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PROTOZOOLOGY - SHORT COMMUNICATION

Ultrastructure Expansion Microscopy (U-ExM) in *Trypanosoma cruzi*: localization of tubulin isoforms and isotypes

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Abstract

Ultrastructure Expansion Microscopy (U-ExM) is a recently developed technique that enables the increase of the spatial resolution within a cell or a tissue for microscopic imaging by physically expanding the sample. For the first time, I report a detailed protocol validating the use of U-ExM in *Trypanosoma cruzi* and quantifying the expansion factors of different subcellular compartments. I was able to determine the localization patterns of different tubulin isoforms, such as α-tubulin and β-tubulin. Also, I immunolocalized acetylated and tyrosinated α-tubulin isotypes in epimastigotes and use mitochondrial cell-permeable dyes to identify this organelle. Finally, U-ExM was also performed in trypomastigotes and amastigotes validating this technique in all life cycle stages of *T. cruzi*.

Keywords Confocal microscopy · Cytoskeleton · Acetylation · Tyrosination · Mitochondria

Introduction

Trypanosoma cruzi, a kinetoplastid parasite and the etiological agent of Chagas disease or American trypanosomiasis, has a complex life cycle that alternates between a mammalian and an insect host (Triatominidae family), which is the biological vector of this disease (Lidani et al. 2019). The World Health Organization classifies Chagas disease as one of the 13 most neglected tropical diseases, constituting a very important social and economic problem, especially in Latin America.

Trypanosomatids are characterized by a precisely organized cytoskeleton constituted by stable microtubules (MT). Such MTs are present in the mitotic spindle (during mitosis), the basal body, the flagellar axoneme, and the subpellicular microtubules. The latter are connected to each other and the plasma membrane forming a helical arrangement along the central axis of the parasite cell body (de Souza 2009). MTs are composed of α/β-tubulin heterodimers forming helical tubes and provide the basis for cytoskeletal architecture. They are regulated by interacting with a variety of MT-associated proteins (MAPs), by a differential expression of α- and β-tubulin genes (tubulin isotypes), and by tubulin isoforms generated by the inclusion of different post-translational modifications (PTMs) in each isotype (Gadadhar et al. 2017). In trypanosomatids, many PTMs were identified across the microtubular array. It is proposed that this could be used to control the differential recruitment of MAPs and motors during the cell cycle (Sinclair and de Graffenried 2019).

Ultrastructure Expansion Microscopy (U-ExM) has been introduced recently as a super-resolution microscopy technique. Biological specimens are included in swellable polymer hydrogels and then are physically expanded to resolve fine details (Chen et al. 2015). This method is very simple, accessible, and compatible with conventional fluorescent probes. Also, it requires inexpensive chemicals. It can achieve a spatial resolution of ~65 nm using conventional (confocal) microscopes, around four times better than the standard resolution of a confocal microscope (~250 nm).

Since 2015 there has been a steady growth of publications involving this technique, especially in protozoan parasites, like *Giardia lamblia*, *Plasmodium falciparum*, and *Toxoplasma gondii* (Tillberg and Chen 2019; dos Santos and Soldati-Favre 2021; Gorilak et al. 2021; Tomasina et al. 2021). This technique was first reported in the kinetoplastid

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Trypanosoma brucei, where a component of the mitochondrial genome segregation machinery was described (Amodeo et al. 2021; Kalichava and Ochsenreiter 2021), but it has not been described for *T. cruzi*.

In this report, I validate the use of U-ExM in *T. cruzi* and quantify the expansion factors of different subcellular structures. I describe the localization patterns of tubulin isoforms, such as α-tubulin and β-tubulin, validating this technique in all life cycle stages of *T. cruzi*. Also, I immunolocalized acetylated and tyrosinated α-tubulin isotypes in epimastigotes to visualize subpellicular microtubules, the flagellar axoneme, and the basal body. Furthermore, I validated the use of the cell-permeable dyes, such as Mitotracker, for U-ExM in *T. cruzi*.

Methods

Parasite culture and infections

T. cruzi Dm28*c* epimastigotes and Vero cells (ATCC CCL-81) were cultured and infected as previously described (Alonso et al. 2016).

U-ExM protocol

I started with 5 million epimastigotes or trypomastigotes, washed the pellets twice with 500 µl PBS and centrifuged for 5 min at 2000 g, then resuspended in 200 µl of PBS, and settled for 20 min at room temperature on poly-D-lysine-coated coverslips (12 mm, Marienfeld GmbH). For mitochondrial staining, epimastigotes were resuspended in PBS and incubated with 1 mM Mitotracker Orange CMTMRos (Invitrogen) for 30 min at 28 °C, washed twice in PBS, and fixed with 3% paraformaldehyde + 0.1% glutaraldehyde (Sigma, 70% solution) in PBS for 15 min at RT and then settled on coverslips as described before. Cells infected with amastigotes in 12-mm coverslips were washed twice with PBS to eliminate culturing media.

For all the conditions stated above, coverslips were carefully submerged into a 24-well plate with 1 ml of 4% formaldehyde (FA; 36.5–38%, Sigma) and 4% acrylamide (AA; 40% stock solution, Invitrogen) in PBS and incubated for 4 to 5 h at 37 °C. For gelation, coverslips were putted, with the cells facing down, into 35 µl of monomer solution: 19% sodium acrylate (Sigma), 10% AA, 0.1% N,N′-methylene bisacrylamide (BIS; Sigma) in PBS, supplemented with 0.5% APS (Sigma) and 0.5% tetramethylethylenediamine (TEMED; Sigma). A drop of the monomer solution was placed over a parafilm in a pre-cooled humid chamber for each coverslip. For gelation, samples were incubated 5 min on ice, and then for 1 h at 37 °C in the dark. Coverslips with gels were then transferred into a six-well plate filled with 2 ml of denaturation buffer (200 mM sodium dodecyl sulfate (SDS), 200 mM NaCl, and 50 mM Tris–HCl in Milli-Q, pH 9) for 15 min at room temperature with gentle agitation to allow the gels to detach from the coverslips. Gels were then moved into a 1.5-ml Eppendorf centrifuge tube filled with 1 ml denaturation buffer and incubated at 95 °C for 1 h and 30 min. After denaturation, gels were placed in 50-ml beakers or Petri dishes filled with Milli-Q water for the first round of expansion. Water was exchanged after 30 min and then gels were incubated overnight in Milli-Q water. The next day, water was exchanged one more time for 30 min and then gels were washed two times for 30 min in PBS (to allow gel shrinkage) and subsequently incubated with primary antibodies (Table 1) (at this point it is possible to cut the gel in smaller pieces to incubate with different antibodies). All antibodies were diluted in 2% PBS/BSA, and gels were incubated for 3 h at 37 °C and then overnight at 4 °C in 24 well plates with 300 µl of the diluted antibodies. Gels were then washed in PBS-T three times for 10 min while gently shaking and subsequently incubated with secondary antibodies (Table 1) using the same incubation times. The next day, gels were washed in PBS-T (three times for 10 min while shaking) and placed into beakers or Petri dishes filled

Table 1 List of antibodies used and working dilutions

	Target	Source	Recommended dilution ExM	Research Resource Identifiers (RRID
Primary antibody				
TAT-1	α-Tubulin	Sigma Aldrich	1:200	Sigma-Aldrich Cat# 00,020,911, RRID:AB_10013740
KMX-1	β-Tubulin	Millipore	1:200	Millipore Cat# MAB3408, RRID:AB_94650
YL1/2	α-Tubulin tyrosinated	Millipore	1:300	Millipore Cat# MAB1864, RRID:AB_2210391
6-11B-1	α-Tubulin acetylated	Sigma Aldrich	1:300	Sigma-Aldrich Cat# MABT868, RRID:AB_2819178
Secondary antibody				
Anti-mouse Alexa 555	Mouse IgG	Thermo Fisher	1:400	Thermo Fisher Scientific Cat# A-21427, RRID:AB_2535848

with Milli-Q water for the second round of expansion. Water was exchanged three times every 30 min. After this step, gels were carefully measured with a caliper showing an expansion factor between 4.2× and 4.6×. The specificity of the antibodies was verified by western blot with epimastigotes' total extracts (Fig. 1a).

Imaging and measurement of the expansion

The gel was then cut with a razor blade into pieces and put into a 35-mm glass-bottom dish (Matsunami) previously coated with poly-lysine for confocal imaging. Confocal microscopy was performed on a Zeiss LSM880 using a 63× oil NA 1.4 objective, with the following parameters: *z* step size at 0.65-µm interval with a pixel size of 0.132 nm. ZEN BLACK 2.1 SP3 software (Zeiss, RRID: SCR_018163) and Fiji (Schindelin et al. 2012) (RRID: SCR_002285) were used to analyze the images.

To determine the expansion factor, the ratio of expanded kinetoplast and nucleus to non-expanded epimastigotes was calculated ($n = 20$ cells) using GraphPad Prism v. 9.0.1 (RRID: SCR_002798). The length of the kinetoplast was determined by measuring its maximum length and the diameter of the nucleus by measuring the widest diameter of each epimastigote using Fiji.

Results and discussion

U-ExM workflow in *T. cruzi*

Although the Ex-M protocols present in the literature follow the same steps, they have differences regarding the concentration of fixative agents, denaturing agents, and incubation times (this also depends on the nature of the protein to be immunolocalized). I tested several conditions and optimized the workflow for *T. cruzi* (Fig. 1), which is described in the "Methods" section. In this report, I used antibodies against cytoskeletal proteins (insoluble proteins) (Table 1) and gave a detailed protocol to perform the U-ExM, but it is important to mention that when targeting soluble or membrane proteins incubation times and temperature during the denaturing step should be optimized.

As observed in Fig. 1, FA and AA (precursor molecules) are introduced in the first step to functionalize the cellular components, which allows the subsequent linkage of swellable polyelectrolyte gel (monomer solution containing SA). The gel formation is accomplished using the oxidizing reagents TEMED and APS (as for standard polyacrylamide gels). Then, the parasite contents are denatured using SDS and high temperature. After this step, the first round of expansion is performed in ultrapure water followed by incubation in PBS, where the gel shrinks again. Finally, standard immunofluorescence techniques are applied followed by the second round of expansion in water before imaging in an epifluorescence or confocal microscope.

Localization of tubulin isoforms

MTs are essential components of the eukaryotic cytoskeleton and cell division machinery. I immunolocalized α- and β-tubulin using specific monoclonal antibodies (Fig. 2a and b). A similar localization pattern was observed for both isoforms at the subpellicular microtubules and the flagellar axoneme. The axoneme is immunostained from the base of the flagellum to its tip, in continuous attachment to the cell body

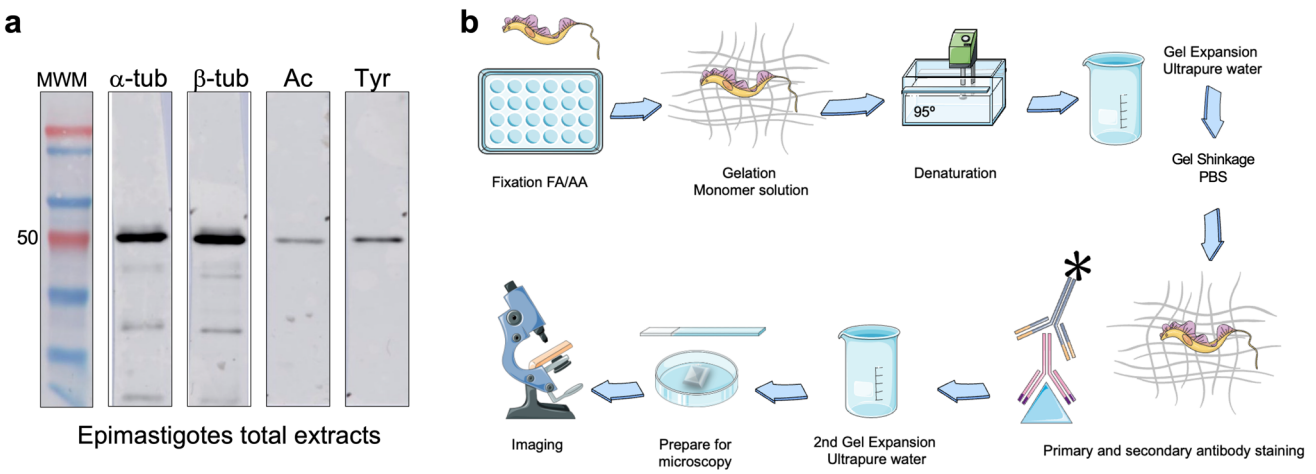

Fig. 1 U-ExM workflow in *T. cruzi*. **a** Specificities of the anti-α-tubulin (49.8 kDa), β-tubulin (49.7 kDa), acetylated (Ac) α-tubulin, and tyrosinated (Tyr) α-tubulin antibodies were verified by western blot against epimastigotes' total extracts. One-million parasites were blotted per lane, and goat anti-mouse IgG conjugated to HRP was used as the secondary antibody. **b** U-ExM workflow in *T. cruzi*. Servier Medical Art was used to create this figure (smart.servier.com)

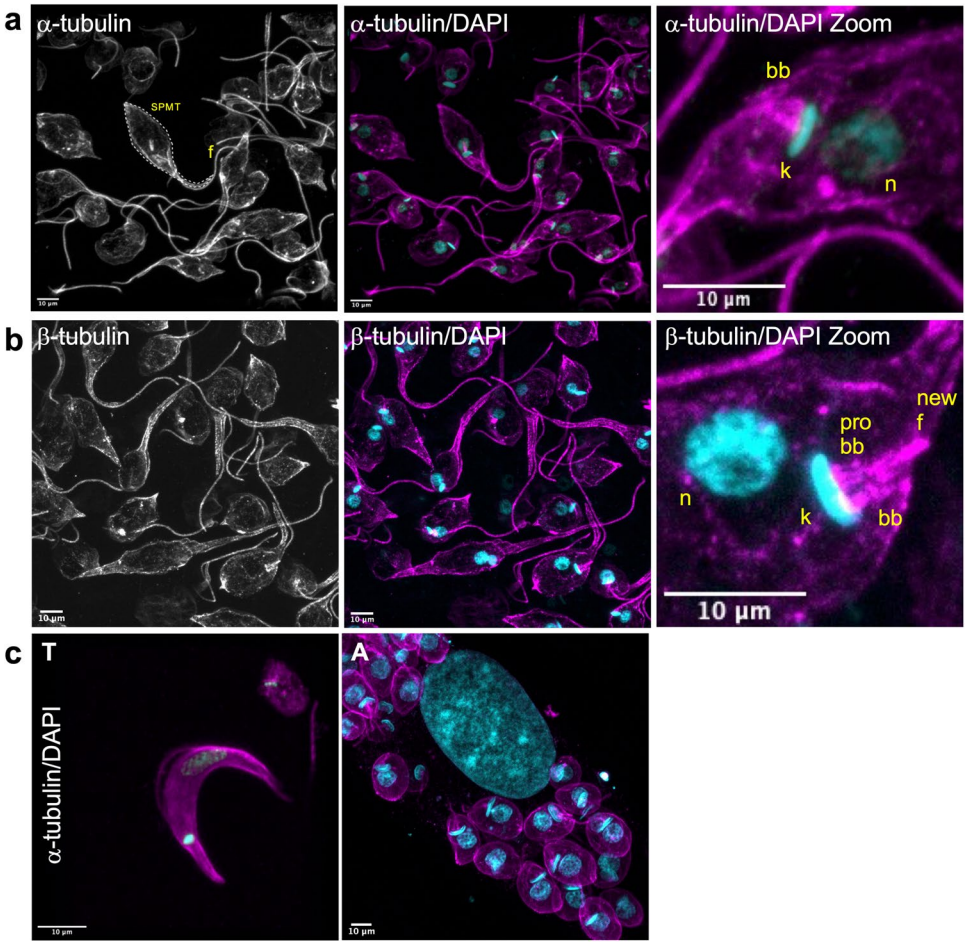

Fig. 2 Tubulin isotypes during *T. cruzi* cell stages. **a** Epimastigotes stained with anti-α-tubulin antibodies (grayscale or magenta; Alexa 555) and DAPI (cyan; kDNA and nuclear DNA). In the grayscale image: f, flagella; SPMT, the corset of subpellicular microtubules is indicated with a dotted line. In Zoom images: bb, basal body; k, kinetoplast; n, nucleus. **b** Epimastigotes stained with anti-β-tubulin antibodies (grayscale or magenta; Alexa 555) and DAPI (cyan; kDNA and nuclear DNA). In Zoom images: bb, basal body; pro-bb, pro-basal body; k, kinetoplast; n, nucleus, F, flagella. **c** Trypomastigotes (T) and amastigotes (A) stained with anti-α-tubulin antibodies (magenta; Alexa 555) and DAPI (cyan; kDNA and nuclear DNA)

as previously described (de Souza 2009). Pro- and mature basal bodies were also clearly observed, in proximity to the kinetoplast in a parasite that is starting to divide (Zoom in Fig. 2b) (Elias et al. 2007).

Alpha-tubulin was also immunolocalized in trypomastigotes and amastigotes, highlighting this technique's value in all life cycle stages of *T. cruzi*. As observed in Fig. 2c the subpellicular array and the flagellar axoneme are clearly immunostained in both stages. It is interesting to note that the anti-α-tubulin antibody used (TAT-1), immunodetects, in our experimental conditions, exclusively amastigotes' α-tubulin and not the host cell protein. I believe this is because TAT-1 was developed by immunizing mice with extracted *T. brucei brucei* cytoskeleton although it is suitable to use in mammalian cells.

Localization of tubulin isotypes in *T. cruzi*

There are many conserved acetylated lysines (K) reported in α- and β-tubulin, but acetylation of the α-tubulin luminal residue K40, discovered over 30 years ago, has been widely characterized. This acetylation mark is associated with stable microtubes and with the resistance of microtubules to depolymerizing drugs (L'Hernault and Rosenbaum 1985). In trypanosomatids subpellicular, mitotic, and axonemal microtubules are extensively acetylated making them interesting models for studying K40 acetylation (Sasse and Gull 1988; Souto-Padron et al. 1993).

Figure 3a shows that acetylated α-tubulin is present in the subpellicular microtubules and flagellar axoneme. Immunodetection of the basal body and pro-basal body in the flagellar pocket region is also observed in great detail using U-ExM (Zoom Fig. 3a). Tyrosinated α-tubulin shows a different localization pattern (Fig. 3b). The subpellicular MTs of the posterior end of the cell body, the nascent flagellum of dividing cells, and the basal bodies are all immunodetected, similar to the localization reported for *T. brucei* (Sherwin et al. 1987). A weak reaction is detected in the subpellicular MTs of the anterior part of the epimastigotes and the old flagellum. This localization correlates with the role of tyrosinated α-tubulin as a marker for newly formed microtubules.

Measurement of subcellular compartments and structures in epimastigotes

Trypanosomatids are characterized by a single flagellum and a mass of mitochondrial DNA organized into a kinetoplast

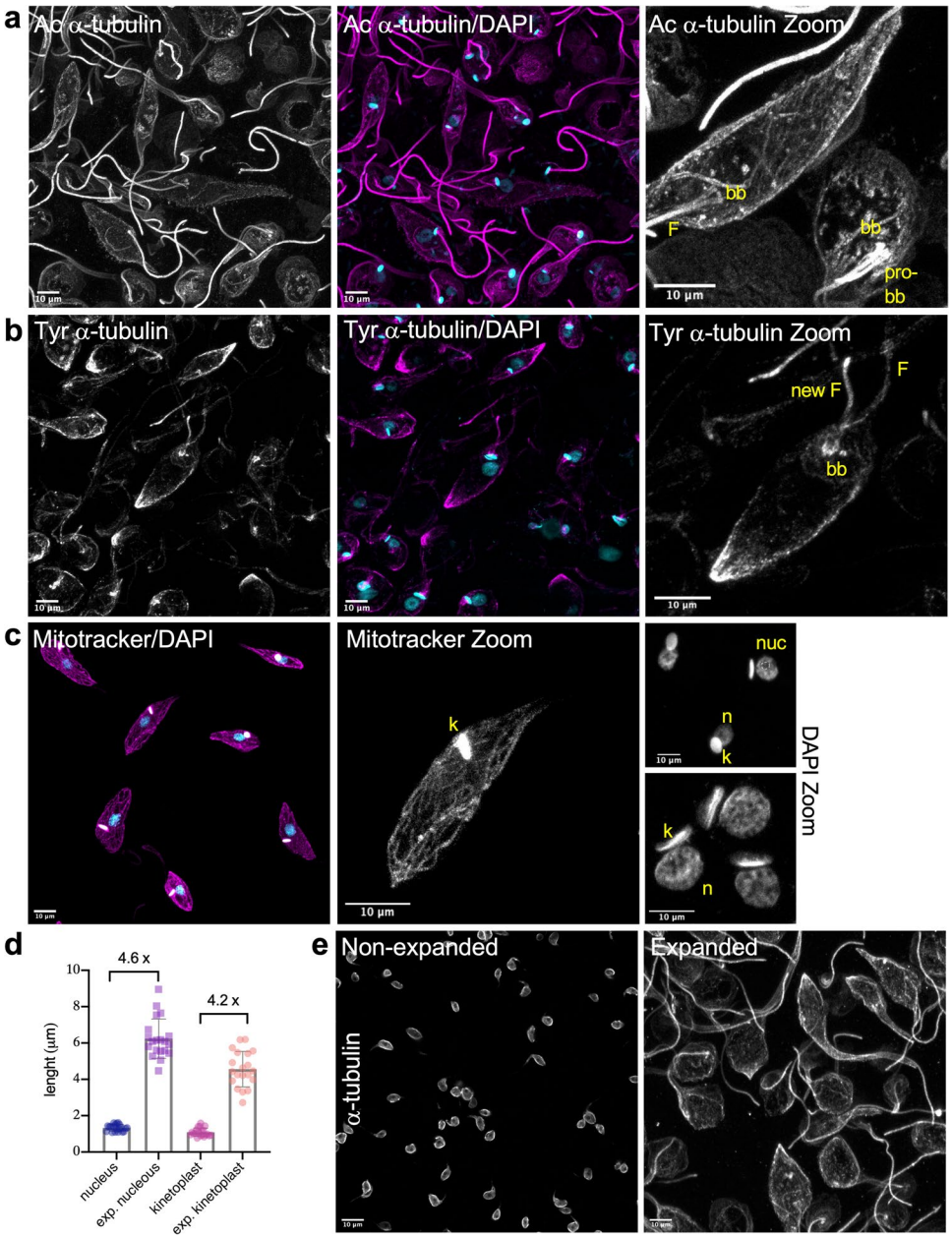

Fig. 3 Tubulin isoforms, mitochondrial staining, and expansion measurements in epimastigotes. **a** Epimastigotes stained with anti-acetylated (Ac) α-tubulin antibodies (grayscale or magenta; Alexa 555) and DAPI (cyan; kDNA and nuclear DNA). In Zoom images: bb, basal body; F, flagella. **b** Epimastigotes stained with anti-tyrosonated (Tyr) α-tubulin antibodies (grayscale or magenta; Alexa 555) and DAPI (cyan; kDNA and nuclear DNA). In Zoom images: bb, basal body; F, flagella. **c** Epimastigotes stained with the cell-permeable dye Mitotracker and DAPI. In Zoom images: k, kinetoplast; n, nucleus; nuc, nucleolus (indicated with a dotted line). **d** Nucleus and kinetoplast measurements in expanded and non-expanded epimastigotes using Fiji. Expansion factor was calculated as the ratio between non-expanded and expanded measurements. **e** Non-expanded and expanded epimastigotes stained with anti-α-tubulin (grayscale; Alexa 555)

connected to the proximal end of the basal bodies and the flagellar pocket (Gull 1999). The kinetoplast was measured in epimastigotes stained with Mitotracker and for nuclear measurements, I stained epimastigotes' DNA with DAPI (Fig. 3c). With Mitotracker staining, the ramifications of the single mitochondria of *T. cruzi* are identified and the kinetoplast is deeply marked; this also allowed us to validate the use of cell-permeable dyes for U-ExM in *T. cruzi*. Using DAPI I was able to discriminate condensed regions of the chromatin as well as the nucleolar domain (identified by the lack of DAPI staining) (Fig. 3c). An expansion factor of 4.2 × and 4.6 × was quantified for the nucleus and the kinetoplast, respectively, which signifies that expansion was homogeneous within the cells (Fig. 3d).

When compared with non-expanded epimastigotes, the resolution gain is clear and allows us to visualize the potential of this technique in *T. cruzi* (Fig. 3e). In conclusion, U-ExM is a powerful approach for studying cytoskeletal structures in *T. cruzi*. It is a robust super-resolution method, but it is very simple and does not need highly specialized reagents or instrumentation. U-ExM allows high-resolution imaging, improving the axial and lateral effective resolution of the molecules to be analyzed. Using regular confocal microscopy, an expansion factor of 4 increases resolution from around 250 to less than 70 nm. In the future, this could

be further improved by super-resolution techniques to visualize biomolecular structures that are near each other, such as the components of the flagellar pocket and basal body, which are poorly described in *T. cruzi*.

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Author contribution V. L. A. has designed, performed the experiments, and wrote the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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