Gliding Motility Assay for *P. berghei* Sporozoites

Important Notes:

1. For all dilutions (including antibodies and sporozoites), always make slightly more than needed. For instance, if you need 200 µl sporozoites in medium, make 210 - 215 µl.

2. 1x PBS pH 7.4 can be used in place of 1x TBS pH 7.4 in all steps of the protocol.

3. A volume of 200 µl is sufficient to cover the bottom of an 8-chambered Lab-Tek well. However, if extra antibody cannot be spared, the volume can be reduced to a minimum of 150 µl per well.

4. Dissection medium can contain less than 3% BSA. Sporozoites will glide in the presence of 1% BSA, however better results are obtained with 3% BSA. If the sporozoites have to be incubated in medium other than 3% BSA/DMEM, add some BSA before adding the sporozoites to the coated Lab-Tek wells.

5. The presence of protease inhibitors in the dissection medium are not necessary and should be avoided. Many protease inhibitors that are cell permeable inhibit gliding motility.

6. For STEP 1, the Lab-Tek wells can be coated with antibody at: 37°C for 1 – 2 hr; RT overnight; or 4°C overnight. Overnight at RT is often best.

7. Streptavidin-FITC can be diluted in 1% BSA/TBS pH 7.4. Alternatively, it can be diluted in 1x TBS pH 8.0 or 1x TBS pH 8.5.

8. The protocol outlined below is using 3D11 (*P. berghei* CSP anti-repeat mAb) to visualized the sporozoites and the trails.

9. When using another antibody for visualizing the trails (i.e., N-terminal antiserum, C-terminal antiserum, etc.), it may be necessary to amend the protocol. For instance:
    a. Coat the Lab-Tek wells overnight at RT.
    b. Use fresh aliquot of 4% PFA.
    c. Increase the incubation time of the 1° Ab from 1 hr to 2 – 3 hr.
    d. Use a 2° Ab which gives brighter fluorescence such as the Alexa Fluor fluorescent conjugates.
    e. If using Streptavidin-FITC, dilute in 1x TBS pH 8.0 or 1x TBS pH 8.5.
    f. Gently shake/agitate the Lab-Teks during antibody incubations.
    g. Perform antibody incubations at 37°C instead of RT.

A. Solutions

1. 1x TBS pH 7.4

	For 1 L:
137 mM NaCl	8.00 g NaCl
20 mM Tris	2.42 g Tris base
	3.80 ml 1 M HCl
	↑ 1 L ddH2O

• pH should be between 7.4 – 7.5. Autoclave or filter sterilize solution.

- This solution can be made as a 10x stock.
- Alternatively, **1x PBS pH 7.4** can be used.
- Store 1x and 10x solutions at RT.

2. 3% BSA/ DMEM

	For 10 mls:
BSA	0.3 g
DMEM (high glucose, no sodium pyruvate) (Gibco #11960-044 or equivalent)	↑ 10 ml with1× stock

- Use high-quality BSA (example: Sigma A-7888)
- Solution will appear orange-red in color. Check the pH to make sure it is between 7 – 8.
- 3% BSA/DMEM can be made beforehand, filter sterilized, aliquoted, and stored at 4°C for a week or so.

3. 4% Paraformaldehyde (PFA))/1x PBS pH 7.4

- Stored in aliquots at -20°C.
- Thaw in 37°C waterbath completely. The solution should be clear; if there is a precipitate, discard.
- Wear gloves when using PFA!
- Unused PFA can be discarded or stored at 4°C for up to week.

4. Blocking Buffer & Antibody Incubation Buffer (1%BSA/1x TBS pH 7.4)

	For 50 mls:
1% BSA	0.5 g
1x TBS pH 7.4	↑ 50 ml with 1x TBS

- Dissolve BSA in TBS, then adjust pH to between 7.4 – 7.5. Do not add BSA to TBS that has already been adjusted to pH 7.4-7.5, because the BSA will change the pH of the solution.

- 1% BSA/TBS can be made beforehand, filter sterilized, and stored at 4°C. Azide can also be added to prevent contamination (usually not necessary).

5. *P. berghei* CSP Antibody (to coat Lab-Teks)

3D11 monoclonal antibody (mouse) recognizes the repeats of *P. berghei* CSP

working concentration = 5 - 10 μg/ml in 1x TBS pH 7.4

6. *P. berghei* CSP-biotinylated Antibody (1°)

Biotin labeled 3D11 (which recognizes the repeats of *P. berghei* CSP)

working stock: make a 1:100 dilution of stock in 1% BSA/TBS pH 7.4

7. **Streptavidin-FITC Antibody (2°)**

   Streptavidin, fluorescein conjugated

   (Amersham Biosciences #RPN1232)

   working stock: make a 1:100 dilution of stock in 1% BSA/TBS pH 7.

8. **Mounting Medium**

   Citifluor Mountant Media, #0 (TED PELLA, INC. #19470),
   VectaShield (Vector Laboratories, #H-1000), or equivalent

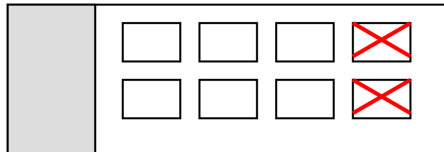
B. <u>Protocol</u>

1. Coat each Lab-Tek well with 200 μl of 5 - 10 μg/ml 3D11 diluted in 1x TBS pH 7.4 and incubate overnight at RT.

   • Use <u>glass</u> LAB-TEK (LPD #177402 NalgeNunc)! Can use plastic LAB-TEK (LDP #177445 NalgeNunc)

   • <u>DO NOT</u> dilute 3D11 in 1% BSA/TBS or else the BSA and not 3D11 will coat the Lab-Tek!

   • Coat enough wells to do triplicates of each experimental condition. If possible do not use the 2 wells farthest from the frosted end.



2. Dissect infected female *Anopheles stephensi* mosquitoes to obtain salivary glands and place salivary glands on ice in 1.5 ml tube(s) containing 3% BSA/DMEM.

3. Grind mosquito salivary glands on ice to release *P. berghei* sporozoites and determine number of sporozoites.

→ At this point, sporozoites can be pre-treated with various compounds, under various conditions, etc. depending on the experiment. After pre-treatment, the protocol resumes with STEP 4.

   • Need 10,000 - 50,000 sporozoites in 200 μl volume for each well of the Lab-Tek.**

• Some sporozoites will wash off, so the more the better. However, too many sporozoites will result in too many overlapping trails.

→ If sporozoites are in a large volume or medium must be changed, spin tube at 12 - 14 krpm (≅16,000×*g*) for 3 - 4 min at 4°C to pellet sporozoites. Remove excess medium or resuspend sporozoites in other medium by pipetting ↑↓ gently and quickly vortexing.

NOTE: The less you handle ("abuse") the sporozoites, the better!

4. Wash Lab-Tek wells 3x with 1x TBS pH 7.4 (1 min per wash).

   •To wash, turn the Lab-Tek upside-down and "flick" to remove liquid in the wells. Gently tap the Lab-Tek upside-down on some paper towels to remove extra liquid. Then using either a P1000 or a Pasteur pipet, gently add 1x TBS pH 7.4 to the top of the well (or ~500 µl). When washing, carefully pipet the TBS along the side of the well in a slow drop-wise fashion at first and then a gentle stream. Do not add too quickly to the middle of the well!

5. Add sporozoites (in 200 µl/well) to washed Lab-Tek wells. Place Lab-Tek in a large covered Petri dish (to prevent evaporation) and incubate at 37°C for 1 hr in culture incubator (Forma Scientific CO_2 Water Jacketed Incubator Hepa Filter).

6. Remove medium (as described for washes in STEP 4). Fix sporozoites by adding ~ 400 µl 4% PFA and incubate at 25°C or 37°C for 30 min - 1 hr or at 4°C overnight.

   • This and any subsequent incubations can be done in any 37°C incubator. The presence of CO_2 is not necessary as sporozoites have already been fixed.

7. Remove PFA and wash 2X with 1x TBS pH 7.4 (1 min per wash).

8. Block by adding 200 µl 1% BSA/TBS in each well and incubating 30 – 60 min at 25 or 37°C.

9. Remove 1% BSA/TBS. Add 200 µl of biotinylated 3D11 diluted 1:100 in 1% BSA/TBS and incubate 1 hr at 25 or 37°C.

10. Wash 3X with 1x TBS pH 7.4 (1 min per wash).

11. Add 200 µl of Streptavidin-FITC diluted 1:100 in 1% BSA/TBS and incubate 1 hr at 25 or 37°C.

12. Wash 3X with 1x TBS pH 7.4 (1 min per wash).

13. Carefully remove plastic upper structure and silicone gasket.

    • It may be necessary to use tweezers to remove the silicone, but be careful not to scratch the well areas or to let the silicone "snap back" onto the wells.

14. Add a drop of mounting medium to each well area of the Lab-Tek used. Cover with cover glass (Corning No. 1 - 22 x 50 mm; Fisher #12-531E), press down with finger and dab with Kimwipe to remove excess mounting medium. Seal with nail polish.

   - A drop ≈ 5 μl. The mounting medium will spread out once the cover glass has been placed on top.

15. View with fluorescence microscope and quantify results as explained on PAGE 7.

   - Sporozoites and trails will appear green in color. Sporozoites will be much brighter than trails.

   - Slides can be saved in the dark at RT (25°C) or at 4°C (if lab is too hot) for later viewing; however, for best results, view within a week of preparation. Trails will still be visible for a few weeks, but intensity will diminish over time.

** Controls:

1. **Positive control**: Sporozoites in dissection medium are added directly to coated Lab-Teks without pre-treatment of any kind and allowed to incubate at 37°C for 1 hr in culture incubator. That is, this protocol is followed exactly as is. These sporozoites will glide and leave trails of CSP.

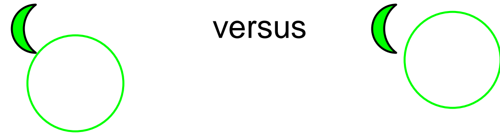
2. **Negative control**: After dissection, Cytochalasin D is added (at a final concentration of 1 μM) to sporozoites in dissection medium, mixed, and incubated for 10 minutes at RT. The dissection medium containing sporozoites with Cytochalasin D is then added directly to coated Lab-Teks and allowed to incubate at 37°C for 1 hr in culture incubator. Since these sporozoites are incapable of gliding, trails of CSP will not be observed.

Quantifying the Results:

1. Total # of sporozoites which have trails = % Motility

   Count the number of sporozoites with and without trails. Be careful, as the sporozoite may not be directly connected to the trail it made but is near it.

   Example:



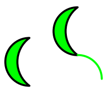
$$\% \text{ motility} = \frac{\# \text{ spz with trails}}{(\# \text{ spz with trails} + \# \text{ spz without trails})} \times 100$$

2. Quality of trails = # of Circles

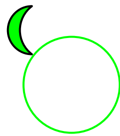
   Of the sporozoites that produced trails, determine the number of trails per sporozoite. Because counting each trail is difficult, divide the results in 4 groups: no trail, 1 trail, 2-10 trails, >10 trails.

   1 trail = 1 complete circle

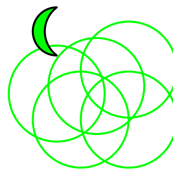
   Example:



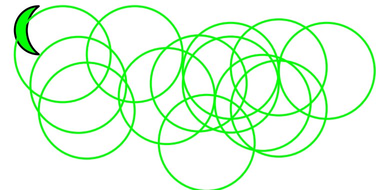
No trail



1 trail



2 – 10 trails



>10 trails