Chapter 23

Light Microscopy in Trypanosomes: Use of Fluorescent Proteins and Tags

Samuel Dean and Jack Sunter

Abstract

Fluorescence microscopy enables the localization of proteins to specific structures within a cell which have either been fused to a fluorescence protein or detected by immunofluorescence. Here, we describe the various procedures that can be used to prepare both the procyclic form and bloodstream form of the human pathogen *Trypanosoma brucei* for fluorescence microscopy. The choice of procedure to be used is determined by various parameters, including protein characteristics and the scientific question being investigated.

Key words Light microscopy, Trypanosomes, Live cell, Fluorescent proteins, Cytoskeletons

1 Introduction

Light microscopy enables images of cells to be acquired; however, unstained cells have little inherent contrast, meaning phase contrast [1] or differential interference contrast (DIC) [2] microscopy is required to visualize cellular features, and these techniques are the foundation on which cellular imaging is based. As informative as these simple phase contrast/DIC images are, they do not provide any information about the localization of specific proteins.

The localization of a protein to a structure or organelle within a cell is informative when considering the function of that protein. The highly structured and polarized cell architecture of *Trypanosoma brucei* makes them an ideal cell type for defining protein localization as each cell has the same pattern of organelle positioning [3]. Fluorescence molecules absorb light of one wavelength and emit light of a longer wavelength. Numerous fluorescent reagents are available to study the localization of a protein, including specific stains, lectins, antibodies, and genetically encoded protein tags including both fluorescent proteins and epitopes [4–8]. Historically, microscopy was performed on fixed cells; however, with the development of fluorescent proteins and other reporters there has

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been a switch to imaging live cells, which has opened up a wide range of experimental possibilities including fluorescence recovery after photobleaching, tracking moving fluorescent particles, and measuring intracellular calcium fluctuations [9–11].

T. brucei has a complex life cycle with many developmental forms; however, only the procyclic form (insect midgut) and the bloodstream form (mammal) are readily culturable and imaging protein localizations in these stages will be the focus of this chapter. The quality of the image acquired during microscopy is dependent on the preparation of the cells for microscopy with poorly handled and prepared samples giving poor quality and inconsistent data. Therefore, we have detailed the protocols we use for preparing trypanosome cells for microscopy and highlighted which approach is most appropriate for which type of protein and which application. This chapter focuses on *T. brucei*, the organism in which these protocols were developed; however, with minor modifications these techniques will be applicable to the other trypanosomatid parasites.

2 Materials

Prepare all solutions using ultrapure water and analytical grade chemicals at room temperature. Store solutions at room temperature, unless indicated otherwise.

1. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. For a 10× stock, add approximately 100 mL of water to a 1 L measuring cylinder. Weigh 80 g NaCl, 1 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4 and transfer to the measuring cylinder. Add water to make volume up to 1 L. Store at room temperature and dilute as necessary.
2. Voorheis modified PBS (vPBS) [12]: 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 3 mM KH_2PO_4, 46 mM sucrose. For a 1× stock, add approximately 100 mL of water to a 1 L measuring cylinder. Weigh 8 g NaCl, 0.22 g KCl, 2.27 g Na_2HPO_4, 0.41 g KH_2PO_4, and 15.7 g sucrose and transfer to the measuring cylinder. Add water to make up to 1 L and then sterile filter through a 0.22 μm filter. Always transfer vPBS from the stock bottle in a sterile environment.
3. 1 mg/mL Hoechst 33342 solution: Weigh 50 mg Hoechst 33342 and transfer to a 100 mL measuring cylinder. Add water to a volume of 50 mL and mix well. Transfer solution to a 50 mL Falcon tube and store at 4 °C. This is a 1000× stock solution.

4. 4% (w/v) formaldehyde in PBS solution: Add 4 mL of formaldehyde 16% (w/v) solution, methanol free (TAAB, F017/3) to 10.4 mL water and 1.6 mL of 10× PBS stock.
5. 1% (w/v) glycine in PBS solution: Weigh 0.5 g glycine and add to 50 mL PBS.
6. 10% (v/v) IGEPAL CA-630 (Sigma-Aldrich, I8896) solution: Measure 5 mL of IGEPAL CA-630 into a 50 mL measuring cylinder and then add water to make up to 50 mL. Mix thoroughly.
7. 0.1% (v/v) IGEPAL CA-630 in PBS solution: Add 500 μL 10% (v/v) IGEPAL CA-630 solution to 50 mL PBS.
8. 1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES)–NaOH pH 6.9: Add approximately 300 mL of water to a 500 mL measuring cylinder. Weigh 151 g PIPES and transfer to the measuring cylinder. Adjust the pH to 6.9 with NaOH and then make up the volume to 500 mL. Sterile filter through a 0.22 μm filter and always transfer PIPES–NaOH from the stock bottle in a sterile environment.
9. 100 mM $MgSO_4$: Add approximately 150 mL of water to a 250 mL measuring cylinder. Weigh 3 g $MgSO_4$ and transfer to the measuring cylinder. Add water to make up to 250 mL.
10. 200 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) pH 8: Add approximately 150 mL of water to a 250 mL measuring cylinder. Weigh 19 g EGTA and transfer to the measuring cylinder. Adjust the pH to 8 with NaOH and then make up the volume to 250 mL.
11. 200 mM ethylenediaminetetraacetic acid (EDTA) pH 8: Add approximately 150 mL of water to a 250 mL measuring cylinder. Weigh 14.6 g EGTA and transfer to the measuring cylinder. Adjust the pH to 8 with NaOH and then make up the volume to 250 mL.
12. PEME buffer: 100 mM PIPES–NaOH pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA, 0.1 mM EDTA. For a 1x stock add approximately 50 mL of water to 100 mL measuring cylinder. Then add 10 mL 1 M PIPES–NaOH pH 6.9, 1 mL $MgSO_4$, 1 mL 200 mM EGTA pH 8, 50 μL 200 mM EDTA pH 8. Make up the volume to 100 mL with water.
13. Fetal calf serum free HMI-9 [13]: Add approximately 400 mL of water to a 500 mL measuring cylinder. Weigh 9 g HMI-9 powder (Gibco), 1.5 g $NaHCO_3$, and 1.7 μL 2-mercaptoethanol. Adjust the pH to 7.5 with NaOH. Make up the volume to 500 mL with water and then sterile filter through a 0.22 μm filter. Always transfer FCS free HMI-9 from the stock bottle in a sterile environment.

14. 1% (w/v) bovine serum albumin in PBS solution: Weigh 500 mg bovine serum albumin and dissolve in 50 mL PBS. Split into 1 mL aliquots and store at −20 °C.
15. 1 M Na_2HPO_4 solution: Add approximately 50 mL water to a 100 mL measuring cylinder. Weigh 14.2 g Na_2HPO_4 and transfer to the measuring cylinder. Make the volume up to 100 mL with water.
16. 1 M NaH_2PO_4 solution: Add approximately 50 mL water to a 100 mL measuring cylinder. Weigh 12 g NaH_2PO_4 and transfer to the measuring cylinder. Make the volume up to 100 mL with water.
17. Mounting medium: Add 448 μL 1 M Na_2HPO_4 solution and 52 μL 1 M NaH_2PO_4 solution to 500 μL water. Then add 9 mL glycerol and mix thoroughly. Split into 1 mL aliquots and store at 4 °C.
18. Slides—high quality, plain glass or poly-L-lysine.
19. Coverslips No. 0.
20. DAKO pen (Agilent, S2002).

3 Methods

3.1 Procyclic Form Cell Preparation

For procyclic form trypanosomes we generate the endogenously tagged protein cell lines using the long primer PCR tagging methodology we have previously described (*see* **Notes 1** and **2**) [4, 14].

Imaging should always be performed on cells that have been in log phase growth for 48 h. It is best to maintain procyclic form cells at between 2×10^6 and 1×10^7 cells/mL. Example images in Fig. 1.

3.1.1 Preparation of Live Procyclic Form Trypanosomes for Imaging

1. Pipet 1 mL of culture into a 1.5 mL microcentrifuge tube and centrifuge at 800 × *g* for 3 min (*see* **Note 3**).
2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL of vPBS containing 1 μg/mL Hoechst 33342 (*see* **Notes 4** and **5**), then centrifuge again at 800 × *g* for 3 min.
3. After centrifugation carefully aspirate the vPBS/Hoechst 33342 solution and resuspend the cell pellet in 1 mL vPBS, then centrifuge again at 800 × *g* for 3 min.
4. After the final centrifugation carefully aspirate the vPBS and resuspend the cell pellet in vPBS to give a cell density of ~1×10^8 cells/mL (*see* **Note 6**).
5. Spray a glass microscope slide with 70% (v/v) ethanol and wipe clean. Pipet 3 μL of cell solution onto the glass slide and carefully place a No. 0 coverslip (50 × 22 mm) on top of the cell solution.

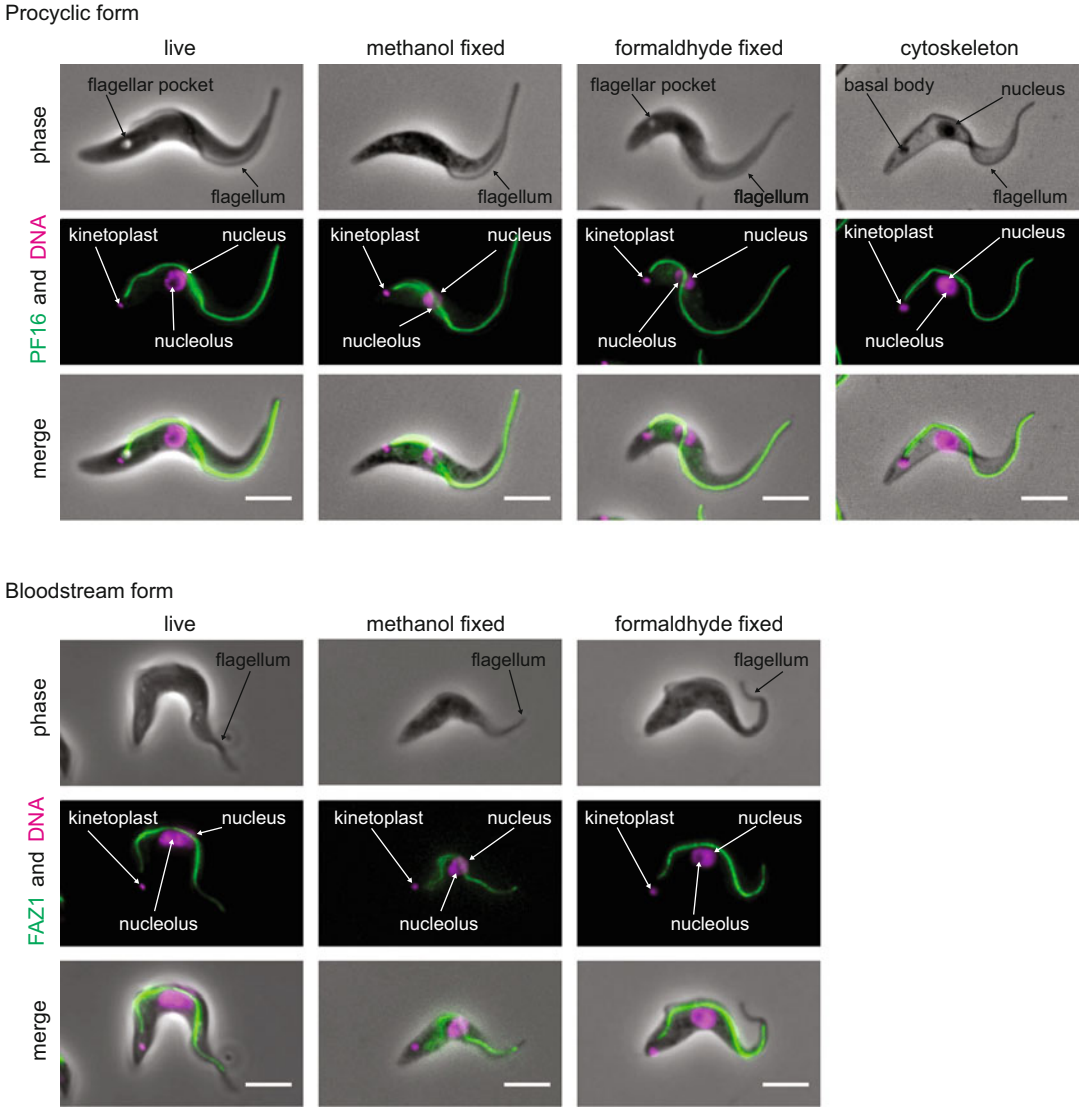

Fig. 1 Representative micrographs of procyclic and bloodstream form *T. brucei* either imaged live or after fixation. Procyclic cells are expressing PF16::mNeonGreen [15] and bloodstream form cells are expressing mNeonGreen::FAZ1 [16]. In the phase channel key cellular structures (flagellum, flagellar pocket) that should be seen if the cell preparation was good are highlighted (black arrows). DNA is stained with Hoechst 33342 and the nucleolus is observable (white arrow) indicating that this channel is not overexposed. Scale bar is 5 μm

6. Check the slide on an inverted microscope using a 20× objective to ensure that the cells are well immobilized on the slide (*see* **Note 7**).
7. Transfer the slides to a microscope suite, ensuring that the transfer is done using the appropriate local health and safety rules for moving pathogens. For imaging instructions *see* Subheading 3.4 below.

3.1.2 Preparation of Methanol Fixed Procyclic Form Trypanosomes for Imaging

Before starting this protocol ensure that there is a Coplin jar in a −20 °C freezer filled with fresh methanol chilled to −20 °C.

1. Pipet 1 mL of culture into a 1.5 mL microcentrifuge tube and centrifuge at 800 × *g* for 3 min (*see* **Note 3**).
2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL of vPBS (*see* **Note 4**), then centrifuge again at 800 × *g* for 3 min.
3. After centrifugation carefully aspirate the vPBS and resuspend the cell pellet in 1 mL of vPBS, then centrifuge again at 800 × *g* for 3 min.
4. After the final centrifugation carefully aspirate the vPBS and resuspend the cell pellet in vPBS to give a cell density of ~1×10^7 cells/mL.
5. Spray a glass microscope slide with 70% (v/v) ethanol and wipe clean. Draw small wells 1 cm × 1 cm with a DAKO pen onto the slide (*see* **Note 8**).
6. Pipet 40 μL of cell solution to each well and allow the cells to settle. Watch the cells adhere to the glass on an inverted microscope using a 20× objective until the adhered cells occupy ~80% of the surface in the field of view (*see* **Note 7**).
7. When the correct density of adhered cells is achieved remove excess cell solution with a pipette and immediately place the slide into the Coplin jar containing the methanol at −20 °C and return to the freezer.
8. Incubate at −20 °C for 20 min.
9. Transfer the slide to a Coplin jar containing PBS and rehydrate cells at room temperature for 5 min.
10. The slide is now suitable for processing by immunofluorescence (*see* Subheading 3.3) or simply staining with a DNA stain, mounting, and imaging (*see* Subheading 3.3, **step 10**).

3.1.3 Preparation of Formaldehyde Fixed Procyclic Form Trypanosomes for Imaging

Before starting this protocol ensure that there is one Coplin jar containing PBS with 1% (w/v) glycine and another Coplin jar containing PBS with 0.1% (v/v) IGEPAL CA-630.

1. Pipet 1 mL of culture into a 1.5 mL microcentrifuge tube and centrifuge at 800 × *g* for 3 min (*see* **Note 3**).
2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL of vPBS (*see* **Note 4**), then centrifuge again at 800 × *g* for 3 min.
3. After centrifugation carefully aspirate the vPBS and resuspend the cell pellet in 1 mL of vPBS, then centrifuge again at 800 × *g* for 3 min.

4. After the final centrifugation carefully aspirate the vPBS and resuspend the cell pellet in vPBS to give a cell density of ~1×10^7 cells/mL.
5. Spray a glass microscope slide with 70% (v/v) ethanol and wipe clean. Draw small wells 1 cm × 1 cm with a DAKO pen onto the slide (*see* **Note 8**).
6. Pipet 40 μL of cell solution to each well and allow the cells to settle. Watch the cells adhere to the glass on an inverted microscope using a 20× objective until the adhered cells occupy ~80% of the surface in the field of view (*see* **Note 7**).
7. When the correct density of adhered cells is achieved, remove excess cell solution with a pipette. Then pipet 40 μL of a 4% (v/v) formaldehyde solution in vPBS into each well and incubate at room temperature for 5 min (*see* **Note 9**).
8. Remove formaldehyde solution with a pipette and immediately place the slide into a Coplin jar containing PBS with 1% (w/v) glycine to block unreacted aldehyde groups. Incubate at room temperature for 5 min.
9. Transfer slide to a Coplin jar containing PBS and incubate at room temperature for 2 min to rinse off glycine solution.

   Steps 10 and **11** are only required if the cells will be processed for immunofluorescence.
10. Transfer slide to a Coplin jar containing PBS with 0.1% (v/v) IGEPAL CA-630 and incubate at room temperature for 5 min to permeabilize the cells.
11. Transfer slide to a Coplin jar containing PBS and incubate at room temperature for 2 min to rinse off IGEPAL CA-630 solution.
12. The slide is now suitable for processing by immunofluorescence (*see* Subheading 3.3) or simply staining with a DNA stain, mounting, and imaging (*see* Subheading 3.3, **step 10**).

3.1.4 Preparation of Methanol Fixed Procyclic Form Cytoskeleton for Imaging

A high quality cytoskeleton is indicated by the kinetoplast and nuclear DNA remaining as discrete structures when visualized using a DNA stain. Before starting this protocol ensure that there is one Coplin jar containing methanol at −20 °C.

1. Pipet 1 mL of culture into a 1.5 mL microcentrifuge tube and centrifuge at 800 × *g* for 3 min (*see* **Note 3**).
2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL of vPBS (*see* **Note 4**), then centrifuge again at 800 × *g* for 3 min.
3. After centrifugation carefully aspirate the vPBS and resuspend the cell pellet in 1 mL of vPBS, then centrifuge again at 800 × *g* for 3 min.

4. After centrifugation carefully aspirate the vPBS and resuspend the cell pellet at 1×10^7 cells/mL
5. Spray a glass microscope slide with 70% (v/v) ethanol and wipe clean. Draw small wells 1 cm × 1 cm with a DAKO pen onto the slide (*see* **Notes 8** and **10**).
6. Pipet 40 μL of cell solution to each well and allow the cells to settle. Watch the cells adhere to the glass on an inverted microscope using a 20× objective until the adhered cells occupy ~80% of the surface in the field of view (*see* **Note 7**).
7. When the correct density of adhered cells is achieved, remove excess cell solution with a pipette and replace with 50 μL of vPBS.
8. If unbound cells are still apparent by microscopy, remove vPBS with a pipette and replace with 50 μL of vPBS.
9. Remove vPBS and add 30 μL of 1% (v/v) IGEPAL CA-630 in PEME solution for 10 s (*see* **Note 10**).
10. Remove IGEPAL CA-630 /PEME solution and transfer the slide to the Coplin jar containing methanol at −20 °C and return to the freezer, incubating for 20 min (*see* **Note 11**)
11. Transfer the slide to a Coplin jar containing PBS and rehydrate cells at room temperature for 5 min.
12. The slide is now suitable for processing by immunofluorescence (*see* Subheading 3.3) or simply staining with a DNA stain, mounting and imaging (*see* Subheading 3.3, **step 10**).

3.2 Bloodstream Form Cell Preparation

For the generation of bloodstream form trypanosome cell lines expressing endogenously tagged proteins we use the CRISPR based method described by Beneke et al. [17] (*see* **Notes 1** and **2**).

Imaging should always be performed on cells that are in log phase growth and have been in log phase growth for the previous 48 h. It is best to maintain bloodstream cells at between 1×10^5 and 1×10^6 cells/mL. Example images in Fig. 1.

3.2.1 Preparation of Bloodstream Trypanosomes for Live Cell Imaging

1. Pipet 8 mL of culture into a 15 mL Falcon tube and centrifuge at 800 × *g* for 7 min (*see* **Note 3**).
2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL of FCS-free HMI-9 containing 1 μg/mL Hoechst 33342 (*see* **Notes 5** and **12**).
3. Transfer cells to a 1.5 mL microcentrifuge tube and centrifuge at 800 × *g* for 3 min.
4. Carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 20 μL of FCS free HMI-9.

5. Lightly fix cells by adding 20 μL of 0.04% (v/v) formaldehyde in FCS free HMI-9 (*see* **Notes 13** and **14**).
6. Remove 2.4 μL with a pipette and place on a clean glass slide and put a No. 0 (50 × 22 mm) coverslip on top.
7. Transfer the slides to a microscope suite, ensuring that the transfer is done using the appropriate local health and safety rules for moving pathogens. For imaging instructions *see* Subheading 3.4 below.

3.2.2 Methanol Fixed Bloodstream Form

Before starting this protocol ensure that there is a Coplin jar in a −20 °C freezer filled with fresh methanol chilled to −20 °C.

1. Pipet 8 mL of culture into a 15 mL centrifuge tube and centrifuge at 800 × *g* for 3 min (*see* **Note 3**).
2. After centrifugation carefully decant the medium without disturbing the cell pellet and resuspend the cells in 1 mL of vPBS (*see* **Note 4**), transfer to a 1.5 mL microfuge tube then centrifuge again at 800 × *g* for 3 min.
3. Aspirate the supernatant and resuspend the cell pellet in 1 mL of vPBS, then centrifuge again at 800 × *g* for 3 min.
4. After the final centrifugation carefully aspirate the vPBS and resuspend the cell pellet in vPBS to give a cell density of ~1×10^7 cells/mL.
5. Spray a poly-L-lysine microscope slide (*see* **Note 16**) with 70% (v/v) ethanol and wipe clean. Draw small wells 1 cm × 1 cm with a DAKO pen onto the slide (*see* **Note 8**).
6. Pipet 40 μL of cell solution into each well and allow the cells to settle for 10 min. Check the slide on an inverted microscope using a 20× objective to ensure that there is a "good" density of cells settled onto the slide (*see* **Note 7**).
7. Carefully remove excess cell solution with a pipette and immediately place the slide into the Coplin jar containing the methanol at −20 °C and return to the freezer.
8. Incubate at −20 °C for 20 min.
9. Transfer the slide to a Coplin jar containing PBS and rehydrate cells at room temperature for 5 min.
10. The slide is now suitable for processing by immunofluorescence (*see* Subheading 3.3) or simply staining with a DNA stain, mounting, and imaging (*see* Subheading 3.3, **step 10**).

3.2.3 Preparation of Formaldehyde Fixed Bloodstream Form Trypanosomes for Imaging

Before starting this protocol ensure that there is a Coplin jar containing PBS with 1% (w/v) glycine.

1. Pipet 8 mL of culture into a 15 mL Falcon tube and centrifuge at 800 × *g* for 8 min (*see* **Note 3**).

2. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 10 mL vPBS, then centrifuge again at 800 × *g* for 8 min.
3. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 1 mL vPBS.
4. Pipet 1 mL of 8% formaldehyde (v/v) in vPBS into the cell solution and mix by inverting the tube several times. Incubate at room temperature for 10 min to fix the cells (*see* **Note 9**)—during this period occasionally invert the tube to avoid the cells settling.
5. Pipet 20 μL of 10% (v/v) IGEPAL CA-630 into the cell solution and mix by inverting the tube four times. Incubate at room temperature for 10 min.
6. Pipet 12 mL of vPBS into cell solution and invert the tube several times. Then centrifuge at 800 × *g* for 8 min.
7. After centrifugation carefully aspirate the medium without disturbing the cell pellet and resuspend the cells in 500 μL vPBS.
8. Spray a poly-L-lysine microscope slide (*see* **Note 16**) with 70% (v/v) ethanol and wipe clean. Draw small wells 1 cm × 1 cm with a DAKO pen onto the slide (*see* **Note 8**).
9. Pipet 50 μL of cell solution to each well and allow the cells to settle for 15 min in a humid chamber (*see* **Note 15**). Check the slide on an inverted microscope using a 20× objective to ensure that there is a "good" density of cells have settled onto the slide (*see* **Note 7**).
10. Place the slide into a Coplin jar containing PBS with 1% (w/v) glycine to block unreacted aldehyde groups. Incubate at room temperature for 5 min.
11. Transfer slide to a Coplin jar containing PBS and incubate at room temperature for 2 min to rinse off glycine solution.
12. The slide is now suitable for processing by immunofluorescence (*see* Subheading 3.3) or simply staining with a DNA stain, mounting, and imaging (*see* Subheading 3.3, **step 10**).

3.3 Immunofluorescence of Fixed Trypanosomes

If using the fixation procedures described above (Subheadings 3.1.2–3.1.4, 3.2.2, 3.2.3) the slides will be in a Coplin jar containing PBS (*see* **Note 3**).

1. Remove the slide from the Coplin jar and carefully dry the back of the slide and between the wells with paper towel. Place the slide within the humid chamber with the lid off (*see* **Note 15**).
2. Pipet 50 μL of 1% BSA (w/v) in PBS (*see* **Note 17**) onto each well on the slide to block nonspecific protein binding sites. Replace the lid of the humid chamber and incubate for 1 h at room temperature.

3. Remove block solution with a pipette and then pipet 50 μL of primary antibody diluted to the appropriate concentration in 1% BSA (w/v) in PBS and incubate for 1 h at room temperature in a humid chamber.
4. Remove the primary antibody solution with a pipette and place the slide into a Coplin jar containing PBS and incubate for 5 min at room temperature.
5. Remove the slide and place into another Coplin jar containing PBS and incubate for 5 min at room temperature (*see* **Note 18**). Repeat this process until the slide has been washed four times in total.
6. Remove the slide from the Coplin jar and carefully dry the back of the slide and between the wells with paper towel. Place the slide within the humid chamber with the lid off.
7. Pipet 50 μL of secondary antibody diluted to the appropriate concentration in 1% BSA (w/v) in PBS and incubate for 1 h at room temperature in a humid chamber.
8. Remove the secondary antibody solution with a pipette and place the slide into a Coplin jar containing PBS and incubate for 5 min at room temperature.
9. Remove the slide and place into another Coplin jar containing PBS and incubate for 5 min at room temperature (*see* **Note 18**).
10. Remove the slide and place into another Coplin jar containing PBS with 0.1 μg/mL Hoechst 33342 (*see* **Note 19**) and incubate for 5 min at room temperature.
11. Remove the slide and place into another Coplin jar containing PBS and incubate for 5 min at room temperature (*see* **Note 18**).
12. Remove the slide from the Coplin jar and carefully dry the back of the slide and between the wells with paper towel.
13. Place the slide onto a clean and dry sheet of paper towel and pipet 3 μL of mounting medium (*see* **Notes 20** and **21**) onto each well.
14. Carefully place a coverslip onto the slide. Invert the slide and press down, squeezing any excess mounting medium onto the paper towel.
15. Invert the slide and carefully wipe any excess mounting medium off the slide.
16. Secure the coverslip in place by adding a drop of nail varnish to each of the 4 corners of the coverslip. The slide is now ready to be imaged (see Subheading 3.4).

3.4 Microscopy of Trypanosomes (See Notes 22–25)

1. Set up Köhler illumination on the microscope (*see* **Note 26**).
2. Focus on the cells using the camera and the transmitted light channel (*see* **Notes 27** and **28**).

3. Optimize the exposure times such that the signal from each channel is maximised but there are no saturated pixels (*see* **Notes 29** and **30**).
4. Capture at least ~100 cells (usually ~3 fields using a 63x objective) to ensure that cells in each stage of the cell cycle are represented.
5. Save the data in the native microscope format or as stacked tiffs that do not discard the data.

4 Notes

1. Development of PCR based methods to generate transgenic cell lines has enabled the localization of proteins using a genetically encoded tag (e.g., fluorescent protein, epitope tag, HaloTag) to be rapidly assessed. Fluorescent proteins are our preferred tool of choice for this. There are a wide variety of fluorescent proteins available that have dramatically different characteristics. mNeonGreen is by far the best choice for most applications because it is bright, stable and very fast folding. Red proteins still lag behind but we find that mScarlet-I works well for most applications. Currently, blue and far red proteins are not bright enough to be useful for imaging in trypanosomes. "Timer" fluorescent proteins and tags (SNAP and HaloTag) that can be labeled with fluorescent ligands offer intriguing experimental possibilities. A comprehensive list of fluorescence proteins and their properties can be found here: http://www.fpvis.org. Fluorescent proteins normally provide simple steady-state information about protein localization and so the dynamics of protein localization is missed. Techniques such as fluorescence recovery after photobleaching can be used to assess this using fluorescent proteins [10]; however, this requires an expensive microscope setup and generally only provides data over a short timescale. We have found the genetically encoded HaloTag can provide information about protein dynamics over a much longer timescale [15].
2. A key experimental consideration when beginning to design an approach for determining the localization of a protein is to examine the characteristics of the protein. A simple bioinformatic analysis of the protein will reveal whether it has any predicted transmembrane domains or targeting signals and this will influence the type of tag used and terminus on which the tag should be fused to the protein [18]. For example, if a signal peptide is predicted it is best to tag on the protein on the C-terminus. Obviously, tagging approaches come with caveats that should be borne in mind such as interfering with protein folding or targeting. If this is a concern, then an antibody

specific to the protein of interest can be used as the basis for an immunofluorescence approach. However, immunofluorescence requires the use of fixatives (formaldehyde and methanol), which can cause fixation artifacts and careful controls are required to show that the antibody signal is specific to the protein of interest. Methanol fixation is not suitable for imaging proteins that localize to membranous structures, such as the mitochondrion or cell-surface membrane, but works well for imaging proteins that localize to the cytoskeleton, such as the flagellum or subpellicular microtubules. However, formaldehyde fixation may reduce the antigenicity of the epitope that the immunofluorescence antibody binds to, meaning that different formaldehyde concentrations and incubation times may need to be trialed. Our initial approach to defining the localization of a protein would be to tag the N and C termini of the protein of interest with mNeonGreen and perform live cell fluorescence microscopy as this usually gives high quality data and can be done rapidly and with high throughput.

3. All steps should be carried out at room temperature unless otherwise stated.
4. We prefer to use vPBS for imaging; however, this does starve the cells of amino acids and will stress the cells leading to the formation of RNA stress granules. As an alternative medium without fetal calf serum can be used for the washing steps as a direct replacement for vPBS; however, we have found there is an issue with imaging in red channel as a background endocytic signal appears due to accumulation of phenol red in the cells especially if the signal from the tagged protein is weak.
5. Hoechst 33342 is used as this DNA stain is cell permeable.
6. When examining cells by live cell microscopy this density of cells gives a good number of evenly spaced cells in each field of view.
7. Tissue culture facilities routinely have a microscope of this nature for monitoring cell cultures. This quick check ensures that only slides with cells suitable for imaging at higher resolutions are used and allows for an assessment of cell density on the slide, ensuring the cells are evenly spaced and not overlapping on the slide but of sufficient density for efficient imaging. In our system using an ORCA-Flash4.0 Digital CMOS camera, we would aim to capture for 20–30 cells per image with a 100× objective and 40–50 cells per image for a 63× objective.
8. Ensure that the hydrophobic boundary of the well has completely dried before adding the cell solution as the solvent will lyse the cells at the edge of the well.
9. We prefer to use electron microscopy grade formaldehyde as this means we do not have to prepare the solution from

paraformaldehyde powder. This is our standard initial fixation conditions when using formaldehyde; however, if this does not give acceptable results then we recommend that the user titers the concentration of formaldehyde from 2 to 4% and time from 5 to 30 min.

10. Small wells are critical for the rapid detergent extraction that is necessary to get high quality cytoskeletons. When drawing the wells, it is convenient to use the holes in a 0.5 mL microfuge tube rack as a template.
11. Extended storage in methanol (~1 week) will quench fluorescent proteins and increase autofluorescence of the nucleus and kinetoplast.
12. For bloodstream form cells washing and resuspending in HMI-9 without FCS helps to maintain the viability of the cells. Bloodstream form cells are very sensitive to changes in osmotic potential and it is therefore important to ensure that the buffers used are at the correct osmolarity.
13. The low concentration of formaldehyde stops flagellum movement but does not initially kill the cell. Moreover, the trapping of the cells between the slide and the coverslip helps to "flatten" the cells.
14. For bloodstream form cells the time for imaging is very short (5–10 min) so only fix and visualize one sample at a time.
15. We construct our humid chambers using square plastic dishes covered in aluminum foil with a piece of damp paper towel in the bottom.
16. Unlike procyclic form cells, bloodstream form cells do not adhere well to glass due to the presence of the glycosylated surface coat. The poly-L-lysine surface will cross-link to the formaldehyde fixed cells ensuring that the cells attach to the slide. Moreover, methanol dehydration will both attach and flatten cells to the glass slide.
17. This is our general purpose blocking solution; however, for specific applications we have found using a 10% serum solution in PBS also to be effective. Especially if the serum is from the same species in which the secondary antibody was raised.
18. We recommend transferring slides between Coplin jars rather than pouring out contents of Coplin jar and refilling with the slide in situ as this will disturb the cells settled on the slide.
19. Other DNA stains such as DAPI or Hoechst 33258 would also work.
20. We normally use our home-made mounting medium, which is simply a phosphate buffer with glycerol; however, commercially available mounting media are also suitable but the user

should test to make sure these do not introduce any background signal.

21. We recommend that microscope slides with fixed cells/cytoskeletons are mounted just before imaging as this reduces the level of background fluorescent signal. This is especially important when imaging with cytoskeletons.
22. For trypanosome imaging we use a widefield epifluorescence microscope and both an upright and an inverted microscope are suitable. However, an upright microscope is preferable as the condenser has a much higher numerical aperture (NA), which gives a higher phase contrast resolution and a reduced depth of field. Generally, we have found that confocal microscopes are not effective for imaging trypanosomes. The resolution of the final image will depend on the magnification and numerical aperture of the objective, and the camera pixel size. We find that a 63× NA1.4 objective combined with a camera pixel size of 6.5 μm ensures Nyquist sampling, which gives optimal resolution over a large field of view.
23. Mercury vapor bulbs, metal halide bulbs and modern LEDs all give high power illumination suitable for fluorescence. Most mercury vapor bulbs need to be replaced every 300 h of usage, whereas metal halide bulbs should be replaced annually regardless of usage. Mercury bulbs need to be aligned upon installation to ensure an evenness of fluorescent illumination.
24. Ensure that the microscope has the appropriate filter sets for each fluorescent protein or fluorophore that you want to image. The standard microscope filter sets for fluorescence microscopy are not well optimized for working with fluorescence proteins and can dramatically reduce the signal–noise ratio of the fluorescence and the subsequent quality of the final dataset. Investing in bespoke filter-sets will therefore reap significant dividends. We use Thorlabs MDF-GFP2 for mNeonGreen and MDF-TOM for mScarlet fluorescence.
25. We recommend that sCMOS cameras are used for acquisition because they have excellent sensitivity, a large field of view and 16-bit linear range.
26. Köhler illumination should be set up at the beginning of each session to ensure high quality transmitted light images—if the microscope is old or needs servicing, it may be necessary to repeat the setup or Köhler illumination mid-session, but it only takes a few seconds once you know how to do it. If DIC is used, adjust the DIC such that the detail of the cells is preserved but the background of the slide remains smooth (nongranular). In our hands, a value of −600 works well, but this will vary depending upon the microscope.

27. A transmitted light (phase contrast or DIC) image should always be acquired so that the quality of the sample can be assessed and as a reference for the fluorescence. Bright field should not be used because the cells do not have enough contrast to be easily visible.
28. If the location of the fluorescent protein is unknown within the cell we focus either on the flagellar pocket, which appears as a bright circle of signal in the phase channel in cells with one flagellum or the flagellum. By focusing on these structures one can ensure a consistent plane of focus between different images (Fig. 1).
29. For general fluorescence imaging, it is best to use the most intense illumination available in your system. However, some fluorescence proteins (e.g., mScarlet) are rapidly bleached at high illumination intensities and superior results are achieved by reducing the illumination to 50% of maximum. Additionally, for time-lapse fluorescence microscopy, reducing the intensity may allow for longer imaging than would be possible using maximum light intensity. Fluorescent channels should be collected longest-wavelengths first (i.e., from red to blue) to ensure that the unimaged fluorescent proteins/labels are not bleached.
30. We find that 3 s is optimal for imaging most proteins tagged with a fluorescent protein, but exposure times when imaging immunofluorescent signals can be much shorter. The nucleolus should be clearly visible in the DAPI/Hoechst-stained nucleus as a less well stained area. Assess the data using captured images as the camera is more sensitive and has better linear range than your eye.

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