mitochondrion from *Plasmodium* [13,14], we have tested various methods to improve the quality of the organelle prepared. Eventually it was shown that the damage of the mitochondrion is much reduced when the parasite cell is disrupted by nitrogen cavitation, a mild but efficient cell disruption method that is often used in preparation of mammalian mitochondria [15,16]. Applying nitrogen cavitation, we have developed a method to isolate the mitochondrion that has been proven useful for the reproducible preparation of active mitochondrial samples from the parasites [17]. This method has been used in biochemical

Abbreviations: Pf, *Plasmodium falciparum*; $Mito_{300}$, mitochondrial sample prepared from the cell lysate of *Pf* disrupted by nitrogen cavitation at 300 psi; $Mito_{reg}$, mitochondrial sample prepared from the cell lysate of *Pf* disrupted by nitrogen cavitation at the regular pressure (1200 psi); *Pf*Ip, iron-sulfur subunit of the mitochondrial complex II of *Pf*; *Pf*HU, bacterial HU-type histone-like protein of *Pf*; $PfHU_{mat}$, matured form of *Pf*HU

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characterisation of *Plasmodium* mitochondria such as those in the Atovaquone-resistant parasites [2,3].

However, the "mitochondrial samples" generated with the method contain not only the mitochondrion but also the apicoplast [17]. The two organelles in the samples are inseparable from each other by conventional cell fractionation or fluorescent activate sorting (FACS), indicating that they associate to each other rather stably [17].

The mitochondrion and the apicoplast are naturally close to each other in the cell of the human malaria parasite *P. falciparum* (*Pf*) [18,19]. However, no apparent connecting structure has been identified between them by electron microscopy [18]. Thus, the natural, weak association of the mitochondrion and the apicoplast may be artificially stabilised to make the two organelles inseparable when they are release from the parasite cell.

To proceed the biochemical characterisation of the mitochondrion and the apicoplast further, it is important to separate the two organelles from each other. After various attempts applying different conditions to the steps in the preparation method, we found that the two organelles exhibit different separation profiles from each other when cells were disrupted with nitrogen cavitation at a lower gas pressure. In this paper, we report the effect of lowering the pressure of the N_2 gas applied for nitrogen cavitation from 1200 psi, the pressure regularly used for this cell disruption method [16,17].

First, the quality of the mitochondrion recovered in the mitochondrial samples prepared from *Pf* ruptured by nitrogen cavitation at a different pressure was compared by measuring the activity of mitochondrial dihydroorotate dehydrogenase (DHODH) and by microscopic observation.

The *Pf* strain Honduras-1, which was used in our previous study [17], was maintained in *in vitro* culture on human type A red blood cells at 3% haematocrit in RPMI 1640 medium (Invitrogen, Waltham, MA, USA) supplemented with human type A serum at 10 vol%. The parasites were released from the red blood cells by saponin treatment and suspended in MSE buffer (3 mM Tris-HCl, 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was loaded in the 4639 cell disruption vessel (Parr, Moline, IL, USA) and the parasite cells were ruptured by nitrogen cavitation applying the N_2 gas for 20 min at 4 °C. After cell debris and the nuclei were removed as pellet by a centrifugation at 800 x g for 5 min at 4 °C, the mitochondria in the lysate were pelleted down with another centrifugation at 5000 x g for 20 min at 4 °C. Recovered mitochondrial samples were suspended in MSE buffer and the activity of DHODH and the protein concentration were measured using the colourimetric methods as described previously [17]. The mitochondrial sample prepared from the cell lysate of the parasite disrupted by nitrogen cavitation at 300 psi ($Mito_{300}$) exhibited almost the same level of DHODH specific activity as the sample prepared applying the gas at the regular pressure, 1200 psi ($Mito_{reg}$); the values (nmol/min/mg protein) determined from three independent experiments for $Mito_{300}$ (this study) and $Mito_{reg}$ [17] were 13.6 ± 4.46 and 12.4 ± 3.01, respectively. This suggests that the *Pf* cell is ruptured sufficiently for preparing the mitochondrion by nitrogen cavitation even when the pressure of the applying N_2 gas is reduced to as low as 300 psi.

The mitochondrion and the DNA in each sample were stained with the fluorescent dye MitoTracker Red CM-H2XRos (Molecular Probes, Eugene, OR, USA) and Hoechst 33342 (Sigma, St. Louis, MO, USA), respectively, and monitored by fluorescence microscopy (Fig. 1). $Mito_{reg}$ was contaminated with a large amount of DNA that formed tightly condensed aggregates. By contrast, the fluorescence signal of DNA detected in $Mito_{300}$ appeared like vague clouds and there were no dense blob-like aggregates observed in $Mito_{reg}$. This indicates that the DNA contaminating in $Mito_{300}$ is less abundant and less aggregated compared to that in $Mito_{reg}$. Probably, carrying out nitrogen cavitation at 300 psi reduced the damages of the DNA-containing organelles, especially the nucleus, and that resulted in the decrease in the amount of DNA contaminating in $Mito_{300}$.

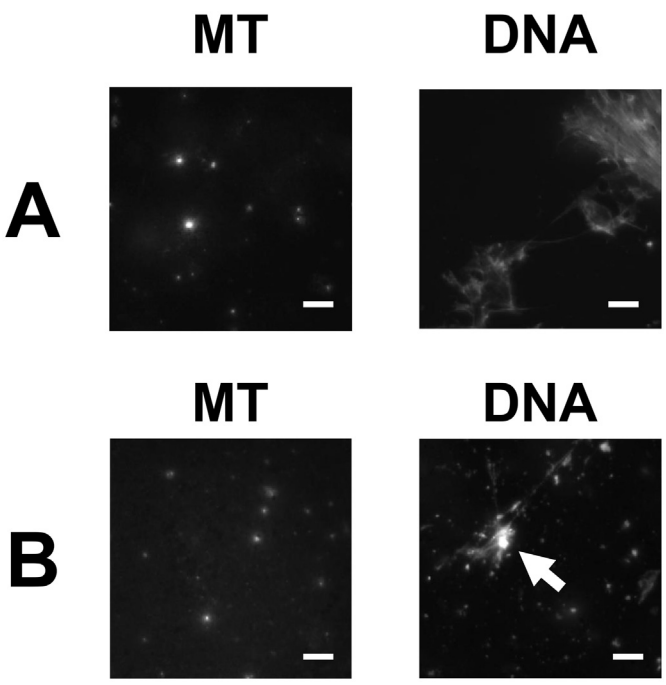

Fig. 1. The mitochondrion and contaminating DNA observed in the mitochondrial samples prepared from disrupted *P. falciparum* cells. A, $Mito_{300}$; B, $Mito_{reg}$. The mitochondrion and the DNA present in each sample were specifically stained with the MitoTracker (MT) and Hoechst 33342 (DNA), respectively, and observed by fluorescence microscopy. Each panel is of a representing image observed at an independent field. Note that tightly condensed DNA aggregates (arrowhead) were observed in $Mito_{reg}$ (B) but were absent from $Mito_{300}$ (A). Scale bars: 10 μm.

Then, we applied $Mito_{300}$ to a cell fractionation with a Percoll density gradient ultracentrifugation and analysed the separation profiles of the mitochondrion and the apicoplast, respectively.

$Mito_{300}$ prepared from the parasites synchronised to the trophozoite-schizont stages was applied to a total volume of 8.5 mL in 28 vol% Percoll PLUS (GE Healthcare, Chicago, IL, USA) in a buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA) and centrifuged at 50,000 ×*g* for 1 h at 4 °C using CP80WX (Hitachi, Tokyo, Japan). After the centrifugation, an aggregating mass of dark brown in colour was observed in the centrifuge tube (Fig. 2). The same aggregate is formed when $Mito_{reg}$ is separated on the Percoll gradient [17]. The aggregate was unable to dissociate without applying shearing forces that would severely damage the organelles. Therefore, the Percoll density gradient formed by the centrifugation was fractionated into 24 fractions (350 μL/fraction) from the top (#1) to the bottom (#24) using the PST-100 peristaltic pump (Iwaki, Tokyo, Japan), after careful removal of the aggregate by pipetting paying attention not to disturb the gradient. This method has been shown to keep the integrity of the mitochondrion [17]; typically 30–80% of the DHODH activity, which is localised only in mitochondria with the intact membrane, loaded on top of the gradient is recovered in the fractions after the protocol depending on the amount of aggregated mass removed.

To evaluate the separation of the two organelles, proteins recovered in each fraction were separated on a polyacrylamide gel by SDS-PAGE and the *Pf* iron-sulfur subunit of the mitochondrial complex II (*Pf*Ip) and the bacterial HU-type histone-like protein of *Pf* (*Pf*HU) localised in apicoplast were detected by immunoblotting using specific polyclonal antisera against each protein. Eighty microliter of each fraction was mixed with sodium deoxycholate (final concentration at 0.015% (w/v)) and trichloroacetic acid (7% (w/v)) in a total volume of 1 mL, and the precipitated proteins were recovered. After wash in the ethanol, the proteins were separated on a 12.5% (w/v) polyacrylamide gel by SDS-

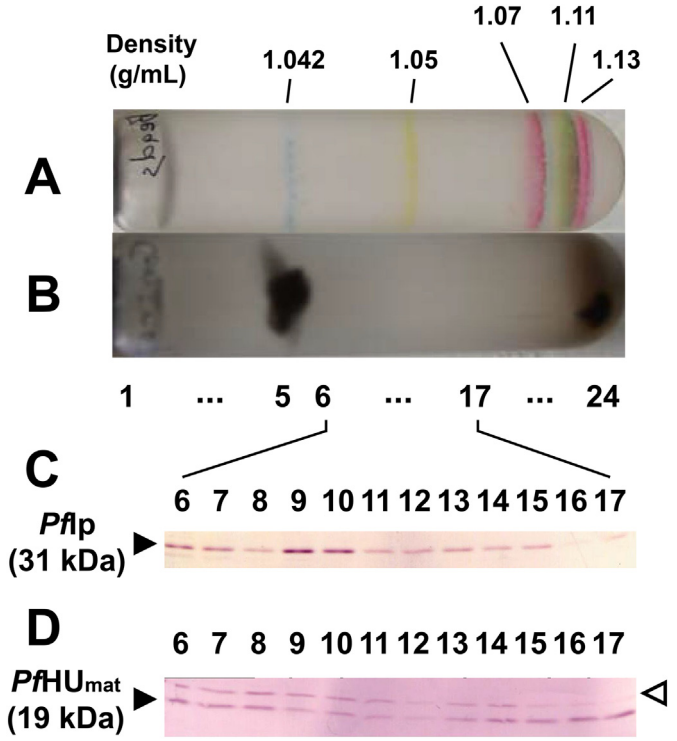

Fig. 2. Separation of the mitochondrion and the apicoplast by Percoll density gradient centrifugation. A, visualisation of the density gradient formed in the centrifuge tube span only with colour coded marker beads for control experiment. The top and the bottom of the centrifuge tube are at the left and the right in this panel. B, the gradient formed in the centrifuge tube loaded with a 300 psi sample after centrifugation. The top and the bottom of the centrifuge tube are at the left and the right in this panel, and the approximate position of the fractions 1 (at the top), 5, 6, 17 and 24 (at the bottom) was indicated below the panel with the number of each fraction. Note the occurrence of a rather large aggregating mass whose apparent density is approximately 1.042 g/mL. C and D, Distribution of the iron-sulfur subunit of mitochondrial succinate dehydrogenase (*Pf*Ip)(C) and the matured form of the HU-like protein (PfHU$_{mat}$)(D) in the fractions. Presence of *Pf*Ip and *Pf*HU were respectively detected by Western blotting using specific antisera raised against the target proteins. The band that appeared above PfHU$_{mat}$ in D (open triangle at the right) is of the transport intermediate of *Pf*HU. For details, see main text.

PAGE and then transferred to a nitrocellulose membrane. After blocking in 3% (w/v) gelatin in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05 vol% Tween-20, pH 8.0), the membranes were probed with specific polyclonal antisera against *Pf*Ip [16] and *Pf*HU [10], respectively. Each antiserum was used at 1:3000 dilution and their specific reaction was detected colourimetrically using the secondary antibody conjugated with alkaline phosphatase (Amersham Bioscience) and the chromogenic substrates, 5-bromo-4-chloro-3-indoryl phosphate (BCIP) and nitro blue tetrazolium bromide (NBT). Like other apicoplast proteins encoded in the nuclear genome [7,20], *Pf*HU is expressed as the precursor that has an apicoplast targeting sequence attached to the N terminus of its matured form (PfHU$_{mat}$). Consequently, two distinct forms of *Pf*HU are detected in the parasite cell: the 19 kDa PfHU$_{mat}$ localised exclusively in the apicoplast and the transport intermediate which is slightly larger than PfHU$_{mat}$ [10].

A remarkable difference between the separation profiles of *Pf*Ip and PfHU$_{mat}$ was observed in the fractions of the Percoll gradient loaded with a Mito$_{300}$ (Fig. 2C and D). *Pf*Ip, the marker of the parasite's mitochondrion, was separated in the fractions between 6 and 15 - most abundantly in fractions 9 and 10. PfHU$_{mat}$ also was separated broadly in fractions from 6 to 17, but the fractions that contained this apicoplast marker most abundantly were not the fractions 9 and 10. This difference in the separation profiles of the two organelles indicates that significant part of the mitochondrion and the apicoplast did not form a stable complex in the lysate prepared with nitrogen cavitation at 300 psi. In addition, this implies that the two organelles are separable using the density gradient method. It is noteworthy that the separation profile of PfHU$_{mat}$ observed had two peaks in both side of fraction 9 and 10. This indicates that there were two populations of the apicoplast which were different in density. Particularly the heavier apicoplast population recovered in fraction 16 and 17 was attractive for obtaining pure apicoplast, because mitochondrial contamination in these fractions was minimum. The apicoplast is surrounded by multiple layers of membrane [18]. Perhaps one of the two populations is of the intact apicoplast and the other have some damage at their membrane caused by mechanical sheering force. Even when this is the case, the presence of PfHU$_{mat}$, a soluble protein localising in the stroma of the apicoplast, implies that each apicoplast in the two populations is sufficiently intact.

Nitrogen cavitation is a useful method for disrupting the *P. falciparum* cell for preparing its organelles. In this study, we showed that the parasite cell is sufficiently processed even when the pressure of the N_2 gas applied was as low as 300 psi. Although the two organelles are known to be difficult to separate from each other, we found that the two organelles in Mito$_{300}$ exhibit different separation profiles on cell fractionation by a Percoll density gradient. This suggests that the mitochondrion and the apicoplast of parasite have a different density and that at least some proportion of them do not associate with each other in Mito$_{300}$. Therefore, the two organelles are separable when the parasite is disrupted by nitrogen cavitation at 300 psi.

Because both the mitochondrion and the apicoplast are involved in metabolisms on which the growth and the survival of the parasite depend, these organelles are promising targets of new antimalarial drugs. In addition, they are attractive research targets of basic science such as cell evolution and cell cycle control. Once each organelle becomes available in uncontaminated form, yet unknown nature of them will be revealed and these preparations will contribute to the progress of both basic researches on the malaria parasite and development of new antimalarials.

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Availability of data and materials

Not applicable.

Authors' contributions

MH designed the study, performed the experiment, analysed data, and participated in manuscript writing. SS assisted designing the study, participated in data analysis and manuscript writing. KK participated in designing the study and manuscript writing, assisted data analysis, and collected funds for research. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This research with human materials was approved by the Research Ethics Committee of the Graduate School of Medicine, The University of Tokyo.

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