Chapter 1

Aspiration techniques and stains

Fine-needle aspiration (FNA) is an important investigation in the management of head and neck masses that requires no specialist equipment or prior patient preparation. It has two important components: sample collection and its cytological assessment. The aspiration technique is critically important for obtaining a suitable sample, on which the reliability of cytological assessment depends heavily [1–3].

Principle of FNA

- Cells dislodged by insertion of a needle and its movement in the mass are collected in the needle shaft/hub by capillary action. This is achieved with or without suction applied by a syringe.

Who can perform the test?

- Any health professional with a clear understanding of the correct technique, and preferably, prior experience can perform the test. Individuals with no knowledge of the principle or technique of FNA should not carry out the procedure without learning it under supervision.
- Proficiency is acquired by regular performance of the test.
- Ultrasound examination helps characterize the location and nature of the mass and when combined with FNA, allows targeting of appropriate areas for aspiration; for example by avoiding cystic areas. This combined assessment is recommended for evaluating thyroid nodules [4].
- Sample collection can be combined with on-site cytological evaluation in rapid-assessment or one-stop clinics.
- There are certain advantages in a pathologist/cytologist performing fine-needle aspirates:
  - Accuracy of FNA diagnosis is higher when the person interpreting the slide is the same as the person performing the procedure [5].
  - It allows appreciation of the clinical context while reporting, which is essential in avoiding diagnostic errors [6].

What lumps to aspirate?

- Any palpable mass in the head and neck region is amenable to FNA.
- FNA plays a limited role in the diagnosis of diffuse swellings of salivary glands and thyroid, where radiology and serology are more helpful.
- Intraoral masses are rarely aspirated; if required, FNA can be performed a few minutes after spraying a local anesthetic (0.5% lignocaine), having excluded allergic sensitization. This is performed ideally in the maxillofacial/dental outpatient clinics, where equipment for oral cavity visualization is available.
- Small (<10 mm) lesions, deep-seated masses, and those in close proximity to the carotid artery are best aspirated under ultrasound guidance.

Contraindications

- There are no absolute contraindications to the procedure.
- Due care must be taken in children and anxious patients in order to avoid accidental injury.
- Anticoagulation medication is not a contraindication as long as due attention is given to achieving hemostasis post-procedure for preventing bruising and hematoma formation.

Where to aspirate

FNA can be carried out in most consulting rooms; some centers have dedicated clinic rooms for FNA. The basic requirements are:

- Adequate ventilation and lighting.
- A couch with an adjustable backrest and pillow.
- A chair with armrests.
- A bench for slide preparation.

Equipment

Essential items

- Frosted glass slides. It is important that slides are dust free, otherwise smear preparation will be compromised.
- Soft lead pencils for marking slides with patient identifiers.
- Disposable syringes: 10 mL or 20 mL.
- Disposable needles: 23G (blue hub) and 25G (orange hub). Finer needles (27G, gray hub) can be used for thyroid nodules. The preferred needle length is 30 mm, rather than the usual 25 mm. For neck lumps, 21G (green hub) needles are rarely required but may be useful in aspirating thick material from cysts/abscesses or when a significant amount of material is required as for cell-block preparation.
- Alcohol swabs for disinfection of aspiration site.
- Cotton wool.
- Small plasters.
- Absolute alcohol, methylated spirit, or spray fixative for fixing smears. Commercially available hair sprays have been used with acceptable results [7].
- Disposable gloves.
- Containers for collecting aspirated fluid.
- Sharps' container.

Desirable items

- Syringe holder (Cameco Syringe Pistol, Cameco AB, Taby, Sweden, or similar device): This is handy when aspirating with suction and for expelling material during slide preparation.
- Wooden applicator sticks (VWR International Ltd., Lutterworth, UK), one end of which has been beveled with a scalpel. These are useful for scraping material stuck in the hub of the needle after expulsion, which then can be smeared on a slide. Alternatively, material can be flushed out in saline or fixative.

Aspiration procedure

Pretest preparation

- No special patient preparation is required for the test.
- Most patients are primed to fast for blood tests and may do so for FNA appointments. In anxious patients, this predisposes to dizziness or feeling faint during or post-procedure. It is useful to notify patients beforehand that fasting is not required.
- Fear of a needle being inserted in the neck and pain are common patient concerns. Giving some measure of discomfort to expect (sharp scratch/needle is finer than that used for blood tests) and duration (test only takes a few seconds) helps allay anxiety.
- Check if the patient is on anticoagulation medication; careful hemostasis is mandated to prevent bruising/hematoma formation.
- Reassure the patient that a properly performed test is virtually complication free. It is advisable to warn patients on anticoagulation medication about the possibility of bruising.
- Give an indication of when the report will be ready.
- Give patients the opportunity to ask any questions they may have.

Positioning of the patient

It is important to ensure patient comfort and unhampered access for sampling. Four possible positions are:

a. *Patient lying supine with a pillow behind the head:* this position is ideal for apprehensive patients and can be used for aspirating most anterior or lateral neck lumps. Lymph nodes in level 2 of the neck, especially those in close relation to the carotid vessels, are most easily aspirated in this position. Ensure that the patient rises up slowly after the test to prevent postural hypotension.

b. *Patient lying supine with a pillow behind the shoulders:* this causes neck extension and is the recommended position for thyroid aspiration. It is also useful for submental masses (lymph nodes, thyroglossal cyst). In elderly patients or in those with cervical spine disorders, this position can be uncomfortable or even impossible to attain, in

which case position (c) is recommended. Ensure that the patient rises up slowly after the test to prevent postural hypotension.

c. *Patient on the couch with the backrest raised (semi-Fowler's position):* this position is useful for aspirating anterior, lateral, and large neck lumps. Ensure that the pillow supports both the upper back and back of the head.
d. *Patient sitting on a chair:* this is particularly useful for posterior triangle or back of neck lumps and in patients with restricted mobility (in a wheelchair). Choose a chair with armrests.

Local anesthesia

This is not required for adults but can be applied in apprehensive patients and children. Injectable anesthesia is not ideal and one of the following two techniques can be used:

1. *Local anesthetic cream:* Ametop gel (active ingredient tetracaine, Smith and Nephew Healthcare Ltd., UK), EMLA cream (active ingredient lidocaine and prilocaine, AstraZeneca UK Ltd.), or similar preparations when applied locally cause numbness. They are effective 30–60 min after application and may be prescribed to the patient before s/he attends for the test.
2. *Ethyl chloride spray* (Cryogesic, Acorous Therapeutics Ltd., UK): this causes numbness of the skin by lowering the surface temperature, which lasts for about 30 s. Caution is required for use in the neck for if it enters the eyes, nose, or mouth, it can cause frostbite-like injury.

Either method carries a potential risk of allergic reaction.

Aspiration techniques

Wearing of gloves on both hands during the procedure is recommended. Care must be taken during aspiration and smear preparation to avoid needle-stick injury and aerosol production, respectively. The test can be performed with or without suction and both techniques are discussed as follows. The term dominant hand is used for the hand holding the needle/syringe and nondominant hand for that stabilizing the lump.

Aspiration without suction

This is carried out using a needle alone and is suitable for most neck masses. Instituted by Zajdela [8], this technique allows better control of the aspiration process and provides an additional dimension of "texture" when the needle moves through the lump.

- Disinfect the skin.
- Stabilize the lump with the index and middle fingers of the nondominant hand. Clasp a cottonwool ball between the ring/little finger and the palm of that hand.
- Holding the needle by its hub between the thumb, and index and middle fingers of the dominant hand, insert it into the lump in an axis perpendicular to the skin.
- Once in the lump, move the needle up and down in the direction of insertion but without withdrawing it completely. At the same time, twirl the hub between the thumb and fingers, creating a rotating movement. Do not move the needle in different directions within the mass, i.e., "probing" movements, as this can cause tissue shearing with pain, increased risk of post-aspiration bruising/hematoma formation, and excessive dilution of the sample by blood.
- Stop needling when material appears in the hub; if no material is seen, stop after about 15 passes (up/down movements).
- Withdraw the needle and apply pressure on the puncture site with cotton wool. Ask the patient (or an assistant) to continue pressing while smears are prepared.
- For large lumps, sampling from multiple separate sites is recommended.

Troubleshooting

- *No material is seen after ~15 passes*:
  - Material is present in the shaft but has not reached the hub: this occurs with thick material and can be seen with pleomorphic adenoma, some Warthin's tumors, colloid goiter with thick colloid, and some reactive lymph nodes.
  - No material has been aspirated: this occurs with densely fibrotic masses (including nodular sclerosis Hodgkin lymphoma) and some soft tissue lesions. Consider repeating the procedure with suction (see next section).

- *Blood wells up almost instantly*:
  - A blood vessel has been punctured; achieve hemostasis and repeat aspiration at a separate site.
  - The mass is a vascular lesion such as a soft tissue hemangioma or vascular malformation. Such lesions characteristically decrease in size on withdrawal of their contents but then refill. Repeat aspiration of these will yield blood only.
  - The mass is highly vascular: in hyperplastic thyroid nodules, thyroid follicular neoplasia, and paraganglioma, blood wells up in the hub after two to three passes. Samples are usually cellular and rapid smear preparation is recommended to avoid clotting and entrapment of cells, which makes their subsequent assessment difficult.
- *Fluid wells up in the hub*:
  - The mass is cystic in nature; withdraw the needle and repeat aspiration with suction (see next section).

Aspiration with suction

This is the preferred technique when cystic masses are present or suspected. For thyroid lesions aspirated without ultrasound guidance, use of suction is recommended as a significant proportion of these are cystic. A syringe holder is used in the following description; it helps maintain steady suction and gives better control over the procedure. If not available, a 10-mL or 20-mL syringe can be used on its own, held in the dominant hand. The piston is withdrawn to create suction when the needle is in the mass.

- Disinfect the skin.
- Stabilize the lump with the index and middle fingers of the nondominant hand. Clasp a cotton wool ball between the ring/little finger and the palm of that hand.
- Withdraw about 2 mL of air in a 10-mL syringe; this helps in subsequent expulsion of the aspirated material. Attach the needle to the syringe.
- Keeping the holder–syringe–needle assembly in the dominant hand, insert the needle in as close a perpendicular axis to the skin as possible.
- Once in the lump, pull the piston slowly to create suction. Maintaining steady suction, move the needle up and down in the direction of insertion until material appears in the hub or for up to 10 passes. Release the suction before withdrawing the needle. Apply pressure on the puncture site with cotton wool and ask the patient (or an assistant) to continue pressing while smears are prepared.
- Remove the needle from the syringe, draw air into the empty syringe, reattach the needle and expel the material collected in its shaft onto slides for smear preparation.
- If the lump is cystic, fluid will start filling the syringe on creation of suction. Maintain full suction and hold the needle steady in the lump. If fluid stops flowing, gently move the needle up and down in the direction of insertion while keeping the suction constant. Gentle pressure on the lump with the fingers of the nondominant hand can help the flow of fluid, but avoid squeezing. If blood starts appearing or when no more fluid is forthcoming, release suction to neutralize the pressure in the syringe, withdraw the needle and apply pressure with a cotton wool ball at the site. Ask the patient (or an assistant) to continue pressing while material is prepared.
- Detach the needle from the syringe and expel fluid collected in it into a clean container. For microbiologic examination, some material can be transferred to a sterile container or culture/transport medium.
- Draw air into the now empty syringe, reattach the needle and expel material collected in its shaft/hub onto slides for direct smears. It is a good practice to prepare these in addition, as exfoliated cells may undergo degeneration during specimen transport and laboratory processing. Examination of direct smears allows assessment of cellular content free from subsequent degeneration.

Smear preparation

This is an important part of the procedure, as poorly prepared smears can be difficult to interpret despite containing adequate cellular material, and at worse, can result in an erroneous diagnosis. Different smear preparation techniques have been described as one-step or two-step techniques [9]. The technique that consistently has produced satisfactory smears is described as follows (Figure 1.1).

- Smears should be prepared quickly once aspiration is completed to prevent clotting of the sample.

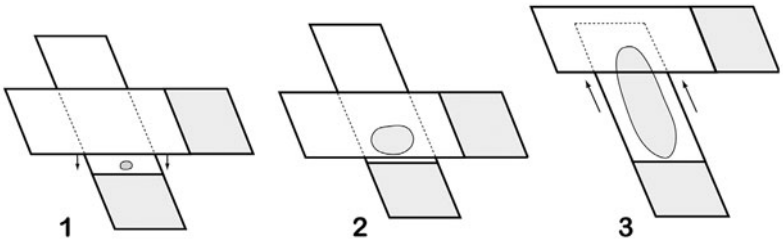

Figure 1.1. Smear preparation: (1) Material is deposited on the "smear" slide and "spreader" slide lowered over it. (2) Material is allowed to spread. (3) Smear is prepared by smoothly sliding the "spreader" over the "smear" slide.

- Deposit a drop of aspirate close to the frosted end of a slide marked with patient identifiers ("smear" slide). If more than one drop of material has been aspirated, place it on additional slide/s.
- Place another slide ("spreader" slide) over the "smear" slide, perpendicular to it and in full contact with the material. With gentle pressure allow the material to disperse between the two. The amount of pressure required comes with experience; too little pressure will result in a thick smear and too much in crushing of cells.
- With a smooth action, pull the "spreader" slide along the length of the "smear" slide, creating an oval shaped smear.
- When aspirated material has been placed on two "smear" slides, a single "spreader" slide can be used for preparing both smears; the slide is turned over after one smear is made. A single "spreader" slide can be used for spreading up to two smears, but this should be from the same aspirate in order to avoid cross-contamination.
- When smears are properly prepared, the "spreader" slide can be discarded as little material remains on it to make a meaningful contribution.
- A small amount of blood in aspirated material acts as a lubricant and helps with the spreading of the smear.

Smear handling

Smears can be air dried or alcohol fixed; it is good practice to prepare both types of smears.

Air-drying

- Smears should be allowed to dry completely and quickly; the process may be assisted by a fan or hair dryer [10].
- Properly air-dried smears can be placed in airtight containers and stored in a cool, dry place for up to seven days without detriment [11].
- Air-dried smears are stained with Romanowsky stains such as May–Grunwald–Giemsa stain (MGG) or other modifications [10].
- Unlike alcohol-fixation, which has to be carried out instantly following smear preparation, air-dried smears require no immediate treatment.
- Air-dried smears are ideal for interpreting the whole range of head and neck cytology.
- If required, air-dried smears can be rehydrated by covering them with normal or buffered saline for 30 s. These can then be alcohol-fixed and stained with the Papanicolaou (PAP) stain [12].

Alcohol fixation

- Smears once prepared should be immersed immediately in alcohol (absolute ethanol or methylated ethanol) or coated with spray fixative (e.g., Cytofixx, CellPath Ltd., Newton, Wales), as air-drying introduces artifact on staining that hampers cytological assessment.
- The time taken for fixation in liquid alcohol is proportional to the amount of blood – hemorrhagic aspirates take longer to fix.
- At least 15 min of fixation time is recommended; longer periods are of no detriment [10].
- Smears should be dried completely of fixative before transportation in slide boxes.
- Alcohol-fixed smears are stained with PAP, or less commonly, hematoxylin–eosin stain.

The differences between air-dried and alcohol-fixed smears are given in Table 1.1.

Cytological assessments in this book are based on conventionally prepared air-dried and alcohol-fixed smears. The former are stained using MGG; alcohol-fixed smears are stained with PAP.

Table 1.1 Comparison of air-dried and alcohol-fixed smears

	Air-dried smears	Alcohol-fixed smears
Smear preparation	Requires rapid air-drying	Requires immediate fixation
Smear fixation	• Post-fixation in methanol prior to staining, for at least 15 min • Longer fixation results in better visualization of nuclear chromatin detail	• In ethanol, methanol, or spray fixative immediately on smear preparation • At least 15 min in liquid fixative • Longer fixation results in better visualization of nuclear chromatin detail
Staining method	Romanowsky stains	Papanicolaou stain
Effect of fixation method on cell size	Cell and nuclear enlargement, which accentuate morphological features	Cell and nuclear shrinkage due to dehydrating effect of alcohol
Nuclear staining	• Purple in color • Differentiation between euchromatin and heterochromatin depends on length of post-fixation • External nuclear membrane abnormalities are seen • Nucleoli indistinct and light to dark blue in color	• Dark blue to bluish purple • Better separation between euchromatin and heterochromatin • Better demonstration of nuclear membrane irregularities including folds within nuclei • Nucleoli are well delineated and dark blue or red in color
Cytoplasmic staining	• Different shades of blue or gray; sometimes amphophilic or light magenta • Keratinocytes appear deep azure or turquoise blue • Neuroendocrine cell cytoplasm and eosinophil granules appear pink • Mast cell granules stain deep purple	• Generally pale green • Keratinocytes are green, pink, or orange in color, depending on keratin content • No differential staining of neuroendocrine cells or eosinophils • Mast cell granules stain pink or red
Hemosiderin pigment	Dark blue or black	Refractile brown or golden brown
Melanin pigment	Dark blue or black	Brown
Stromal material	Metachromatic staining (magenta colored)	Green
Colloid	Various shades of blue	Green or orange-red
Cholesterol crystals	Dissolution of crystals post-fixation shows the space occupied by them as a negative image	Dissolution of crystals on fixation means they are not visible on smears
Red blood cells	Pale or dark gray	Green or red
Ideal for visualizing	• Lymphoid morphology • Colloid in thyroid aspirates • Stroma in salivary gland neoplasms • Neuroendocrine differentiation (medullary thyroid carcinoma, paraganglioma) • This is a robust, all-round technique for head and neck cytological assessment	• Keratinization in squamous cells • Nuclear membrane irregularity • Nuclear chromatin detail • Nucleoli

Artifacts on smears

- When smears are slowly air-dried, nuclear staining is pale/suboptimal with MGG.
- Air-drying in alcohol-fixed smears results in:
  - Cytoplasmic eosinophilia.
  - Loss of nuclear chromatin detail and "blurry" nuclei.
- Nuclear streaking is seen when excessive pressure is applied during smear preparation (overspreading). This artifact affects lymphoid cells and is also observed in the "spreader" slide.
- Delay in smear preparation initiates clotting of blood in the needle, resulting in uneven smears with poorly displayed cells entrapped within the clot.
- Ultrasound jelly artifact on ultrasound-guided FNA:
  - Ultrasound jelly appears as granular magenta-colored material with MGG (Figure 1.2).
  - It is cytolytic and cells appear indistinct or swollen with pale nuclei (Figure 1.3) [13].
  - When extensive, it will hamper cytological assessment.

Liquid-based preparations in head and neck cytology

Liquid-based preparations (LBP) are designed to produce homogenized, cell-enriched samples. Aspirates are transferred directly to a fixative; inconsistencies in smear preparation are removed and immediate fixation prevents air-drying artifact with PAP. Additionally, material is available for cell-block or for preparing multiple slides on which immunocytochemistry and/or molecular tests can be performed. Material can be stored in the fixative for between 3 wk and 3 mth for later use [14].

However, LBP has significant shortcomings in the morphological assessment of head and neck FNA samples:

- Air-dried smears with their enhanced cellular, nuclear, and stromal characteristics with MGG cannot be examined.
- Sample processing in LBP results in:
  - Disruption of architectural features with breakage of papillae, cell groupings, and increased cellular dyscohesion.
  - Loss, reduction, or alteration of important background material such as colloid, chondromyxoid matrix, stroma, mucus, and necrotic debris.

By the correct FNA and smear preparation technique achieved, by education if necessary, optimal conventional smears can be prepared countering the advantage of LBP and without compromising cell group architecture, cell morphology, and extracellular component detail. Material for ancillary immunocytochemical or cytogenetic analysis can be collected separately at the time of FNA, if indicated. With simple attention to detail, the additional cost incurred by LBP can be avoided, ensuring that FNA cytology remains a cost-effective and widely available investigation.

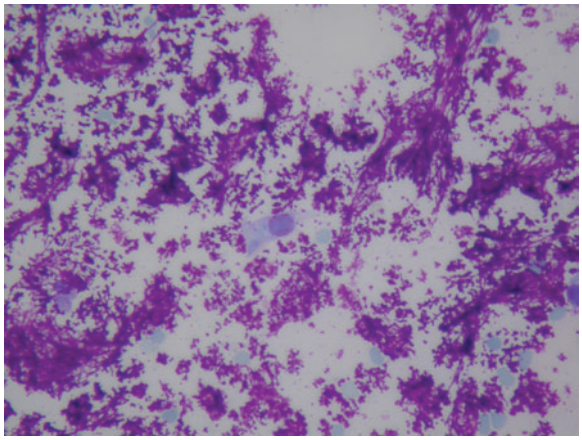

Figure 1.2 Granular ultrasound jelly. MGG x200.

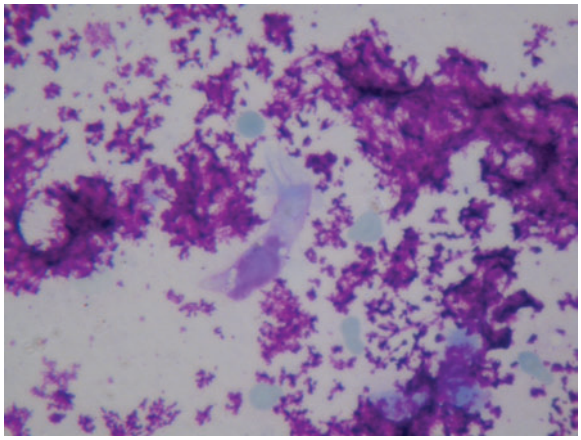

Figure 1.3 Ultrasound jelly causing cellular ballooning. MGG x400.

Complications of FNA

FNA is a safe procedure, and when carried out with due care and asepsis, is virtually free from complications. Possible complications that can occur are [2, 15]:

- *Bruising and hematoma formation*:
  - Increased risk when a lump is "probed" during needling.
  - Follows inadequate hemostasis in patients on anticoagulation medication.
  - Seen more frequently when wide-bore needles are used.
- *Infection*:
  - There is a small risk of infection of cystic masses following FNA, as a potential channel with the exterior is established. Appropriate asepsis and informing the patient to keep the area clean post-procedure will minimize this risk.
- *Spread of tumor*:
  - Isolated case reports exist of tumor seeding along the needle tract [16]. Use of 23G or 25G needles makes the risk negligible, especially when compared with the benefits of this investigation.
- *Syncope*:
  - This usually occurs in apprehensive patients; engaging them in general conversation during and post-aspiration will minimize its occurrence.
  - Can be due to postural hypotension when patients get up quickly from a recumbent position.
- *Infarction*:
  - This is seen occasionally with thyroid and salivary gland neoplasms and in lymph nodes subsequently excised for histological assessment [17–20].
  - Avoiding the use of large-bore needles and the correct aspiration technique prevents this complication.
- *Site-specific complications*:
  - The trachea can be punctured during thyroid FNA, which triggers the cough reflex. If this happens, withdraw the needle and reassure the patient.
  - Puncturing the carotid artery: this extremely uncommon complication may occur when aspirating masses in close proximity to the vessel, commonly lymph nodes. Arterial blood spurts out, whereupon withdraw the needle immediately and apply steady pressure to the site. It is important not to panic as bleeding from the puncture site will be controlled by external compression. Ultrasound-guided FNA of masses at this site will prevent this occurrence.

One-stop/rapid assessment FNA clinics

- These are run in tandem with routine outpatient clinics and a cytological diagnosis is made available in the clinic.
- Aspiration may be carried out by:
  - Pathologists/cytologists.
  - Radiologists under ultrasound guidance.
  - Physicians/surgeons.
- Cytological assessment may be carried out in the:
  - Outpatient clinic itself.
  - Pathology laboratory where smears are transported to and treated in a rapid manner similar to frozen sections.
- Cytological reports generated may be:
  - Definitive (requires a well-resourced service and may involve longer waiting times for patients).
  - Provisional, followed by a final report within 24 hours. This happens when not all material (e.g., cyst fluid) is examined immediately.
- *"Pros" of one-stop/rapid assessment clinic*:
  - Patients are given a diagnosis and management plan in most cases.
  - Patients with benign diagnoses can be reassured. Those with clinically and cytologically assessed reactive lymph nodes may be discharged from further care.
  - There is a potential saving in time for both patients and clinicians, as a repeat visit to discuss the result is avoided.
  - A potential cost saving of over $400 000 per year was predicted by utilizing on-site evaluation by avoiding repeat FNA for each nondiagnostic sample despite additional initial costs [21].
- *"Cons" of one-stop/rapid assessment clinic*:
  - Appropriate supportive teams of cancer nurse specialists and counselors are required to help

patients deal with an unexpected diagnosis of malignancy.
- The amount of time spent per patient in the initial consultation, discussion of diagnosis, formulation of a management plan, and institution of additional investigations could limit the number of patients seen when compared to ordinary clinics.
- For thyroid masses, cytological diagnoses and management options are well defined and frequently protocol driven. However, general neck lump clinics would also deal with lymph node, salivary gland, and miscellaneous neck masses with a range of potentially complex cytology. A definitive diagnosis may not be reached, diluting the benefit of such clinics.
- There are significant resource implications in terms of dedicated time for pathologists, clinical/biomedical/health scientists, and radiologists as per the model employed. In the UK, the 2004 National Institute for Health and Clinical Excellence recommendation of establishing such service for management of patients with neck lumps acknowledged significant cost implications, estimated to be £20 000 per clinic per year [22].

While the role of one-stop/rapid assessment FNA clinics is well established for thyroid masses, the case for a general head and neck FNA clinic is less clear and depends on local need and available resources.

FNA and ancillary investigations

FNA is a valuable tool for obtaining cellular material for additional investigations.

- *Immunocytochemistry*:
  - This may be carried out after destaining MGG- or PAP-stained slides, but this has a limitation in the number of antibodies that can be applied and technical issues related to background staining.
  - A separate FNA sample for immunocytochemical assessment can be collected in:
    - Buffered saline for cytological Cytospin preparations, or
    - 10% buffered formalin/ethanol-carbowax for the agarose cell-block histological technique.
- *Flow cytometry*:
  - Flow cytometry is used to measure cell size, DNA ploidy, clonality, and expression/coexpression of antigens. In conjunction with cytological morphology, it is utilized in lymphoma diagnosis.
  - The number of cells required for accurate flow cytometric analysis ranges from 300 000 to 1 000 000, which can be achieved by collecting three adequate aspirates in RPMI or other media [23].
- *Microbiological analysis*:
  - In case of suspected infection (bacterial, mycobacterial, or fungal), aspirated material can be transferred directly to sterile saline or transfer/culture medium for microbiological analysis.
- *Molecular and cytogenetic analysis*:
  - These tests are used to detect alterations including mutations, translocations, and chromosomal/gene rearrangement.
  - Techniques employed include polymerase chain reaction (PCR), reverse-transcriptase PCR, and fluorescence *in situ* hybridization.
  - The availability of these techniques is limited but they could play an important role in the future, both in primary diagnosis and prognostic stratification of the disease process.

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