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Essential Practice Lab Tips, Tricks and Techniques Mini Series

Session Three: Microscopy: Does it Always Have to be Blurred?

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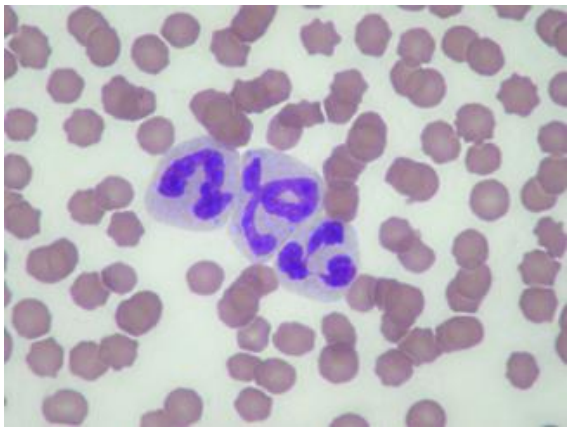


Cytology and Skin Preparations

Skin and Membrane Preparations:

These are most commonly performed for local or generalised, often itchy, skin diseases. They can often be done on conscious animals, but light sedation can be advisable for certain procedures, such as scrapes, and locations, such as interdigitally, to ensure optimal sampling. Preparations vary from impression smears, adhesive strips and skin scrapes.

Impression Smears:

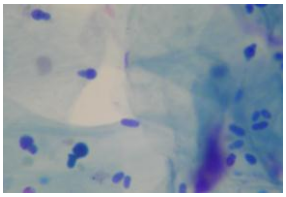
These are done on superficial moist skin structures or areas, such as the mouth, eyelids, vagina or ear canal that can only be sampled using an implement. If accessible, a clean slide is labelled and then simply placed gently over the area. The material naturally sticks to the slide. This 'spotting' can be repeated several times, but care should be taken not to drag the material along as this may damage the cells. This process can then be repeated if required after the area is cleaned with saline to provide representative but slightly deeper samples. By their very nature though, impression smears are very superficial, and so deeper, possibly more significant changes can be missed.

With inaccessible sites, such as the external ear canal, it is best to use a saline moistened swab or cotton bud. This is rubbed/rotated against the area in question and then the swab/bud is **gently rolled** along a clean labelled slide. It should not be dragged as it damages the cells. In this fashion, 2-3 tracks can be placed onto one slide.

Both these styles are left to air dry before staining, most commonly with 'Diff Quik'. They are then examined as for cytological preparations. If speed is essential, after drying, the slide can be dipped once in the blue dye, rinsed and then examined. This produces sufficient staining for certain preparations such as ear smears.

- **Ears** - These are mainly examined to identify infectious agents. Ear mites can be seen grossly and confirmed via this method or adhesive strips. Malassezia yeasts are seen as large circular blue-purple structures, often budding and appearing like 'peanuts/footprints or skittles'. Bacteria, both cocci (small blue-purple dots in pairs, chains or bunches) and rods (small to medium sized blue-purple 'rice grains') can be seen. Inflammatory cells such as neutrophils are uncommonly seen but when present, are often associated with bacteria such as Pseudomonas. Gram staining can be performed but is often confounded by overlying debris and false reactions/staining. It is really only appropriate on cultured organisms for this reason and is not recommended in practice. Typically bacterial cocci are Gram positive whereas rods are Gram negative. Identifying these aids appropriate use of antibiotic therapy.

- Squames are superficial epithelial skin cells that have no (or a very small) nucleus within smooth, turquoise and polygonal cytoplasm. They are commonly seen in ear preps.



Adhesive strips

These are very useful for flakey skin disease, especially with surface parasites such as Cheyletiella. The tape, which should be clear (such as “Sellotape”), is applied repeatedly to the area until sufficient debris material has been harvested. Tacking the ends together to form a circle which is gently rotated as it’s applied helps to sample many areas well. This is then stuck onto a labelled microscope slide and examined as for urine sediment (p58). These preparations can be stained by forming a loop that is then dipped into the stains before being applied to the slide. However, this is generally messy and gives very variable staining, the adhesive glue often dissolving whilst staining so the material is lost. If staining is desired, skin scrapes or impression smears are generally recommended.

Skin scrapes

These are ideal for investigating skin disease, especially deeper ectoparasites such as Demodex and Sarcoptes. Fresh or new lesions should be sampled rather than traumatised skin areas. Multiple sites/areas should be sampled. They can often be done conscious but sedation can be advisable in difficult areas such as the digits to ensure good technique. The following guidelines provide a brief technique.

1. Shorten the hair shafts by cutting with scissors, but do not damage the skin. Squeeze the skin gently as if trying to push out the hairs.
2. Dip a scalpel blade (no.10 or 15 are ideal) in liquid paraffin or apply a drop of paraffin to the skin to be sampled.
3. Scrape with the direction of the hair until capillary ooze is seen. If blood is not seen, the scrape is often too shallow and unrepresentative. If too deep, excessive blood can hamper examination and the animal becomes resentful.
4. Transfer the material to as many slides as necessary, ensuring the material is not too thick.
5. A drop of liquid paraffin or potassium hydroxide (KOH) is mixed in (see later) and a coverslip applied. The coverslip improves discrimination by flattening the material into a single plane and is essential for dry x40 lens examination.
6. Scan at low power (x4 or x10) and use higher power magnification if required to identify the species seen. Hair bulbs and shafts can be examined for fungal spores.

The use of KOH is debatable. Many use liquid paraffin alone, but often do not use enough to spread the material out and areas can be too thick for parasitic examination. Potassium hydroxide is useful (and is the author's preference) as it 'dissolves' skin debris, making parasites easier to see. Various strengths are used (5-20%), but at least 10-20 minutes are required after application before complete 'clearing'. Longer times are needed for weaker solutions. Gentle warming can speed this process. A drawback is that KOH, especially stronger concentrations, kills the parasites, so movement cannot be used to 'draw your eye' to the parasites.

Hair plucks taken in the direction of hair can be good for Demodex and fungal spores.

CYTOPATHOLOGY

This is a huge area that cannot be completely covered today, but sampling considerations, fundamental principles and specific common examples will be discussed.

Cytology is very useful as it is minimally invasive and quick, and with good sampling and accurate interpretation, is often diagnostic. Further more invasive, expensive and potentially risky investigations are therefore not always required and therapy can be initiated immediately. Alternatively it can reduce the number of differential diagnoses and therefore help guide further investigations. It is commonly used for superficial or palpable masses (*e.g.* skin) and is good for fluid preparations. Internal structures can be sampled under ultrasound guidance. **Whenever samples are taken, the size and (palpable or sonographic) nature of the mass should be taken into account.** A small needle in a large mass will sample only very small areas of the mass, so useful or diagnostic material can be missed. Therefore aspirates from various locations within the mass are recommended. If large enough, ultrasound guidance can used to target areas. **Both** the centre and periphery of a mass should be sampled.

Sampling

Superficial/Palpable masses:

Impression smears can be used for ulcerated lesions as described above, but this is not recommended, as there if often a lot of debris and the sample will only represent the surface of the lesion. Deeper, and usually more significant or diagnostic material, is not therefore seen. These masses are best sampled by fine needle aspiration, the material harvested then being prepared in various ways. The area can be clipped and cleaned, but this is often not required or can be confined to alcohol swabbing only. If the aspirate is for deeper bacterial culture though, the site should be aseptically prepared and allowed to dry thoroughly before aspirating.

Fine needle aspiration

The mass is first isolated and 'fixed' using one hand and then one of the techniques described below is used to harvest cellular material.

1. **Needle only** - initially it is recommended that the needle is used alone. A 21-23 gauge needle is preferred. It is held between the thumb and index finger, quickly introduced and moved to and fro in the mass without exiting the skin or mass itself. Spinning/rotating the needle on its axis helps to harvest tissue cells. The needle is then withdrawn. An air-filled 2.5 ml syringe is attached and the material briskly expelled onto one end of a clean labelled slide. Diagnostic material often appears as slightly 'creamy' to blood tinged fluid. Grossly the material can appear fatty if a lipoma or adipose tissue is sampled. This material is then prepared (spread) as outlined later (p43). The sampling process is ideally repeated with a fresh needle. This technique minimises both cell damage and blood contamination. If no material is obtained, it is then best to try a suction method.

2. **Suction –** this is identical to that described above, but when the needle is in the mass, suction is applied by withdrawing the plunger on a (2.5-5ml) syringe attached before or after needle insertion. It is much easier (and better) to have two people doing this (one holding the mass and one aspirating), as otherwise the needle can move out of the mass when suction is applied. The suction can either be:

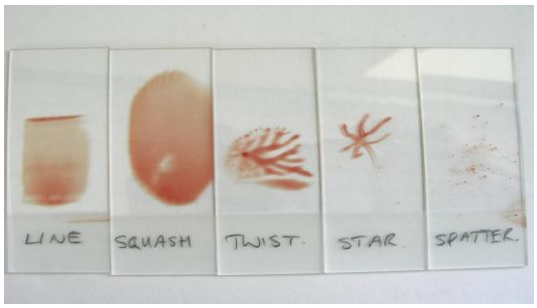
   intermittent – move the needle, apply suction and release. Redirect the needle and reapply suction, repeating several times.
   continuous – suction is applied and maintained whilst the needle is moved to and fro within the mass.

Suction must be released before removing the needle from the mass or the material is lost into the syringe itself. The syringe is removed and filled with air to expel the material as outlined above. Suction methods increase the risk of both cell damage and blood contamination. This is especially true of continuous suction, which can also increase the risk of other tissues around the mass being sampled as the needle is moved. This method though is often essential for firmer masses, especially if they are derived from soft tissue components.

Sample Preparation:

Once the material has been obtained, there are several ways of dispersing/spreading it to allow staining and examination. Crucially the cells must form a monolayer and not become damaged.

Strengths and weaknesses vary, and very much depend upon the skill of the operator. As with blood films, **only practise and adaptation allow a consistently good smear to be made.**



- **blood film technique** – this is identical to that for a blood film and is very good for more liquid, blood contaminated preparations, especially if the tissue cells are known to be fragile. The advancement speed should generally be slower though. For example lymph node aspirates can be prepared like this.
- **line concentration –** this is also the same as the blood film technique above, but when the material has been advanced about 2/3-3/4, the spreader slide is stopped and lifted off. This leaves a line of concentrated cells at one end. It is very useful for fluid and lavage/wash preparations, especially when they have low cellularity. Urine sediment cytology can be prepared this fashion.
- **Squash –** If you have steady hands, this can be freestyle. However if not, it can be better to hold (with thumb and index finger at either end) and rest the slide that has the material (A) on a flat bench before starting. Another labelled slide (B) is held at right angles with one end (that nearest your hand) over the droplet of material and lowered onto the material. As slide B touches, the material will naturally spread out. Without exerting any downward pressure, slide B is then smoothly and briskly pulled across over slide A. The majority of material is transferred to slide B, but it is worth using slide A too. Some people perform this with a flourish but this will only damage the cells. Others do it too slowly, allowing the slides to actually ‘stick’ to each other which also damages the cells. Downward pressure is only used when actual tissue fragments are seen grossly, when gentle pressure is used to squash the fragments and create a thin layer of cells.
- **Star –** here the aspirate needle is used to drag the material into radiating streaks from the central droplet of material. This is minimally traumatic but unfortunately rarely spreads the cells well, so many areas are too thick for examination. It is not generally recommended, but can be good for (centrifuged/concentrated) fluid preparations that are moderately cellular or grossly turbid in appearance.

Internal organs and structures

If internal structures - ideally under ultrasound guidance - are being sampled the principles outlined above are the same, but the skin should be aseptically prepared. If a vascular organ such as the liver or spleen is being sampled, some argue that coagulation (haemostasis) screening should be performed. However, detectable bleeding following aspirates is very rarely seen, and as the spleen has a muscular capsule, this often 'closes' over the point of entry. Checking platelet numbers are 'normal' is recommended, coagulation times being justified if significant liver disease or clinical bleeding is seen.

If biopsy tissue is taken, trucut or wedge, this can be used to make very good and valuable impression smears. Before making the smear, the tissue section must be blotted dry with a gauze swab to remove seeping blood. A clean labelled slide is then gently pressed onto the tissue as described earlier. If the blood is not first removed, tissue cells will not adhere to the slide and you will get only fresh blood. If the tissue appears very firm, scrapes can be taken using a scalpel blade (after blotting) with the material gently smeared directly onto a slide.

Staining:

The majority of in house labs and commercial external labs use water based stains (such as 'Diff Quik' and Wrights) for cytology *etc.* It is therefore vital that any slides or fluid taken are not exposed to formalin liquid or even the fumes from biopsy material. If being sent in the post, **they should be sent separately**. Formalin is designed for wet fixing cells and stains such as Papanicolauo. Otherwise it introduces cell artefacts and prevents interpretation using water based stains. A few labs do use formalin stabilised fluids, but this should be checked first with the lab.

Staining is the same as that for blood films but as the smears are often thicker, 'dunking' times are often variable and longer or more repeated.

PRINCIPLES OF CYTOLOGY

Cytology uses only a handful of cells to try and diagnose a huge range of diseases and conditions. It therefore requires:

1. good quality samples – is there too much artefact or contamination for a diagnosis?
2. confidence that the sample examined is representative – have we got enough cells to diagnose a disease?
3. a detailed knowledge of normal anatomy and cell appearance - what should we be seeing if this was normal healthy tissue?
4. a detailed knowledge of disease processes – is this sample inflammatory or proliferative, and if proliferative, is it benign or malignant?
5. does the diagnosis 'fit' with the clinical history, signalment *etc*?

1.

One of the commonest pitfalls of cytology is sample artefact. This is often in the form of damage to the cells. Nuclei may remain intact, but without the surrounding cytoplasm, they are difficult if not impossible to interpret. Even the nuclei can be damaged, with only streaks of material remaining. **Optimal sampling and preservation cannot be over-emphasised.** Contamination can be in the form of adjacent tissues as already mentioned, which can also hamper other diagnostic tests. For example, a tracheal lavage can be contaminated with oropharyngeal material. If inflammation is seen, it is not possible to say whether the inflammation is in the trachea or pharyngeal area. Similarly, this material often contains commensal bacteria, and so culture results can be difficult to interpret. Ultrasound guided aspirates can have too much gel which obscures the cells, or the stain may have too much precipitate which again obscures the underlying cells.

2.

Representation and therefore confidence in a diagnosis is very difficult to assess. It certainly in part reflects experience, but the sampling itself is very influential. The more cells we have, the more likely we are to make the correct diagnosis. Certain clinicians produce very good samples. The history and description of the lesion is very important here. A large mass that has only one smear made from an aspirate is unlikely to be entirely represented in that preparation. Conversely small masses can allow the aspirating needle to exit the mass and aspirate adjacent tissue. This combination of abnormal and normal tissue can make a diagnosis difficult if not impossible.

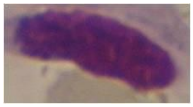
3.

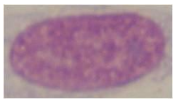
Specific cells and organs are too numerous to describe in a single day. However, all cells and tissues have common features that should always be examined.

Nucleus

This stains purple with most commonly used stains. It is composed of DNA in the form of heterochromatin (dark inactive DNA) and euchromatin (light active DNA). The varying combination of these two gives the nuclei of cells characteristic textures or appearances (*e.g.* dotted/stippled, smudged or reticular/ropey). Cells in many tissues are 'resting' and so mainly have inactive (heterochromatin) nuclei which appear smooth and dark purple. As the nucleus becomes active and the cell produces (more) substances, whiter areas (euchromatin) are seen. Proliferative tissue cells therefore have more 'open' chromatin, with whiter and darker areas. This activity can be great enough to show as a focal 'hyperactive' point – the nucleolus. This is the area where DNA is actually being read and translated to produce proteins etc. Very active cells have large nucleoli or multiple nucleoli. Nucleolar appearance varies from indistinct clear 'bubbles' to discrete dark patches within the nucleus.

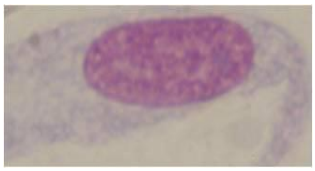
The nucleus is surrounded by a nuclear membrane that is usually invisible and smooth. This membrane is often oval to circular, but can have characteristic shapes.

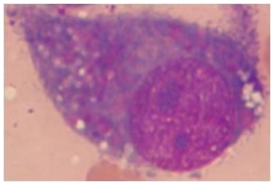




Cytoplasm

This is surrounded by an invisible membrane. However it forms certain shapes that are very useful and whether this shape is discrete and very clear, or poorly demarcated is also crucial. Inside very variable features can be seen. Conversely this time (compared to the nucleus), resting or non proliferative cells have pale smooth cytoplasm. Active cells have darker blue cytoplasm that in some cells can show focal intensity in the form of a pale zone near or adjacent to the nucleus (the Golgi zone). If the cell is productive, product can be seen, as distinct granules (blue, pink, purple etc), indistinct smudges, as a diffuse hue, or in the form of clear vacuoles. If the cell is phagocytic (able to ingest and digest stuff), other cells, infectious agents like bacteria, and debris *etc.* may be seen inside.





Populations

No tissue has only type of cell in it. Therefore the combination and arrangement of cell types can be very important, not only in identifying the tissue/organ, but also whether any of the populations are absent or increased. Certain populations, such as inflammatory cells can be the disease process. Certain tissue types have cellular arrangements that are characteristic.

Background

It should never be underestimated how useful this can be. The appearance of the background is often affected by contamination but can be very useful. Proteinaceous material typically appears variably blue (basophilic) and smooth, whilst glycoproteins/carbohydrates are pinker (eosinophilic). For example, joint fluid is lightly pink and stippled, whereas lymph nodes are lightly basophilic. Chylous effusions have a very light blue background that has numerous irregular vacuoles or clear white spaces.

All of these features will be present in some form in every single cell sample examined. The key is that normal tissue has similar features to proliferative tissue, whether it is benign or malignant. **There is (probably) no single cell feature that tells you a cell is malignant or not. In other words, malignant features are features that can be seen in 'normal' tissue, but not at that time, to that degree or with such variation**. Normal tissue though is uniform, with cells appearing very similar and regular. Benign proliferation can be almost

identical this, but may appear more crowded or ‘active’. Malignant tissue can have many similar features, but crucially there will be more variation from cell to cell, in size, shape and features because the proliferation is now uncontrolled and excessive. Unfortunately though, some malignancies (such as leukaemia or lymphoma) are the direct opposite. Here the neoplastic process is very uniform (clonal) and similar from cell to cell (monomorphic).

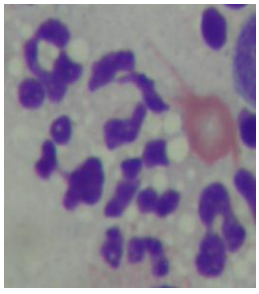
2.

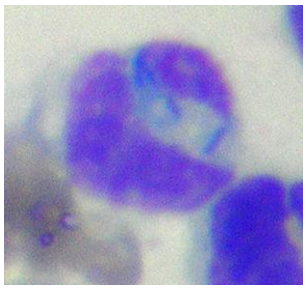
One of the main reasons for cytology is to identify whether a lesion/mass/process is inflammatory, infectious or proliferative (neoplastic). Whenever inflammation or infection is present within tissue, it will cause the surrounding tissue to react and proliferate. As outlined above, physiological reaction to inflammation (hyperplasia and dysplasia) can be very similar to neoplasia (inappropriate or excessive proliferation). When inflammation is present diagnosing neoplasia can be very difficult by cytology alone. Being able to diagnose neoplasia will in part depend on the relative proportions of inflammatory cells to tissue cells. If mostly inflammatory cells are seen and the sample is representative, the lesion is diagnosed as inflammatory. Within this there may be (very) atypical (tissue) cells, but only a few are seen. However, if many atypical cells are seen or they dominate the sample, even if inflammation is present to cause tissue reaction and proliferation, neoplasia can be diagnosed, and if the cells are very variable (pleomorphic), the proliferation is malignant.

Inflammation is commonly broken into three categories based on the dominant or mixture of cells present. This classification can help identify the cause of the inflammation, but can be non specific. Inflammatory cells include: neutrophils, macrophages, eosinophils, lymphocytes, mast cells and plasma cells. Basophils are rarely seen/identified.

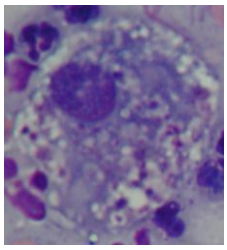
INFLAMMATION

Acute or suppurative – this is dominated by neutrophils, with only low numbers of other mixed inflammatory cells. The neutrophils should be checked for infectious agents, such as bacteria, within their cytoplasm. Bacteria may not be seen, especially if antibiotics have recently been administered. So called ‘degenerate’ changes though can provide support for an infectious process. These changes involve the nucleus that becomes pale and indistinct, swelling and losing its lobulated appearance. They are not the same as ‘toxic’ changes seen in the peripheral blood and described earlier.

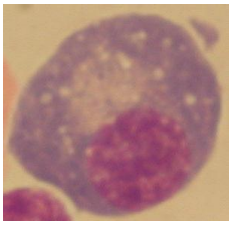




Pyogranulomatous or chronic active – this is when the inflammatory population is very mixed, with macrophages being prominent and about 30-50% of cells seen. Other inflammatory cells can also be present in low numbers. It is non specific, but more commonly seen in foreign body reactions or inflammation/infection that has been present for a while.



Macrophage



Plasma cell

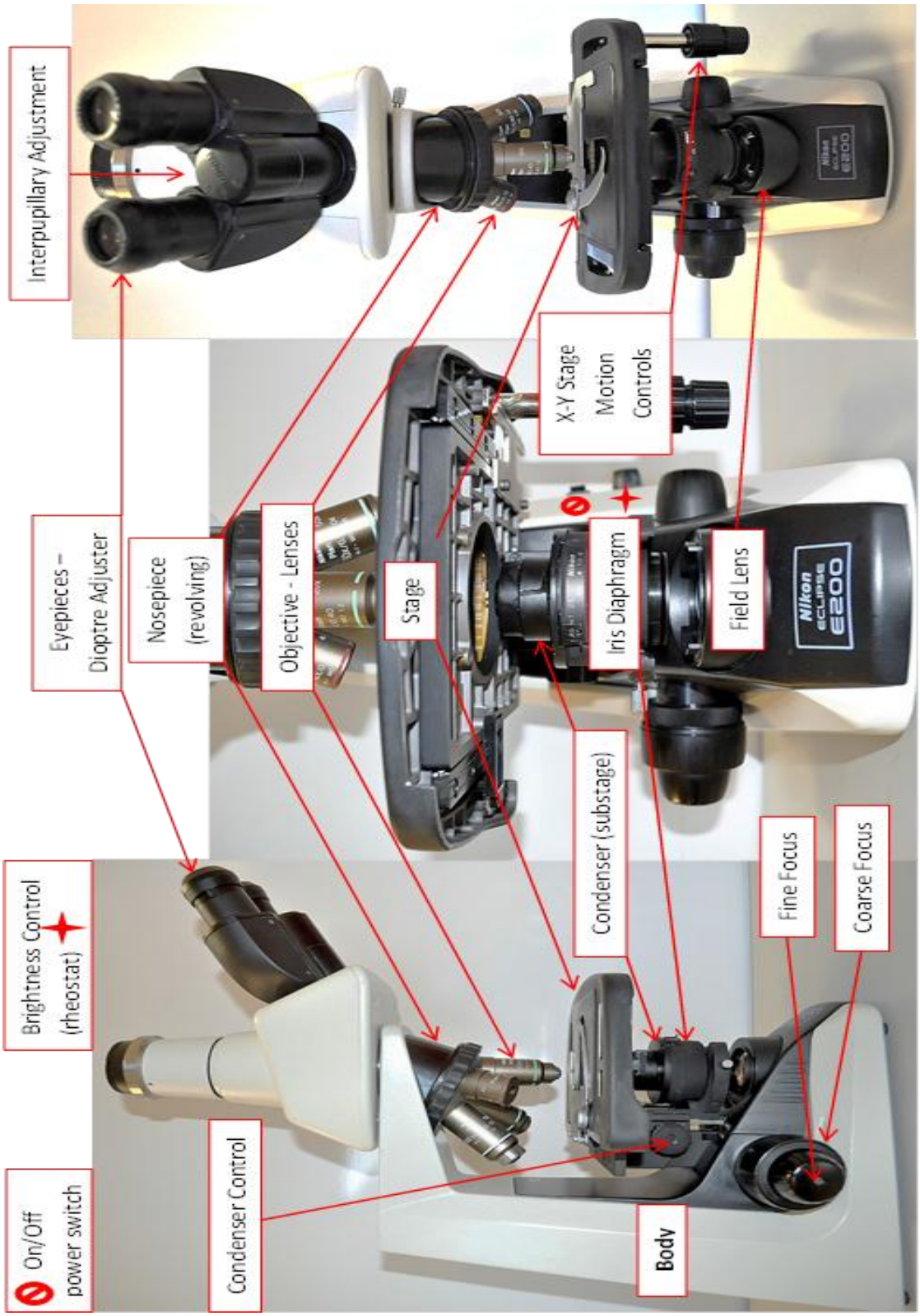
Granulomatous – this is dominated by mononuclear cells, especially macrophages, along with lymphocytes and plasma cells. Other inflammatory cells are also present, especially neutrophils, but these appear mature and 'non degenerate'. Macrophages are very variable in appearance. Key features include an eccentric nucleus, low nuclear:cytoplasmic ratio, reticular chromatin and abundant very irregular cytoplasm that can contain debris, cells and miscellaneous material. With time and especially foreign body lesions, macrophages can become giant and multinucleate. They can also form loosely cohesive sheets with more blue and smooth cytoplasm, similar in appearance to epithelial cells. These are termed epithelioid macrophages. Granulomatous inflammation can be characteristic of certain infections such as Leishmania and Mycobacteria and also very chronic foreign body reactions (such as around a retained swab).

Eosinophilic – this can be dominated by eosinophils, with low numbers of mixed inflammatory cells, often including mast cells. However, if significant numbers (arbitrarily >10%) are present, they are significant and suggest underlying parasitic or allergic diseases most commonly. In cats, focal eosinophilic granulomas can be seen.

5.

Whenever you make a cytological diagnosis, it should be made within the context of the history, signalment *etc.* This not only gives more confidence in the representation as already outlined, but is also an internal quality control check. However, it is also very easy to use this information to over diagnose, providing a diagnosis when actually there are too few cells or other differential diagnoses for the same cell populations. This factor again relies in part on self confidence, but self confidence also plays a role.

Setting up a microscope:



Microscopes without an adjustable field iris diaphragm

1. Turn the lamp on, (adjust the brightness for comfort) and adjust the interpupillary distance so only one image is seen.
2. Place a coverslipped slide onto the microscope stage.
3. Using the x10 objective lens, focus on the slide material. Ensure the eyepiece focus settings are equal and set at 'baseline'.
4. Using the x40 lens, focus on the slide material. Then change back to the x10 lens, and using only your right eye, change the eyepiece focus settings until the image is focussed. Do **not** use the fine/coarse focus control knob. Repeat this procedure using only your left eye.
5. Repeat step 4 using the x40 lens to ensure the eyepiece focuses are perfectly set for your eyes.
6. Set the condenser to just below its highest setting using its focus control knob.
7. Use the desired lens for examination. If the microscope has an adjustable aperture diaphragm on the condenser, this should be set to the same figure as the magnification of the lens (*e.g.* 4, 10, 40 or 100). This aperture should be changed each time the objective lens is changed ideally. If not, a compromise setting for most objective lenses is 40.

The above guidelines allow each microscope to be used to the best of it ability. They do not have to be adhered to, but if not, image quality will be lost and this may hamper examination of material. Once they have been followed once, only steps 1-5 need to be repeated for the microscope to be adjusted to each user, because each person has slightly different strength eyes and interpupillary distances.

The above settings are designed to optimise the microscope for examining cells and cytological preparations. **However, when examining unstained fresh urine or scrapes *etc* for parasites, it is advisable to lower the condenser to its lowest setting and close the field iris diaphragm down**. This may require increasing the brightness, but will maximise the refraction and therefore make sediment and parasites very easy to see.

Some References:

BSAVA Manual of Canine and Feline Clinical Pathology 2nd Ed. Editors L. Blackwood and E. Villiers.

Small Animal Clinical Diagnosis by Laboratory Methods 3rd Ed. Editors Willard, Tvedten & Turnwald

Diagnostic Cytology and Haematology of the Dog and Cat. 3rd Ed. Editors Cowell, Tyler and Meinkoth

Veterinary Haematology – Jain and Schalm 2nd Edition

Veterinary Haematology and Clinical Chemistry – Editor M. **Thrall**