

Tissue Recombination, Urogenital Sinus Preparation and Renal Capsule Grafting

Equipment List

Dissecting instruments (all from Fine Scientific Tools – 1-800-521-2109)

Large Scissors	14054-13
Small Scissors	14060-09
Vanna scissors	15100-09
Large forceps	11021-12
Medium Forceps	11027-12
No 3 Forceps	11231-30
No 5 Forceps	11252-30

Dissecting scope/light source.

Many potential sources. Some light sources are built in, other scopes require an external source. These techniques require low to medium magnification and a wide field of view.

100mm and 30mm petri dishes – bacteriological dishes are fine for this. They are cheaper than tissue culture coated plates.

Microconcavity slides – These are an off-catalog item from Fisher - number 1537 . In the United States they usually arrive in 3-4 weeks. I know that in Europe they can be hard to obtain.

Pregnant Animals: For urogenital sinus dissection.

Timed pregnant – plug date is day 0.

Mice ideally 16 days gestation

Rats ideally 18 days gestation

In the case of both species urogenital sinus can easily be dissected at plus or minus one day from the ideal date – as this time increases the dissection becomes progressively more difficult.

Note: The rat and mouse urogenital sinus dissections are essentially identical. The use of rat UGM with mouse epithelium is a common tissue recombination control, since this allows confirmation of the species of origin of epithelium.

Medium in which to dissect (sterile Hanks is fine, alternatively date expired tissue culture medium is a cheap and easy alternative).

Syringes

Tuberculin type with attached needles. Alternatively 1ml syringes and 25 gauge needles (these are used to inject anesthetic and also as scalpels during dissection).

Sterile Pasteur pipettes

Bunsen burner

Blue (1ml) pipette tips

Calcium/magnesium-free Hanks balanced salt solution

Agar plates:	1% agar (Difco)	5ml
	2X DME/H16	3.8ml
	Serum (fetal bovine)	1ml

Trypsin – Sigma or Difco 1:250 (Sigma T-4799)
Make a 10mg/ml solution in calcium/magnesium-free Hanks

Silastic tubing. Internal diameter about 1.5mm (Note; this can be purchased from Fisher – it used to be sold as “medical tubing” but is now marketed as “laboratory tubing”. The exact size isn’t crucial, essentially what we need is soft rubber tubing into which we can shove the pointed end of a blue pipette tip.

DNase type 1 – Sigma (DN-25)

EDTA – 20mM in calcium/magnesium free Hanks

Medium with 10% fetal bovine serum (this is used to neutralize trypsin after separation of tissue layers).

Anesthetic (Note will vary with local IACUC Preferences, Avertin was used historically generally now we use isoflurane)

Avertin for Mouse Anesthesia

Stock Solution:

25g 2-, 2-, 2-Tribromoethanol (Aldrich T4, 840-2)
15.5ml tert-Amyl alcohol (Aldrich 24, 048-6)

Mix and warm to 40°C to dissolve solid. Do not heat. Tribromoethanol is light sensitive, so cover with foil. When completely dissolved wrap in foil and store at 4°C. If the tribromoethanol recrystallizes warm again to redissolve. Stock is good for many months.

To make final solution, mix 19.75ml Hanks salt solution with 0.25ml stock (can also use any growth medium, but phenol red is needed to monitor pH). The mixture will have to be gently warmed and stirred to dissolve the stock (45°C in a water bath is fine). Do not heat excessively as the alcohols will vaporize, and the mixture will not work. Heating should be the minimum required to achieve solution. If the mixture becomes acid (yellow), it has been heated too much and should be discarded. When dissolved store at 4°C. This working stock is good for up to 14 days.

To use, inject i.p. Dosage is 0.02ml/g body weight (a 25g mouse gets 0.5ml, a 30g mouse 0.6ml). Mice should go down in 2-3 mins and will remain asleep for 30-40 mins. Check for response to paw squeezing. Individual responses to anesthetic agents do vary slightly, if necessary administer extra anesthetic (0.05 to 0.1 ml).

Animal Hosts to receive tissue recombinants.

Ideally use hosts around 60 days of age. Animals less than 45 days old are rather small for this work, though in experienced hands there is no real problem with animals as young as 35 days – however the endocrine status of young animals should be borne in mind. For work on prostate development and function hosts are normally intact males, although this may vary with specific experiments.

Depending upon the specifics of the experiment hosts can be syngeneic with the experimental material, or can be either athymic (nude) or severe combined immunodeficient (SCID). For preference use outbred strains of immunocompromized mice (such as CD-1 nudes) since these are generally larger and more robust than inbred strains such as Balb/c nude. In general if rodent or benign human tissue is involved nude mice are the best host option while if malignant human tissue is involved SCID mice can offer significant advantages.

Note: If athymic rats are used as hosts these should ideally be around 80-100g in weight. As they age rats can become very fat leading to problems with anesthesia and producing problems in locating the kidney.

Sterile gauze swabs – (Johnson and Johnson 2318)

Betadine – required by some IACUCs to sterilize the skin before surgery. Other committees will accept the use of 70% ethanol.

70% Ethanol

Sutures – For preference number 3 silk with a small curved needle. The use of silk provides a visual confirmation that a kidney has been grafted. However, please note that some IACUCs will not approve the use of non-resorbable sutures.

Wound clipper and clips

Beckton Dickenson	Clipper	427630
	Clips	427631

Heating pad (Gaymar T/Pump TP-500)

Tutorial

An illustrated tutorial for the sub-renal capsule grafting method (and a refresher for the memory) can be found at:

<http://mammary.nih.gov/tools/mousework/Cunha001/index.html>

Preparation of Urogenital Sinus Mesenchyme

Sacrifice pregnant mice or rats.

The preferred methods of sacrifice will vary with local regulations. In general **both** a chemical and a physical method of sacrifice is required. For pregnant hosts the use of anesthetic overdose is preferable since this will also lead to the death of the fetuses.

Remove Fetuses

Open mother with a midline incision through the ventral body wall. Remove the uterus containing the fetuses.

Carefully open the uterus by cutting from one end with small scissors. The fetuses are in individual amniotic sacs which may or may not be damaged by this process.

Individually remove the fetuses by opening the amniotic sacs. Pick up the fetus with forceps and cut through the umbilicus using scissors. Transfer the fetus to a 10cm petri dish. Note that it is important to lift the fetuses gently and to cut the umbilical cord carefully. The umbilical veins and arteries run along the side of the bladder and, if pulled, can remove the bladder and attached urogenital sinus.

If required by local regulations fetuses can be decapitated at this point.

Dissect Urogenital Tract

There are a number of approaches to removing the urogenital tract. As with most of these techniques no method is right or wrong – as long as the end result is the removal of an intact tract. The important thing is find a method which works well for each individual. Therefore the methods which will be demonstrated are those which we personally prefer. We will discuss other approaches.

Lay the intact fetus on its back on a piece of paper towel. The fetus should be arranged with its head away from you. The back legs and tail will stick to the paper and can be placed to allow a good view of the ventral surface of the animal.

The umbilicus can be trimmed back to the body surface with scissors. In most cases the umbilical artery (which runs along the side of the bladder) can be visualized through the skin of the fetus. Open the body wall of the fetus by rubbing number 5 forceps across the skin from the end of the umbilicus towards the front end of the back leg. Carefully cut the body wall on the opposite side with Vanna scissors. Lift the flap of skin and body wall away from the fetus with forceps and cut through the urachus (connection between the top of the bladder and the body wall). It may be helpful at this point to push the visceral organs away to give a clearer view of the urogenital tract.

Gently hold and lift the bladder. Cut the urethra as far into the pelvis as possible. Pull out the urogenital tract and remove the testes from male samples by cutting through the ductus deferens.

At this point you should have the major portion of the urogenital tract consisting of the urinary bladder, the urogenital sinus and a portion of the urethra. There will also be some attached Wolffian duct-derived structures including parts of the ureters and ductus deferens. Tracts are stored in either Hanks BSS or medium on ice while awaiting further processing. For a picture illustrating this stage see figures in:

Cunha, G. R. and Donjacour, A. A. Mesenchymal-epithelial interactions: Technical considerations. *In*: D. S. Coffey, N. Bruchofsky, W. A. Gardner, M. I. Resnick, and J. P. Karr (eds.), *Assessment of Current Concepts and Approaches to the Study of Prostate Cancer*, pp. 273-282. New York: A.R. Liss, 1987.

And/or:

Staack A, Donjacour AA, Brody J, Cunha GR, Carroll P. Mouse urogenital development: a practical approach. *Differentiation; research in biological diversity* 2003;71:402-13

Isolate the Urogenital Sinus

The urogenital sinus is isolated by removing the bladder using Vanna scissors. The sinus is then cleaned by cutting away the urethra and the attached Wolffian duct-derived structures to leave a “doughnut” (taurus) shaped structure. Sinuses are stored in either Hanks BSS or medium on ice while awaiting further processing. The most convenient tool to use as a scalpel in this procedure is a 1ml syringe with either a tuberculin or a 25 gauge needle. Location of cuts and the appearance of the various products are illustrated in the appended paper:

Cunha, G. R. and Donjacour, A. A. Mesenchymal-epithelial interactions: Technical considerations. *In*: D. S. Coffey, N. Bruchofsky, W. A. Gardner, M. I. Resnick, and J. P. Karr (eds.), *Assessment of Current Concepts and Approaches to the Study of Prostate Cancer*, pp. 273-282. New York: A.R. Liss, 1987.

Separation of Urogenital Sinus Epithelium and Mesenchyme.

Sinuses are submerged in a 10mg/ml solution of 1:250 trypsin in calcium/magnesium-free Hanks solution. Digestion is for approximately 75 minutes either on ice or at 4°C. Tryptic digestion is terminated by washing the sinuses three times in medium containing 10% fetal bovine serum.

The epithelial and mesenchymal tissue layers are separated mechanically using no 5 forceps and a hypodermic needle.

Notes on digestion and separation of tissues

Separation of mesenchymal and epithelial components is organ and age specific. Thus the best methods will vary. Tryptic digestion followed by mechanical separation works well for many fetal tissues including the urogenital sinus and the newborn seminal vesicle. For adult tissues other methods such as collagenase, or collagenase/hyaluronidase digestion are often better. Separation can be achieved in some organs, notably urinary bladder, by using 20mM EDTA in calcium/magnesium-free Hanks solution. Immersion in this solution for 30-60 minutes at room temperature will loosen urothelium enough that it can be easily separated from the surrounding bladder stroma. In the case of the bladder separation is helped by opening the bladder up to allow maximal penetration of the EDTA solution.

Preparation of Recombinants

There are two common methods to prepare tissue recombinants. The most commonly used is direct association of mesenchyme and epithelium on agar plates. The second is the recombination of single cell suspensions.

To make tissue based recombinants solidified agar plates are used as a support and nutrient source. Mesenchyme is transferred using a pulled Pasteur and oral suction (applied via a flexible tube) to form a mound on the surface of the agar. It is important to keep this structure as tightly knit as possible and to avoid excessive moisture. It can be helpful to suck a "moat" in the agar immediately surrounding the mesenchyme so that medium can drain off. The desired epithelial fragments can be transferred to the top of the pile of mesenchyme. The agar plate is then incubated overnight in a tissue culture incubator and the recombinants grafted.

To make recombinants based on single cell suspensions UGM must be reduced to single cells by a 90 min digestion at 37°C with 187u/ml collagenase (Gibco-BRL17028-029) in RPMI-1640 tissue culture medium containing 10% fetal bovine serum. Shake the tube frequently during this digestion to break up cell clumps. Following digestion the cells must be washed extensively with RPMI-1640 tissue culture medium to remove traces of collagenase. Viable cells are then counted using a hemacytometer, with viability determined by trypan blue exclusion.

Cell recombinants are prepared by mixing 100,000 epithelial cells (as a single cell suspension) with 250,000 mesenchymal cells. Cells are pelleted and resuspended in 50 μ l of neutralized type 1 rat tail collagen prepared as previously described. Alternatively gels containing only mesenchymal cells can be prepared and pieces of epithelial tissue introduced mechanically. The cell recombinants are allowed to gel at 37°C for 15 minutes and then covered with growth medium containing 5% fetal bovine serum and testosterone (10⁻⁸M) and cultured overnight. Following overnight culture the cells will form a mesh within the gel allowing it to be easily handled with forceps. Prior to grafting gels should be shrunk slightly by brief contact with a dry gauze pad.

Note: A stock solution of the collagenase can be made at 1500u/ml and stored in 1ml aliquots at -20C. Before use this can be diluted to a final volume of 8ml with RPMI-1640 + 10% fetal bovine serum

Sub-renal Capsule Grafting

Anesthetize hosts (Note: Avertin is an excellent anesthetic. However, its use is not accepted by all IACUCs. The use of inhalable anesthetics can be problematic for this technique as considerable manipulation of the host is required, therefore injectibles are often the preferred method). To anesthetize rats as hosts either sodium pentobarbital or ketamine/xylazine are possible agents.

Aseptic Preparation:

Prior to surgery the wound site should be cleaned with 70% ethanol and betadine. Note that in nude mice and the absence of hair makes this a simple process. In mice with fur shaving should be considered, and may be required by some IACUCs.

Make incision through skin (midline along the back); and bodywall. This incision is through a single layer of the body wall, cutting along the line of fat which runs parallel to the spine. Note that the kidney is retroperitoneal and that it is not necessary – and is in fact undesirable – to cut into the peritoneum. The cut should start approximately 1cm forward of the position of the knee and should be long enough (6-8mm) for the kidney to be “popped out” along its long axis without a risk of it falling back into the body cavity. Manually exteriorize kidney, this requires a firm but gentle touch – squeezing a mouse hard will kill it! Prepare graft site by making small hole in renal capsule using Vanna scissors and a blunted pasteur pipette. It is important to keep the capsule moist during this process otherwise it will be easily fragmented. Insert graft using two pairs of forceps (one to hold the capsule and one to insert the graft). Replace kidney, suture body wall (silk or resorbable suture), close skin with wound clips. Mice should be placed on a heating pad set at approximately 42°C until sternally recumbant.

Analgesia:

Drug:	Buprenorphine
Dosage:	0.01-0.05 mg/kg
Route:	sc
Frequency:	Once at surgery, additionally as needed

A full illustrated tutorial for this methodology can be viewed at:
<http://mammary.nih.gov/tools/mousework/Cunha001/index.html>