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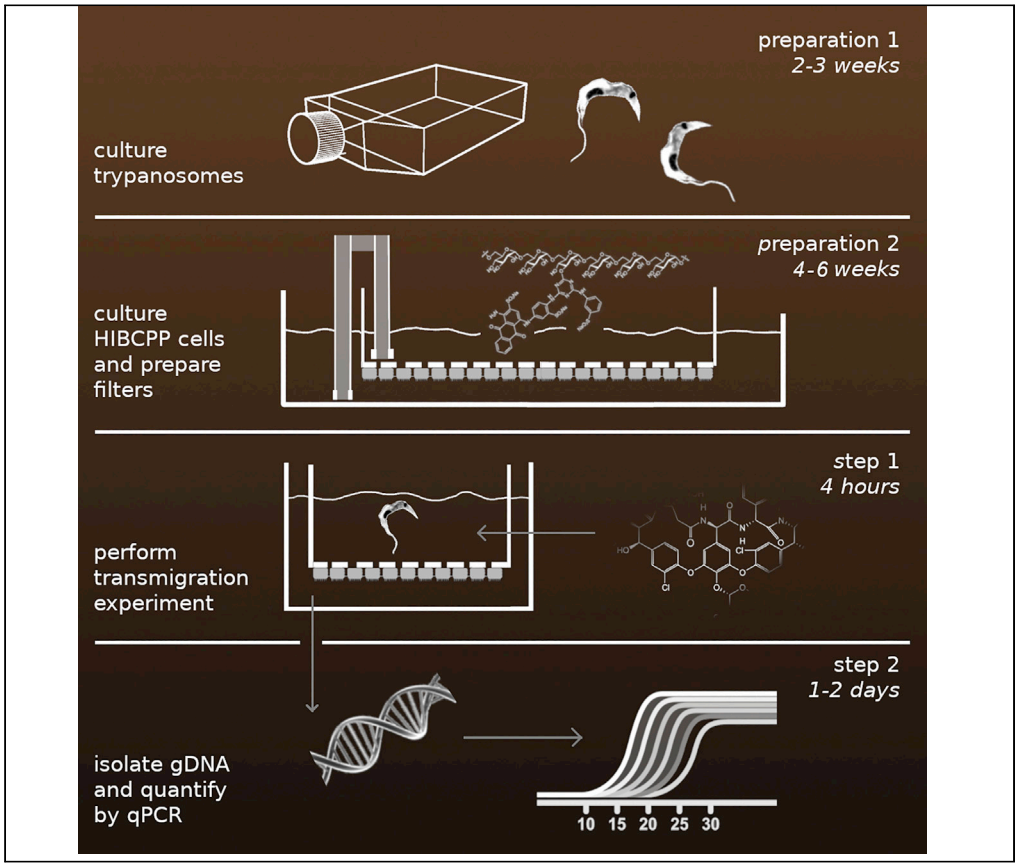
Protocol to analyze the transmigration efficiency of *T. brucei* using an *in vitro* model of the blood-cerebrospinal fluid barrier



At present, the only approach to investigate the transmigration of *Trypanosoma brucei*, the causative agent of human African trypanosomiasis, from blood to cerebrospinal fluid is through animal experiments. This protocol details how to analyze the transmigration efficiency using an *in vitro* model of the blood-cerebrospinal fluid (blood-CSF) barrier. We describe how to grow human choroid plexus epithelial cells on cell culture filter inserts to form the barrier, followed by isolating and quantifying genomic DNA of transmigrated parasites by qPCR.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Use of HIBCPP cells on transwell filters as an *in* vitro model of the blood-CSF barrier

Assess barrier function by transepithelial electrical resistance & molecule flux

Co-cultivate HIBCPP filters with *T. brucei* to analyze transmigration

Isolate and quantify genomic DNA of transmigrated trypanosomes by qPCR

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Protocol to analyze the transmigration efficiency of *T. brucei* using an *in vitro* model of the blood-cerebrospinal fluid barrier

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SUMMARY

At present, the only approach to investigate the transmigration of *Trypanosoma brucei*, the causative agent of human African trypanosomiasis, from blood to cerebrospinal fluid is through animal experiments. This protocol details how to analyze the transmigration efficiency using an *in vitro* model of the blood-cerebrospinal fluid (blood-CSF) barrier. We describe how to grow human choroid plexus epithelial cells on cell culture filter inserts to form the barrier, followed by isolating and quantifying genomic DNA of transmigrated parasites by qPCR. For complete details on the use and execution of this protocol, please refer to Speidel et al. (2022).

BEFORE YOU BEGIN

⚠ CRITICAL: You will have to start the experiment once the Human Choroid Plexus Papilloma (HIBCPP) cells on the cell culture filter inserts reach a Transepithelial Electrical Resistance (TEER) of > 600 Ω (200 Ω·cm^2). At that time, you will need 5e6 trypanosomes per filter. Ensure that both preparation steps (“trypanosomes” and “*in vitro* blood-cerebrospinal fluid barrier”) are coordinated in time to each other.

Note: Unless stated otherwise, trypanosomes and HIBCPP cells are incubated at 37°C in a water-saturated atmosphere (> 95% relative humidity) with 5% (v/v) CO_2.

Preparation of trypanosomes

Timing: 2–3 weeks

1. Preparation of HMI-9 Medium.
   a. Prepare the basic Iscove’s Modified Dulbecco’s Medium (IMDM) as described in the materials and equipment section. Immediately continue to the next step.
   b. Prepare the Hirumi’s Modified Iscove’s medium 9 (HMI-9) as described in the materials and equipment section.

c. Store the medium in a sealed flask (e.g., a 500 mL Schott bottle) overnight at 4°C to allow the dissolved air to exhaust. Medium can be stored up to 3 months at 4°C in the dark.

2. Cultivation of *T. brucei brucei*.
   a. Take frozen cells (containing 5e6 cells in PBS pH 7.7 / 20 mM glucose / 10% DMSO) into culture. Cells can be stored up to 6 months at −80°C or for multiple years in liquid nitrogen.
      i Thaw frozen cells quickly (e.g., in a 25°C–30°C water bath).

⚠ CRITICAL: After thawing, the following steps should be performed on ice.

      ii Wash cells in 10 mL pre-chilled HMI-9.
      iii Centrifuge for 10 min at 1,500 × *g* and 4°C and discard the supernatant.
      iv Resuspend the pellet in 1 mL pre-chilled HMI-9.
      v Determine cell density with a counting chamber (Neubauer improved 0.01 mm).
      vi Inoculate 2e5 trypanosomes in 1 mL pre-warmed HMI-9 per well in a 24 well plate.
      vii Culture cells at 37°C in a water-saturated atmosphere with 5% (v/v) CO_2.
   b. Maintain permanent culture.
      i Count cell density daily (Neubauer improved 0.01 mm).
      ii Dilute trypanosomes with pre-warmed HMI-9 to 1e5 / mL into a new well (24 well plate).

⚠ CRITICAL: You can expect a generation time between 7–8 h (i.e., a tenfold increase per day). Always keep cell density between 1e5 / mL and 1e6 / mL. Do NOT use trypanosomes if cell density was higher than 1e6 / mL, as the parasites might have differentiated into a cell-cycle arrested "short stumpy" form (Hamm et al., 1990).

      iii Incubate diluted cells at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2.
      iv RECOMMENDED: In order to bridge the weekend, a 1:10 dilution series can be created (down to 1e3 / mL). On Monday, determine the latest well with a cell density close to 5e5 / mL to continue daily dilution.
   c. 2–3 days before experiment: increase volume.

Note: You will need 5e6 trypanosomes (5 mL culture at 1e6 / mL) per filter. Scale the protocol to your needs. An example is provided below on how to proceed for 7–8 filters. Plan reserves in case the cells do not grow as well as expected.

      i If you expect 7–8 filter inserts with HIBCPP cells ("HIBCPP filter inserts") to be ready for use within two days, pre-warm 10 mL HMI-9 in a T-25 flask with filter cap.
      ii Inoculate 1e5 trypanosomes per mL.
      iii Incubate culture flask lying at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 24 h.
      iv Repeat steps i-iii with 50 mL HMI-9 (in a T-125 flask with filter cap).

Preparation of the *in vitro* blood-cerebrospinal fluid barrier

Timing: 4–6 weeks

3. Preparation of HIBCPP Medium.
   a. Prepare the HIBCPP Medium with 10% FBS as described in the materials and equipment section. This medium will be used for HIBCPP cultivation.
   b. Prepare the HIBCPP Medium (0% FBS) as described in the materials and equipment section. This medium will be used for HIBCPP filter inserts when a TEER of 300 Ω (100 Ω•cm^2) is reached.

Note: Cultivation in serum-free medium causes formation of a higher TEER by choroid plexus epithelial cells *in vitro* (Haselbach et al., 2001).

4. Cultivation of HIBCPP cells.
   a. Take frozen cells (containing 1-2e6 cells in PBS pH 7.7 / 20 mM glucose / 10% DMSO) into culture. Cells can be stored up to 6 months at −80°C or for multiple years in liquid nitrogen.
      i Thaw the aliquot carefully while pipetting up and down with 1 mL pre-warmed HIBCPP medium (10% FBS) and transfer the suspension into 10 mL medium.
      ii Pipet cell suspension in a T-75 flask with filter cap.
      iii Incubate culture flask lying at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 24 h.
      iv Change the medium to 10 mL fresh pre-warmed HIBCPP medium (10% FBS) to remove DMSO-remnants / dead (not attached) cells.
      v Change medium every second day (i.e., on Monday, Wednesday, Friday).
      vi Grow the cells in this flask until they reach 70%–80% confluency (which will take around 2–4 weeks).
   b. Trypsinization of HIBCPP cells.

⚠ CRITICAL: Only use HIBCPP cells up to passage 38.

      i Remove medium from T-75 flask and wash twice with 10 mL pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS). Discard DPBS after both washing steps.
      ii Add 3 mL 0.25% trypsin-EDTA and incubate for 20 min at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2.

Optional: Check under the microscope if all cells have detached after 15 min. In order not to over-trypsinize the cells, it is not necessary to wait until the cells are completely singled.

      iii Stop the reaction with 10 mL pre-warmed HIBCPP medium (10% FBS).
      iv Cells will appear slimy; try to get them suspended as much as possible.
      v Centrifuge for 10 min at 50 × *g* (room temperate) and discard the supernatant.
      vi Resuspend the pellet in 1 mL pre-warmed HIBCPP medium (10% FBS).
      vii Determine cell density with a counting chamber (Neubauer improved 0.01 mm).
      viii Inoculate 1e6 HIBCPP cells in 10 mL medium (10% FBS) in a new T-75 flask.
      ix Grow the cells in this flask at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 until they reach 70%–80% confluency (see Figure 1).

5. Preparation of inverse culture HIBCPP filter inserts.

RECOMMENDED: Seed cells on Thursday, turn filter inserts on Friday and measure TEER daily beginning on Monday.

   a. Seed and cultivate HIBCPP cells on filter inserts (Dinner et al., 2016).
      i Optional: Normally, we use translucent filters (pore density 2e6 cm^{-2}) with 3 μm pore size. If necessary, 8 μm filter inserts or transparent filters can be used.
      ii Use sterile forceps to place filter inserts upside down into a 12 well plate. Hold the insert slightly tilted with the forceps and fill the well to the brim with HIBCPP medium (10% FBS): The air will escape upwards through the filter. The liquid level must reach the membrane and there must no air be left inside the insert (see Methods video S1).

⚠ CRITICAL: Once the filter membrane had contact to any fluid, it is no longer permeable to air. Thus, premature wet filters can hardly be filled correctly.

      iii Remove half of the medium from the well. The insert remains filled.
      iv Put a drop of HIBCPP medium (10% FBS) (approx. 100 μL) on top of the insert to wet also the second side of the filter and wait until the drop is completely absorbed.
      v Close the lid of the plate and put it into the incubator at 37°C (water-saturated atmosphere) and 5% (v/v) CO_2 to pre-warm.
      vi Trypsinize HIBCPP cells (see step 4b) and bring cell suspension to a stock concentration of 1e6 / mL. From this suspension pipet 100 μL on the filter membrane.

Note: Usually younger passages of the cells need less cells to be put onto a filter insert. This initial cell number can vary from time to time and needs to be adjusted due to the TEER values measured in the last filter batch (see 5b vii).

vii Incubate HIBCPP filter inserts at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 24 h.

viii **After 1 day,** put 1 mL pre-warmed HIBCPP medium (10% FBS) into each well of a 24 well plate (see Methods video S2 for steps viii-x).

ix Lift the inserts with sterile forceps out of the 12 well plate dropping the medium inside the insert. Turn the insert and put it in standard orientation (i.e., the filter membrane points downwards) into the prepared 24 well plate.

x Add 500 µL pre-warmed HIBCPP medium (10% FBS) into the upper compartment and incubate HIBCPP filter inserts at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2.

Optional: In steps viii-x, medium can be reused: Once the first filters are transferred, you may pipet the remaining medium (12 well plate) into subsequent wells of the 24 well plate (see Methods video S2).

xi **At day 4,** measure TEER (see step 5b). If cells reach a TEER of 300 Ω (100 Ω•cm^2), put them to HIBCPP medium (0% FBS). If TEER is lower than 300 Ω, put them to fresh HIBCPP medium (10% FBS) (see next step).

xii Place 1 mL pre-warmed HIBCPP medium (either 0 or 10% FBS) in a new 24 well plate, empty the inserts and put them into the new plate. Add 500 µL HIBCPP medium (either 0 or 10% FBS) into the upper compartment.

Optional: If experiments deviating from this protocol are planned, we note that HIBCPP cells also form a barrier function at FBS concentrations between 0%–10%. Under these conditions, it may take longer to reach a certain TEER.

xiii Incubate HIBCPP filter inserts in a water-saturated atmosphere at 37°C and 5% (v/v) CO_2 for 24 h. If TEER was lower than 300 Ω, re-do steps xi-xiii the following day(s).

xiv The day after changing medium to 0% FBS, measure TEER daily and change medium (0% FBS) every second day.

xv Inserts are taken for experiments when TEER is higher than 600 Ω (200 Ω•cm^2). Filters should not exceed 1200 Ω, since higher TEER values indicate that HIBCPP cells have formed a multilayer.

b. Measurement of transepithelial electrical resistance (TEER).

i Start measuring TEER four days after seeding the HIBCPP filter inserts.

ii For TEER measurements you may use a commercially available (Millipore MillicellERS2) or self-assembled (Theile et al., 2019) voltammeter with a MERSSTX01-electrode.

iii Place the electrode for 15 min into a 50 mL Falcon tube with approx. 10 mL of 80% ethanol. Take the electrode under the sterile hood, let it dry and equilibrate in HIBCPP medium (0% FBS) for 5 min (see Methods video S3 for steps iii-v).

iv Put the long arm of the electrode into the lower reservoir and the short arm into the filter insert. Read TEER.

⚠ CRITICAL: Pay attention that the electrode is used in the same position / angle in each measurement. Do not touch the filter membrane.

v Place the electrode into 80% ethanol and subsequently store it in a dry Falcon tube.

⚠ CRITICAL: We considered filters with a TEER of more than 600 Ω (200 Ω•cm^2) ready for transmigration experiments. If TEER remains below 600 Ω one week after seeding the

cells, the filters should be discarded. Reasons for this could be that seeded cells were either not vital or applied in an insufficient concentration to form a monolayer.

Note: It may be necessary to regularly adjust the amount of HIBCPP cells that are seeded. If HIBCPP cells are between passage 21 and 25, 7e4 cells per filter should suffice. Ideally, filters that have been prepared on Thursday reach a TEER of 300 Ω (100 Ω•cm^2) and can be transferred into HIBCPP medium (0% FBS) on Monday. If TEER values are higher (800 Ω), the amount of HIBCPP cells should be reduced to 5e4. If TEER values are lower (150 Ω) and raise rather slowly during the next days, the starting amount of HIBCPP cells should be adjusted to 1.5e5 cells.

c. Determination of barrier function by measuring molecular flux.

RECOMMENDED: To validate the barrier integrity at a defined TEER value, we highly recommend measuring the flux of dextran blue in regular intervals (or when this method is to be newly established).

i Prepare a 133 mg / mL (26.6 mM) dextran blue (5 kDa) stock solution in DPBS.

ii One day after seeding HIBCPP filter inserts (i.e., at the time point filters are turned into standard orientation) transfer a duplicate of filters into new wells with 1 mL pre-warmed HIBCPP medium (0% FBS) in a 24 well plate.

iii Add 375 µL pre-warmed HIBCPP medium (0% FBS) together with 75 µL dextran blue solution to the upper reservoir.

iv Incubate the plate at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 2 h.

v Measure absorbance (A) of the medium in the lower compartment at 595 nm. HIBCPP medium (0% FBS) is defined as blank (A := 0). For measuring, we used a 96 well Dynex MRX plate reader and a sample volume of 200 µL.

vi Define the absorbance one day after seeding the HIBCPP cells as 100% permeability.

vii Repeat steps ii-v with the filters of interest, e.g., filters that have reached a TEER of 600 Ω (200 Ω•cm^2) or higher.

viii Calculate the molecular flux as $A_{\text{filter of interest}} / A_{\text{one day after seeding}} \cdot 100\%$.

Note: The formula gives the quotient of the absorbance of the medium in the lower well at a given time point related to the absorbance one day after seeding. It is a measure of the relative permeability of the filter. Example data is provided in Table 1.

⚠ CRITICAL: Do not use the HIBCPP filter inserts after addition of dextran blue, as we cannot exclude that dextran blue will interfere with subsequent transmigration experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Iscove's modified Dulbecco's Medium (IMDM)	Sigma	I7633
Sodium bicarbonate	Sigma	S5761
Hypoxanthine	Sigma	H9636
Pyruvate	Sigma	P5280
Thymidine	Sigma	T1895
Bathocuproinedisulfonic acid	Sigma	B1125
L-Cysteine	Sigma	C7352
2-Mercaptoethanol	Sigma	M3148
Pen/Strep	Sigma	P4333

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal bovine serum (FBS)	Life Technologies	#10270106
Serum Plus II ™	Sigma	14009C
DMEM/F12	Gibco	#31330-038
Insulin	Sigma	I9278
Trypsin-EDTA	Gibco	#25200056
Dextran blue	Sigma	90008-1G
Critical commercial assays		
QIAamp DNA Mini Kit	QIAGEN	#51304
Fast SYBR Green MasterMix	Applied Biosystems	#4385610
Experimental models: Cell lines		
T.brucei brucei Lister 427 MiTat 1.2 VSG 221	(Speidel et al., 2022)	none
HIBCPP cells, use between passage 21 and 38	(Schwerk et al., 2012)	none
Oligonucleotides		
TbAOX fwd	Invitrogen	5′-AAACGGCCTCGTTGATACAC-3′
TbAOX rev	Invitrogen	5′-TGCTGAGGTTCCAGTACACG-3′
Software and algorithms		
QuantStudio™ Design & Analysis software v1.4.3	Thermo Fisher Scientific	download
Other		
Counting chamber Neubauer improved 0.01 mm	Carl Roth	PC73.1
Cell culture plates CELLSTAR® sterile 24 wells	Carl Roth	CE56.1
ThinCert cell culture filter insert 3 µm	Greiner	#662631
TEER chopstick electrode	Merck	MERSSTX01
TEER Millicell electrical resistance system	Merck	MERS00002
QuantStudio™ 3 Real-Time PCR System	Thermo Fisher Scientific	A40393

Alternatives: gDNA can alternatively be isolated with the DNeasy Blood and Tissue Kit (-QIAGEN) or the Monarch Genomic DNA Purification Kit (New England Biolabs). Phenol / chloroform extraction can also be performed. For qPCR, the FastStart Universal SYBR Green Master Rox (Merck) can be used. TEER measurement can also be performed with an EVOM2 voltammeter from World Precision Instruments (WPI) together with an STX2 TEER electrode (WPI).

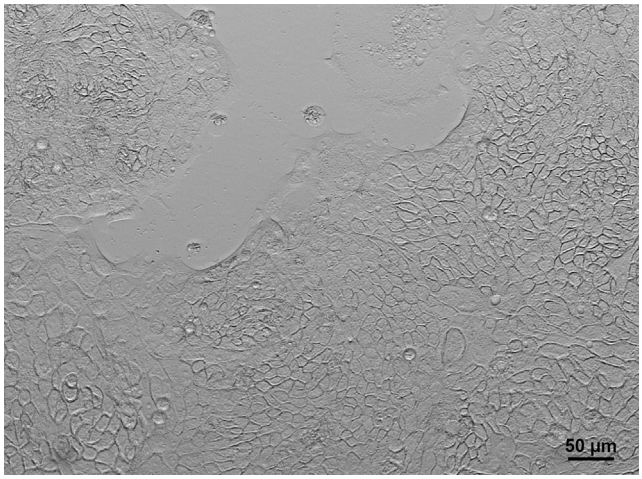

Figure 1. Light microscope image of a HIBCPP cell layer: Once the cells reach 70%–80% confluency in a T-75 flask, they can be harvested by trypsinization and used for the preparation of inverse culture HIBCPP filter inserts (preparation step 5)

Image has been obtained with a 20× objective lens. Scale bar represents 50 µm.

Table 1. Example data of dextran blue measurement

TEER	A (595 nm)	A (595 nm) blank	Delta A	Permeability[a]
118 Ω (one day after seeding)	0.405	0.049	0.356	:= 100%
319 Ω (HIBCPP Medium 0% FBS)	0.071	0.049	0.022	6%
649 Ω	0.056	0.049	0.007	2%
697 Ω	0.054	0.049	0.005	1%
769 Ω	0.054	0.049	0.005	1%
888 Ω	0.052	0.049	0.003	1%

200 µL of the medium in the lower compartment were pipetted in each well of a 96 well plate. HIBCPP medium (0% FBS) was incubated at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 2 h and used as reference (blank). Absorbance was measured at 595 nm in a Dynex MRX plate reader.

[a]Permeability describes the amount of dextran blue diffusing across the HIBCPP filter as measured by the absorbance at 595 nm. Permeability is given in percentage values with 100% being defined as the amount of dextran blue diffusing across HIBCPP filters one day after seeding the cells.

MATERIALS AND EQUIPMENT

Iscove's modified Dulbecco's Medium (IMDM)		
Reagent	Final concentration	Amount
Iscove's modified Dulbecco's Medium (IMDM)	N/A	powder for 1 L
ddH_2O	N/A	900 mL
Sodium bicarbonate	3.02 g / L	3.02 g
Titrate pH (7.1–7.3) with 1 N HCl / NaOH		
ddH_2O	**N/A**	**Ad 1000 mL**
Use immediately for HMI-9 preparation.		

HMI-9 medium		
Reagent	Final concentration	Amount
Iscove's modified Dulbecco's Medium (IMDM)	N/A	800 mL
Hypoxanthine (13.6 mg / mL)*	136 mg / L	10 mL
Pyruvate (110 mg / mL)	110 mg / L	1 mL
Thymidine (39 mg / mL)	39 mg / L	1 mL
Bathocuproin disulfonic acid (28.2 mg / mL)	28.2 mg / L	1 mL
L-Cysteine (18.2 mg / mL)	182 mg / L	10 mL
2-Mercaptoethanol	N/A	14 µL
Pen/Strep (100 ×)	1 ×	10 mL
Control pH (7.4)		
Sterilize by filtration (0.22 micron)		
FBS (inactivated 30 min @ 56°C)	10% (v/v)	100 mL
Serum Plus II ™	10% (v/v)	100 mL
Total	**N/A**	**1033 mL**
Store HMI-9 medium at 4°C up to 3 months in the dark.		
* Hypoxanthine stock: dissolve 0.4 g of NaOH in 100 mL of water and add 1.36 g hypoxanthine.		

Alternatives: For cultivation of trypanosomes, HMI-9 can be replaced with Duszenko's Medium (Hamm et al., 1990) with modifications (Hesse et al., 1995).

HIBCPP medium (10% FBS / 0% FBS)		
Reagent	Final concentration	Amount
DMEM/F12	N/A	450 mL*
Insulin (10 mg / mL)	5 µg /mL	250 µL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Pen/Strep (100 ×)	1 ×	5 mL
Sterilize by filtration (0.22 micron)		
FBS (inactivated 30 min @ 56°C)	10% (v/v)	50 mL **
Total	N/A	505 mL

Store HIBCPP medium at 4°C up to 3 months in the dark.
* for HIBCPP medium (0% FBS) add 500 mL DMEM / F12.
** for HIBCPP medium (0% FBS) do NOT add FBS.

STEP-BY-STEP METHOD DETAILS

Conduction of transmigration experiment

Timing: 4 h

This section describes the actual transmigration experiment. Trypanosomes are incubated on HIBCPP filter inserts. After a defined time period, filter inserts are removed, and the genomic DNA of transmigrated parasites is isolated for quantification.

1. Setting up the transmigration experiment.
   a. Determine cell density of trypanosome culture with a counting chamber (Neubauer improved 0.01 mm).
   b. Harvest trypanosomes by centrifugation (10 min at 1,500 × *g* and 4°C) and resuspend cells in pre-warmed HMI-9 medium to a final concentration of 1e7 cells / mL.

   Note: Keep 2.5e6 trypanosomes to isolate genomic DNA (gDNA) as qPCR standard (step 3).

   c. Identify filters that have reached a TEER of > 600 Ω (200 Ω•cm^2), empty the inserts and place them into 1 mL pre-warmed HMI-9 medium in a new 24 well plate.

   ⚠ CRITICAL: High TEER values (> 1200 Ω) indicate that HIBCPP cells have formed a multilayer, which does not represent the correct anatomical structure of the blood-cerebrospinal fluid barrier (where the choroid plexus epithelium is single-layered).

   d. Add 500 µL trypanosome suspension (i.e., 5e6 trypanosomes / filter) into the HIBCPP filter inserts. Set up all experiments in biological duplicates.

   ⚠ CRITICAL: Do not add more than 500 µL medium to the upper reservoir in order to prevent spilling. If desired, apply only 450 µL to ensure that no medium passes along the outside of the filter into the lower chamber due to capillary action.

   e. If desired, add chemical substances whose influence on transmigration is to be tested to the upper reservoir.
   f. For single time point measurements: Incubate filter inserts at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2 for 2 h.
   g. For kinetic experiments: Prepare additional wells containing 1 mL HMI-9 in which the filters can be transferred after each time interval. Incubate filter inserts at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2.
2. Collection of samples.
   a. For single time point measurements: Carefully remove and discard the filter inserts.
   b. For kinetic experiments: After each time interval, transfer the HIBCPP filter insert into the next well of the 24 well plate (as previously prepared; see 1g).

⚠ CRITICAL: Handle the plates with HIBCPP filter inserts with care to prevent spilling.

c. Resuspend transmigrated trypanosomes and transfer 500 μL of each well into a 2 mL microcentrifuge tube.
d. For kinetic experiments: Repeat steps 2b–d for each time interval.

⚠ CRITICAL: Try to keep plates always at 37°C in a water-saturated atmosphere and 5% (v/v) CO_2.

e. Perform gDNA isolation immediately or flash-freeze samples at −80°C.

3. Isolation of gDNA.
   a. Isolate gDNA both from transmigration samples (see step 2c) and from standard concentrations. To prepare standard concentrations, you will need around 2.5e6 trypanosomes:
      i Dilute trypanosomes from steps 1b to 1e6, 1e5 and 1e4 cells / mL (2 mL each).
      ii Use 500 μL of the trypanosome standards for gDNA isolation.
      iii Produce standards at least as duplicates.
   b. In general, gDNA is isolated as described in the QIAamp manual (version 05/2016, page 26, DNA Purification from Blood or Body Fluids – spin protocol). As we start with a higher sample volume than stated in the manual, the protocol has to be slightly modified. Changes from the manufacturer's protocol are highlighted in bold:
      i Pipet **50 μL** proteinase K into the bottom of a 2 mL microcentrifuge tube.
      ii Add **500 μL** sample to the microcentrifuge tube.
      iii Add **500 μL** Buffer AL to the sample and mix by pulse-vortexing for 15 s.
      iv Incubate at 56°C for 10 min.
      v Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the inside of the lid.
      vi Add **500 μL** ethanol (96%–100%) to the sample and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge to remove drops from the inside of the lid.
      vii Carefully apply **half of the lysate** to the spin column (in a 2 mL collection tube) without wetting the rim. Close the cap, and centrifuge at 6,000 × *g* for 1 min. **Discard the filtrate and repeat the step with the remaining lysate.** Place the spin column in a clean 2 mL collection tube.
      viii Carefully open the spin column and add 500 μL Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6,000 × *g* for 1 min. Place the spin column in a clean 2 mL collection tube and discard the collection tube containing the filtrate.
      ix Carefully open the spin column and add 500 μL Buffer AW2 without wetting the rim. Close the cap and centrifuge at 20,000 × *g* for 3 min.
      x Place the spin column in a new 2 mL collection tube and discard the old collection tube with the filtrate. Centrifuge at 20,000 × *g* for 1 min.
      xi Place the spin column in a clean 1.5 mL microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the spin column and add 200 μL Buffer AE or distilled water. Incubate at room temperature (15°C–25°C) for 1 min, and then centrifuge at 6,000 × *g* for 1 min.

Quantification of transmigrated trypanosomes

Timing: 1–2 days

The number of transmigrated trypanosomes is analyzed by real-time PCR in relation to gDNA standards.

4. Setting up qPCR.
   a. Calculate the number of samples to be analyzed.

Note: Perform 2–3 technical replicates for each sample, standards, and negative probes.

b. Prepare qPCR master mix for all samples, standards, and negative probes. The master mix will contain all PCR ingredients except DNA templates.

PCR reaction master mix	
Reagent	Volume [µL] per sample
Primer TbAOX fwd (10 µM)	1
Primer TbAOX rev (10 µM)	1
Fast SYBR green MasterMix	10
ddH_2O	6

c. Dispense 18 µL of the master mix in each well of a 96 well qPCR plate.
d. Add 2 µL template DNA per well.

⚠ CRITICAL: Avoid cross-contamination by aerosol formation during tip shedding. We recommend working in a sterile bench and changing pipette tips outside the hood.

e. Seal the plate and remove air bubbles by centrifugation.
f. Set up qPCR cycler (also see Figure 2).

Note: We used the QuantStudio™ 3 Real-Time PCR System together with QuantStudio™ Design & Analysis software v1.4.3. The experiment type was set to "standard curve".

PCR cycling conditions				
Steps	Temperature	Time [min:sec]	Gradient	Cycles
Uracil-DNA de-glycosylation	50°C	2:00	1.6°C /s	1
Enzyme activation	95°C	10:00	1.6°C /s	1
Denaturation	95°C	00:15	1.6°C /s	40 cycles
Annealing/extension	60°C	01:00	1.6°C /s	
Final denaturation	95°C	00:15	1.6°C /s	1
Final annealing/extension	60°C	1:00	1.6°C /s	1
Melt curve	60°C–95°C	–	0.1°C/s	1

Alternatives: For quantification of transmigrated trypanosomes, cells collected from the lower well (see step 2) can be directly counted with a Neubauer improved (0.01 mm) counting chamber if the cell density is higher than 5e4 cells / mL. For lower concentrations, cells can be centrifuged and resuspended in a lower volume before counting. Quantification can also be performed by flow cytometry, either with fluorescent parasites, or parasites stained with a nuclear or membrane dye. This method is sensitive enough to detect 50 parasites in 100 µL using an Accuri™ flow cytometer (BD Biosciences).

EXPECTED OUTCOMES

Dextran blue measurement

Table 1 provides reference values of the molecular flux. For this experiment, translucent HIBCPP filter inserts with 3 µm pore size were used. Note that these filters have a background of about 90 Ω. The given TEER values are direct measurement values, including background impedance. We considered HIBCPP filter inserts ready for experimentation when TEER was higher than 600 Ω. It should be noted that TEER values and absorbance values (595 nm) can vary slightly from experiment to experiment.

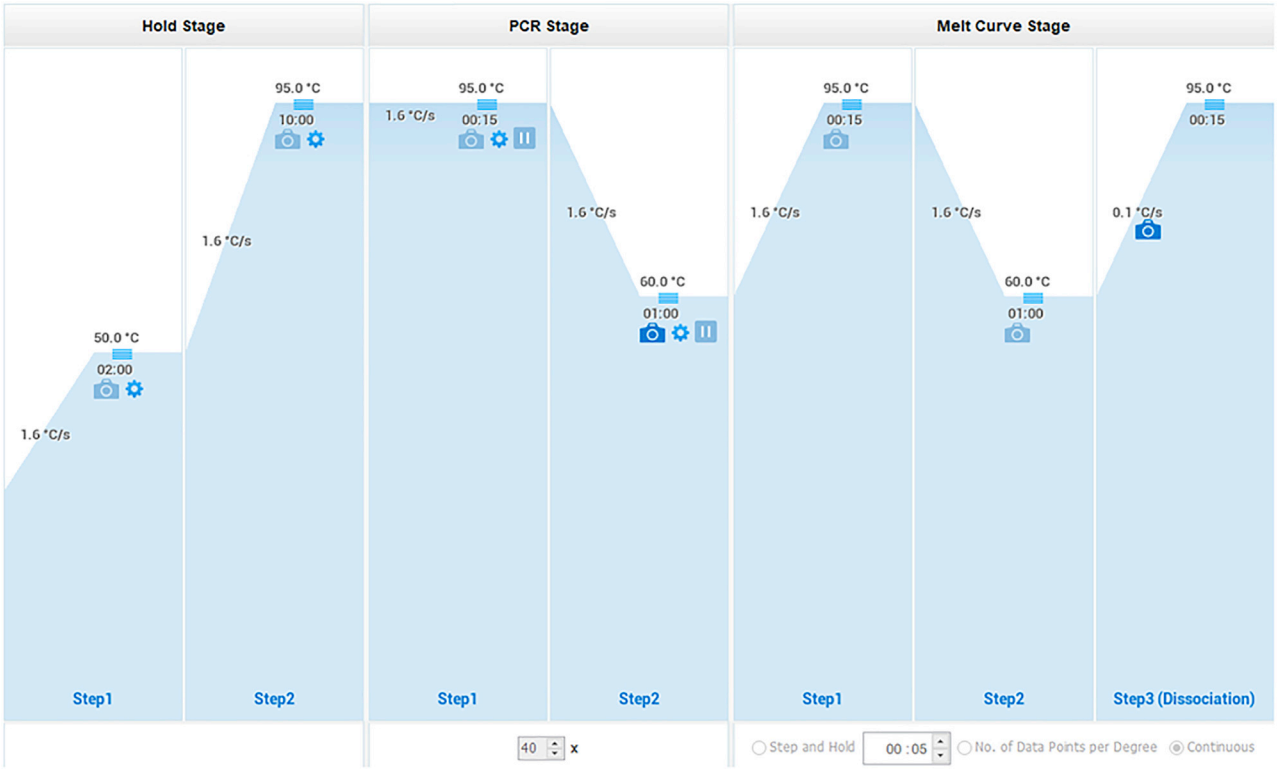

Figure 2. Screenshot from QuantStudio™ Design & Analysis software v1.4.3: Example how the cycling conditions could be programmed
Data collection was activated in "PCR Stage", step 2 and "Melt Curve Stage", step 3.

Transmigration experiment

Data can be analyzed by QuantStudio™ Design & Analysis software v1.4.3. Take care to correctly define standards and samples. The software will calculate an appropriate threshold (i.e., the signal that reflects a statistically significant increase over the baseline signal). The threshold meets the following criteria: a) being above the background, b) being below the plateau and linear phases of the amplification curve, and c) being within the exponential phase of the amplification curve. Threshold cycle values (Ct) (i.e., the PCR cycle number at which the fluorescence level meets the threshold) are determined (see Figure 3). The number of trypanosomes in the samples will be calculated by interpolation of the standard curve.

Table 2 shows example data of Ct and melting temperature (Tm) values of the standard probes. A hypothetical sample is listed with a mean Ct of 24.076, which is automatically calculated as 2.7e5 trypanosomes per mL. For manual calculation, Ct values of standard concentrations can be plotted against the mean quantity (as shown in Figure 4). An exponential regression curve can be calculated, and sample quantity can then be derived from the respective equation.

LIMITATIONS

This method has limitations: 1) As in all *in vitro* models, the complex processes within a living system cannot be completely simulated. Since they were derived from a choroid plexus epithelial papilloma, HIBCPP cells recapitulate many, but not all properties of primary choroid plexus epithelial cells (Schwerk et al., 2012; Bernd et al., 2015). Contrary to primary choroid plexus epithelial cells, HIBCPP tend to form multilayers because of lost contact inhibition. 2) It is important to note that even if HIBCPP cells recapitulated fully primary choroid plexus epithelial cells, other cell types are involved in the blood-cerebrospinal fluid barrier. Their presence may also affect transmigration behavior and

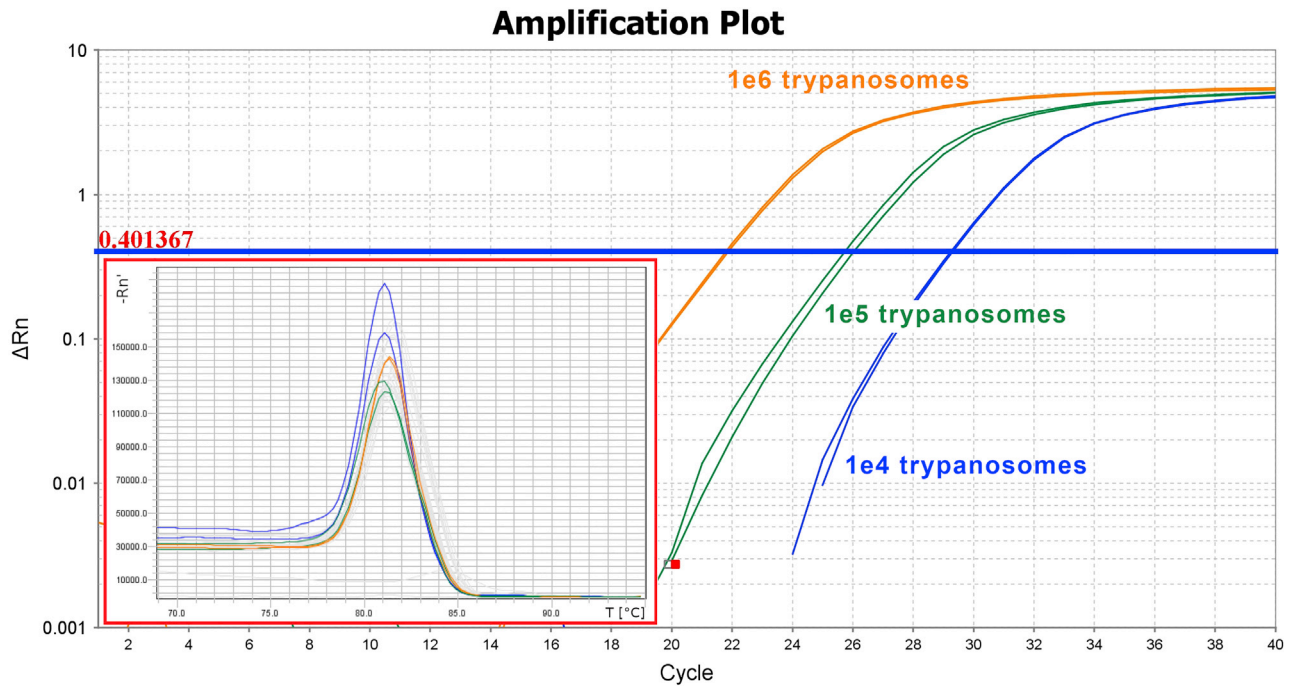

Figure 3. Partial screenshot from QuantStudio™ Design & Analysis software v1.4.3: Possible outcome of a real-time PCR measurement

The amplification plot shows 3 standard curve duplets (1e6 trypanosomes / mL in orange, 1e5 / mL in green, 1e4 / mL in blue) with a threshold of 0.401367. Cycle number is plotted against ΔRn (i.e., the magnitude of the normalized fluorescence signal Rn generated by the reporter at each cycle during the PCR amplification. Rn is the fluorescence signal from the reporter dye normalized to the fluorescence signal from the passive reference). Using the Fast SYBR green MasterMix, the reporter signal was generated by SYBR green, and the passive reference signal was generated by ROX dye. The inlay shows a melt curve plot of the respective samples. Temperature (°C) is plotted against the negative first derivative of Rn.

the protein expression of choroid plexus epithelial cells. 3) As HIBCPP cells and trypanosomes will proliferate during the transmigration experiment, the timespan for a longitudinal experiment is limited. We suggest an incubation time of 2 h (which can be extended up to 16 h).

TROUBLESHOOTING

Problem 1

Trypanosomes do not grow after passaging. They look short and stumpy under the microscope and die within a few days. This problem is related to preparation step 2.

Potential solution

If the cell density at some point was higher than 1e6 / mL (e.g., having forgotten to dilute the cells), the parasite differentiates from the proliferating "long slender" form into a cell-cycle arrested "short stumpy" form (Hamm et al., 1990). In this case, start a new culture from frozen cells.

Problem 2

HIBCPP cells do not grow (or grow slowly) after thawing or passaging. This problem is related to preparation step 4.

Potential solution

Do not use HIBCPP cells after passage 38. Cultivate cells in HIBCPP medium with 10% FBS. Change medium every second day. After thawing, it may take 2–4 weeks to reach 70%–80% confluency. For preparation of DMSO stocks, harvest cells at 70%–80% confluency, resuspend 2e6 cells in 500 µL PBS pH 7.7 / 20 mM glucose / 10% DMSO, and freeze the cells at −80°C with a cooling rate of −1°C per minute.

Table 2. Example data calculated with the QuantStudio™ Design & Analysis software v1.4.3

Sample name	CT	CT mean	Quantity	Quantity mean	Tm1
standard 1e6	21.785	21.828		defined as 1e6	81.331
standard 1e6	21.871				81.331
standard 1e5	25.732	25.873		defined as 1e5	81.194
standard 1e5	26.013				80.920
standard 1e4	29.228	29.253		defined as 1e4	81.057
standard 1e4	29.278				81.057
sample	23.921	24.076	292583	266919	81.194
sample	24.232		241256		81.057

Data can be exported into an excel file and used for further analysis.

Problem 3

TEER measurement values are not reliable. This problem is related to preparation step 5b.

Potential solution

Make sure that the electrode is used in the same position and angle within each measurement. Check the TEER measurement device using a 1 kΩ test resistor (MERSSTX04, Merck Millicell).

Problem 4

TEER of HIBCPP filter inserts is too low/high. This problem is related to preparation steps 5a and 5b.

Potential solution

If HIBCPP filter inserts reach a TEER of 300 Ω (100 $\Omega \cdot cm^2$), put them into HIBCPP medium (0% FBS). Keeping the cells in HIBCPP medium (10% FBS) may prolong the time to reach a certain TEER. Usually, younger passages of HIBCPP cells need less cells to be seeded onto a filter insert. The cell number can vary from time to time and needs to be adjusted due to the TEER values measured in the last filter batch. If HIBCPP cells are between passage 21 and 25, 7e4 cells per filter should suffice. Ideally, filters that have been prepared on Thursday reach a TEER of 300 Ω (100 $\Omega \cdot cm^2$) by Monday. If TEER values are higher (800 Ω), 5e4 cells should be seeded on filter inserts; if TEER values are lower (150 Ω) and raise rather slowly during the next days, 1.5e5 cells should be seeded.

Problem 5

Dextran blue stock solution precipitates during storage. This problem is related to preparation step 5c.

Potential solution

Freshly prepare a dextran blue stock solution for each set of experiments or store the solution at 4°C in the dark for up to 1 month. If a precipitate has formed, you can purify the solution by centrifugation before beginning a new set of experiments. Do not change the dextran blue stock or centrifuge within an experimental setup.

Problem 6

Trypanosomal DNA can be detected by qPCR in negative controls (e.g., HIBCPP filter inserts that had not been in contact with trypanosomes). This problem is related to step-by-step method details, step 4.

Potential solution

The primer pair TbAOX fwd and TbAOX rev targets the trypanosomal alternative oxidase (Tb427.10.7090), which is present in *T. brucei*, but absent in HIBCPP cells. Thus, no background due to detached HIBCPP cells is expected. In most cases, a trypanosome DNA contamination will occur by aerosol formation during pipetting or tip shedding. We recommend setting up the qPCR experiment in a sterile bench and changing pipette tips outside the hood.

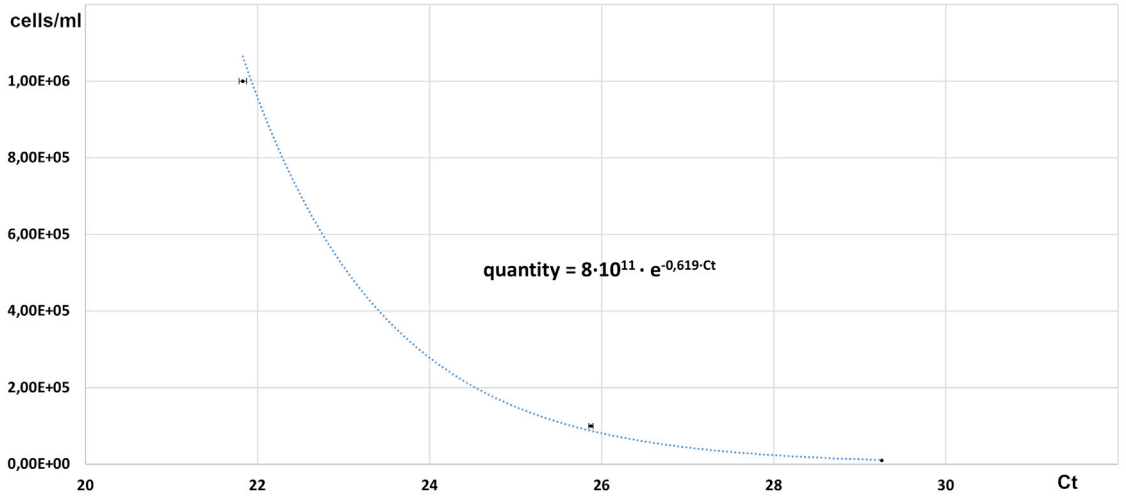

Figure 4. Manual calculation of sample quantity from qPCR derived Ct values: Mean Ct values of standard probes have been plotted against standard concentrations (1e6, 1e5 and 1e4 cells/mL)

An exponential regression curve has been calculated and the regression equation is given inside the figure. Subsequently, trypanosome concentration of probes can be calculated from the qPCR derived mean Ct values of the respective samples.

Problem 7

No transmigration can be detected by qPCR. This problem is related to step-by-step method details, steps 1–4.

Potential solution

You can check whether transmigrated trypanosomes can be found in the lower well by light microscopy. Be sure to use filter inserts with a pore size of 3 μm or 8 μm. Filters with a pore size of 0.4 μm are commercially available but hinder the passage of trypanosomes. Use the gDNA standards (that have been prepared in step-by-step method details, step 3) as template to check if the qPCR is working.

Problem 8

The number of transmigrated trypanosomes is higher than expected (e.g., using a knockdown strain where no transmigration should occur). This problem is related to step-by-step method details, steps 1–4.

Potential solution

Do not add more than 500 μL medium to the upper reservoir of a filter insert in order to prevent spilling. Handle the plates with HIBCPP filter inserts with care (e.g., do not shake). If desired, apply only 450 μL to ensure that no medium passes along the outside of the filter into the lower chamber due to capillary action. As a negative control, you may fix trypanosomes with 0.25% formaldehyde for 10 min before washing the cells with DPBS and applying them onto a HIBCPP filter. Fixed trypanosomes do not transmigrate. For details see Speidel et al. (2022).

RESOURCE AVAILABILITY

Lead contact

Requests for further information should be directed to the lead contact (Stefan Mogk, stefan.mogk@uni-tuebingen.de).

Materials availability

General materials and reagents used in this study are commercially available with no restrictions. Trypanosome strains are available from the lead contact. HIBCPP cells are available from C.S. and H.S. with a completed Material Transfer Agreement.

Data and code availability

This study did not generate/analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101676.

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AUTHOR CONTRIBUTIONS

A.H. and S.M. wrote the protocol. C.S.S. performed experiments to provide example data. C.S., H.S., and H.I. contributed to materials and methodology. C.S. and H.S. revised the protocol.

DECLARATION OF INTERESTS

There are no conflicts to declare.

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