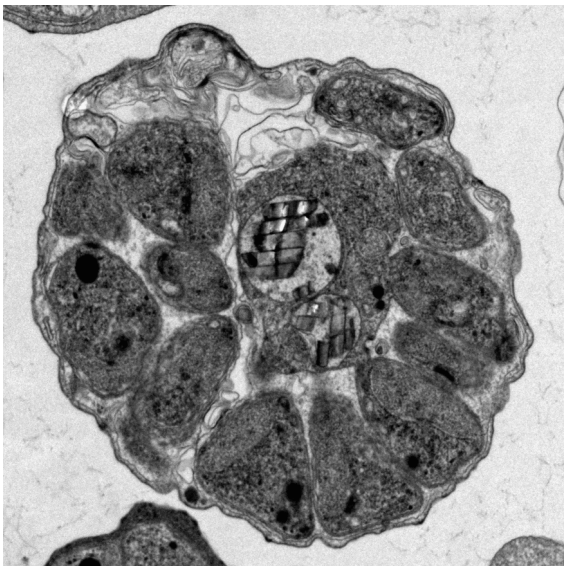
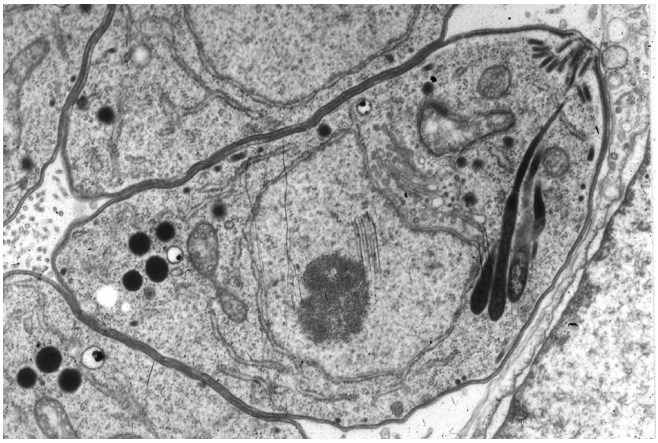
Middle East Biology of Parasitism 2016

Module II

Asparyl proteases in *Toxoplasma gondii* and *Plasmodium falciparum*





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Goals of the module

- Build up a working hypothesis based on minimal information
- Design experimental strategies to confirm or infirm the hypothesis
- Familiarize with the inducible systems to investigate the function of essential genes and the diverse assays to elucidate gene function

Exploit the experimental tools/strategies to investigate gene function

The objectives are to learn how to:

- Propagate and manipulate the parasites (Theory only)
- Identify by immunofluorescence assay the subcellular compartments of the parasite
- Familiarize with the assays available to perform a phenotypic analysis of parasite mutants

Experiment 1: *T. gondii* Tet-inducible conditional knock-out system. Immunofluorescence assays to quantify intracellular growth, egress, and invasion and to visualize the different organelles.

Experiment 2: Identification of potential substrates of the gene product studied and investigation by western blots in *T. gondii*.

Experiment 3: *P. falciparum* DiCre conditional knock-out system. Egress and invasion assays with FACS.

Experiment 4: Luciferase-based parasite growth inhibition assay for measurement of drug susceptibility and calculation of IC_{50}.

Lab Schedule: overview

* *Toxoplasma* work in black

* *Plasmodium* work in blue

Sunday July 31st
Introduction to the Module
Genome database and literature mining

Tuesday August 2d
Introduction to parasite culture and assays
Formulation of the working hypothesis
Design of the experimental approaches
Prepare solutions and reagents
Immunofluorescence assays (IFAs) organelles, invasion, egress, intracellular growth

Wednesday August 3rd
Observation of the IFAs and counting
Western blot: running, transfer and blocking
Discussion

Thursday July 4th
Western blot: incubation with antibodies and revelation
Quantification of the invasion and egress assays (FACs)
Blood smears and observation of development stages

Friday July 5th
Drug assay with luciferase (IC50)
FACs drug
Discussion and questions

MeBoP Module – Detailed Protocols

A. Culturing HFF (Human Foreskin Fibroblast) cells and *T. gondii*

B. Culturing Vero (African Monkey Kidney) cells and *T. gondii*

C. Transient Transfection of *Toxoplasma gondii* by electroporation

D. Plaque Assay

E. Immunofluorescence Assay (IFA)

F. Intracellular Growth Assay

G. Ionophore Induced Egress Assay

H. Invasion assay

I. SDS-PAGE gel running, Western blotting

J. Culturing *Plasmodium falciparum* (blood smears, staining, counting parasitemia, Sorbitol synchronization of ring stage parasites)

K. Percoll enrichment of schizonts

L. Egress assay / Time lapse video microscopy

M. Invasion assay

N. Schizonts/merozoites transfection Missing

O. Luciferase-based parasite growth inhibition assay for measurement of drug susceptibility & IC50 Missing

P. Parasite strains

A. Culturing HFF (Human Foreskin Fibroblast) cells

Reagents:

- 10% Media (500ml)
  450 ml DMEM (Dulbecco's Modified Eagle Medium) high glucose, no glutamine
  50 ml FCS (fetal calf serum)
  2 mM L- Glutamine
  25 µg/ml Gentamycin

- 5% Media (500ml)
  485 ml DMEM
  25 ml FCS
  2 mM L-Glutamine
  25 µg/ml Gentamycin

- 0.25% Trypsin/1mM EDTA

Splitting HFF cells:

1. Add 0.25% Trypsin/EDTA to a confluent monolayer of HFF (3 ml for T175)
2. Incubate 5 min, 37°C
3. To release the cells from the plate gently tap the bottom of the dish. Check under the microscope if all of the cells have detached before continuing
4. Resuspend cells in 10% media

Culture *Toxoplasma gondii*:

When the HFF cells are confluent, replace the 10% media with 5% media. The cells will remain happy for a couple of weeks, but will not continue to grow (contact inhibition). They can be used now for infection with *T. gondii* parasites. Note, the amount of parasites used for infecting a new monolayer of HFF allows to estimate at which point the HFF will be fully lysed by the parasites. For example if 300 µl of freshly egressed parasites are passed into a new 6 cm dish, it will take 2 days before the HFF of the new dish are fully lysed.

B. Culturing Vero (African monkey kidney) cells

Reagents:

- 5% Media
- 0.25% Trypsin/EDTA

Splitting Vero cells:

1. Add 0.25% Trypsin/EDTA to a 80-100% confluent monolayer of Vero cells (1 ml/T25 flask, 2 ml/T75 flask). Incubate for 5 min at 37°C
2. For cell detachment, gently tap the bottom of the dish and check under the microscope if all the cells are detached before continuing
3. Transfer the trypsinized cells to a 15 ml falcon tube and add an equal volume of 5% media. Spin 1500 rpm, 10 min
4. Resuspend cells in 5% media.
5. Place the cells into a new flask or dish

Culturing *Toxoplasma gondii*:

When the Vero cells are 50-80% confluent, they can be infected with parasites. Do not let the Vero cells reach complete confluency because they do not stop growing when a monolayer is formed like HFF but make multilayers (no contact inhibition).

Vero cells are only used to perform large-scale experiments and not for routine passage because they are highly susceptible to mycoplasma contamination. In addition, selection of newly transfected parasite is not optimal in these cells as the Vero will continue to grow whereas only a small fraction of parasite will survive the selection process.

C. Transfection of *Toxoplasma gondii* by electroporation

Cytomix

FOR 500ml	Final Concentration	Stock Solution	Volume/Amount Add
KCl	120 mM	1 M	60 ml
$CaCl_2$	0.15 mM	1 M	75 ml
K_2HPO_4/KH_2PO_4 pH 7.6	10 mM	100 mM *	50 ml
HEPES pH 7.6	25 mM	1 M	12.5 ml
EDTA	2 mM	Weigh in	0.372 g
$MgCl_2$	5 mM	1 M	2.5 ml

1. Mix 86.6 ml 1 M K_2HPO_4 with 13.4 ml 1 M KH_2PO_4, fill up with water to 1 l to make 100 mM stock

2. Adjust final pH to 7.6 with KOH (cytomix has to be Na free), add water to 500 ml

3. Filter the solution and keep it at 4^0C

Protocol:

A. Prepare the parasites to be transfected:

Infect a confluent 6 cm dish with 0.5 ml of freshly egressed tachyzoites 2 days prior the experiment and wash a few hours later. One completely lysed 6 cm dish produces enough parasites for 4 transfections.

B. The day of the transfection, prepare a plate with fresh medium to receive the transfected parasites.

C. Get the plasmid DNA to be transfected. For transient transfections: 20-30µg of circular plasmid DNA, for stable transfection of a second copy 60-80 µg of linearized (in the backbone) plasmid DNA, for single or double homologous integration 20-30 µg of linearized plasmid DNA.

D. Transfection protocol:

1. Harvest the freshly lysed parasites and centrifuge them in a 15 ml Falcon tube at 1000 g, 10 min in a swing bucket rotor.
2. Resuspend the parasite pellet in 2.5 ml of Cytomix supplemented with 30 µl ATP [2mM] and 20 µl GSH [5mM] per ml of Cytomix.
3. Transfer 700 µl of resuspended parasites into the electroporation cuvette and add the DNA.
4. Electroporation using the settings *T. gondii* in the BTX ElectroCell Manipulator 600.
5. Immediately after electroporation add the transfected parasites to the new dish
6. Add the corresponding drug the day after.

D. Plaque Assays

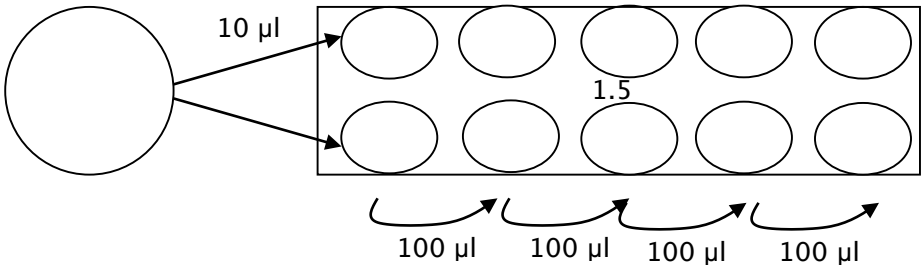
Reagents:

- Crystal violet
- 4% Paraformaldehyde (PFA)

This assay will reveal if the parasites are able or not to propagate in culture. This will give you a general idea of the fitness but you obviously will not know which step of the lytic cycle is affected. Here only the sizes of the plaques are informative not theirs numbers. Set up duplicate dishes or wells for each condition.

Protocol:

1. Prepare the IFA plate to receive the parasites: add to each well fresh medium and ± drug (if necessary).
2. Transfer 10 µl of parasites from a totally lysed dish into the first well of an IFA plate and pass 100 µl to the neighboring well. Continue the dilution process up to the end of the line.



3. Incubate for 6-7 days without disturbing the wells (don't bump them, move them or slam incubator doors - this is very important).
4. After incubation, aspirate the medium, rinse the infected monolayer with PBS, fix for 10 minutes with 4% paraformaldehyde, and stain with crystal violet for 15 min (1/5 dilution of Crystal violet in water, filter the solution with 0.22 µm filters).
5. Aspirate the crystal violet solution and rinse twice with water.
6. Carefully remove the coverslips and mount them (without Fluoromount G) on the slide with the cell layer facing down.

E. Immunofluorescence Assay (IFA)

Reagents:

- 4% paraformaldehyde (PFA) or PFA + 0.005 % Glutaraldehyde (PFA/GA) in PBS, depending on the Abs used
- 1X PBS/0.1M glycine
- 1X PBS/0.2% (w/v) Triton X-100
- 2%BSA/1X PBS/0.2% (w/v) Triton X-100
- Primary antibodies
- Secondary antibodies
- DAPI
- Fluoromount G

Protocol:

1. Remove the medium from the IFA plate and fix with PFA or PFA/GA for 10 min (250 µl of fixative per well).
2. Quench the reaction by adding 1X PBS/0.1M glycine, incubate for 5 min
3. Permeabilize with 1X PBS/0.2% Triton X-100, 20 min on the shaker
4. Block with 2%BSA/1X PBS/0.2% Triton X-100, 20 min on shaker
5. Incubate with primary antibodies in 2%BSA/1X PBS/0.2%Triton for 1 h (250 µl/well)
6. Wash 3 times with 1X PBS (5-10 min each wash)
7. Incubate with secondary antibodies (1:3000 dilution; 250µl/well), 45 min The plate should be kept in the dark from now on!
8. Wash 3 times with 1X PBS (5-10 min each wash)
9. Dilute DAPI stock in PBS. Stain cells 5-10 min.
10. Wash with 1X PBS
11. Carefully mount coverslips on the slide with cell layer facing down using Fluoromount G

Keep slides in the dark at RT in order to let them dry. Then check them using a fluorescence microscope. For long-term storage, keep them at 4°C, in the dark.

F. Intracellular growth/replication assay

T. gondii divides synchronously within a vacuole but asynchronously across the different vacuoles. Counting the number of parasites/vacuole is thus an easy and efficient way to determine the replication rate of the parasites.

Reagents:

- ATc
- PFA/GA
- α-GAP45
- Secondary anti-rabbit
- PBS Triton-X100
- BSA

1. Transfer 15 µl of parasites from a fully lysed dish to three IFA wells ± ATc
2. Incubate for 30-24 h, 37°C
3. Fix with PFA/GA.
4. Perform IFA using α-GAP45 Abs and secondary α-rabbit Abs and count the number of parasites/vacuole (only vacuoles with at least 2 parasites are counted). Count 100 vacuoles per coverslip.

G. Ionophore Induced Egress Assay

In *T. gondii*, egress is an active process relying on the parasites ability to sense that their host cell is dying. Treatment with the Ca^{2+} ionophore (A23187) leads to an increase in the intra-parasitic level of Ca^{2+}, which artificially induces parasite egress as early as two hours post-infection. During this process, the conoid protrudes, the micronemes discharge their content and the parasites become motile.

Reagents:

- Calcium ionophore A23187 (from *Streptomyces chartreusensis*)
- DMEM w/o serum
- DMSO
- ATc
- PFA/GA
- α-GAP45
- α-GRA3
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and Triton-X100
- BSA

Protocol:

1. Resuspend parasites from a 6 cm dish.
2. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium).
3. Inoculate IFA plate with 30-50 µl of the diluted parasites and incubate for 30 h (± ATc) at 37°C. Don't forget a control.
4. Aspirate the medium and wash with DMEM **w/o serum**
5. Incubate the cells with DMEM **w/o serum** containing either 3 µM A23187 or DMSO, for 10 min, 37°C
6. Fix the cells with PFA/GA and perform IFA with α-GAP45 and α-GRA3 Abs.

H. Invasion assay

In *T. gondii*, invasion like egress is an active process. This process follows sequential steps: i) Microneme discharge and attachment of the parasite to the host cell. ii) apical reorientation of the parasite iii) rhoptries secretion within the host cell iv) formation of the moving junction (AMA1-RONs) v) translocation of the junction propelling the parasite forward inside the host cell.

The principle of the assay consists to let the parasite invade for 30 min before fixation with PFA. Then, IFAs is performed without permeabilization using a parasite surface marker. The absence of permeabilization will block the antibodies outside the host cell, leaving the intracellular parasites unlabelled. Samples will then be permeabilized and stained with a second marker to label the total population. The invasion rate can be then be easily calculated.

Reagents:

- ATc
- PFA/GA
- 1% formaldehyde
- α-GAP45
- α-SAG1
- Secondary anti-rabbit
- Secondary anti-mouse
- PBS and PBS Triton-X100
- BSA

Protocol:

1. Resuspend parasites from a 6 cm dish.
2. Dilute parasites 1:10 in pre-warmed medium (100 µl parasites + 1 ml medium)
3. Inoculate IFA plate with 100 µl of the diluted parasites and incubate for 30 min (± drug) at 37°C.
4. Fix the cells with PFA/GA. **Do not was the cell** otherwise you can detach non-invaded parasites.
5. IFAs are then process as follow:

   Fixed cells were blocked 30 min with 2% BSA/PBS, incubated with α-SAG1 antibodies diluted in 2% BSA/PBS for 20 min and washed 3 times with PBS. Cells were then fixed with 1% formaldehyde/PBS for 7 min and washed once with PBS. Permeabilization using 0.2% Triton X-100/PBS was performed for 20 min. A second incubation using α-GAP45 antibodies diluted in 2% BSA/0.2% Triton X-100/PBS was performed. Cells were washed 3 times with 0.2% Triton X-100/PBS and incubation with secondary antibodies was performed as described previously.

I. SDS-PAGE gel running, western blotting

Preparation of parasite protein lysate:

A fully lysed well from a 6 well plate produces enough parasites ($2x10^7$) to run on a gel

1. Harvest the freshly lysed parasites and centrifugation at 1000 rpm, 10 min
2. Wash the parasite pellet in 1X PBS
3. Resuspend the parasite pellet in 120µl 1X PBS
4. Add 80 µl 2xSDS loading buffer and 20 µl of DTT (1 M), boil at 95°C for 10 min
5. The lysate can be used directly or stored at -20°C

SDS-PAGE gels:

1. **Use the precast gels**
2. **Run each gel at constant 35 mA per gel (approx. 45 minutes running)**

Western Blotting with ECL detection kit

Transfer the proteins from the gel to the membrane

Reagents:

- 1xPBS/0.05% Tween20
- Milk powder
- 5% milk powder in 1X PBS/0.05% Tween20
- Primary antibodies
- Secondary antibodies, HRP conjugated
- ECL plus kit

Protocol:

1. Blot the membrane with 5% milk/PBS/Tween solution 45 min, RT or o/n 4°C
2. Incubate the membrane with the appropriate dilution of the primary antibody in 5% milk/PBS/Tween for 1 h, RT
3. Wash the membrane 3 times in 1xPBS/0.05% Tween, 10 min, RT
4. Incubate the membrane with secondary antibodies (1:3000 dilution) in 5% milk/PBS/Tween for 1 h, RT
5. Wash the membrane 4 times in 1xPBS/0.05% Tween, 10 min, RT
6. Briefly dry the membrane, then incubate it with the ECL plus detection kit
7. Expose to capture the chemiluminescent signal

The membrane can be stripped by incubating for 1 h, RT with stripping solution (PBS/2%SDS and 7µl/ml of β-Mercaptoethanol). Wash extensively with PBS and PBS/0.05% Tween and re-block membrane with 5% milk before incubation with antibodies.

J. Culturing *Plasmodium falciparum*

1) Reagents:

- RPMI 1640 with L-glutamine and 25mM HEPES (liquid) – Gibco 52400-025
- RPMI 1640 with L-glutamine and 25mM HEPES (powder) – Gibco 13018-015
- Gentamycin (50mg/mL solution).
- AlbuMAX II from Invitrogen (20% solution in RPMI)
- D-Glucose
- Sodium Bicarbonate
- Sorbitol (5% solution in water)
- Percoll – GE HealthCare 17-0891-01
- Human blood
- Giemsa stain – Sigma GS500-500ML
- Hypoxanthine 100mg/mL in 1N NaOH
- Methanol

2) Making up complete medium (for 1 L):

5. RPMI 1640 with L-glutamine and 25mM HEPES (powder) – Gibco 13018-015
6. 2g D-Glucose
7. 2g sodium bicarbonate
8. 25mL of 20% Albumax II solution
9. 1mL of 50mg/mL gentamycin
10. Hypoxanthine 100mg/mL in 1N NaOH
11. Filter sterilize, store at 4°C, up to 2 weeks

3) Washing blood:

Blood should be drawn into packs/tubes containing anticoagulant citrate/EDTA. Parasites generally do not appreciate heparin-based anticoagulants.

White cells/buffy coat can be removed in 2 ways:

1. Spin down blood at 2000 rpm for 5 mins, 4°C, no break.
2. Aspirate off serum and as much of the white buffy coat layer on the surface of the red cells as possible.
3. Wash 3x in Incomplete RPMI medium (Gibco 52400-025) aspirating, re-suspending and mixing between each wash. Use 100% or more of the volume of cells for washing each time.
4. After the third wash, re-suspend the red cells at 50% hematocrit in RPMI liquid medium. (Gibco 52400-025).
5. This can be stored in the fridge and used up to 2 weeks from the date of drawing.

4) Asexual parasites culturing (candle jar technique)

5-8 mL culture in 6 cm dishes

20 mL culture in 10 cm dishes

Change culture medium every 2 days, 5% hematocrit. Check parasitaemia using Giemsa-stained thin blood smear. For routine culture, keep parasitaemia between 5-10% which correspond to a dilution of around 1/20 every two days (prepare 18 ml of fresh medium add 1 ml of blood and pass 1 ml of the previous culture)

Parasite must be kept at 37 °C in a sealed jar. Light a candle within the jar before closing. The burning candle will produce an atmosphere inside the jar mimicking the blood environement. Within a healthy culture, parasitaemia will increase 8 time every 48h. If you see gametocytes, you've done something wrong, dilute the culture at least 10 times and wait!

5) Giemsa staining of thin blood films and counting parasitemia

Prepare a new solution for each staining session or at least every three hours.

Phosphate buffer, 6.7 mM (pH 7.1):0.41 g KH_2PO_4 0.65 g Na_2HPO_4 H_2O Bring to 1 liter with water.

1. Prepare a thin blood film for each culture
2. Air-dry the thin film.
3. Fix the film in methanol for 3 sec.
4. Prepare a fresh 5 -10% Giemsa solution in phosphate buffer
5. Stain the slides for 20 min.
6. Rinse carefully and thoroughly under running tap water.
7. Dry.
8. Observe the film with immersion oil and objective at 100.
9. Choose an area of a Giemsa-stained thin blood film where the erythrocytes are in a monolayer and evenly distributed.
10. Count *all* erythrocytes in the small square plus two of the borders.
11. Without moving the slide, also count the number of *infected erythrocytes* in the whole area of the big square.
12. Repeat the counting at least twice for a total examination of three different parts of the slide. Take the mean number of infected erythrocytes per 1000 erythrocytes and divide by 10 to get the percent infected erythrocytes.

6) Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes

Take parasites when they are mostly at the ring stage. They must not be later than 10 to 12 h post-invasion when the sorbitol treatment is done.

1. Spin down the parasite culture at 600 × *g* to a pellet.
2. Add 1 volume (same volume as culture) of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Shake 2 or 3 times.
3. Centrifuge the culture at 600 × *g,* and wash it 3 times in malaria culture medium, and dilute it to 5% hematocrit.
4. Count parasitemia and subculture as usual.
5. Repeat the procedure after one cycle (approximately 48 h).

To keep the parasites synchronized, the sorbitol treatment must be performed once a week.

7) Freezing and thawing of asexual *Plasmodium* spp.

Freezing medium: 28% glycerol, 3% sorbitol, 0.65% NaCl in distilled water.

To make 250 mL: Mix 180 mL of 4.2% sorbitol in 0.9% NaCl with 70 mL of glycerol. Filter-sterilize; store frozen.

Freezing

1. Transfer a 4-mL culture into a Falcon tube.
2. Pellet erythrocytes by centrifugation 600g, 8 min, and discard the supernatant; the pellet is approximately 0.2 mL.
3. Add 0.3 mL (1.5 vol) of human serum.
4. Add 0.5 mL (2.5 vol) of the freezing medium, drop-by-drop, while shaking the vial gently; the addition should take approximately 1 min.
5. Transfer the medium into a sterile cryovial.
6. Drop the vial gently into the liquid nitrogen tank.

Thawing

Materials and reagents sterile 1.6% NaCl, sterile 12% NaCl, malaria culture medium, fresh blood.

1. Remove a vial from cold storage and thaw it up quickly at 37 °C for 1 to 2 min.
2. Transfer blood to 50-mL centrifuge tubes with a sterile pipette. Measure blood volume, V.
3. Add 0.1 x V of 12% NaCl slowly, dropwise, while shaking the tube gently.
4. Let the tube stand for 5 min.
5. Add 10 x V of 1.6% NaCl slowly, dropwise, swirling the tube.
6. Fill the tube with culture medium
7. Centrifuge the tube at 600*g* at 20 °C for 5 min.
8. Aspirate the supernatant and add blood and medium, transfer to a culture flask.

K. Percoll enrichment of *Plasmodium falciparum* schizonts

Use tightly synchronized cultures (within a 6 hour spread of developmental age) 10 to 20% parasitemia in early schizont.

1. Spin down the culture 8 min 600g (for 20mL culture)
2. Resuspend the pellet in 5 mL RPMI
3. Prepare 60% Percoll solution (25 ml):
    a. 15ml percoll sterile
    b. 1.75ml 10X PBS sterile
    c. 8.25ml RPMI
4. Carefully load the resuspend culture (5 mL) on 5 mL diluted Percoll
5. Centrifugation 8 min 1400g, no break
6. Recover the supernatant and the interface
7. Centrifugation 8 min 600g
8. Discard supernatant and resuspend schizonts in complete culture medium

These “purified” schizonts can be used for egress assay, invasion assays, protein preparations….
Depending on the assay, egress can be blocked with C1, C2, E64 or combinations of compounds.

L. Egress assay / Time-lapse video microscopy

Homemade viewing chambers (internal volume 80 µl) for observation of live schizonts are constructed by adhering 22×64 mm borosilicate glass coverslips to microscope slides with strips of double-sided tape, leaving 4 mm gaps at each end.

1. Prepare a schizont suspension as above (blocked with C1 or C2)
2. Pre-warm the viewing chambers to 37°C
3. Pellet the fully mature schizonts in Falcon tubes and remove as much medium as possible (important!).
4. Resuspend each schizont sample in 1 ml warm, pre-gassed protein-free RPMI (with glutamine added) and transfer them to 1.4 ml Eppendorf tubes.
5. Quickly pellet the cells at 5,000 rpm for 1 minute in a microfuge, and remove and discard the entire medium.
6. Immediately resuspend them once more in 1 ml warm protein-free RPMI and pellet them again in exactly the same way (these two washes are to remove the C1 and the Albumax).
7. Remove and discard the medium once again, and resuspend them once again in 1 ml warm, gassed protein-free RPMI.
8. Load the schizonts into the viewing chambers
9. Transfer the slides to a temperature-controlled microscope stage held at 37°C.
10. Take differential interference contrast (DIC) images at 5 s intervals over a total of 30 min.
11. Fluorescent signal can also be analyzed depending on the parasite strain used.

M. Invasion assay

100 ml of tightly synchronized culture, 8-10% parasitemia, 2-3% hematocrit will typically result in approximately 2 X 10^8 isolated merozoites; this amount is sufficient for approximately 20-40 aliquots of 50 µl volume samples for invasion inhibition assays.

1. Prepare a schizont suspension as above (blocked with C1 or C2)
2. When schizonts are ready to egress, add E64 to 10 µM final concentration while washing out the C2. (Note, if E64 is added to parasites <~40 hrs post invasion, this kills the parasites).
3. Pellet at 1900 g for 5 min.
4. Resuspend pellet in a minimal volume of ~1000 µL warm incomplete culture medium (higher if sample appears very dark black to prevent clogging during filtration). Carry over of a small amount of complete culture media is fine.
5. Push the resuspended parasites through a 1.2 µm filter. Some loss in the filter is inevitable. The resulting filtered merozoites in solution will survive best at RT and have a half-life of around 5 min.
6. Add 50 µL of this mix to 96 wells plates containing 50 µL of 2% hematocrit washed blood
7. Put back to culture for 2-6 hours.
8. Fix by addition of 100 µL PAF (4%)/Glu (0.005 %)
9. Stain with a DNA dye (DAPI, Hoechst…)
10. Quantify invasion by FACs

Alternative for IFAs or quantifications on blood smears.

4. Resuspend pellet in a minimal volume of ~750 µL warm incomplete culture medium (higher if sample appears very dark black to prevent clogging during filtration). Carry over of a small amount of complete culture media is fine.
5. Push the resuspended parasites through a 1.2 µm filter. Some loss in the filter is inevitable. The resulting filtered merozoites in solution will survive best at RT and have a half-life of around 5 min
6. Add 500 µL of this mix to a 1.5 mL microtube containing 50 µL (or less for higher percentage of invasion) of 2% hematocrits washed blood and briefly mix.
7. Place immediately in shaker at ~1000 rpm at 37℃.
8. After desired period (1 min 45 sec for invading parasites, longer for post-invasion).
9. Blood smear.
10. Or remove from shaker, add 2x fixative solution (550 µL) to the tube(s), mix by inverting and place on a roller at room temperature (RT) for 30 min.
11. Perform IFA.

N. Schizonts/merozoites transfection

1) DNA preparation:

1. Precipitation DNA 40-50ug by transfection: 800ul ETOH + 40ul Acetate-Na 3M. I want to try with much less DNA, but I didn't do it yet!
2. -20°C 1h
3. Centrifugation 10min 14000rpm at 4°C
4. Wash DNA in ETOH 70%
5. Centrifugation 10min 14000rpm at 4°C
6. Discard supernatant
7. Let dry in the hood at least 1h
8. Re-suspend to have about 40-50ug in 10ul of water [4-5ug/ul]

2) Pf culture preparation:

Synchronized culture 10 to 20% parasitemia in early schizont (20mL for 2-3 transfections).

3) Transfection of Pf merozoites:

9. Prepare 60% Percoll solution (25ml):
    a. 15ml percoll sterile
    b. 1.75ml 10X PBS sterile
    c. 8.25ml PBS sterile

    2 options: Percoll on late troph/early schizonts or on mature schizonts; If on "early stages", you'll get a 40-100 µL pellet to put back into culture (20 mL) until ready. If on mature schizonts procede directly to transfection.
10. Resuspend parasite in 10ml medium and put them carefully on the percoll 60% solution
11. Centrifugation 8min 1800rpm, no break
12. Recover the supernatant and the interface
13. Centrifugation 8min 1800rpm
14. Discard supernatant and resuspend merozoites with Lonza/Amaxa buffer (primary human T cells buffer)
15. Mix merozoites/Lonza-buffer with 10ul of digested DNA to transfect (40-50 µg of linearised/circular DNA in water) in an eppendorf.
16. Transfer the parasite/DNA/buffer solution to a cuvette
17. Transfect using the Amaxa gene pulser using protocol **U33**
18. Immediately after electroporation, transfer the content of the cuvette to an eppendorf containing 300 µl of fresh erythrocytes (haematocrit 50%).
19. Leave the suspension for 15 min at 37°C on a shaker (strong, 1400rpm) to allow reinvasion of erythrocytes by the merozoites. check on smear.
20. Add 20 ml Pf medium
21. drug selection 24h after transfection and add 2-3 drops of fresh blood every day.

O. Luciferase-based parasite growth inhibition assay for measurement of drug susceptibility & IC_{50}

First, you need to define the kind of assay you want to run, time series or end point assay; here we use a end point assay at 4 days.

It is also critical to determine the number of parasites needed to have no more than 80% lysis of the host cells at the end of the assay. This number depends on the duration of the treatment! In our case, we determined that 400 parasites per well gave about 80% lysis after 4 days.

T. gondii tachyzoites expressing luciferase (RH-pTub-CBG99-luciferase) were added in HFF monolayers in 96-well plate. Drugs (49c, 49b, 49f) were diluted in supplemented DMEM and added to the monolayers at various concentrations in triplicates along with no drug treated control for each set. The assay was performed in a 150µl final volume. The plates were incubated for 4 days at 37°C.

Plates can be stored at -80°C.

The lysis is done directly in the plate by adding 150µl of 2X lysis buffer.

Transfer 20µl of the lysate to a special light blocking plate (white) and add 20µl of substrate buffer.

Reading: That depends on the reader, but be quick, the 560 nm chemiluminescence from this reaction peaks within seconds.

Lysis buffer

20 mM	Tris HCl pH 7.5
10%	Glycerol
1%	Triton X-100
2 mM	DTT
Qsp	Water

Filter in sterile conditions (0.22 µm). Store at -20°C

Luciferase solution

1 mM	Luciferin
3 mM	ATP
15 mM	$MgSO_4.7H_2O$
30 mM	Tris HCl pH 7.5
Qsp	Water

Filter in sterile conditions (0.22 µm). Store at -20°C hidden from light

P. Parasite strains

RHΔHX strain

The *T. gondii* hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) locus has been deleted from the RH strain and thus can now be used for both positive and negative selection. HXGPRT is not an essential enzyme in *Toxoplasma,* because AMP and IMP are interconvertible. In the absence of HXGPRT, the parasite depends on the interconversion of AMP into GMP and the rate-limiting enzyme in this pathway is IMPDH (inosine monophosphate dehydrogenase). IMPDH can be inhibited by mycophenolic acid (MPA) treatment combined with xanthine (XAN) supplementation. This treatment can thus be used to positively select parasites transfected with a plasmid containing the HXGPRT selection marker, because the parasites will have to rely on the HXGPRT pathway. Alternatively, parasites can be negatively selected with xanthine analogues such as 6-thioxanthine (TX), which converts HXGPRT into a toxic compound. Alternatively, parasites can be selected with chloramphenicol, pyrimethamine or bleomycin.

- **RHΔKu80 strain**

The most successful method to generate genetic knockouts and incorporate reporter proteins or tags to endogenous loci is via the use of a parasite strain (RHΔHX) in which the *Ku80* gene was disrupted. Ku80 is normally implicated in the non-homologous end-joining repair (NHEJ) machinery of DNA double-strand break. In the *T. gondii ΔKu80* strain, the high rate of random integration is abolished, thus leading almost exclusively to homologue recombination.

- **TgASP3-iKD (TATi)**

Parasite strain in which *TgASP3* is expressed under the tetracycline-inducible promoter and its expression can therefore be turned off upon addition of anhydrotetracycline (ATc).

- **Pf3D7-PV1-GFP**

Parasite strain in which *PV1-GFP* is expressed on an episomal plasmid. PV1 is a soluble protein residing in the PV.

- **Pf3D7-DiCre-PM9Ty-LoxP**

Parasite strain expressing DiCre, where the *PM9* gene contain a LoxP site in an intron and a second LoxP after its 3'utr.