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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.

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 pre-sorted 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

using the light strand primer and BigDye V1.1 terminator cycle sequencing kit (Applied Biosystems). After purification with Dye Ex 96 kit or Dye Ex 2.0 (both: Qiagen), the sequencing products were detected on an ABI 3130xl or 3100*Avant* automated DNA sequencer (Applied Biosystems). Detected sequences were aligned with Sequencher Software (Gene Codes, Ann Arbor, MI, USA).

2.9. Real-time quantitative PCR (qPCR)

Supernatant of OT and LCM plates was dried by incubation for 2 h at 60 °C. Primers K1 (5′-ctctacctcaccatctcttgctaa-3′) and K2 (5′-cattggctacaccttgacctaacg-3′) were designed to amplify a 122 bp fragment of the murine mitochondrial 12S rRNA gene. qPCR reactions contained 2.5 µl of Power SYBR Green Master Mix (Applied Biosystems) and 0.2 µM of each primer in a total volume of 5 µl. At least 40 cycles of a two-step qPCR (annealing and extension at 60 °C) were performed. Each plate included at least 6 positive controls in different dilutions to test qPCR efficiency and to serve as plate-to-plate controls. Specificity of amplified products was assured by melt curve analysis.

In preliminary experiments, most no-template controls showed signals with cycle threshold values (C_T values) slightly higher than 40. Therefore, a C_T of 40 was used as cut-off value.

Sample C_T values were subtracted from the cut-off C_T of 40 resulting in ΔC_T values, which were used to compare qPCR results.

2.10. Statistical analysis

As a prerequisite for accurate statistical analysis, at least three plates of mitochondrial particles were analysed by qPCR as well as by sequencing analysis to evaluate deposition by LCM, FCM, and OT, respectively (Table 1). The analysed plates contained mitochondrial particles prepared from at least three different animals and three different mitochondrial extractions. Using LCM or FCM, particles were deposited on at least three different occasions. Regarding OT, they were deposited on two different occasions.

qPCR data were checked for significant differences using the exact non-parametric Mann–Whitney-U-Test at a two-sided significance level of 5% (Mann and Whitney, 1947; Wilcoxon, 1945).

From the sequencing analysis data, a maximum probability (w_{max}) for the deposition of a single mitochondrion was calculated as:

$$w_{max} = (1-(x/n))*100$$

where (x) is the number of sequences showing a mixture of cytochrome b-fragments from *Mus musculus* and *Sus scrofa*, and (n) is the sample size.

Using the ratio of samples showing pure (either murine or porcine) sequences (y) with

$$y = 1-(x/n),$$

the number of mitochondrial particles (m) that have most probably been deposited can be calculated as:

$$m = log_{1/2}(y/2).$$

2.11. Controls

On every 96-well plate for either qPCR or sequencing analysis 4 to 6 no-template PCR controls were performed. In addition, technique-based controls were performed as follows: For FCM, 15 µm beads were added to the mitochondrial suspensions (diluted 1:30), and only beads were sorted into the wells. For LCM, smear regions without any fluorescence were cut out and transferred to the capture plate. For OT, samples were taken from the wells of the Ibidi-Slide containing only water without prior trapping of particles from the sample-containing wells. For each of the three techniques, at least one 96-well plate with controls was analysed with qPCR and sequencing analysis, respectively. Further controls for FCM (e. g. sorting buffer checks) were performed as described previously (Lutz-Bonengel et al., 2008).

Also, multiple human and porcine mtDNA controls were analysed to ensure specificity for the murine target.

3. Results

3.1. Mitochondrial isolation and staining

Successful isolation of mitochondria was shown by EM. The detected globular particles were 0.5–2.5 µm in diameter, and mainly showed structures of physically intact mitochondria. There were no structures ≥3 µm in diameter (Fig. 1).

Fluorescence microscopy verified successful incorporation of MTG FM dye and revealed mostly globular and insular structures. To some extent, clustered particles were detected.

3.2. Sequencing analysis

Sequence data for single mitochondrial particles was obtained with all three methods tested (Table1). The success rate was 52.68% for FCM, 4.76% for LCM, and 26.53% for OT.

At least 141 bp of cytochrome b sequence — referring to positions 14975–15115 (Anderson et al., 1981) — were aligned and used for determination of either pure or mixed sequences of *Mus musculus* and *Sus scrofa*. Deposition of "single mitochondrial particles" by FCM resulted in 32 mixed sequences out of 177 successfully analysed samples. For deposition by LCM and OT, 1 mixed sequence out of 16, and 0 out of 13 detected sequences were found, respectively. All other detected sequences showed unambiguous, "pure" sequences of either murine or porcine origin (Fig. 2). In total, 47.4% and 52.6% of all pure sequences were of murine and porcine origin, respectively.

The highest maximum probability w_{max} for deposition of a single mitochondrion was calculated for OT (100%) followed by LCM (93.75%), and FCM (81.92%). The most probable number of deposited particles m was 1 for OT, 1.09 for LCM, and 1.29 for FCM, respectively.

3.3. Real-time quantitative PCR (qPCR)

Specificity of qPCR was assessed by melt curve analysis, which clearly distinguished the murine amplicons (T_m between 73.6 °C and 75 °C) from any artificial and non-specific products. Only specific signals with C_T of ≤40 were included into further analysis. Average efficiency of qPCR was 98.84%. Regarding C_T, the standard deviation of the plate-to-plate control (±0.46) was in the range of the intra-plate standard deviation of mitochondrial particles irrespective of the technique used for deposition.

The median ΔC_T was 4.9 for FCM (in a range of 0.57–8.09), 3.26 for LCM (range: 1.45–4.93), and 3.64 for OT (range: 0.46–7.34).

When testing data with the Mann–Whitney-U-Test, less mtDNA was detected in mitochondrial particles deposited with LCM compared to FCM ($P<0.001$) (Fig. 3). No significant differences were found when comparing ΔC_T values of FCM and OT ($p=0.056$) or LCM and OT ($p=0.434$).

3.4. Controls

For the sequencing analysis, altogether 134 no-template controls were performed. Only in one of the FCM plates (two controls) and one of the OT plates (one control), contaminating human mtDNA was detected. Regarding all plates processed, human mtDNA was (co-)

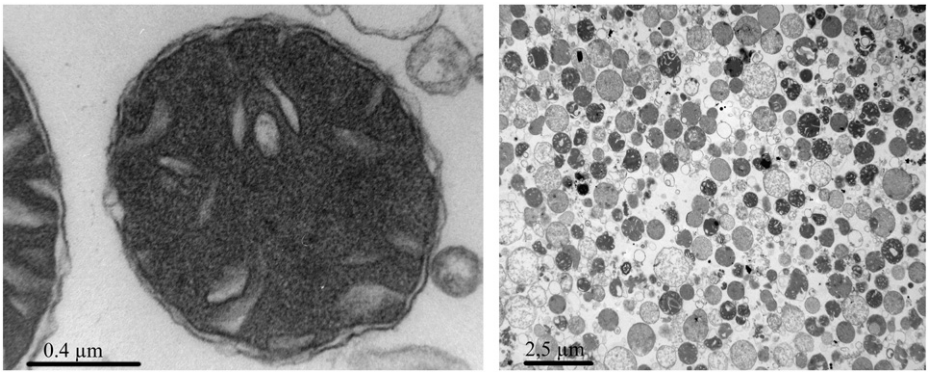

Fig. 1. Isolated mitochondria. On the left: Picture of a globular mitochondrial particle obtained by EM. On the right: EM of a mitochondrial suspension after isolation of mitochondria with the Qproteome Mitochondria Isolation kit (Qiagen, Hilden, Germany).

amplified in 16 out of 721 wells provided with a single porcine or murine mitochondrial particle.

In qPCR, one out of 90 no-template controls showed a fluorescence signal (ΔC_T 0.48). The respective plate had been processed with OT.

3.4.1. Technique-based negative controls

When pooling data from sequencing analysis and qPCR, 37.7% of FCM-plate wells showed positive signals. In LCM and OT, these signals were extremely rare with rates of 2.38% and 1.11% respectively.

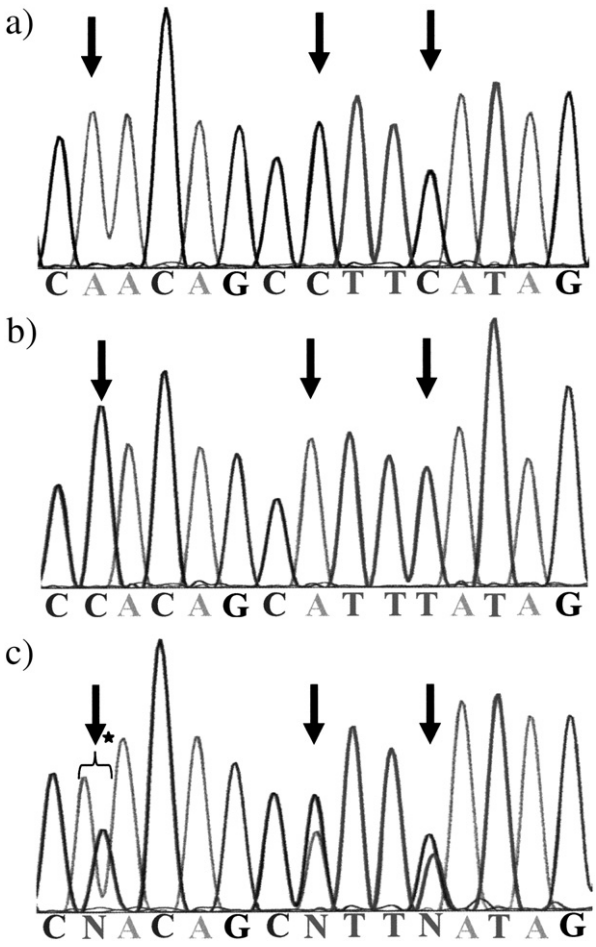

Fig. 2. Sequencing analysis — electropherograms. Representative section of electropherograms showing a) a pure porcine, b) a pure murine, and c) a mixed sequence (porcine/murine). The asterisk indicates a base shift of mixed peaks. These electropherograms were obtained from single mitochondrial particles deposited by LCM.

Drop-out rates were pooled for sequencing analysis and qPCR, and were 94.9% for LCM, 60.54% for FCM, and 68.6% for OT.

4. Discussion

4.1. Mitochondrial isolation and staining

Due to its high content of mitochondria (Alberts et al., 2002), we chose liver tissue to serve as starting material for the mitochondrial preparations in order to obtain high quantities of isolated mitochondria for the methodological developments.

Most structures found by EM seemed to be derived from intact mitochondria. To some extent structures (≤2.5 µm in diameter) of unclear origin were found. These may be mitochondria damaged especially during the preparation procedure for EM analysis due to additional centrifugation steps as well as microtome cuts. No structures ≥3 µm in diameter were found, suggesting that all cells had been lysed and disrupted during isolation of mitochondria. In addition, fluorescence microscopy identified no intact cells. However, aggregated fluorescent structures were detected. Deposition of aggregated mitochondria can lead to a false overestimation of the mtDNA content by qPCR. Three possibilities may account for this observation. First, repeated pelleting by centrifugal forces during the isolation process may lead to a clustering of mitochondria. Second, the fluorescence dye itself may lead to aggregation of mitochondria. Third, the aggregates may correspond to persisting mitochondrial networks as found *in vivo* (e.g. Bereiter-Hahn et al., 2008).

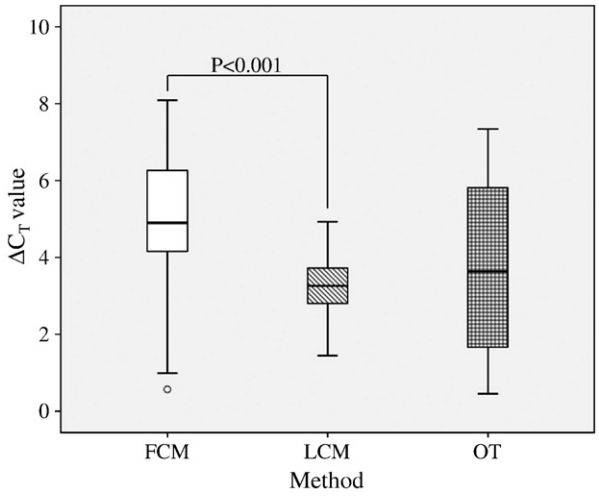

Fig. 3. qPCR — results. Box-and-Whisker plot of ΔC_T values as calculated from qPCR results. Statistical significance is given. Circles (FCM) depict outliers. FCM: fluorescence cytometry. LCM: laser capture microdissection. OT: optical tweezers.

Nevertheless, the isolation process of mitochondria has a significant impact on their structure, since almost all particles observed with EM or fluorescence microscopy were of globular shape and did not resemble the tubular mitochondria existing in live cells (e.g. Frazier et al., 2006; Xu et al., 2005).

4.2. Sequencing analysis and qPCR

The similar percentages of pure sequences from murine or porcine origin (47.4% and 52.6%, respectively) indicated sufficient accuracy of the murine/porcine mixtures (1:1). Although the occurrence of a pure mtDNA sequence of either murine or porcine origin may suggest that in fact only one mitochondrion was deposited, two additional aspects have to be considered. First, clusters consisting of many mitochondria (e.g. mitochondrial networks) of the same species may have been deposited. Second, stochastic events in early stages of PCR may lead to "false" pure sequences. This is especially the case when only a few molecules serve as template for PCR. This phenomenon of "low copy number" DNA typing is well known in forensics (e.g. Budowle et al., 2009).

To overcome these objections, we calculated a maximum probability w_{max} for the deposition of single mitochondria. According to w_{max}, the occurrence of only pure sequences of either murine or porcine origin in OT-deposited particles provides strong evidence that in fact only one mitochondrion was deposited and analysed.

Regarding qPCR, the lowest overall mtDNA content was found in wells containing LCM-deposited particles. It seems likely that the preparation of mitochondria for LCM alters their morphology and affects their mtDNA content. Especially the drying of smears seems to be responsible for such an effect since desiccation affects the membrane integrity (Liu et al., 2005) and may lead to a leakage of intra-organelle mtDNA. Such a leakage of mtDNA plausibly explains both the (though not significantly) lower mtDNA content of LCM samples and their lower w_{max} compared to OT samples. Regarding the latter, extra-mitochondrial, "free" mtDNA can lead to contaminations, if it is co-deposited with another mitochondrion. This mechanism may account for the single mixed sequence found in LCM samples, which was also responsible for the lower w_{max} in LCM. Perhaps "free" mtDNA is also responsible for the positive signals found in the technique-based negative controls.

Interestingly, sequencing analysis revealed that the OT had a much higher w_{max} compared to FCM, but regarding qPCR the significance niveau of 5% was missed ($p = 0.056$). Nevertheless, two samples of FCM-deposited particles showed higher ΔC_T values (7.64 and 8.09) compared to the highest ΔC_T value when using OT (7.34). The most satisfying explanation may be that exactly such cases represent a co-deposition of mtDNA — either resulting from deposition of multiple mitochondria or from co-deposition of "free" mtDNA sticking to the membrane of a respective mitochondrion. The rest of the ΔC_T values found in FCM and OT analyses actually seem to represent single mitochondria. Their variation may reflect the variation of mtDNA content in single mitochondria. For example, Cavelier et al. (2000) found between 0 and 11 mtDNA molecules per mitochondrial particle in fibroblasts. They also detected 25% of heteroplasmic mitochondrial particles when trying to separate single mitochondria from a mitochondrial suspension containing wild and mutant type mtDNA. However, they did not take into account that a "heteroplasmic" particle may well reflect that actually more than one mitochondrion or extra-mitochondrial mtDNA was co-deposited and analysed. Our FCM based technique yielded 18.08% of mixed sequences, which most likely resulted from such co-depositions.

Using FCM, a medium ensuring mitochondrial intactness can be used. The same medium can be used for LCM, but since the suspension must be dried, mitochondria may be damaged. When working with OT in combination with the 1µ-Ibidi-Slide, the medium of choice is water, which also does not affect downstream PCR applications. All media that are isotonic for isolated mitochondria contain excessive concentrations of sugar or salt and behave as strong PCR inhibitors. When working with LCM and FCM, only very small amounts of medium are co-deposited, so an inhibition of PCR is not to be expected (Lutz-Bonengel et al., 2008). However, a swelling of mitochondria upon transfer into water was observed and it cannot totally be ruled out that water treated mitochondria might have released mtDNA to some extent (van Bruggen et al., 1968). But the relatively high number of successfully analysed samples suggests that at least the inner membrane stayed intact thus preventing a relevant leaking of mtDNA molecules. This is in agreement with reports on mtDNA being anchored to the inner membrane via interactions with its non-coding region (Albring et al., 1977; Boesch et al., 2010; Jackson et al., 1996).

The high drop-out rates in LCM can be explained by the deposition technique itself. Very small areas containing mitochondria are transferred to capture plates by a laser shot. They are then transferred into the PCR plates by centrifugation. In addition to the procedural steps that may fail, "leaked out" mitochondria may be transferred.

OT seem to generate slightly higher drop-out rates compared to FCM. But since FCM can evidently lead to a co-deposition of more than one mitochondrion, the greater success of FCM may most probably result from higher amounts of mtDNA template improving downstream analyses.

4.3. Controls

Human contaminations were found in 22 out of 1191 sequencing reactions. All deposition techniques and analysis methods have been performed carefully to prevent external human contaminations. Despite general precautions (e. g. gloves, masks, laboratory coats etc.) and due to the high sensitivity of downstream analyses, human contaminations occurred in rare cases. The problem of human contamination has also been addressed by Tobe and Linacre (2008). Nevertheless, we could differentiate these contaminations from "real" positive results according to the respective species-specific polymorphisms of the analysed cytochrome b fragment or by melt curve analysis, respectively.

In qPCR, 1 out of 90 no-template controls showed a positive signal close to the cut-off C_T value of 40 (ΔC_T: 0.48). Therefore, the nature of this fluorescence signal is not certain. Other than from contamination, this signal may result from an unspecific "false" product having occurred randomly at early stages of PCR. In very rare events, unspecific products may be misinterpreted as a signal originating from a mitochondrion. Almost all qPCR results of deposited mitochondria showed higher ΔC_T values (range: 1.35–8.09) than the respective signal, but some samples that were detected in qPCR were of a similar dimension (ΔC_T: FCM: 0.57 and 0.99; OT: 0.46 and 0.69). Accordingly, these may also represent signals originating from "false" products. With FCM, 95 out of 252 of the technique-based negative controls (deposited beads) showed positive results. The most probable explanation for this high rate seems to be that mitochondria stick to the surface of deposited beads. Using LCM and OT, only 2.38% and 1.11%, respectively, of the technique-based negative controls showed (contaminating) mtDNA. Thus, the problem of "free" mtDNA in the respective carrier media seems to be of minor relevance.

5. Conclusions

In conclusion, we tested three different methods, which show different benefits and drawbacks regarding the deposition of single mitochondria.

FCM is the fastest method followed by LCM. OT require the most time, since transportation of mitochondria through the channel between wells of the 1µ-Ibidi-Slide is demanding. Compared to FCM, LCM as well as OT allow for optical control of the whole process

of separation. A major advantage of FCM is that a medium ensuring mitochondrial intactness can be used.

The OT showed the highest maximum probability w_{max} for the deposition of single mitochondria as well as the lowest drop-out rates. Thus, this method should be preferred whenever one single particle is to be focused, e.g. if heteroplasmy in single mitochondria or plastids is to be studied. Deckman et al. (2008), who also applied a similar technique using OT, actually measured heteroplasmy at the single-mitochondrion level. In contrast to this group, which analysed single mitochondria originating from a respective cell, we were able to analyse single mitochondria from bulk preparations.

If analysis of singular mitochondria or particles is not absolutely necessary faster techniques like FCM, which are easier to handle, are the better choice.

The analysis of single mitochondria especially with OT might improve the early diagnosis of mitochondrial respiratory chain disorders. For that purpose this technique should also be adapted to mitochondria isolated from other tissues, e.g. from skeletal muscle, but also from white blood cells (Wong et al., 2010).

Beside the presented qPCR analysis and the mathematical approach using w_{max} we also tried to verify the deposition of single mitochondria using microscopy based techniques. Due to the small size of mitochondria all these attempts failed, so the experimental proof is still lacking. Nevertheless, at IMTEK, we tested OT combined with quadrant diodes, which can detect small positional variations of a light beam. This technique has the potential to verify that actually one single particle is trapped at a given point of time, but further research has to be done. Optical trapping in combination with quadrant diodes may considerably enhance the reliability of singular (mitochondrial) particle analysis.

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