E12.5 Embryo and Adult Wholemount Processing Using X-gal staining

E12.5 embryo wholemount processing using X-gal staining 1.0-3.2

Adult wholemount processing for X-gal staining 4.0-6.4

1.0 Equipment

1.1 pH Meter

1.2 Functional fume hood

1.3 Dissection scissors

1.4 Forceps

1.5 Fine forceps

1.6 Spring scissors

1.7 Stereomicroscope

1.8 Rocking platform shaker at 4°C

1.9 Rocking platform shaker at RT

1.10 Rocking platform shaker at 37°C

1.11 Ice Bucket

2.0 Supplies

2.1 50ml falcon tube

2.2 12 well Costar plate

2.3 96 well genotyping plate

2.4 1 x PBS pH 8.0

2.5 4% PFA pH 8.0 freshly prepared on the day of embryo harvest.

2.6 Pins

2.7 Sylgard lined petri dish

2.8 *LacZ* staining solution

2.9 For solutions see appendix 1

3.0 Procedure

3.1. Dissection

3.1.1. Within LAF cabinet place the cage containing mice and a paper tissue on the base. Ensure the mice in the cage cannot see the mice being sacrificed.

3.1.2. Cull the mouse by cervical dislocation and place onto its back onto the paper towel and wet the abdominal fur with 70% alcohol. Check for secondary confirmation of death before proceeding.

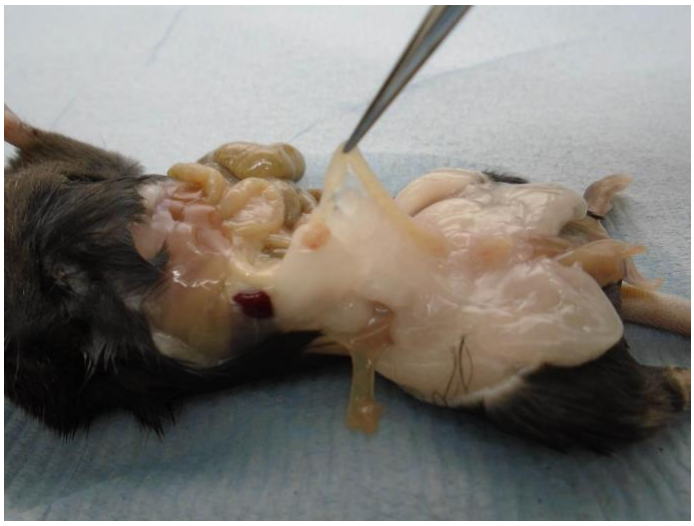
3.1.3. Using forceps and scissors pinch the skin and make a small lateral incision at the midline with scissors. Firmly hold the skin above and below the incision, pull the skin in opposite directions towards the head and tail until the abdomen is exposed.

3.1.4. Using forceps and scissors make an incision in the peritoneum just below the sternum in the direction of the extremities and down to the tail, to expose the abdominal organs.

3.1.5. Use the forceps to gently push the viscera up towards the head, exposing the reproductive organs.

3.1.6. Use fine forceps to grab the junction between the oviduct and the uterus (Picture 1).

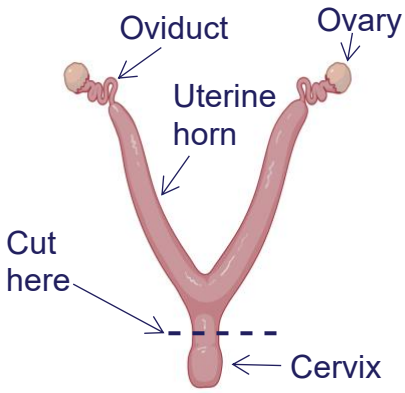
Picture 1



3.1.7. Insert the tip of a closed pair of scissors where the membrane and uterus meet to remove adherent tissue and fat.

3.1.8. **Whole uteri**:
Cut around the ovary and then just above the cervix to remove the uterus. (Picture 2).

Picture 2

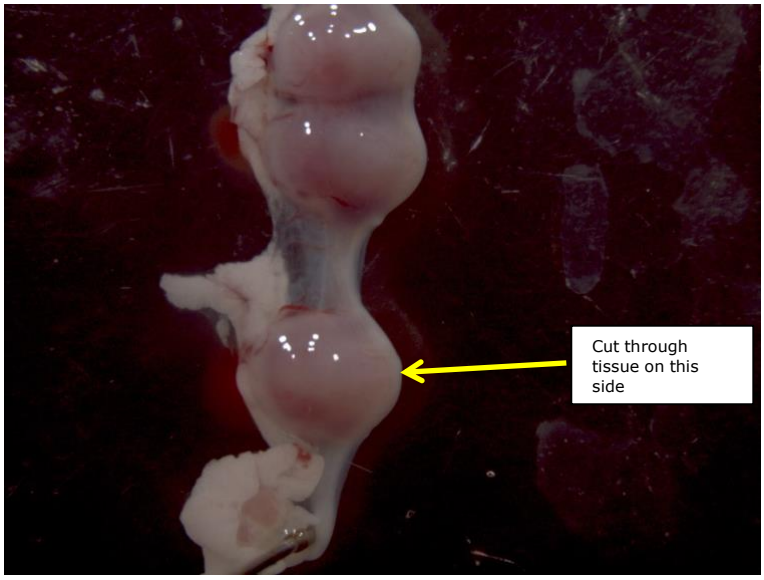


Oviduct:
Cut around the ovary and then ~5mm down the uterus from the oviduct-uteri junction.

3.1.9. Place the uterine horn into a 50ml falcon tube filled with 1 x PBS pH 8.0. Transfer to a Stereo microscope and gently tip uterine horn out of 50ml falcon tube into a sylgard lines petri dish.

3.1.10. Pin one end of the uterine horn using the forceps, arrange placentas so that they all face the same way then pin into place ensuring that there is no damage to the placentae during pin placement. (Picture 3).

Picture 3

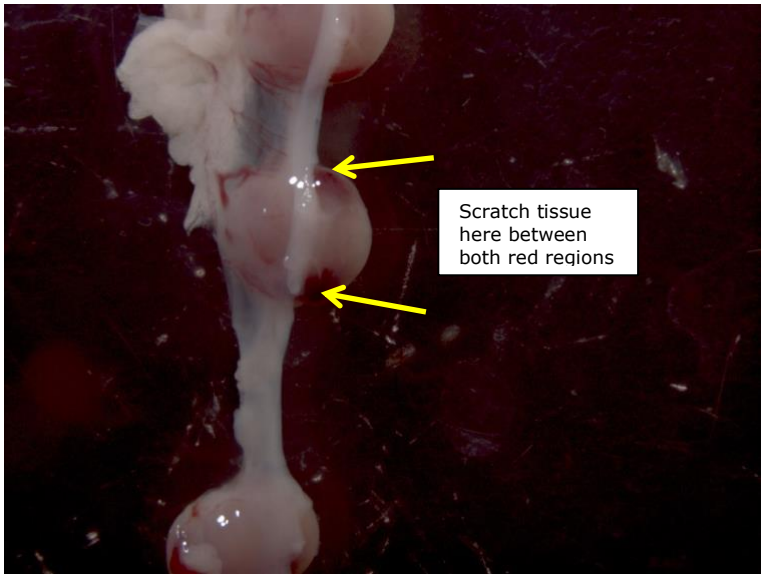


3.1.11. Once pinned count embryos (and resorption if necessary) and record.

3.1.12. Using forceps to hold the tissue steady use spring scissors to cut through the uterine horn muscle at the edge of each placenta being carefully not to damage the yolk sac or embryo inside.

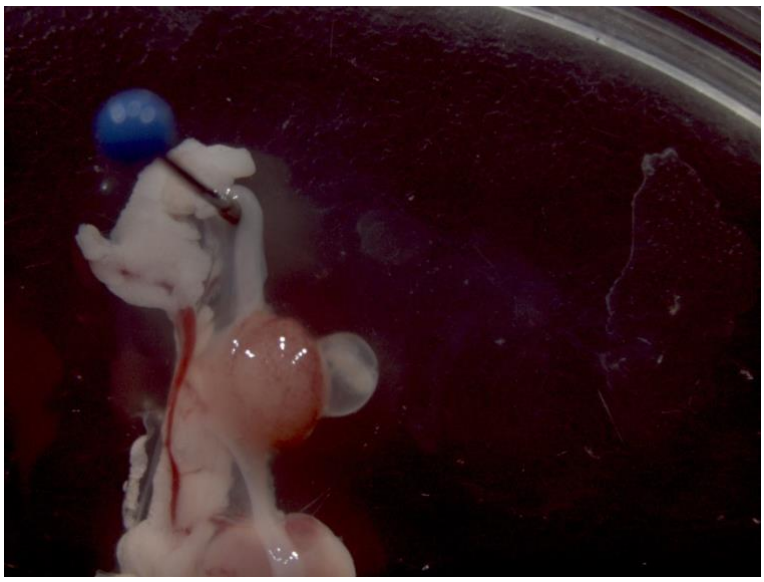
3.1.13. Using forceps scratch the thin covering of the placenta away from the yolk sac. (Picture 4).

Picture 4



3.1.14. Carefully use forceps to pull yolk sac away from the placenta wall ensuring the embryo inside is not damaged. (Picture 5).

Picture 5



3.1.15. Remove some yolk sac using either spring scissors or fine forceps and place in a 96 well genotyping plate.

3.1.16. Remove remaining yolk sac and cut any umbilical veins to allow blood to drain. Examine embryo under stereomicroscope for obvious morphological abnormalities. Record number of abnormal embryos.

3.1.17. Take a 3ml Pasteur pipette with the tip cut off and transfer the embryos from the petri dish to a pre-labelled 12 well costar plate. Fill with ice cold PBS pH 8.0 and place on ice.

3.1.18. Let embryos bleed out for 5 mins in the 1 x PBS as haemoglobin inhibits the staining reaction.

3.2. Washing and X-gal staining of embryos

3.2.1. When all embryos are dissected rinse with 1 x PBS pH 8.0.

3.2.2. In a functional fume hood add 4% PFA pH 8.0 to all embryos. Seal the 12 well Costar plate using a clear adhesive PCR film. Incubate on ice or in a cold room on a rocking platform. For an E12.5 embryo fixation should be for 20mins. (Longer than 20 minutes starts to impact the B-galactosidase action and in turn your staining quality).

3.2.3. After fixation, return to the fume hood and discard the 4% PFA into an appropriately labelled waste bottle. Rinse with 1 x PBS. Dispose of the PBS used to rinse the embryos.

3.2.4. The remaining washes can be done outside of the fume hood.

3.2.5. Carry out 3 x 30 minute washes in PBS incubating the 12 well plate either on ice or in a cold room on a rocking platform.

3.2.6. Once the washes are complete remove the PBS from the 12 well Costar plate and replace with X-gal staining solution. Cover with a clear adhesive PCR film and the Costar lid and protect from the light by wrapping in aluminium foil.

3.2.7. Incubate on a rocker at room temperature and check after 3 hours. If no staining is yet observed then incubate for 48hrs at room temperature. If staining is observed after 2hrs transfer to a rocker in a 4°C cold room and check overnight then up to 48hrs depending on depth of staining wanted.

3.2.8. Following staining discard X-gal solution into an appropriately labelled waste bottle and rinse with 1 x PBS.

3.2.9. Then carry out 2 x one hour 1 x PBS washes on ice to remove excess staining solution.

3.2.10. In fume hood add enough 4% PFA to cover embryos, cover again with a clear adhesive PCR film and post fix on a rocking platform at 4°C over-night.

3.2.11. Once embryos have been fixed discard the 4% PFA appropriately and rinse with 1 x PBS. Re-fill wells with as much 1 x PBS as the Costar well can hold, re-apply adhesive PCR film and Costar lid and store.

3.2.12. Embryos can be stored in 1 x PBS at 4°C for 3-6 months before imaging. After this time risks evaporation and fungal growth damaging your stained embryos. If you need to keep for longer but are unable to image then either a small amount of anti-fungal agent can be added to the 1 x PBS or the embryos can be stored in 1% PFA. The PFA can dehydrate the embryo over time and cause shrinkage but the embryos can then be stored for 1-2 years.

3.2.13. Imaging can be done in 1 x PBS (double filtered to remove dust and PBS salt crystals).

3.2.14. For the best imaging results take a 10cm petri dish and put a cross shape magnetic stir bar in centre of dish and fill half way with 3% agarose. When set remove magnetic bar and you have a perfect groove to put you embryos in so that imaging can be done vertically and dorsally. The side of the dish is good for left and right facing images. If possible use a ring light to reduce shadows (Picture 6).

Picture 6

Appendix 1 Solutions

PBS

PBS from 10x stock (laboratory services)

For 1L of 1x PBS mix 100ml of 10x PBS with 900ml of dH_2O

Adjust to pH 8.0 with sodium hydroxide (1/100)

Adjust to pH 7.4 (for making glycerol) with hydrochloric acid

4% PFA

(PFA is toxic, mutagenic and teratogenic. It must only be used in an effective fume hood or on a functional down draft table. Appropriate PPE **MUST** be worn.)

Per 100mls

12.5mls 32% PFA stock solution

10mls 10xPBS

77.5mls dH_20

2mM $MgCl_2.H_2O$

Sodium deoxycholate 10% stock solution

Dissolve 5g in 50ml of dH_2O

Protect from light and store at room temperature

IGEPAL CA-6302% stock solution

For 100ml mix 2ml of IGEPAL with 100ml of dH_2O.

Mix well on stirrer

Potassium ferrocyanide II 100mM stock solution

For 100ml use 4.22g of potassium ferrocyanide and make up to 100ml with PBS pH 8.0

Keep in amber bottle and store at 4°C

Potassium ferricyanide III 100mM stock solution

For 100ml use 3.29g of potassium ferricyanide and make up to 100ml with PBS pH 8.0

Keep in amber bottle and store at 4°C

2.5% X-Gal stock solution in Di-Methyl Formamide (DMF)

(DMF is toxic, mutagenic and teratogenic. It must only be used in an effective fume hood and appropriate PPE **MUST** be worn.)

Dissolve 1 g in 40 ml of DMF

Protect from light and store at -20°C

LacZ Staining Solution

2mM $MgCl_2.6H_2O$

0.01% Sodium Deoxycholate ($C_{24}H_{39}NaO_4$)

0.02% IGEPAL CA-630

5mM Potassium Ferrocyanide ($K_4Fe(CN)_6.3H_2O$)

5mM Potassium Ferricyanide ($K_3Fe(CN)_6$)

0.1% (1mg/ml) X-Gal in DMF

Make up in cold freshly made PBS pH 8.0

	Stock	Final		
LacZ Staining Solution			~100ml	~200ml
2mM $MgCl_2.H_2O$	1M	2mM	0.2	0.4
0.02% IGEPAL	10%	0.02%	0.2	0.4
5mM Potassium Ferrocyanide	100mM	5mM	5	10
5mM Potassium Ferricyanide	100mM	5mM	5	10
4°C PBS pH 8.0			83	166
10% Sodium Deoxycholate	10%	0.01%	0.1	0.2

0.1% (1mg/ml) X-Gal in DMF	25mg/ml	1mg/ml	4	8

Extra solutions needed for Adult X-gal staining

70 % Glycerol (200ml/ adult mouse)

50% Glycerol (200ml/ adult mouse)

Euthatal *(200mg/ml) solution provided in house. Appropriate anaesthetic (Pentobarbital Sodium)*

Adult Wholemount Processing for X-gal staining

4.0 Equipment

4.1 Functional down draft table

4.2 REGLO Digital MS-4/8 ISM 834 (peristaltic pump)

4.3 Peri-pump tubing TYGON 1STOP Type 1.02d

4.4 Student Surgical scissors

4.5 Extra fine Bonn Scissors

4.6 Narrow pattern Forceps

4.7 Delicate forceps

4.8 Kelly Hemostat

4.9 Scalpel

4.10 Rocking platform shaker at 4°C

4.11 Brain matrix

4.12 Matrix blades

4.13 Histology Cassette

4.14 Ice Bucket

5.0 Supplies

5.1 Blue towel

5.2 Body Bags

5.3 96 well genotyping plate

5.4 Brown jars

5.5 Needle winged 25GX3/4.1

5.6 Steritop-GP 250ml filer, Express plus PES 0.22um 45mm RS

5.7 Needle 26G ½"

5.8 1 ml syringe

5.9 Sieve

5.10 Funnel greater than 100mm

5.11 For solutions see appendix 1. You will need to make up 200ml 4% PFA and 200ml *LacZ* staining solution for each mouse you will perfuse. This is the volume that can go into the brown jars. You will also need to make an additional 400mls of 4% PFA for flow through during the perfusion.

6.0 Procedure

6.1 Set up the peristaltic pump and switch the flow direction button to the appropriate position. Prime the tubing with 4% PFA so that no air bubbles are in the system. Ensure that the intake end of the tubing will remain below the surface level of the PFA.

6.2 Check that the cannula is not blocked and that the flow rate is set to 2.83ml/min.

6.3 Fill 1ml syringe with Euthatal.

6.4 Remove mouse from transport box and check mouse ID against cage card.

6.5 Weigh mouse and give appropriate amount of Euthatal by intraperitoneal injection (see table below) or whichever anaesthetic that is in use at your institute. Following injection the mouse should be placed in a separate cage.

6.5.1

0.05ml	Mice up to 5g
0.10ml	Mice up to 24g
0.15ml	Mice up to 30g
0.20ml	Mice over 30g

6.6 Attach label to brown jar and fill approximately half way with 4% PFA (~150mls).

6.7 Observe animals breathing and wait until it starts to gasp and check reflexes.

6.8 Collect a 2mm ear punch and place in well of a 96 well genotyping plate.

6.9 Place animal in a perfusion tray.

6.10 Damp down hair on abdomen using 1 x PBS.

6.11 Open the abdomen to expose the rib cage. Carefully cut through the diaphragm avoiding cutting any internal organs.

6.12 Cut up towards the lower jaw along both sides of the rib cage to keep it intact.

6.13 Attach hemostat to the Xiphoid process and reflect it back. Ensure hemostat is placed to avoid damage to the ribs.

6.14 Grasp the heart gently between blunt ended forceps and carefully push the left ventricle onto the cannula. DO NOT try pushing the cannula into the heart muscle as it is too easy to push it through and out the other side of the heart.

6.15 Once the left ventricle has been cannulated carefully snip the right atrium with a pair of fine scissors. Switch on the pump once blood starts to flow.

6.16 Check mouse rigidity and colour (the paws should go white). You can tell if it has been done correctly as the mouse will begin to move as the 4% PFA flows around the body. Perfuse the mouse for 15 minutes.

6.17 Switch off the pump and remove the cannula and hemostat.

6.18 Cut across the top of the rib cage and remove the skin. Place rib cage in brown jar.

6.19 Remove an ear at its base and separate the two halves to expose cartilage and muscles to the staining solution. Place in brown jar.

6.20 From initial incision cut down either side of the animal between the skin and abdominal wall ensuring mammary tissue remains attached to skin. Place in tea bag and keep for addition of back skin. (A tea bag is used to contain the hair that falls off so that there is less clean up needed before imaging).

6.21 Cut approximately 1cm of tail cleanly with a scalpel. Place in brown jar.

6.22 Cut through abdominal wall.

6.23 Dissect all tissues from the tongue to end of the colon and all the associated tissues and viscera. Make sure to include the thoracic and abdominal aorta. Avoid any damage to the intestine. Also, gently hook the genital area and pull up organs in one piece. Place in brown jar. You will need to make a cut either side of the jaw between the teeth to separate the upper and lower jaw in order to remove all internal organs in one go. Place in brown jar.

6.24 To allow better penetration of the staining solution cut the liver, spleen and kidneys with a scalpel as follows:

6.24.1 Spleen - transverse cut in two separate halves.

6.24.2 Kidneys - Coronal cut in two separate halves.

6.24.3 Liver – Remove the upper lobe along with the gall bladder from the rest of the liver.

6.25 Keep **a piece of skin from the back** and open it to spread it flat. Place in tea bag in brown jar.

6.26 Remove brown adipose tissue from between shoulders. Place in brown jar.

6.27 Cut a piece of spinal cord in the lumbar region, approximately 1cm with a scalpel. Then cut in half and place in brown jar.

6.28 Remove **the left hind limb** and pull the skin off. Slit the paw with a pair of scissors all the way to the tip of the digit. Place in brown jar.

6.29 Remove **brain** from skull and place both in brown jar.

6.30 Any tissues not used must be placed in body bag and disposed of in accordance with local rules.

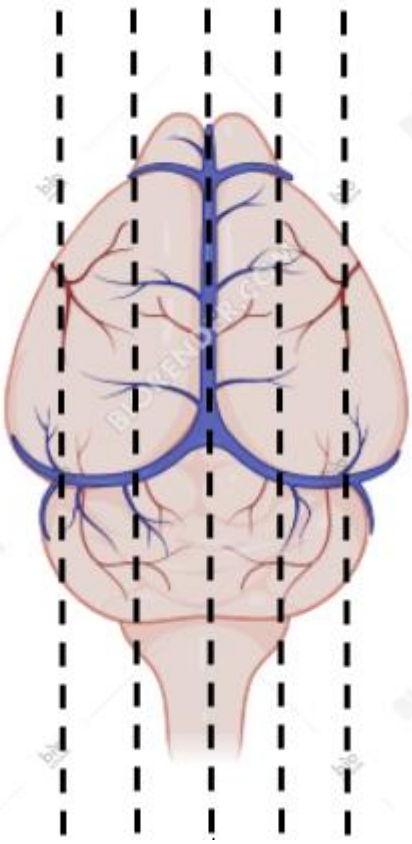
6.31 Place brown jar on ice (on rocker if possible). Leave to fix for 30 minutes. **DO NOT ALLOW TO FIX FOR MORE THAN 1 HOUR as this will affect the staining.**

6.32 Wash three times for 20 minutes in excess volume of 1 x PBS pH 8.0 at 4°C.

6.33 Place brain ventral side up in sagittal brain matrix. Using a new razor blade cut along the midline, either side of the olfactory bulb and then halfway to the edge. (This will give you six sections). Place sections in histology cassette and place in brown jar (Picture 7).

Picture 7

Cuts to make on brain
in brain matrix



6.34 Incubate organs in *LacZ* staining solution (see appendix 1) at 4°C with gentle agitation for up to 48 hours. Check the stain after 24 hours.

6.35 Wash 2 x PBS pH 8.0 30 minutes each.

6.36 Post-fix with 4% PFA pH 8.0 overnight at 4°C with gentle agitation.

6.37 Rinse with PBS pH 8.0.

6.38 Clear with 50% glycerol overnight at 4°C with gentle agitation.

6.39 Final clearing and storage in 70% glycerol in the dark at 4°C.

6.40 Images should be taken within 3 months for best quality but tissues can be stored for 2+ years and still be in good order.

6.41 When taking images transfer tissues into imaging dish with 50% glycerol in. This will ensure all tissues sink to the bottom for imaging and do not float. Therefore less pinning is needed. (For a list of tissue images taken see appendix 2 and examples of adult images see appendix 3 and picture 8).

Appendix 2

Organ System	Annotated Entity

Adipose tissue	Brown adipose tissue
	White adipose tissue
Cardiovascular system	Heart
	Vascular system
Digestive system	Stomach
	Small intestine
	Large intestine
	Colon
	Liver
	Oesophagus
	Gall bladder
	Oral epithelia
Endocrine glands	Adrenal gland
	Thyroid
	Parathyroid
Endocrine/exocrine gland	Pancreas
Immune system	Thymus
	Spleen
	Peyers Patch
	Mesenteric lymph node
Integumental system	Skin
Musculoskeletal system	Cartilage
	Skeletal muscle
	Bone
Nervous system	Olfactory bulb
	Cerebral cortex
	Striatum
	Hippocampus
	Hypothalamus
	Cerebellum
	Brainstem
	Brain
	Spinal cord
	Peripheral nervous system
	Pituitary gland

Renal/Urinary system	Kidney
	Lower urinary tract
Reproductive system	Mammary gland
	Ovary
	Uterus
	Testis
	Prostate gland
Respiratory system	Trachea
	Lung
Visual system	Eye
Cardiovascular system	Aorta

Appendix 3

1-3 Brain, brainstem, olfactory bulb, cerebral cortex, hippocampus, hypothalamus, cerebellum, striatum, **4** spinal cord, **5** pituitary gland, peripheral nervous system, **6** skin, **7** small intestine, peyer's patch, **8** large intestine, **9** skeletal muscle, cartilage, bone, **10** liver, gall bladder, **11** blood vessel, **12** heart, aorta, **13** trachea, thyroid, parathyroid, **14** adrenal gland, **15** lung, **16** eye, **17** kidney, **18** stomach, oesophagus, **19** thymus, **20** mammary gland, **21** uterus, ovary, oviduct, **22** mesenteric lymph node, **23** bladder, prostate gland, **24** testis, white adipose, **25** brown adipose, **26** pancreas, **27** oral epithelium, **28** spleen

Picture 8