



**Cytological sample
collection & preparation**
for veterinary practitioners

Cytology sample collection and preparation for veterinary practitioners

by Brett Stone and George Reppas

Introduction:

Since the mid-1960s, when the first reports of cytology appeared in the veterinary literature, cytology has become an extremely useful diagnostic aid in veterinary medicine.

Cytology has many advantages over histopathology:

- Cytology samples can be easily obtained pre-operatively, often without general anaesthesia and sometimes even without sedation, and can be used to screen patients for more comprehensive diagnosis.
 - Fine needle aspiration cytology is less costly than surgical biopsy in both sample collection and laboratory analysis.
 - The fine needle aspiration procedure is less likely to result in adverse effects when compared to tissue biopsy.
 - Because less sample processing is required, cytology results are available sooner than histopathology results.
 - 'Quick' checking for recurrence of local malignancies or regional lymph node metastases.
 - Pathologic micro-organisms involved in microbial infections of various organs (eg: canine and feline leprosy, subcutaneous mycoses, bacterial prostatitis) diagnosed initially by cytology have a greater chance of being cultured successfully.
- Techniques are being developed whereby the aspiration of neoplastic lymphoid cells from suspected malignant lymphomas can be immunocytochemically phenotyped into T & B cell populations either directly from FNA smears of lymph nodes or via flow cytometry - thereby avoiding costly and sometimes contraindicated general anaesthesia to perform an incisional/excisional surgical biopsy
 - Cytology aspiration of particularly 'hard to get to' organs (eg: pancreas, heart base), can be successfully sampled with the use of ultrasound guided techniques.

Cytology also has its limitations however. As the cells/material being evaluated are 'outside' their normal environment, an assessment of cellular organisation, arrangement or architecture is often not possible by cytology. Therefore, adequate sample collection and preparation is of considerable importance when it comes to cytological interpretation, as this will provide the pathologist with as much information as possible on which to base an interpretation.

Sample Collection and Preparation:

With cytology, unlike histopathology, it is the clinician's responsibility to not only collect the specimen appropriately, but to also adequately prepare the specimens that are to be presented for cytological examination.

Regardless of the experience of the clinician or veterinary pathologist examining the smears, poor sample collection and preparation will most likely result in interpretations or diagnoses such as; 'Open', 'Equivocal' or 'Non Diagnostic'. Improving the quality of cytological submissions will maximise the likelihood of a meaningful cytological description and a more accurate cytological diagnosis. Even if a definitive cytological diagnosis cannot be made, cytological findings may be of value in determining which additional diagnostic laboratory tests may be of further value (eg: histopathology or culture) or directing further investigative procedures such as ultrasonography or radiography (eg: when skin lesions may be involving bone or joints).

It is extremely frustrating for clinicians and owners alike, when submitted cytological samples are non-diagnostic. Our aim here is to outline the basic principles of cytological sample collection and preparation and to also provide advice on ways to reduce the frequency of non-diagnostic cytological results.

In our experience, cutaneous and subcutaneous lesions are the most commonly sampled for cytological evaluation and therefore where appropriate in this article, we have preferentially addressed collection and sample preparation techniques from such lesions. However, many of the comments that follow are relevant to all aspects of veterinary cytology specimen collection and preparation.

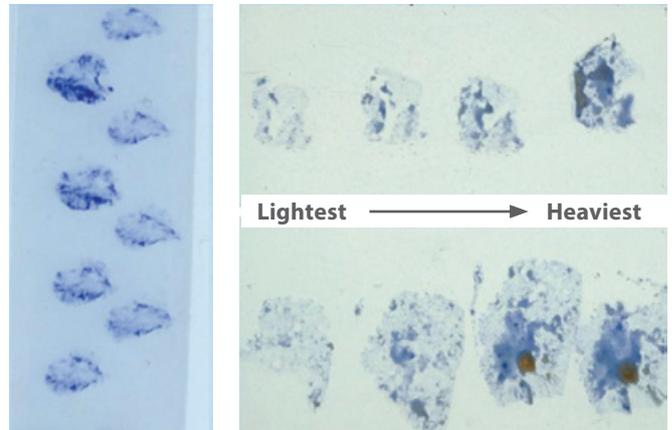
Cytology Collection Methods:

1) Imprints

Making quality smears of impression smears:

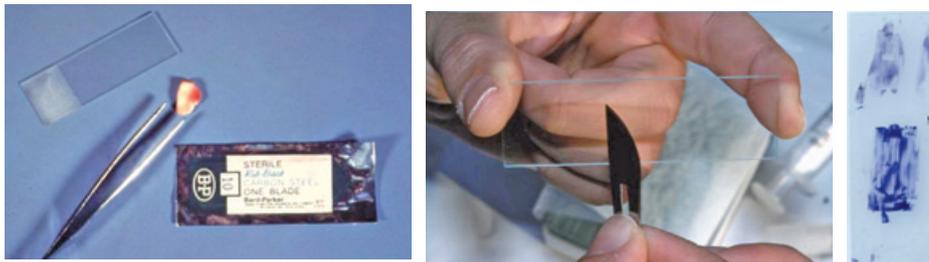
Touch imprints may be made directly from crusted and ulcerative skin lesions or from impressions of deeper surgical biopsies gently rolled onto a glass slide prior to placement in formalin. Dry scabs/crusts should be removed manually prior to impression smears being made, as cells in these scabs/crusts will generally reveal poor cellular morphologic preservation and poor staining characteristics. The tissue should be blotted dry (using paper towel) to remove surface fluid or blood as these may impair adhesion of cells to the slide and dilute the cytological material. Following this, the biopsy sample or lesion being examined is firmly pressed several times onto a clean glass slide. Do not rub the tissue on the slide as this will result in distorted cellular morphology by causing cell rupture and nuclear stranding. Imprints from ulcerative lesions often only yield superficial inflammation and infection and any underlying/primary neoplastic process may be missed. This is a good technique for investigating the presence of inflammation with or without infectious agent involvement, and it may also be useful in assessing the presence of superficial neoplasia such as that often seen with squamous cell carcinoma.

2 slides, 4 touches/slide,
different degrees of pressure



2) Scrapings

Making quality smears by tissue scraping:

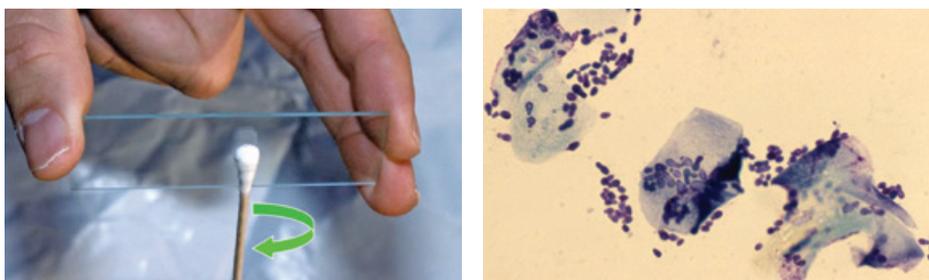


The back (blunt edge) of a scalpel blade or edge of a glass slide is used to gently scrape across the lesion or tissue biopsy until a small amount of material is collected. This material is then gently spread/buttered across a slide (*see preparation*

of slides). This method has similar uses to imprinting, but may also be used where imprinting is likely to yield too few cells for complete assessment (eg: conjunctiva, mesenchymal neoplasia).

3) Swabs

Making quality smears with cotton-tipped swab:



This technique is useful for the sampling of fistulous tracts, ear canals, exudates and for vaginal cytology. Unless the location is very moist, lightly moistening the swab with 1 or 2 drops of sterile saline prior to collection is advised as this will minimise cell damage. Once the area to be investigated

has been sampled by using the moistened swab, smears are prepared by gently rolling the swab over a glass slide. Do not smear the swab on the slide in a side-to-side motion as this causes cell rupture and poor cell preservation.

4) Fine Needle Biopsy / Fine Needle Aspirate Biopsy (FNAB)

The best and most commonly used method for sampling proliferative lesions and masses. We recommend using a 22-25 gauge needle and a 2-5ml syringe and as a general rule, the softer the tissue, the smaller the needle and syringe required to obtain an adequate sample.

Making quality smears by fine needle aspiration and non-aspiration techniques:



Remember to redirect needle 3–4 times when seated in the mass and if using “vacuum” don’t pull back on plunger whilst redirecting needle.



“Pin cushion” whatever you need is in the needle and the hub.

ASPIRATION PROCEDURE:

Once the mass is stabilised between the operator’s fingers, the fine gauge needle is inserted into the mass. When the needle is seated comfortably in the mass, negative pressure is applied to the plunger/syringe. Try and avoid redirecting the needle or moving it back and forth within the mass whilst vacuum (negative pressure) is applied as this generally results in increased blood contamination of samples. This procedure should be repeated at least 3 – 4 times at different angles within the lesion to obtain a representative cell population from the lesion in question. Smaller syringes attached to the needle offer the operator better control during the aspiration process, particularly when aspirating smaller lesions. A minimal

amount of material within the hub of the needle is adequate and generally this is sufficient for cytological interpretation. Attempted further aspiration often leads to unwanted blood contamination. If blood is encountered during aspiration attempts, then the exercise should be ceased and repeated a little further away from the initial puncture site. Negative pressure should be released before the needle is removed from the mass and skin. Once the needle is removed from the syringe, air is drawn into the syringe and the needle is firmly re-attached to the syringe. The material within the hub of the needle is then expelled onto a couple of slides for smear preparation as described below.

NON-ASPIRATION PROCEDURE:

This technique also utilises a fine gauge needle (22 – 25g). Here a syringe is not attached to the needle for aspiration of skin masses. The needle is briskly redirected within the mass at several different angles. This technique works well for most masses and may be superior to the aspiration technique when

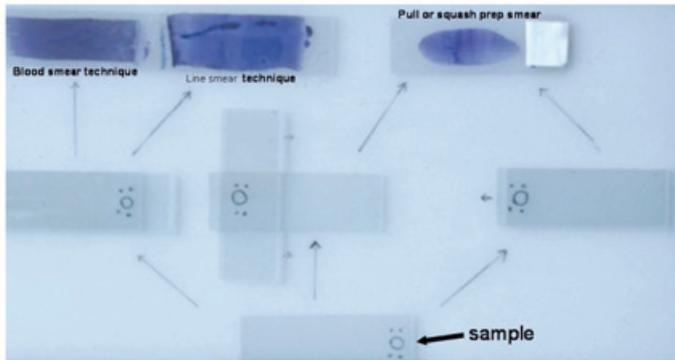
sampling highly vascular masses, as blood contamination is often reduced. Once the needle is removed from the lesion, it is attached to an air-filled syringe and the material within the needle is gently expelled onto clean glass slide/s for smear preparation as described below.

Collection Tips and Advice for Cytology Specimens:

1. Avoid blood contamination. Possibly use the non-aspiration needle biopsy technique for soft, highly vascular and small lesions.
2. Do not prolong the period of aspiration (should take less than 30 seconds) and make smears immediately after collection to optimise cell preservation.
3. Attempt 2-3 separate collections (if the lesion/mass is large enough).
4. Make 2 slides from each collection (see preparation of slides).
5. If cell yield appears poor via FNAB: use a larger needle and syringe (within the limits indicated above) and/or increase the amount of negative-pressure within the syringe.
6. Material within the hub of the needle is usually sufficient and further sampling often results in unwanted blood contamination.
7. Should blood appear in the syringe during FNAB – stop the procedure immediately and start again with a new needle and syringe.

Preparation of Slides

The aim of slide preparation for cytological evaluation is to achieve a monolayer of well-preserved cells. This can be achieved by several methods:



1) Squash Preparation (A misnomer?)

This is the preferred method for preparing slides from needle biopsies, FNAB and scrapings. Material collected by fine needle biopsy or scraping is placed towards one end of a 'frosted edge' glass slide (the region adjacent to the frosted region of a slide is preferred). A second slide is aligned perpendicular to the first and is allowed to rest on the slide containing the expelled material. This slide is then gently and smoothly drawn over the length of the first slide

whilst concurrently rotating them from a perpendicular to a parallel position. This results in the simultaneous preparation of two smears. Avoid physically squashing the material which causes excessive pressure, leading to cell rupture and a non-diagnostic preparation. Often the fluid material will 'suck' the second slide down onto the first and together with the weight of the second slide, further downwards pressure is not required.

2) Needle / Starfish Preparation

Material collected by fine needle biopsy is placed in the centre of a glass slide and the needle is used to drag/tease the material outwards – in multiple directions - to produce a star / starfish shaped smear with multiple projections. Many areas of the smear will be too thick for evaluation, however there are usually multiple cell monolayer regions present on the smear that should be acceptable for cytological assessment.

3) Blood Smear Technique

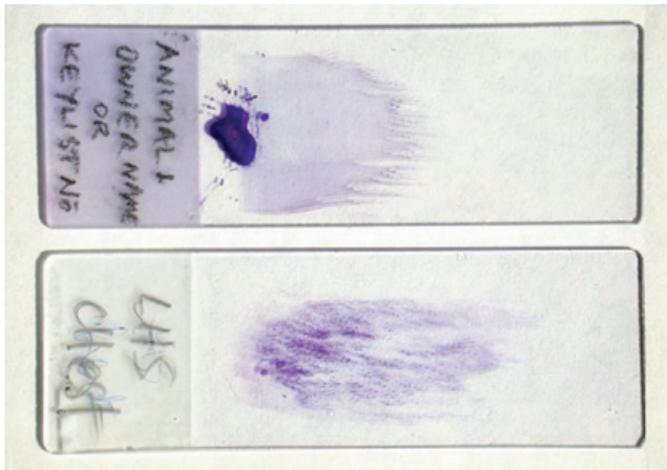
Aspirated material may contain enough blood and/or liquid to allow smearing of the material in a similar fashion to that used to make a peripheral blood smear. The material is expressed towards one end of a 'frosted edge' glass slide (again, the region adjacent to the frosted region of a slide is preferred) and the short edge of the spreader slide is placed in front of the sample. The spreader slide is tilted to an angle of approximately 45 degrees, pulled backwards into the material and once the material has dispersed along the width of the spreader slide, the spreader slide is smoothly, steadily and rapidly slid forward. The smear ends with a feathered edge of material. As a general rule, the more material placed on the specimen slide, the slower the spreader slide is slid forward and the more acute the angle between the spreader and specimen slide, the longer the smear will be.

Slide Preparation Tips and Advice for Cytology Specimens:

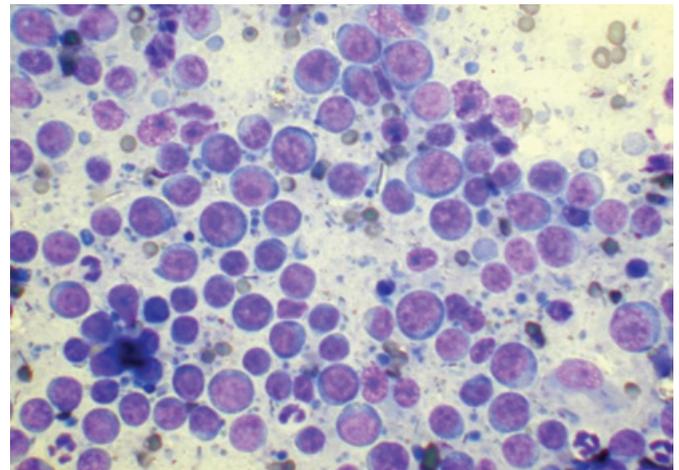
1. Use frosted slides to clearly label slides with the animal's name and the location aspirated.
2. Use pencil to write on the frosted section of the glass slide, as ink may wash off during staining.
3. When using single-sided frosted slides, it helps to ensure material is placed on the same side of the slide as the frosting/label.
4. Label the slides and not the slide carriers. This is especially important when multiple sites are sampled and sent to the laboratory for cytological assessment.
5. A diamond pen for etching/circling particular areas of note on glass slides which you would like the cytopathologist to examine, can also be used (contact your laboratory or drug wholesaler to enquire about purchasing these).
6. Ensure clean glass slides are used and minimise handling of glass slides to reduce collection artefacts (eg. squames off your fingers).
7. Prepare 2 slides from each collection.
8. Be gentle when preparing squash preparations. The weight of the slides together with the 'suction' produced from the fluid material is usually adequate and further downwards force is not required.
9. Rapidly drying the slides (hair-drier or by 'waving' in the air) after preparation, reduces cell crenation and slow-drying artefact, resulting in superior cell preservation.
10. Simply spraying material onto the slide often produces thick cell clumps which will impede individual cell cytological evaluation.
11. The presence of a large amount of blood or the preparation of thick smears also severely hinders cytological cell evaluation.
12. Avoid placing material near the edges of the slides, particularly towards the shorter edges, as these regions may be difficult to assess microscopically and automatic stainers may not adequately stain these regions. Furthermore, there is an increased likelihood that superficial squames from human fingers will have contaminated these areas during smear preparation.

Collection and Preparation of Slides from Fluid Samples

WHAT IS A GOOD CYTOLOGICAL SMEAR?



Well labelled slide (use frosted edge glass slides to pencil on owner and animal name).



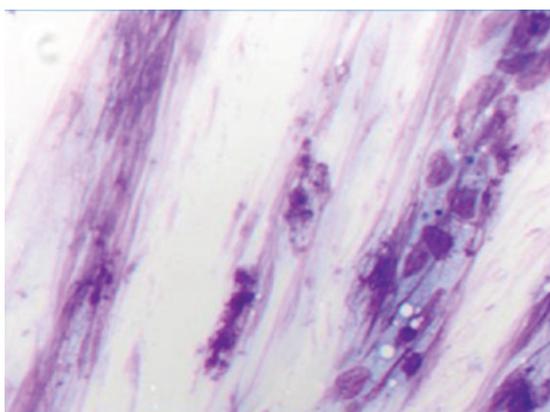
Smear reveals good cell yield and cells are arranged in monolayers displaying good cellular morphological preservation with minimal cell damage.

Fluid samples are obtained from sampling body cavity effusions, cysts, joints, cerebrospinal fluid, seromas, urine and when performing various types of washes (eg: bronchoalveolar lavage, transtracheal wash). Volume permitting, fluid aliquots should be collected into EDTA-containing tubes and sterile/plain tubes. Smears should also be made at the time of sampling. EDTA prevents coagulation and therefore allows for accurate cell counts to be performed when required. EDTA helps preserve cell morphology during transit to the laboratory, however morphology is best preserved by making smears at the time of sampling. This is particularly true with respect to urine cytology as prolonged contact with urine often causes severe cellular swelling and degeneration. Making smears at the time of sampling also helps better determine the relevance/significance of certain cytological features such as erythrophagocytosis and the presence of intra-cellular bacteria (phagocytosis of both red cells and bacteria by leukocytes may occur post collection during transit). EDTA is bacteriostatic and culturing from EDTA-containing fluid is generally not advised. Concurrent

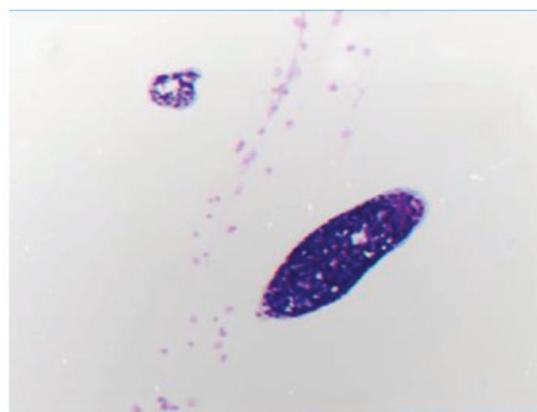
submission of a plain fluid sample collected into a sterile container, allows for culture to then be performed if initial cytology indicates that this may be warranted.

Smears from cloudy, highly cellular, well-mixed fluids can be made directly via the blood smear technique or line smear technique. If there are any floccules of particulate matter grossly visible in the fluid at the time of collection, then these should be included in the smears as well. Aliquots of clear or slightly turbid fluids should be concentrated via centrifugation to increase the cellularity of prepared smears. Following centrifugation, the majority of the supernatant is decanted, the pellet (cellular material) is then resuspended in the minimal remaining supernatant, and smears are made via either the blood smear or line smear techniques. The line smear technique is similar to the blood smear technique, however the spreader slide is abruptly stopped and lifted off the specimen slide prior to creating a feathered edge, resulting in a higher concentration of cells present in the terminal line, than within the remainder of the smear.

WHAT IS A POOR CYTOLOGICAL SMEAR?



The majority of cells are ruptured leaving linear nuclear strands and debris.



Material is present in thick clumps and individual cell evaluation is impossible.

Submission Tips and Advice for Cytology Specimens:

1. Submit 2-4 well prepared smears from each location sampled.
2. Label all smears appropriately (see above).
3. Air-dried smears made at the time of sampling do not require further fixation and will survive transit to the laboratory 'as is'.
4. Submit slides in rigid plastic slide-holders and not in cardboard holders or by any other method (eg: wrapped in surgical swabs or tissues).
5. Submitting unstained smears is preferred, however it is often advantageous to stain one smear in-house (see below) to assess the adequacy of cellularity and smear preparation prior to submission to the laboratory. If samples appear inadequate, further specimens can be obtained and re-assessed. If multiple attempts at sampling yield poor cellularity and/or poor cell preservation, then tissue biopsy and histopathology may prove more beneficial than cytology.
6. If staining one or more smears in-house, please also submit these with the unstained smears.
7. **Do not submit unstained smears with formalin-containing specimens. Formalin vapour will 'fix' unstained material and will severely adversely affect subsequent staining intensity and quality, and will often render the smears non-diagnostic. Ensure formalin and cytological specimens are 'bagged' separately when submitted together to the laboratory.**
8. When submitting fluid samples; submission of in-house made smears, EDTA-containing fluid and fluid in a plain/sterile container is recommended.

Staining of Cytology Smears:

Diff Quik® Stain

Disadvantages: may not stain mast cell granules & requires regularly changing as it may grow contaminants (eg fungi) and does lose potency.



Coplin Jar.

Several different types of stains are used for cytology; Romanowsky type stains (Wright's, Giemsa, Diff-Quik®), Supravital stains (toluidine blue, New Methylene blue) and Papanicolaou stains.

Papanicolaou stains provide excellent nuclear detail and adequate cytoplasmic detail, however they are time consuming and impractical for in-clinic usage.

Supravital stains provide excellent nuclear detail but poor cytoplasmic detail and are typically reserved for evaluation of reticulocyte identification (peripheral blood smear) or for evaluating the presence of poorly-granulated mast cells.

Romanowsky stains are inexpensive, easy to use and they are readily available to veterinary practitioners. They provide good nuclear detail, excellent cytoplasmic detail and infectious organisms are readily visualised. In clinical practice, the most cost effective, quickest and easiest stain to use is the Diff-Quik stain®. The recommended staining procedures outlined on the product should be followed, however as a general rule; the thinner the material on the smear, the less time needed for staining and the thicker the material, the more time required for staining.

Staining Tips and Advice

1. Sealing staining solution containers properly (ie: can use cling-film between the lid and container) will minimise the loss of stain fluid due to vaporisation (particularly the first 'fixing solution').
2. Make sure that the smears are completely dry before staining.
3. Take care not to over-stain smears. Under-stained smears can be re-stained, for further periods to increase staining intensity.
4. If bacteria and/or fungi are regularly noted within examined smears (particularly if sampled sites are not superficially ulcerated lesions), then the staining solutions are likely contaminated and need to be replaced. This can be confirmed by staining an unused, clean glass slide and then examining this slide for the presence of organisms.
5. Stain precipitate excess typically presents as extracellular blue granular material that is often clumped. The stains should be mixed thoroughly or replaced.
6. Stains will lose potency over time and need to be replaced.
7. Practice makes perfect. Evaluate the smears for the thickness of material and the staining intensity and quality and adjust future staining times appropriately.

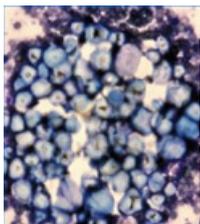
Determining the adequacy of a cytology smear to be presented for cytological examination:

To ensure whoever (either the attending veterinarian or the cytopathologist to whom the slide has been sent for review) is going to look at the cytology smear you have made has the best opportunity of arriving at a useful conclusion, you must ensure that the slide being sent is representative of the lesion in question.

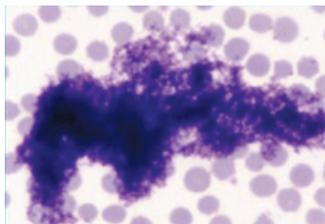
The smear you make must contain a reasonable number of cells to be of any diagnostic value and free of artefacts. You need a reasonable microscope (with at least a clean 10x and 40x objective) to be able to assess the cellularity of a slide adequately, as well as some knowledge of the common artefacts that are most frequently encountered in cytological preparations.

Common artefacts include:

stain precipitate, talc powder granules (eg from gloves), keratin bars (squames – from collectors fingers), water/moisture (due to inadequate drying), ultrasound gel, cotton threads and formalin vapour effects upon cells.



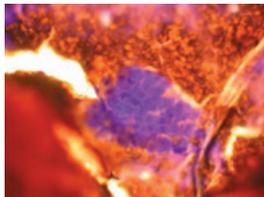
Glove powder



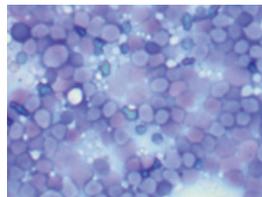
Stain precipitate



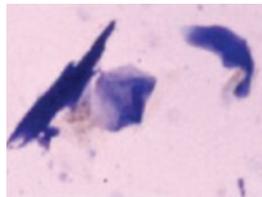
Contaminants



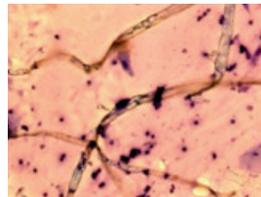
Very thick & blood contaminated



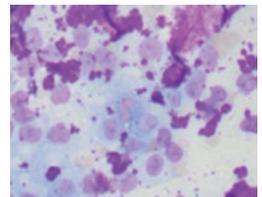
Formalin vapour effects



Squames



Cotton threads



Ultrasound gel – magenta coloured material

Tips and advice for determining that a smear is adequate for cytological examination and interpretation:

1. Allow the smear to dry completely prior to examination. This may be enhanced by using a hair drier set on the "low heat" level.
2. Scan the smear at low power (4x or 10x objective) to assess overall cellularity and to find areas within the smear that contain a monolayer of well-preserved and adequately stained cells.
3. Examine smear using 'high dry' technique (40x objective) by placing a coverslip directly on the dry stained smear. Oil is not required.
4. Use high power (x 40 objective) to evaluate individual cells within the monolayer.
5. Immersion oil is usually only required to investigate the presence and morphology of infectious agents such as bacteria.

History... History... History

The importance of supplying a clear, concise and relevant history cannot be over emphasised. Together with the experience of the veterinary pathologist and alongside good sample yield and cellular morphological preservation, the provision of adequate clinical information is a very important factor affecting the cytological diagnosis. Although a cytological diagnosis will be based on examination of the material provided, patient history and clinical information is often used to improve the likelihood of a successful diagnostic cytological outcome.

References:

Christopher, MM & Hotz, CS. Cytological diagnosis: expression of probability by clinical pathologists. *Vet Clin Path.* 2004; 33(2): 84-95.
 Meinkoth, JH & Cowell, RL. Sample collection and preparation in cytology: increasing diagnostic yield. *Vet Clin Small Anim Prac.* 2002; 32: 1187-1207.
 Meinkoth, JH, Cowell, RL, Tyler, RD & Morton RJ. Sample collection and preparation. In: *Diagnostic cytology and hematology of the dog and cat.* 3rd edn. Mosby Elsevier, 2008. 1-18.
 Rakich, PM & Latimer, KS. Cytology. In: *Duncan & Prasse's veterinary laboratory medicine: clinical pathology.* 4th edn. Blackwell Publishing, 2003. 304-306.
 Sharkey, LC., Dial, SM. & Matz, ME. Maximizing the diagnostic value of cytology in small animal practice. *Vet Clin Small Anim Prac.* 2007; 37: 351-372.

Summary:

The submission of good quality, highly cellular, well-labelled specimens together with a complete, concise and relevant history are paramount for an accurate cytological interpretation.