

CELL INJURY AND DEATH: NECROSIS AND APOPTOSIS
INTRODUCTION: AIMS OF THE PRACTICAL CLASSES

The aim of the practical classes is to help you understand the biological processes that lead to disease. You will be shown tissue changes in histological sections and basic techniques in bacteriology, parasitology, virology and immunology.

In all of the class sheets there are references (links) to digital images of the specimens and slides that are shown in the classes. Electronic versions of all of the practical class sheets can be accessed from the Departmental Web Site (http://www.path.cam.ac.uk/partIB_pract/) and following these links using a Web Browser (e.g. Microsoft Explorer) will show this material. Note that in all cases there are small (low resolution) and large (high resolution) images provided. Please note that there will be a delay of one week before any given class sheet is available.

The practical component of the Part I course will be introduced and a short video highlighting various aspects of safety and operating procedures in the Part I laboratory will be shown.

The remainder of the class will introduce you to the ways of looking at tissue sections in order to understand pathological processes and introduce two examples of cell injury and two modes of cell death: necrosis and apoptosis.

The practical classroom is a potentially hazardous environment and it is essential that everyone using the classroom understands and observes Safety Regulations. Please read the following carefully.

SAFETY IN THE LABORATORY

- (1) Safety is important as you will handle bacteria, viruses and blood.
- (2) Laboratory gowns must be worn for all practical classes, but should be left on a hook in the cloakroom when you go to any other part of the Department.
Outdoor clothes and unnecessary belongings should be left in the cloakroom but the Department cannot accept responsibility for loss of valuable articles.
- (3) Eating (and chewing gum) is not allowed: no food or drink in the classroom.
- (4) No smoking. Switch off mobile phones.
- (5) Hazards of infection. When using cultures of bacteria and viruses, never touch your face or hair or go to the lavatory until you have washed your hands. Never pipette by mouth.
Do not wave wire loops in the air. Do not contaminate oil immersion lenses on the microscope.
- (6) Spillages. Tell a demonstrator or staff member if infected material is spilled. Prevent others from walking into the contaminated area (e.g. block the aisle with a laboratory stool).
- (7) Daily disinfection. After practical classes involving blood or infectious agents, wipe the bench with the disinfectant and a paper towel; wash your hands and forearms thoroughly before leaving, (operate the taps with your elbows). If you leave the classroom at any time, wash your hands before you go.
- (8) Hazards of human blood. Read the yellow notice on the bench.

TO GET THE BEST OUT OF THESE CLASSES

- (1) Ask for help from a demonstrator or the classroom staff, (e.g. when you have a problem with your microscope or you cannot see the monitor screen very well or you have a question).
- (2) Learn to use your microscope properly, familiarise yourself with use of the computer to view scanned images and get to grips with writing a brief description of the histological sections as soon as possible. You are given guidance on how to write descriptions in practical 2.
- (3) Refer to normal tissues (kept in the boxes on the end of every bench). An example of every slide used in class will be kept in other boxes on the end of the bench. These will be available throughout the year and you may refer to them at any time.
- (4) Study demonstrations when included.
- (5) Specimens (fixed organs in pots) or illustrative photographs of diseased organs will sometimes be available. This material is valuable because it illustrates how diseases evolve in the context of the whole body. Throughout this year we will be attempting to relate molecular and cellular processes to the mechanisms and manifestations of disease. You will find it helpful to integrate the information you acquire from microscopy with these macroscopic images.
- (6) Some of the teaching is in the form of problems for you to solve, working in groups.

USE OF THE MICROSCOPE

It is very important that you know how to use the microscope properly and feel comfortable using it. The following brief instructions will help. If, however, you are uncertain about how to use the microscope, or you have difficulty in obtaining a clear image of a slide, please ask either a demonstrator or the classroom staff.

- (1) If you are tall, put a wooden block under the microscope so that you are more comfortable. (There are wooden blocks on the side of the screen which is near bench F).
- (2) Before switching on the light, ensure that:-
 - (i) Light intensity is at minimum
 - (ii) Low power (x4) objective is in position
 - (iii) Condenser is at upper 'stop' position
 - (iv) Stage is at upper "stop" position [use coarse (larger) focus knob]
- (3) Switch the light on and then increase the light intensity. If the image is too dark even with the light control at maximum, check the aperture diaphragm of the condenser underneath the slide. It may be closed right down (lever to the right). Move the lever to the left.

In general, use the light at the lowest comfortable setting. Please turn the light on and off at low intensity. Always turn it down to the minimum before switching off.
- (4) Adjust the distance between the eye-pieces for comfortable binocular vision and note the reading this gives on the central scale. Correct each eye-piece scale to the same reading and then focus accurately. (You may find you have to re-adjust one of the eye-pieces if your eyes are of unequal focal length).
- (5) Changing objectives

There are four objectives:

x4 - red (low power objective)

x10 – orange/yellow (medium power objective)

x40 – green/blue (high dry objective)

x100 – blue/black & white (oil-immersion objective). It is rarely necessary to use this objective.

Secure a well focused image with the x40 objective using the coarse and fine adjustment knobs. Then turn to the x4 objective and adjust the focus with the eye-pieces only. Thereafter, little adjustment should be required on changing from one objective to another.

The oil-immersion (x100) objective: Use only when absolutely necessary with a very small drop of oil. Do not allow the oil to get on any other objective lens.

Having selected a field with the x40 (high dry objective), turn the nose-piece to the low power (highest above the slide) so you can place the drop on the slide. Now gently swing the oil-immersion objective into the oil. The field will not be in exact focus, so you will have to use the fine focus with great care.

Afterwards, turn the nose-piece in the reverse direction, so the low power objective is again high over the oil. Do not accidentally bring the x 40 objective into the oil as you do this. Remove the slide gently and wipe it with a tissue but use lens paper to remove the oil from the oil objective. This should prevent the oil from being transferred to the high dry objective.

INTERPRETATION OF PATHOLOGICAL PROCESSES

LOOKING AT TISSUE SECTIONS

Before starting to look at the sections illustrating injury, death and Acute Inflammation, read the notes on Interpretation of Pathological Process. These notes will also be provided in future classes to help guide you in making deductions about Pathological Processes and also in Writing Reports.

- (1) Look with the naked eye first against a white background. See if you can identify distinct areas? If so, note their overall shapes, e.g., circular/wedge/cone, distributions, patchy, general and size.
- (2) Look at the section with the low power objective. Scan across the section from one edge of the tissue to the other in order to get an overall impression of the different areas and identify patterns of cells.
- (3) Using a higher power objective (x10, then x40) home in on representative areas of normal appearance or very abnormal looking regions. At this stage, try to identify cellular and other details. Always return to low power to finish scanning the section, drawing a sketch at low power and interpreting the overall picture at low power.
- (4) If you can't remember what the normal tissue should look like, have a look at the edge of the section. Often there are small residual areas of normal tissue there.

Microscope sections present two-dimensional images, harvested in a moment of time, from three-dimensional tissues in which dynamic processes are going on continuously. Sections contain a great deal of information about such processes, but skill and caution are required to extract it appropriately.

Observe the following:

- (1) Distinguish the parenchymatous from the stromal elements in the tissue represented.

Parenchyma is an umbrella term for the main functional cell type in the tissue – hepatocytes in the liver, cardiac-type muscle in the heart, secretory epithelium in the intestinal glands. Stroma in a

tissue is the equivalent of service ducting in a building, and consists of fibroblasts and the collagen they produce, blood vessels, fine nerve bundles and sometimes adipose tissue. Each of these has a characteristic appearance in a section, which you should learn to recognise. Stroma is often called connective tissue.

(2) From the appearance of the parenchymatous element, try to determine which organ you are studying. Sometimes this information will be given, but you can still identify clues in the section as to its exact original whereabouts. Thus, does your section of lung come from the periphery of the organ (plentiful alveolar spaces, only small respiratory passages, perhaps also a pleural surface), or from nearer the centre (larger bronchi and blood vessels, possibly lymph nodes)?

(3) Determine if the tissue reveals the presence of a pathological process. Look for neutrophils, macrophages and lymphocytes in substantial numbers outside of blood vessels, as indicators of inflammation. In sections stained with haematoxylin & eosin, as most of yours will be, the nuclei of these migrating cells appear as dark-blue dots peppering the section. As you will find later, tumour growth also has the effect of producing a clustered excess of cells (with blue nuclei), but this time they bear a crude similarity to the normal cells from which they arose. In contrast, in necrosis the dead tissue appears as sheets of formless, pink material, the nuclei of the dead cells having been destroyed.

(4) Bearing all this in mind, first, look with the naked eye; then scan the section at low power. Make sure you do not miss out any part of the section. Once you have scouted out the whole territory covered by the section, swoop down selectively on areas that interest you, using the higher power lenses (professional pathologists never scan whole sections with the high power lens). Compare the appearances of the sections given with sections from corresponding normal tissue. These are sometimes also given, but there are always examples in a box at the end of your bench.

	Colour of stain	Terminology	Examples
Haematoxylin	Blue/purple	Basophilic (Haematoxyphilic)	Nucleic acids, eg. DNA of nucleus RNA of ribosomes All bacterial clumps
Eosin	Pink	Acidophilic (Eosinophilic)	Proteins, eg. Cytoplasmic proteins Extracellular matrix proteins Deposited plasma proteins

SCANNED DIGITAL SLIDE IMAGES OF TISSUE SECTIONS: VIEW USING NDP

Instructions for using NDP software:

1. Plug and switch on the PC.
2. Double click on the NDP.view icon on the desktop. NDP.view software is a dedicated viewer for displaying digital slide files (NDPI, VMS files) & lets you open the scanned digital files so you can navigate around the specimen as well as view the specimen at different magnifications.
3. Navigate to a digital slide image file in the appropriate Practical Class sub-folder which is in the Pathology Pt 1B folder & double click on it. NDP.view will open the file.

4. You can navigate around a digital scanned image by using the keys as well as the mouse. The HELP list has all the key functions listed and can be displayed by clicking on the “Toolbar > Icon”, “Tool menu > HELP”, or by simply pressing the “F1” key.

NDP.view Help:

- Drag the image with the left mouse button to move around.
- Click the left mouse button to centre that point.
- Drag an area to view with the right mouse button
- Zooming in and out of a scan can be done by using mouse’s wheel. Push the mouse wheel forward to zoom into the cursor location, pull back to zoom out.
- Hold down the mouse wheel or control key and scroll the mouse wheel to focus (if available)

Keyboard Commands:

- Spacebar: Show overview image.
- S: Show/hide current status
- M: Show/hide maps windows.
- T: Show/hide toolbar
- B: Show/hide scalebar
- F: Enable/disable sharpen filter
- Backspace/Alt+left: To go back to the previous screen.
- Alt+right: To go forward.
- Arrow keys: Move whole screen in that direction.
- C: Copy the current image to the clipboard
- Alt-C: Copy current coordinates to clipboard.
- I: Show/hide image controls
- L: Change language.
- Page Up/Page Down: Focus Up/Down if available
- R: Rotate image (press and release for 90° steps, hold and move mouse for free rotation)
- O: Open a new image
- E: Export the current image to a file
- Q/Escape: Quit
- Number keys change lens:
0: Overview, 1= 1.25x, 2: 2.5x, 3: 5x, 4: 10x, 5; 20x, 6: 40x, 7: 63x, 8: 100x.
- +/-: Change lens up/down

When a new scanned image is opened in the viewer the toolbar is turned on and visible.

5. You can turn the Toolbar on or off by pressing the “T” key or clicking on the **X** in the upper right of the toolbar.
6. The tool menu is displayed by clicking the right mouse button.
7. The magnification can be changed from 1.25x to 100x with the “Toolbar”
8. Multiple scanned files can be opened at once in the viewer by simply selecting “Tool menu > Open”. You have the choice to open another scanned image above, below or on either side of the already open scanned image.
9. The scanned images can be locked together (synchronised) so that when one scanned image is moved or zoomed in, the other opened scanned image will also follow the same commands.
10. The current displayed image can be exported as a JPEG, BMP or TIFF or copied by “Toolbar > icon” or “Tool menu > Export or Copy” or “E” or “C” keys respectively.

CELL INJURY AND DEATH

AIMS

- (1) To look at an example of reversible cell injury
- (2) To see the two types of cell death: APOPTOSIS and NECROSIS

REVERSIBLE INJURY

Cells have means of responding to injury, many of which are reversible. Amongst them is the shutdown of metabolic processes. These reversible reactions are seldom visible in tissue sections, but the handling of lipid does sometimes produce visual clues to the underlying cellular processes. Traditionally called FATTY CHANGE, it is the accumulation of lipid droplets in the cytoplasm. These droplets dissolve during routine processing and appear as sharp-edged vacuoles in paraffin sections, which vary in size from the barely visible to those which distend the cell. If it is severe, even the macroscopic appearance of the organ may be affected, e.g., a liver containing large amounts of fat may appear very pale.

Fatty change is reversible, despite the appearance, even a cell distended with lipid can return to normal if the injurious stimulus is withdrawn. The most extreme examples usually occur in liver cells, but it is also seen in cardiac and occasionally kidney cells, and other metabolically active tissues. It is believed that this is related to the disruption of aerobic glycolysis and beta-oxidation of fat. Under such circumstances, fatty acids are not broken down and the cells esterify glycerol to form triglycerides which form the fat droplets within the cytoplasm.

1.1 Liver: fatty change 87.117 & 65.141

Catalogue Number	Small Image	Image Map	Large Image
A_CI_FC_LV_10.jpg	Fatty liver		Fatty liver
A_CI_FC_LV_01.jpg	Fatty liver	Image map	Fatty liver
A_CI_FC_LV_04.jpg	Fatty liver		Fatty liver

Macroscopically, this liver was pale, greasy and enlarged, weighing 2,000g. (Liver weights vary with body build but this is heavier than normal). These sections were taken from a patient with heavy alcohol use. Alcohol (and other causes) can result in reversible injury leading to fatty change in the hepatocytes.

Q (i) What are the colourless vacuoles in the cytoplasm of the cells?

Q (ii) Why are they colourless?

IRREVERSIBLE INJURY

More severe injury to cells may be irreversible and causes the cells to die. This may be effected by two pathways, by NECROSIS or APOPTOSIS.

NECROSIS

In necrosis there is complete breakdown of cellular homeostasis, with a largely uncoordinated breakdown of all cellular constituents. Under the microscope this appears as changes in the nucleus and cytoplasm.

Nuclear changes:

There is fading and eventual loss of normal basophilic staining of DNA, so that the nucleus eventually disappears and this is called KARYOLYSIS.

Cytoplasmic changes:

The cytoplasm becomes more avid for eosin (i.e. it becomes more eosinophilic) as proteins are denatured. This denaturation of proteins is described as coagulative necrosis. Necrosis is frequently caused by vascular occlusion or exposure to toxic compounds. It often affects sheets of contiguous cells. The cells swell and the plasma membranes burst open releasing cellular contents which usually excites an inflammatory reaction from the surrounding viable tissue.

1.2 Rat liver: necrosis 70.624A

Catalogue Number	Small Image	Image Map	Large Image
A_EX_CJ_LV_02.jpg	Cold injury	Image map	Cold injury
A_EX_CJ_LV_03.jpg	Cold injury	Image map	Cold injury
A_EX_CJ_LV_04.jpg	Cold injury	Image map	Cold injury

At one edge of the liver is a sharply demarcated pale zone of necrosis, the result of freezing with a cold probe. The animal was killed 24 hrs after this injury.

The cytoplasm of the necrotic cells is pale pink and contrasts with the healthy hepatic cells which normally contain abundant organelles. Some of the liver cell nuclei have disappeared entirely but others are still present, although small and pale.

Remember to use the short-hand terminology to describe the histological changes in necrosis such as karyolysis and cytoplasmic eosinophilia.

APOPTOSIS

In apoptosis, the death process is driven from within the cell as a reaction to stimuli that may or may not be intrinsically lethal. It usually affects individual cells surrounded by viable neighbours. Both

intracellular stimuli (e.g. DNA damage) and extracellular stimuli (e.g. signals from surrounding cells including cytokines) can lead to apoptosis. Apoptosis may be seen as a form of cell suicide, whereas necrosis is more akin to murder!

The cytological changes of apoptosis include cell shrinkage, loss of cell-to-cell contact and condensation of chromatin at the periphery of the nucleus. These nuclei are smaller and denser and strongly basophilic and are described as pyknotic. The condensed nucleus fragments into several small round balls of condensed chromatin. Fragmentation of the cell into small membrane bound bodies occurs as an explosive event. These fragments (or apoptotic bodies) are then phagocytosed either by the cell's neighbours or by macrophages already present in the tissue. Please take time to look at the demonstration photographs.

Note that apoptosis allows individual cells to vanish from the scene without causing an acute inflammatory reaction

**H5 Normal Lymph Node with Germinal Centres
85.163, 75.221 & 58.444**

Catalogue Number	Small Image	Image Map	Large Image
N_HL_LN_91.jpg	Lymph Node		Lymph Node
N_HL_LN_50.jpg	Lymph Node	Image map	Lymph Node
N_HL_LN_79.jpg	Lymph Node		Lymph Node
N_HL_LN_83.jpg	Lymph Node		Lymph Node

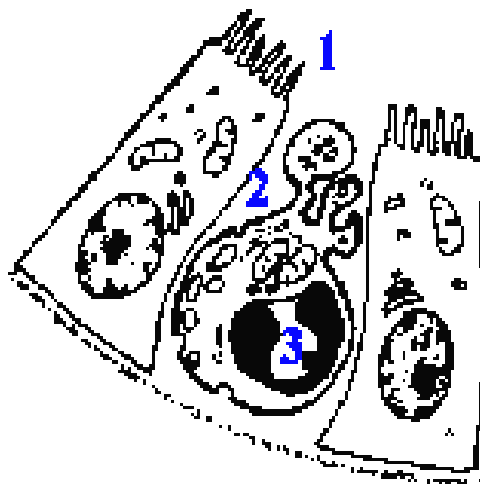
Examine the slide of the normal lymph node, identifying the follicles with germinal centres (round or oval aggregates of cells with a group of pale cells in the centre [the “germinal centre”] and a rim of darker appearing lymphoid cells) at low power. Having found the germinal centres, move to high power and you can usually identify examples of apoptotic cells (these occur at a higher frequency in germinal centres) in most of the germinal centres, but occasionally you may have to look at more than one germinal centre at high power to find apoptotic cells. In comparison, in the darker staining lymphoid cells around the follicles there are hardly any apoptotic cells to be found. The most easily recognisable feature of the apoptotic cells is the condensed nuclear chromatin that either appears as a darkly staining single nucleus or a fragmented nucleus (several small round balls of dark blue/black staining condensed chromatin). The apoptotic cytoplasm is often a deeper orange/pink. The apoptotic cells may also be seen within vacuoles (with an apparently empty space surrounding the apoptotic bodies within the vacuole) inside neighbouring cells (macrophages in the germinal centres), having been phagocytosed rapidly after the onset of apoptosis.

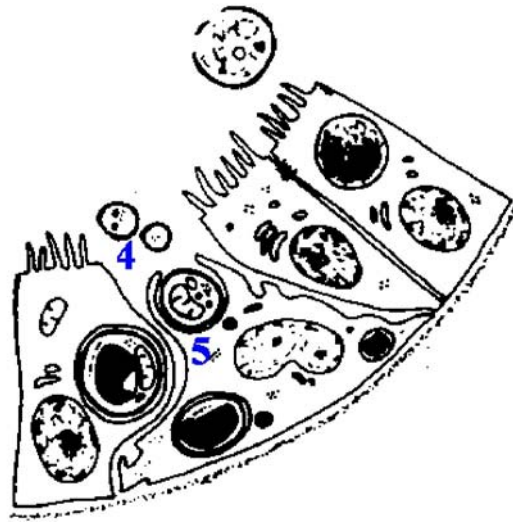
The apoptotic cells should not be confused with mitotic figures, which are also plentiful in the germinal centres. Mitotic cells usually have condensed chromosomes lining up (in metaphase) for separation (in anaphase) into two groups during cell division. The mitotic condensed chromosomes also stain a dark blue/black colour, but tend to appear as a “hairy caterpillar” in metaphase, or as two “hairy caterpillars” being pulled apart in anaphase.

N.B. Please note that we will return to studying the structure of the lymph node in a later practical – the intention here is only to identify apoptotic cells.

APOPTOSIS: KEY MORPHOLOGICAL FEATURES

1. Cell shrinkage.
2. Loss of surface contact with neighbours.
3. Chromatin condensation (dark blue/black staining) around edge of the nucleus or fragmentation into small balls of condensed chromatin.
4. Fragmentation of the apoptotic cell into small apoptotic bodies.
5. Phagocytosis by neighbours or macrophages.





DEMONSTRATIONS

A. Preparation of tissue for histological examination

The many steps required to process fresh tissue to the stained sections we are studying are shown. It is worth looking quickly at how it is done because it will help you understand some of the artefacts which affect the sections.

For example, once thin sections of the paraffin wax impregnated block have been cut, they are floated on the surface of a warm water-bath, so they can be picked up on a glass slide. You will often see tiny folds present where the section failed to flatten out completely before it was mounted.

B. Apoptosis: Photographs

The following changes are often described in the nucleus in cells undergoing apoptosis:

PYKNOSIS	Smaller and denser nucleus. Strongly basophilic staining of condensed chromatin.
KARYORRHEXIS	Nuclear fragmentation into many balls of condensed chromatin.

Catalogue Number	Small Image	Image Map	Large Image
N_HL_LN2_37.jpg	Apoptosis	Image map	Apoptosis
N_CS_AF_01.jpg	Apoptosis	Image map	Apoptosis
N_CS_AF_02.jpg	Apoptosis	Image map	Apoptosis
N_CS_AF_03.jpg	Apoptosis	Image map	Apoptosis
N_CS_AF_04.jpg	Apoptosis	Image map	Apoptosis
N_CS_AF_05.jpg	Apoptosis		Apoptosis
N_CS_AF_06.jpg	Apoptosis		Apoptosis

QUESTIONS

- (iii) What do you understand by the term eosinophilia applied to injured cells and how is it brought about?
- (iv) How can you tell the injury to the liver shown in Section 1.2 was inflicted whilst the rat was still alive (but anaesthetised)? After all, freezing the surface of dead liver might cause changes.
- (v) Following tissue injury, what major types of tissue reaction might one expect ?
- (vi) How are necrosis and apoptosis different from each other?

WORDS USED TODAY (all Greek)

Pneumon	Lung	PNEUMONIA
Necros	Dead	NECROSIS
Pyknos	Compact	PYKNOSIS
Karyon	Nut (as in nut-shaped nucleus)	KARYORRHEXIS
Rhexis	Wrecking, bursting out	KARYORRHEXIS
Lysis	Dissolution, breaking down	KARYOLYSIS
Chromatos	Colour	CHROMATOLYSIS
Apo	from	APOPTOSIS*
Ptoxis	A fall	APOPTOSIS*
Pathogenesis	Sequence of events in evolution of a disease	
Aetiology	Assignment of the cause of disease	

* Apoptosis: this term was originally coined to represent the “programmed death” typified by leaves falling from trees in the Autumn.

TIDYING UP

*Before you leave, please remember to:
Dim and then switch off the microscope light.
Cover the microscope and leave it on the bench.
Return the wooden block if you used one.
Push your stool under the bench.
Thank you!*