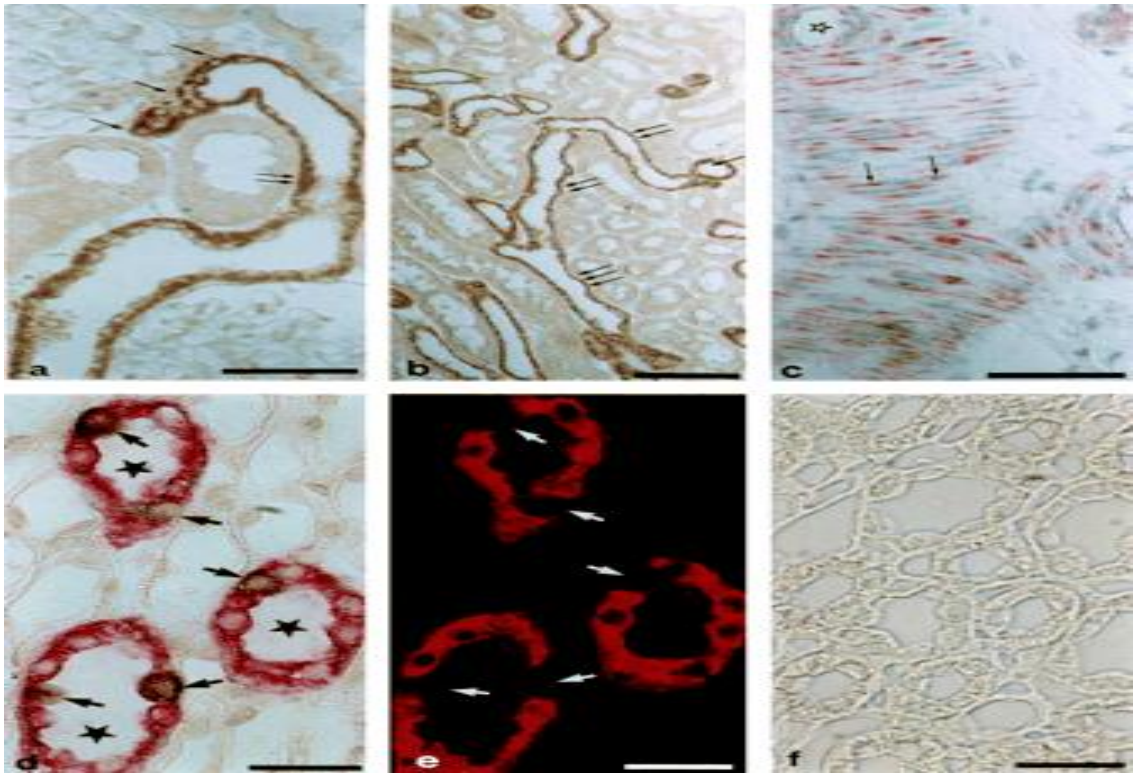
PRACTICAL: Immunohistochemistry (IHC)

Techniques we will cover:

- IHC technique
- Microscopy
- Basic primary and secondary lymphoid organ structure

J Am Soc Nephrol 13:1-9, 2002



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Contents Page

Time Plan

Exp. / Time	Discussion & Analysis	Protocol 1 Spleen Section IHC	Protocol 2 Blood Cell Populations	Breaks
10.00	Lab re-cap			
10.30	Introduction			
11.00		Add 1° Ab		
11.30		Incubate 1° Ab	Stain blood cells & view	
12.00		Wash slides		
12.30		Add 2° Ab		LUNCH
13.00		Incubate 2° Ab		
13.30		Wash slides		
14.00		Develop & Mount		
14.30	Lecture			
15.00				
15.30		View & analyse slides		
16.30		View demo slides		
17.00				

N.B. **Please read through the entire practical before the day of the practical:**

1. Complete Exercise 1 before arriving at the practical.
2. Read over the introduction and protocols.
3. Think about the questions for discussion.
4. Fill in the abbreviation list and glossary on page 13.

Introduction

Immunohistochemistry (IHC) is one of the principle methods employed in immunological science. IHC is a method of identifying cell types within tissue sections based on the detection of antibodies bound to specific components of cells. This technique provides us with a window into the cellular world *in vivo.* Many modern techniques provide essential information, but few reveal in such detail what is happening in the context of living tissue. In this practical you will be using a panel of unconjugated primary antibodies that bind to various cellular antigens of immunological significance in spleen sections. The fact that the primary antibodies are unconjugated means that you will need to use a conjugated secondary antibody to detect where the primary antibodies have bound; Indirect IHC (**Figure 1A**). The technique we will use is described in Protocol 1. In this technique a primary layer monoclonal antibody (mAb) is applied to a tissue section to detect a cellular antigen (e.g. CD4). The specifically bound primary antibody is then detected using a secondary antibody which specifically recognises the primary antibody. The secondary layer antibody is conjugated to an enzyme. When the enzyme's substrate is added to the tissue section, the antibody-conjugated enzyme acts on its substrate to produce a coloured precipitate of the substrate – known as a chromogen. The coloured precipitate is formed where the antibody is bound to the tissue, thus allowing the antibody position to be visualised by light microscopy. The technique of *direct* IHC staining is similar, except the primary Ab itself is directly conjugated to the enzyme (**Figure 1B**).

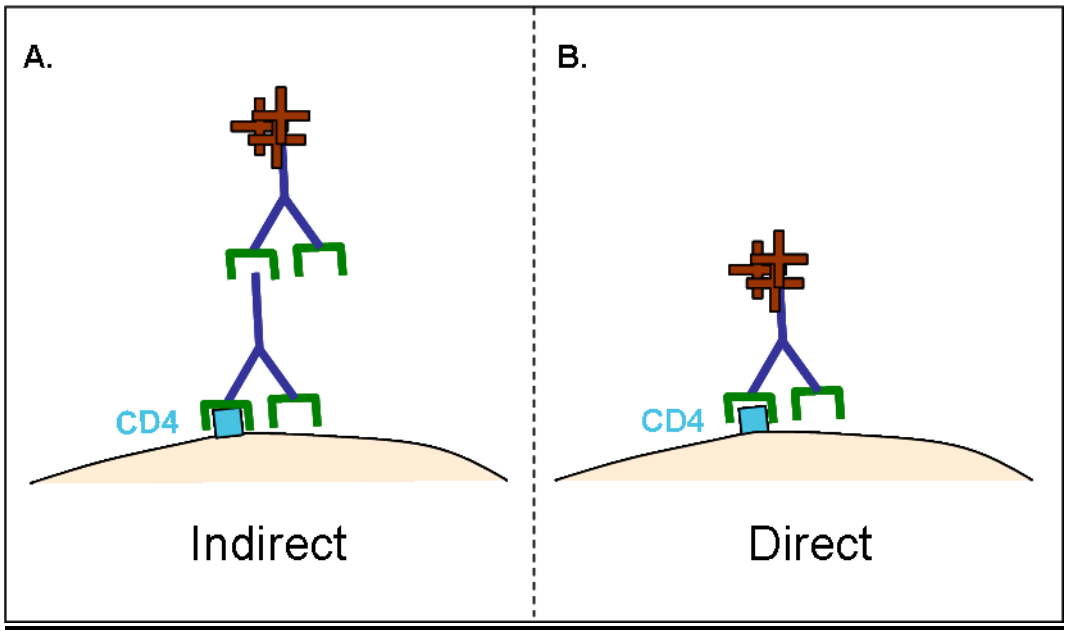


Figure 1. Indirect (A) and Direct (B) Immunohistochemistry.
See above text for details.

In this practical the secondary antibody is conjugated to the enzyme Horse Radish Peroxidase (HRP). The chromogenic substrate of HRP is 3,3'-Diaminobenzidine tetrahydrochloride (DAB)/H_2O_2. The chromogen formed when HRP acts on DAB is brown. Wherever the primary and secondary antibodies bind, a brown precipitate will form. In protocol 2 you will stain cells found in whole blood (ICC. Also called Cytology) and learn to distinguish between cell types based on their staining patterns.

Experimental Aims

To perform Immunohistochemistry (IHC) and cytology in order to i) observe the presence or absence of specific leukocytes in the spleen, ii) to observe spleen architecture and iii) to distinguish between different cells found in whole blood.

Learning Objectives

By the end of this practical you will be able to:

- Understand the methodologies of IHC and cytology
- Understand the potential pitfalls and the reasons for controls
- Relate the technique of IHC to other immune detection techniques
- Visualise stained tissue sections and cells by light microscopy
- Identify splenic germinal centres in stained tissues
- Identify different populations of cells in a blood smear

Context

The techniques we will use and discuss in this practical relate directly to other immunological techniques such as ELISA and Flow Cytometry. By staining tissue sections yourselves you will be able to combine theoretical knowledge of germinal centre formation and thymic development with the practical technique used to visualise these events.

Questions to Consider for Discussion

1. How does direct staining differ from indirect staining?
2. Which controls are required for IHC staining and what do they tell us?
3. Why does the PBS, that the primary and secondary antibody reagents are diluted into, contain 5% sera?
4. Can you name any other immunological techniques which use the same principle (i.e.: labelled antibodies) to detect molecules of interest?

Materials & Reagents

Universals
Staining trough (Coplin jar)
Humidifying box: Transparent box with wet filter paper and sticks
Phosphate buffered saline pH 7.4 (PBS)
Spleen section slides (prefixed with acetone)
Mouse blood smear slides
Pipettes
Tissues
3,3'-Diaminobenzidine tetrahydrochloride (DAB) solution
Haematoxylin
Coverslips
DePex Mountant
Diff-Quik® Staining Kit:
Diff-Quik® Solution I (Xanthene dye, pH buffer, Sodium Azide),
Diff-Quik® solution II (Thiazine dye, pH buffer)
Diff-Quik® Fixative Reagent (Triarylmethane dye, Methanol)
Primary Antibodies: See Exercise 1.
Pre-blocking solution: PBS containing 5 % serum
No antibody, negative control: PBS
Secondary antibody:
Rabbit anti-Rat Ig conjugated to Horse Radish Peroxidase (HRP) (in 5% serum). The secondary antibody is diluted 1:50 in PBS with 5% serum: The serum reduces background caused by the secondary antibody cross-reacting with murine Ig in the tissue.

Recipes

PBS

8 g NaCl

0.2 g KCl

1.44 g Na_2HPO_4

0.24 g KH_2PO_4

Make up to 1 litre (pH 7.4)

Exercise 1: Primary Antibody Dilutions

Complete Table 1 below before the practical using the example in the top row (shaded grey) as a guide.

Antibody Target	Initial Concentration	Final Concentration	Dilution Factor	Marker of:
CD3	100 μg/ml	5 μg/ml	1:20	T cells
CD4	50 μg/ml		1:50	
CD8	50 μg/ml	5 μg/ml		
CD19	0.02 mg/ml		1:4	
F4/80	1 mg/ml	1 μg/ml		
MHC Class II (M5/114)	Neat	Neat	None	
Isotype control IgG2a	0.5 mg/ml	1 μg/ml		

Table 1. Antibody Dilutions.

Protocol 1: Spleen Section - IHC

The samples you have been provided with are murine spleen sections, they were prepared in the following way: Spleen tissue, removed from a mouse, was embedded in OCT matrix and frozen in liquid nitrogen. Tissue sections were then cut (6 µM thick) using a cryostat, carefully transferred onto a slide and left to dry for a minimum of 2 hours (preferably overnight). When dry the sections were fixed in ice-cold acetone, wrapped in tin-foil (whilst taking care not to disturb the section) and frozen at -20°C.

Important points to note before starting:

- Wear gloves at all times when working with murine tissue
- Keep the tissue sections wet at all times (do not allow to dry). The staining is therefore performed in a humidified box.
- Make sure slides have warmed up to room temperature before starting the staining procedure.
- Do not touch the tissue sections themselves at any time with fingers or pipettes!
- Where you see this sign: 

please wait for demonstration of the technique before attempting it yourself.

A diagram of the steps you will perform is shown on the next page in Figure 2 (N.B. You need to complete part of it yourself!). This is followed by text describing exactly how to do each step.

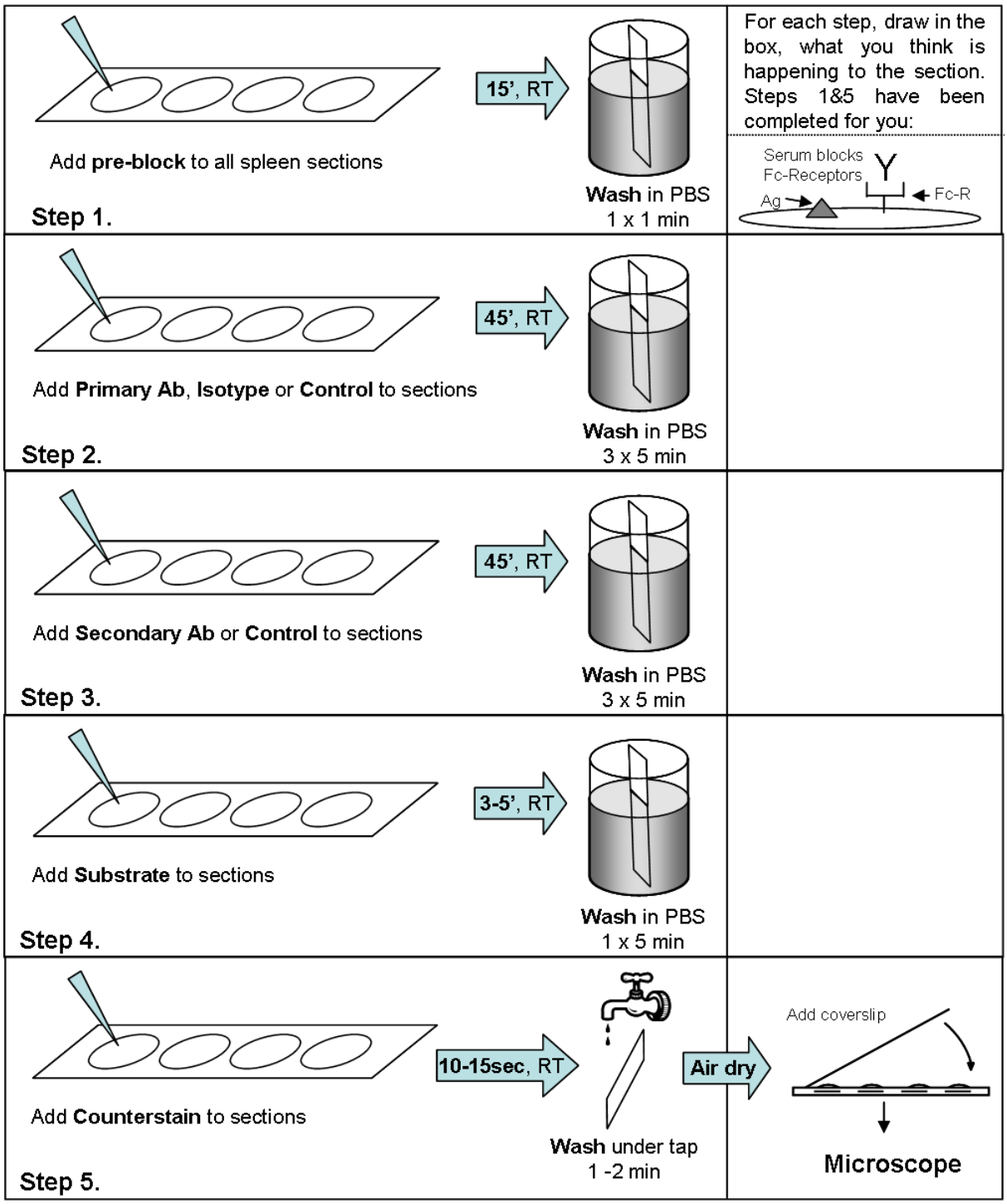


Figure 2. Diagram of the IHC protocol.

Protocol 1: In detail…

Pre-blocking

1. Label slides using a pencil on the white edges (see diagram overleaf). Place slides into the humidifying box on the sticks provided in the box.

2. Carefully add 50 µl of pre-blocking solution to each section. Incubate for 15 min at room temperature (RT). What is in pre-blocking solution and why?

Washing

3. Tap the excess pre-blocking solution off the slide onto paper towels. Wash the slide once in PBS: Fill the Coplin jar with PBS and carefully place the slide in the grooves of the Coplin jar. Leave for 1 minute then slowly pour out the PBS whilst holding the slides in the jar.
   Remember not to touch the tissue section.

4. Carefully dry around each well using a piece of folded tissue, be careful not to wipe off the sections. Do not allow the sections to dry out.

Addition of Primary Antibody

5. Add 50 µl of the appropriate primary antibody (already diluted in 5% serum/PBS) to each section as shown below in Figure 3.

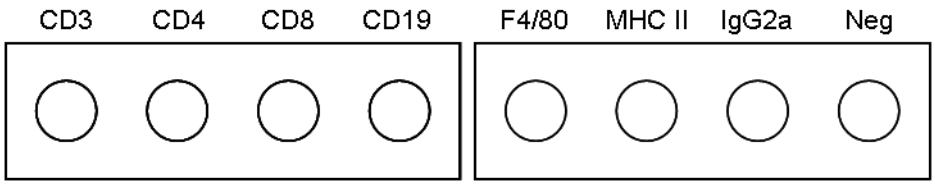


Figure 3. Layout of slide.

6. Incubate at room temperature for 45 minutes.

7. Tap the excess antibody solution off the slide onto paper towels. Wash the slide in PBS; fill the Coplin jar with PBS and carefully place the slide in the grooves of the Coplin jar. Leave for 5 minutes then slowly pour out the PBS whilst holding the slides in the jar.
   Remember not to touch the tissue section.
   Repeat this two more times; a total of 3 washes.

Addition of Secondary Antibody

8. Apply 50 µl of the secondary antibody (Rabbit anti-Rat Ig HRP) to the sections. *Remember: Do not add any antibody to your Neg but do add PBS to the Neg to stop it drying out.*

9. Incubate at RT for 45 minutes to 1 hr.

10. Wash 3 times in PBS as before, 5 minutes per wash.

Addition of Substrate

11. Prepare the chromogenic substrate: Add all of the DAB Liquid Chromogen Solution (B) to the DAB Liquid Buffer Solution (A). Mix well.

12. Add 100 µl substrate per section. Incubate for 3-5 minutes at RT.

13. Wash once in PBS, for 5 minutes, as before.

Application of Counterstain

12. Counterstain in haematoxylin for 10-15 seconds: ***WEAR GLOVES*** (haematoxylin will permanently stain your skin blue and is toxic when absorbed through skin). Apply 100 µl of haematoxylin per section, count 10-15 seconds whilst watching the tissue on the slide change colour, then carefully rinse the slide in slowly running tap water until blue (about 1-2 min). The haematoxylin will stain all nucleated cells and will allow you to see the general tissue structure.

Mounting Sections: *N.B.* This will be done in the fume hood.

13. Mount your sections using a drop of mountant (See Figure 4): Set your p1000 pipette to 1 ml, draw up 1 ml of mountant into the pipette and gently squeeze the plunger until a drip forms, allow the drop to fall onto the slide, 1 drop per section. Hold the coverslip at a 45° angle to the slide as shown below, then supporting it with a pipette tip, slowly lower the slide:

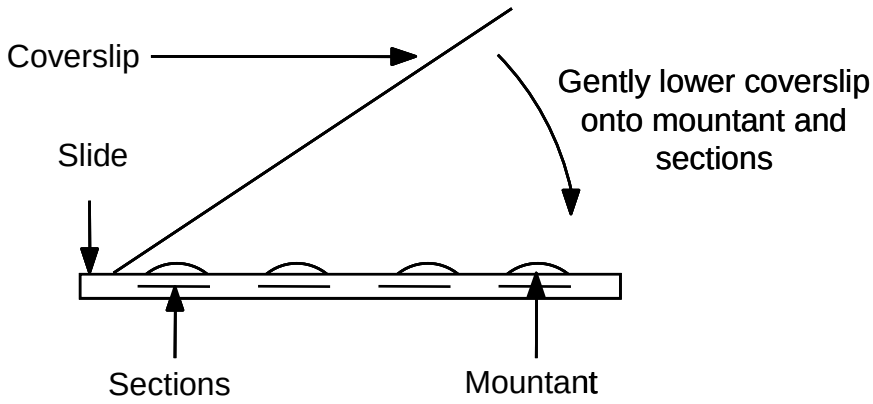


Figure 4. Schematic representation of method used to mount coverslips.

13. Allow the mountant to dry.

14. View slides under the light microscope

15. See Appendices 1 – 4 at the back of the handout for examples of what you should expect to see.

Protocol 2: Blood Cell Populations - ICC

You have been provided with murine blood smears that have been allowed to air dry and then fixed to microscope slides using Diff-Quik® fixative. You will now stain and mount the cells these to reveal the morphology of the different cell populations within murine peripheral blood. Diff Quik® is a proprietary mixture of the thiazine dyes methylene blue and azure A in a water solution with a buffer. Different cells stain a variety of colours depending on their cellular constituents. This allows you to distinguish between cell types. Looking at individual cells stained in this way is an example of cytology.

By the end of this component of the practical you will be able to identify and describe the morphology of immune cells. Appendix 5 at the back of the handout (adapted from Wheater's Functional histology 5th Edition) will help with the identification process.

You will have these techniques demonstrated to you.

1. Place the slide into Diff-Quik Solution I (red) for 5 seconds. Then drain off excess fixative onto a paper tissue
2. Counter-stain the cells on the slide using Diff-Quik Solution II (blue) for 6 seconds. Then drain off excess fixative onto a paper tissue.
3. Rinse gently with tap water to remove excess stain. Air dry until mostly dry.
4. Mount using oblong coverslips and mountant (See also Figure 4).
5. Allow to dry for 10 - 15 mins before viewing (this allows the mountant to set so that the coverslip will not slide around when using the 100 X objective on the microscope).
6. Observe under the light microscope, use Table 2 below and Appendix 5, to help you identify the different cell populations. You may be able to see: Lymphocytes, NK cells, Neutrophils, monocytes, mast cells, eosinophils, basophils and erythrocytes. Have a look at Table 3 and fill in the gaps below before the practical so that you know how likely you are to see each cell type (there is approximately 100 μl blood/smear).

1L = _____ ml = _____ μl

Structure	Colour
Erythrocytes	Pink/yellowish red
Platelets	Violet/purple granules
Neutrophils	Blue nucleus, pink cytoplasm, violet granules
Eosinophils	Blue nucleus, blue cytoplasm, red granules
Basophils	Purple/dark blue nucleus, violet granules
Monocytes	Violet nucleus, light blue cytoplasm

Table 2. Cell histology revealed by Diff-Quik® staining

Cell Type	Erythrocyte	Lymphocyte	Neutrophil	Eosinophil	Basophil	Monocyte	Platelets
Cell Size (μM)	6.7 - 7.7	6 - 15	12 – 14	12 - 17	14 - 16	16 - 20	1.5 – 3.5
Number/L	3.9 - 6.5 $x10^{12}$	0 - 0.1 $x10^{9}$	2 - 7.5 $x\ 10^{12}$	1.3 - 3.5 $x10^{9}$	0 - 0.44 $x10^{9}$	0.2 - 0.8 $x10^{9}$	150-400 $x10^{9}$
Number/100 μl							
Percentage of total leukocytes	-	20 - 50%	40 - 75%	1 – 6%	<1%	2 - 10%	-
Development	5-7 days	1-2 days	6-9 days	6-9 days	3-7 days	2-3 days	4-5 days
Lifespan of mature cell	120 days	?	6hrs – few days	8-12 days	?	Months-years	8-12 days

Table 3. Peripheral blood cell population characteristics

Abbreviations

DAB:
ELISA:
HRP:
IHC:
mAb:
PBS:
RT:

Glossary

Direct immunofluorescence:
Ex vivo:
Immunocytochemistry:
Immunofluorescence microscopy:
Immunohistochemistry:
Indirect immunofluorescence:
In vivo:
In vitro:

References:

1. Janeway and Travers, Immunobiology. 6th Edition, (2004) Garland Publishing.
2. Posters in teaching laboratory

Data Interpretation

1. Page 14 – 16 of this handout.

COSHH form

Substances: Tissues of murine origin, murine serum and rabbit serum
Sodium Azide
3,3'-Diaminobenzidine tetrahydrochloride (DAB)
Haematoxylin
Diff Quik®
Faramount
DePex

Data Interpretation

1. How does direct staining differ from indirect staining?

2. Which one is likely to be more sensitive?

3. Which controls are required for immunohistochemical staining and what do they tell us?

4. Why does the PBS, that the primary and secondary antibody reagents are diluted in, contain 5% sera?

5. Can you think of any other immunological techniques which use the same principle (i.e.: labelled antibodies) to detect molecules of interest?

6. On the *basis of today's experiment*, fill in the gaps relating to the figure below:

   Primary Ab(s) =…….
   Secondary Ab =……
   Conjugate =……..
   Substrate =……
   Detection method =…….

6. If you changed the experiment to use a fluorochrome conjugated antibody instead of the HRP conjugated one, which steps of the experiment would you have to modify?

7. If you changed the experiment to use a biotinylated secondary antibody instead of the HRP conjugated one, which steps of the experiment would you have to modify/add?

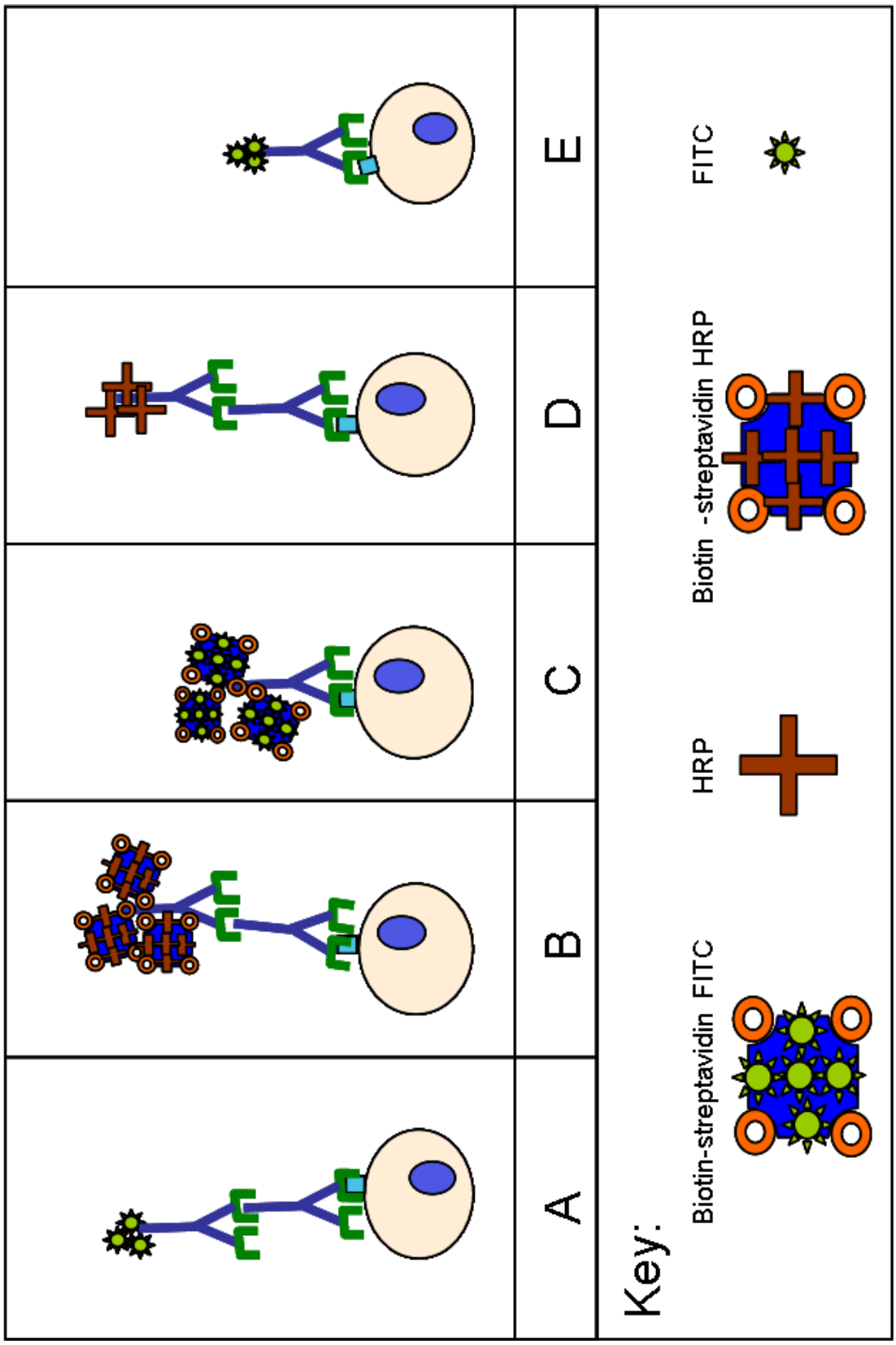
8. Which of the following diagrams illustrate:

Direct immunofluorescent staining:

Indirect immunocytochemical staining:

Fluorochrome detection method:

Enzyme detection method:



9. From the list of reagents below, which of the reagents (including the appropriate controls; more than 1 option in some cases) would you use to perform the following experiments*:

A) To detect human CD3 on splenic T cells and visualise under a fluorescent microscope
B) To detect murine CD19 on splenic B cells and visualise using DAB
C) To detect murine macrophages in spleens and visualise using DAB
D) To detect rat cytotoxic T cells and visualise under a fluorescent microscope
E) To detect Class II on murine splenocytes and visualise by fluorescence
F) To detect rat CD19 and visualise using DAB

Reagents:

Hamster anti-human (IgG) CD3 - Biotin
Rat anti-mouse (IgG1) CD3
Mouse anti-human (IgG2b) CD19
Rat anti-human (IgG2b) CD19
Hamster anti-mouse (IgG) CD19
Rabbit anti-rat (IgG2b) CD19 - HRP
Rabbit anti-rat (IgG2a) CD8 - FITC
Sheep anti-hamster - FITC
Sheep anti-hamster - Biotin
Sheep anti-mouse - FITC
Streptavidin - HRP
Streptavidin - FITC
Human IgG
Human IgG2a
Hamster IgG
Rat IgG2b
Rat IgG1
Rat IgG2a
Rabbit IgG2b - FITC
Rabbit IgG2b - HRP
Rat IgG1 conjugated to: HRP, Biotin or FITC
Rat IgG2a conjugated to: HRP, Biotin or FITC
Rat IgG2b conjugated to: HRP, Biotin or FITC
Hamster IgG conjugated to: HRP, Biotin or FITC
Mouse IgG1 conjugated to: HRP, Biotin or FITC
Mouse IgG2a conjugated to: HRP, Biotin or FITC
Mouse IgG2b conjugated to: HRP, Biotin or FITC
Mouse IgG3 conjugated to: HRP, Biotin or FITC

F4/80 (see practical handout)
M5/114 (see practical handout)
Mouse anti-human (IgG2a) CD8
Human anti-mouse (IgG2a) CD8
Rabbit anti-rat (IgG2b) CD8
Rat anti-rabbit CD19-HRP (IgG2b)

Goat anti-rat - FITC
Goat anti-rat - HRP
Goat anti-rat - Biotin
Goat anti-rabbit - Biotin
Goat anti-mouse - Biotin
Rabbit IgG2a
Rabbit IgG2b
Mouse IgG2a
Mouse IgG2b

* You may need to refer to your Practical and Lecture handouts for some of the antibody information