Protocol

In Vitro Induction of *Xenopus* Embryonic Organs Using Animal Cap Cells

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The animal cap—the presumptive ectoderm of the blastula embryo—can differentiate into a variety of tissues belonging to the three germ layers following exposure to specific inducers. The “animal cap assay” was devised based on the pluripotency of presumptive ectodermal cells and enabled many important discoveries in the field of embryonic induction and cell differentiation. Using this system, investigators can test multiple factors in solution simultaneously to determine their inducing activities qualitatively, quantitatively, and synergistically. Furthermore, after dissociation and induction, reaggregated animal cap cells can be induced to form higher-order organs. This protocol details preoperative preparations, followed by the basic animal cap assay. Advanced protocols for the induction of kidney, pancreas, and heart are also described.

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

Activin solution (0.5–100 ng/mL human recombinant activin A [e.g., Merck Millipore, 114700] in BSA-CM)

Store stock solutions (>100 ng/mL) at –80°C. Avoid repeated freeze–thaw cycles.

BSA-CM (0.1% [w/v] bovine serum albumin [BSA] in CM)

Adding BSA to CM reduces adsorption of inducing substances or embryonic tissues to the surfaces of plastic culture plates.

BSA-CMFCM

Ca²⁺/Mg²⁺-free BSA-CM is used to dissociate animal cap cells.

Dejelling solution

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Dissolve 4.5% (w/v) cysteine-HCl or 1% (w/v) sodium thioglycollate in CM. Adjust the pH to 7.8–8.0 with NaOH. Prepare fresh before use.

Embryonic *Xenopus* culture media (CM) <R>

There are a number of media suitable for culturing embryonic *Xenopus* tissues.

Retinoic acid stock solution (10 mM all-trans retinoic acid [e.g., Sigma-Aldrich, R2625] in dimethyl sulfoxide) (RA)

Store 1-mL aliquots at -80°C . Avoid repeated freeze–thaw cycles.

Xenopus laevis embryos

Detailed descriptions of the methods used to obtain embryos are presented in Ariizumi et al. (2000, 2009).

Equipment

Beakers, 50- and 100-mL, sterilized

Illuminator

Fiber-optic LED lights are preferred.

Incubator, 20°C – 22°C

Microscope, binocular, equipped with 10 $\times$ oculars and 1 $\times$ to 4 $\times$ objectives

Operation dishes

To prevent embryonic tissues from adhering to glass or plastic surfaces, line Petri dishes with autoclaved 3% (w/v) agar.

Pipette bulb, silicon

Sterilize in 70% ethanol before use.

Tissue culture plates, low-adhesion, 24-well (e.g., Sumitomo Bakelite, MS-80240Z)

Tissue culture plates, low-adhesion, round-bottomed, 96-well (e.g., Sumitomo Bakelite, MS-3096UZ)

Transfer pipettes

To prepare, flame a Pasteur pipette at its center and draw it out at a 30° – 40° angle. Cut pipettes such that the opening is 0.5–2-mm diameter. Flame the tip briefly to smooth the cut edges. Heat-sterilize for 2 h at 180°C .

Tungsten needles

Insert a 0.2-mm tungsten wire (2-cm length) into a transfer pipette (2-mm i.d.). Flame to seal the wire in place. Bend the wire at a right angle; cut 3–5-mm from its end. Sharpen the wire electrolytically using 5 M NaOH and a 9V-dry cell (submerge the negative pole on a carbon point in the NaOH solution; attach the positive pole to the tungsten wire). Heat-sterilize for 2 h at 180°C .

Watchmaker's forceps (e.g., Fontax no. 5)

Heat-sterilize for 2 h at 180°C .

METHOD

Membrane Removal

To access the animal cap, the jelly coat (see online Movie 1 at cshprotocols.cshlp.org) and vitelline membrane (see online Movie 2 at cshprotocols.cshlp.org) surrounding the embryo must be removed.

1. Collect embryos in a sterilized 100-mL beaker. Add 50 mL of dejelling solution.
2. Swirl for 5–10 min. Decant the dejelling solution when the jelly-free embryos begin to pack together.
3. Rinse 10 times by swirling gently with the CM of choice.
4. Select blastula embryos at the desired stage of development (see Nieuwkoop and Faber 1994). Use a 2-mm diameter transfer pipette and a sterilized pipette bulb to place the embryos into an agar-lined Petri dish containing CM.

5. Holding the embryo upside-down, use fine forceps to grasp the vitelline membrane. Gently tear the membrane from the embryo.

Animal Cap Dissection

The procedure is shown in online Movie 3 at cshprotocols.cshlp.org.

6. Place the blastula embryo with the animal pole facing upward in an operation dish filled with the CM of choice.
7. Using a tungsten needle, trim both sides of the embryo.
8. Insert the needle into the blastocoel from one side. Divide the vegetal hemisphere (i.e., the endodermal region) by pushing down the needle.

This produces a sheet of blastocoel roof with cell masses at each end.

9. Reverse the sheet. Trim away vegetal yolky cells and marginal zone cells.
10. Trim the sheet (i.e., the animal cap) into a 0.5 × 0.5-mm square.

Animal Cap Culture

The procedure is shown schematically in online Movie 4 at cshprotocols.cshlp.org. Use a transfer pipette (0.5- to 1-mm diameter) to handle the animal caps.

11. Transfer five to 10 animal caps into activin solution of the desired concentration. Place them such that the inner blastocoel side faces upward. Incubate for a defined period (e.g., 3 h).

Depending on the concentration used, activin can induce a variety of tissues and organs (Table 1).

12. Transfer the animal caps to a dish filled with BSA-CM. Wash them by pipetting gently.
13. Incubate the caps in fresh BSA-CM for 3–4 d at 20°C–22°C.

The explants will show obvious histodifferentiation patterns at the end of the culture.

Kidney Induction

Simultaneous treatment with RA and activin can induce the generation of the pronephros (i.e., the embryonic kidney) (see online Movie 5 at cshprotocols.cshlp.org). The original protocol (Moriya et al. 1993) used Steinberg's Solution as the CM.

14. Transfer 10 animal caps from late blastulae (i.e., stage 9) to a well of a 24-well tissue culture plate containing "Test Solution K" (i.e., 10 µL of RA stock solution and 990 µL of 10-ng/mL activin solution). Incubate for 3 h at 20°C.
15. Wash the caps in BSA-CM. Culture in fresh BSA-CM for 3 d at 20°C.

After 3 d, pronephric tubules should be observable inside the thin epidermal vesicle.

Pancreas Induction

Sequential treatment with activin and RA can induce formation of the pancreas (see online Movie 5 at cshprotocols.cshlp.org).

16. Transfer 10 animal caps from late blastulae (i.e., stage 9) to a well of a 24-well tissue culture plate containing "Test Solution P1" (100-ng/mL activin solution). Incubate for 1 h at 20°C.
17. After washing briefly with BSA-CM, incubate in fresh BSA-CM for 5 h at 20°C.
18. Transfer the animal caps to "Test Solution P2" (i.e., 10 µL of RA stock solution in 990 µL of BSA-CM). Incubate for 1 h at 20°C.
19. Wash the animal caps with BSA-CM. Culture in fresh BSA-CM for 3 d at 20°C.

*Pancreatic differentiation can be characterized by histological examination and/or the expression of molecular markers (e.g., *pdx1*, *insulin*).*

TABLE 1. Examples of tissues and organs induced from *Xenopus* animal caps by activin and other factors

Tissues and organs	Methods	Notes	Markers	References
Ectoderm				
Epidermis	Animal cap assay (Steps 11–13)	Induced by 0.5–50 ng/mL activin	<i>xk81a1</i>	Asashima et al. 1990; Ariizumi et al. 1991
Brain (forebrain, hindbrain); Spinal cord	Combination of treated and untreated animal caps	Induced from untreated animal caps by animal caps treated with >50 ng/mL activin	<i>otx2, sox2, foxg1</i>	Ariizumi and Asashima 1994
Eye (lens, neural retina, retinal pigment epithelium); Ear vesicle	As above	Induced secondarily from brain	<i>pax6, six3, rax</i>	Ariizumi and Asashima 1994; Sedohara et al. 2003
Mesoderm				
Notochord	Animal cap assay (Steps 11–13)	Induced by high concentrations (>50 ng/mL) of activin	<i>chrd, not</i>	Asashima et al. 1990; Ariizumi et al. 1991
Skeletal muscle	As above	Induced by intermediate concentrations (5–50 ng/mL) of activin	<i>myod, myf5, mrf4</i>	Asashima et al. 1990; Ariizumi et al. 1991
Coelomic epithelium	As above	Induced by low concentrations (0.5–5 ng/mL) of activin	—	Asashima et al. 1990; Ariizumi et al. 1991
Blood cells; Blood vessel	As above	Induced by low concentrations (0.5–1 ng/mL) of activin	<i>runx2, spib, mpo</i>	Asashima et al. 1990; Ariizumi et al. 1991; Miyanaaga et al. 1999; Nagamine et al. 2007
Pronephros (glomus, tubule, duct)	Multiple treatments with activin and retinoic acid (Steps 14–15)	Induced by simultaneous treatment with 10 ng/mL activin and 0.1 mM retinoic acid	<i>wnt4, pax2, sall1, wt1</i>	Moriya et al. 1993; Chan et al. 1999; Osafune et al. 2002
Heart	Dissociation and reaggregation of animal cap cells (Steps 21–24)	Individual cells are treated with 100 ng/mL activin during reaggregation	<i>nkx2.5, gata4, tbx5, tnni3</i>	Ariizumi et al. 2003
Craniofacial cartilage	Combination of treated and untreated animal caps	Induced from untreated animal caps by animal caps treated with 100 ng/mL activin	<i>dll4, col2a1, alx1</i>	Furue et al. 2002
Endoderm				
Pharynx; Intestine	Combination of treated and untreated animal caps	Animal caps treated with >50 ng/mL activin form pharynx and intestine in recombinants	<i>fabp2, darmin</i>	Ariizumi and Asashima 1994
Pancreas	Multiple treatments with activin and retinoic acid (Steps 16–19)	Induced by sequential treatment with 100 ng/mL activin and 0.1 mM retinoic acid	<i>pdx1, mist1, ins</i>	Moriya et al. 2000
Liver	Animal cap assay (Steps 11–13)	Yolk-rich endoderm induced by 100 ng/mL activin forms liver when transplanted into larvae	<i>hhex, ambp, transferrin, albumin</i>	Ariizumi et al. 2003

Dissociation and Reaggregation of Animal Caps for Heart Induction

Cell-to-cell interactions can be studied by examining the activity of animal caps dissociated into individual cells. Here, we describe a dissociation/reaggregation protocol to induce heart formation (see online Movie 6 at cshprotocols.cshlp.org). The original method (Ariizumi et al. 2003) used Holtfreter's Solution as the CM.

20. Dissect five to 10 animal caps from mid-blastulae (i.e., stage 8) in an operation dish filled with CM.
21. Transfer the caps to a 35-mm diameter Petri dish containing BSA-CMFCM.
This is crucial to eliminate Ca^{2+} and Mg^{2+} cations that might be transferred from the operation dish.
22. Transfer five to 10 animal caps into a single well of a 96-well tissue culture plate containing 100 μL of BSA-CMFCM. Incubate for 20 min.
23. Replace the BSA-CMFCM with 100 μL of 100-ng/mL activin solution. Disperse the cells by pipetting gently. Incubate for 5 h.
24. Transfer the newly formed spherical “reaggregates” into a single well filled with 200 μL of BSA-CM.

The reaggregated cells will begin to beat rhythmically within 3 d at 20°C.

DISCUSSION

When treated with a mesoderm-inducer such as activin, animal caps are competent for in vitro organogenesis from stage 7 (early blastula) up to stage 9 (late blastula). Thus, accurate staging of embryos is vital to the success of in vitro organogenesis. The late blastula (stage 9) is used as the standard for the animal cap assay, although for the heart induction described in this protocol, mid-blastula embryos (stage 8) are most suitable. The size of the animal caps is also critical. The number of cells in the explant will affect the efficacy of tissue differentiation and organ formation. A large cap might be contaminated with cells from the marginal zone, which can differentiate autonomously into mesodermal tissues. The most reliable animal cap size is 0.5×0.5 mm. Finally, the duration of the exposure of the animal caps to an inducer can influence the differentiation pattern. For example, a brief exposure (10 min) to 10-ng/mL activin causes the differentiation of ventral mesoderm, whereas a longer exposure (>3 h) to the same dose induces dorsal mesoderm differentiation (Ariizumi et al. 1991).

RECIPE

Embryonic Xenopus Culture Media (CM)

Components (mM)	Holtfreter's solution	Modified Barth's solution ^a	Marc's modified Ringers ^a	Normal amphibian medium ^a	Steinberg's solution
NaCl	60.00 ^b	88.00	100.00	110.00	60.00 ^b
KCl	0.67	1.00	2.00	2.00	0.67
CaCl ₂	0.90	0.41	2.00	—	—
Ca(NO ₃) ₂	—	0.33	—	1.00	0.34
MgCl ₂	—	—	1.00	—	—
MgSO ₄	—	0.82	—	1.00	0.83
EDTA	—	—	—	0.10	—
Sodium phosphate, pH 7.50	—	—	—	2.00	—
NaHCO ₃	0.20 ^d	2.40	—	1.00	—
HEPES	4.60 ^d	10.00	5.00	—	10.00
pH	7.60	7.40	7.40	7.40	7.40

^aUse at half-strength (i.e., 0.5×) for culture media.

^bTo prevent animal caps from curling, increase the NaCl concentration from 60 mM to 90 mM. However, note that animal cap cells will differentiate into neural tissue autonomously when exposed to high NaCl concentrations.

^cOmit these components when preparing Ca²⁺/Mg²⁺-free culture media (CMFCM).

^dFor Holtfreter's Solution, use *either* NaHCO₃ *or* HEPES.

Sterilize the CM of choice by autoclaving for 20 min at 121°C. Add antibiotics such as kanamycin sulfate (0.1 g/L) to avoid bacterial contamination.

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