CHAPTER 8

Ultrastructural Investigation Methods for *Trypanosoma brucei*

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Abstract

Trypanosoma brucei is a unicellular parasite causing African sleeping sickness in cattle and humans. Due to the ease with which these cells can be cultured and genetically manipulated, it has emerged as a model organism for the kinetoplastids. In this chapter we describe the preparation of *T. brucei* for transmission electron microscopy. A thorough explanation of conventional sample preparation through chemical fixation of whole cells and detergent extracted cytoskeletons followed by dehydration and Epon embedding is given. We also introduce a novel high-pressure freezing protocol, which followed by rapid freeze substitution and HM20 embedding generates *T. brucei* samples displaying good cell morphology, which are suitable for immunocytochemistry.

I. Introduction

Trypanosoma brucei is a eukaryotic single-celled, flagellated protozoan parasite, which is the causative agent of African sleeping sickness. It is spread through the bite of the tsetse fly (*Glossina sp*.), and has a complex lifecycle. Each of the different life cycle stages of *T. brucei* have different cell shapes and internal organization (Gull, 1999; Sharma *et al*., 2009; Vickerman, 1985). Laboratory studies tend to focus on two easily cultured forms: the procyclic form (PCF), normally found in the fly midgut, and the form found in the bloodstream of humans and cattle (BSF).

T. brucei has emerged as the model organism of the Kinetoplastida, a protozoan order named after a DNA-containing structure found within their single mitochondrion. Other kinetoplastids include *Leishmania spp*. (causing cutaneous and/or visceral leishmaniasis) and *T. cruzi* (cause of Chagas disease). All three pose major public health problems in their respective endemic areas. Kinetoplastids have a large evolutionary distance to yeast, worms, and other common model systems, making the study of their cellular mechanisms also a study of conservation and divergence in biological systems. Studies of trypanosomes have brought insight into many areas of biology including mitochondrial DNA, RNA editing, GPI anchors, antigenic variation and mono-allelic exclusion, flagellum and cytoskeleton, glycolytic metabolism and gene expression systems.

African sleeping sickness is lethal if untreated in humans and the drugs available have severe side effects, and drug resistance is emerging. The need for new, cheap drugs that can be easily administered is large but only one such drug has been brought into use since the 1950s (Bacchi, 2009; Delespaux and de Koning, 2007). Therefore, it is increasingly important to better understand this parasite's cell biology.

The subspecies *T. brucei brucei* is not human infective (Pays *et al*., 2006) and the procyclic and bloodstream forms are easily kept cultured. Molecular genetic approaches are very tractable since homologous recombination is a dominant

phenomenon. Gene knockouts, inducible RNAi for gene silencing, and inducible expression of tagged (GFP/epitope) or mutant proteins are well established (Beverley, 2003; LaCount *et al*., 2000; Shi *et al*., 2000). The *T. brucei* genome sequencing has been completed, as well as the genomes of the related *L. major* and *T. cruzi* (Berriman *et al*., 2005; El-Sayed *et al*., 2005; Ivens *et al*., 2005). It was found that these three species have similar genomic architecture and their genomes are now gathered in a common database named tritrypDB (Aslett *et al*., 2009).

For electron microscopic (EM) purposes, these cells are ideal, because of their small size (~4 μm wide and ~20 μm long). Their internal architecture is defined by a subpellicular microtubule array that maintains the cell shape. The other organelles are often in single copies and occupy distinct subcellular locations (Gull, 2003). This invariant architecture and reproducibility in position and division facilitates the description of mutant phenotypes.

Excellent EM methods have been developed including those for chemically fixed samples and, more recently, results were obtained using high pressure freezing (Gadelha *et al*., 2009; Grunfelder *et al*., 2002; Lacomble *et al*., 2009; Overath and Engstler, 2004; Sherwin and Gull, 1989; Weise *et al*., 2000, 2003). In this chapter we detail some existent methods and their uses and present a new high-pressure freezing protocol that has yielded very good cell morphology, and is useable for immunolocalization of proteins.

II. Rationale

This chapter seeks to provide a toolbox for performing electron microscopy of *T. brucei*. The established methods of chemical fixation, positive staining of cytoskeletons, and detection of nucleic acids using EDTA is reiterated, and a new protocol using high-pressure freezing is introduced. The improvements made when using high-pressure freezing, followed by freeze substitution (FS) and plastic embedding for studying *T. brucei* have already provided novel views of these parasites. The largest structural preservation improvements of high-pressure freezing are to be found when studying membranous cellular compartments such as the Golgi.

III. Methods

A. Cell Culture

The procyclic form of *T. brucei* is grown in SDM-79 (Brun, 1979) medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum (HIFCS) at 28°C in closed top tissue culture flasks. The procyclic culture is kept between 10^5 and 10^7 cells/ml, and best harvested at ~8×10^6 cells/ml. Bloodstream form *T. brucei* is grown in HMI-9 medium supplemented with 15% (v/v) HIFCS at 37°C in vented top culture flasks in a 5% CO_2 environment (Hirumi and Hirumi, 1989), and best harvested at ~8×10^5 cells/ml. Cell line stocks are kept in liquid nitrogen in a medium containing 10% glycerol.

B. Chemical Fixation for Morphology Studies

Chemical fixations provide a means for rapid crosslinking and immobilization of cellular structures. The most widely used fixative for ultra structural studies is glutaraldehyde. Primary fixation with an aldehyde is typically followed by postfixation in osmium tetroxide, en bloc staining with uranyl acetate, dehydration in ethanol or acetone, and embedding in Epon resin. The following is a standard protocol for the processing of cultured trypanosomes. This protocol has been optimized for *T. brucei* procyclic forms, but will give good results for a number of trypanosomatid species and life cycle stages. Where the processing of *T. brucei* bloodstream forms requires modifications, this is specifically indicated. Procyclic *T. brucei* prepared with this method are shown in Fig. 1.

1. Primary Fixation of Cells in Suspension Culture

To minimize manipulation of cells prior to fixation, the initial fixation occurs in the culture flask by adding one volume of 25% EM-grade glutaraldehyde to nine volumes of cell culture (2.5% final glutaraldehyde concentration). Cells are fixed for 5–10 min at room temperature. The fixed cells are then harvested by gentle centrifugation (800 g for 5–10 min) and the supernatant decanted. The cells are resuspended in 1 ml buffered fixative (2.5% glutaraldehyde, 2% formaldehyde in 100 mM phosphate buffer pH 7–7.4) and centrifuged again to form a pellet and left to continue fixation for at least 2 h at room temperature (the samples can be left for longer fixation at 4°C). The buffer should maintain the physiological pH and osmolarity of the sample and the choice of buffer will therefore vary depending on the type of cell used. To maintain the osmolarity of the fixative, sucrose, glucose or NaCl can be added to the buffer. For *T. brucei* bloodstream forms, the addition of 50 mM sucrose to the buffered fixative enhances the ultrastructural preservation.

To avoid losing cells during the subsequent steps they are processed in a pellet, rather than in suspension. It is most convenient to continue sample processing in a 1.5 ml Eppendorf tube, using ~1 ml volumes for each of the following steps. After each centrifugation step the pellet can be dislodged from the wall of the tube by gentle tapping. The fixative is removed and the sample washed extensively in buffer (200 mM phosphate buffer pH 7–7.4). It is important to remove any remaining free aldehyde, which otherwise react with osmium and cause formation of small electron dense particles. This requires a minimum of three buffer changes over a period of 2–3 h at room temperature (samples may be left at 4°C overnight).

2. Preparation of Trypanosomes Isolated from the Tsetse Fly

Dissect the trypanosome-infected tsetse flies (Sharma *et al.*, 2008, 2009), isolate the gut, proventriculus, or salivary glands as required and transfer the organs directly into buffered fixative (Section III.B.1). Cut the organs into small pieces (<1 mm) to facilitate penetration of the fixative and then process in the same way as the cell pellets described in Section III.B.1.

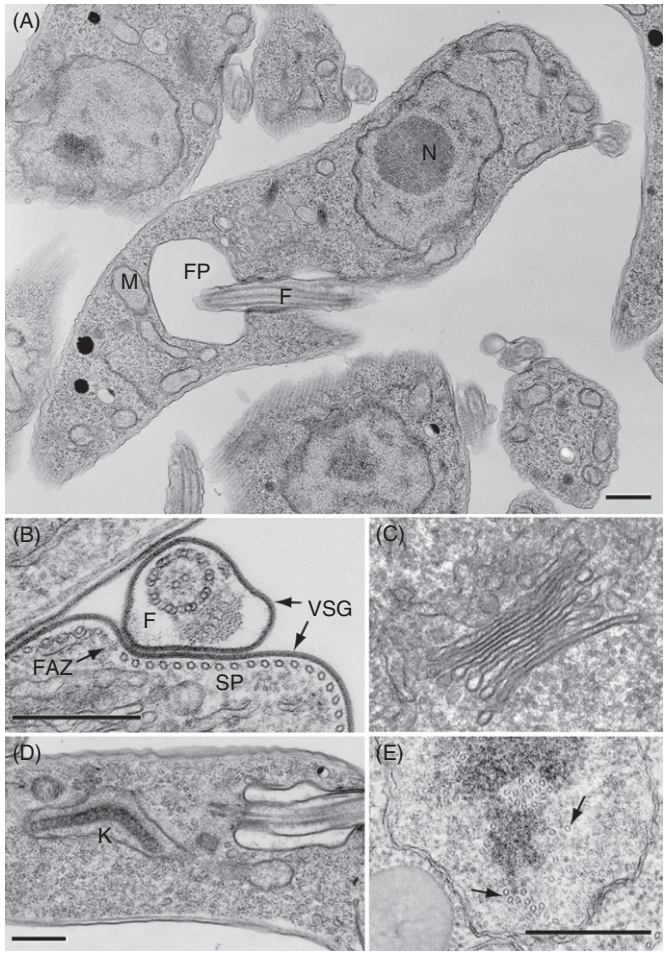

Fig. 1 Chemical fixation of *T. brucei* (A) Low magnification view of procyclic form (PCF) showing nucleus (N), flagellar pocket (FP), flagellum (F), and mitochondrion (M). (B) Bloodstream form (BSF), cross-section of flagellum, subpellicular microtubules (SP), flagellum attachment zone (FAZ), VSG surface coat (arrows). (C) BSF Golgi and (D) BSF dividing kinetoplast (K), flagellar pocket. (E) PCF nucleus showing spindle microtubules (arrows) in cross-section fixed in 2.5% glutaraldehyde and embedded in Epon. Scale bars: 500 nm.

3. Postfixation, Staining, and Dehydration

Glutaraldehyde-fixed samples are postfixed in 1% osmium (in 100 mM phosphate buffer or distilled water) for 1–2 h in the dark. Osmium tetroxide acts as a fixative as well as a stain. It reacts with lipids and oxidizes unsaturated bonds of fatty acids, adds electron density, which gives contrast, and it permeabilizes the cells instantly. It is important not to leave the samples in osmium too long because this will result in

the extraction of cellular material. Osmium fixation will cause the pellet to go dark brown. After osmium fixation, the samples are washed multiple times in distilled water. It is important to remove excess phosphate ions to prevent the precipitation of uranyl acetate in the subsequent step. To provide en-bloc stain, the cell pellet is immersed in 2% magnesium uranyl acetate in water for 2 h at room temperature or 4°C overnight. Uranyl acetate compounds are photo reactive and will precipitate when exposed to light, so samples need to be kept in the dark. Samples are then washed with water. Dehydration enables penetration of resin, and is typically performed in a series of washes with increasing concentration of dehydrated ethanol or acetone: 30, 50, 70, 90% and three times 100% for 10 min each.

4. Resin Infiltration and Embedding

In the final step, the solvent is gradually replaced by resin monomers, which are then polymerized to form a solid resin block in which the sample is embedded. A typical infiltration consists of submersion of the cell pellet two times into 100% propylene oxide for 15 min each, then several hours in 1:3, 1:1, and 1:0 resin : propylene oxide baths. The choice of resin will depend on individual preference and the nature of the experiment. For routine preparation of trypanosomes, Epon or Spurr's resin gives good results. Propylene oxide is highly miscible with the plastic embedding medium, and thus facilitates infiltration. However, it is very volatile and dehydration of the sample should be prevented by keeping tube lids closed and the sample immersed. The pellets are then transferred to silicone embedding moulds containing fresh resin. These are placed at 60°C for 24–48 h during which the epoxy monomers that permeate the sample polymerize until a solid block is formed that can be sectioned.

C. High-Pressure Freezing for Morphology Studies

High-pressure freezing is a physical means to cryoimmobilize samples very rapidly by exposure to liquid nitrogen under a pressure of over 2000 bar. The pressure is applied to prevent the formation of cubic and hexagonal ice (Richter, 1994) (see also chapters by O'Toole, Giddings, Müller-Reichert, this volume). Ice crystals are known to damage the cell's fine structure. Using high-pressure freezing, "large" samples with a thickness of up to 200 μm can be routinely vitrified, i.e., frozen without such ice-crystals).

A sample transfer follows the cryoimmobilization from the high-pressure freezer into a freeze substitution (FS) machine, where the water is extracted using a solvent, such as ethanol or acetone and a simultaneous slow fixation occurs at very low temperatures through addition of fixatives to the FS "cocktail". The FS is then followed by resin infiltration and the sample preparation is completed with resin embedding/polymerization.

The benefits of high-pressure freezing are usually most apparent in membrane morphology, which appear wrinkled after dehydration but are smooth when using cryoimmobilization before fixation and dehydration at low temperatures. Although some aspects of cell morphology are greatly improved in high-pressure frozen

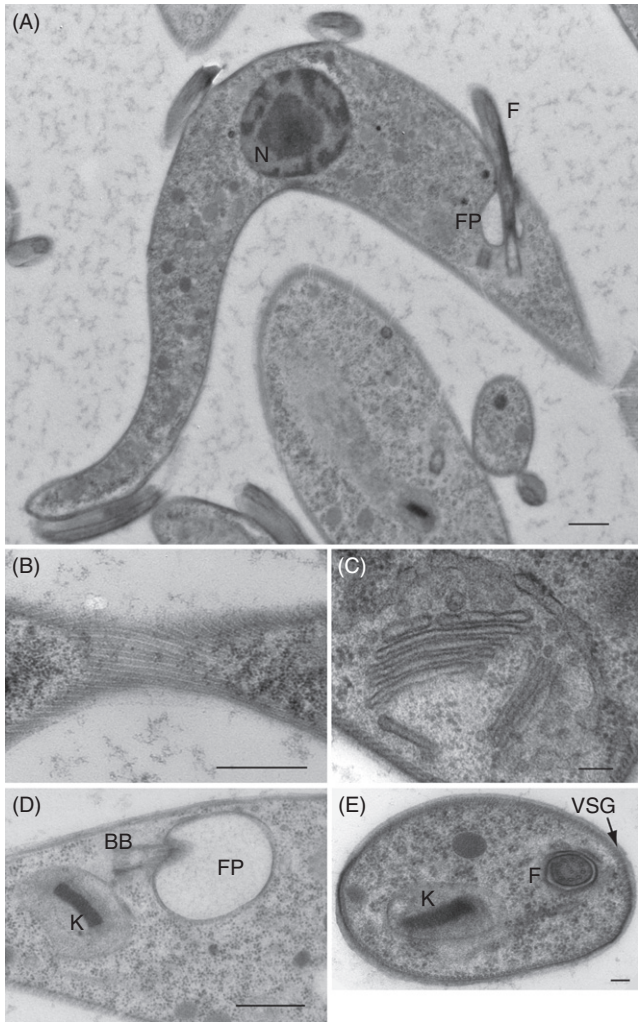

Fig. 2 High-pressure frozen *T. brucei* embedded in HM20 resin. (A) Low magnification of a PCF, note the round nucleus (N), well-defined chromatin, and smooth membranes around the cell and in the flagellar pocket (FP), where the flagellum (F) originates. (B) PCF subpellicular microtubule array. (C) Golgi apparatus in a BSF. (D) The FP is plump and the kinetoplast (K) DNA is an electron dense disc without clear filamentous circles as seen in chemical fixation. BB, basal body. (E) BSF in cross-section showing a VSG-coat, a flagellum in cross section, and a kinetoplast. Scale bars: 500 nm, in (C) 100 nm.

T. brucei (Fig. 2), these cells are not straight forward to freeze (Fig. 3). This is probably due to a cytoplasm relatively "empty" of solutes that would act as internal cryoprotectants. Freezing is variable inside the pellet, so taking time to search for a well-frozen area is recommended.

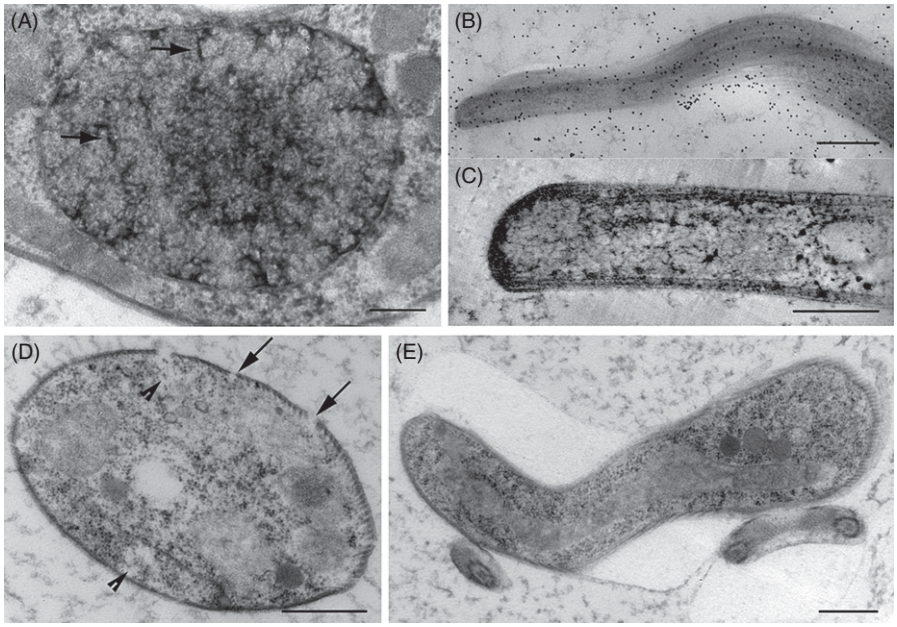

Fig. 3 Sample preparation problems (A) A severely ice-damaged *T. brucei* nucleus. Note the chicken-wire appearance of the nucleoplasm (arrows). (B) A zero-tilt low-magnification image of an anterior end in a semi-thick section shows no traces of ice damage. The section displays colloidal gold particles to be used for image alignment when calculating a tomogram. (C) The tomographic reconstruction of the same anterior end shows extensive ice damage by smaller ice crystals. (D) A cell with cracks in the membrane and underlying sub-pellicular microtubule cytoskeleton (arrows). These fractures could have been caused by centrifugation prior to freezing or by ice-crystal formation since ice damage is also apparent (arrowheads). (E) Poor infiltration of resin is often seen around narrow structures, such as the flagellum. Here the whole cell has "fallen out" of the plastic. Scale bar: 200 nm in (A) and (C), 500 nm (B, D) and (E).

1. Procyclic Form (PCF)

To increase the amount of well-frozen cells, the amount of solutes in the medium is increased by using 20 or 50% heat-inactivated FCS instead of the regular 10%. The increased FCS medium does not inhibit growth. This is likely to work as an external cryoprotectant (McDonald *et al.*, 2007). Other cryoprotectants tested but with less successful results were 20% bovine serum albumin (McDonald *et al.*, 2007) and 2% gum arabic.

For high-pressure freezing, cells need to be concentrated in a pellet. It is convenient to use a ~50 ml culture of procyclic forms at a density of $0.8–1.0\times10^7$ cells/ml (seed cultures at 1.0×10^6 cells/ml 24 h prior freezing) and prepare pellets by gently spinning down 10–15 ml of culture at a time (2–3 min at 600 g; $\sim1.0\times10^8$ cells/pellet).

Prepare the high-pressure freezing and FS machines and have everything ready for freezing before harvesting the cells.

2. Bloodstream Form (BSF)

Since the bloodstream form must be kept at low density to ensure high viability the culture needs to be expanded to > 200 ml to get enough cells for freezing. Use healthy looking cells just below 1×10^6 cells/ml. Spin down a full 50 ml falcon tube for 2–3 min at 600 g. Proceed immediately to freezing. Since the bloodstream form *T. brucei* is more sensitive to temperature changes than the procyclic form, they may not benefit from centrifugation before freezing and an alternative method harvesting cells from colonies growing on agar plates may prove superior (Carruthers and Cross, 1992; Gadelha, 2008). However, we have found good cell preservation using centrifugation as a means to concentrate the cells.

3. High-Pressure Freezing

Without disturbing the pellet, gently remove the supernatant and pipette up a few µl of sample from inside the pellet. White lumps of cell pellet should be visible in the pipette tip (but this might not be possible for the bloodstream form). Fill the smallest freezing carriers available with cells; smaller volumes increase the efficiency of the subsequent freezing. For a thorough description of high-pressure freezers and carriers, see McDonald *et al*. (2007).

Once the cells have been frozen, be extremely careful to precool all the tools in liquid nitrogen before using them anywhere near the sample. When moving the sample, if there is any doubt whether it has been raised above the nitrogen surface, discard and do another. This might appear wasteful but it saves a lot of sectioning and microscopy time. Freeze as fast as possible after centrifugation to ensure cells remain healthy, no longer than 5 min after centrifugation. Then spin down a new pellet. Try to make at least 2–3 samples of each condition. Once all samples have been frozen, transfer them under liquid nitrogen into the precooled (–90°C) FS container.

4. Freeze Substitution and Embedding into HM20 Resin

This protocol is an adaptation of a protocol worked out for mammalian cells (Hawes *et al*., 2007), but we have seen it work well on *Schizosaccharomyces pombe* as well as *T. brucei* (Fig. 2). It has a much shortened FS and excludes many of the most toxic chemicals, such as glutaraldehyde and osmium tetroxide, in the FS solution. The contrast is good, the HM20 resin is more electron transparent than Epon, which is especially beneficial for electron tomography when thick sections are used. In addition, it can also be used for immunolocalization of proteins.

We find increased electron beam induced shrinkage in x- and y-axis when cells were imaged in HM20 compared to Epon. This might not be a disadvantage, since the shrinkage is more uniform at around 25–30% in all directions (as measured from MT shrinkage), compared to Epon that has only 12% shrinkage in x and y but up to 40–60% in the z-axis (Luther *et al*., 1988). These considerations are especially important if you prepare the sample for electron tomography. If the increased shrinkage in x and y would pose a problem, use the alternative protocol using Epon (see below).

The FS solution is made up of 2% uranyl acetate (UA; from a 20% stock in dehydrated methanol kept at –20°C) in dehydrated acetone. The solution is precooled and immediately applied to the samples after the transfer. After 1 h of FS at –90°C, the UA is washed out with two changes of precooled dehydrated acetone. Whilst the acetone washes occur, the temperature is slowly increased (15°C/h) to –50°C where HM20 infiltration starts. Infiltration occurs in steps with increasing resin to acetone ratio; start with 3:1 acetone to resin, increase to 2:1, 1:1, 1:3 ratios. Then do at least three 100% HM20 exchanges, with at least one overnight. The whole infiltration protocol should take 2–4 days, but the longer the better. Start UV polymerization at –50°C for 48 h, heat to room temperature (20°C/h), and continue polymerization for a further 48 h.

5. Freeze Substitution and Embedding into Epoxy Resin

We have better experience with using HM20 resin than Epoxy resins, probably due to the low infiltration temperatures. However, cells can also be embedded into Epon which is a standard resin for morphology studies. Since Epon infiltration occurs at higher temperatures, an FS solution containing more fixatives is used to preserve cell morphology.

FS takes place for 50 h at –90°C by adding precooled 0.01% OsO_4, 0.25% uranyl acetate, 0.1% glutaraldehyde in acetone containing 1% H_2O (Muller-Reichert *et al.*, 2003). The temperature is then raised by 10°C/h to –30°C where the samples are left for 6–8 h and then washed twice with precooled dehydrated acetone. The infiltration starts at –30°C with a 1:1 ratio of acetone : Epon for 4 h. Change solution to a 1:2 acetone : Epon mixture and leave for another 3 h. Warm up to room temperature and do several pure Epon changes before starting to polymerize at 60°C for 50 h.

D. Sectioning and On-Section Stain

It is very practical to use serial thin sections to gain 3D understanding of the structures studied.

To gain ultra thin or semi-thick serial sections, trim the block surface into a trapezoid shape (Fig. 4A). The section area can be changed depending on how many sections one has to follow. We have found that it is much easier to shrink the area of each section, by reducing the height of the trapezoid, and fitting many sections onto the same grid, than it is to keep the large area and have a series of sections over many grids. In cases where many serial sections are needed, the block surface can start as a "line", with a pyramid below (Fig. 4B). With this geometry, a ribbon of ~50 sections can fit onto a single slot grid. Two or even three ribbons may be picked up onto the same grid, so one can follow over a hundred serial sections without changing the grid.

If serial sections do not stick together in a ribbon, a drop of rubber cement diluted 1:20 in xylene can be applied onto the corner of the block and allowed to dry. This will even allow poorly trimmed sections to form nice ribbons.

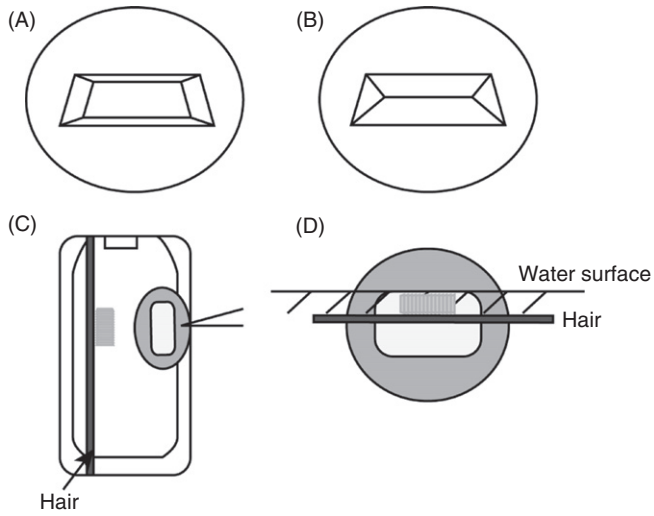

Fig. 4 Preparing serial sections. (A) The trapezoid block surface trimmed to fit few to medium amount of sections on a grid. (B) This "long pyramid" trimming of a block will yield increasing size of sections, starting with extremely thin ones. This enables fitting 30–50 sections in a single ribbon on the slot. (C) To pick up the sections we use a hair that has been spanned across the water surface, next to which the ribbon can be parked. (D) The slot grid is lowered to the top bar of the slot and pushed in close to the sections, angled to catch the sections and pulled out of the water.

Picking up the sections is made easier by "parking" them next to a hair that has been put in place over the knife's boat with a piece of tape on each side of the knife (Fig. 4C–D). The sections are then picked up using the hair to trap them on one side, and the grid on the other.

Finally, the sections are stained for 8 min in 2% aqueous UA (this time may be increased up to 15 min if contrast is bad), followed by 3 min in Reynold's lead citrate (Reynolds, 1963).

E. Immunolocalization and Cytochemistry for the Study of Cellular Compartments or Proteins

There are a large number of cytochemical and immunocytochemical methods to visualize specific cellular structures or proteins within cells.

In *T. brucei* cytochemical techniques have been particularly informative in the study of the functional domain organization of the cell nucleus (Fakan, 2004; Moyne, 1980), and have recently been used to provide novel insights into the ultrastructural organization of the kinetoplast (Gluenz *et al*., 2007).

For immunolocalization of proteins on sections to work, the antigen needs to survive the fixation and embedding process, and be exposed on the section surface where the antibody has free access. Alternatively, antigens can be detected before embedding

(i.e., the so-called preembed labeling), using plastic-embedded detergent-extracted cytoskeletons (Stephan *et al.*, 2007), or in whole-mounted cytoskeletons (Sherwin and Gull, 1989). The antibody ideally needs to be highly specific and have a high affinity for the antigen. Here, we present a method where we used antibodies on the HM20 embedded high-pressure frozen samples with great success.

1. Immunolocalization of Proteins on High-Pressure Frozen Samples

To test the immunogenicity of the high-pressure frozen samples embedded in HM20 we detected *T. brucei* β-tubulin using 3B15.9 and 4A1, two monoclonal antibodies raised against sea-urchin and *Drosophila melanogaster* β-tubulin (Piperno and Fuller, 1985; Scholey *et al.*, 1984) (Fig. 5A–C).

Thin sections (80 nm) on nickel mesh grids are postfixed for 10 min using 1% paraformaldehyde in phosphate-buffered saline (PBS) buffer. The grids are then washed three times in PBS. To avoid unspecific binding the grids are then floated for 1 h on drops of blocking buffer (0.1% fish skin gelatin, 8% bovine serum albumin in PBS). The excess blocking buffer is blotted off with a filter paper, and the grids are floated on drops with the primary antibody (in blocking buffer, dilution dependent on the antibody used) in a wet chamber overnight at 4°C. The grids are then washed three times with PBS for 20 min each before they are floated on drops of blocking buffer containing the secondary antibody (dilution 1:20) conjugated to gold particles for 1 h in room temperature. Wash the grids three times 20 min in PBS again. The labeled sections are then postfixed in 1% aqueous glutaraldehyde for 10 min before the final three washes in water. The sections are then stained in UA and Reynold's lead citrate as in Section III.D.

2. EDTA Regressive Staining—To Visualize DNA Containing Structures

Uranyl acetate, which is used as a general stain, can be specific to nucleic acids when used under certain conditions. Uranyl ions react with phosphate groups, including those in the backbone of nucleic acids, and with amino groups. UA staining therefore contributes to the stabilization of nucleic acids and confers electron density, staining nucleic acids and certain proteins. Bernhard's EDTA regressive staining method differentiates between ribonucleoprotein and deoxyribonucleoprotein particles (Bernhard, 1969). The *T. cruzi* kinetoplast was among the original test materials used by Bernhard to demonstrate the specificity of this method to a wide range of DNA-containing structures (Bernhard, 1969). Using Bernhard's EDTA regressive staining has recently enabled us to define a new domain in the kinetoplast, the inner unilateral filaments of the tripartite attachment complex (TAC) of *T. brucei* and the insect trypanosome *Crithidia* (Gluenz *et al.*, 2007).

Samples are fixed in glutaraldehyde (as described in Section III.B), without osmium postfixation and en-bloc stain using UA. Samples are dehydrated using ethanol and embedded into Epon resin. Thin sections are then stained with 5% aqueous uranyl acetate for 5 min, followed by three washes in filtered distilled water (1 min each). The

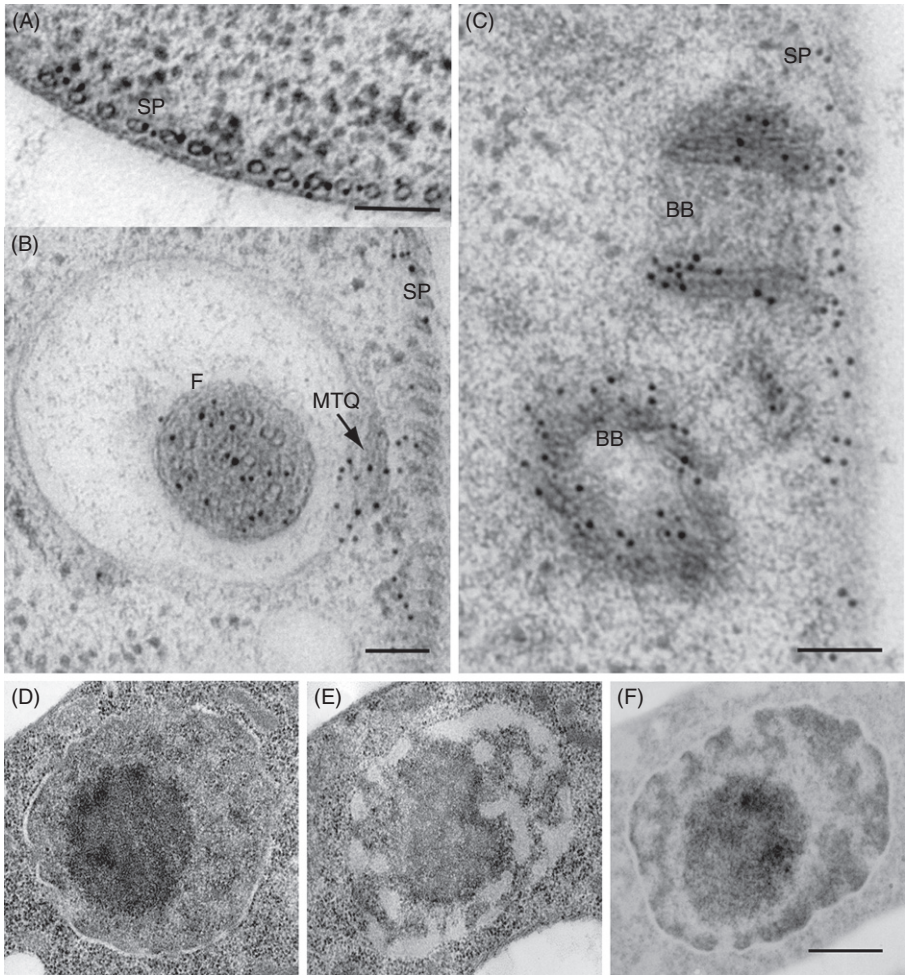

Fig. 5 Immunolocalisation and cytochemistry to localize proteins or nucleotides. Anti-β-tubulin antibody detecting (A) subpellicular microtubule array (SP), (B) flagellum (F), microtubule quartet (MTQ) and SP, (C) basal bodies (BB) and SP in high-pressure frozen cells embedded in HM20, (D) uranyl acetate-stained nucleus in chemically fixed cells, (E) nucleus stained with uranyl acetate and then treated with EDTA (30 min) to bleach areas containing DNA, and (F) nucleus treated with E-PTA to reveal location of basic proteins. All images are of procyclic cells. Scale bars: 100 nm in (A–C), 500 nm in (D–F).

sections are then treated with EDTA (0.2 M EDTA in distilled water; pH 7) for 15 min to 1 h (timing is critical and must be determined experimentally), and grids washed three times in filtered distilled water (1 min each). This results in preferential removal of uranyl stain from DNA, giving the DNA-containing structures a bleached appearance while RNA-containing particles remain strongly stained. The EDTA bleaching effect is

reversible; restaining with UA will again contrast the DNA-containing structures. Add more contrast with a lead citrate stain (1 min), followed by three filtered water washes. This technique is applied to a *T. brucei* nucleus (Fig. 5D–E).

3. Ethanolic Phosphotungstic Acid Staining of Basic Proteins

Phosphotungstic acid (PTA) selectively binds to the basic groups (lysine and arginine residues) of proteins (Sheridan and Barrnett, 1969). This property has been used in electron microscopy to study the ultrastructure of synapses and chromosomes (Bloom and Aghajanian, 1966; Sheridan and Barrnett, 1969), and the flagellar attachment zone of *Trypanosoma cruzi* (Rocha *et al.*, 2006). Trypanosome nuclear DNA is associated with histones, which stain strongly with ethanolic PTA (E-PTA) and the kinetoplast DNA is also associated with basic proteins. E-PTA staining is therefore a useful reagent to reveal ultrastructural details in the nucleus and kinetoplast that are not seen in samples stained with osmium and uranyl acetate (Gluenz *et al.*, 2007; Souto-Padron and De Souza, 1979).

Cells are fixed in 2.5% glutaraldehyde, as above (Section III.B), without postfixation in osmium and without uranyl acetate staining. Samples are dehydrated in ethanol. After the first change of 100% ethanol, pellets are immersed in 2% PTA in 100% ethanol at 4°C overnight. Samples are then rinsed twice more in 100% ethanol and embedded in Epon, as above (Section III.B). No on-section stain is necessary (Fig. 5F).

F. Detergent Extraction of Cells

The following methods are used to visualize the detergent insoluble cytoskeleton of trypanosomes, which includes both the subpellicular and flagellar microtubules and associated structures.

1. Whole-Mount Cytoskeletons

Detergent extraction of whole cells on an EM grid is an excellent protocol that preserves the cell shape and form as a whole and allows visualization of individual cytoskeletal elements including microtubules and their connections (Fig. 6A).

To enable cells to attach to the formvar, coated copper mesh grids were carbon coated and plasma etched. Grids should ideally be used immediately after plasma etching, but can be used for up to 1 h afterwards. Grids can be reetched if needed.

Centrifuge approximately 5×10^7 cells for 5 min at 800 g. Carefully remove most of the supernatant, leaving 500 µl of cells and carefully resuspend. Hold grid (coated side up) in forceps and place a drop of cell suspension on it so that the grid is completely covered. Allow cells to adhere for 5 min. Mutant cell lines that produce very small, aflagellate, or very big cells have more difficulty adhering, so extend the time allowed for the cells to adhere accordingly.

Transfer grid (cell side down) to a 100 µl drop 1% (v/v) NP-40 (Sigma) in PEME (100 mM PIPES, 2mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA, pH 6.9) for 2–3 min.

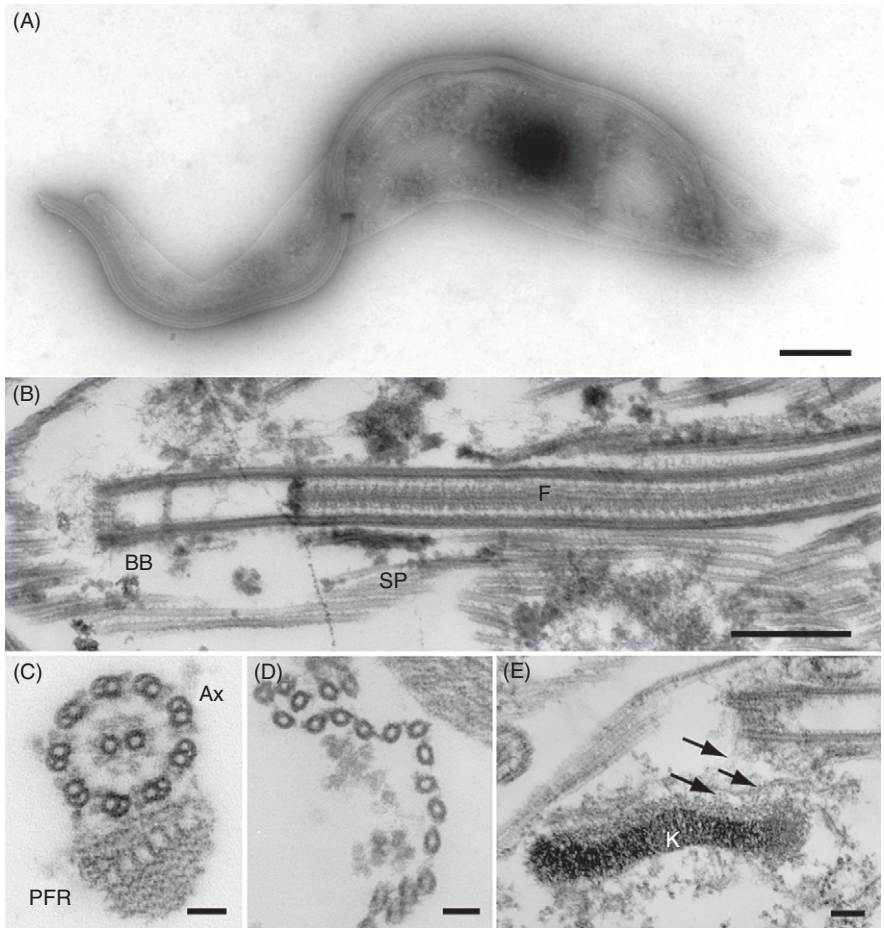

Fig. 6 Cytoskeletal preparations of *T. brucei*. (A) Whole mount cytoskeleton negatively stained using aurothioglucose. Plastic-embedded cytoskeleton showing (B) a longitudinal view of flagellum (F) with basal body (BB) and subpellicular microtubules (SP), (C) axoneme (Ax) and the paraflagellar rod (PFR), and (D) subpellicular microtubules. (E) Simultaneous detergent extraction and glutaraldehyde fixation preserves some cellular features, such as the kinetoplast (K) and tripartite attachment complex (arrows). (E, Courtesy of Emmanuel Ogbadoyi). Scale bars: 1 μm in (A), 500 nm in (B), 100 nm in (E), 50 nm in (C–D).

Repeat with a fresh drop. Gently transfer the grid using forceps onto the surface of the detergent droplet. NP-40 is a nonionic detergent that solubilizes both the plasma membrane and the internal membranous organelles, leaving the cytoskeleton intact (Sherwin and Gull, 1989). Keep the back of the grid dry to avoid sinking the grid – this is important to extract the cells fully. Larger or denser cells may need to be extracted for longer. To avoid problems in picking up and releasing grids, clean and dry forceps with filter paper regularly.

Fix cells by transferring to a drop of 2.5% (w/v) EM grade glutaraldehyde in PEME for 3 min. Transfer into double distilled water (ddH_2O) for 1 min to wash, repeat on fresh water drop. Pick up grids and blot off excess water, but do not allow drying out.

Apply negative stain with a 15 µl drop of 0.5% aurothioglucose in ddH_2O and immediately remove with a plastic pipette tip attached to a vacuum pump or, alternatively by blotting the side of the grid with filter paper. The staining reaction is nonspecific and occurs immediately. Aurothioglucose is supplied in powder form, but is best made up as a stock solution (2.5–3% w/v) in ddH_2O and stored in the fridge where it will last at least a year.

This protocol can be used for immunolocalization of antigens as it is useful to see labeling in a whole cell environment (Sherwin and Gull, 1989). Prepare cells on grids and fix as above, then instead of applying the negative stain transfer the grid using forceps to a 100 µl drop of 100 mM glycine in PBS for 5 min to neutralize free aldehyde groups. Incubate the grids in 1% (w/v) BSA, 0.1% (v/v) Tween 20 in PBS for 30 min to block nonspecific binding of antibody, then incubate with primary antibody diluted in 1% (w/v) BSA + 0.1% (v/v) Tween 20 in PBS for 30 min. Wash grids at least 5 times in 1% (w/v) BSA in PBS for 5 min each. Incubate secondary antibody (gold conjugated) 1% (w/v) BSA + 0.1% (v/v) Tween 20 in PBS for 30 min. Wash grids at least 5 times in 0.1% (w/v) BSA in PBS. Finally fix in 2.5% (w/v) glutaraldehyde in PBS and then apply a negative stain as above.

2. Plastic-Embedded Cytoskeletons

Centrifuge trypanosomes (800 g for 5–10 min) and resuspend in 1% NP-40 in PEME buffer pH 6.9 for 5 min. Wash the cells twice with PEME buffer and fix with 2.5% glutaraldehyde and 2% formaldehyde in 100 mM phosphate buffer pH 7–7.4 and follow the description for chemically fixed cells, starting with the second fixation step in buffer (Section III.B; Fig. 6B–D).

3. Simultaneous Fixation and Detergent Extraction for Plastic-Embedded Cytoskeletons

This method to prepare cytoskeletons has been developed to visualize the set of filaments linking the kinetoplast DNA to the basal body of the flagellum, a structure termed tripartite attachment complex (TAC; Fig. 6E) (Ogbadoyi *et al.*, 2003). Harvest procyclic cells from 5–10 ml of a log phase trypanosome culture by centrifugation (800 g for 5–10 min), and discard the supernatant. Resuspend cells in the residual small volume of media before adding 5 ml fixative (1% glutaraldehyde, 1% formaldehyde, 0.1% NP-40 in 1× PEM [0.05 M PIPES, 1 mM EGTA, 0.5 mM $MgSO_4$, pH 6.9]), which is mixed gently with the cells by inverting the tube a few times. Cells are then left to fix for 1 h at room temperature, before they are collected by centrifugation and resuspended in 1ml PBS. Continue washing cells in PBS three times and collect cells by centrifugation (1800 g for 3 min), without resuspending the cells (just tap on the tube gently to dislodge the pellet from the tube wall). Let it stand for 3 min.

To postfix the cytoskeletons, add 1% OsO_4 in PBS to pellets and leave for 30 min in the dark, at room temperature. The pellet should turn dark brown. Remove the osmium and wash the pellet thoroughly in distilled water (add 1 ml of distilled water to tube, leave for a few minutes, remove; repeat at least seven times). Stain the pellet by adding 1 ml 2% UA in water, leave overnight at 4°C in the dark. Wash with distilled water, dehydrate in ethanol, and embed in Epon resin as for standard fixation, described above (Section III.B).

IV. Materials

A. Cells, Media, and Buffers

Lister 427 *T. brucei* cells were used throughout, see Section III.A.
PEME (100 mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 0.1 mM EDTA, pH 6.9)

B. High-Pressure Freezing and Freeze Substitution

Cells were frozen using 100 µl deep membrane carriers (McDonald *et al*., 2007) in a Leica EM PACT2 (Leica Microsystems GmbH, Vienna, Austria). The small volume of these cell carriers is essential for good freezing. Carriers were placed in flow-through rings inside reagent bath holders (both by Leica Microsystems GmbH, Vienna, Austria), in which they were freeze substituted, infiltrated, and embedded. FS was performed in a Leica EM AFS device.

C. Chemicals/Reagents

Paraformaldehyde (Sigma Aldrich Company Ltd., Gillingham, UK)
Formaldehyde (EM-grade, TAAB Laboratory Equipment Ltd.)
Glutaraldehyde (Science Services GmbH, Berlin, Germany)
Uranyl acetate (Amsbio, Abingdon, UK)
Dehydrated methanol (Sigma Aldrich Company Ltd., Gillingham, UK)
Dehydrated acetone (Science Services GmbH, Berlin, Germany)
Osmium tetroxide (Amsbio, Abingdon, UK)
General chemicals (i.e., PBS and BSA; Sigma-Aldrich Company Ltd, Dorset, England)
Goat α-mouse F(ab′) conjugated to 10 nm gold (Ted Pella, Inc., Redding, CA, USA)

D. Resins

We embed samples in Agar 100 Resin (equivalent to Epon 812), Spurr's, or HM20 (all from Agar Scientific, Standsted, UK).

E. Microtomy

Sections were cut using a Leica Ultracut E Microtome or a Leica Ultracut UCT (Leica Microsystems GmbH, Vienna, Austria), and collected on Cu–Pd slot grids (Agar Scientific, Standsted, UK). For cytoskeletons, copper mesh grids from TAAB Laboratories Equipment Ltd, Berks, UK, were used.

F. Electron Microscopy

Micrographs were acquired using the AMTV600 software operating an Advantage HS-B camera (2000×2000 pixels; AMT, Danvers, MA, USA) on a FEI CM100 microscope or DigitalMicrograph software operating a Gatan Ultrascan 1000 CCD camera on a FEI Tecnai 12 microscope (FEI Company, Eindhoven, the Netherlands). Alternatively, micrographs were acquired on Kodak electron microscopy film 4489 (Agar Scientific Ltd).

V. Discussion

A fixative should preserve the morphology and structure of the cell as close to the living state as possible without altering the volume and spatial relationships of subcellular structures. Moreover, the cellular ultrastructure must remain intact during the harsh conditions encountered during postfixation treatment and withstand the electron beam in the microscope.

In this chapter we have described both chemical fixation and cryoimmobilization using high-pressure freezing. The most widely used fixation method for ultrastructural studies is chemical fixation using glutaraldehyde. Glutaraldehyde has two terminal aldehyde groups that react with amino groups in proteins, some carbohydrates, and other molecules. This results in irreversible crosslinking of proteins, creating a dense meshwork of immobilized intracellular structures (Bozzola and Russell, 1998).

Variants of this method have been used to study trypanosome fine structure since the late 1950s and provided the basis for discovery of many fundamentally important aspects of trypanosome biology and virulence. Among these are the discovery of the variant surface glycoprotein (VSG) coat in bloodstream form *T. brucei* and its role in antigenic variation in the mammalian host (Vickerman and Luckins, 1969), the structure of the single mitochondrion and changes that occur during metabolic adaptation in different life cycle stages (Brown *et al*., 1973), the structure of the kinetoplast (Meyer *et al*., 1958), the flagellar pocket as the site of endocytosis (Brown *et al*., 1965), the cell cycle cytoskeletal changes (Sherwin and Gull, 1989), the flagella connector (Moreira-Leite *et al*., 2001), and this method is still used today (Lacomble *et al*., 2009).

Glutaraldehyde can be added directly to the culture medium of trypanosomes, where fixation can be seen as immobilization of cells within seconds. Handling of cells before fixation is thus kept to an absolute minimum, without subjecting live cells to centrifugation or temperature shifts. This facilitates experiments such as time series, where

the intervals between samples are within the second to minute range, since high-pressure freezing individual samples cannot be frozen faster than ~1 min one after the other. Furthermore, fixation of cells is achieved without the need for specialized equipment, thus providing a routine method for preservation of trypanosomes collected in field studies.

However, the process of dehydration and crosslinking of proteins using chemical fixation causes aggregation artifacts, where molecules in solution will cluster and form matrices not representative of the undisturbed cellular environment (Dubochet and Sartori Blanc, 2001). In micrographs, such dehydration artifacts often show as extracted cells with wrinkled membranes (Vanhecke *et al*., 2008). Therefore, striving toward visualizing the most native state of the cell is important especially with a view to building faithful 3D reconstructions of cellular structures, and ultimately whole cells, using electron tomography.

High-pressure freezing followed by FS provides a step toward better cellular preservation since the cells are dehydrated and fixed slowly at very low temperatures (i.e., at –90°C in the FS "cocktail"). The cells may still have some extraction, but membranes are smooth and the cytoplasm is more homogenous than in chemical fixation. In budding yeast (*Saccharomyces cerevisiae*) it has been shown that the volume of organelles such as endoplasmatic reticulum, nucleus, and vacuole, and their interactions changes significantly between chemical fixation and high-pressure freezing (Perktold *et al*., 2007). We have shown that in *Chlamydomonas reinhardii* and *T. brucei*, axonemal microtubules at the distal end of the flagellum were depolymerized in chemically fixed samples but were well preserved in high-pressure frozen cells (Höög *et al*. manuscript in preparation). In general, trypanosome cell morphology benefits greatly from using cryoimmobilization.

The next step toward a more native cell preservation is to freeze the cells and then image them in ice directly, completely avoiding dehydration, resin infiltration, and embedding. However, the cells are too thick to be imaged without sectioning (3–4 μm), therefore cryomicroscopy of whole cells will prove challenging. Methods for cryomicrotomy of hydrated bacteria (Matias *et al*., 2003), human skin cells (Al-Amoudi *et al*., 2005, 2007), and frozen rat hepatoma (HTC) cells (Bouchet-Marquis and Fakan, 2009) have been developed. In the future, cryomicroscopy of such frozen hydrated sections may provide new insights into trypanosome cell biology.

VI. Summary

T. brucei is an excellent model system to study cell motility, morphology, and gene expression because of the many tools available for its genetic manipulation, ease of growing cells in culture, and suitability for electron microscopy studies. We summarize a range of well-tested methods to prepare cells for electron microscopy using chemical fixation, and introduce a protocol using rapid freezing followed by FS, which yields much improved cellular morphology. The ease with which genes can be silenced in this

organism combined with good structural preservation makes *T. brucei* a powerful tool when examining structure–function relationships in cells.

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