Materials and Methods
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3.1 P. falciparum culture

Blood stages of *P. falciparum* 3D7 were cultured in O+ve human red blood cells (RBCs). Whole blood was supplied by blood banks for parasite culture. Blood was washed with RPMI-1640 (Invitrogen, USA) medium supplemented with 0.2 % sodium carbonate (incomplete RPMI) and stored at 50 % hematocrit in incomplete RPMI at 4°C until use. Parasite cultures were grown in RPMI-1640 media supplemented with 0.5 % Albumax I (Invitrogen, USA), 0.2 % sodium bicarbonate (Sigma, USA), 27.2 mg/L hypoxanthine (Sigma, USA) and 10 µg/ml gentamicin (Gibco, USA) (complete RPMI). The cultures were maintained at 3-4 % hematocrit and were incubated at 37°C in a mixed gas (5 % CO₂, 5 % O₂ and 90 % N₂) environment as described previously (Trager et al., 1992). Different stages of parasites were observed under light microscope by making thin smears of cultures on glass slides and staining with Giemsa stain for 10-15 mins.

3.2 Synchronization of parasite cultures

Parasite cultures were synchronized using sorbitol as described earlier (Lambros and Vanderberg, 1979). Cultures containing majority of ring stage parasites were washed with incomplete RPMI by centrifuging at 1,200 rpm (Sorvall, RT7) and incubated with 20 pellet volumes of 5 % sorbitol solution (w/v) at room temperature with intermittent shaking for 10 mins. Sorbitol treatment lysed trophozoite and schizont infected erythrocytes leaving uninfected and ring infected erythrocytes unaffected. After sorbitol treatment, parasite cultures were washed two times with incomplete RPMI by centrifugation at 1,200 rpm (Sorvall, RT7), resuspended in complete RPMI and incubated at 37°C in mixed gas environment for further growth.

3.3 Enrichment of trophozoites and schizonts by Percoll treatment

Parasite cultures with majority of trophozoite and schizont stage parasites were washed with incomplete RPMI by centrifugation at 1,200 rpm (Sorvall, RT7). Parasite pellets were resuspended in incomplete RPMI at 10 % hematocrit and 5 ml of parasite culture suspension was layered on top of equal volume of 65 % Percoll (Sigma, USA) solution in incomplete RPMI in a 15 ml centrifuge tube (Falcon, USA).
This was centrifuged at 2,000 rpm (Sorvall, RT7) for 20 mins at room temperature. The interphase containing trophozoites and schizonts was collected. Ring and uninfected erythrocytes settle at the bottom of the tube. Cells collected from interphase were washed three times with incomplete RPMI. Thin smear of parasites were made on glass slide, stained with Giemsa stain, and observed under light microscope. The purity of trophozoite and schizont preparation was scored.

3.4 Isolation of *P. falciparum* merozoites

*P. falciparum* 3D7 or 3D7Δ175 cultures were synchronized by treatment with sorbitol as described above in at least two successive cycles to obtain tight synchronization. Progress of synchronized *P. falciparum* schizonts was periodically monitored by light microscopy of Giemsa stained smears prepared from culture samples. When majority of infected erythrocytes reached the mature schizont stage with segmented merozoites (as shown in Figure 3.1), cultures were resuspended in complete RPMI or buffer mimicking intracellular ionic conditions (IC buffer – 5 mM NaCl, 140 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 5.6 mM Glucose and 25 mM HEPES). Schizonts were allowed to rupture and release merozoites over a period of 1 hour. Cultures containing unruptured schizonts and released merozoites were centrifuged at 2,000 rpm (500g on Sorvall RT7 with RTH-250 rotor) for 5 mins to separate released merozoites from unruptured schizonts and uninfected erythrocytes. Supernatants containing free merozoites were centrifuged at 5,000 rpm (3,300 g on Eppendorf 5810R with F-34-6-38 rotor) for 5 mins to collect merozoites preparation. The merozoites were resuspended in incomplete RPMI or IC buffer for use in experiments. Figure 3.2 shows the purity of the merozoites. Merozoite preparations had less than 0.5% contaminating schizonts. Number of schizonts that ruptured was scored by Giemsa staining culture samples before and after collection period. Merozoite yields were scored by flow cytometry as described below.
Figure 3.1. Giemsa stained smear of mature schizont stage of *P. falciparum*

Figure 3.2. Giemsa stained smear of purified *P. falciparum* merozoites
3.5 Counting of number of merozoites

Fluorescent liquid counting beads (Becton deckinson, USA) of known concentration were added to merozoite suspension and samples were acquired on a BD FACS Calibur™ brand flow cytometer equipped with 488-nm laser excitation and BD CellQuestPro™ software. Dot plot of fluorescence in FL1 region verses fluorescence in FL2 region were plotted and analyzed using BD CellQuestPro™ software. Beads are fluorescent in FL1 as well as in FL2 region and merozoites are negative for both. Figure 3.3 shows a representative dot plot of merozoites and fluorescent beads where upper right quadrant is the bead region and lower left quadrant is the cell region.

Figure 3.3. Dot plot of merozoites and fluorescent counting beads acquired by flow cytometry
Concentration of merozoites was calculated using following formula:

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\text{Concentration of merozoites} = \frac{\text{No. of events in cell region} \times \text{No. of beads/test} \times \text{Dilution factor}}{\text{No. of events in bead region} \times \text{Test volume}}
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3.6 Invasion assay with merozoites

Approximately \(1 \times 10^6\) merozoites were added to \(1-2 \times 10^7\) RBCs in final volume of 200 \(\mu\)l of complete RMPI in 96 well flat bottom microtitre plates and incubated for 18-20 hours in mixed gas environment (5 % CO\(_2\), 5 % O\(_2\) and 90 % N\(_2\)) at 37°C. After incubation RBCs were washed twice with 1 ml incomplete RPMI at 1,200 rpm for 3 mins in Sorvall RT7 with RTH-250 rotor. Thin smears of cells were made on glass slide, stained with Giemsa stain and percentage of infected cells was scored under light microscope.

3.7 Invasion assay with schizonts

Density of RBCs in Percoll purified schizonts suspensions and fresh RBCs were scored using hemocytometer. About \(1 \times 10^5\) schizonts were added to \(1 \times 10^7\) RBCs in a final volume of 200 \(\mu\)l of complete RPMI in a 96-well flat bottom microtiter plate. Schizonts were incubated with target RBCs for 18-20 hours in mixed gas environment (5 % CO\(_2\), 5 % O\(_2\) and 90 % N\(_2\)) at 37°C to allow rupture of schizonts and invasion of RBCs by released merozoites. Thin smears were made on glass slide, stained with Giemsa and percentage of ring stage infected RBCs was scored under light microscope.

3.8 Image of Fluo4-AM loaded merozoites

Merozoites were isolated in complete RPMI and loaded with 10 \(\mu\)M of fluorescent calcium indicator Fluo4-AM (Calbiochem, USA) in incomplete RPMI for 20 mins at 37°C. Fluo4-AM loaded merozoites were washed two times with 1 ml of incomplete RPMI. Nuclear DNA was counterstained with 40 nM 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, USA). Bright field and fluorescence images of Fluo4-AM loaded merozoites were acquired under confocal microscope (Nikon TE 300) to study the distribution of fluo-4AM in merozoites.
3.9 Treatment of *P. falciparum* merozoites with calcium modulating agents and transfer to ionic conditions mimicking intracellular and extracellular conditions prior to analysis of cytosolic calcium levels or surface expression of merozoite proteins

*P. falciparum* merozoites isolated in RPMI 1640 were treated with 10 μM A23187 (Calbiochem, USA) for 15 mins at 37°C with or without pre-treatment with 50 μM BAPTA-AM for 15 mins at 37°C. Merozoites isolated in IC buffer (5 mM NaCl, 140 mM KCl, 1 mM EGTA) were transferred to EC buffer (140 mM NaCl, 5 mM KCl, 1 mM CaCl, 1 mM EGTA), IC-\textsubscript{low} buffer (5 mM NaCl, 5 mM KCl, 135 mM choline-Cl, 1 mM EGTA), EC w/o Ca\textsuperscript{2+} buffer (140 mM NaCl, 5 mM KCl, 1 mM EGTA), EC buffer containing glyA (1 mg/ml; Sigma, USA) or EC buffer containing RBC ghosts with or without prior treatment with calcium modulators BAPTA-AM (50 μM; Calbiochem, USA), U73122 (10 μM; Calbiochem, USA) or its inactive analog U73343 (10 μM; Calbiochem, USA) for 15 mins at 37°C.

RBC ghosts were prepared as follows. RBCs were lysed by resuspending RBC pellets in lysis buffer (cold, 10-fold diluted PBS). Lysed RBCs were collected by centrifugation at 15,000 g for 5 mins and resuspended again in lysis buffer. This process was repeated 4-5 times to obtain RBC ghosts devoid of hemoglobin. RBC ghosts were finally resuspended in EC buffer and sonicated for 5 cycles with sonicator on or off for 0.5 seconds each before use. Protein content of RBC ghosts was measured by the bicinchoninic acid assay (BCA). RBC ghosts containing 250 μg of protein were added to 200 μl of merozoite suspension to stimulate rhoptry release.

3.10 Determination of free cytosolic calcium levels in *P. falciparum* merozoites by flow cytometry

*P. falciparum* merozoites isolated in complete RPMI or IC buffer as described above were loaded with 10 μM Fluo-4AM for 20 mins at 37°C, washed, resuspended in same buffers and used for experiments within 5 mins. Fluo-4AM loaded *P. falciparum* merozoites were treated with calcium modulating agents and/or transferred from ionic environments mimicking intracellular conditions to extracellular conditions as described above prior to analysis of fluorescence signal on FACSCalibur (Becton Dickinson, USA) using CellQuest software. The Fluo-4AM
loaded merozoites were excited at 488 nm and fluorescence signal was detected with a 430/30 nm band pass filter for periods of 2-3 mins. Merozoites were gated on the basis of their forward scatter and side scatter. Histograms of mean fluorescence intensity (MFI), which reflects cytosolic calcium levels in merozoites, were plotted against time using FlowJo software.

3.11 Determination of in situ dissociation constants for Fluo-4AM in *P. falciparum* merozoites under intracellular and extracellular ionic conditions

In situ dissociation constants for Fluo-4AM were measured in *P. falciparum* merozoites under different ionic conditions using methods described earlier for mammalian cells (Thomas et al., 2000). Calcium Calibration Buffer Concentrate Kits (Molecular Probes, USA) containing 100 mM Ca$^{2+}$-EGTA, pH 7.2 and 100 mM K$_2$EGTA, pH 7.2 were used to generate IC and EC buffers containing a range of known concentrations of free calcium (17 nM to 39 μM) as described by the manufacturer. Merozoites were loaded with 10 μM Fluo-4AM for 20 mins at 37°C and washed with IC or EC buffers. Loaded merozoites were resuspended in IC and EC buffers containing range of calcium concentrations. Ionomycin (10 μM) (Calbiochem, USA) was added to the merozoite suspension to equilibrate intracellular and extracellular calcium concentrations. The fluorescence signal was measured at 485 nm using a Perkin Elmer Victor$^3$ fluorimeter. Measured mean fluorescence intensity (MFI) was used to plot $\log[(F - F_{\text{min}})/F_{\text{max}} - F]$ against $\log[\text{Ca}^{2+}_{\text{free}}]$ where $F$ is the fluorescence intensity at different known free calcium concentrations, $F_{\text{min}}$ is the fluorescence intensity at zero free calcium concentration and $F_{\text{max}}$ is the fluorescence intensity at saturating free calcium concentration (39 μM free Ca$^{2+}$). The x-intercept of the plot is $\log K_d$ of Fluo-4AM. The $K_d$ values determined represent affinity of Fluo-4 for calcium in the merozoite cytoplasm under ionic conditions that mimic intracellular and extracellular ionic environments.

3.12 Detection of proteins on *P. falciparum* merozoite surface by flow cytometry and fluorescence microscopy

*P. falciparum* merozoites isolated in complete RPMI or IC buffer were treated with calcium modulating agents and/or transferred from ionic environments mimicking intracellular conditions to extracellular conditions as described above prior
to analysis of expression of microneme proteins (EBA175 and AMA1), rhoptry proteins (CLAG3.1 and RH2a/2b) or merozoite surface protein (MSP4) on the merozoite surface. Merozoites were fixed with 0.15% chilled p-formaldehyde for 1 hour and used for detection of parasite proteins on the merozoite surface. Fixed merozoites were washed with phosphate buffered saline (PBS), incubated with sera raised against EBA175 (mouse sera at 1:50), AMA1 (rabbit sera at 1:50 dilution), CLAG3.1 (rabbit sera at 1:50 dilution), PrRH2a/2b (rabbit sera at 1:50 dilution) and MSP4 (rabbit sera at 1:50 dilution) for 1 hour on ice. Merozoites were washed two times with PBS, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma, USA) or FITC-conjugated anti-rabbit IgG (Sigma, USA) for 1 hour on ice, washed two times with PBS and used for analysis by flow cytometry using a Becton Dickinson FACS Calibur. Merozoites were excited at 488 nm and fluorescence signal was detected with a 430/30 nm band pass filter. Merozoites were gated on the basis of their forward scatter and side scatter. For each sample, data for 50,000 merozoites was acquired and analyzed using CellQuest software. Histograms showing logarithmic green fluorescence intensities (FL1) were plotted for each sample. Merozoites were also labeled with mouse sera raised against the cytoplasmic protein NAPL at 1:50 dilution as control. Merozoites were permeabilized by adding 0.05% saponin to primary and secondary sera to detect intracellular proteins. Expression of EBA175, CLAG3.1 and NAPL was also analyzed by IFA using a Nikon TE 300 fluorescence microscope following staining with primary and secondary sera as described above. Nuclear DNA was counterstained with 40 nM DAPI in IFA.

3.13 Detection of proteins secreted from *P. falciparum* merozoites by Western blotting

To study the effect of calcium modulating agents on secretion of microneme proteins, *P. falciparum* merozoites were isolated in complete RPMI and incubated for 15 mins at 37°C in incomplete RPMI with 10 μm A23187 with or without prior loading with BAPTA/AM for 20 mins at 37°C. Merozoites incubated in incomplete RPMI without any treatment were used as control.

*P. falciparum* merozoites isolated in IC buffer were incubated in either IC or EC buffer for 15 mins at 37°C with or without prior treatment with BAPTA-AM for
20 mins at 37°C to evaluate the role of ionic conditions in secretion of microneme proteins.

Merozoite supernatants from above experiments were separated by centrifugation and used for detection of microneme proteins, EBA175 and AMA1, by Western blotting using specific rabbit antisera. Anti-EBA175 rabbit sera were used at a dilution of 1:200, anti-AMA1 rabbit sera were used at 1:2,000 dilution and horse radish peroxidase (HRP) conjugated anti-rabbit IgG goat sera (Sigma, USA) were used at a dilution of 1:2,000 for Western blotting. Anti-NAPL rabbit sera were used at a dilution of 1:2,000 as control for Western blotting with merozoite pellets as well as with merozoite supernatants for each condition. Densitometry analyses of Western blots of merozoite pellets and supernatants using anti-NAPL sera were used to normalize levels of EBA175 and AMA1 detected in merozoite supernatants under different conditions.

3.14 Measuring effect of calcium modulating agents on erythrocyte invasion by *P. falciparum* merozoites

Merozoites isolated from 2 ml of *P. falciparum* 3D7 cultures with 5% parasitemia were mock-treated or treated with 1 mM BAPTA-AM (Calbiochem, USA), 10 μM U73122 (Calbiochem, USA) or 10 μM U73343 (Calbiochem, USA) for 15 mins at 37°C, washed with IC buffer, resuspended in EC buffer and incubated with 1x10⁷ erythrocytes in EC buffer at 37°C under mixed gas environment for 2 hours to allow invasion. EC buffer was then replaced with complete RPMI. After 18-20 hours of incubation in complete RPMI under mixed gas environment to allow development of ring stages, the percentage of infected erythrocytes was scored by microscopy of Giemsa stained smears to determine invasion rates.