Isolation of mitochondria from procyclic *Trypanosoma brucei*

André Schneider*, Fabien Charrière, Mascha Pusnik and Elke K. Horn

Department of Biology/Zoology, University of Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland

*corresponding author: email, andre.schneider@unifr.ch; phone, +41263008877; fax, +41263009741.

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Abstract

The mitochondrion of the parasitic protozoon *Trypanosoma brucei* shows a number of unique features many of which represent highly interesting research topics. Studies on these subjects require the purification of mitochondrial fractions. Here we describe and discuss the two most commonly used methods to isolate mitochondria from insect stage *T. brucei*. In the first protocol the cells are lysed under hypotonic conditions and mitoplast vesicles are isolated on Percoll gradients, whereas in the second method lysis occurs isotonically by N_2 cavitation and the mitochondrial vesicles are isolated by Nycodenz gradient centrifugation.

Key Words: trypanosome, *Trypanosoma brucei*, mitochondria, subcellular fractionation, N_2 cavitation, Percoll, Nycodenz

1. Introduction

The parasitic protozoon *Trypanosoma brucei* is not only an important pathogen but has also proven to be an excellent model for basic science in general. Two main reasons for this are that (i) it is amenable to a wide range of molecular genetic, cell biological and biochemical techniques and (ii) that it has a very unique biology (1, 2).

The first point is illustrated by the fact that transfection of *T. brucei* by homologous recombination has already been achieved in 1990 (3). Furthermore, in 1995 transfection of *T. brucei* was used to establish a highly inducible gene expression system (4) and thus greatly expanded the repertoire of molecular genetic techniques. Most importantly, shortly after the discovery of RNA interference (RNAi) in *Caenorhabditis elegans* in 1998 (5) it was shown that the process is also operational in *T. brucei* (6). In the year 2000, finally, inducible gene expression was combined with RNAi (7, 8). The resulting system has since revolutionized research on all aspects of *T. brucei* biology, as it allows stable and inducible downregulation of any desired mRNA.

The unique biology of trypanosomes has and still is attracting a lot of interest. That this interest is justified, is illustrated by the fact that processes such as transsplicing, glycosyl phosphatidylinositol anchoring of membrane proteins and RNA editing were originally discovered in *T. brucei* and only later on shown to occur in essentially all eukaryotic cells.

Phylogenetic analyses based on rRNA sequences suggest that *T. brucei* belongs to the deepest branching eukaryotes having bona fide mitochondria involved in oxidative phosphorylation (9). Thus, it might not be a surprise that the *T. brucei* mitochondrion shows many unique features which represent highly interesting research topics (10). Unlike in most other eukaryotes, *T. brucei* has a single mitochondrion. Its genome is not distributed all over the matrix but localized to a specific region inside the organelle opposite the basal body of the flagellum (11). The genome itself is also very unusual: it consists of a large structure of two highly concatenated genetic elements the maxi- and the minicircles. Thus, the replication of the mitochondrial genome and how it is segregated during cell division represents a fascinating problem (12). The maxicircle encodes typical mitochondrial genes. However, many of these represent cryptogenes, meaning that their primary transcripts need to be processed by RNA editing in order to become functional mRNAs. The intriguing process of RNA editing has been the focus of much research during the last decade and is still very actively investigated (13). It is known since many years that the trypanosomal mitochondrial DNA does not encode any tRNA genes

indicating that, unlike in most other eukaryotes, all mitochondrial tRNAs have to be imported from the cytosol (14). Synthesis of mitochondrial encoded proteins in *T. brucei* shows interesting deviations to other translation systems. Not only does it require edited mRNAs (at least in some cases) and imported tRNAs but it also uses mitochondrial ribosomes which have among the shortest known rRNAs (15). Finally, it is known that the trypanosomal mitochondrion has some unusual metabolic pathways, such as a plant-like alternative oxidase and the ATP producing acetyl:succinate CoA-transferase cycle, which normally is only found in hydrogenosomes (16).

The examples above represent some of the topics of mitochondrial biology which are actively investigated in *T. brucei* and serve to illustrate the rich and unusual biology of the *T. brucei* mitochondrion. It is clear that research on any of these problems requires at some point the purification of mitochondria. It is the aim of this review to summarize and discuss the two main methods, which are used for this. The main difference between the two protocols is that in one the cells are lysed under hypotonic conditions, whereas in the other one lysis occurs in an isotonic buffer. The hypotonic protocol, which is based on the publications (17, 18), is the method of choice if purity of the preparation is the main concern, whereas if functionality is the main issue, it is recommended to use to the isotonic protocol (19).

2. Materials

2.1. Isolation of mitochondria, hypotonic procedure

2.1.1. Growth and harvesting of cells

1. Procyclic *Trypanosoma brucei* cells (*see* **Note 1**).
2. SDM-79 medium supplemented with 5% heat inactivated fetal bovine serum (20).
3. Centrifuge; fixed angle rotor, 6 x 500 ml capacity; 6 centrifuge bottles.
4. Disposable counting chamber: KOVA Glasstic Slide 10 with grid chamber (cat. No. 22-270141; Hycor Biomedical).
5. Wash buffer: 20 mM NaPi, pH 7.9, 20 mM Glucose, 0.15 M NaCl. Prepare as 4x stock (*see* **Note 2**).

2.1.2. Hypotonic cell breakage and DNase digestion

1. Hypotonic lysis buffer: 1 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0. Prepare as 10x stock.
2. 40 ml Dounce Tissue Homogenizer with a large clearance pestle.
3. 5 liter pressure vessel (cat. No. XX6700P05; Millipore).
4. Luer Lok syringe and hypodermic needle No. 26 (brown) and No. 25 (orange).
5. Sucrose stock: 1.75 M.
6. Centrifuge; fixed angle rotor, 8 x 50 ml capacity; 8 centrifuge tubes 50 ml.
7. STM buffer: 20 mM Tris-HCl, pH 8.0, 0.25 M Sucrose, 5 mM $MgCl_2$. Prepare as 4x stock (sterilize by filtration).
8. DNase I, from bovine pancreas, grade II (cat. No. 104159; Roche).
9. STE buffer: 20 mM Tris-HCl, pH 8.0, 0.25 M Sucrose, 2 mM EDTA, pH 8.0. Prepare as 4x stock.
10. EDTA stock: 0.5 M, pH 8.0.

2.1.3. Percoll step gradients

1. Ultracentrifuge; large swinging bucket ultra centrifuge rotor, 6 x 38.5 ml capacity; 6 Ultra-Clear centrifuge tubes 38.5 ml (25 x 89 mm)(cat. No. 344058; Beckmann). Tubes can be washed and reused.
2. Percoll 100% (cat. No. P-1644; Sigma), keep at 4^0C.

3. STE buffer containing 20, 25, 30, 35 and 75% of Percoll (v/v) each, keep at 4^0C.
4. 40 ml Dounce Tissue Homogenizer with the small clearance pestle.
5. 10 ml syringe with attached 100 μl glass capillary (*see* **Note 3**).

2.1.4. Removal of Percoll and storage

1. Centrifuge; fixed angle rotor, 8 x 50 ml capacity; 8 centrifuge tubes 50 ml.
2. STE buffer.
3. BCA protein assay kit (cat. No. 23227; Pierce).
4. Fatty acid free BSA (cat. No. A-6003; Sigma), prepare 100 mg/ml stock.

2.2. Isolation of mitochondria, isotonic procedure

2.2.1. Growth and harvesting of cells

See **Subheading 2.1.1.**

2.2.2. Isotonic cell breakage, DNase digestion and low speed spins

1. SoTE buffer: 20 mM Tris-HCl, pH 7.5, 0.6 M Sorbitol, 2 mM EDTA, pH 7.5. Prepare as 2x stock.
2. Cell disruption bomb for N_2 cavitation, capacity 920 ml (cat. No. 4635; Parr Instrument Company).
3. Centrifuge; fixed angle rotor, 8 x 50 ml capacity; centrifuge tubes 50 ml.
4. SoTM buffer: 20 mM Tris-HCl, pH 8.0, 0.6 M Sorbitol, 5 mM $MgCl_2$. Prepare as 4x stock (sterilize by filtration).
5. Luer Lok syringe and hypodermic needle No. 25 (orange).
6. DNase I, from bovine pancreas, grade II (cat. No. 104159; Roche).
8. EDTA stock. 0.5 M, pH 7.5.

2.2.3. Nycodenz step gradients

1. Ultracentrifuge; large swinging bucket ultra centrifuge rotor, 6 x 38.5 ml capacity; Ultra-Clear centrifuge tubes 38.5 ml (25 x 89 mm)(cat. No. 344058; Beckmann). Tubes can be washed and reused.

2. Nycodenz powder (cat. No. 1002424; Nycomed), prepare 80% (w/v) stock (*see* **Note 4**).
3. SoTE buffer containing 18, 21, 25, 28% and 50% of Nycodenz (w/v) each.
4. 40 ml Dounce Tissue Homogenizer with the small clearance pestle.
5. 10 ml syringe with attached 100µl glass capillary (*see* **Note 3**).

2.2.4. Removal of Nycodenz and storage

1. Centrifuge; fixed angle rotor, 8 x 50 ml capacity; 8 centrifuge tubes 50 ml.
2. SoTE buffer.
3. BCA protein assay kit (cat. No. 23227; Pierce).
4. Fatty acid free BSA (cat. No. A-6003; Sigma), prepare 100 mg/ml stock.

3. Methods

T. brucei contains a single large mitochondrion which cannot be isolated as an intact structure. Thus, independent of the chosen isolation method the mitochondrion will fragment into vesicles. However, while the native mitochondrial morphology gets disrupted during purification, the isolated vesicles retain many mitochondrial functions.

3.1. Isolation of mitochondria, hypotonic procedure

This procedure represents a modified version of the one described in (18). It relies on an initial cell lysis under hypotonic conditions by passage through a narrow hypodermic needle. Subsequently the extract is treated with DNAse and separated on Percoll gradients. Hypotonic cell lysis is very efficient and thus the hypotonic procedure is the method of choice to get biochemically pure mitochondria (e. g. for isolating mitochondrial RNAs). However, during lysis not only the cell membrane but also the mitochondrial outer membrane becomes ruptured and thus the purified mitochondrial vesicles represent mitoplasts (*see* **Note 5**)(21). These mitoplasts show little if any activity when assayed for mitochondrial protein import. Thus, for studying mitochondrial functions it is better to use mitochondrial vesicles purified by the isotonic method (*see* **Subheading 3.2.**).

3.1.1. Growth and harvesting of cells

1. Procyclic *T. brucei* are grown in suspension at 27°C in a total volume of 1 to 5 liters of SDM-79 medium containing 5% FCS. Cells are harvested at a density of 2.5-5.0 x 10^7 cells/ml corresponding to late log phase (*see* **Note 6**).
2. Except noted all further steps are on ice.
3. Spin cells in 500 ml centrifuge bottles in a fixed angle rotor at 4^0C for 10 min at 11'000 g (*see* **Note 7**).
4. During centrifugation determine cell concentration by microscopic counting using a disposable counting chamber.
5. After centrifugation immediately remove the medium (*see* **Note 8**). Add fresh cell culture to the same set of centrifuge bottles and repeat step 3.
6. Resuspend pellets in a small volume of wash buffer, combine pellets in one bottle, add wash buffer to approx. 450 ml and spin as above (*see* **Note 9**).

3.1.2. Hypotonic cell breakage

1. Resuspend cell pellet in hypotonic lysis buffer at 1.2 x 10^9 cells/ml (see **Note 10**). Add only little hypotonic lysis buffer first and homogenize in a 40 ml glass Dounce Tissue Homogenizer using the large clearance pestle to prevent clogging of the hypodermic needle. Check under the microscope whether as expected under hypotonic conditions the cells have rounded up.
2. Poure suspension into pressure vessel sitting in an ice bath and containing a magnetic stirrer. Close vessel, lock the outlet valve and apply 5 bars of pressure using N_2 (*see* **Note 11**).
3. Attach hypodermic needle (No. 26, brown) to the outlet of the pressure vessel.
4. Open outlet valve, collect suspension in a glass beaker on ice and measure volume.
5. Add 1/6 volume of 1.75 M sucrose stock. Mix well to re-establish isotonic conditions (*see* **Note 12**).
6. Examine lysis microscopically (*see* **Note 13**).

3.1.3. DNase digestion

1. Spin lysate in 50 ml centrifuge tubes in a fixed angle rotor at 4^0C for 10 min at 17'500 g.
2. Poure out supernatants (*see* **Note 14**). The cloudy supernatants represent the cytosolic fraction and depending on the experiment may be kept.

3. Resuspend pellets by vortexing in 1/6 volume of STM buffer. Pool in a single tube and estimate total volume.
4. Add solid DNase I to 0.1 mg/ml final (*see* **Note 15**).
5. Push the extract through a hypodermic needle (No. 25, orange) by using a Luer Lok syringe.
6. Incubate for 45 min at 4ºC. During the incubation repeat Step 5. The viscosity should drop during DNase digestion.
7. Add an equal volume of STE buffer.
8. Add 1/125 of the total volume of EDTA stock (0.5 M)(*see* **Note 16**).
9. Spin in 50 ml centrifuge tubes at 4^0C for 10 min at 17'500 g. The resulting pellet will be very soft. Thus the cloudy supernatant should be removed with a pipet.

3.1.4. Percoll step gradients

1. Precool large swinging bucket ultra centrifuge rotor.
2. Determine the number (2, 4 or 6) of gradients you will need. Each gradient should be loaded with lysate corresponding to 2.0-3.5 x 10^{10} cell-equivalents (*see* **Note 17**). In order to simplify the balancing an even number of tubes should be used.
3. During the DNase digestion prepare Percoll step gradients: pipet 8 ml of cold 35% Percoll containing STE buffer into each of the 38.5 ml ultracentrifuge tubes. Carefully overlay 8 ml each of cold 30%/25%/20% Percoll containing STE buffer (the gradients can also be prepared the day before and should in this case be kept at 4^0C).
4. Resuspend pellets (*see* **Subheading 3.1.3.** , **step 9**) in a total volume of 3 to 6 ml of 75% Percoll containing STE buffer per gradient, pool them and homogenize in a 40 ml glass Dounce Tissue Homogenizer using the small clearance pestle (*see* **Note 18**).
5. Place equal volumes of the samples (3-6 ml) at the bottom of each gradient. Practically this is done by using the 10 ml hypodermic syringe with the attached glass capillary (*see* **Note 19**).
6. Balance gradients with the 20% Percoll containing STE buffer on an electronic balance.
7. Spin gradients in the large swinging bucket ultra centrifuge rotor at 4^0C for 45 min at 100'000 g.
8. After centrifugation the gradients should show three bands. The middle one, at the 25%/30% Percoll interphase, is the most diffuse and may account for up to a third of the total gradient volume.

Microscopic examination shows that it is this band which is most enriched for mitoplast vesicles (*see* **Note 20**).

9. Collect 10 to 15 ml of this zone (25%/30% Percoll interphase) using the same 10 ml hypodermic syringe with the attached glass capillary which was used for loading.

3.1.5. Removal of Percoll and storage

1. Distribute the collected mitoplast fractions of all 6 gradients into eight 50 ml centrifuge tubes and dilute to 50 ml each with STE buffer.
2. Cap tubes with two layers of parafilm and mix vigorously by inversion (*see* **Notes 21**).
3. Spin in a fixed angle rotor at 4^0C for 15 min at 33'000 g. The resulting pellet will be very soft, thus discard supernatant with a pipet leaving approx. 3 ml of STE in the tube.
4. Combine two pellets into a single tube each. Dilute the combined pellets in the resulting four tubes to 50 ml with STE buffer and repeat step 2 and 3. The obtained pellets will be tighter now and essentially all STE can be removed.
5. Combine all four pellets into one tube dilute to 50 ml with STE buffer and repeat step 2 and 3.
6. Resuspend the mitoplast pellet in a small volume of STE buffer and examine it in the microscope. The fraction should look as shown in **Fig. 1A**.
7. Take a small aliquot and determine the protein concentration by the BCA protein assay kit (*see* **Note 22**).
8. Aliquots of mitoplasts can directly be flash frozen in liquid N_2 and stored at -70^0C. However, the best way to preserve the membrane integrity of the mitoplast is to add 1/9 volume of 100 mg/ml fatty acid free BSA before freezing (*see* **Note 23**)(22).

3.1. Isolation of mitochondria, isotonic procedure

This procedure is based on the one described in (19), the low speed spins were modified from (23). In this method the cells are lysed in an isotonic buffer by N_2 cavitation. Subsequently the extract is treated with DNase, intact cells are removed by low speed spins and organellar vesicles are separated on Nycodenz gradients. Cell breakage by N_2 cavitation is less efficient than hypotonic lysis. Thus, the obtained mitochondrial fraction is often less pure, than the one obtained by the hypotonic lysis procedure. However, isotonically isolated mitochondrial vesicles have an intact outer membrane (*see*

Note 4)(21). Furthermore, it was shown that for many functional studies, such as investigating mitochondrial protein import, the isotonic procedure is the method of choice (19).

3.2.1. Growth and harvesting of cells

See **Subheading 3.1.1.**

3.2.2. Isotonic cell breakage

1. Resuspend pellet in SoTE buffer at 2 x 10^9 cells/ml. Take a small sample as a control for the microscopic examination of the extent of the cell lysis (*see* below, **step 7**).
2. Homogenize in a 40 ml glass Dounce Tissue Homogenizer using the large clearance pestle.
3. Put cell suspension into the Cell disruption bomb sitting in an ice bath. Close bomb, lock the outlet valve.
4. Apply 55 bars by using N_2 and close the inlet valve. Incubate for 30 min under constant stirring while bomb sits in an ice bath (*see* **Note 24**).
5. Depressurize the Cell disruption bomb and collect the foamy suspension.
6. Let the foam settle for few minutes.
7. Examine lysis microscopically by comparing samples before and after lysis (*see* **Note 25**).
8. Spin lysate in 50 ml centrifuge tubes in a fixed angle rotor at 4^0C for 10 min at 24'000 g. Pour out supernatants (*see* **Note 14**). The supernatants represent the cytosolic fraction and depending on the experiment may be kept.

3.2.3. DNase digestion

1. Resuspend pellet in equal volume of SoTM buffer.
2. Add solid DNase I to 0.1 mg/ml final (*see* **Note 15**)
3. Push the extract through a hypodermic needle (No. 25, orange) by using a Luer Lok syringe.
4. Incubate for 45 min at 4ºC. During incubation repeat **step 3**. The viscosity should drop during DNase digestion.
5. Add an equal volume of STE buffer.
6. Add 1/125 volume of EDTA stock (0.5 M)(*see* **Note 16**).

3.2.3. Low speed spins

1. Spin lysate in 50 ml centrifuge tubes in a fixed angle rotor at 4^0C for 10 min at 490 g. Fill tubes to the top, if necessary add SoTE buffer.
2. Transfer supernatant to a beaker and keep on ice. The pellet will be very soft, thus leave 1-2 ml of the supernatant in the tube.
3. Resuspend each pellet in ca. 10-15 ml of SoTE by homogenizing in a 40 ml glass Dounce Tissue Homogenizer using the large clearance pestle. Pool supernatants.
4. Spin in 50 ml centrifuge tubes in a fixed angle rotor at 4^0C for 10 min at 375 g. Fill tubes to the top, if necessary add SoTE buffer (*see* **Note 26**)
5. Pool supernatants with the previous ones (*see* **step 2**).
6. Distribute the pooled supernatants to 50 ml centrifuge tubes and spin in a fixed angle rotor at 4^0C for 10 min at 24'000 g. Discard supernatants.

3.2.5. Nycodenz step gradients

1. Precool large swinging bucket ultra centrifuge rotor.
2. Determine the number (2, 4 or 6) of gradients you will need. Each gradient should be loaded with lysate corresponding to 3.5-6.5 x 10^{10} cell-equivalents. In order to simplify the balancing an even number of tubes should be used.
3. During the DNase digestion prepare Nycodenz step gradients: pipet 8 ml of cold 28% Nycodenz containing SoTE buffer into each 38.5 ml ultracentrifuge tube. Carefully overlay 8 ml each of cold 25%/21%/18% Nycodenz containing SoTE buffer.
4. Resuspend pellets (*see* **Subheading 3.2.6.** , **step 6**) in a total volume of 3 to 6 ml of 50% Nycodenz containing SoTE buffer per gradient, pool them and homogenize in a 40 ml glass Dounce Tissue Homogenizer using the small clearance pestle (*see* **Note 18**).
5. Place equal volumes of the samples (3-6 ml) at the bottom of each gradient. Practically this is done by using the 10 ml hypodermic syringe with the attached glass capillary (*see* **Note 19**).
6. Balance gradients with the 18% Nycodenz containing SoTE buffer on an electronic balance.
7. Spin gradients in the large swinging bucket ultra centrifuge rotor at 4^0C for 45 min at 100'000 g.

8. After centrifugation the gradients should show three bands. Microscopic examination shows that the middle band at the 25%/28% Nycodenz interphase, which generally is the most prominent one, is enriched for mitochondrial vesicles (*see* **Note 27**).
9. Collect approx. 5 ml of this band (25%/28% Nycodenz interphase) using the same 10 ml hypodermic syringe with the attached glass capillary which was used for loading.

3.2.6. Removal of Nycodenz and storage

1. Distribute the collected 25%/28% Nycodenz interphase mitochondrial fractions of all gradients into 50 ml centrifuge tubes and dilute at least 5fold with SoTE buffer.
2. Cap tubes with two layers of parafilm and mix by inversion.
3. Spin in a fixed angle rotor at 4^0C for 15 min at 33'000 g and discard supernatants.
4. Resupend pellets in approx 1 ml per gradient of SoTE buffer and pool. Distribute the resulting suspension into 1.5 ml Eppendorf tubes.
5. Spin in a Eppendorf centrifuge at approx. 10'000 g and discard as much supernantant as possible.
6. Resuspend the mitochondrial pellet in a small volume of SoTE buffer and examine in the microscope. The fraction should look as shown in **Fig. 1B**.
7. Take a small aliquot and determine the protein concentration by the BCA protein assay kit (*see* **Note 28**).
8. Aliquots of mitochondrial vesicles can directly be flash frozen in liquid N_2 and stored at -70^0C. However, the best way to preserve the membrane integrity of the mitochondrial fraction is to add 1/9 volume of 100 mg/ml fatty acid free BSA before freezing (*see* **Note 23**)(22).

4. Notes

1. The procedures appear to work for any *T. brucei* cell line. We have used it for the T. brucei 427 and 29-13 strains, as well as for many transgenic cell lines including induced RNAi strains.
2. If not indicated otherwise the buffers and solutions described in this chapter were not sterilized. EDTA containing solutions are not prone to microbiological contaminations and thus for short term storage were kept at 4^0C; longterm storage was done at -20^0C. All other solutions were kept at -20^0C and thawed overnight before use.
3. The glass capillary can easily be attached to the syringe by using Parafilm.

4. Weigh out 80 g of Nycodenz, add aliquots to approximately 45-50 ml of H_2O mixed by a magnetic stirrer. 80% Nycodenz will take a long time to solubilize, to accelerate the process the solution can be warmed up to approx. 37^0C. After complete solubilization add H_2O to 100 ml.
5. This has been demonstrated by direct comparison of the mitochondrial ATP production pathways in organellar vesicles isolated by either the hypotonic or the isotonic purification protocols (21).
6. Both the hypotonic and the isotonic procedures work best on a large scale (5 liters of culture) and are not recommended for less than one liter of well grown cells. The indicated cell densities are for *T. brucei* 427 grown in SDM-79 and may be different for other cell lines or media. Large scale cultures are grown at 27^0C in 2 liter Erlenmeyer flasks containing 1-1.3 liter culture each on a shaking incubator set at 115 rpm. Some transgenic strains may be more fragile than wildtype cells, in this case shaking needs to be reduced.
7. Indicated g forces always refer to g_{max} at the bottom of the tube.
8. *T. brucei* cells are highly motile which results in soft pellets. Thus, the medium needs to be poured off immediately after centrifugation. This should always be done with the pellets facing downwards.
9. 1 x PBS can be used instead of wash buffer. The total cell number can be estimated by weighing and be compared to the one obtained by microscopic counting: 5.8 g wet weight of cells correspond to approx. 10^{11} cells.
10. This is the highest recommended concentration. Breakage of cells using the pressure vessel works best for volumes $\geq$100 ml. For smaller preparations the cells can be diluted 2 to 4 fold more.
11. If no pressure vessel is available the cells can be lysed manually by using a 20-40 ml Luer-Lok syringe and pushing them once or twice with as much force as possible through a No. 26 hypodermic needle.
12. The time the lysed cells remain in the hypotonic lysis buffer before the sucrose is added is critical and should be minimized since otherwise the mitochondrial vesicles will lyse as well.
13. Lysis is expected to be complete. Thus cell fragments, flagella and floating vesicles but no live cells are observed.
14. A white floating layer will appear on the solution. This layer probably represents broken membranes and is indicative of efficient cell lysis, it can most easily be removed by using a paper tissue.

15. DNase digestion is essential in order to allow efficient separation on either Percoll or Nycodenz gradients.
16. Addition of EDTA complexes the magnesium and thus stops the DNase digestion. Furthermore, addition of EDTA also serves to prevent aggregation of mitochondrial preparations which is observed in the presence of magnesium.
17. We have never loaded more the 3.5 x 10^{10} cell equivalents, however, we expect the gradients to tolerate higher loadings.
18. The dilution of the pellet with 75% Percoll containing STE buffer in the hypotonic procedure or with the 50% Nycodenz containing SoTE buffer in the isotonic preparation needs to be sufficient to allow the suspension to sink beneath the lowest layers of the step gradients.
19. It is best to insert the capillary into the gradient along the tube wall and to keep it there till the whole sample has been applied. First, load a small volume of the sample only and wait few seconds to make sure it remains at the bottom of the tube. If it floats up, remove the syringe, add more of the 75% Percoll containing STE buffer for the hypotonic procedure or 50% Nycodenz containing SoTE for the isotonic preparation and try again.
20. Microscopic examination shows that the top band (20%/25% Percoll interphase), which is the most intense one, mainly contains flagella and some cell fragments, whereas the lowest band (35%/75% Percoll interphase) which normally is the least intense one contains a uniform population of vesicular structures of unknown origin which are much smaller than the ones observed in the mitoplast fraction. Large mitoplasts vesicles are seen in all three fractions but are most enriched in the central part of the gradient (25%/30% Percoll interphase)(**Fig. 1A**). The main contaminants of the central mitoplast fraction are flagella. Depending on the cell line and the efficiency of the cell lysis there can be variations in the relative intensities of the three zones. Furthermore, if only little material is loaded on the gradient, it is possible that no clear accumulation of material is seen in the central zone. While the yield will be low in this case, it is still worth to collect the zone and to proceed with the purification, as a mitoplast pellet may only become visible after the washes.
21. Percoll does not mix easily so vigorous mixing is important. The more the Percoll is diluted the more tight pellets will be obtained after centrifugation.
22. The obtained yields can be quite variable, but are typically in the range of 20 mg protein or 30-50 μg of RNA per 10^{11} cells.

23. While the integrity of the mitoplasts or the mitochondrial fractions during storage at -70^0C is best maintained in the presence of BSA, it should be considered that the presence of BSA might interfere with some downstream applications such as Western blots analysis.
24. Pressure will slightly drop during incubation, therefore re-adjust to 55 bar after the first 5 min.
25. Besides cell ghosts and floating vesicles a significant number of intact live cells are observed. Thus, cell lysis is less efficient - typically around 80-90% - than with the hypotonic protocol.
26. The resulting pellet should be smaller than the one obtained in the first low speed spin.
27. Microscopic examination shows that the top band (18%/21% Nycodenz interphase) mainly contains intact cells, whereas the lowest band (28%/50% Nycodenz interphase) is enriched for flagella. Mitochondrial vesicles are seen in all three fractions but are most enriched in the central part of the gradient (25%/28% Nycodenz interphase)(**Fig. 1B**). The main contaminants of the central mitochondrial vesicle fraction are flagella. Occasionally few intact cells may be observed as well. As for the hypotonic procedure depending on the cell line and the efficiency of the cell lysis there can be variations in the relative intensities of the three zones.
28. The obtained yields can be quite variable, but are typically in the range of 15-30 mg protein per 10^{11} cells.

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References

1. Gull, K. (2001) The biology of kinetoplastid parasites: insights and challenges from genomics and post-genomics. *Int. J. Parasitol.* **31,** 443-52.
2. Cross, G. A. (2001) African trypanosomes in the 21st century: what is their future in science and in health? *Int. J. Parasitol.* **31,** 427-33.
3. tenAsbroek, A. L. M. A., Ouellette, M., and Borst, P. (1990) Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of Trypanosoma brucei. *Nature* **348,** 174-75.
4. Wirtz, E., and Clayton, C. (1995) Inducible gene expression in trypanosomes mediated by a prokaryotic repressor. *Science* **268,** 1179-83.
5. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806-11.
6. Ngo, I., Tschudi, C., Gull, K., and Ullu, E. (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* **95,** 14687-92.
7. Shi, H., Djikeng, A., Mark, T., Wirtz, E., Tschudi, C., and Ullu, E. (2000) Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6,** 1069-76.
8. Wang, Z., Morris, J. C., Drew, M. E., and Englund, P. T. (2000) Inhibition of *Trypanosoma brucei* gene expression by RNA interference. *J. Biol. Chem.* **275,** 40174-79.
9. Sogin, M. L., Elwood, H. J., and Gunderson, J. H. (1986) Evolutionary diversity of eukaryotic small-subunit rRNA genes. *Proc. Natl. Acad. Sci. USA* **83,** 1383-87.
10. Schneider, A. (2001) Unique aspects of mitochondrial biogenesis in trypanosomatids. *Int. J. Parasitol.* **31,** 1403-15.
11. Ogbadoyi, E. O., Robinson, D. R., and Gull, K. (2003) A high-order trans-membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. *Mol. Biol. Cell.* **14,** 1769-79.

12. Morris, J. C., Drew, M. E., Klingbeil, M. M., Motyka, S. A., Saxowsky, T. T., Wang, Z., and Englund, P. T. (2001) Replication of kinetoplast DNA: an update for the new millennium. *Int. J. Parasitol.* **31,** 453-58.

13. Koslowsky, D. J. (2004) A historical perspective on RNA editing: how the peculiar and bizarre became mainstream. *Methods Mol. Biol.* **265,** 161-97.

14. Schneider, A., and Marechal-Drouard, L. (2000) Mitochondrial tRNA import: are there distinct mechanisms? *Trends Cell Biol.* **10,** 509-13.

15. Horvath, A., Nebohacova, M., Lukes, J., and Maslov, D. A. (2002) Unusual polypeptide synthesis in the kinetoplast-mitochondria from *Leishmania* tarentolae. Identification of individual de novo translation products. *J. Biol. Chem.* **277,** 7222-30.

16. vanHellemond, J. J., Opperdoes, F. R., and Tielens, A. G. M. (1998) Trypanosomatides produce acetate via a mitochondrial acetate:succinate CoA transferase. *Proc. Natl. Acad. Sci. USA* **95,** 3036-41.

17. Braly, P., Simpson, L., and Kretzer, F. (1974) Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. *J. Protozool.* **21,** 782-90.

18. Harris, M. E., Moore, D. R., and Hajduk, S. L. (1990) Addition of uridines to edited RNAs in trypanosome mitochondria occurs independently of transcription. *J. Biol. Chem.* **265,** 11368-76.

19. Hauser, R., Pypaert, M., Häusler, T., Horn, E. K., and Schneider, A. (1996) In vitro import of proteins into mitochondria of *Trypanosoma brucei* and *Leishmania tarentolae*. *J. Cell Sci.* **109,** 517-23.

20. Brun, R., and Schönenberger, M. (1979) Cultivation an in vitro cloning of procyclic culture forms of *Trypansoma brucei* in a semi-defined medium. *Acta Tropica* **36,** 289-92.

21. Allemann, N., and Schneider, A. (2000) ATP production in isolated mitochondria of procyclic *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **111,** 87-94.

22. Kozlowski, M., and Zagorski, W. (1988) Stable preparation of yeast mitochondria and mitoplasts synthesizing specific polypeptides. *Anal. Biochem.* **172,** 382-91.

23. Priest, J. W., and Hajduk, S. L. (1996) In vitro import of the rieske iron-sulfur protein by trypanosome mitochondria. *J. Biol. Chem.* **271,** 20060-69.

Legend

Fig. 1. **Nomarski microscopy of purified mitochondrial fractions.**

A. Final mitoplast vesicle fraction isolated by the hypotonic purification procedure. **B**. Final mitochondrial vesicle fraction using the isotonic purification method. Vesicles isolated by the hypotonic procedure are larger in size than the ones purified by the isotonic method. Bar. 20 μm.

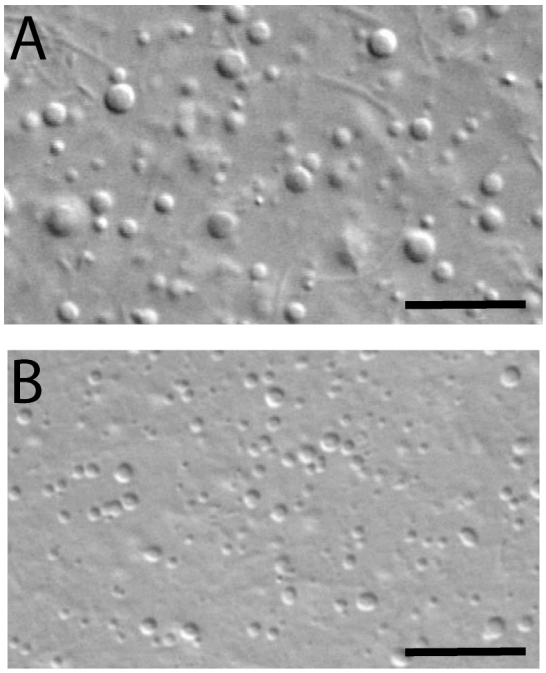

Fig. 1