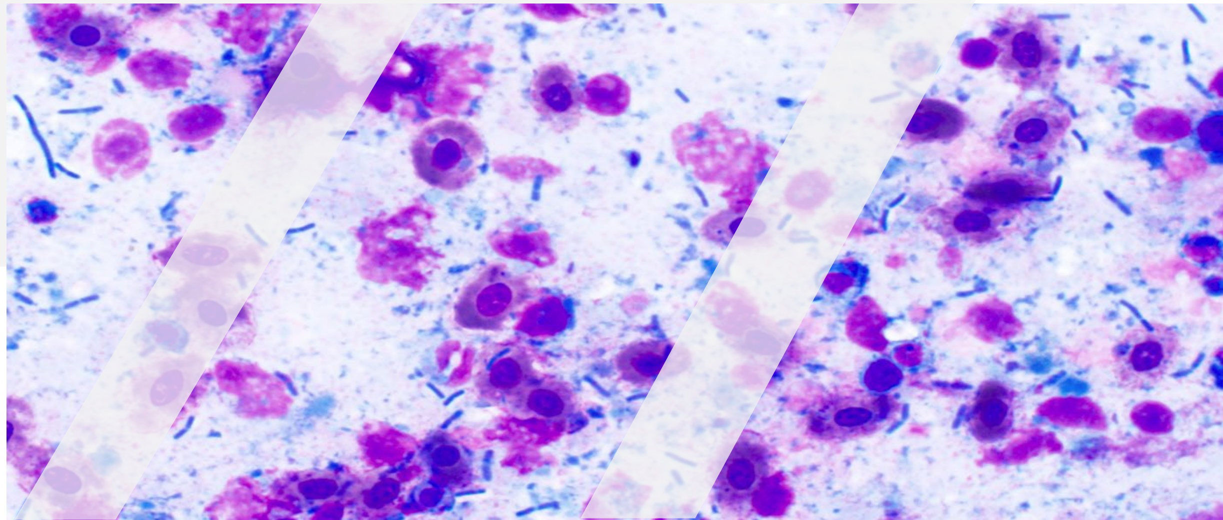
2024 Wildlife Disease Association Conference

Cytology 101



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MediDivert

TARONGA
CONSERVATION SOCIETY AUSTRALIA
Australian Registry of Wildlife Health

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Introduction

Welcome to the 2024 WDA Wildlife Disease Investigation Workshop.

Today we aim to share techniques and tips based on many years of wildlife diagnostic experience in hopes that you will leave feeling confident to “try this at home”.

The Australian Registry of Wildlife Health was established in 1985 as a collaboration between Taronga Conservation Society Australia and University of Sydney. Today’s workshop is an example of that ongoing collaboration as we work together to provide a diagnostic service for free-ranging wildlife, conduct research to identify, characterise and understand the ecology of new pathogens, and shape the outputs of those activities into educational programs.

With your participation we can expand and expedite wildlife diagnostic capabilities across Australia, to protect biosecurity, biodiversity, wildlife care and welfare.

Goals of workshop

1. Identify your learning priorities for the day
2. Review effective means to create, evaluate, and examine cytologic preparations
3. Provide an approach to gross post-mortem examination
4. Discuss record keeping and methods of describing your findings
5. Review appropriate sample collection, storage and shipment

LABORATORY SAFETY

Biosafety

- No eating or drinking is permitted in the laboratory.
- All laboratory personnel must wear protective laboratory coats or gowns, and disposable gloves, while performing tests in the laboratory.
- Gowns should not be worn outside the laboratory.
- Protective, covered, non-slip footwear should be worn at all times.
- Protective eyewear should be worn when biological sample or chemical splatter is a risk.
- N95 face masks should be worn when handling higher infection risk samples i.e. birds and mammals.
- Hand washing, with a disinfectant hand wash, should be performed after sample handling.
- Samples for microbiology should be processed in a biological safety cabinet (minimum Class I). Cabinet should be serviced and tested annually.
- All biological waste should be discarded into dedicated waste bags.
- Benches should be wiped down daily with 70% alcohol or any laboratory grade neutral detergent.

Biological Spill Clean-up

- If lab gown and gloves become contaminated, remove gloves into contaminated waste and place gown in a dedicated tub for disinfection.
- Scrub any bare skin thoroughly with warm water and an appropriate disinfecting soap.

- If eye was splashed, use eyewash (as per manufacturer recommendations).
- Report incident to supervisor (as per institutions incident reporting system) as soon as possible.
- To disinfect benches, Biological Safety Cabinet or floor, soak up any liquid with vermiculite and place in biological waste. Then spray with F10 (or other appropriate disinfectant) following manufacturer's instructions.
- If broken glass is involved, disinfect glass first then sweep and place in sharps container.

Disinfectants

F10 or CaviCide

Can be used for decontaminating surfaces where a small biological spill has occurred. Can be used to disinfect liquid biological samples prior to disposal (less corrosive on stainless steel surfaces if used long term).

Sodium hypochlorite (10%)

Can be used for decontaminating surfaces where a small biological spill has occurred. Can be used to disinfect liquid biological samples prior to disposal (may be corrosive on stainless steel surfaces if used long term).

Virkon S (1.0%)

Used for disinfecting instruments or decontaminating surfaces (may be corrosive on stainless steel surfaces if used long term).

70% Alcohol

Used for disinfecting laboratory surfaces.

Chemical Hygiene

- All chemicals should be stored appropriately as per their Safety Data Sheets (SDS). This may include a Chemical Hazard Cupboard.
- SDS should be updated every 5 years.
- Appropriate chemical personal protective equipment should be made available as per SDS recommendations.
- Suitable spills kits need to be made available for the types of chemicals used.

CYTOLOGY 101

Goals: Our ultimate goal is the creation of a monolayer of well-preserved cells.
Steps to achieve our quest:

1. Sampling
   - Techniques and equipment
   - Equipment
   - Number of aspirates
2. Smearing
3. Staining
4. Smear Submission/Evaluation
   - Microscope set up
   - Preparing slides
   - History/clinical information
   - Quality control – check the smear!

Sampling Techniques:

- Fine needle aspiration
- Impression smears
- Squash preps
- Scrapings
- Swabs and cyto-brushes
- Sticky tape preparation
- Hair plucks
- Washes

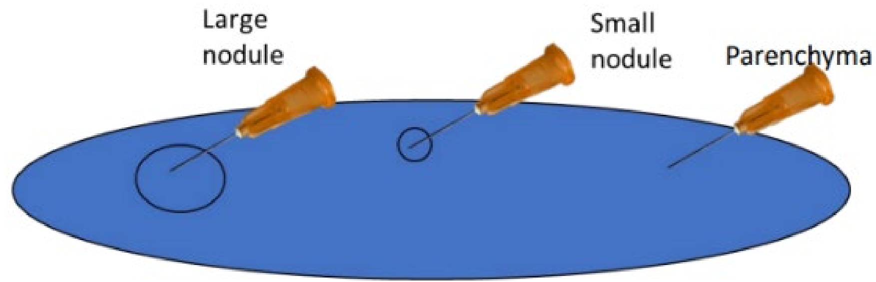
Fine Needle Aspiration (FNA)

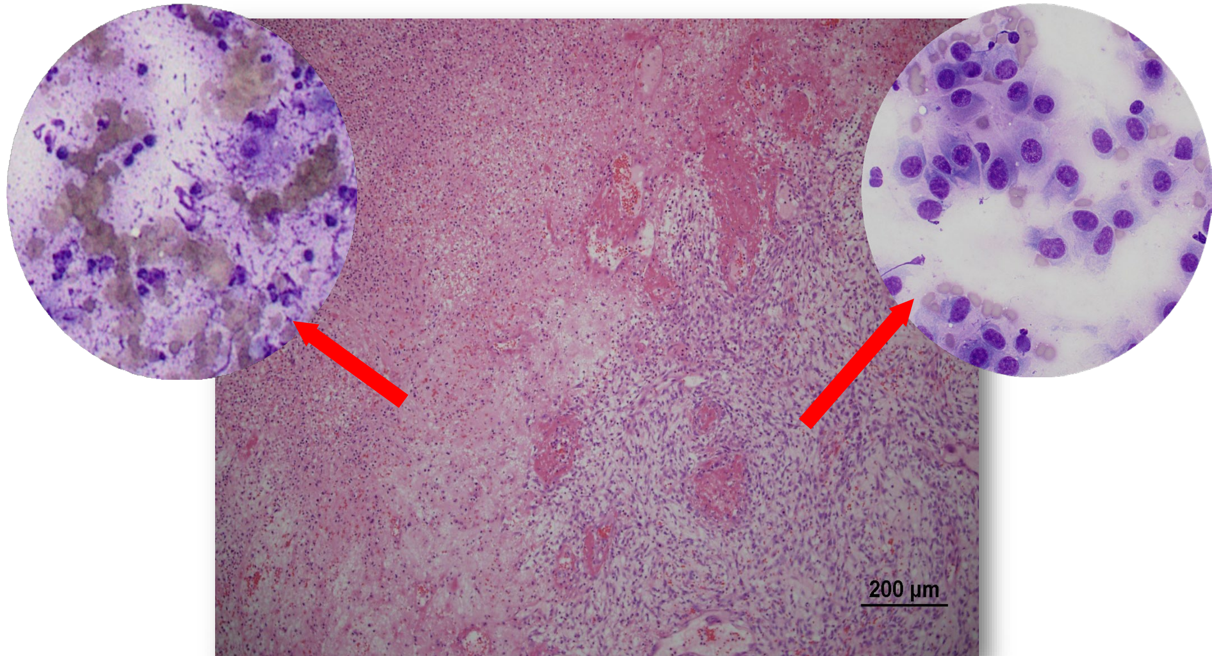
- Non-aspiration vs Aspiration technique
  - Less haemorrhage with non-aspiration
  - Often similar cell yields (but tissue dependent)
- Degree of suction – 1-2 ml is usually sufficient.
- Needle movement
  - 3-4 quick multi-plane movements
  - No need to twist
  - Release plunger before withdrawing needle
- Ideally 3-4 aspirates per lesion
- Gently extrude material onto slide at label end then immediately spread the material

FNA Equipment

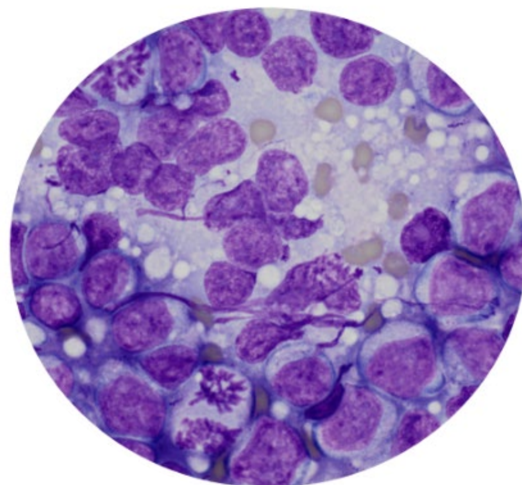
- Needle gauge: 23-25G
- Needle length:
  - 2.5-3.8cm needle usually suffice
  - 6.3-8.9cm spinal needle for deeper internal organs or larger animals
- Syringe size 5-10ml +/- extension set
- Frosted glass slides for easy labelling with pencil

Why Aspirate Multiple Areas?





FNA of Lymph Nodes



- Non-aspiration technique is best with large nodes
- Minimum of 3 aspirates recommended
- Gentle smearing – cells are very fragile
- If generalised lymphadenomegaly sample multiple nodes - mandibular lymph nodes are least preferable
- Use blood smear technique for smearing if fluid aspirate

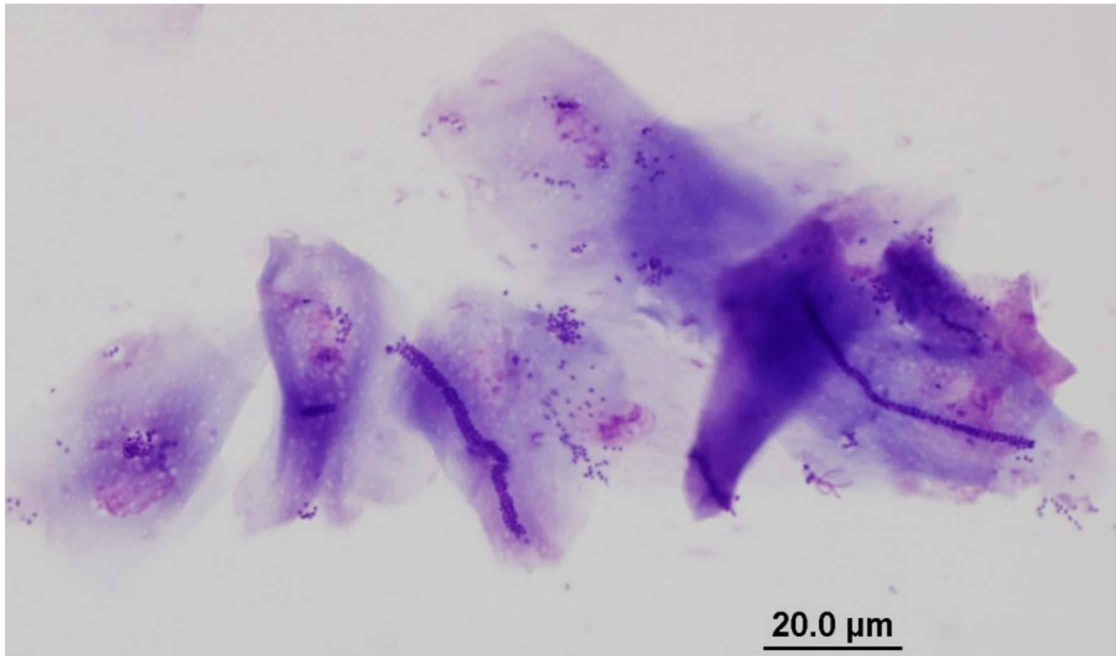
Impression Smears of Superficial Lesions

Useful for screening for infectious agents e.g. *Dermatophilus*, *Cryptosporidium*, secondary infection of mass lesions

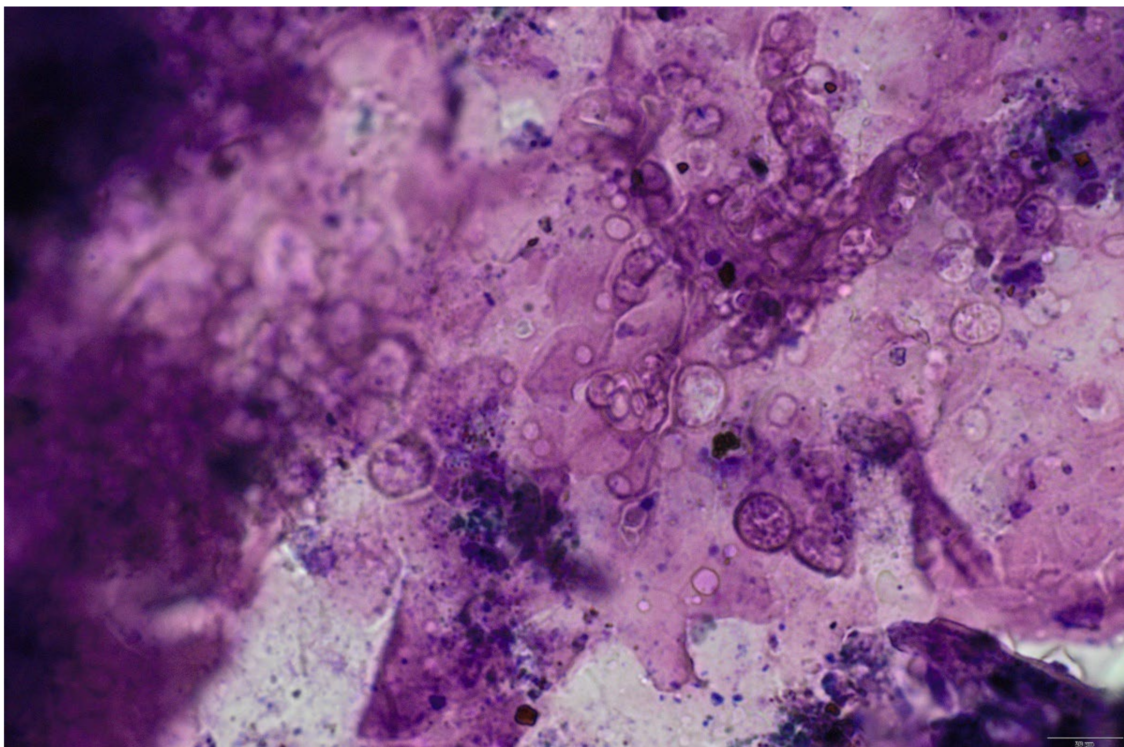
Steps:

- Remove large crusts (save these in a sterile pot)
- If sampling suspected tumours, also gently blot the surface with a **saline** moistened gauze swab then with a dry gauze swab to remove blood
- Touch the slide on the surface of the lesion multiple times

Example: Dermatophilus congolensis



Example: Chytrid fungus in a frog

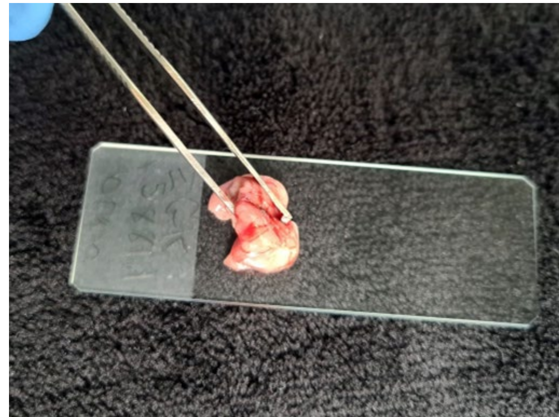


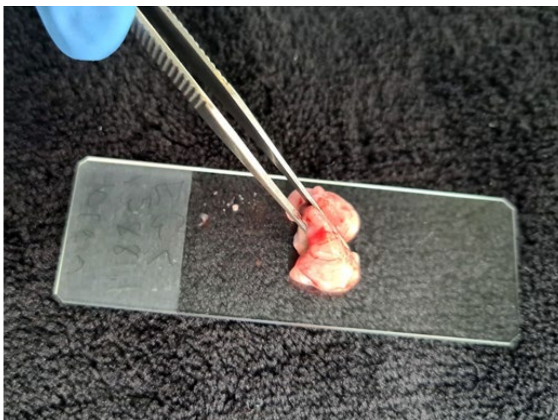
Impression Smears of Organs and Tissues

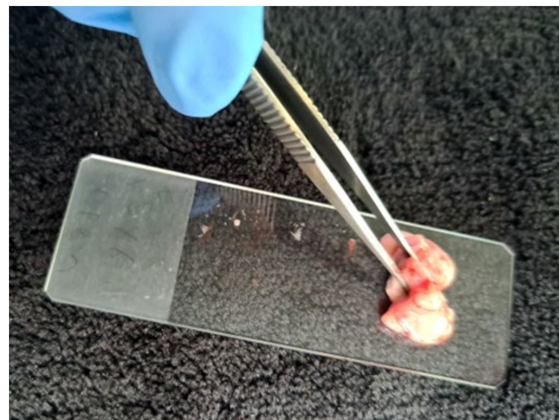
Useful for screening for neoplasia, infectious agents e.g. *Dermatophilus*, *Cryptosporidium* species

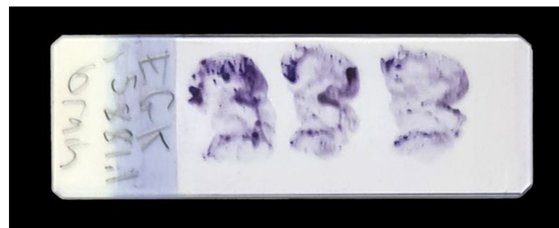
Steps:

- Make a fresh cut in the tissue
- Blot on gauze swab
- Press the surface onto the slide multiple times
- Label smears
- Air dry then stain



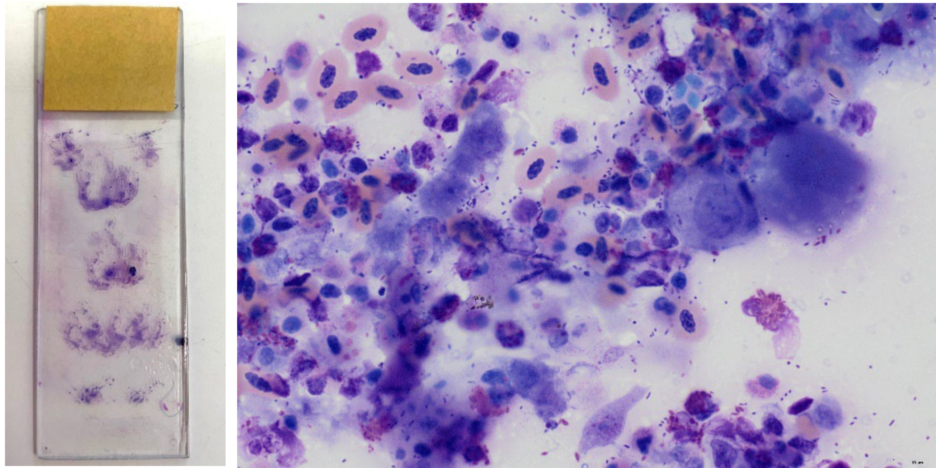






Steps for creating an impression smear of organs and tissues

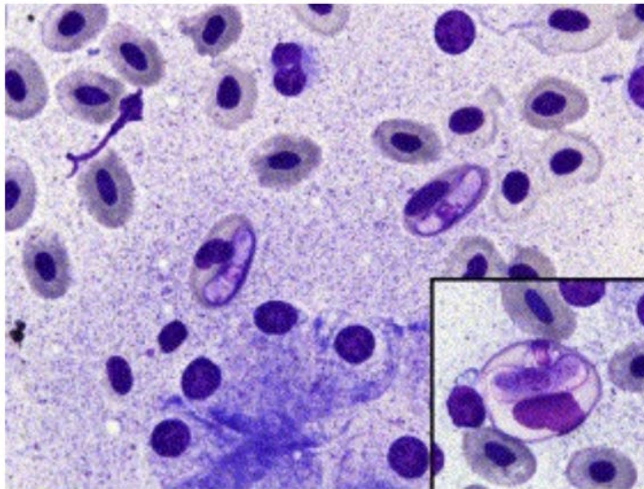
Example: Wing mass in a cockatoo



Example: Hepatozoon sp. in a frog

- Liver impression smear

Pessier A. P. (2007). Cytologic diagnosis of disease in amphibians. *The veterinary clinics of North America. Exotic animal practice*, *10*(1), 187–vii.

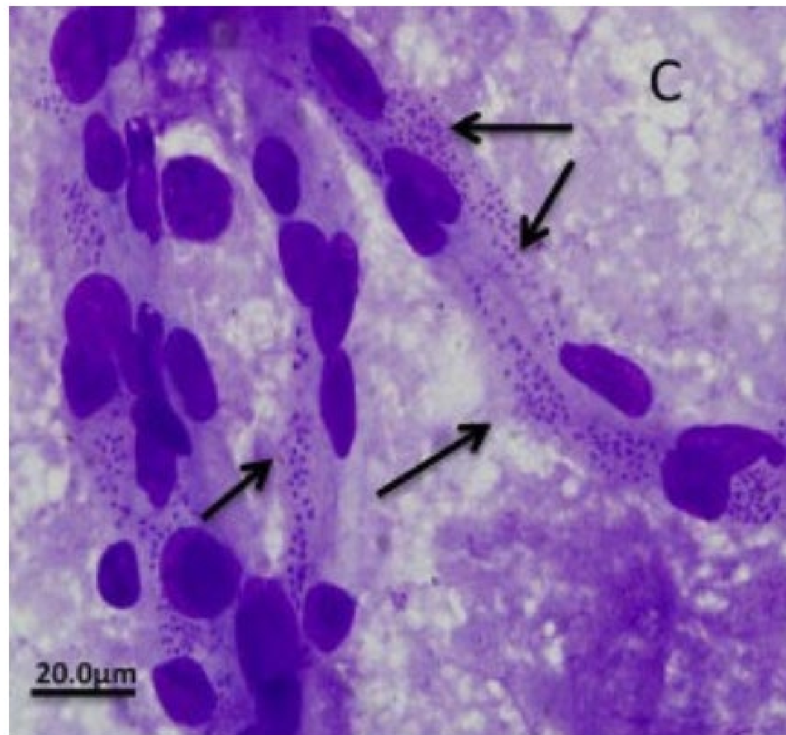


Squash Preparation

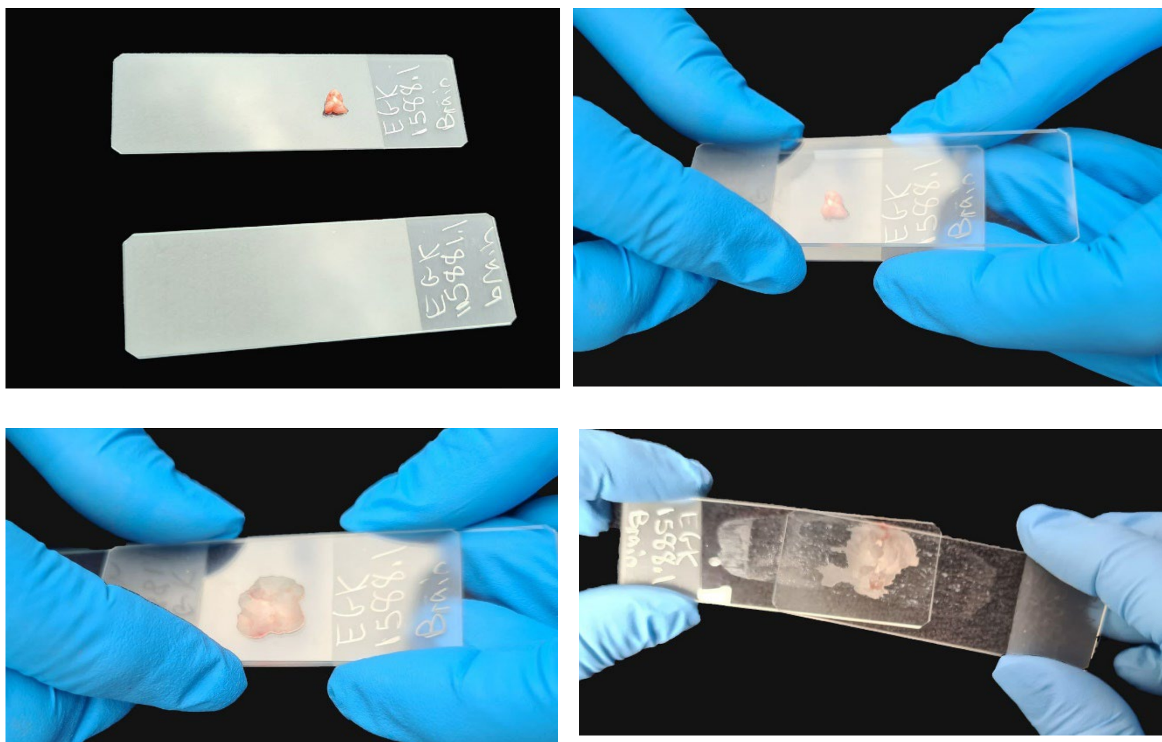
- Great for evaluating macropod brain tissue for *Babesia* sp..
- Useful for masses, lymph nodes, organs
- Place aspirated material at label end of the smear
- Place another slide on top to spread the material
- Gently draw the slides apart
- Liffman R, Courtman N. Fine needle aspiration of abdominal organs: a review of current recommendations for achieving a diagnostic sample. J Small Anim Pract. 2017 Nov;58(11):599-609.

Steps

- Cut 1mm^3 piece of tissue
- Smear using squash preparation

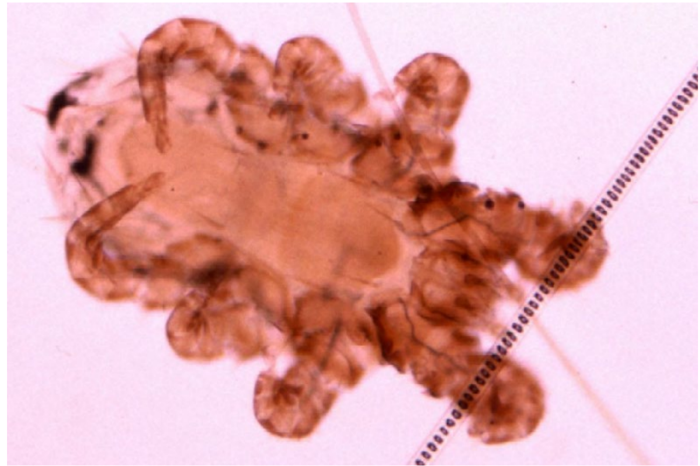


Babesia in a brain squash prep of a kangaroo (Donahoe et al 2015 A retrospective study of Babesia macropus associated with morbidity and mortality in eastern grey kangaroos (Macropus giganteus) and agile wallabies (Macropus agilis). IJP: Parasites and wildlife, 4(2), 268–276. https://doi.org/10.1016/j.ijppaw.2015.02.002)



Brain squash prep

Scrapings



Useful for sampling incisional biopsies if impression smears are poorly cellular.
Also useful for infectious agents and parasites.

Steps

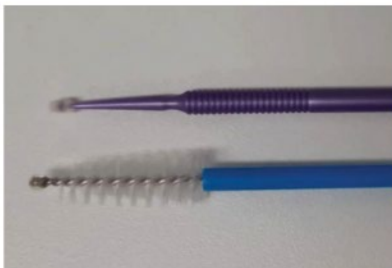
- Remove large crusts (save these in a sterile pot)
- Scrape the surface with a scalpel blade
- Transfer the material to the glass slide
- Make a squash preparation
- If looking for parasites disperse the scraped material in paraffin oil on a glass slide then coverslip

Swabs and Cyto-brush preparations

- Useful for sampling ears, conjunctiva, cornea, endometrium, vagina, fistulas, nasal cavity.
- Do not use culture gel swabs for cytology.
- Do not send dry swabs for cytology (great for PCR).

Steps

- Use local anaesthetic if required
- Roll swab over surface
- Roll over the glass slide



Kovalcuka L, Sarpio L, Nikolajenko M. **Comparison of five conjunctival cytology sampling methods in normal cat eyes**. Vet World. 2023 Apr;16(4):779-785. doi: 10.14202/vetworld.2023.779-785. Epub 2023 Apr 15. PMID: 37235165; PMCID: PMC10206965.

Ripolles-Garcia, A., Sanz, A., Pastor, J., & Abarca, E. M. (2021). **Comparison of the use of a standard cytology brush versus a mini cytology brush to obtain conjunctival samples for cytologic examination in healthy dogs.** *Journal of the American Veterinary Medical Association*, *259*(3), 288–293. https://doi.org/10.2460/javma.259.3.288

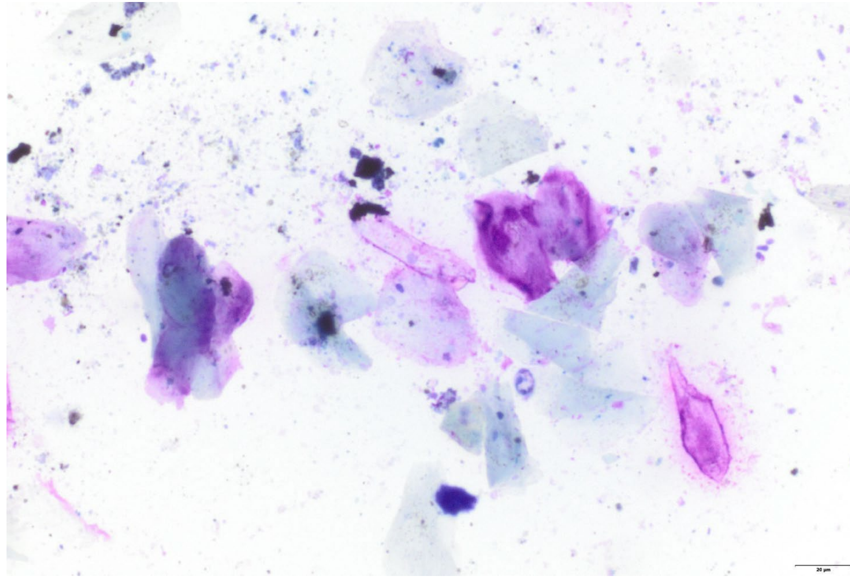
Sticky-tape preparations

Useful to screen for ectoparasites, fungi.

Steps

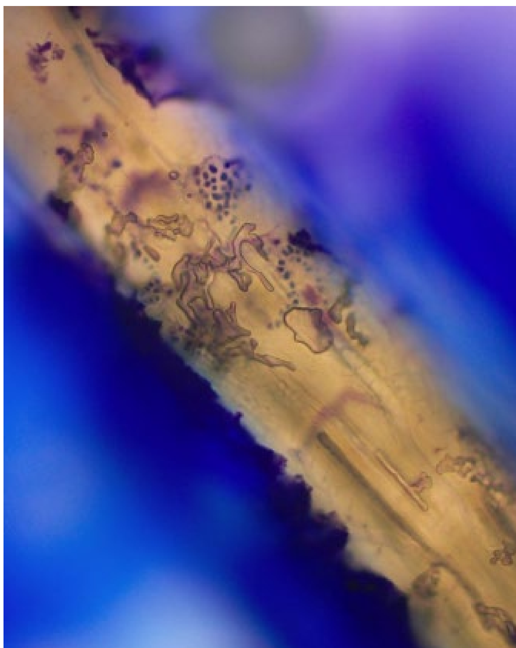
- Separate the hair

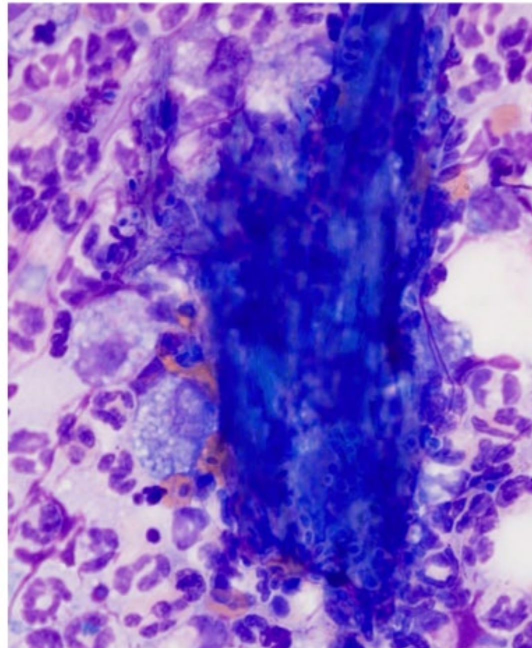
- Press the tape to the skin surface multiple times
- Stain
- https://www.bristol.ac.uk/medialibrary/sites/vetscience/documents/clinicalskills/Tape%20Strip.pdf



Hair/Quill Pluck

Useful to evaluate for Dermatophytes and for fungal culture

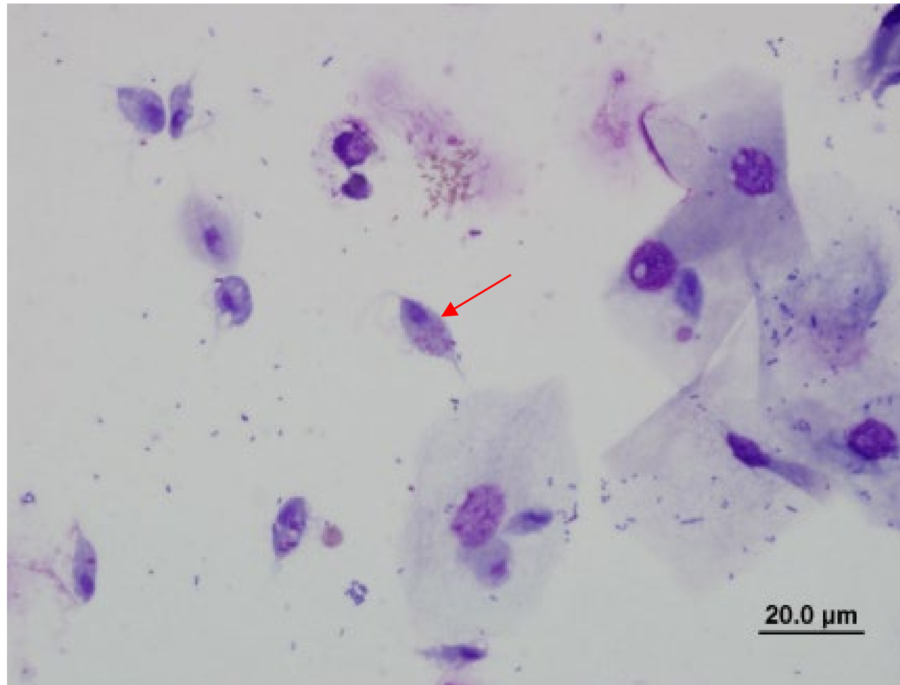




Washes

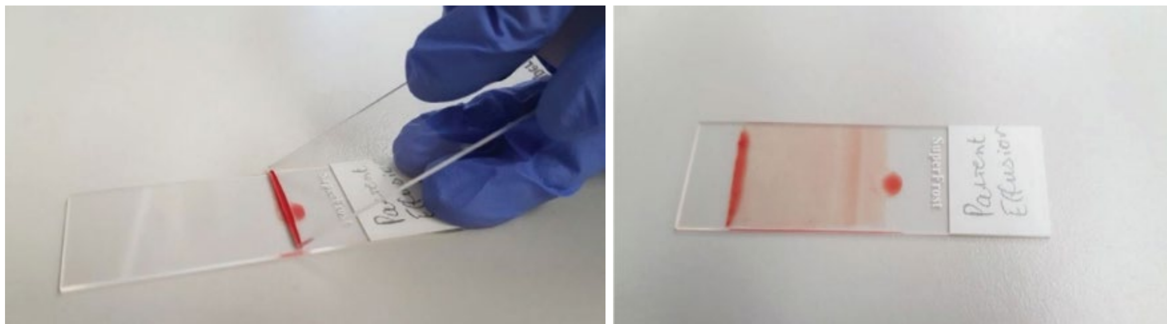
- Use normal saline at body temperature.
- Make fresh smears.

Example: Crop wash, trichomoniasis



Line Preparation

Useful for fluid, bloody aspirates.



Submitting fluid samples

Use EDTA (cytology) and plain sterile tube (culture, biochemistry)
Make fresh smears especially for BAL

- Smear techniques
- Blood smear technique
- Line preparation
- Sediment preparation

Do not use clot activator tubes for fluids.
Place fluids in EDTA or plain tubes for cytology (EDTA is better for cell preservation especially if there is blood contamination)
DON'T use SST or gel tubes or serum Z tubes.

Optimising staining

Use a high-quality rapid stain.

University of Sydney VPDS prefer Rapid Diff™ from Australian Biostain, available at:
[Australian Biostain - a CSA Pathology brand (chemsupply.com.au)]

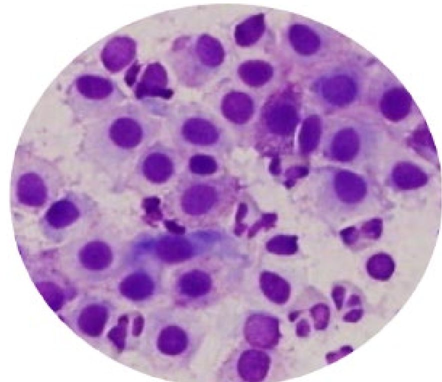
The following procedure can be used for most commercially available rapid stains:

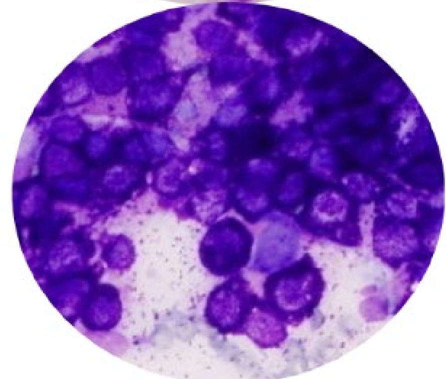
- Place the slide in fixative (methanol) for 1-2 minutes
- Stain I and II: 6-8 one second dips (adjust for smear thickness)
- Gentle water rinse both sides of smear for 20sec
- Dry vertical or use a fan

Optimising outcomes

- Ensure smears are not exposed to formalin or freezing
- Don't heat fix smears - low heat hair dryer is OK
- Thoroughly air dry before staining.

Diff Quick is fine for most preparations:
  - Ensure fresh stain with no precipitate (change every 1-2 weeks)
  - Potential of poor staining of mast cell and lymphocyte granules
  - Adjust timing to thickness of smear
  - Avoid overstaining





Excellent staining (top), excessive staining (bottom)

SLIDE PREPARATION FOR EXTERNAL LABORATORIES

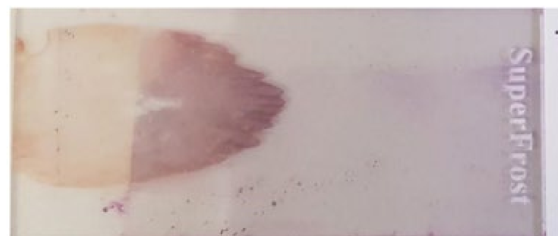
Label the slide first, so that the smear is made on the frosted side of the slide

Smear material at label end or in centre of the slide.

Use PENCIL to label the smear with 2 patient identifiers, site and lesion e.g. liver nodule, liver parenchyma.

Avoid using sticker labels – if using don't wrap the sticker around the slide

Avoid condensation



Faded smear at wrong end of slide (top), condensation damage (bottom)

Submitting samples to external laboratory

- Check slide is of diagnostic quality BEFORE submitting
- Leave some smears unstained

- Provide a comprehensive history.
- Lesion location, growth rate, size, shape, colour texture etc.
- Imaging findings for organ aspirates.
- Relevant clinical history (drug Rx, other lesions, concurrent diseases).
- If bloody aspirate provide CBC details if possible.
- Provide your main differential diagnoses.
- Talk to your pathologist.
- Pay attention to modifiers (levels of probability/certainty).

Communicate any concerns regarding potential zoonotic diseases among the differential diagnoses.

Smear Evaluation

- Check staining with 10x and adjust.
- Use a coverslip (use oil as "temporary glue").
- Always check a smear for cell yield before submitting to a laboratory.
- Label with 2 patient identifiers and sample site in pencil.
- Keep your microscope clean!

There are published guidelines to help use and interpret modifiers to express certainty.

See: Rishniw, M., & Freeman, K. P. (2023). Clinical pathologists should limit modifier terms used to denote probability of a diagnosis: a survey-based study. Journal of the American Veterinary Medical Association, 261(4), 1–8. https://doi.org/10.2460/javma.22.11.0488

Trouble Shooting

Reasons for poor diagnostic cell yield:

- Poorly exfoliative mass e.g. mesenchymal masses
- Geographical miss
- Highly vascular lesion
- Poor cell preservation
    - Too thick
    - Cell damage during smearing
    - Poor staining
    - Ultrasound gel
    - Formalin fume exposure

Excess blood:

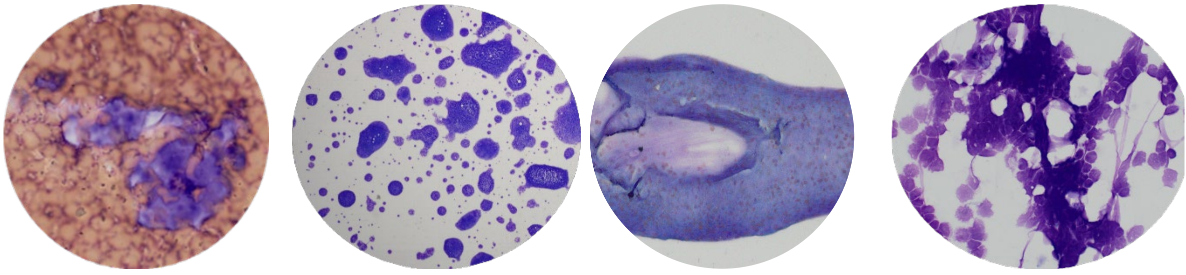
- Try non-aspiration technique if your aspirate is bloody and ensure you smear the material (use blood smear technique).

Smear is too thick:

- Avoid splattergrams
- Don't delay smearing
- Ensure you smear the cell material to create a monolayer of cells

Ruptured cells:

- Be gentle when aspirating, spraying and smearing cells to avoid rupturing cells



Excess blood (far left). Splatergram (left). Delayed smear – thick and stringy (right), cell rupture (far right).

Stain precipitate:

- Rinse slide well
- Change stain weekly

Weird staining:

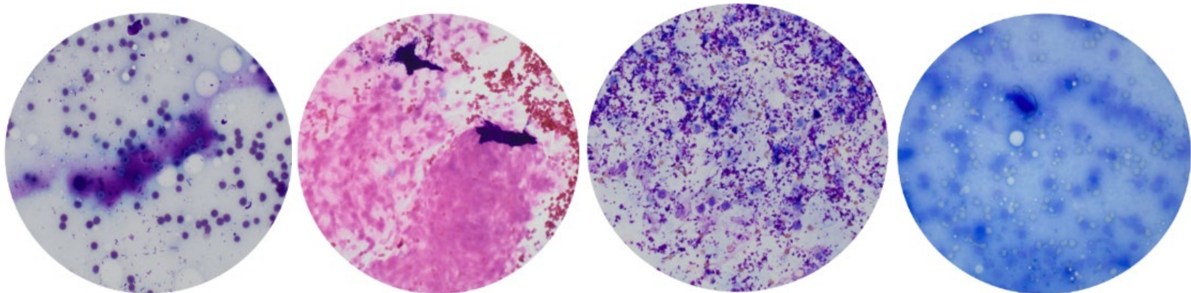
- Time slide in each stain jar

Purple debris:

- Minimise lubricant/ultrasound gel

Washed out cells:

- Formalin artefact
- keep cytology samples away from formalin
- **NEVER package cytology samples and formalin together**



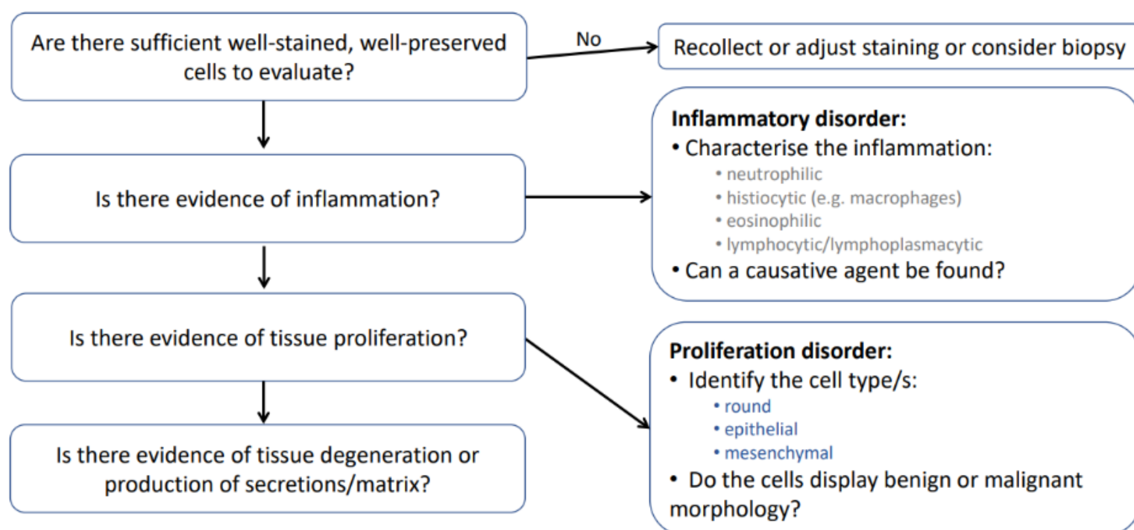
Stain precipitate (far left). Weird staining (left). Purple debris (right), cell wash out (far right).

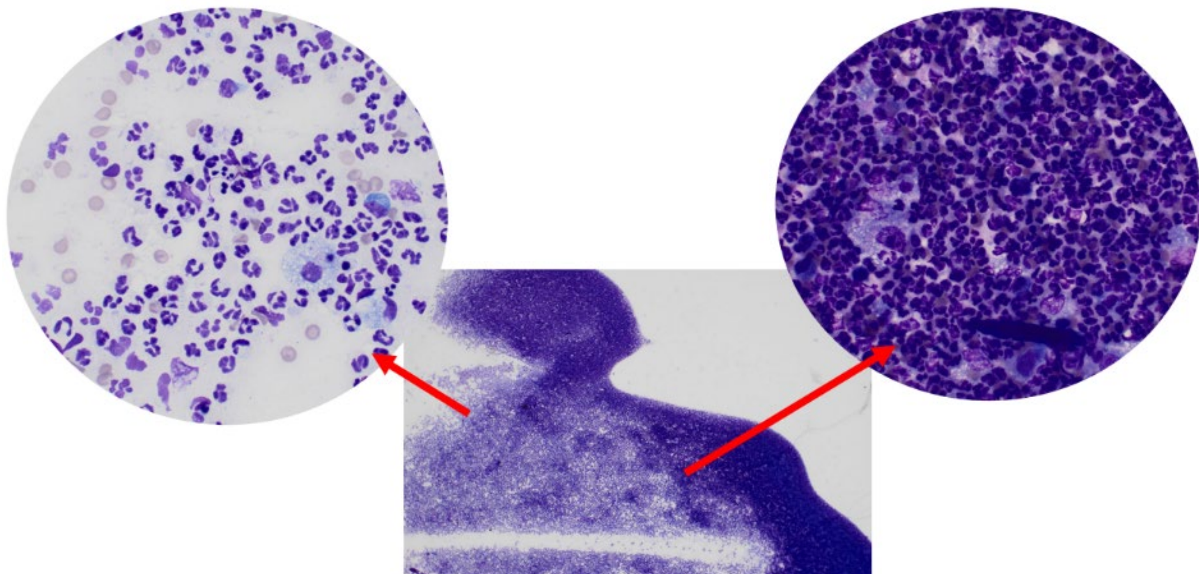
EXAMINATION OF STAINED SMEARS

- Place a coverslip on the slide (40x lens will focus better) - a drop of oil under the coverslip helps to hold it in place like a glue
- Check the condenser position is up
- Check the field diaphragm is open
- Adjust the iris diaphragm to match the objective
- Only use oil for the 100x objective
- If you get oil on the 40x immediately remove with alcohol
- Find the monolayer
- Assess cell preservation

Condenser – yellow arrow

Approach to cytology





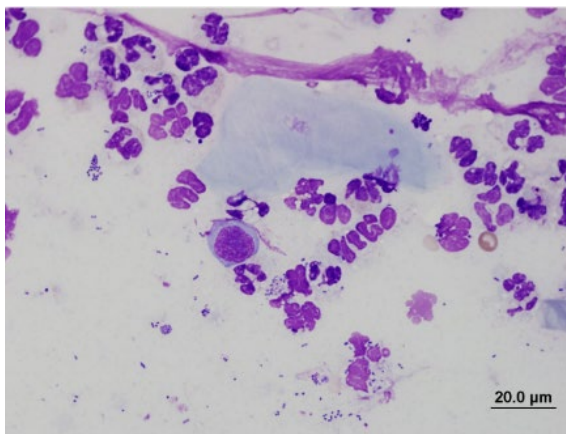
Evaluating smears - find the monolayer

Describing cytologic findings

- Cellularity
- Associations (i.e. individual or in clumps, sheets or bundles)
- Size – use RBCs (6-8um) and neutrophils (12-14um) as your micrometer
- Shape e.g. round, polygonal, spindle
- Cytoplasm quantity, colour and texture – granules, inclusions, vacuoles
- Nuclear size and shape, chromatin pattern and nucleoli
- Criteria of malignancy
- Background matrix/stroma

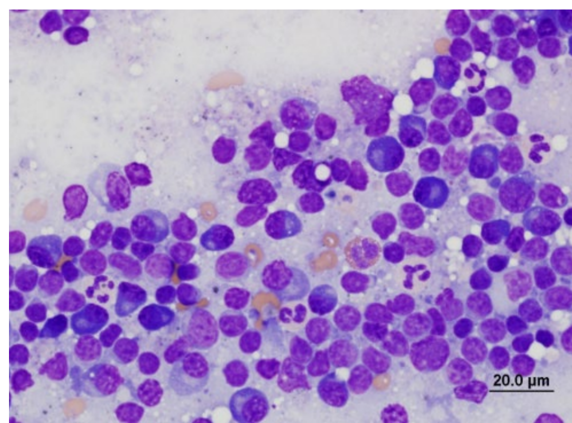
Inflammatory Lesions

Neutrophilic

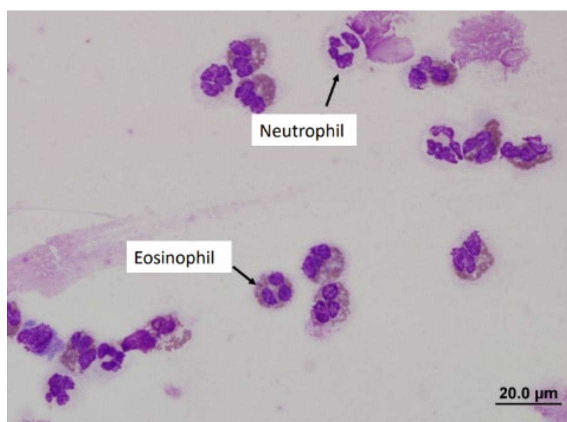


Assess if neutrophils are degenerate = swollen nuclei

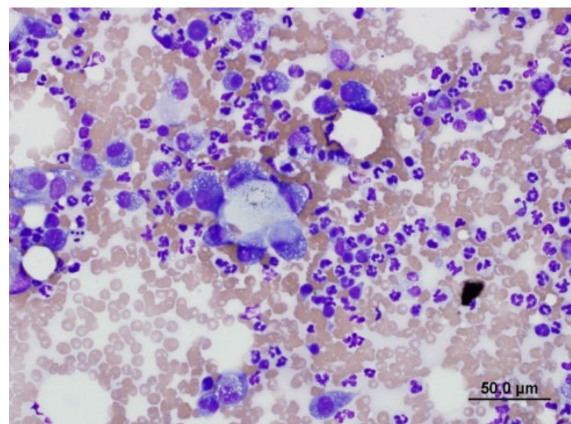
Lymphoplasmacytic



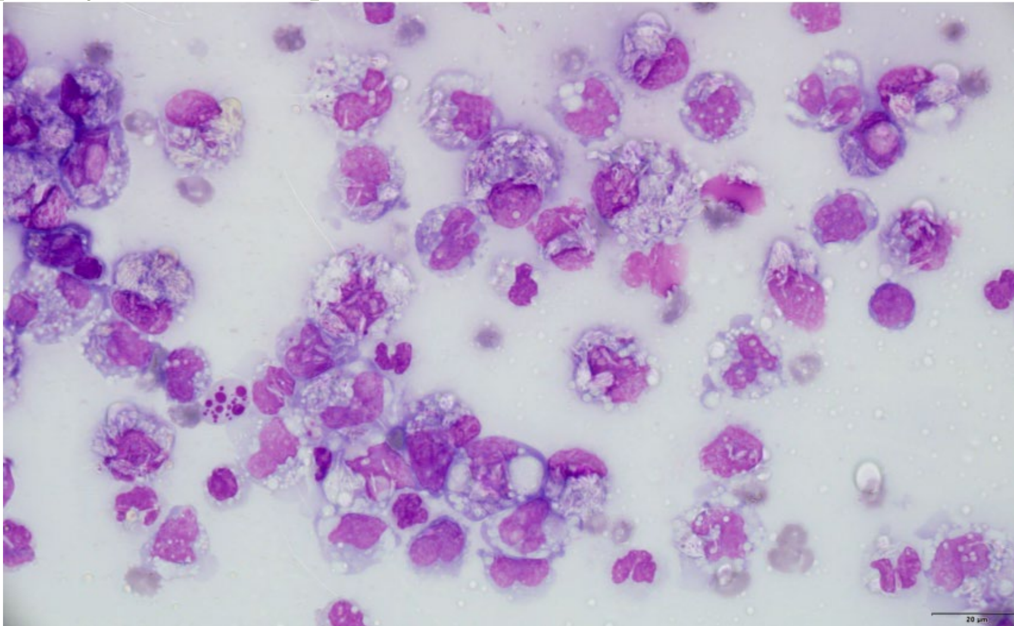
Eosinophilic



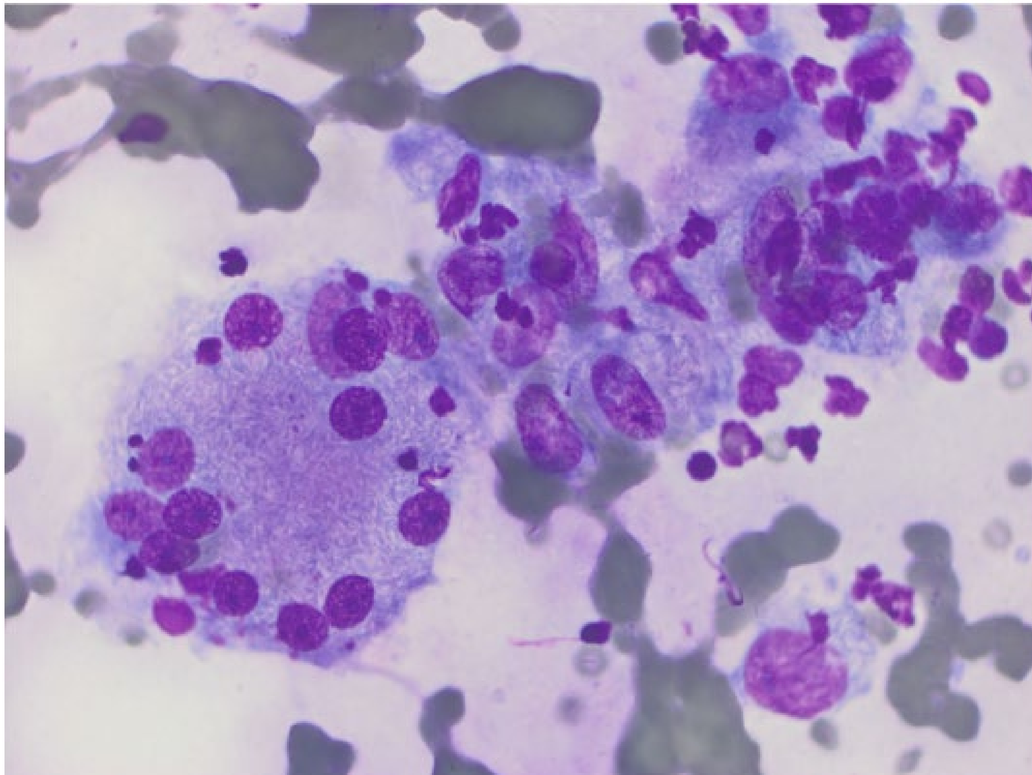
Pyogranulomatous



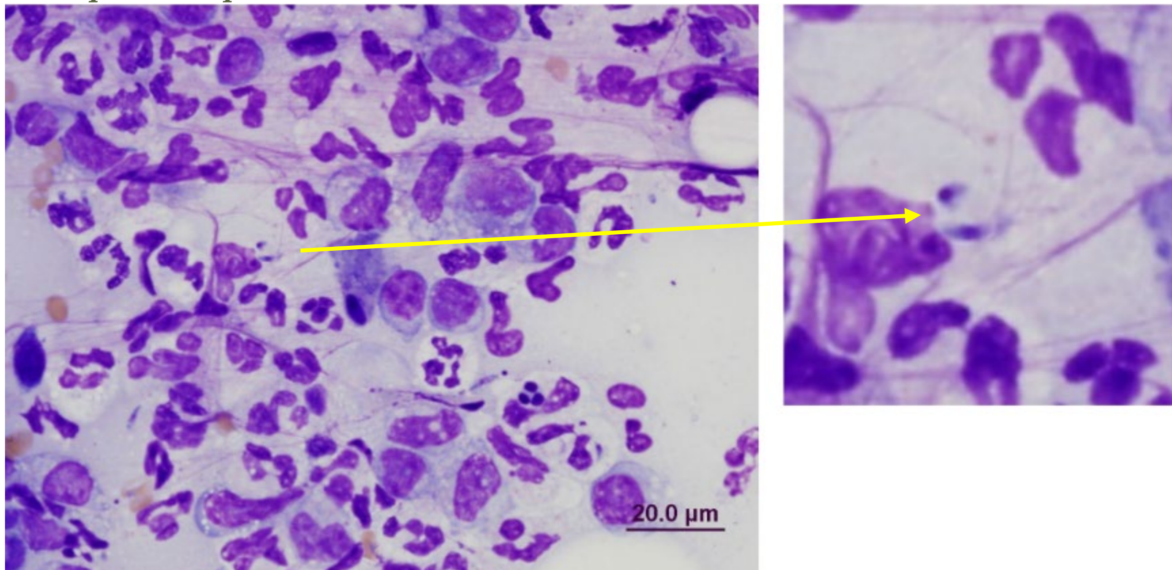
Example: Mycobacterium sp.



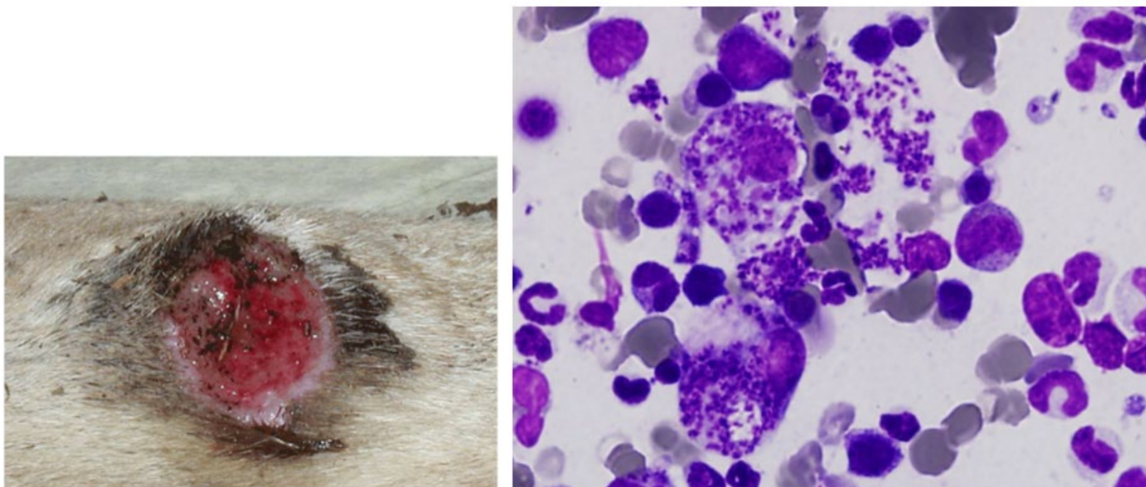
Example: Inflammatory lesions – histiocytic/granulomatous



Example: Toxoplasmosis



Example: Leishmania sp.



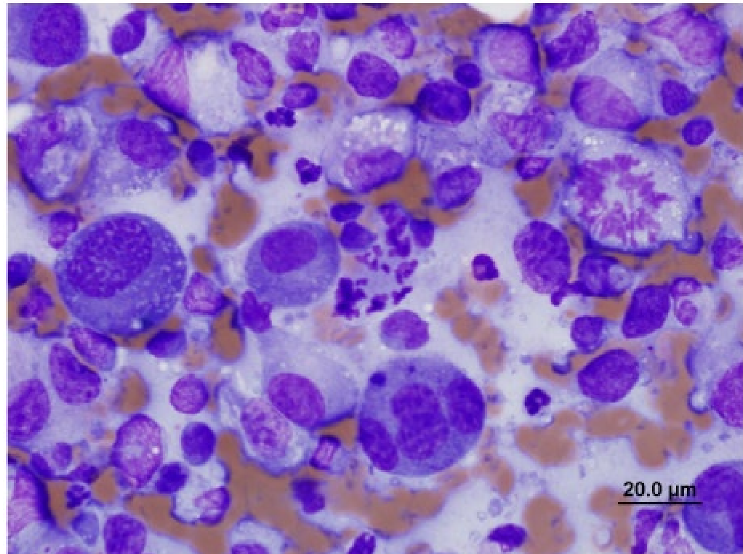
Rose, K., Curtis, J., Baldwin, T., Mathis, A., Kumar, B., Sakthianandeswaren, A., Spurck, T., Low Choy, J., & Handman, E. (2004). Cutaneous leishmaniasis in red kangaroos: isolation and characterisation of the causative organisms. *International journal for parasitology*, *34*(6), 655–664. https://doi.org/10.1016/j.ijpara.2004.03.001

DISORDERS OF GROWTH

- Round Cell
- Epithelial
- Mesenchymal
- Neuroendocrine

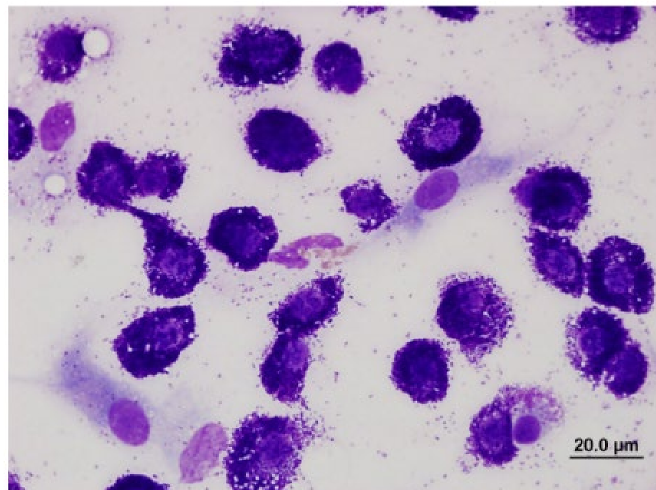
Criteria of Malignancy

- Anisokaryosis
- Karyomegaly
- High N:C ratio
- Increased mitoses (atypical)
- Coarse chromatin
- Macronucleoli
- Anisonucleolosis
- Angular nucleoli
- Multinucleation
- Nuclear moulding
- Basophilic cytoplasm
- Anisocytosis



Round Cell Neoplasia

- Small, discrete round cells, round to oval nuclei with distinct cell margins
- Exfoliate well on FNA
- Highly cellular samples can mimic cohesion - look for gaps between the cells
- Cytoplasmic characteristics used to differentiate type of round cell

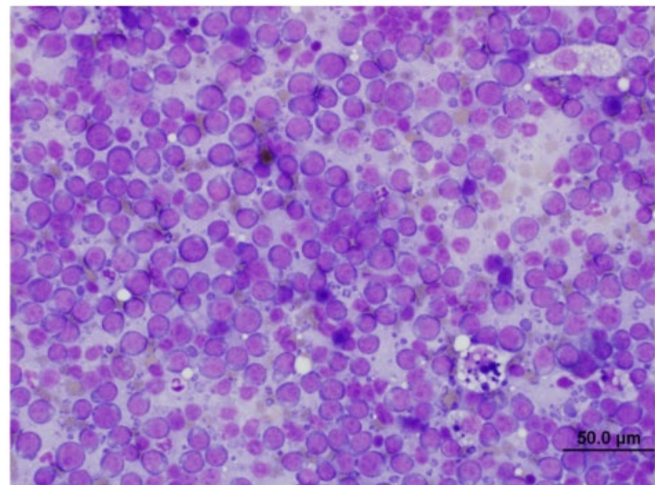


5 main differentials:

- Plasma cell tumour
- Mast cell tumour
- Histiocytic tumour – histiocytoma, histiocytic sarcoma
- Lymphoma
- Transmissible venereal tumour
- (Melanoma, anaplastic tumours)

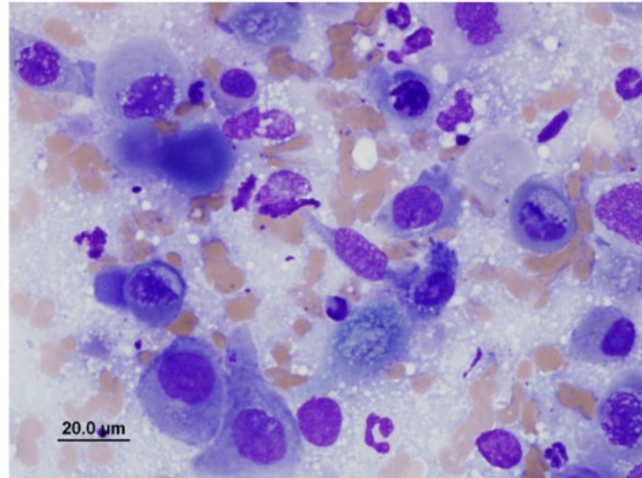
Lymphoma (image):

- Round cells with round nuclei and high N:C ratio
- Monomorphic lymphocytes - look clonal

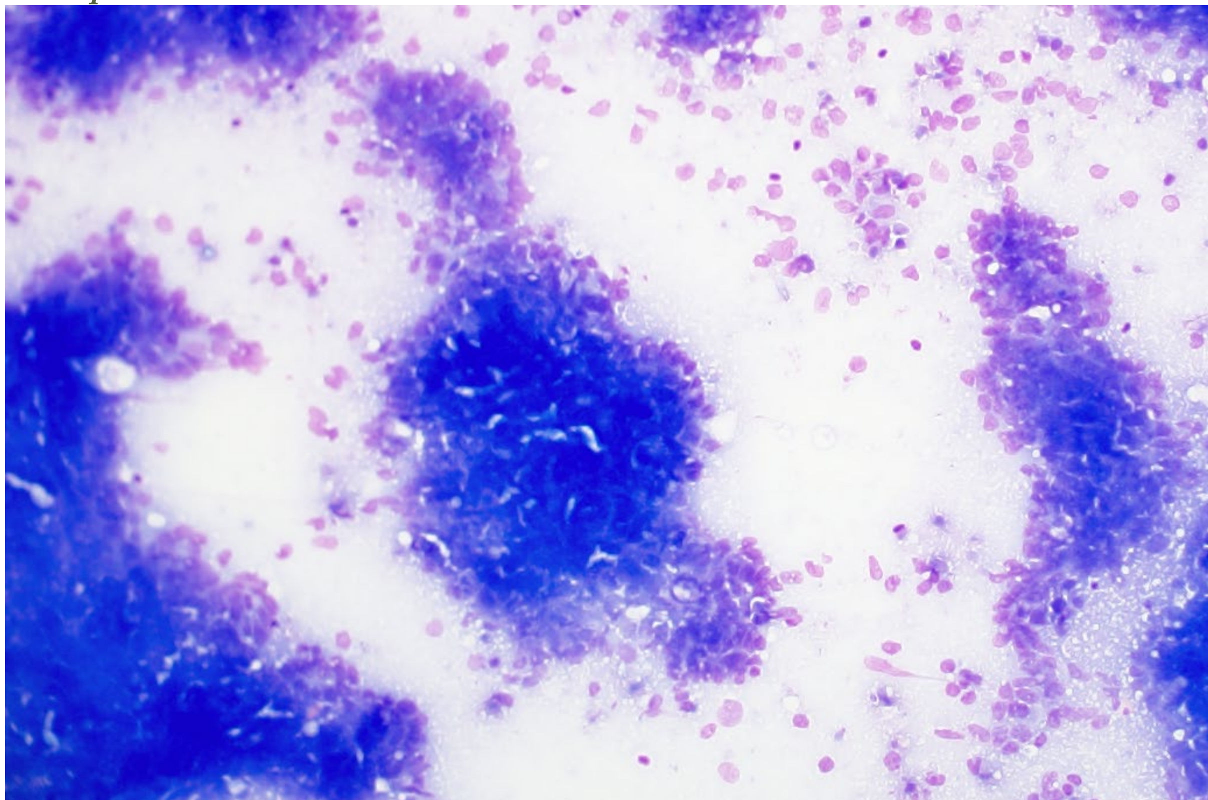


Epithelial Tumours

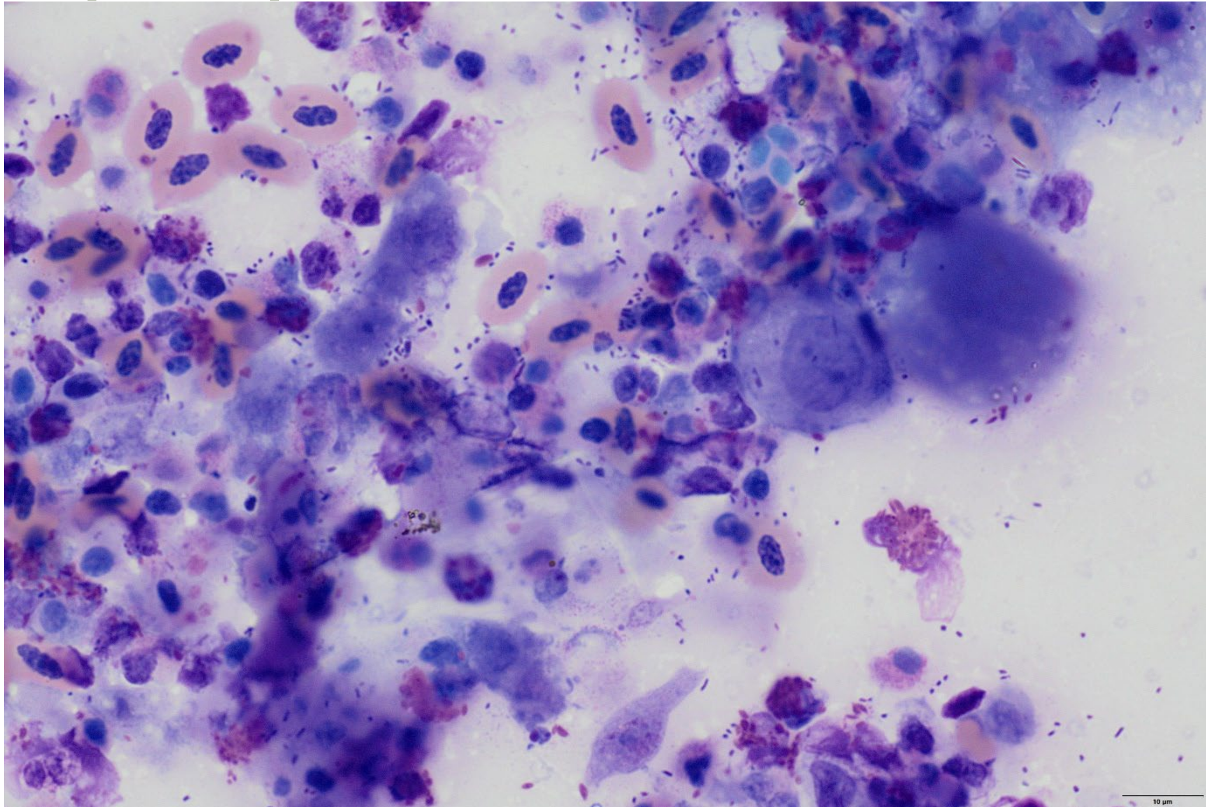
- Large cells – usually abundant cytoplasm, round to ovoid nuclei
- Cohesive sheets or clusters often with orderly
- arrangement e.g. rows, even cell spacing
- May see acinar like structures if glandular origin
- Exfoliate well on FNA
- May show differentiation into cells resembling normal tissue
- Can lose cohesion and become more spindyloid with malignant transformation "epithelial-mesenchymal transition"



Example: Avian oviductal adenocarcinoma

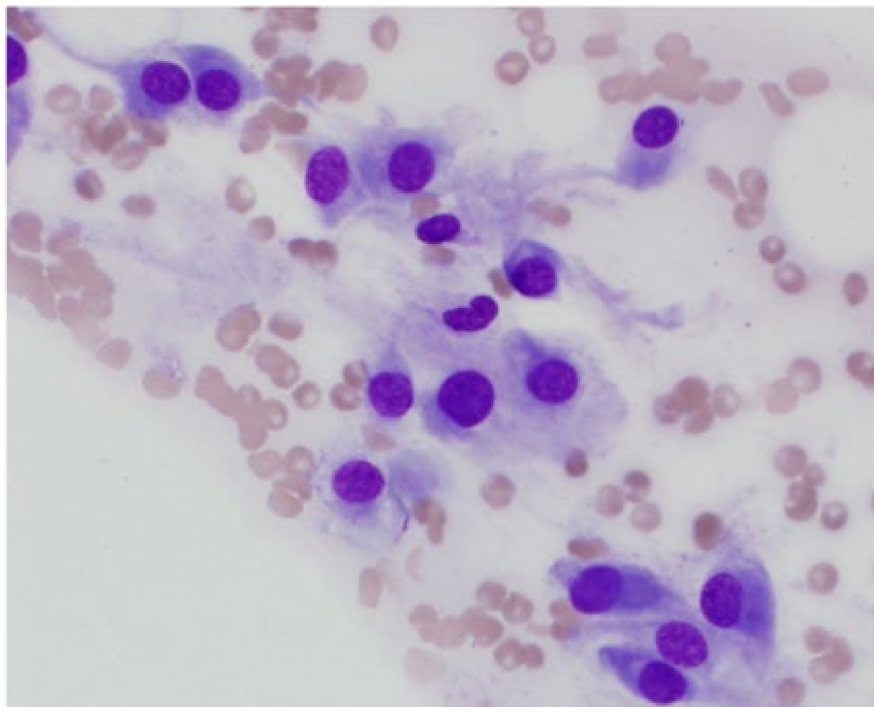


Example: Avian squamous cell carcinoma



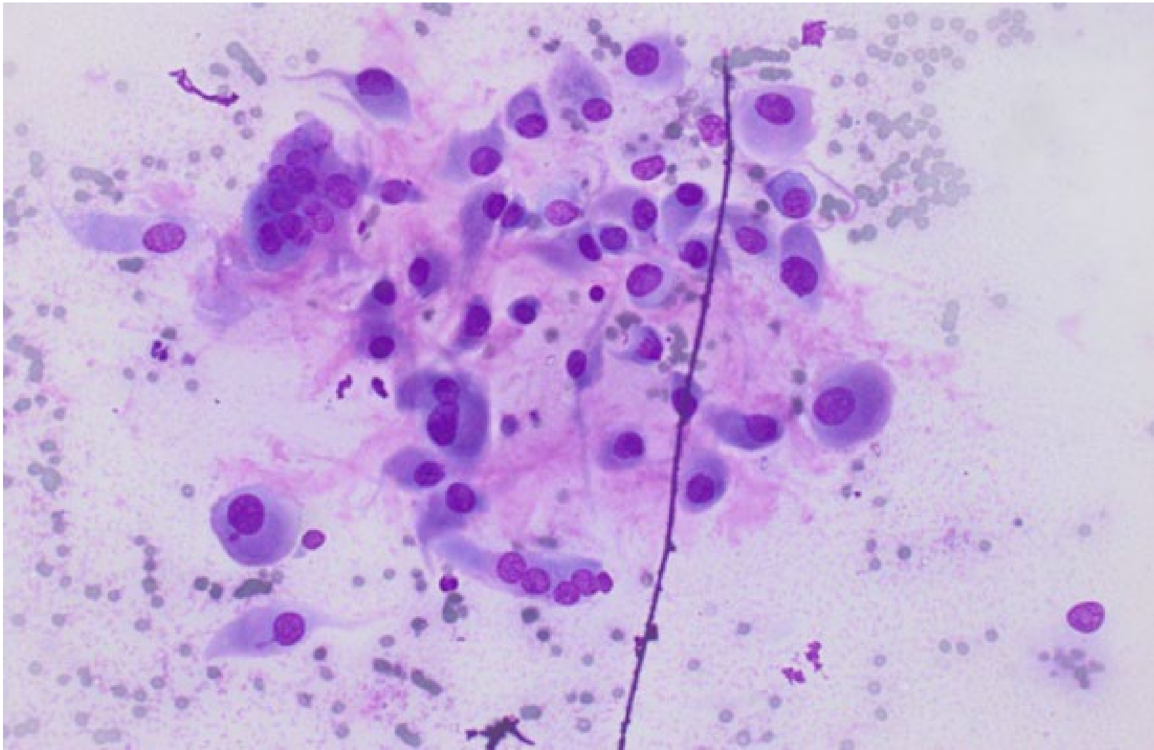
Mesenchymal tumours

- “spindle” shaped cells, indistinct wispy cell borders
- Often poorly exfoliative
- Loose disorderly cohesion in bundles
- Background matrix or arrangements can suggest tumour type
- Difficult to define tissue origin as mesenchymal cells appear similar cytologically
- **Beware: Mesenchymal cell atypia can occur with both neoplasia and inflammatory dysplasia**

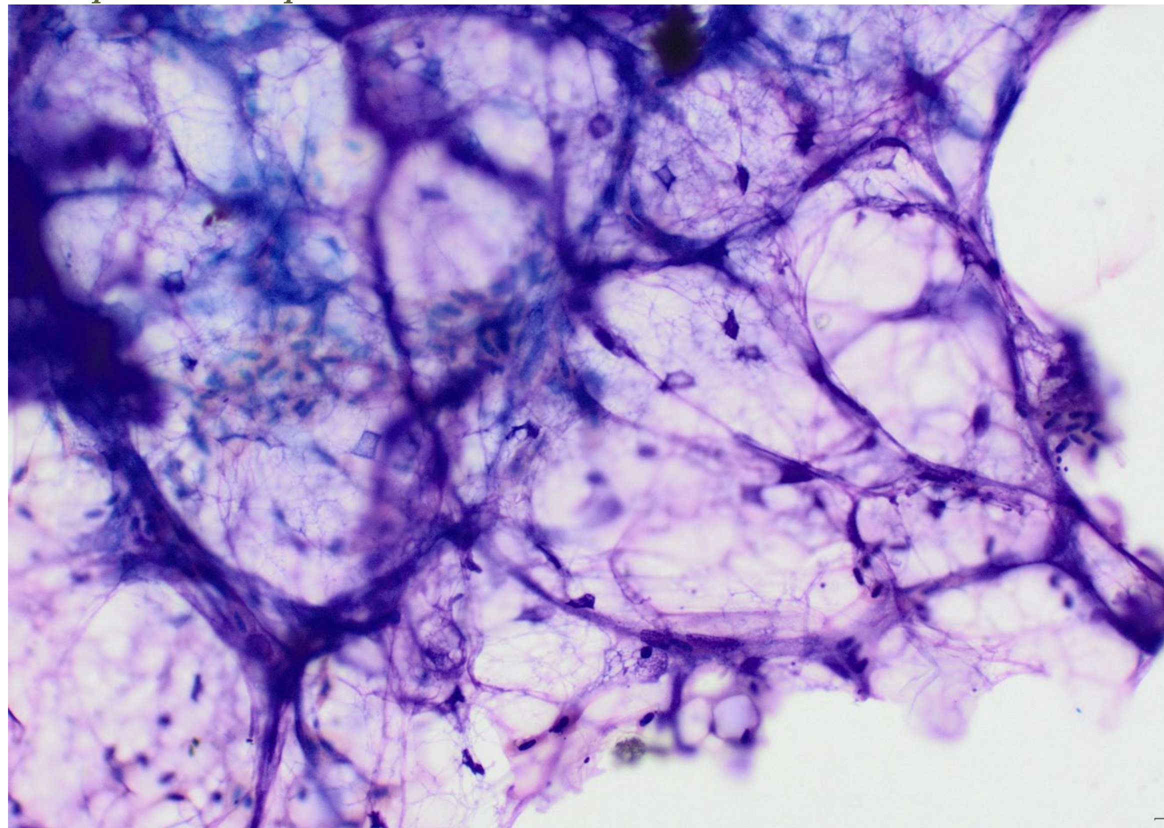


Example: Soft tissue sarcoma

Spindle cells with pink stroma



Example: Avian Lipoma

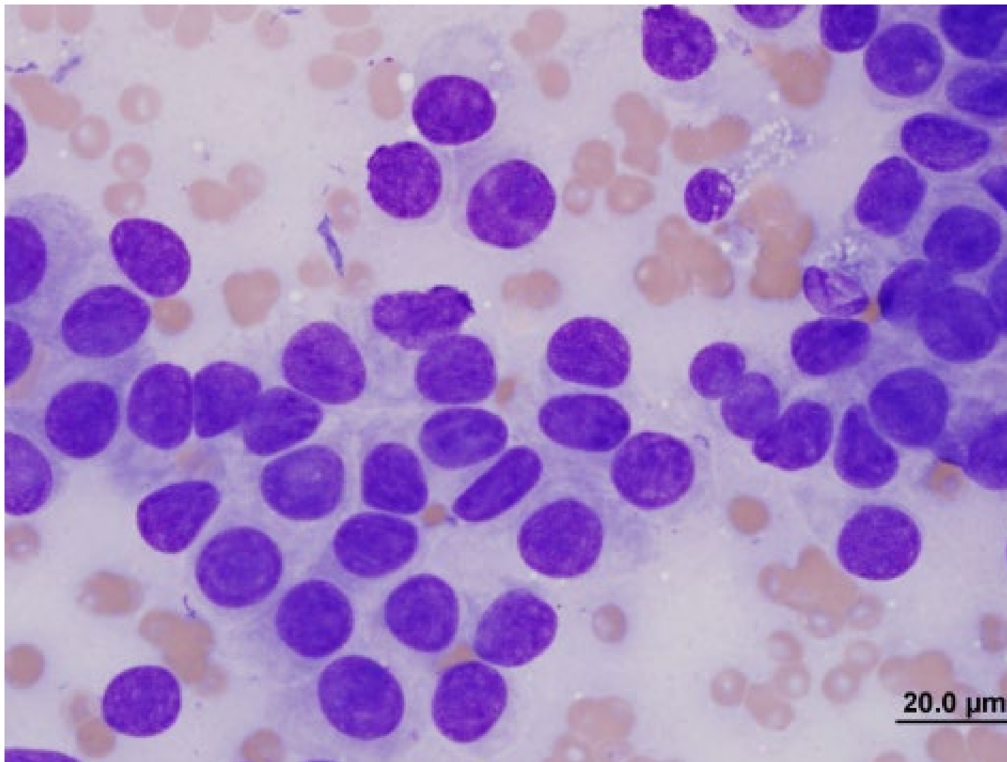


Neuroendocrine tumours

- Cohesive cells in acinar arrangements
- Fragile – naked nuclei in a sea of cytoplasm

- Bland round nuclei, fine chromatin, indistinct nucleoli

Example: Neuroendocrine tumour

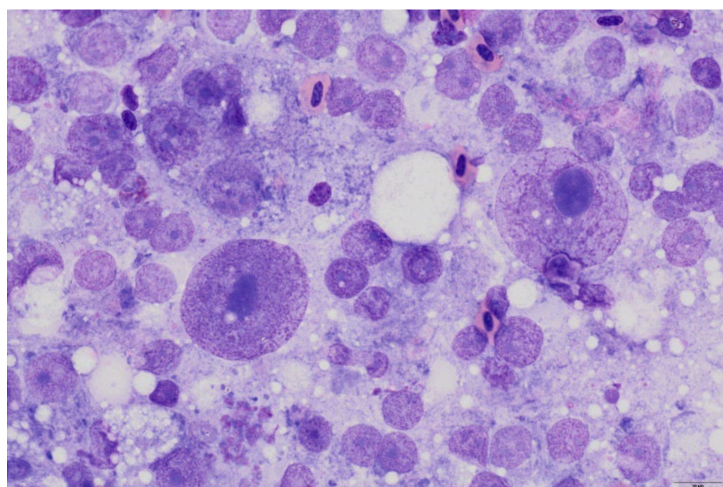


Anaplastic neoplasia

- Pleomorphic cells with strong criteria of malignancy
- Do not resemble any normal tissue so origin is unclear

Criteria of malignancy include:

- Anisokaryosis
- Karyomegaly
- High N:C ratio
- Increased mitoses (atypical)
- Coarse chromatin
- Macronucleoli
- Anisonucleolosis
- Angular nucleoli
- Multinucleation
- Nuclear moulding
- Basophilic cytoplasm
- Anisocytosis

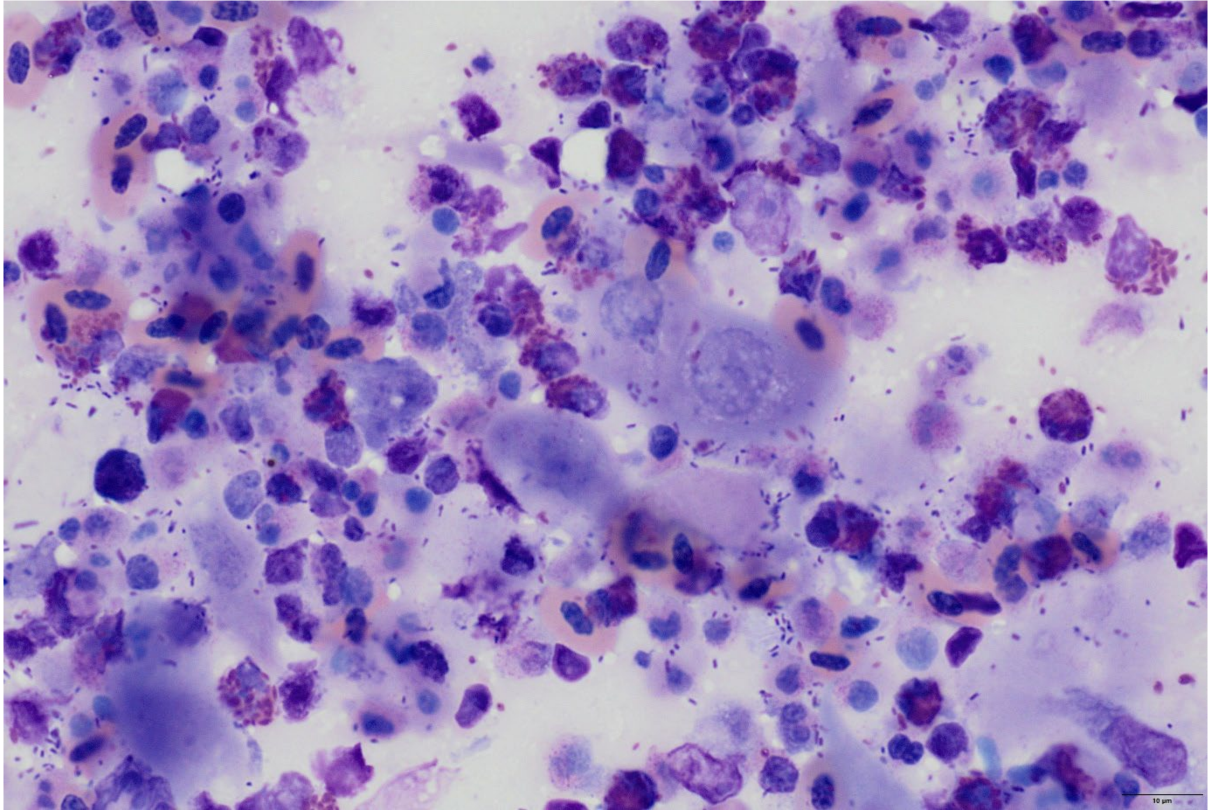


Example: Budgerigar renal carcinoma

Inflammation + Dysplasia/Atypia

Dysplasia in the presence of inflammation can mimic neoplasia

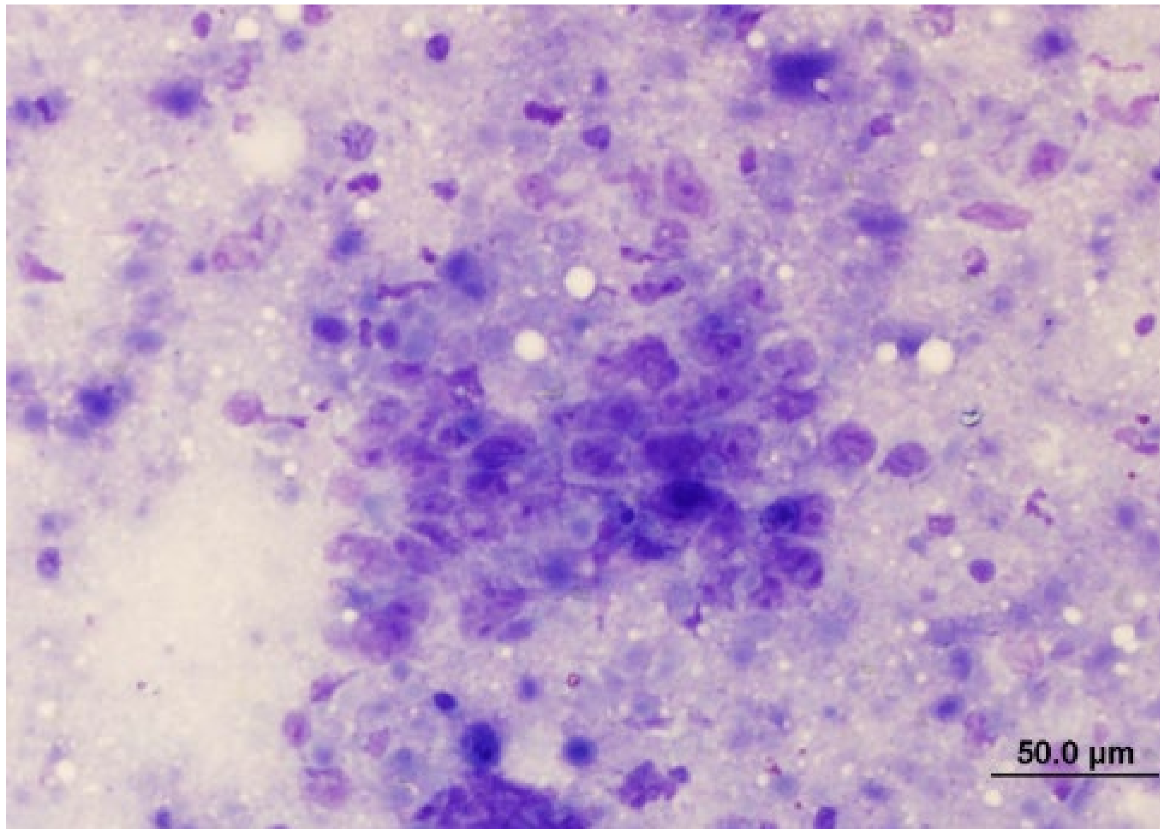
Example: Cockatoo with inflamed squamous cell carcinoma



Necrosis

Smudged blue-grey material

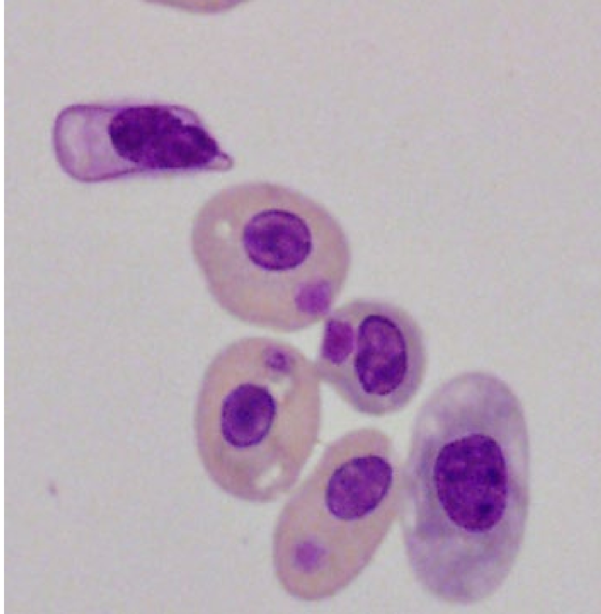
Nuclear ghosts



Check the surrounding blood too!

Look for parasites and inclusions

Example: Python with Iridovirus inclusions



Example: Microfilaria in ringtail possum