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Making the Most of Your Microscopy Mini Series

Session Three: Haematology: Preventing Automated Analyser Misdiagnosis

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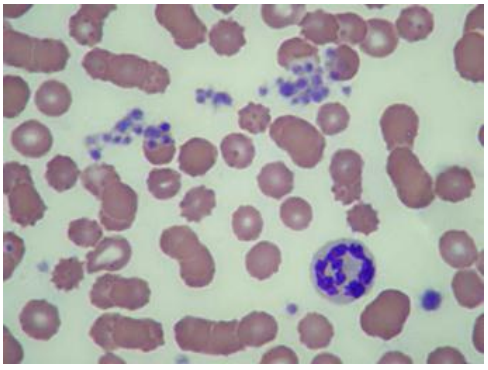


This course will focus on the sampling, processing and examination of samples for haematology, but will only briefly address the clinical interpretation or significance of any findings or tests.

Sampling

As a general rule, venupuncture should be **clean and quick**, using as **large** a needle as possible with **minimal** or gentle suction only. **Typically this involves using the jugular vein with a 21-23 gauge needle and 2.5-5 ml syringe**. A peripheral vein, especially the cephalic, can be used in medium to large breed dogs as it can be easier to locate and sample (in overweight animals), with some animals also being easier to restrain for this sampling site. It is better to get a clean and quick sample than make repeated attempts at any single vein. If the first attempt fails, it is advisable to use a new needle (and possibly syringe), changing the venupuncture site slightly or using a different vein. Unless the animal is very small (*e.g.* less than 2.5 kg) a 21 gauge needle is recommended.

All these guidelines aim to minimise trauma to the cells and therefore the risk of artefact and cell lysis/haemolysis. Haemolysis will reduce red cell numbers, PCV and artificially elevate the cell haemoglobin concentrations. A clean technique also minimises tissue damage and activation of platelets during sampling. Therefore platelet clumping and clot formation is prevented. If either is present, platelet numbers will be artificially low and potentially white cells too, especially neutrophils.

The blood is typically placed into EDTA (ethylenediamine tetra-acetic acid) anticoagulant for many veterinary species. **The tube should be filled, without the needle attached, and to the correct level** for several reasons:

- underfilling results in a relative anticoagulant excess, especially if it is in liquid form. This excess shrinks the cells and alters their morphology. It can thus also artificially reduce the MCV and PCV. **If the tube is less than half full, the PCV can be reduced by 5%.** These effects are compounded if the animal is actually anaemic or cytopoenic (reduced cell numbers).
- overfilling will allow clotting to occur, which can prevent automated analyser analysis, and alter cell numbers as outlined above.
- if the needle is still in place, the cells are traumatised and more likely to lyse when in the tube.

The blood should be gently mixed with the anticoagulant by inversion and rolling in your hand, **not** shaking**. At least one fresh blood smear/film should now be made**. This is especially true if the sample is going via the post to an external lab. Cells begin to degenerate as soon as they leave the body, with significant changes being seen after 12 hours. Whilst one can

nearly always interpret morphology in aged samples, subtle features are often lost, artificial features are created or clinical interpretation is hampered. Once the smears are made, the sample can be processed or stored in the fridge if being sent to an external referral lab. The slides should **not** be stored in the fridge though, as condensation following removal can damage the cells or wash the sample off.

If chemical restraint or sampling occurs under general anaesthesia, this must be noted. Changes in blood pressure can have very significant effects on circulating cell numbers. This is seen naturally/physiologically in animals that are agitated during sampling. Increased blood pressure (and splenic contraction) results in increased numbers of circulating white cells (especially neutrophils) and red cells. Conversely, sedation or anaesthesia lowers systemic blood pressure and causes splenic sequestration that can reduce the number of circulating cells, both red cells (falsely low PCV) and white cells (false leukopaenia).

In summary:

Haemolysis is caused by:

- excessive suction
- narrow gauge needle (especially if left on when filling the tube)
- shaking the tube
- prolonged storage, especially at high temperature or with large temperature fluctuations
- lipaemic samples, especially over time / with postage

Sampling technique:

1. Label EDTA tube.
2. Use 2.5-5 ml syringe and 21 gauge needle in the jugular vein.
3. Ensure the venupuncture is clean and quick.
4. Remove the needle and gently expel the blood into the EDTA tube.
5. Fill to the correct level (indicated by a small black line commonly). Filling >50% at least is recommended.
6. Gently mix by rolling and inverting the tube.
7. Make a fresh blood film from the EDTA tube.
8. Store the tube in the fridge if not analysing immediately

N.B. Ideally, leave the blood in the EDTA at least 10 mins before analysing. This prevents unknown artifacts being introduced as the blood and EDTA have time to equilibrate.

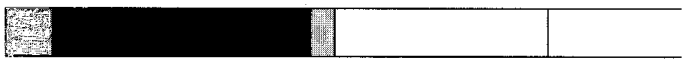
Complete / Full Blood Count (CBC / FBC)

In house analysis varies from a simple microhaematocrit and plasma protein measurement to a full analysis - five part white cell differential (neutrophils, lymphocytes, monocytes, eosinophils and basophils), red cell parameters (RBC, Hb, HCT/PCV, MCV, MCH and MCHC) including reticulocytes, along with platelets - all depending on the equipment available. **Both scenarios though must involve assessment of a fresh blood film.** A huge amount of information can be obtained from only a microhaematocrit tube and blood film examination.

Packed Cell Volume (PCV)

This refers to the volume of red cells that is produced after high speed (11-15000 rpm) centrifugation of whole blood in a microhaematocrit tube for 5 minutes. Whilst not identical, it is equivalent to the calculated Haematocrit (HCT) value produced by an automated haematology analyser. Unlike the PCV that can be affected by excess EDTA significantly (see earlier), HCT from an analyser can be less affected as the cells are diluted and 're-expand' when being analysed. However, HCT can be affected artificially by factors such as agglutination and prolonged EDTA exposure, as these affect the measured MCV.

Centrifugation produces a variable column of cells in the form of bands, as shown later. At the bottom (A) is the clay plug.; band B is the red cells; band C is the buffy coat - white cells and platelets – and band D is the plasma. The width of band B is expressed as a percentage of the total length (B + C +D) to give the PCV (*i.e.* B / (B+C+D) x 100). A crude estimate of the white cell count can similarly be obtained from expressing band C as a percentage of the total length. The first 1 % equates to 10 x 10^9/l, with every additional 2 % being an extra 20 x 10^9/l.



A B C D

When performing a manual PCV in this fashion, the microhaematocrit tube **must be filled 2/3 to 3/4** as the centrifugal force produced and required is based on this volume of fluid. If there is too little, the relative force is increased, whereas if there is too much, the relative force is reduced. This is often not clinically significant, but can be, artificially altering the PCV value when compounded by changes in cell numbers of the animal itself. For example, an anaemic animal (< 25%) that is also under filled can result in an artificially lower PCV. Similarly, if the red cell numbers are increased (PCV >50-55%), the PCV can be artificially high as the cells are not packed very well by standard centrifugation.

Two other important pieces of information are also readily available once the tube has been spun. These are the plasma protein level and plasma colour. The plasma protein is measured after the tube is gently broken (with a diamond cutter commonly) at the mid-level of the plasma column. A drop of plasma is then placed onto a refractometer. As with urinary specific gravity, light is bent to varying degrees depending upon the amount of substances dissolved in the liquid or plasma. A different scale is used from urine, with the value/level being multiplied by 10 to give the plasma protein in g/l. As with urinary SG, if the liquid is not 'clear', the level/value will be artificially raised. Thus, haemolysis, lipaemia and to a lesser degree, icterus, can all falsely elevate the protein level measured via refractometry. Measuring the biochemical total protein level is often more accurate, but can be similarly affected, although to a lesser degree.

Assessing the plasma colour is therefore useful to assess the accuracy of your plasma protein level. If the plasma is grossly haemolysed or icteric and the animal is anaemic, haemolytic diseases are immediately suggested. Assessing the blood film can then provide further evidence. Similarly, if the plasma is lipaemic, and the animal has been fasted appropriately, underlying hormonal diseases may be suggested. It must be emphasised that even if the plasma looks clear, free haemoglobin (haemolysis), increased lipids (lipaemia) or bilirubin (icterus) can still be present. For example it requires about 20 µmol/l of bilirubin (healthy levels typically being <7 µmol/l) to be visible to the naked eye. Therefore biochemical analysis will detect increased bilirubin before we can see it.

Whilst these may not seem useful, or better evaluated via an analyser, if the plasma is discoloured or the protein levels elevated, they can provide a cheaper (and quicker) way of monitoring disease states (such as icterus in liver disease or hyperglobulinaemia in multiple myeloma).

Finally, the colour of the plasma also allows you to assess whether there is any potential interference to automated analyser analysis.

- **Haemolysis** – this falsely reduces the red cell (RBC) numbers (and potentially PCV), whilst falsely elevating the Hb, MCH and MCHC.
- **Lipaemia** – can induce haemolysis. In itself, it can falsely elevate the haemoglobin parameters and artificially elevate platelet numbers due to lipid droplets or cell fragments being miscounted as platelets
- **Icterus** – this does not usually have any significant effect.

Automated analyser cell counting

Before any blood is run through an analyser, it should be:

- checked for clots using either a microhaematocrit tube or cocktail stick run around the tube to locate any clotted material. If not done, cell counts can be artificially affected or the analyser blocked/damaged.
- gently and evenly resuspended, especially if the tube has been left standing for a period of time. If this is not done, cell counts can be artificially affected.

There are a large number of haematology analysers available, **none of which can provide results that can be interpreted without also looking at a fresh blood film**. Most analysers provide 6 red cell parameters, a white cell count and platelet count. Most also provide a variable white cell differential that can be very inaccurate or misleading, although newer laser cytometer analysers giving a 5 cell differentiation (neutrophils, lymphocytes, monocytes, eosinophils and basophils) can be good in many healthy and some diseased situations. Even haematology analysers in commercial referral labs, whilst costing many thousands of pounds more, can and do make differential errors. For this reason, **confirming white cell differentials and assessing cell morphology in a blood film is a vital part of the complete blood count**.

However, if you have a good analyser, especially the newer laser cytometers, as the analyser counts thousands of cells, the numerical accuracy of the analyser is superior to any manual count that typically uses only 100-500 cells. An automated differential from such analysers is therefore only altered if your manual film differential is significantly different. Other analysers that cannot reliably differentiate cells should only be used to provide total cell counts and then the differential has to be provided by your film examination.

Quantitative buffy coat (QBC) analysers

White cell counts in these analysers are determined from the width and fluorescence of various cell layers in a buffy coat that is artificially expanded by a float. If the distinction between the bands is lost, due to haemolysis or lipaemia for example, this counting can be wrong or prevented. Whilst the layer width and degree of fluorescence reflects cell numbers (presuming normal sized cells), the differentiation of white cells is based on the variable fluorescence of white cells when stained with acridine orange – DNA glows green whilst RNA and lipoprotein glow red. This creates a differential of mononuclear cells (lymphocytes and monocytes) from granulocytes (neutrophils and eosinophils, primarily in dogs). This variable fluorescence is very reliant on no background interference and healthy normal cells. Often in diseases the size, nature and RNA content of the cells changes, and thus this discrimination is lost or wrong. **Therefore whilst these analysers give an accurate total white cell count, unless healthy, the differential count is often unreliable and misleading**. For

example, certain (granular) lymphocytes can be mistaken for eosinophils. The PCV is measured automatically as with a manual PCV, with the MCHC being inversely proportional to the distance to which the float sinks in the red cell layer. The other red cell parameters are then calculated as previously outlined, with Hb also now being calculated: Hb (g/dl) = PCV (%) x MCHC / 100. Therefore if the PCV is artificially altered by EDTA effects, the haemoglobin calculation can also now be affected.

Impedance Counters

These analysers count cells using impedance, whereby the cells are diluted in an electrolyte solution and then drawn through an aperture within an electrode. As each cell passes through, it creates a pulse of resistance – the more pulses, the more cells there are (Coulter principle). The size of each resistance (impedance) pulse equates to the size of cell. Impedance counters must therefore be adjusted for each species so that the threshold size for each cell type is known. If there is overlap between two cells types, impedance counters can fail to distinguish the cells and ascribe them to the wrong type. In this fashion, the red cells are counted and only distinguished from platelets by platelets being smaller than red cells. This is true for many veterinary species, especially dogs. However, cats have smaller red cells and larger platelets, and thus the analyser can confuse platelets for red cells, or vice versa. Similarly, if a disease creates small red cells or very large platelets (such as iron deficiency or inflammation respectively) this error can be exaggerated.

The white cells are also counted in this fashion, but only after the red cells have been lysed in another solution. This lysing solution can be used to produce variable effects on white cells allowing white cell differentials to be automatically produced. However, this lysis works primarily on anucleate cells (such as red cells), but some are resistant, especially immature nucleated red cells. The analyser therefore 'assumes' that only white cells are present which may not be true, especially in diseased states. In this example, a nucleated red cell appears 'identical' to a mature lymphocyte. Therefore most impedance 'white cell counts' are in fact a nucleated cell count, and so if nucleated red cells are present in significant numbers, the white cell count is artificially elevated and needs correcting. Newer, very expensive commercial analysers can inherently do this, but otherwise it relies on blood film examination.

The calculation is as follows:

corrected (true) white cell count = 100 / (nRBC + 100) x nucleated cell count.

Once cells are counted and the sizes and haemoglobin (Hb) levels measured, the analyser calculates several red cell parameters. The HCT, Mean Cell Haemoglobin Concentration (MCHC) and Mean Cell Haemoglobin (MCH) are calculated as follows:

HCT (l/l) = MCV (fl) x RBC (x 10^{12} /l) / 1000

MCHC (g/dl) = Hb (g/dl) / HCT (l/l)

MCH (pg) = Hb (g/dl) x 10 / RBC (x 10^{12} /l)

Laser flow cytometers

These are the basis for most commercial referral lab analysers, but are only recently available as affordable bench top in house analysers. A stream of single file cells is passed through a laser beam, resulting in scattering of the light. This scatter is both forward (related to the size of the cell) and also to the side (related to the complexity/granularity of the cell). This scattering effect can be enhanced by use of certain lysing solutions to leave only a few cell types for analysis, and also the use of dyes for granules that fluoresce under laser light. A number of graphical plots are produced to aid analysis and interpretation. These produce reliable red and white cell analysis in some situations, but are still subject to artefacts or problems during many diseases.

All the analyser problems outlined above are more significant and dramatic the more diseased an animal is, and especially if the disease is haematological (*e.g.* leukaemia). A blood film must therefore **always** be examined to check that what the analyser is telling you is correct. **Blood film examination also allows you to assess cell morphology that can provide additional valuable information about disease processes.**

MCHC is a useful parameter for quality control and monitoring the analyser's performance. This parameter is calculated from the Haemoglobin (Hb) and haematocrit (HCT) in many machines (excluding the QBC) by using two independent techniques/systems. As the concentration is very similar from species to species, it therefore provides a good marker. Values less than 28 are likely artifactual, as even diseases such as iron deficiency rarely produce values this low. Similarly if the value is elevated, artefact is again supported, such as agglutination, haemolysis or lipaemia. If this value falls outside the reference values, assessing for potential artefact or interference would be recommended initially, before ascribing the value to a disease.**A good general rule related to this is that the Hb value should be one third the HCT/PCV +/- 3.**

Blood film Examination

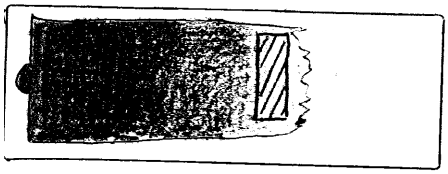
Preparation:

This is made from a drop of anticoagulated blood placed at one end of a clean glass slide and then dragged with another slide to create a thin film of blood with an even feathered edge and cell monolayer. This is the 'wedge' or 'pull & drag' technique. The slide should be labelled with the patient name or number *etc.* at one end.

1. Slides: Label one slide with the identification and date. A 'manufactured' spreader slide is ideal to create the film, although a normal slide can be used at an angle to create the same effect. It ensures the blood is not spread over the edge of the labelled slide. It can be created by breaking the corner off a normal slide.

N.B. The spreader slide should be cleaned after each use and intermittently with water, being replaced on a regular basis. Otherwise dried deposits and gradual roughening of the spreading edge occurs, creating an uneven and poor blood film.

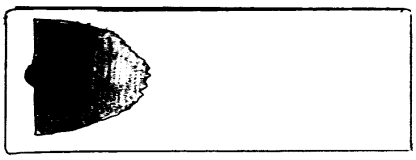
2. The EDTA sample is gently resuspended and mixed and then a small drop of blood is placed at one end of the labelled slide using a fine pipette or capillary tube.
3. The spreader slide is held between your thumb and second finger, your index finger being placed gently on top to ensure even pressure is maintained. It is then held at **about 30°** and slid back to meet the drop of blood.
4. The blood is allowed to spread along this edge. When fully along the edge, the spreader slide is advanced **smoothly and briskly**. An even 'square' feathered edge should naturally be produced about 2/3 to 3/4 of the way along the labelled slide. Do not lift the spreader slide off until the feathering is produced. If this does not occur, please see troubleshooting later.
5. Allow the smear to air-dry fully before staining. This process can be quickened if necessary by gently heating the reverse side of the slide with warm air from a hairdryer. Ensure the air is not hot or directly onto the smear itself. Generally though this quickening is not required.



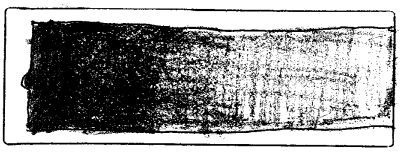
Slides with a frosted end are ideal as they allow easier labelling. The slide should be clean and handled only at the edges. Grease from fingerprints will impair even blood dispersal and contaminants (such as bacteria, fungi or skin cells) from the slide box or environment can complicate interpretation. **Producing a good quality film requires practice and consistency, but is essential for reliable examination and subsequent interpretation**. Artefacts can be introduced both in numbers, due to uneven dispersal, and cell morphology, such as slow drying due to an excessively thick film. Examples of poor films are shown below, alongside possible reasons and solutions.

Troubleshooting:

Film too short: too little blood – use a slightly larger drop
spreader slide angle too steep – check angle is near 30°
advancement is too fast – slow speed down.



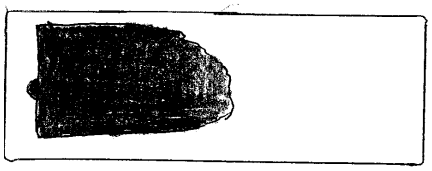
Film too long: too much blood – use a slightly smaller drop.
spreader slide too shallow – check angle is near 30°
advancement is too slow – speed up slightly



Film too thick: too much blood – use a slightly smaller drop.

spreader slide angle too steep – check angle is near 30°

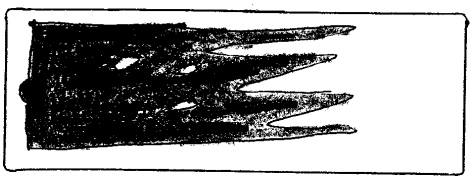
advancement is too fast – slow speed down.



Film has holes or streaks:

greasy slide – clean slides (with acetone/alcohol), ensuring no fingerprints and only sides are handled.

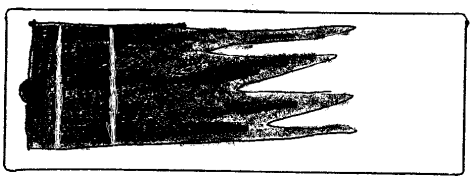
spreader slide is dirty or damaged. Clean or replace it.



Thick line(s) seen:

Uneven pressure, allows blood to escape in front of the spreader slide. Ensure index finger is placed on top of the spreader slide.

Advancement not smooth – ensure constant speed is maintained.



If the animal is anaemic or has increased numbers of red cells then the above technique, especially the angle, may need modifying to produce a good quality film. Anaemic blood has a tendency to spread too fast and far, whereas increased numbers of red cells make the film excessively thick or short. If the problem is severe enough, it may not be possible to produce a standard good quality film.

Staining

In house staining invariably involves three separate liquids into which the slide is 'dunked' a variable number of times ('Diff Quik'). There are a large number of these stains available. They should be replaced (not just topped up) and the containers (*e.g.* Coplin jars) cleaned on a regular basis, as the staining material becomes depleted with use and stain precipitates form with time. Both these factors will prevent or hamper examination of the film and its cells.

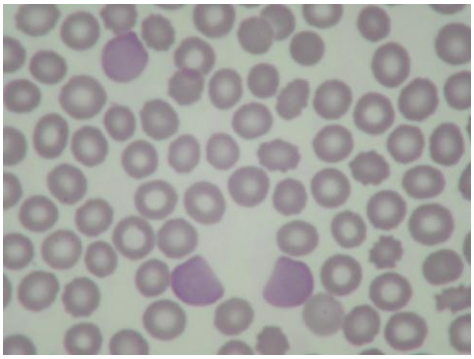
The first clear liquid is a fixative, the second is an orange dye and the third a blue dye. Typically the manufacturer's guidelines should be followed for the timing and number of 'dunks' required. However, it is worth checking that the colour scheme produced is acceptable for you. For example, eosinophil granules should be orange to pink, neutrophil cytoplasm clear and nuclei dark purple. Often people use too little blue dye or too much orange dye. As a rough rule, after fixing in the first liquid, dunk in the orange dye until an even orange covering is produced on the slide, and then similarly with the blue dye.

Many people rinse any remaining dye off with tap water. This can be fine, especially if gently applied to the back of the slide and not directly to the cells. However, it is better to use specific buffered or distilled water as this is gentler to the cells, is consistent and creates little artefactual change. Tap water can wash out the dyes and alter the cell shape artificially. Buffered water is easily made by adding buffer tablets to distilled water. Once rinsed, the slide is allowed to air-dry. As with initial smearing, this can be quickened by judicial use of a hairdryer.

Slide examination

The slide is now ready for examination at low power (x4, x10 or x20). Many practice microscopes have a x40 objective lens that is designed to be used with mounted slides. Without this covering, cells viewed at this magnification will appear blurred and you will be unable to see fine detail within the cells. Whilst mounting slides with glued coverslips is an approach, few, if any, practices will have the time or inclination to do this. A slightly messier alternative, but as good, is to place a drop of immersion oil on the slide and gently lower a coverslip onto this, ensuring no air bubbles are trapped in between. As long as the immersion oil is good quality, you will still be able to place oil on the coverslip itself when using the oil immersion lens (x100). Unfortunately unless carefully used, this often leads to oil contaminating the x40 lens itself, which can eventually damage it and hampers its use elsewhere. Also, if the oil is too thick and poor quality, you will find it difficult to use the x100 lens on an oil mounted coverslip, as moving the slide, moves the coverslip too, blurring the picture viewed. It will not affect use of the low power objectives (x4, 10 or 20). Please see the appendix for setting up the microscope correctly.

Any slide examination starts with a low power (x4) scan, followed by sequentially higher powered examination. Much of the blood film examination can be performed at x40, or even x10 or 20 with experience, with x100 only needed for specific detailed examination. Find a way that suits you which is consistent and logical, but covers examination of cell numbers and morphology, from red cells to white cells and platelets. **Examination of cell morphology (size and shape *etc.)* must take place in the monolayer –** the area just behind the feathered edge where the red cells are just touching each other (see hatched area on model blood film earlier and picture below.



If examined in the feathered edge, cells are often distorted, whereas in the thicker areas the cells are poorly spread out and contracted. The following is one way of examining a blood film, with specific cell lines and factors being discussed later.

1. At low power, check the feathered edge for platelet clumps primarily and rarely abnormal cells and parasites such as Dirofilaria larvae.
2. At higher power (x40 or x100), first look at the red cells, checking cell shape and colour. Look for any inclusions and check for clumping.
3. Roughly estimate white cell numbers and perform a white cell differential count. Then look at each white cell line specifically for any abnormality or changes. Check for inclusions and cell clumping.
4. At high power (x100) estimate platelet numbers, and check their size and shape. Check for any inclusions.
5. Check the periphery of the smear for any atypical or abnormal cells. These are often larger and therefore more commonly found towards the edge of the film, with smaller cells remaining nearer the centre.
6. Check the background colour and appearance. Lipaemic samples often appear slightly blue with fine vacuoles, the cells appearing slightly blurred or out of focus. Elevated protein levels, especially if marked, will give a smooth light blue background, often with increased rouleaux.

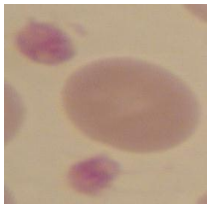
Any changes or features noted, such as variation in size, should be subjectively quantified. This allows accurate serial monitoring and detailed clinical interpretation. A standardised practice scheme should be adopted that everyone follows. This may simply be from “occasional, mild, moderate to marked”, or take the form of a grading system like + to ++++. As long as everyone follows and understands it, the exact nature doesn’t matter. Often red cell features are done at x40 or x100 with the affected numbers seen in an average field equalling the grade; whereas white cell features are based more specifically on the numbers or percentages affected, say per 100 cells examined.

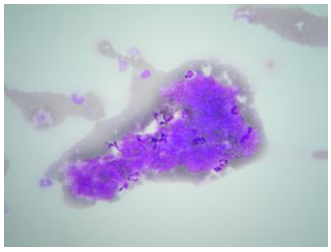
Platelets

These do not have a nucleus. They are smaller than erythrocytes, often less than a quarter the diameter. They are cytoplasmic fragments, which have an irregular circular outline with a pale purple-pink, granular appearance. In healthy dogs and cats, numbers can be estimated from the blood film. At x100, every platelet seen roughly equates to a platelet count of 15 x 10^9/l. Numbers are counted per five x100 fields and then averaged. Healthy animals typically have between 10-30 platelets per x100 field.
As already stated, platelet clumping must be excluded initially – large clumps at the feathered edge and small (micro-) clumps within the monolayer. The size of the platelets should be assessed. In inflammation or infection, or following increased production of platelets, larger forms can be seen. These so called shift or macro- platelets can be as large, or larger, than an erythrocyte, especially in cats.

Cavalier King Charles dogs are somewhat unique because many have relatively low numbers of very large platelet naturally in health. Automated analysis may give a falsely low count as the platelets are miscounted as red cells. However, they often have low platelet numbers compared to other breeds and usual reference values. This though is not associated with any bleeding tendency.

Certain breeds naturally have lower platelet counts, although the size and shape are similar to other breeds. Greyhounds are an example.





Red cells (erythroid)

The first recognisable red cell is in the bone marrow and is an **erythroblast.** This matures into a **prorubricyte**, to a **rubricyte**, then **metarubricyte**. At this stage, further cell division cannot occur, the nucleus is lost, and visible haemoglobinisation occurs. This creates a polychromatic **reticulocyte** (see later) that in 24-48 hrs matures into an **erythrocyte**.
Typically the maturation and cell division up to the reticulocyte stage occurs in the bone marrow. In health, small numbers of reticulocytes are released into the circulation, often sequestering in the spleen whilst they mature. During periods of increased demand, when anaemic following haemolysis or haemorrhage for example, the bone marrow releases earlier precursors into the circulation to compensate for the loss of erythrocytes. This maturation

process can therefore be seen occurring in the circulation and its features are identified when evaluating anaemic patients for expected and appropriate regeneration. If the erythrocyte loss is minimal, no early release is seen, but as the severity of loss worsens, the bone marrow will start to release more and earlier immature precursors. This maturation process though should still occur in an 'orderly and regular' fashion, so reticulocytes should dominate, with reducing numbers of earlier immature stages. If this is not seen, the anaemia is inappropriately regenerative and often means bone marrow disease. **Thus the presence of nucleated immature cells alone, without appropriate and associated polychromatic reticulocytes, is not a regenerative process.**

Erythrocytes

In dogs and cats, mature red cells (erythrocytes) do not have a nucleus. They appear as a smooth circle and are slightly orangey pink to brown. In dogs they are slightly larger and have a central pale area (central pallor) that is usually not seen in cats. In dogs red cells are normally roughly equal in size, whereas in cats there is always mild variation in their relative sizes. The central pallor in dogs is usually less than 50% of the cell volume. Descriptively they are called **normocytic** (normal size) and **normochromic** (normal colour). **Hypochromic** erythrocytes have a rim of haemoglobin only, the central pallor filling more than 75% of the cell volume.

Dog

Cat

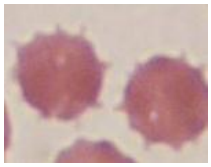
Anisocytosis –this is variation in cell size. The more relative variation there is in red cell sizes, the greater the anisocytosis. Immature red cells are typically larger than mature ones, and thus increased anisocytosis is often seen during regeneration. This size change encompasses both small (microcytic) and large (macrocytic) erythrocytes.

- **microcytic** – this is seen as a low MCV from automated analyser analysis. In the film these appear as small erythrocytes that may appear normochromic or slightly pale (hypochromic). As a guide, they are about half the size of a lymphocyte.
- **macrocytic** – conversely this is seen as an elevated MCV. In the film, these cells are normo- to hypo- chromic but larger than normal. They can be difficult to identify specifically, but as a guide are twice as large as a lymphocyte.

Poikilocytosis – this is variation in erythrocyte shape. Erythrocytes are circular, so any uneven projections, cell fragments, loss of central pallor *etc.* should be noted. Certain cell shapes, such as schistocytes, can be seen in certain diseases, but many shapes are not

disease specific but do indicate processes occurring that are significant or warrant further investigation. If many different shapes are seen, the general term poikilocytosis is used and the severity graded. Some more common examples are outlined below.

- **Crenation / Echinocyte –** these have regular even projections over the surface of the erythrocyte. Most commonly they are artifactual due to drying effects, excessive or prolonged EDTA storage. They are one of the commonest shape changes seen.

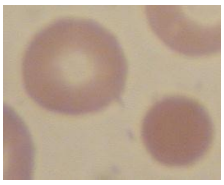


- **Acanthocyte ('spur cell') –** these have rounded, irregular and uneven projections from the surface and are similar in size to an erythrocyte. They are seen in vascular organ diseases (*e.g.* liver and spleen), lipid disorders and following erythrocyte reabsorption with internal haemorrhage.

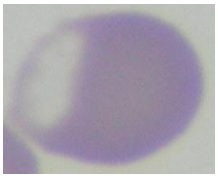
- **Codocyte ('target cell') –** these have expanded, corrugated membranes resulting in varying cell thickness and haemoglobin 'rings'. They are non specific but often associated with regenerative anaemias and liver disease.

- **Schistocyte** – these are red cell fragments, appearing smaller than erythrocytes and very irregular in shape. They are seen with microvascular diseases and DIC due to fibrin formation and red cell shearing.

- **Spherocyte** – these are smaller than erythrocytes, appearing darker with no central pallor. They are seen in immune mediated haemolytic disease. Their appearance reflects incomplete loss of membrane due to antibody mediated phagocytosis.



- **Eccentrocytes** – these are the same size as erythrocytes, but one portion/side of the membrane is fused, appearing as a circumscribed white crescent. These reflect oxidative damage, more so in dogs, often due to toxins such as onion.

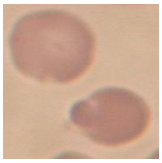


Inclusions

These are seen inside erythrocytes and must be identified, especially with regard to infectious organisms.

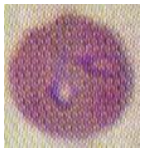
- **Howell-Jolly bodies** – these are small, dark purple and circular, often located eccentrically. They are the remains of the red cell's nucleus. They are often seen during regeneration, but if regeneration is not seen, they can be a feature of splenic or bone marrow disease. Occasional examples can be seen in healthy animals, especially dogs.

- **Heinz bodies** – these are small circular blebs which protrude and have a clear white crescent on one side next to the cell surface. They are more commonly seen in cats with oxidative toxin damage but are seen in all species. New methylene blue staining (see reticulocyte counting later) will confirm Heinz bodies as they appear as dark blue peripheral blebs with this stain.



- **Mycoplasma (Haemobartonella)** – these are small, regular purple dots, often seen in a small chain inside or attached along the cell membrane. **They must not be confused with stain precipitate that is more irregular in size and more refractile.**

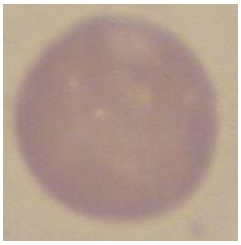
- **Babesia** – these are medium to large, teardrop to circular organisms, often present in pairs, seen within the cell. Typically they have a clear white centre with a dark purple rim.



Polychromasia

This means 'many coloured', and causes the cell to appear blue-purple. It is a feature of immature red cells and specifically reticulocytes in the circulation. Reticulocytes are slightly larger than erythrocytes, have this blue-purple colour, often without any central pallor. Only occasional reticulocytes are seen in healthy dogs (1%), with even fewer to none in cats (<0.5%). Increased numbers are seen during regenerative anaemia. Polychromasia grading is essential when assessing whether the anaemia is regenerative or not, presuming 2-3 days has elapsed for the bone marrow to respond.

Normal



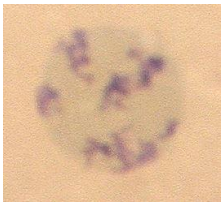
Polychromasia

If the anaemia does not appear regenerative because no polychromasia is seen, and presuming the clinical history is more than 2-3 days, the next step may be to perform a reticulocyte count. This is a more sensitive and quantified way of monitoring and evaluating regeneration, but if polychromasia is present, there is little point in performing a reticulocyte count, especially as it is time consuming.

Reticulocyte counting

1. Mix equal amounts of EDTA blood with 0.5% new methylene blue. Typically one drop of each in a sterile tube is fine. Alternatively specific and special tubes or slides can be used that are lined with new methylene blue and simply incubated with the blood (film).
2. Leave at room temperature for about 20 minutes.
3. Gently resuspend the cells and make a blood film as normal (see earlier).
4. Presuming a good smear (it will appear very pale) the reticulocytes should be evenly distributed.
5. In the monolayer area, count at least 500 red cells, including reticulocytes. Ideally a 1000 is better. The percentage of reticulocytes is then calculated.
6. Calculate the absolute reticulocyte count to prevent relative distortion due to the anaemic severity. Absolute reticulocytes (x 10^9 /l) = % observed x RBC (x 10^{12} /l) x10.

New methylene blue (NMB) stains the proteinaceous reticulum and ribosomes of the reticulocyte blue. Nuclei, white cells and platelet will also stain. In dogs and cats, polychromatic cells are aggregate reticulocytes. NMB therefore shows irregular large clumps of blue dots within an otherwise pale and clear red cell. Cats are different because they also have punctate reticulocytes that form when aggregate reticulocytes mature in the circulation. They then persist in the circulation for 10-12 days, and so are not accurate markers for acute regenerative responses. They are **not** included in the standard reticulocyte count in cats. They appear different by only having a few single scattered blue dots rather than larger clumps.

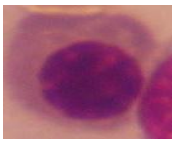


Reticulocyte counts can also be reported either as a corrected percentage based on PCV or by a reticulocyte production index based on maturation times. These are rarely calculated, inaccurate, unvalidated and provide no further useful information beyond the absolute count already counted.

Nucleated Red Cells

There are four types of nucleated red cell precursor (outlined earlier), the commonest seen in the blood being the metarubricyte. (shown below). The nucleus is typically more central and a neat circle, being a denser more black-purple colour that has fine white fracture lines, occupying about 50% of the cell's volume. Their cytoplasm varies from red cell colour and

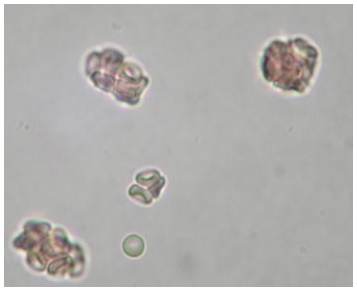
browny-red to more blue-purple and polychromatic. They are occasionally in health and also in low numbers alongside polychromasia during regenerative anaemias.

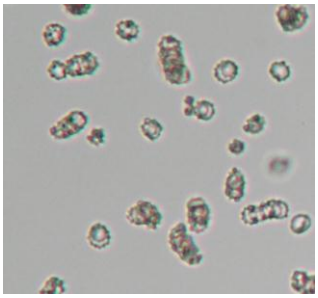


Rouleaux & Agglutination

Rouleaux describe a physiological phenomenon when erythrocytes are clumped together in a chain, similar to a stack of coins. It is commonly seen incidentally in cats but only rarely in dogs. In both, increased and marked rouleaux formation can be seen when the plasma protein levels increase due to globulin production in inflammation, infection or gammopathies associated with diseases like myeloma. **Rouleaux must be distinguished from agglutination.**

Agglutination describes when erythrocytes are seen in tight groups, similar to a bunch of grapes and is associated with immune mediated haemolytic disease (IMHA). It can be visible grossly as flecks or clumps of red cells within the tube. If present, there is no need for further diagnostic testing for IMHA. However, grossly in the tube rouleaux formation can mimic this (to the naked eye). To confirm and distinguish, the sample is examined microscopically after the addition of saline. Rouleaux formations disperse, but agglutination persists. Initially a drop of saline is mixed gently with a drop of EDTA blood on a slide. Coverslipping aids dispersal and examination microscopically to confirm agglutination. This process should be repeated with greater saline dilution (up to 1:10, so 1 drop of blood to 9 drops of saline) to confirm genuine agglutination and therefore IMHA.





There are many breed specific red cell idiosyncrasies that should be excluded before diagnosing disease. For example: Poodles typically have macrocytic erythrocytes, whereas Akitas have microcytic erythrocytes; several breeds, such as Greyhounds, have naturally 'high' PCVs (55-65%).

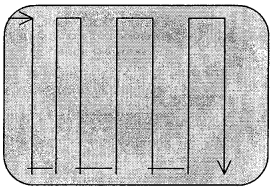
White cells/Leukocytes

White cells derive from two cells of origin – lymphoid and myeloid, myeloid stem cells also generating erythroid/red cells as described earlier. Lymphoid maturation is very different and will not be discussed. The other cell lines eventually differentiate into specific and identifiable precursors in a variable fashion. The granulocytes mature similarly, the earliest recognisable cell being a **myeloblast**, which divides and matures into a granular **promyelocyte**. From here, the three lines split, with distinctive secondary granules forming at the next **myelocyte** stage (so neutrophils become clear (neutral), eosinophils red-orange and basophils blue-purple). **Metamyelocytes** are seen next, which then mature further into **band** forms and finally the mature leukocyte (*i.e*. eosinophilic myelocyte, eosinophilic metamyelocyte, band eosinophil and eosinophil). Cell division continues until the myelocyte stage, so with maturation and storage, there are many more mature cells than immature cells.

Neutrophils are different in having a significant storage pool/buffer within the bone marrow whereas other leukocytes are produced on demand. Excluding lymphocytes, which are essentially recycled/recirculating, leukocytes exist in healthy blood for only a few hours, either destined for tissue pools (such as eosinophils and monocytes) or mucosal loss in the case of neutrophils. Neutrophils also differ in having a significant marginating pool (especially in cats) whereby they transiently adhere and roll along the blood vessel walls. It is this marginating pool that accounts for the sometimes marked physiological increase and decrease that is seen with blood pressure changes as mentioned earlier. It must be remembered that blood sampling typically only involves assessment of the circulating pool at that time, which will be affected or altered by a number of mechanisms.

It is useful initially when evaluating leukocytes in the blood film to estimate at low power (x10) by counting the number of white cells seen per field. An average from at least five such fields should be taken. If the count falls between 18-50 WBCs/x10 field, the total WBC count is likely normal, and correlates well with the automated count. However, at lower and higher numbers, this estimation becomes inaccurate and unreliable. Alternatively the average number of nucleated cells is counted at x20 per field and then multiplied by 0.5 to give the WBC (NCC) count (x 10^9/l).

A differential count is performed from the edge of the monolayer towards the centre, moving in a progressive and systematic fashion, ensuring the same areas is not revisited. This can take the form of a so called ‘battlement meander’ method (shown below) within the examination area/monolayer (hatched area shown earlier- p13)



A minimum of 100, or ideally 200 or more, cells are counted. Nucleated red cells are included in this differential but then numbers are adjusted as outlined earlier (p10). The percentage of white cells for each type is then multiplied by the total white cell number to given the absolute number of that type. It is this absolute number that is crucial and should be compared to healthy reference values. If you compare and interpret the percentages, these are inherently relative and therefore reduction in one line must 'increase' another cell line, even if that cell line hasn't altered at all. In healthy dogs and cats, mature neutrophils are the dominant white cell, followed by lymphocytes, with low numbers of monocytes and eosinophils, with rare to no basophils seen in most differential counts.

When assessing cell morphology, identifying cell types and any potential abnormality, the following three features must be assessed:

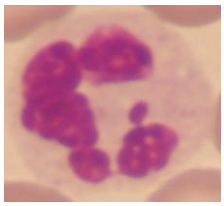
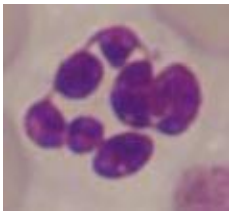
- cell size – relative to the mature erythrocyte
- nuclear shape, texture and colour
- cytoplasmic shape, colour and features

Neutrophil:

These are granulocytes and are also known as polymorphonuclear leukocytes. They are 1.5-2 times the size of an erythrocyte. They have:

- nucleus – dark purple with indistinct white lines. It is elongated and forms thick segments joined by thinner sections. Typically there are 3-5 segments in cats and dogs.
- cytoplasm – this is smooth and light grey to clear. With some stains it can appear pale pink (due to the granules), but typically the granules do not stain. Inflammation or infection can lead to 'toxic' changes – see p.
- Birman cats can have prominent red-pink granules in the neutrophil cytoplasm. This is incidental, but must be distinguished from polysaccharide storage diseases that can produce similar changes. Toluidine blue staining is negative for Birman granules but positive for storage disease granules.
- Pelger Huet anomaly – this is when the neutrophils have hypolobulated nuclei appearing similar or identical to band neutrophils. Functionally this has no significance though.
- Chediak-Higashi syndrome – this is a granular defect, resulting in large abnormal red-pink granules within the neutrophils (also in platelets), primarily in Persian cats with yellow-green eyes.

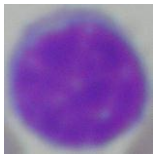
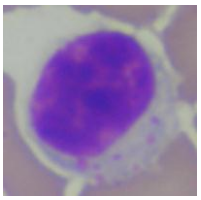
- hypersegmentation (>5 lobes, usually joined by a thin line only) can be seen with steroid effects or following antibiotic use, the neutrophils ageing in the circulation – see right hand cell below.

Lymphocyte:

These are the smallest white cell, being about 1-1.5 times the size of an erythrocyte. They have:

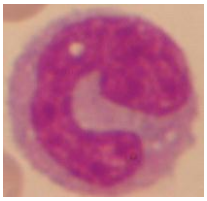
- nucleus – circular to cleaved or indented with smudged to smooth dark purple colour. It is eccentric (off centre).
- cytoplasm – minimal and on one side of the nucleus. Typically light blue and smooth.
- There are many types of lymphocytes that have characteristic features and can be seen (re)circulating in healthy animals in low numbers. For example, certain lymphocytes have a small volume of pale cytoplasm with red-pink (azurophilic) granules. A key feature is noting a heterogeneous or mixed population of lymphocytes. If the lymphocyte population looks very monomorphic and homogeneous (similar), especially if numbers are increased, it becomes suspicious of neoplasia and leukaemia.

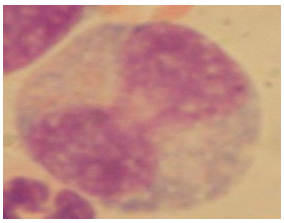



Monocyte:

These are the largest leukocyte and the most variable in appearance. They are at least twice the size of an erythrocyte. They have:

- nucleus – paler purple with more open white lines. Its shape is very variable, from U shaped to dumbbell, convoluted or bean shaped.
- cytoplasm – this is irregular. It is often a variable blue-grey with a pale pink hue and often contains small white vacuoles (holes).

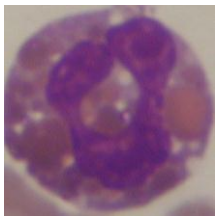




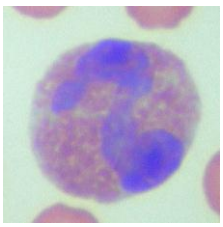
Eosinophil:

These are also granulocytes, and their granules are visible. They are 1.5-2 times the size of an erythrocyte. They have

- nucleus – moderately purple with indistinct white lines (lighter than a neutrophil). It appears segmented but much less so, with thicker segments. Usually only 1-3 lobes are seen.
- cytoplasm – this is variably filled with distinct orange-pink granules, the background being pale grey-blue. Dog granules are usually circular but very variable in size. Cat granules are very uniform, finer and rod shaped, filling the cytoplasm.
- certain dogs, especially Greyhounds, have so called 'grey' eosinophils where the typical orange-pink granules are seen instead as clear white vacuoles. This is incidental as long as the vacuoles are not seen in other cell lines to suggest storage diseases.



Dog

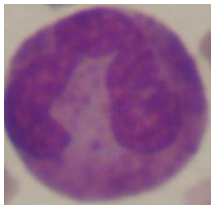


Cat

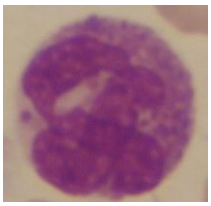
Basophil:

These are also granulocytes and their granules are also visible. They are 1.5-2 times the size of an erythrocyte. They have:

- nucleus – moderately purple with indistinct white lines. It is variably lobed or convoluted, with thicker segments.
- cytoplasm – this is variably filled with distinct purple granules, and the background is pale blue-grey. In the dog they are dark purple granules, but finer lilac/lavender in cats.



Dog



Cat

Inflammation or Infection

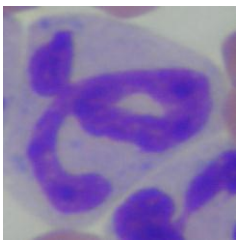
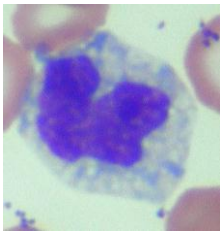
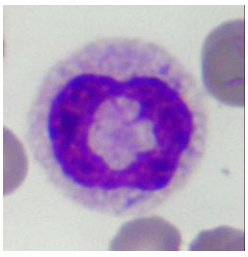
When inflammation or infection is present, one of the primary features can be an increase (or decrease) in the number of certain white cells seen (*e.g.* neutrophilia with increased numbers of mature neutrophils). This change can give an indication as to the disease process (*e.g.* increased eosinophils - eosinophilia – suggests parasitic or allergic disease most commonly). However, changes in the cell morphology can also be useful. This primarily focuses on neutrophils and lymphocytes, but monocytes can show changes to a lesser degree. Increases in certain cell lines (such as neutrophils and lymphocytes) do not necessarily indicate disease.

Neutrophils:

These can show changes that are termed 'toxic' changes when more neutrophils are needed during inflammation or infection. These changes are mainly cytoplasmic, but rarely and if severe enough, can affect the nucleus. They are:

- Dohle bodies can be seen. These are irregular, indiscrete darker blue deposits. In cats, they are often seen in low numbers in healthy animals so must be prominent and seen in many or all neutrophils to indicate toxicity.
- cytoplasm becomes more blue (basophilic)
- cytoplasmic vacuolation, with a foamy appearance.
- toxic granulation with dark pink indistinct granules.
- nucleus can become paler purple or form ring/doughnut shapes.

These changes actually reflect maturation defects that are seen as the bone marrow produces neutrophils so fast that maturation does not occur normally. They are not a specific product of inflammatory or infectious toxins, and are rarely seen in inflammatory/infectious tissue aspirates such as an abscess.

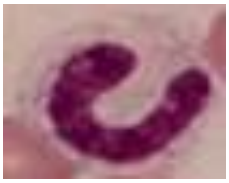
Band neutrophils:

These are immature neutrophils that are only rarely seen in healthy animals. They can be seen during inflammation or infection as demand is so great that the bone marrow has to release immature cells earlier than normal – a so called left shift. This principle is similar to reticulocytes (polychromasia) seen in regenerative anaemia. In these cells and situations,

toxic changes can also be seen. The principal defining feature of a band neutrophil is its nucleus.

- nucleus – dark purple with indistinct white lines. It is classically U or C shaped with parallel sides. Uneven indentations can be seen, but these should not form distinct segments or be greater than 50% of the width of the nucleus. If any segments or prominent/full indentations are seen, it is classified as a mature neutrophil.

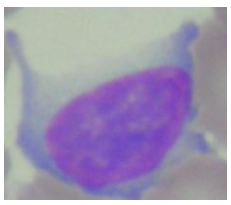
The number of band neutrophils is usually appropriate and less than the number of mature neutrophils, indicating that whilst increased, the body is able to meet the demand. Neutropoenia or dominant band neutrophilia is significant, especially if persistent, as it indicates that the demand is too great for marrow production and the disease is more severe.

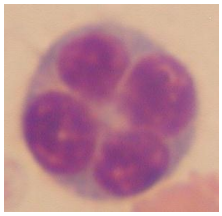


Lymphocytes:

When inflammation or infection is present, especially if viral, lymphocytes can show quite dramatic and variable changes. In these situations they are called reactive lymphocytes. Typically you should see variation from 'normal' lymphocytes through to these reactive forms. Possible features include:

- cytoplasm becomes much more basophilic (dark blue)
- the amount of cytoplasm increases and can 'wrap around' adjacent erythrocytes.
- the nucleus can vary from circular, to clover leaf, indented or cleaved.
- the nucleus stays dark purple, but more whiter/paler areas are seen.
- examples can have expanded pale cytoplasm with distinct red-pink (azurophilic) granules.
- rarely more active forms are seen, similar to lymphoblasts in the marrow or lymph nodes. This is especially true in young animals and cats when virally infected. These should be rare though in the circulation. They are much larger (>2 times an erythrocyte size), with very dark blue cytoplasm. Their nucleus is paler with more white lines and less dark areas. It can also contain a nucleolus.





Monocytes:

These become even more variable than normal. The main inflammatory/infectious changes are more vacuoles in darker cytoplasm and a lighter purple nucleus with more open white lines.

When performing a differential, especially in severely diseased animals, it is not always possible to identify and define every single leukocyte seen. This should only happen rarely though. If many cells seen are unknown (*e.g.* leukaemia) consider sending the film/blood to a specialist haematologist for an opinion, or using further tests that may only be available at a few specialist referral labs.

Leukaemia

Diagnosing leukaemia can be very easy when the cells are obviously immature and unrecognisable especially when present in very high numbers (*e.g.* lymphoblastic leukaemia). However, identifying which lineage these cells are from can be very difficult or impossible from the morphology, requiring further testing such as immunophenotyping or special stains. Early or developing leukaemia can be very difficult to diagnose too, especially if it involves mature cells that are identical to those seen in healthy/normal blood (*e.g.* chronic granulocytic leukaemia). **The key feature with leukaemia is a monomorphic population of (atypical) cells present inappropriately or in excessive numbers. Diagnosis relies on a fresh peripheral blood film, CBC/FBC and bone marrow examination.** Bone marrow examination is a specialist discipline that will not be addressed here.

Most commonly, leukaemias are acute, involving immature or stem cells that should only be seen in the bone marrow in very low numbers. A characteristic feature of immature cells is the nucleus. It is much paler than mature cells, looking more pink than purple. Dense dark areas and white lines are not seen, the texture appearing much finer and smooth, with stippled white and pink/purple areas. They often (but not always) have variably prominent nucleoli that appear as blurred 'bubbles' or circles within the nucleus. These nucleoli can be single or multiple and variably sized.

Whilst the nuclear features are critical, the cytoplasmic features are also useful when trying to identify the specific cell type. Myeloid cells often have a pale pink hue or discrete granules, whereas lymphoid cytoplasm is smoother, sometimes with distinctive projections or blebs.

With mature or chronic leukaemias, whilst the morphology is useful, especially very subtle features such as nuclear clefts, diagnosis can rely on either monitoring for increasing cell counts and exclusion of potential inflammatory or infectious diseases that may produce a similar response (so called 'leukaemoid' reactions).