Cyst Detection in *Toxoplasma gondii* Infected Mice and Rats Brain

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[Abstract] Toxoplasmosis caused by the intracellular parasite *Toxoplasma gondii*, is characterized by a life-long chronic infection. The parasite is an efficient neurotropic infectious agent that establishes its "safe" life by forming intracellular cysts in chronically infected animals and humans. This protocol describes the specific recipes and method to stain brain cysts from infected mice and rats for further quantification using epifluorescence microscopy. This method provides the possibility to scan the entire brain and thus to numerate all cysts.

Materials and Reagents

1. Human foreskin fibroblasts (HFFs) cells culture (ATTC, catalog number: SCRC-1042)
2. Dulbecco's modified Eagle medium (DMEM) (Life Technologies, catalog number: 41966-029/052)
3. Fetal bovine serum (FBS) (Eurobio®, catalog number: CVFSVF0001)
4. Penicillin/streptomycin (PAN Biotech GmbH, catalog number: P0607-100)
5. L-Glutamine (200 mM) (Life Technologies, catalog number: 25030-024)
6. Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies, catalog number: 14190-094/069)
7. Proteinase K (molecular biology grade) (Bio-Rad Laboratories, catalog number: P8107S, Lot: 0051310)
8. TRIS (Euromedex, catalog number: 26-128-3094-B)
9. EDTA (Euromedex, catalog number: E013)
10. SDS (Euromedex, catalog number: EU0660)
11. NaCl (Euromedex, catalog number: 1112-A)
12. HCl (37% ACS reagent) (Sigma-Aldrich, catalog number: 258148-2.5ML)
13. Phenylmethylsulfonylfluoride (PMSF) (Euromedex, catalog number: 1111-C)
14. Hoescht 33258 (Life Technologies, Molecular Probes®, catalog number: H-3569)
15. Formaldehyde methanol free (Polysciences, catalog number: 04018)

16. *Dolichos biflorus* lectin coupled to fluorescein isothiocyanate (FITC) (Clinisciences, catalog number: FL-1031)
17. Complete DMEM medium (see Recipes)
18. Phenylmethylsulfonylfluoride (PMSF) solution (see Recipes)
19. Lysis buffer (see Recipes)

Equipment

1. 37 °C/5% CO_2 cell culture incubator
2. 6-well plates
3. Scissors and forceps
4. Glass homogenizers (Potter-Elvehjem PTFE, 15 ml) (Dutscher, catalog numbers: 057009 and 057021)
5. 15 ml polystyrene tubes
6. Epifluorescence microscope

Procedure

A. Preparation of Human Foreskin Fibroblasts cell culture in 6-well plates

Plate HFFs cells in complete DMEM medium and culture them for 4 days at 37 °C in presence of CO_2. HFFs have to be at 100% confluence in each well (8 x 10^5 cells) before staining.

B. Staining of HFFs

1. Wash twice the cells culture with 1 ml 1x DPBS.
2. Fix cells with 1 ml formaldehyde diluted at 2.5% in DPBS 1x for 20 min at 4 °C.
3. Incubate 20 min, in the dark and at RT, the cells with 1 ml of Hoescht diluted 1/50,000 in 1x DPBS.
4. Wash twice the cells culture with 1 ml 1x DPBS.
5. Add 1 ml 1x DPBS.
6. Stored at 4 °C until addition of the stained homogenized brain.

   Note: The stained cells are stored at 4 °C for a maximum of 24 h.

C. Isolation of brains from infected mice or rats

1. Anesthesize animal with isofluoran and euthanize it by cervical dislocation.
2. Soak the head with 70% (v/v) ethanol.
3. Cut the skin at the base of skull and remove it as much as possible.
4. Cut with scissors the dorsal and lateral part of the skull and take the top off.
5. Collect the brain tissue in 5 ml DPBS 1x solution.

D. Brains homogenization

Put each mouse brain in 2 ml DPBS 1x into a glass homogenizer, homogenize at RT and adjust final volume to 4 ml. For rat brain, homogenize in 4 ml and adjust to 8 ml final.

Note: Brains can be stored at 4 °C until the staining for no more than 2 days.

E. Brains staining

1. Prepare 5x lysis buffer and proteinase K at 8 U/ml (stock at 800 U/ml, 1/100 dilution in 5x lysis buffer just before use).
2. For mice, take 1 ml of homogenized brain (¼ brain), add 398 µl of 5x lysis buffer, 2 µl of proteinase K (see step E-1, so final concentration at 0.008 U/ml) and 600 µl of 1x DPBS (final volume of 2 ml).

   For rats, take 2 ml of homogenized brain (¼ brain), add 995 µl of 5x lysis buffer, 5 µl of proteinase K (see step E-1, so final concentration at 0.008 U/ml) and 2 ml of 1x DPBS (final volume of 5 ml).
3. Incubate at 56 °C for 15 min, homogenize every 5 min by vortexing.
4. Stop the proteinase K activity by adding PMSF to a final concentration of 2 mM (stock at 200 mM), homogenize and incubate at RT for 5 min.
5. Centrifuge the sample for 15 min at 1,250 *x g* (RT).
6. Gently eliminate the supernatant by pipetting.
7. Resuspend in 1 ml of *Dolichos biflorus* lectin diluted at 1/250 in DPBS 1x (996 µl 1x DPBS + 4 µl of *Dolichos biflorus* lectin).
8. Incubate 30 min at RT in a mechanical wheel placed in the dark.
9. Add 3 ml DPBS 1x and homogenize to wash the sample.
10. Centrifuge 15 min at 1,250 *x g* (RT).
11. Gently eliminate the supernatant by pipetting.
12. Resuspend each sample in 1 ml in DPBS 1x and transfer onto Hoescht-stained HFFs (see part A and B of the procedure) by pipetting (1 ml/well).

    Note: It is important to transfer the homogenized brain pellet onto HFF cells to facilitate microscope focus when there are no or very few cysts.

F. Count the cysts using epifluorescence microscopy (Representative data).

Note: Samples can be stored at 4 °C until microscope observation for no more than 10 days. To know the cysts quantity in entire brain, multiply the counted number by 4.

Representative data

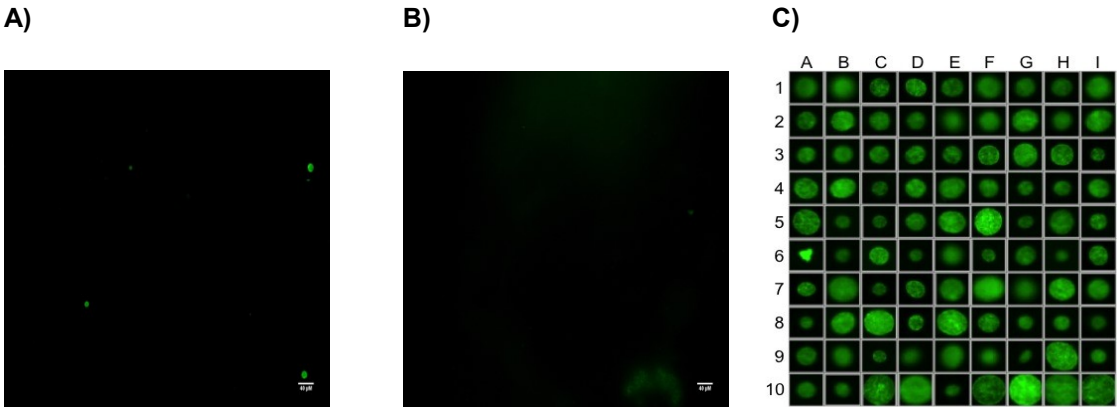

Figure 1. Representative cysts staining from uninfected and infected CBA mice brain with *Toxoplasma gondii* parasites. Two months after i.p. infection of CBA mice with 1,000 ΔKu80-ΔHXGPRT (PRU) tachyzoïtes, brains were collected, homogenized in 4 ml of PBS and ¼ of each brain suspension was used for the labeling of cyst walls with the *D. biflorus*-FITC lectin and analyzed by high content screening microscopy (Scan^R Olympus). A) Photo (4x objective) of a CBA infected mouse brain. Three stained cysts are present in this field. B) Uninfected CBA mouse brain (4x objective) no cysts are visible but only brain debris. C) Representative panel of stained cysts obtained from infected CBA mouse and detected by (4x objective) microscopy. Cysts are zoomed to detect potential false positive as in A6.

Recipes

1. Complete DMEM medium
   10% (v/v) FBS
   0.5% (v/v) penicillin/streptomycin
   2 mM glutamine
2. Phenylmethylsulfonylfluoride (PMSF) solution (stock concentration of 200mM)
   0.35 g of PMSF powder to dissolve in 10 ml of isopropanol
   Stored at -20 °C in 1 ml aliquots
   Note: PMSF will take some time to dissolve.
3. Lysis buffer (500 ml)
   10 M TRIS: 2.5 ml of 2 M TRIS at pH 8.0 (60 g in 250 ml H_2O, adjust pH)
   1 mM EDTA: 2 ml of 250 mM EDTA at pH 8.0 (23.27 g in 250 ml H_2O, adjust pH)
   0.2% (w/v) SDS: 5 ml of 20% (w/v) SDS
   100 mM NaCl (1.165 g)
   Up to 500 ml H_2O
   Note: The 8.0 pH is adjusted using HCl 3 M (HCl 3 M: 25 ml HCl 37% added to 75 ml of H_2O).

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