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Quick Fixation of Eukaryotic Cell Suspensions for Routine Transmission Electron Microscopy

Fijación Rápida de Células Eucarióticas en Suspensión para Microscopía Electrónica de Transmisión Rutinaria

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Abstract: A rapid protocol for quick fixation of eukaryotic cell suspensions for routine transmission electron microscopy is presented. Duration of the fixation, washing and dehydration steps is extremely reduced, so that this pre-embedding process can be accomplished in about 60 minutes. The methodology was successfully applied to different single cell suspensions, such as trypanosomatid protozoa and mice peritoneal cells. Ultrastructural observation showed that the fine morphology of the samples could be finely preserved.

Key words: Quick fixation; transmission electron microscopy, trypanosomatids, ultrastructure

Resumen: Se presentó un protocolo breve de fijación rápida de células eucarióticas en suspensión para microscopia electrónica de transmisión rutinaria. Durante la fijación, los pasos de lavado y deshidratación fueron extremadamente reducidos, para que el proceso de pre-inclusión pudiera lograrse en aproximadamente 60 minutos. La metodología se aplicó con éxito a las diferentes suspensiones celulares sencillas, como protozoarios tripanosomatidos y células peritoneales de ratones. La observación ultrastructural mostró que la morfología de las muestras puede preservarse finamente.

Palabras clave: fijación rápida, microscopía electrónica de transmisión, tripanosomatidos, ultraestructura.

INTRODUCTION

Routine fixation and dehydration processing of cell suspension samples for transmission electron microscopy (TEM) is a time-consuming methodology, which involves centrifugation of the samples in each step of the protocol. Development of a rapid fixation methodology for routine TEM would be helpful when fast results are needed (such as for diagnosis purposes), when experiments with long incubation times before the routine glutaraldehyde fixation are made, or simply to optimize the time spent in the experimental work.

Some rapid processing protocols for transmission electron microscopy have been already described. However, some require the use of a special microwave device (1), while others use non-conventional embedding media (2) or accelerate the Epon resin hardening by raising the polymerization temperature to 100°C (3).

In this work we present a simple, quick fixation protocol for eukaryotic single cell suspensions that has been developed in our laboratory, whereby the fixation, washing and dehydration times have been reduced to a minimum, thus allowing resin embedding of the material af-

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ter only 60 minutes. Although primarily developed for trypanosomatid protozoa (unicellular organisms), the quick fixation was also applied with good results to mice peritoneal cells.

MATERIALS AND METHODS

Trypanosomatid flagellates of different genera (*Blastocrithidia, Crithidia, Herpetomonas, Leptomonas* and *Trypanosoma*) were maintained at 28°C with serial passages in LIT medium supplemented with 10% fetal calf serum (2). Two-day-old culture forms (five days for *Trypanosoma*) were used in the experiments. Mice peritoneal cells were collected by needle aspiration and resuspended in Dulbecco's Minimum Essential Medium (DMEM).

Culture cells were transferred to 1,5 mL Eppendorf microfuge tubes and a pellet was obtained by centrifugation for 30-60 seconds at 10,000 g. To allow better penetration of the fixatives, small pellets (1-3 mm) were used. After discarding the supernatant, the pellets were processed according to the following protocol:

1. Fix for 20-30 minutes with 2.5% glutaraldehyde in 0.1 M cacodylate/phosphate buffer, pH 7.2.
   Note: During steps 1-4 suspend the pellet after adding the solutions. After the incubation time, pellet the cells by centrifugation for 30-60 seconds at 10,000 g. Repeat the suspension and centrifugation procedure up to dehydration in acetone 70%.
2. Rinse 2x1 minute in the same buffer.
3. Post-fix for 15 minutes with 1% osmium tetroxide / 0.8% potassium ferricyanide / 5 mM $CaCl_2$ in 0.1M cacodylate buffer (4), and then rinse 2x1 minute in the same buffer.
4. Dehydrate in acetone 50%, 70%, 90% and 100% (2x), 3 minutes each (including the centrifugation step). When in acetone 70%, centrifuge the cell suspensions for 1 minute at 10,000 g and then carefully remove the whole condensed pellet from the microcentrifuge tube bottom with the aid of a wooden toothpick (tip: press the toothpick against the tube bottom and then slowly **rotate the tube** until the pellet is released). Carefully pour the pellet into a glass Petri dish containing acetone 70% and mince it into smaller pieces, if necessary. Transfer the samples with the aid of a large Pasteur pipette to glass vials containing 90% acetone. From this point on, the centrifugation step is no longer required.
5. Infiltrate the pellets for 2 hours (alternatively: overnight) in an Epon / acetone solution (1:1), 4 hours in pure Epon and then embed in new Epon. Polymerize for 48 hours at 60°C.

Ultra-thin sections were stained for 30 minutes with 2% aqueous uranyl acetate and for 2 minutes with lead citrate, and then observed in a Zeiss EM10C transmission electron microscope.

RESULTS AND DISCUSSION

Observation of eukaryotic cell suspensions processed according to this rapid fixation protocol for transmission electron microscopy showed that the proposed reduction in the processing time did not result in cell damage or bad preservation. The minimal fixing time (20-30 minutes for glutaraldehyde and 15 minutes for osmium tetroxide) was obtained after testing progressively decreasing fixation times (120, 60, 45, 30 and 20 minutes for glutaraldehyde and 60, 30 and 15 minutes for osmium tetroxide). The fine structure was well preserved, including cell membranes and cytoplasmic organelles such Golgi complex, nuclei and mitochondria. Excellent cell preservation was obtained with the several trypanosomatid protozoa tested, e.g. *Leptomonas colossoma* (Fig. 1), and *Herpetomonas muscarum muscarum* (Fig. 2), as well as with mice peritoneal cells (Figs. 3 and 4).

Some alternative protocols, using different reagent concentrations and temperatures, have been proposed in order to improve ultrastructural preservation. For instance, potassium ferrocyanide [$K_4Fe(CN)_6$] or ferricyanide [$K_3Fe(CN)_6$] have been used in combination with osmium tetroxide (OsO_4) to enhance membrane contrast, or in combination with ruthenium red (RuO_4) to reduce damage to cellular components (4, 5, 6). However, the main focus of these works was to obtain a better fixation quality, sometimes even leading to an increase in the fixation time. Our data show that the use of potassium ferricyanide/OsO_4 in a quick fixation methodology improves the visualization of the cell membranes even when short OsO_4 fixation times are used.

Our quick fixation protocol, by combining a reduction in some incubation times and the use of a microfuge, allowed a significant decrease in time of fixing and dehydrating single cell suspensions for transmission electron microscopy, without the need of additional refined equipment, such as the microwave ovens designed for TEM. An alternative procedure to eliminate the centrifugation steps was proposed by Rittenbourg et al. (7), who collected microorganisms on membrane filters. Our quick fixation procedure is routinely used in our laboratory with reproducible results for both scanning (data not shown) and transmission electron microscopy. Further studies are being carried out to verify whether this simplified fixation/dehydration schedule is effective on other unicellular organisms (prokaryotic/eukaryotic cells), cell suspensions, or even tissues.

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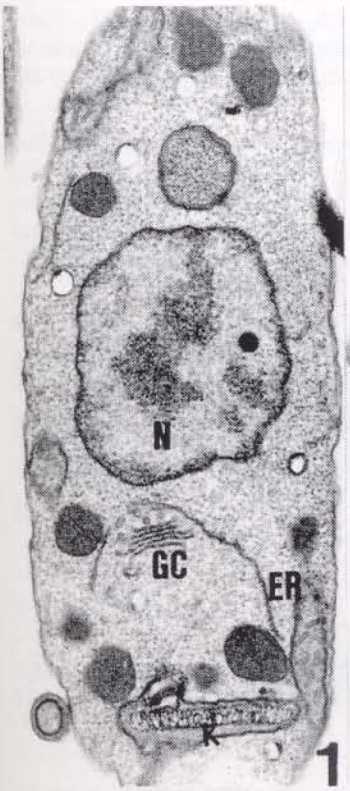


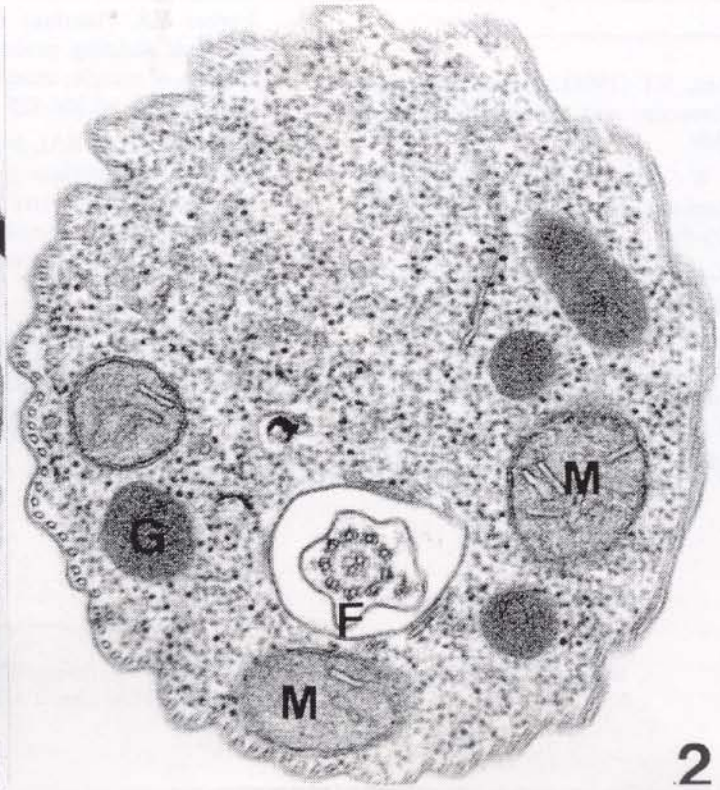
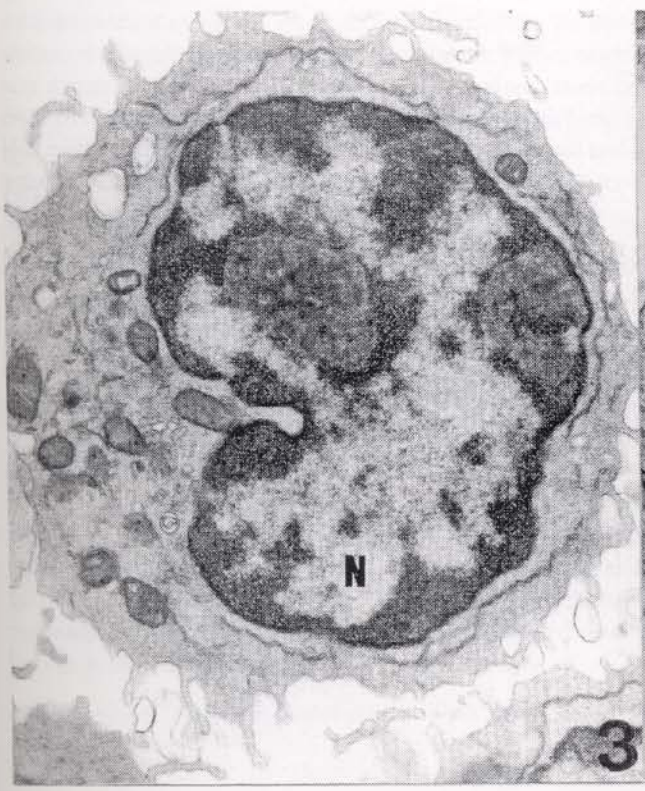
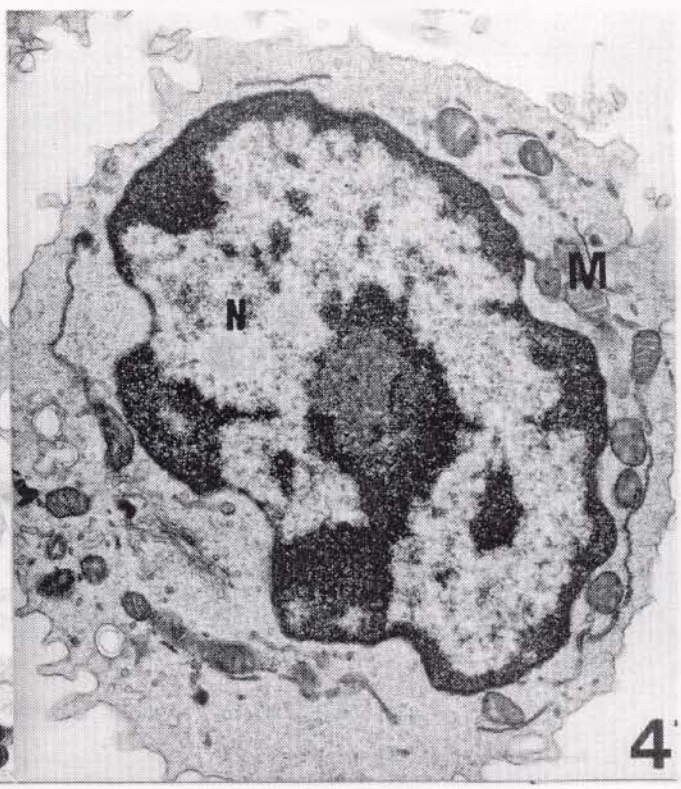


Figure 1. Culture promastigote form of the trypanosomatid protozoa *Leptomonas colossoma*, N, Nucleous, GC, Golgi Complex, ER, Endoplasmic reticulum; K, Kinetoplast. X 24,700

Figure 2. Culture promastigote form of the trypanosomatid protozoa *Herpetomonas muscarum muscarum*, M, Mitochondria, F, Flagellum; G, Glicosome. X 58,800.





Figures 3 and 4. Mice peritoneal macrophages M, mitochondrion; N, nucleus. X 10,000.

Figures 1-4. Cells fixed according the quick fixation protocol, showing the excellent fine structural preservation obtained.

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