Protocol

Xenopus Tadpole Tissue Harvest

Matthew D. Patmann,[1] Leena H. Shewade,[1] Katelin A. Schneider,[1] and Daniel R. Buchholz[1,2]

[1]*Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221*

The procedures described here apply to *Xenopus* tadpoles from the beginning of feeding through the major changes of metamorphosis and are appropriate for downstream postoperative snap freezing for molecular analysis, fixation for histological analysis, and sterile organ culture. To the uninitiated, the most difficult aspects of tadpole tissue dissections are likely knowing the appearance and location of organs, and the difficulty manipulating and holding tadpoles in place to carry out the oftentimes fine and precise dissections. Therefore, images and stepwise instructions are given for the harvest of external organs (tail, head, eyes, tail skin, back skin, gills, thymus, hind limbs, forelimbs) and peritoneal organs (intestine, pancreas, liver, spleen, lungs, fat bodies, kidney/gonad complex), as well as brain, heart, and blood. Dissections are typically done under a dissection stereomicroscope, and two pairs of fine straight forceps, one pair of fine curved forceps, and one pair of microdissection scissors are sufficient for most tissue harvests.

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

Ethanol (70%)

Frog water

Remove chlorine/chloramine from tap water by carbon filtration (Alternatively, reconstitute reverse-osmosis water to 800 µS using Instant Ocean or equivalent). Adjust the pH to 7.5 using sodium bicarbonate.

Ice

MS-222 solution (0.1% tricaine methanesulfonate [Sigma-Aldrich], in frog water)

Add sodium bicarbonate to 0.1% to adjust the pH to 7.0–7.5.

Phosphate-buffered saline (0.6×) <R>

Dilute 1× PBS 60:40 with reverse-osmosis or distilled water. Dilution is required because the osmolarity of amphibian blood is ~62% that of mammalian blood.

Xenopus tadpoles

[2]Correspondence: buchhodr@ucmail.uc.edu

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

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Tadpoles at stages from the beginning of feeding through tail resorption (i.e., NF stages 45–66 [Nieuwkoop and Faber 1994]) can be obtained from suppliers listed at Xenbase (www.xenbase.org/other/obtain.do). Full-sized tadpoles around the stage of forelimb emergence (NF stages 57–58) are ideal for this protocol. Smaller tadpoles from the beginning of feeding (≤NF45) or tadpoles during metamorphosis (NF 61–66) will be more challenging for some tissues.

Equipment

Capillary tubes, micro-hematocrit, heparinized, 0.5-mm i.d., 75-mm length (Kimble-Chase, #40C505)
Dissection stereomicroscope
Finger bowl, 350-mL
Fish net (or tea strainer)
Forceps, curved (Dumont #7)
Forceps, straight (Dumont #5 or #55)
Kimwipes
Microdissection scissors, straight, w/ sharp points, 3.25-in.
Petri dishes, 100- and 150-mm diameter, plastic or glass
Razor blade
Syringe, 3-mL, fitted w/ 30-gauge needle

METHOD

Dissection tools are shown in Figure 1A. The forceps and scissors used in these procedures are delicate and damage easily. Care must be taken not to drop them, or even tap the tips into hard surfaces such as the surface of the Petri dish during dissection. Use straight forceps in the following procedures unless specifically instructed otherwise. When harvesting tissues from tadpoles that have undergone hormone or chemical treatments, avoid cross-contamination by rinsing the Petri dish, dissection tools, and tea strainer between treatment groups or samples.

Anesthetizing the Tadpole

1. Transfer tadpole from a large stock tank using a fish net (or from a small treatment tank with a tea strainer) into a 0.1% MS-222 solution in a finger bowl or 100-mm Petri dish.
2. After 1–2 min, verify that the tadpole is anesthetized by gently pinching the tadpole's tail with forceps.

   A properly anesthetized tadpole will not respond to a tail pinch but will still have a heartbeat.

3. Scoop the anesthetized tadpole with curved forceps. Place it in a 100-mm Petri dish under the dissection stereomicroscope to perform tissue harvest.

   Placing anesthetized tadpoles on a slightly damp Kimwipe tissue can help prevent them from sliding during dissections. Tissues for gene expression analysis need to be dissected on ice; place the 100-mm Petri dish containing the tadpole on top of a 150-mm Petri dish filled with ice. For genomic analyses, clean the Petri dish and dissection tools with Kimwipes and 70% ethanol between each individual.

Harvesting External Tissues

Tail and Head (Fig. 1B,C)

The tail and head are the easiest and most common tadpole organs to harvest. The tail has fewer tissues than the head, and these organs have different gene expression responses during metamorphosis. The size of the tail increases during growth and the ratio of tail skin to tail muscle changes through ontogeny; both of these features need to be taken into account when collecting tissue to maintain comparable results across treatments or developmental stages. Unlike the other tissues, a stereomicroscope is not needed to harvest the tail or head.

4. Use forceps to hold the tadpole in place while harvesting the tail and head.

   i. Using a razor blade, cut off the tail with a transverse cut posterior to the hind limbs.

   ii. Using a razor blade, cut off the head posterior to the eyes and anterior to the thymus glands (solid line in Fig. 1B).

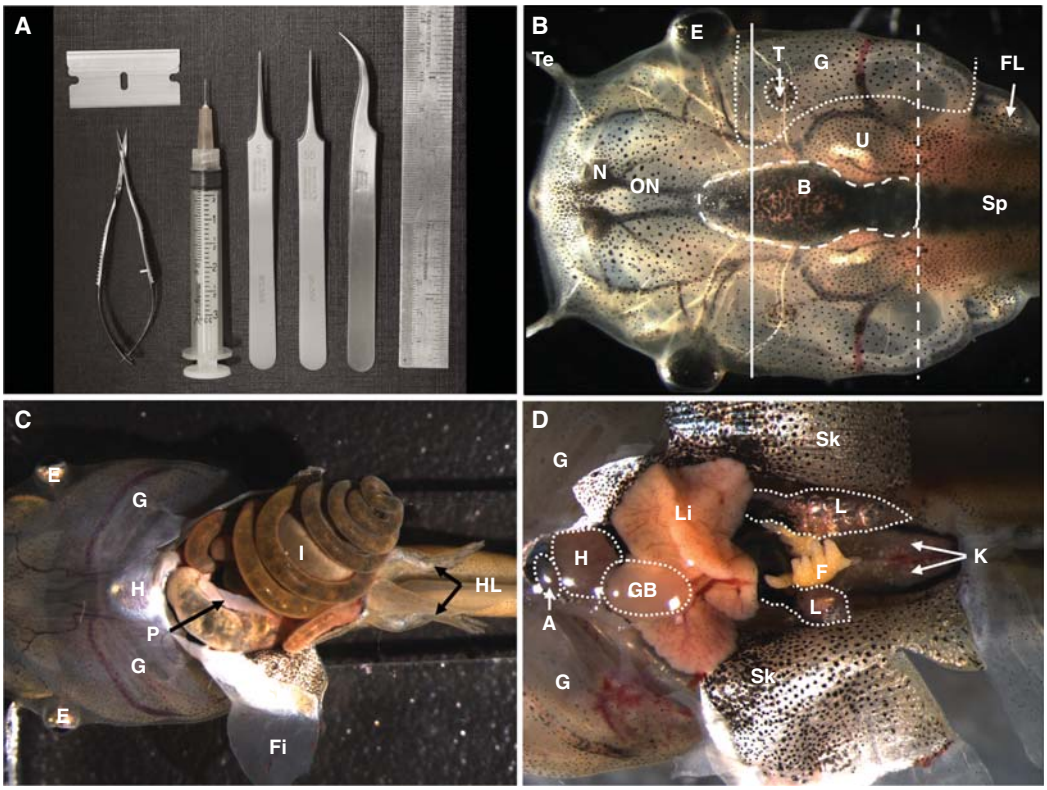

FIGURE 1. Dissection tools and tadpole organs in situ. (*A*) The dissection tools pictured are sufficient to harvest all tissues in this protocol except blood, which also requires heparinized capillary tubes (ruler shown for size). (*B*) In this dorsal view of the tadpole head, the organs are labeled only on the *right* side for clarity. The solid line is the cut site to harvest the head. The straight dashed line is the first cut site to harvest the brain, and the curved dashed line outlines the location of the brain. The dotted line is the cut site to harvest the gills, and the dotted circle outlines the thymus. (*C*) Ventral view of tadpole with skin and abdominal muscle reflected to the sides. Heart is still in pericardial cavity. (*D*) Ventral view of tadpole with intestine, pancreas, and spleen removed. The *right* lung is sharply curved upward and thus appears shorter than the left lung. The tadpole is Nieuwkoop and Faber (NF) stage 56 (Nieuwkoop and Faber 1994). Abbreviations: (A) aorta, (B) brain, (E) eye, (F) fat bodies, (Fi) fin on abdomen, (FL) forelimb, (G) gill, (GB) gallbladder, (H) heart, (HL) hind limb, (I) intestine, (K) kidney/gonad complex, (L) lung, (Li) liver, (N) nostril, (ON) olfactory nerve, (P) pancreas, (Sk) skin, (Sp) spine, (Te) tentacle, (T) thymus, (U) ultimobranchial body.

Eyes (Fig. 1B)

Eyes change during metamorphosis in position and in photopigment expression.

5. Secure the anesthetized tadpole by piercing the head along the midline with the two points of a pair of forceps.
6. Use curved forceps to partially pinch below the ball of the eye.
7. Slowly separate the eye from the body.
8. Carefully complete the pinching action below the eye as the eye is being removed.
   Take care while lifting the eye out to ensure that it separates cleanly from the optic nerve and connective tissue remains held by the curved forceps.

Tail Skin

Tail skin is more challenging to obtain than whole tail, but a highly uniform tissue (i.e., almost exclusively epidermis) is the result. The tail muscle (with spinal cord and notochord) can be harvested at the same time, if desired.

9. Use a razor blade to obtain the tail as described in Step 4.i.
10. Secure the tail by inserting open forceps into the muscle of the tail through the cut site, being careful not to pierce or break the skin.

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Maintain a secure hold of the tail muscle with open forceps for the rest of the procedure.

11. Using curved forceps, pinch the tail skin at the cut end. Slowly pull a few millimeters of the skin toward the posterior end of the tail.
12. Turn the tail over (without releasing the muscle). Slowly pull the skin toward the posterior of the tail.
13. Repeat Steps 11–12, turning the tail over periodically to release the skin evenly down the length of the tail.

If done carefully, the skin will release from the muscle like pulling off a glove. The skin becomes difficult to separate from the muscle at the posterior tip of the tail and thus is not collected.

Back Skin (Fig. 1B)

The back skin has a distinct developmental fate compared with the tail skin and skin covering the gills (Watanabe et al. 2002; Suzuki et al. 2009). The back skin will transform from the larval to adult skin by the end of metamorphosis rather than being resorbed like the skin covering the tail and gills.

14. Pinch up an edge of back skin to be harvested with forceps.
15. Use scissors to cut a small hole through the skin.
16. Reposition the forceps to hold the skin at the hole.
17. Use scissors to cut out the desired patch of skin, repositioning the forceps as necessary.

Make sure to avoid the skin covering the gills (Fig. 1B) because that skin remains larval in character and is resorbed during metamorphosis.

Gills (Fig. 1B)

Gills—along with tail, pronephros, tentacles, and the overlying integument—are strictly larval structures that resorb completely at metamorphosis. The gills are comprised of a fine cartilaginous network covered with epithelium all enclosed within larval skin. The forelimbs and thymus are found in the posterior and central portion, respectively, within the gill compartment. The thymus is embedded in a space in the gill cartilage and will remain with the gills unless specifically removed.

18. Secure the anesthetized tadpole by piercing the head along the midline with the two points of a pair of forceps.
19. Using microdissection scissors, cut from the posterior side of the eye toward the brain (dotted line in Fig. 1B).
20. Hold the tadpole with forceps through the mouth. Cut posteriorly along the side of the brain to release the gills.

The ultimobranchial bodies can be easily pierced, releasing a milky substance. The skin and thymus (see below) can be removed to obtain a less complex tissue mixture.

21. Repeat Steps 18–20 to harvest gills on the other side.

Thymus (Fig. 1B)

The thymus is particularly delicate and extremely easy to burst if not handled with caution. It decreases in size dramatically during metamorphosis, likely in response to high glucocorticoid levels. Repositioning of the thymus after gill resorption makes the thymus more difficult to locate in later stages of metamorphosis.

22. Secure the anesthetized tadpole by piercing the head along the midline with the two points of a pair of forceps.
23. Using microdissection scissors make two transverse cuts completely through the gill, one anterior and the other posterior to the thymus.

These cuts should begin at the lateral edge of the gill and extend to a point toward the midline beyond the thymus, but not into the brain.

24. Using microdissection scissors, make a longitudinal incision connecting the end points of the first two transverse cuts, going straight down with the scissors through the dorsal surface.
25. Fold back the skin/cartilage flap created to expose the thymus, which is still encapsulated by internal gill tissue.
26. Use forceps to secure the opened gill area.
27. Using microdissection scissors, carefully trim away the jelly-like gill tissue from the surface of the thymus.

    It is very difficult to remove 100% of the gill tissue without causing the thymus to burst.

Hind and Forelimbs (Fig. 1B,C)

Limb buds form early in the larval period but require thyroid hormone produced during metamorphosis to grow and develop beyond the paddle stage. Larger limbs are less fragile and can be gripped anywhere along the length of the limb and cut off with scissors.

28. With two pairs of forceps positioned next to each other, pinch at the base of each hind limb to separate the limbs from where they join the posterior part of the abdomen. Expose the forelimbs from underneath the gill skin with forceps or scissors, then remove them as for hind limbs.
    i. Use the forceps to pinch at the base of the hind limb to separate it from where it joins to the posterior part of the abdomen.
    ii. Expose the forelimbs from underneath the gill skin using forceps or scissors.
    iii. Use the forceps to pinch at the base of the forelimb to separate it from the torso.

Harvesting the Brain, Heart, and Blood

Brain (Fig. 1B)

The brain is composed mostly of neurons and glia, but is highly heterogeneous in gene expression (Yao et al. 2008; Denver et al. 2009). Also, the pituitary is found in a pocket in the ventromedial region of the brain and is not readily separated by accident from the rest of the brain in this procedure. The spinal cord can also be harvested (Muñoz et al. 2015).

29. Secure the tadpole with forceps. Using a razor blade, make a single transverse cut at the base of the hindbrain, just anterior to the spinal cord (dashed line in Fig. 1B).

    For the next three steps, maintain hold of the head dorsally with a pair of forceps inserted through the anterior gills on both sides of the brain.

30. Using scissors, make straight cuts through the dorsal skin and cartilaginous braincase along the left and right sides of the brain.
31. Expose the brain by lifting the skin/cartilage flap up with forceps.
32. Insert closed forceps under the brain. Gently push the brain upward, taking care to allow the cranial nerves to break in the process.

    The ultimobranchial body can be pierced easily and release a milky substance.

Heart (Fig. 1C,D)

The heart is located along the midline, enclosed in a silver-colored pericardium just anterior to the peritoneal cavity. Very few studies have been performed on the heart larval-to-adult transition.

33. With the ventral side up, use two pairs of forceps to carefully tear and peel away the clear epidermis and silvery pericardium that covers the heart.
34. With the heart exposed, gently slide the forceps underneath it. Grasp it at the point where it connects to major arteries.
35. Pinch the forceps to cut the heart free of the arteries. Lift the heart out of the heart cavity, ensuring that it tears free cleanly.

    Assist the process by pinching any attached arteries with a second pair of forceps.

Blood

Because of the small blood volume of tadpoles, it is typical to obtain heparinized plasma rather than serum. This procedure should be done in <3 min to avoid altered stress hormone levels.

36. Expose the heart as described in Step 33.

    The heart should still be beating.

37. Use an absorbent tissue to blot dry the cardiac area.

38. With all interstitial fluid removed, position a heparinized capillary tube at a 45° angle to the aorta (Fig. 1D).

39. Nick the aorta with forceps to allow the blood to flow into the tube by capillary action.

    As the blood rises into the capillary, it might be necessary to decrease the angle to continue collection.

40. Transfer the blood into a fresh Eppendorf tube on ice by blowing it from the capillary tube with a P200 pipettor fitted with a yellow tip.

41. Centrifuge at 4000–6000 rpm for 20 min at 4°C.

42. Carefully remove the supernatant plasma using a P200 pipette. Store at −80°C.

    To collect a sufficient volume for measurements, blood from 2–3 tadpoles can be pooled before centrifugation.

Harvesting Peritoneal Tissues

Access the Peritoneum

The peritoneal cavity is accessed through two tissue layers: the ventral skin and the abdominal muscle wall. Both layers are thin and can be cut one at a time or simultaneously in the steps below. Sufficiently long cuts will allow the skin and muscle wall to be reflected back to expose the peritoneal organs, although only the large coiled intestine will be visible initially.

43. With forceps, pinch/grasp the ventral fin connected along the surface of the peritoneal cavity. Maintain this hold for the next step.

    The ventral fin regresses at later stages of metamorphosis. It is thus necessary to pinch the posterior abdominal skin directly at later stages.

44. Snip open a small hole into the posterior end of the peritoneal cavity.

    Care must be taken not to lacerate the intestines that lie just beneath the thin skin and muscle wall covering the peritoneal cavity.

45. While still holding the skin with forceps at the cut site, make lateral cuts with scissors through the skin and muscle wall along the posterior border of the peritoneum.

46. Maintaining hold with the forceps, make a posterior to anterior cut with scissors through the skin and muscle up the midline of the peritoneal cavity.

    Two pairs of good quality forceps can also serve to make several tears to similarly open the cavity.

Intestine (Fig. 1C)

The gastrointestinal tract consists of the esophagus, manicotto glandulare, intestine, and rectum. The manicotto is a tadpole-specific organ that is resorbed, and the stomach develops in that location during metamorphosis (Griffiths 1961). After intestinal remodeling that occurs during metamorphosis, a mesentery binds the loops of the greatly shortened intestine such that it cannot be straightened without many small cuts to the mesentery. For gene expression and histology, it is often important to flush the intestine of its contents.

47. Without damaging it, gently push but not uncoil the intestinal coil out of the cavity with forceps.

    The coil should roll out of the cavity while remaining attached at each end.

48. Clip the posterior end of the intestine with forceps where the intestine meets the rectum.

49. Clip the anterior end of the intestine around the level of the pancreas, where the pancreatic duct enters the gastrointestinal tract.

50. To flush the intestine, gently grip the open end of the intestine with forceps without pinching it closed.
51. Holding the intestinal opening at a ~45° angle, insert a 30-gauge needle attached to a 3-mL syringe filled with 0.6× PBS into the opening.

    It is important to get the angle of needle and intestinal opening aligned to avoid piercing the side of the intestine with the needle's point.

52. Carefully secure the intestine onto the needle by gently squeezing the forceps. Use the syringe to slowly flush the gut contents from the intestine with 0.6× PBS.

    The intestine can be cut into two or more pieces to facilitate flushing the gut contents which will help avoid rupturing the intestinal wall.

Pancreas (Fig. 1C)

The white translucent pancreas is closely associated with the anterior loop of the intestine at the manicotto glandulare. The pancreas decreases greatly in size as it remodels to the adult version during metamorphosis. Like the liver, the pancreas breaks apart easily when pulled on with forceps.

53. Using two pairs of forceps, gently separate the pancreas from where it joins the intestine.
54. Once completely free of the intestine, gently grasp the pancreas with the forceps. Lift it out of the cavity.

    Do not pull on the pancreas directly with forceps as it will just tear and remain attached to the intestine.

Liver (Fig. 1D)

The tadpole liver is yellow/orange in color, has three lobes, and is located in the anterior portion of the peritoneal cavity. The liver is fairly small at early developmental stages but greatly increases in size toward the end of metamorphosis. Like the pancreas, it breaks apart easily when pulled with forceps. Embedded within the tadpole liver is the larval hematopoietic tissue, which is gradually replaced by adult hematopoietic stem cells during metamorphosis.

Because bile can interfere with procedures associated with gene expression analysis, care must be taken not to pierce the gallbladder while removing the liver.

55. Push the intestine out of the peritoneal cavity to reveal the anteriorly located liver.
56. Gently position curved forceps underneath the liver. Squeeze the curved forceps forcefully beneath, but not on, the liver.
57. While holding the curved forceps firmly, lift the liver out of the cavity by tearing the connections grasped by the forceps.

    If the liver does not come out in one piece, it can be removed in multiple pieces. Nevertheless, care should be taken not to shred the organ.

58. Use two pairs of forceps to clear away any extra tissue that might have been removed with the liver (e.g., the gallbladder, which is closely associated with the liver).

Spleen

The spleen is a tiny red ball found among the intestinal coils secured in place by two thin, black strands of connective tissue. It is not closely associated with any organ.

59. Carefully lift out coils of intestine until the spleen is located.
60. Using forceps, grasp the black strands of connective tissue that hold the spleen in place. Pull out the spleen.

    Do not use forceps to grasp the spleen directly, as it will rupture and release lymphocytes.

Lungs (Fig. 1D)

The lungs are small and inconspicuous in small tadpoles but grow in later staged tadpoles to extend the entire length of the peritoneal cavity and fill with air (Rose and James 2013).

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61. Push the intestine out of the peritoneal cavity.
62. Remove the liver as described in Steps 56–57 to reveal the lungs in the anterior dorsolateral parts of the peritoneal cavity.

    The anterior end of the intestine will remain situated medially between the two lungs adjacent to the trachea.

63. To better access the lungs, use forceps to clip the anterior end of the intestine.
64. Secure the tadpole in place by piercing the head with the two points of the forceps.
65. Grasp the trachea with forceps. Gently pull upward, from anterior to posterior, to remove the lungs from the peritoneal cavity as a pair.

    The connective tissue of the lungs is fairly strong such that pulling the trachea can be done without damage to the lungs in nearly all cases.

66. Use forceps to trim any nonlung tissue away from the harvested lungs.

    Do not mistake the bronchial diverticula (Wassersug and Souza 1990) for nonlung tissue. These structures exist as small offshoots of the lung bronchii, which often contain small air bubbles in the anterior region of each lung.

Fat Bodies (Fig. 1D)

The abdominal fat bodies are yellow, finger-like structures that emanate from a pair of bilateral stalks at the anterior end of the kidney/gonad complex. When fat globules are released from the fat bodies, they spread quickly and glisten in the light. The size of the fat bodies generally increase during the larval period correlated with food intake.

67. Push the intestine out of the peritoneal cavity to reveal the fat bodies.
68. Grasp the stalks connecting the fat bodies to the kidney/gonad complex with forceps. Pull out to harvest.

    The kidneys might come out with the fat bodies, but they can be separated using forceps.

Kidney/Gonad Complex (Fig. 1D)

The kidney/gonad complex is located at the dorsal posterior region of the peritoneal cavity and is fairly well attached to the ventral side of the dorsal trunk muscles. The gonads are inconspicuous, thin translucent strands running nearly the length of the ventral side of kidney. The gonads are fragile and are not easily separated from the kidneys in the tadpole stages. Steroidogenic tissue producing glucocorticoids and mineralocorticoids (called "interrenal tissue" and homologous to the adrenal gland of mammals) is contained within the kidney capsule.

69. Push the intestine out of the peritoneal cavity. Remove the liver as described in Steps 56–57 to reveal the fat bodies and kidney/gonad complex.
70. Remove the fat bodies from the kidney/gonad complex by pinching and breaking the stalk of the fat bodies with two pairs of forceps.
71. Carefully separate the kidney/gonad complex by running a closed pair of forceps between the kidney and the back muscles.

    The kidney/gonad complex can break easily if pulled with forceps before this step.

72. Once separated from the back muscles, gently lift the kidneys from the peritoneal cavity.

DISCUSSION

The *Xenopus* tadpole is a well-established and valuable vertebrate model for hormone-dependent growth and development, molecular mechanisms of gene expression in vivo, and regeneration in tail, limbs, and spinal cord (Beck et al. 2009; Buchholz 2015; Sachs and Buchholz 2017). Most tadpole tissues have been examined to some extent to determine their function during tadpole life and to determine their hormone-dependent changes throughout metamorphosis (Pouyet and Beaumont 1975; Dodd and Dodd 1976; Fox 1983; Gilbert et al. 1996; Brown and Cai 2007). Many organs have been cultured in vitro, including tail, hind limbs, skin, intestine, liver, pancreas, lung, fat

bodies, spinal cord, and gills (Derby 1968; Hanke 1978; Derby et al. 1979; Richmond and Pollack 1983; Mathisen and Miller 1989; Ishizuya-Oka and Shimozawa 1991; Tata et al. 1991; Helbing et al. 1992; Buchholz and Hayes 2005; Veldhoen et al. 2015). Supplementary anatomical aspects of tadpole skeletal and soft tissue structures are available (McDiarmid and Altig 1999).

Virtually all *Xenopus* tadpole tissues can be readily harvested. After harvest, tissues can be used in numerous types of analyses. For gene expression or hormone analysis, tissues must be harvested on ice and snap frozen immediately by placing the tissue with forceps into a prelabeled 1.5 mL Eppendorf tube that has been in direct contact with dry ice and then kept at −80°C for storage until assay. For histology and in situ hybridization, tissues can be fixed in ethanol, neutral buffered formalin, MEMFA, or other fixatives (Sive et al. 2000). Standard procedure for organs to be processed for immunohistochemistry is fixation in 4% paraformaldehyde at room temperature for 2 h (or overnight at 4°C) with rotation.

For organ culture, the sterility of internal organs such as liver and spleen must be maintained during the tissue harvest procedures. Before incision, treat the dissection tools and ventral integument with 70% ethanol. Care should be taken during dissection to avoid rupturing the intestine and thus releasing bacteria into the cavity. For external structures and the intestine, tadpoles can be pretreated overnight in sulfadiazine to inhibit bacterial growth, and harvested tissues are placed in sterile 0.6× L15 culture medium supplemented with antibiotics and antimycotics (Derby 1968; Buchholz and Hayes 2005).

RECIPE

Phosphate-Buffered Saline (PBS)

Reagent	Amount to add (for 1× solution)	Final concentration (1×)	Amount to add (for 10× stock)	Final concentration (10×)
NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM
If necessary, PBS may be supplemented with the following:				
$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1× solution or as a 10× stock. To prepare 1 L of either 1× or 10× PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.

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Matthew D. Patmann, Leena H. Shewade, Katelin A. Schneider and Daniel R. Buchholz

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