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A novel and effective separation method for single mitochondria analysis

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ABSTRACT

To investigate the set of mtDNA molecules contained in small biological structures, powerful techniques for separation are required. We tested flow cytometry (FCM[1]), laser capture microdissection (LCM[2]) and a method using optical tweezers (OT[3]) in combination with a 1μ-Ibidi-Slide with regard to their ability to deposit single mitochondrial particles. The success of separation was determined by real-time quantitative PCR (qPCR[4]) and sequencing analysis.

OT revealed the highest potential for the separation and deposition of single mitochondrial particles. The study presents a novel setup for effective separation of single mitochondrial particles, which is crucial for the analysis of single mitochondria.

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1. Introduction

In many applications it is important to analyse subcellular components separated from each other with the guarantee that only one particle is being focused. In heteroplasmy research, trying to identify the DNA composition of a single mitochondrion is particularly challenging. Heteroplasmy describes the situation of a co-existence of more than one mtDNA haplotype within an organism. A lot of research has been done to analyse mitochondrial heteroplasmy on different species, tissues and levels (e.g. Ashley et al., 1989; Cavelier et al., 2000; Deckman et al., 2008; He et al., 2010; Jacobs et al., 2007; Lehtinen et al., 2000; Lutz-Bonengel et al., 2008; McLeod and White, 2010; Woloszynska, 2010). We have chosen single mitochondria, which are approx. 0.5–5 μm in diameter (Whittaker and Danks, 1978), to investigate the limits of three different techniques potentially able to separate and deposit these small organelles (e.g. Cavelier et al., 2000; Deckman et al., 2008; Kuroiwa et al., 1996). These are flow cytometry (FCM), laser capture microdissection (LCM) and optical tweezers (OT). Control of successful separation and deposition of organelles can be achieved by sequencing analysis and real-time PCR (qPCR) of the intra-organelle mtDNA. The definition of a single mitochondrion is difficult, since mitochondria are linked to the cytoskeleton and behave as an entire network within a cell, regulated by fusion and fission (e.g. Bereiter-Hahn et al., 2008; Chan, 2006; Chen and Chan, 2009; Kuznetsov et al., 2009; Meeusen and Nunnari, 2005). In this study, the term "single mitochondrion" refers to a globular mitochondrial structure as mainly generated using the isolation technique applied in this study.

FCM is a powerful tool in quantitative analysis and deposition of particles and therefore is an established method for many applications (for review e.g. Comas-Riu and Rius, 2009; Czechowska et al., 2008). It has been widely used to analyse features of isolated mitochondria under different conditions, e.g. reactive oxygen species (ROS) generation (Wakabayashi et al., 2000) or membrane potential (e.g. Cazzalini et al., 2001), see Medina et al. (2002) and Fuller and Arriaga (2003) for a review. It has also been applied to study the mtDNA composition of single mitochondrial particles (Cavelier et al., 2000).

LCM is used to cut out specific tissue, cells or other structures under optical control (Edwards, 2007). In mitochondrial research, this technique has been used to analyse mtDNA of cancer tissue (e.g. Aldridge et al., 2003), and of individual cells (Kraytsberg et al., 2009).

OT generated by an infrared laser beam have been shown to leave cells intact when they get trapped (Ashkin et al., 1987). The technique was also used in combination with LCM to analyse mtDNA in small groups of mitochondria (Kuroiwa et al., 1996). Recently, they have been applied to deposit single mitochondria from single cells (Deckman et al., 2008) allowing detection of heteroplasmy at single-mitochondrion level. In addition, OT combined with near

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[1] Flow cytometry.
[2] Laser capture microdissection.
[3] Optical tweezers.
[4] Real-time quantitative PCR.

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infrared Raman spectroscopy have been used to analyse the chemical composition of single mitochondria (Tang et al., 2007).

The goal of this study was to develop a methodical setup well suited for further research on single mitochondria (e.g. mitochondrial heteroplasmy). For this purpose, we compared the above three techniques as to their respective limits regarding the separation and deposition of small biological particles. The suggested method may also be useful for the early diagnosis of heteroplasmic mutations involved in mitochondrial disease.

2. Material and methods

2.1. Material

For all mitochondrial preparations fresh murine or porcine liver tissue was used. Murine tissue was drawn from the livestock breeding of the University of Freiburg, and porcine liver was obtained from the Faber slaughterhouse, Freiburg.

2.2. Mitochondrial isolation

The Qproteome Mitochondria Isolation kit (Qiagen, Hilden, Germany) was used following the manufacturer's protocol, but 60 mg of liver tissue was washed in storage buffer (2000 g for 1 min at 4 °C) and the tissue was crushed with a tissue grinder (T10 basic, IKA, Staufen, Germany) at minimum speed for 15 s. Cells were disrupted using a 2-ml glass dounce homogenizer (Kimble Chase, NJ, USA) performing 5 strokes with a large clearance pestle and 25 strokes with a small clearance pestle. The mitochondrial pellet was resuspended in 400 µl of storage buffer. At least two liver samples each of murine and porcine origin were processed in parallel. After mitochondrial staining, the resulting mitochondrial pellets were combined according to size in order to yield approx. (1:1) mixtures of murine and porcine organelles for further analysis. Suspensions of purely murine mitochondria were produced accordingly.

2.3. Electron microscopy (EM)

Exemplary isolates of porcine liver mitochondria were fixated with 2% paraformaldehyde and 0.5% glutaraldehyde, and EM was performed as previously described (Walker et al., 2002), but ultrathin sections were additionally incubated in lead citrate (Reynolds, 1963).

2.4. Mitochondrial staining

Mitotracker Green (MTG) FM (Molecular Probes, Eugene, OR, USA) was used to stain isolated mitochondria. This mitochondrial selective dye is a substrate of P-glycoprotein (Marques-Santos et al., 2003) and labels a subset of proteins found in mitochondrial enriched fractions (Presley et al., 2003). This dye was previously used for FCM analysis of mitochondria (Teranishi et al., 1999; Wakabayashi et al., 2000). Using fluorescence microscopy, dye concentration was adapted to 400 nM. Mitochondria were incubated for 20 min at 37 °C.

2.5. FCM

A MoFlo cell sorter (Beckman Coulter, Brea, CA, USA) was used. Beads with a diameter ranging from 2 to 15 µm were used for instrument adjustment. For sorting single mitochondria, FSC, SSC and fluorescence were used in logarithmic adjustments. Laser power was 200 mV, and fluorescence was the trigger signal. The first sort region (R1) was placed in the SSC/FSC window where particles with a diameter of 2 µm and smaller were expected (according to the presorted beads). The second sort region (R2) included weakly positive stained particles found in the PE/FITC window and the third sort region (R3) was placed in the pulse width/FSC window. For FCM, all mitochondrial suspensions were diluted 1:30 in storage buffer (Qiagen). For sequencing analysis, 96 well-plates were provided with a single mitochondrial particle per well from the (1:1) mixture of murine and porcine mitochondria. For qPCR, single mitochondrial particles from a pure murine mitochondrial suspension were deposited.

2.6. LCM

MTG stained mitochondria were diluted 1:400 with Mitochondria Storage Buffer (Qiagen) and smears were prepared on standard microscopy glass slides. Using a Palm MicroBeam (Carl Zeiss, Jena, Germany), fluorescent particles were observed and cut out using either laser pressure catapulting (LPC) only or, alternatively, the auto circle function (diameter: 5 µm). Settings for the laser cut were modified depending on the amount of crystallized salt. Average instrument settings were: Cut energy: ~75; cut focus ~10; LPC energy: ~84; LPC focus: ~9. Mitochondrial particles were catapulted into CapturePlates 96 (Carl Zeiss) (Table 1) containing 20 µl HPLC-water in each well and were transferred to 96 well-plates by centrifugation.

2.7. OT

A Palm CombiSystem (Carl Zeiss) and OT at IMTEK (Department of Microsystems Engineering, University of Freiburg, Germany) were used to separate single mitochondrial particles. A 1064-nm infrared laser was used in combination with an Ibidi-Slide (1µ-Slide VI flat ibiTreat, Ibidi, Martinsried, Germany). The slide shows 6 pairs of wells, each connected by a small channel. For this application, one of the two wells was filled with DNA-free HPLC-water whereas the other well was filled with HPLC-water and 1 µl of mitochondrial suspension (diluted 1:200 with HPLC-water). By moving the microscopy stage, single trapped particles were transported from the well containing the probe to the second well containing only water, and were then transferred to 96-well plates by pipetting of 20 µl of water. On each plate, 4 to 5 samples were transferred (Table 1). At IMTEK, we used a 40x C-APOCHROMAT (Carl Zeiss) with a numerical aperture of 1.2, and at Zeiss laboratories (Munich, Germany) an EC Plan-Neofluar 100× oil (Carl Zeiss) with a numerical aperture of 1.3 was used.

2.8. Sequencing analysis

Supernatant of OT and LCM plates was dried by incubation for 2 h at 60 °C. A 357 bp fragment of the mitochondrial cytochrome b gene was amplified as previously published (Parson et al., 2000), but 5-µl reactions were performed using 0.2 µM of each primer, 200 µM each dNTP and 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). All amplifications were carried out in 96-well plates as received from the respective deposition techniques. Cycling was performed on a PTC 200 thermal cycler (MJ Research, Waltham, MA, USA) with 45 cycles (45 s at 95 °C, 1 min at 50 °C and 1 min at 72 °C). The amplification products were purified using ExoSAP-IT (USB, Cleveland, OH, USA). Sequencing was performed

Table 1
Analysed samples. x: number of 96-well plates used for sample deposition; y: number of samples processed; z: number of successfully analysed samples. FCM: fluorescence cytometry. LCM: laser capture microdissection. OT: optical tweezers.

Deposition technique	Analysis method	x	y	z
FCM	Sequencing analysis	4	336	177
	qPCR	4	333	87
LCM	Sequencing analysis	4	336	16
	qPCR	3	252	14
OT	Sequencing analysis	11	49	13
	qPCR	8	37	14

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