

Aplysia Cell Culture

See our JOVE video at:

<http://www.jove.com/index/Details.stp?ID=1355>

Necessary solutions/reagents/supplies:

To anesthetize animals:

0.35 M MgCl₂

To prepare culture dishes:

0.1M Na Borate pH 8.2

0.5 mg/ml poly-l-lysine in 0.1 M Na Borate pH 8.2: can make up 100-200 mls, filter sterilize with 0.2 um filter, and keep at 4°C. Poly-l-lysine should be MW approximately 350,000 daltons (not below 180,000 or about 500,000 daltons): from Sigma use catalog #P-1524.

Artificial Sea Water (ASW): use Instant Ocean

Culture medium: L15 medium (Sigma catalog #P-4333). Need to add salts to this (remember, Aplysia lives in sea water, so osmotic strength of any solution needs to be around 1000 mOsmoles).

To 500 mls of L15 add, in the following order:

NaCl	6.25 g.
Dextrose	3.12 g
MgSO ₄ *7H ₂ O	3.12 g
KCl	172 mg
NaHCO ₃	96 mg
MgCl ₂ *6H ₂ O	2.85 g
CaCl ₂	744 mg

The pH should be about 6.9. Once into solution, add 5 mls of 100X Pen/Strep solution, and filter sterilized through a .22 u filter. Label L15 + salts+ pen/strep and put date. Keep at 4°C.

To digest connective tissue sheath:

10 units/ml Protease XIV in L15 plus salts: from Sigma use catalog # P-5147. Must be made fresh each day

For culture medium:

L15 with salts (see attached sheet): keep at 4°C. From Sigma, use L15 catalog #P-4333

Hemolymph: must be stored at -80°C—not at -20°C. See below re: hemolymph.

200 mM glutamate: keep frozen at -20°C, and add to medium day of culture (has half-life of only a few days). From Sigma use catalog # G-7513, aliquot into 1 ml aliquots for freezer and discard after thawing.

100X penicillin/streptomycin: keep frozen at -20°C , and add to 500 ml L15 with salts. From Sigma use catalog # P-4333. Aliquot into 5 ml aliquots for freezer.

Animals:

For sensory neurons: **80-100 g *Aplysia californica***

For L7 neuron: **1-4 g. *Aplysia californica***

Dishes:

1. Large **dissecting pans** for anesthetizing animals and removing ganglia (Fisher catalog # 09-002-20)
2. **60 mm dishes with Sylgard** for pinning down ganglia (Buy Sylgard from World Precision Instruments, catalog number Sylg184. 60 mm dishes from VWR are catalog #25384-060—any 60 mm Petri dish will do(does not have to be expensive tissue culture dish!!!)
3. **Culture dishes** with glass well (Mattek cat. # P50G-0-14-F...note these are expensive, \$1.00 a dish).

Electrodes/Pins for pinning down ganglia and pulling out cells:

Insect Pins for pinning down ganglia on sylgard dishes: use thinnest, finest pins available--from Fine Science Tools, use Catalog # 26002-10 (0.1 mm diam., 10 mm long). Most useful when cut in half—put in sylgard and cut off top half with scissors.

1.5 mm capillaries: from A-M systems use catalog #5968, 1.5 mm OD, 0.86 mm ID, 100 mm length. Pull on P-97 Sutter puller, using parameters to pull so that have a long, wispy fine tip of such high resistance that there is no capillary intake of fluid when placed in solution. The presence of a fluid meniscus means that the electrode tip is broken and will damage cell (do not use).

Tools:

Forceps with teeth for holding *Aplysia* skin while cutting open with scissors: e.g. Sigma cat #

Fine forceps for holding ganglia: from Fine Science tools cat # 11252-20 (Dumont #5 forceps--\$27.50 each).

Scissors for cutting animal open:

Small scissors for cutting out ganglia: from Fine Science tools Cat. # 14084-08 (\$55.50 each).

Extra delicate mini-vanna scissors for desheathing ganglia: from Fine Science Tools catalog # 15000-00 (\$241.50 each).

Very easy to rust after use with salt water. Rinse with ddH₂O and then with ethanol, dry carefully with Kimwipes.

Equipment:

Incubator for protease treatment, kept at 34.5°C .

Stereo Microscope with base illumination (I use a Zeiss Stemi 4000)

Fiber Optic Cable Illumination (I use a

Incubator to keep cultures at 18°C. This should be a very sturdy incubator so that the cultures do not experience vibrations/bumps etc. (I use a Forma Scientific Model)

Procedure:

For sensory cells: Remove the animal gently from the tank, always keeping it in sea water. I do not like to leave an excessive amount of time between taking the animal out of the tank and anesthetizing (usually 5-15 min). Anesthetize 80-100 g animal by injecting 0.35 M MgCl₂ into the animal using a 60 ml syringe and an 18 gauge needle (I change the needle every few days so that it doesn't get too dull). Enter the animal at an angle of about 35°, and do not enter too deeply. You do not want to enter the gut of the animal. Remember that the animal is basically a sac of hemolymph, and that is where you want to place the MgCl₂. The animal will become very distended and relaxed.

Pin the animal on a dissecting dish, with a pin at the head and the tail, and the foot facing up. Hold the foot with a toothed forceps and cut open with a surgical scissors. Cut through the skin and underlying connective tissue, but do not open the gut. Cut the full length of the foot. Pin the two sides down. Using a fine forceps and a fine scissors, cut out the pleural pedal ganglia (see drawing). I like to leave a fair amount of nerve (see drawing).

Place the ganglia in Protease, and put in an incubator that is set for 37 °. Usually this incubation is for 1 hr 50 min, but it is variable depending on the animal. The point of the protease treatment is to allow the connective tissue to be removed. If it is very difficult to desheath the ganglia, incubate for a longer time. If it is extremely easy and the cells are very “soft”, reduce the incubation time.

To wash out the protease, transfer the ganglia through several washes with L15 containing salts, or artificial seawater. Place in L15/50% hemolymph, which is the culture medium. All the protein in the hemolymph will immediately stop the protease action.

While the ganglia are being digested in protease, prepare culture dishes. The glass in the culture dish needs to be coated with poly-l-lysine, for at least one hour. I usually leave it in poly-l-lysine for overnight. The poly-l-lysine must be thoroughly removed by rinsing multiple times in artificial seawater (4-5 times). After removing the last artificial seawater wash, put 2 mls of 50% L15 (with salts)/50% hemolymph. This must be in the dish for at least one hour prior to plating cells, so that the hemolymph coats the dish. The mix of 50% L15 plus salts/50% hemolymph is historically referred to as 1:1.

A note about hemolymph: Lore has it that hemolymph collected from animals in the spring (mid-March to June) is best. I prefer to collect from large animals, around 1 kg, so that I can get at least 500 mls from an individual animal. I swaddle the animal in a

disposable underpad so that only a small portion of the animal is exposed, and then hold it while another person uses a sterile razor blade to make an incision in the exposed area. I then squeeze the animal so that the hemolymph squirts into a clean beaker, making sure that it does not contact the dirty skin of the animal. I usually rewrap the animal once or twice, make a new incision and really squeeze hard to get out as much hemolymph as possible (when doing a second incision, collect in a new beaker so that if it becomes contaminated e.g. with ink, you can still use the first beaker). Hemolymph from each animal is kept separately (i.e., do not pool hemolymph from different animals). Spin the hemolymph at about 2000 x g for 10 min to spin down any blood cells. When I have large volumes, I use a large Sorvall bottle and spin in the high speed centrifuge, when I have smaller volumes I spin in 50 ml tubes on a table top centrifuge. Aliquot the supernatant in 10 ml aliquots, label by animal (eg 1, 2, 3 or A,B,C), and store at -80. This is very important. When hemolymph has been stored at -20, it forms precipitates in medium. The growth factors in the hemolymph do not appear to have very long half lives, so make the 1:1 fresh each time you make cultures.

Transfer the protease treated ganglia to a 60 mm dish containing sylgard and 1:1. Under the dissecting microscope, using illumination from fiber optic cables, pin out the pleural pedal ganglia as shown below. Note that it is not necessary to pin the ganglion down at this point, and to desheath an unpinned ganglion. Use whichever way works best for you. Similarly, it is possible to use the base illumination as opposed to the light from the fiber optic cables.

Using a fine forceps and small Vanna scissors, lift carefully the connective tissue from the pleural ganglion, and cut away to expose the nerve cells. Be very gentle—make sure not to touch or damage the nerve cells. Cut the connectives between the pleural and the pedal ganglia and release the pleural ganglion...you may need to gently tease away the connective tissue. Using a glass pipette, transfer the ganglion to a new Sylgard containing dish with 1:1. It is very important that the desheathed ganglion never be exposed to air (ie that it always be in medium), otherwise the cells die. Using a fine insect pin (I like to cut mine in half so that they are shorter), pin the pleural ganglion to the Sylgard as shown below. Break the tip of an electrode to make a “post” at a short distance from the ganglion.

At this point, I switch to the base illumination. Using a sharp electrode pulled to a fine, wispy point (see above), pull out the cells one by one. Touch the cell body just off center (see picture) and slowly and steadily pull out of ganglion. Gently tap against the glass “post” to remove the cell from the electrode.

To transfer cells from the sylgard dissecting dish to a culture dish, use a microcapillary with a bulb (Drummond microcaps micropipets from Fisher Catalog No: 21-170F). I like to fire polish my own capillary glass for this purpose. This step requires a lot of practice as the cells are easily killed, most frequently by air bubbles. As a result, many people prefer to use a P10 pipetman.

When making sensory-motor connections, the motor cell is removed from the abdominal ganglion of 1-4 g animals (for the L7 motor neuron) or from the abdominal ganglion of 80-100 g animals (for lfs neurons). The ganglia are protease treated as above, except that when the ganglion is from the 1-4 g animal, the digestion should only be done for 1 hr 45 min. the ganglia are pinned to the sylgard dish as drawn below, and only partially desheathed to expose the L7 or lfs neurons. This is best observed by video.

When making sensory-motor cocultures, the motor cell is dissected first, and transferred to the culture dish first. Using a microelectrode, the axon of the motor neuron is manipulated so that it lies flat on the coverslip. It must sit on the stage undisturbed for at least 30 min prior to adding the sensory neuron in order to adhere to the coverslip. The sensory neurons are then (gently) added to the dish and manipulated so that their axons make contact with the motor neuron.

Cocultures must sit on the microscope stage undisturbed overnight before being moved to an 18 degree incubator. It is essential that the cultures not be disturbed during this period (I usually leave a big sign to warn against bumping the hood etc.). For studies of long-term facilitation, I use cultures between days 4-6 after plating. I do not change the medium.