Histology Protocols

The following text is the actual protocol that you would follow if you were carrying out the practical work in a real laboratory. You should be able to identify most of the steps in the instructional videos. This is provided as supplementary information only.

Safety and Good Laboratory Practice (GLP)

Although this is a virtual experiment, you should be aware of Health and Safety issues and Good Laboratory Practice, as if you were working in a real lab.

Most of the chemicals used in this laboratory are relatively harmless. All have their risks however. These risks are explained in the COSHH guidelines, which should be available in each physical laboratory. If working in a real lab, it is your responsibility to be aware of the risks that each chemical carries in the protocols you will be using; it is also your responsibility to ensure that you have read the COSHH assessments properly. Be especially sure that you are aware of the risks that any chemicals pose if you are using them. There are also important guidelines to follow in disposing of some chemicals.

Laboratory rules

These rules are absolute, and incorporate the OU safety rules:

- You must wear a lab coat in the laboratory at all times - however hot it gets. Clothing under the lab coat should be sensible but remember if you wear shorts or a short skirt, your knees are more exposed to anything that you happen to spill.
- Open-toed sandals are absolutely forbidden in the laboratory - as are bare feet. It is just too easy for something that has been spilled to seep through the gaps in sandals.
- Absolutely no eating, drinking or smoking in the laboratory.
- Do not apply cosmetics in the laboratory. As with smoking and eating, there is a high risk that you will contaminate your skin with a chemical during the application.
- Where the experiment specifies, you must always comply with the instruction that you should wear safety spectacles or gloves, or that you should work at the fume cupboard.
- If you have an accident or injure yourself, or even if you spill something that you are not sure about, tell the local safety officer immediately. Make sure that any open cut is bandaged with a plaster before you re-enter the laboratory.
- When you leave the laboratory, remove your lab coat. Lab coats must not be worn outside the laboratory, especially in rest areas or eating areas.
- Absolutely no practical jokes in the laboratory.

Anatomical and histological study of adipose tissue

Overview of the method

Freshly killed animals will be delivered to the laboratory.

The first step is to observe animals from the control and experimental groups, and weigh them to measure their body mass.

The next step is to dissect out the entire interscapular BAT depot (iBAT) and a representative sample of WAT. You should weigh the tissues and take a small quantity (specimen) for histological analysis.

Histological preparation includes:

Fixation

The purpose of fixation is to halt putrefaction (the destruction of tissue by bacteria from the air and non-sterile vessels) and enzyme-induced autolysis (the destruction of tissue by lysosomal action from within the cell). Moreover, fixation hardens the tissue, makes it more chemically stable and (depending on the fixative) enhances staining.

Processing and embedding

The tissue is brought into a state that allows preparation of thin sections. This process has three steps:

1 Dehydration: the removal of water from the tissue by soaking in increasing alcohol concentrations: 70% alcohol; 90% alcohol; absolute (i.e. pure) alcohol.

2 Clearing: removal of dehydrating agent with a substance (toluene or xylene) that is miscible with the embedding medium (paraffin wax).

3 Embedding: impregnation of tissue with melted embedding medium (paraffin wax at 58 °C) to allow cutting of thin sections.

Section cutting

Thin sections (5–10 μm) of tissue embedded in paraffin wax are cut and placed on a glass slide. The sections are thin enough for the outlines of the adipocytes to be visible.

Staining

In order to perform the microscopic analysis of the structure of the tissues within the BAT and WAT, you must first carry out a number of steps to make the cells visible. Sections are stained to introduce colour and contrast to the almost transparent cells. Before staining, slides are usually re-hydrated. Once stained, they are again dehydrated.

Mounting

The stained sections are covered with a resin and coverslip.

To perform these different stages and to examine the stained sections under the microscope requires two full days. It is usual to fix the specimens during the morning of day 1 and leave them in the automatic tissue processor (Histokinette) to dehydrate and infiltrate overnight. The tissue is then ready for embedding and sectioning on the morning of day 2. Once sections are stained and mounted, they can be examined on the microscope in the afternoon.

Materials and methods

Animals and dissection

1 When the animals are delivered to the laboratory, examine them and record any observations you make. Do you observe any gross (macroscopic) differences in the anatomy and condition of the animals, e.g. quality of the animals' fur?

2 Weigh all the animals on the electronic balance provided and record your results. Make sure that the number of the rat and its treatment are carefully recorded and affixed to all records and pieces of tissue from the animal.

3 Dissect out the iBAT pad and a discrete unit of the perirenal or epididymal WAT. The iBAT pad is the largest and most clearly defined area of BAT in the body of adult rats.

4 You will need to weigh the BAT and the WAT, take a small sample and remember to label all samples clearly. Unlabelled samples are useless!

5 Record the total weight of iBAT and use this value to work out the mass of tissue as a percentage of the whole body mass. Keep careful notes and record the data as you collect it. In working out the mass of iBAT as a percentage of body mass, remember that body mass was measured in grams (g), whereas BAT mass was measured in milligrams (mg = 1/1000 of a gram).

Further notes on safety and good laboratory practice (SGLP) in histology

Some of the histology reagents are toxic, most are harmful, and some are of uncertain toxicity. Remember:

- Solvents (toluene, DPX, Histoclear and ethanol) are volatile and so are toxic by inhalation as well as by skin absorption. Keep the lids on coplin jars, do not breathe vapours and do not splash on skin.
- The pigments in the stains are toxic or harmful. You should be wearing gloves throughout the procedure.
- Wash your hands after using histology reagents.

To obtain good results, histology requires care.

- Many steps require two separate baths of the same solution. The first is to remove the excess of the previous solution; the second is to give a solution that is ultra-clean.
- Be especially careful of the Histoclear baths; if the Histoclear appears milky, or takes too long to clear the sections, then it contains too much water and should be changed.

Disposal of waste:

- All organic solvents should be disposed of into the organic waste bottles in the fume cupboard.
- 10% Formalin (formaldehyde) should be washed down the sink in the fume cupboard.

Preparation of tissue for sectioning

Fixation

1 You will take a small specimen of BAT and WAT for histological examination. Use a scalpel or a single-edged razor blade to cut the specimen into small pieces, no more than 5 mm long in any direction.

2 Place the trimmed tissue in one of the small processing cassettes. Remember to label the cassette before you begin processing. Write in pencil; some of the histology reagents (e.g. Histoclear) remove any pen marker.

3 Immediately place the cassette in a small beaker containing 10% formalin fixative in the fume cupboard and leave to fix for 30–60 min.

Processing and embedding

As mentioned above, the collected and fixed tissue should be dehydrated and infiltrated with paraffin wax.

These stages are done automatically in the Histokinette. After processing in the Histokinette, the tissue specimen has to be made suitable for thin sectioning, by embedding the tissue specimen in freshly melted paraffin wax, in the shape of small block. Whilst embedding the tissue, take care to minimize any spillage of molten wax as it makes the floor very slippery and hazardous.

The embedding process has to be done quickly, so get everything ready beforehand. Make sure there is:

- Fresh melted wax in the oven. Place clean paraffin wax in the embedding oven at 58–60 °C to keep warm.
- A pair of pre-warmed forceps. Place forceps in the oven for few min to pre-warm; cold forceps harden the wax, making embedding difficult.
- Be especially careful while handling hot forceps – use blue roll to protect your hands.

Stages 1–5 below should all be carried out on a warm plate.

1 Place a clean white plastic mould on the hot plate and add a few drops of liquid paraffin wax to each well.

2 Take a tissue specimen from its cassette and place it in the paraffin wax at the bottom of the well of the plastic mould. Repeat for each tissue specimen, noting which specimen goes into which well.

3 Make sure that the specimens are orientated as required. A flat side of the specimen should be parallel and close to the bottom of the white plastic mould.

4 Add wax gently to each well of the mould without disturbing the specimen or introducing air bubbles.

5 Split the empty cassette in which the tissue was processed, keeping the part with the label on. Take the part of the cassette with the label on and place it directly on top of the wax-filled mould. Add wax if needed.

6 Allow the wax to cool, and then place the mould in a bath containing crushed ice in water. Alternatively, place the mould in the fridge for an hour.

7 Trim the block so that it is ready for sectioning on the microtome.

Procedure for sectioning with the microtome

Safetywarning

The microtome moves the specimen block across a fixed razor blade. The block is heavy and the razor is sharp, and it is possible for the block to push your finger onto the blade when you are working on the specimen. There is a lever that operates a lock on one side of the microtome, which should be kept locked whenever you are not sectioning!

1 With the microtome arm at the top of its travel (rotate the big handle clockwise to move the arm), lock the arm with the lock lever on the right side of the microtome.

2 Remove the wax block containing the specimen from the plastic mould and place it within the block holder, with the wax block facing towards the blade. Tighten the screw on the block holder.

3 If the blade needs changing, slide a new razor blade into the knife holder and tighten the screws firmly.

4 Use the coarse control to bring the specimen close to the knife. If the specimen is too far from the knife edge when you start turning the handle, it takes a long time to get any worthwhile sections (since each turn of the handle is only advancing the specimen by 7 μm). However, if the specimen is too close at the start, there is a risk of taking an extra-thick slice, which blunts the knife and may damage (or entirely dislodge) the specimen, requiring you to re-embed it.

5 Unlock the microtome arm and slowly lower it onto the knife blade, controlling it by means of the big handle. When the specimen is just above the knife, stop the arm. If the specimen is a long way from the knife edge, use the coarse control lever to advance the specimen. If the specimen is overlapping the knife edge, use the coarse control to retract the specimen.

6 Check the dial indicating the section thickness to be cut. For a new block, set the dial at 7 μm. Start rotating the handle until slivers of wax fall from the knife blade.

7 Clean the blade with the paint brush. Brush the wax debris UPWARDS and away from the blade (brushing downwards cuts the brush and blunts the blade). Continue to cut until entire sections appear. Further sections should stick to each other to form a ribbon. Lift the end section with a needle, and draw out the ribbon. When you have 5–8 sections in a ribbon, you are ready to mount some on a slide.

8 When you have collected as many sections as you need, lock the microtome mechanism and remove the specimen. Clean off wax slivers before progressing to the next specimen. When you have finished sectioning, clean the area around the microtome to remove any stray wax and take care not to let wax get on the floor.

Collecting, mounting and staining sections

1 Make sure that the water bath is on and the water temperature is set at 50 °C. Draw some paper tissue across the surface to remove accumulated wax and fluff.

2 Choose clean slides, and with the diamond marker, mark the details of the sections on the slide. For example, C17W may denote 'cold rat', number 17, WAT sample. If the slides have frosted or coloured ends these may be written on directly in pencil.

3 As a means of sticking the sections to the slide, take a small amount of glycerine egg albumin solution on your little finger and smear a little of it across the slide (not too much because albumin also takes up stain). Place the slides on the hot plate until ready for use.

4 With the water bath at 50 °C, lift the ribbon of sections between two mounted needles, paint brushes or forceps and lower it gently into the water bath allowing the sections to spread on the water surface. The sections should immediately uncurl and flatten. If they do not, the water bath is too cold. If they appear glassy at the edges, the wax is melting and the bath is too hot.

5 After the sections have flattened, lower the appropriate marked slide at an angle of 45 degrees into the water, at a spot remote from the sections. Manoeuvre under the sections, and then lift the slide clear of the water, with the sections attached. Use a mounted needle to hold the sections on the slide until they are clear of the water. Drain and leave to dry. *Note*: An alternative method is to place some 20% alcohol onto the slides sitting on the hot plate and directly transfer the ribbon to this.

6 Place in a 60 °C oven to promote sticking of the sections to the slide. Ideally, slides would be left in a 40–50 °C oven for 1–2 h to dry off.

7 The stains we use are water-soluble. As paraffin wax is immiscible with water, you must remove the wax by soaking the dry slides in Histoclear and gradually rehydrate the sections in decreasing concentrations of alcohol, as summarized in Table 1.

Table 1 **Dewaxing and rehydrating slides.**

Stage	Process	Time
1	Place the slides in a Histoclear bath.	1 min
2	Move the slides to a second Histoclear bath.	1 min
3	Transfer the slides to an absolute alcohol bath.	1 min
4	Transfer the slides to a second absolute alcohol	1–3 min
5	Rinse the slides in 90% ethanol.	1–3 min
6	Rinse the slides in 70% ethanol.	1–2 min
7	Rinse the slides in running tap water.	1–2 min

8 Start by staining some sections from each rat using the haematoxylin and eosin (H & E) staining method. Haematoxylin stains DNA and RNA a blue colour, whilst eosin stains the cytoplasm and mitochondria pink. Once stained, the sections must be dehydrated once more, before they are cleared in Histoclear and mounted. The whole procedure is outlined in Table 3.

Table 3 **Staining, dehydrating, clearing and mounting.**

H & E staining method

1	Stain in Harris's haematoxylin for 5 min.
2	Wash and 'blue' in running tapwater for 1 min (i.e. wash out)
3	Differentiate in acid alcohol for 30 sec (this step increases contrast)
4	Wash and blue in running tap water for 1 min.
5	Stain with eosin for 4 min.
6	Wash in running tap water for 1 min

Dehydration

7	Immerse in 90% alcohol for 1 min.
8	Immerse in 95% alcohol for 1 min.
9	Immerse in absolute alcohol for 2 min.
10	Immerse in absolute alcohol for 2 min.

Clearing

11	Clear in Histoclear for 5 min.

Mounting

12	Using a glass rod, place a drop of DPX mountant on the coverslip
13	Press the coverslip down gently with a needle.

Your section is now stained and preserved and should last for years without deterioration. You can now inspect it under the microscope.

Microscopy and calibration

You now need to examine your stained sections using the light microscope and determine the average cross-sectional area of cells in the preparations from control and cold-adapted animals. One way to determine the average cell size would be to measure the area of a number of different individual cells in a particular specimen and calculate the average of these values. However, in practice, this approach would be very difficult. Instead, we count the number of cells in a 'field' of known dimensions. We can calculate the average cell area by dividing the area of the field by the number of cells that occupy that field.

It is possible to count cells by viewing the specimen through the eyepiece of the microscope and using an in-built graticule that provides a scale. Another method and the one described here, counts cells by displaying the image on a monitor that is connected to the microscope. The protocols are outlined below.

Place a processed slide on the microscope stage and set up your slide using the microscope eyepiece, when you can see it clearly in focus switch over to monitor viewing and re-establish focus on the monitor. Begin by selecting an objective that provides a microscope magnification that will show twenty to thirty cells when viewed on the monitor. You will be provided with a graticule slide, marked with a scale measuring 1mm and subdivided into smaller units, it is important that you identify what these smaller units are for later stages. Place the graticule on the microscope stage in place of your slide and centre and focus the scale being sure not to change the objective. The graticule slide is very expensive, and racking an eyepiece lens through it can easily break it!

1 Place a sheet of acetate over the screen and fix it in place. Using the graticule scale on the monitor draw a square on the acetate that provides the largest viewing area for cell counting; it may be for example that the

drawn square will represent a field of view of 300µm x 300µm. Use a permanent marker pen for this process **but do not mark the actual monitor screen**

2 Mark on the acetate sheet sub-divisions of the graticule lines at least to the 50 µm level but it probably is not necessary to go down to 10µm. You would then end up with a square that is made up of three major sub-divisions along each edge representing 100µm and six minor sub-divisions along each edge representing 50µm. You may find it easier to draw all the lines on the acetate on a flat surface using a ruler after first marking dots to scale using the graticule as a guide.

3 Remove the graticule slide; you will no longer need it from this point since you will be using your acetate scale. Insert the tissue slide you are interested in and examine the specimen at low power, identifying irregularities and positions of blood vessels. Following this, be sure to change to the magnification that you used to make your acetate prior to counting.

4 Using a non-permanent whiteboard marker pen draw the outlines of adipocytes within the rectangle as accurately as possible. This task is fairly easy for WAT, but more difficult for BAT. Count the number of cells entirely within the field (i.e. the square); cells at the edge of the field should only be counted if more than half of their outline lies within the rectangle. To ensure that you do not count the same cell twice, place a dot on each cell as you count it. Other strategies may be used for counting cells.

5 Wipe off the outlines and repeat on at least five fields per specimen, ideally some on different sections. Remember to record you results in your lab notebook, clearly indicating the details of each specimen examined.