Protocol

Elicitation of *Xenopus laevis* Tadpole and Adult Frog Peritoneal Leukocytes

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Peritoneal lavage of *Xenopus laevis* tadpoles and adult frogs is a reliable way of isolating resident and/or recruited innate immune populations. This protocol details the isolation of tadpole and adult amphibian (*Xenopus laevis*) peritoneal leukocytes. The isolated cells are comprised predominantly of innate immune populations and chiefly of mononuclear and polymorphonuclear granulocytes. As described here, these cells are typically elicited by peritoneal injections of animals with heat-killed *Escherichia coli*, causing peritoneal accumulation of inflammatory cell populations, which are then isolated from the stimulated animals by lavage. *E. coli*-mediated elicitation of tadpole and adult peritoneal leukocytes greatly enhances the total numbers of recovered cells, at the cost of their inflammatory activation. Conversely, lavage may be performed on naïve, unstimulated animals to isolate nonactivated cells with much lower yield. This protocol represents a reliable means of deriving tadpole and adult frog innate immune cell populations, and the conditions of the stimulation may be amended to suit the specifics of a given experimental design.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Amphibian phosphate-buffered saline (APBS) <R>
Complete-amphibian medium (C-AM) <R>
E. coli (DH5α or alternative laboratory strain)
LB medium <R>
Tricaine mesylate (0.1%) buffered with sodium bicarbonate (0.5 g/L)
Tricaine mesylate (1%) buffered with sodium bicarbonate (0.5 g/L)
Trypan blue
Xenopus laevis tadpoles (stage of choosing) or adult frogs (Nieuwkoop and Faber 1967)

Equipment

Aquarium filter floss or sponge
Bunsen burner

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From the *Xenopus* collection, edited by Hazel L. Sive.

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Centrifuge (preferably refrigerated)
Centrifuge tubes (1.5, 15, and 50 mL)
Hemocytometer
Needles (25G, 1 inch; 18G, 1.5 inch)
Parafilm
Pasteur pipettes
Plastic spoon
Rubber bulb (small)
Shaking incubator (37°C)
Syringe (1 mL and 10 mL)

METHOD

Preparation of Heat-Killed *E. coli*

1. Inoculate 40 mL of LB medium with DH5α (or alternative laboratory strain) *E. coli* and grow while shaking overnight at 37°C.
2. The following day collect the bacteria by centrifugation at 6000*g* for 10 min at 4°C, resuspend the pellet in 4 mL of APBS, and boil for 1 h at 100°C to heat-kill the bacteria.
3. Cool the heat-killed *E. coli* on ice and make 0.5 mL aliquots. Use immediately or store at −20°C.
4. Proceed to Step 5 to inject tadpoles and Step 21 to inject adult frogs.

Injection of *Xenopus laevis* Tadpoles with Heat-Killed *E. coli* and Peritoneal Leukocyte Collection

5. The day before injections, isolate the tadpoles to be used in the experiment from general housing and house separately overnight.
6. Prepare glass injection needles by pulling Pasteur pipettes to a fine point over a flame of a Bunsen burner.
7. The following day, anesthetize the tadpoles by immersion in 0.1% tricaine mesylate solution buffered with sodium bicarbonate (0.5 g/L).

   Tadpoles will cease movement after 2–5 min. Wait an additional 1–2 min after cessation of movement to ensure that animals are fully sedated.

8. Pipette the heat-killed *E. coli* in 5 (for stage NF 50 tadpoles) to 10 µL (for stage NF 54+ tadpoles) droplets onto a wide section of parafilm on a sturdy surface.
9. Gently place anesthetized tadpoles on their side on top of moist aquarium filter floss or a moist sponge.
10. Attach a finely pulled needle to a small rubber bulb and carefully take up the heat-killed *E. coli* droplet into the tip of the needle, taking care not to take up the suspension past the tip of the needle.

    Make sure that you are comfortable taking up and expelling small volumes with a glass needle and a rubber bulb before you begin this procedure.

11. Using the thumb and forefinger of your nondominant hand, gently distend the tadpole's abdomen (Fig. 1A).
12. Using your dominant hand, carefully insert the *E. coli*-containing needle 1–2 mm into the tadpole's abdomen and carefully expel 95% of the volume into the animal by gently squeezing the bulb.

    Take care not to inject the entire volume into the tadpole, thus avoiding possible introduction of air into the animal.

13. Using a plastic spoon, pick up the injected tadpole and place into water. Monitor until the animal regains movement.

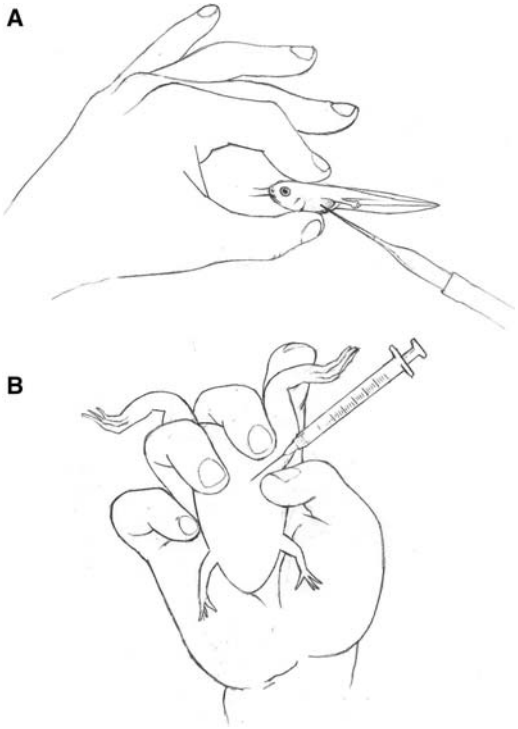

FIGURE 1. (*A*) Peritoneal injection and lavage of a *X. laevis* tadpole. (*B*) Peritoneal injection and lavage of adult *X. laevis*.

14. Three days after *E. coli* injection, anesthetize the tadpole(s) by immersion in 0.1% tricaine mesylate solution buffered with sodium bicarbonate (0.5 g/L).

    Tadpoles will cease movement after 2–5 min. Wait an additional 1–2 min after cessation of movement to ensure that animals are fully sedated.

    Note that peritoneal leukocytes begin to accumulate within the first 24 h of injection (primarily granulocytes) and isolating cells 3 d after injection is a suggested time.

15. Pipette APBS in 50 µL droplets onto a wide section of parafilm.
16. Gently place anesthetized tadpole(s) on their side on top of moist aquarium filter floss or a moist sponge.
17. Following the procedures described in Steps 10–12, take up approximately one-third of the APBS droplet into the tip of the needle (using a small rubber bulb) and inject it into the tadpole, preferably into the same needle entry site.
18. Gently pull the liquid back into the needle and eject it into a 1.5 mL microfuge tube. Repeat twice, with fresh APBS volumes and collect the total 50 µL of lavaged fluid into the same tube over ice.
19. Collect the isolated leukocytes by centrifugation at 600*g* for 10 min at 4°C. Wash the cells with 1 mL APBS and collect by centrifugation at 600*g* for 10 min at 4°C. Discard supernatants.
20. Resuspend the cells in a small volume (100–200 µL) of complete amphibian medium and enumerate by hemocytometer counts using trypan blue exclusion viability stain.

    Using stage NF 54 tadpoles, the yield should be ~1–5 × 10^6 cells.

Injection of Adult *Xenopus laevis* with Heat-Killed *E. coli* and Peritoneal Leukocyte Collection

21. The day before injections, isolate the animals to be used in the experiment from general housing and house separately overnight.
22. The following day, anesthetize the adult *X. laevis* by immersion in 1% tricaine mesylate solution buffered with sodium bicarbonate (0.5 g/L).

Frogs will cease movement after 2–5 min. Wait an additional 1–2 min after cessation of movement to ensure that animals are fully sedated.

If you are comfortable with handling the animals, you may inoculate them with E. coli without sedation, if injections are performed quickly.

23. Take up an appropriate amount of the prepared heat-killed *E. coli* suspension into a 1 mL syringe fitted with a 25G 1 inch needle and place within reach.
24. Pick up individual anesthetized frogs with your nondominant hand so that the top of their head is resting in the palm of your hand and their legs are dangling on either side of your middle finger (Fig. 1B).
25. Distend the frog's abdomen by gently putting pressure on their sides with your thumb and pinky finger (Fig. 1B).
26. With your dominant hand, pick up the syringe with the heat-killed *E. coli*, gently pierce the frog's lower abdomen (2–3 mm), and make sure not to insert the needle too far into the animal. Inject 100 µL of the *E. coli* suspension into the animal.
27. Immediately place the animal in a shallow container of water, so that its nostrils are exposed to air but the majority of its body is submerged underwater.
28. Monitor the animal(s) as they recover from sedation, making sure that the exposed areas of their bodies are moist (to prevent desiccation) and that their nostrils are above water at all times. Alternatively, you may keep the sedated frogs covered with wet filter floss, to ensure that they do not dry out.
29. Once the animals recover movement, place them back into separate housing and monitor.
30. Three days after *E. coli* injection, anesthetize the adult frogs by immersion in 1% tricaine mesylate solution buffered with sodium bicarbonate (0.5 g/L).

    Frogs will cease movement after 2–5 min. Wait an additional 1–2 min after cessation of movement to ensure that animals are fully sedated.

    Note that peritoneal leukocytes begin to accumulate within the first 24 h of injection (primarily granulocytes) and isolating cells 3 d after injection is a suggested time.
31. Fill a 10 mL syringe fitted with an 18G 1.5 inch needle with APBS and place within reach.
32. Repeat Steps 24–26, injecting 5 mL of APBS (rather than *E. coli*) into the frog's peritoneum, preferably into the same needle entry site.
33. Carefully unscrew/unhinge the needle from the syringe while making sure that the needle remains lodged in the frog's abdomen.

    When detaching the syringe, take care not to move the needle further into the animal.
34. Hold the animal above an opened 15 mL conical tube (on ice) so that the wide part of the needle is directly above the tube. While firmly holding the animal, gently massage its sides, forcing the injected APBS to drip out of the needle. If necessary, gently twist and adjust the positioning of the needle within the incision to promote the leukocyte-containing APBS recovery.
35. When 4.5–5 mL of APBS has been collected from the animal, gently reattach the 10 mL syringe containing the other 5 mL of APBS to the needle while it is still in the animal.
36. Inject the frog with the remainder of the APBS and recover the peritoneal leukocytes as above. Collect both 5 mL fractions into the same 15 mL tube.
37. Monitor the animal as in Steps 27–29.
38. Collect the isolated leukocytes by centrifugation at 600*g* for 10 min at 4°C, wash with 1 mL APBS and again collect by centrifugation at 600*g* for 10 min at 4°C. Discard supernatant.
39. Resuspend the cells in complete amphibian medium (300–500 µL) and count using a hemocytometer and trypan blue exclusion viability stain.

    Using a 2–2.5 inch adult X. laevis*, the yield should be 1–2 × 10^7 leukocytes.*

DISCUSSION

The use of peritoneal lavage of *Xenopus laevis* tadpoles and adult frogs to isolate resident and/or recruited innate immune populations is described further in De Jesús Andino et al. (2012), Fites et al. (2013), and De Jesús Andino et al. (2016). Both tadpole and adult frog resident cells appear to be comprised predominantly of mononuclear phagocytes. Elicitation of peritoneal cells with heat-killed *E. coli* accumulates both macrophage- and granulocyte-lineage cells in animal peritonea, so these are the populations that are retrieved upon peritoneal lavage of these animals (Du Pasquier et al. 1985; Morales et al. 2010). Elicitation with heat-killed *E. coli* before lavage results in significantly greater cell yields than achieved when performing peritoneal lavage on unstimulated animals. However, it should be noted that the derived cells are activated and no longer represent the resident peritoneal leukocyte populations but rather inflammatory infiltrates, with elevated expression of inflammatory immune genes. These cells may be returned to relatively more baseline states by incubating them in complete amphibian medium for 24 h at 27°C with 5% CO_2 before commencing subsequent experimentation. Alternatively, animals can be lavaged without the heat-killed *E. coli* elicitation. While the total peritoneal leukocyte yields will be substantially lower ($1–5 \times 10^5$ cells for tadpoles and $1–5 \times 10^6$ cells from adult frogs, respectively), these cells will be representative of respective tadpole and adult frog resident peritoneal populations and not activation-biased by exposure to an inflammatory stimulus (heat-killed *E. coli*). Finally, animals may be injected with putative leukocyte chemo-attractants and/or growth factors. In this case injections and lavage would be performed using the methods and volumes specified above.

RELATED INFORMATION

Related reagents and resources are available via the *Xenopus laevis* research resource for immunology: https://www.urmc.rochester.edu/microbiology-immunology/xenopus-laevis/protocols.

RECIPES

Amphibian Phosphate-Buffered Saline (APBS)

Sodium chloride (NaCl)	6.6 g/L
Sodium phosphate (Na_2HPO_2)	1.15 g/L
Potassium phosphate (KH_2PO_4)	0.2 g/L

Adjust pH to 7.7 with 10 N NaOH, and filter-sterilize through a 0.2-µm filter. Store at room temperature for up to 6 mo.

Complete-Amphibian Medium (C-AM)

Mammalian serum-free medium <R>	200 mL
Triple-distilled H_2O (purchased or from filtering system)	60 mL
Fetal bovine serum	5 mL
Penicillin-streptomycin (10,000 units/mL; Gibco, 15140122)	5 mL
Gentamycin (50 mg/mL)	80 µL

Combine all reagents, filter-sterilize through a 0.2-µm filter, and store at 4°C.

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LB (Luria-Bertani) Liquid Medium

Reagent	Amount to add
H_2O	950 mL
Tryptone	10 g
NaCl	10 g
Yeast extract	5 g

Combine the reagents and shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~0.2 mL). Adjust the final volume of the solution to 1 L with H_2O. Sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle.

For solid medium, see the recipe entitled "Media containing agar or agarose." <R>

Mammalian Serum-Free Medium (MSF)

Reagent	Quantity (for 1 L)
Iscove's modified Dulbecco's medium, powder	1 pkg
Penicillin-streptomycin (10,000 units/mL; Gibco, 15140122)	10 mL
Non-essential amino acids solution (10 mM; Gibco, 11140050)	10 mL
Insulin from bovine pancreas (5 mg/mL)	10 mL
2-mercaptoethanol	3 µL
Peptone Primatone RL (10% in H_2O)	3 mL
$NaHCO_3$	3.02 g

Adjust pH to 7.7 with 10 N NaOH, and filter-sterilize through a 0.2-µm filter. Store at 4°C.

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