Head and Neck Pathology: Practical Points to Ponder

Bridget Mitchell, Jing F. Kee, Lisa K. Peart, and Duncan Mitchell

Core Messages

- Pathologists need information from clinicians and radiologists.
- Taking better specimens leads to better feedback to clinicians.
- Preparing the optimum specimen for your differential diagnosis.
- Being aware of all the testing modalities at the pathologists' disposal.
- Forming a symbiotic relationship with your pathologist.

1 Introduction

Anatomical pathology is the branch of medicine that deals with the examination of cells, tissues and organs, mainly for diagnostic purposes. It is a self-standing medical discipline, and its practitioners require specialist knowledge and skills. It best serves the needs of patients if it is conducted not in isolation but in harmony with the clinicians who are in direct patient contact [1]. The aim of our chapter is not to review the pathology of malignancies of the head and neck but rather to give the pathologists' perspectives of how best to achieve that harmony. We shall cover some practical information about what clinicians can do to enhance the subsequent contribution that pathologists can make to the team, and we shall cover how best to interpret the pathological report. Particularly in the field of head and neck pathology, providing appropriate clinical and radiological information, and the best possible specimens, massively assists what the pathologist can do to ensure the best outcome for the patient.

The specimens submitted by clinicians managing head and neck pathology most frequently are biopsy specimens. For the pathology laboratory to perform all the relevant testing and analysis required for the most accurate diagnosis, the most suitable specimen needs to be submitted, in the most appropriate transport medium. Pathologists have little leeway if the specimen is unavoidably small, and non-ideal handling of a small specimen before it reaches the laboratory can have irrecoverable consequences. So we will offer the pathologists' perspectives on specimen collection techniques and transport media.

Once a specimen has been received and processed through the laboratory, samples are examined by pathology technicians and pathologists. The main tool of the anatomical pathologist remains the optical microscope, but today pathologists have access to a large range of special stains. Immunohistochemical stains and other molecular studies have vastly improved their diagnostic and prognostic capabilities, as well as their contribution to treatment planning. We shall offer a brief overview of some of these ancillary procedures.

If a cancer is identified in the specimen, the pathologist usually will submit a synoptic report that ensures that vital information about each cancer is recorded. Some of the important prognostic factors from these synoptic reports are tumour thickness and depth of invasion, pattern of invasion, perineural invasion and surgical margins, and we shall discuss how those factors are determined.

Not just at the report stage but throughout the process, we urge clinicians in direct contact with patients to interact with their pathologists. Discussions between pathologists and clinicians can help resolve a challenging diagnosis and encourage pathologists to review cases where initial pathological interpretations do not reconcile with the clinical picture.

B. Mitchell (✉) · J. F. Kee
Anatomical Pathology Services, Auckland, New Zealand
e-mail: bmitchell@adhb.govt.nz; DennisK@adhb.govt.nz

L. K. Peart
Auckland DHB Anatomical Pathology Services, Panmure, New Zealand
e-mail: LPeart@adhb.govt.nz

D. Mitchell
Brain Function Research Unit, School of Physiology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

School of Human Sciences, University of Western Australia, Perth, WA, Australia
e-mail: duncan.mitchell@wits.ac.za

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Also, when you really are in a bind and need something special, like rapid turnaround time, a pathologist, who you have established as an ally, can move mountains.

2 Obtaining a Specimen

We offer pathologists' views on the procedures that clinicians need to undertake to obtain and submit the best specimens. Practices in the hands of clinicians determine the capacity of pathologists to derive information from the specimen and consequently the value of the report submitted back to the clinicians. The procedures that we shall discuss are fine needle aspiration (FNA), biopsy for frozen section and biopsy for routine histology.

3 Fine Needle Aspiration

Fine needle aspiration, which can be freehand or ultrasound guided, trumps other procedures with regard to rapid diagnostic capability. When used in the appropriate setting, a fine needle aspirate may provide material for a quick, sometimes immediate diagnosis as well as material for ancillary testing. The procedure is technically simple, usually has few side effects, and is a great addition to the diagnostic armoury of the surgeon/pathologist team. Cellular tumours, lymph nodes, metastatic skin lesions, salivary tumours and thyroid lumps are examples of lesions that are good candidates for cytology. Certain other lesions, for example, fibrotic lesions, small ill-defined lesions, exclusively cystic lesions and stromal tumours, may not be amenable to, or easily diagnosed by, FNA.

Here is how to get the most from an FNA.

3.1 Sample Procurement

Whilst fine needle aspiration is a simple technique, there is an art to taking a good sample and preparing a beautiful slide.

1. Have all the necessary equipment, such as needles, clean syringes, labelled glass slides and transport medium, at hand before starting the procedure. This preparation reduces the time between aspiration of the material and its transfer to the slide. The longer the lag time, the greater the risk of creating a drying artefact (see Fig. 5) or allowing the material to clot in the needle, preventing expulsion.
2. Positioning the patient is equally important, especially if the fine needle aspirate is to be done freehand. Aim to achieve good access to the lesion and easy immobilisation of the lesion with your non-aspirating hand. Improving positioning can be as simple as tilting the chin or hyperextending the neck, the latter a useful technique for thyroid FNAs.
3. There is no universal agreement on the right needle gauge. As a general guideline, larger bore needles do not always yield better results. Avoid large needles such as 1.27 mm outer diameter (18 gauge) needles because they can promote bleeding, leading to blood clot obscuring target cells on the slide. Our preference is either 0.64 mm (23 gauge) or 0.52 mm (25 gauge) and a 10 mL syringe.
4. Tissue procurement can be done with or without suction. Both techniques have advantages and disadvantages. Suction may produce more tissue volume but at the risk of generating more bleeding. Our recommendation is to stop suction if there is bleeding at the site of sampling.
5. It is the fine backward and forward motion of the needle bevel that cuts loose target cells. The repeated forward

motion forces the loose cells to move up the needle shaft, even without suction. FNA expert Susan Rollins suggests that the ideal needle oscillation is three per second with an intralesional dwell time of between 3 and 5 seconds [2].

3.2 Smear Preparation

If you are making the smear yourself, using a reliable technique will ensure that the pathologist can use the material to your mutual best advantage. Whilst there are many possible techniques, we shall describe one [3].

1. Immediately after aspiration has been completed, remove the needle from the syringe, and pull back on the plunger to provide sufficient force to express the material onto the slide (Fig. 1).

   Pitfall: Failure to disconnect the syringe barrel from the needle may pull all the aspirated material back into the syringe, making transfer of the specimen onto the slide difficult or impossible.
2. Re-attach the needle, place the needle onto the upper third of the glass slide, and depress the plunger of the syringe to express the material (Fig. 2).

   Pitfall: If your needle is held too far above the slide, the material will splatter. Worse than splatter is the dart-like needle projectile after excessive force is applied to express material clotted in the hub.
3. Invert a second slide over the first so that the material spreads from the weight of the slide only (Fig. 3).

   Pitfall: Additional pressure creates the risk of introducing crush artefact (see Fig. 6).
4. Pull the slides apart with a gentle horizontal motion, creating two slides for every needle pass, facilitating Pap and Giemsa stains (Fig. 4).
5. Air-dry one of the slides with the assistance of a fan or hair dryer if necessary, and wet fix the other slide immediately using 95% alcohol or spray fixative.

Fig. 1 Remove the needle containing the aspirated material from the syringe and then pull the plunger back

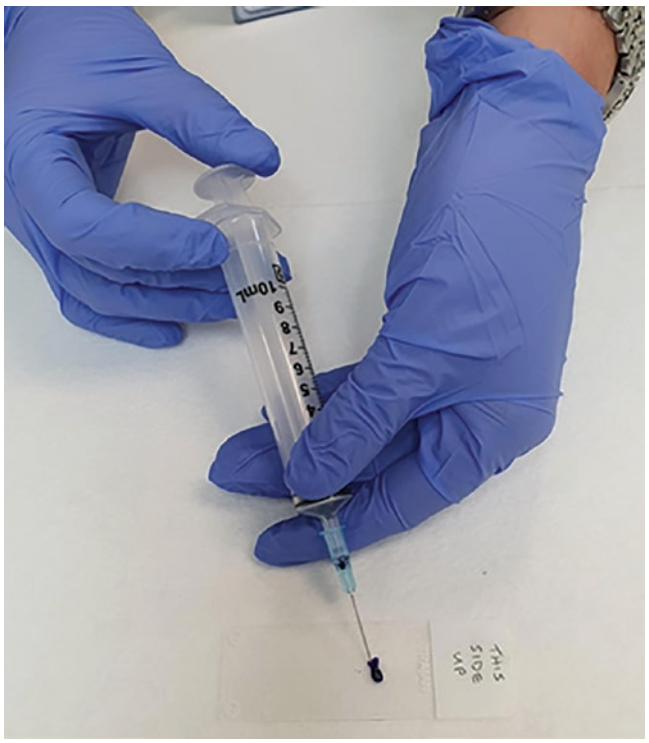


Fig. 2 Express the material onto the upper third of the slide

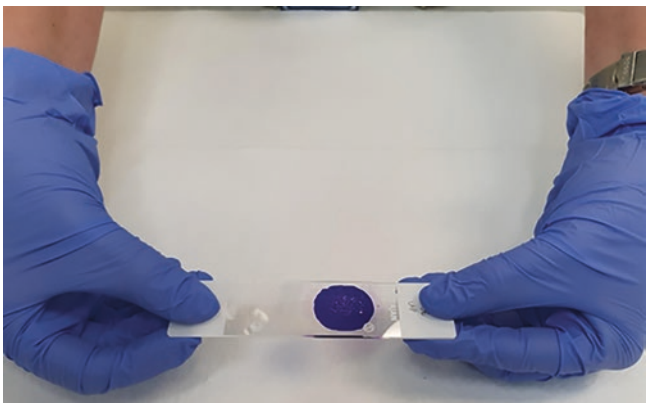
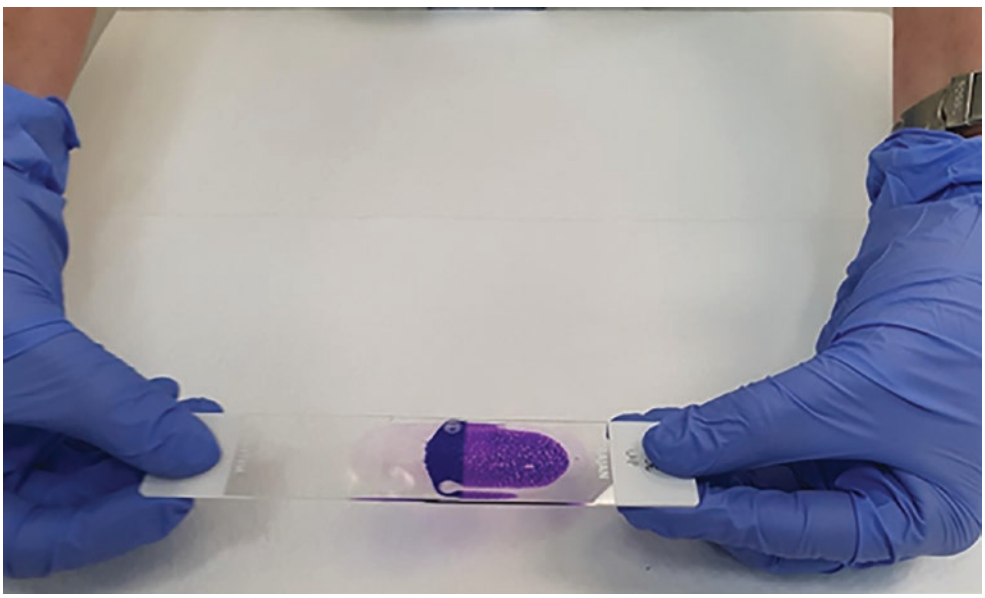


Fig. 3 Invert a second slide over the first, so that the material spreads from the weight of the slide only

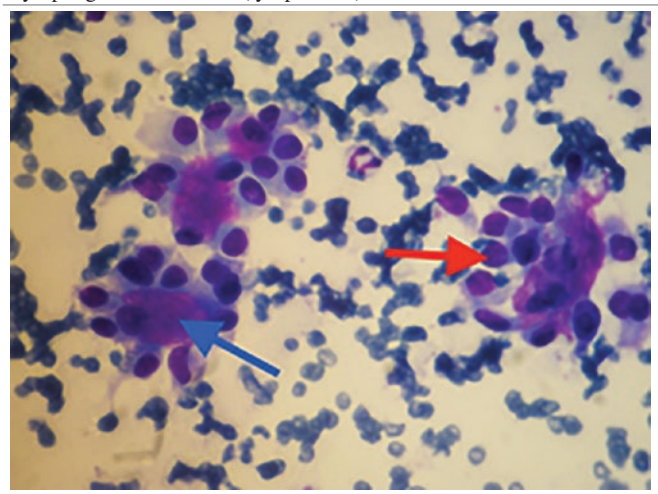
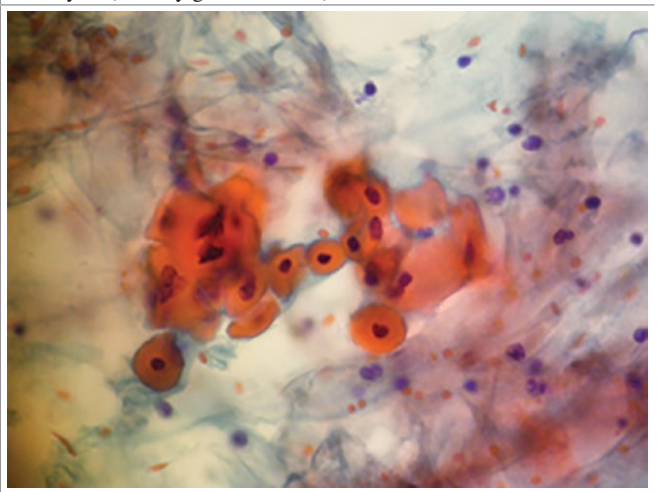
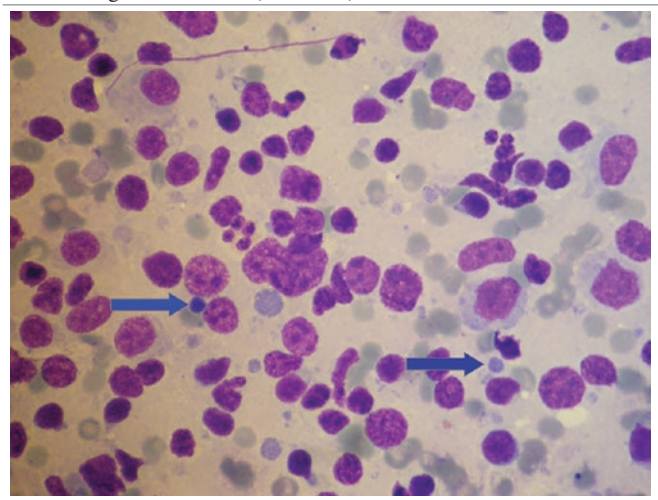
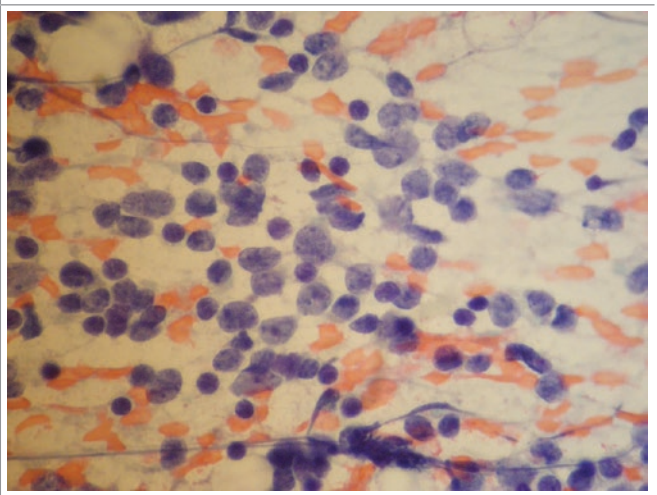
Fig. 4 Gently pull the slides apart completely using a horizontal motion



3.3 Papanicolaou Stain vs Giemsa/Diff-Quik Stain

The stains usually employed for histopathological examination of FNA smears are the Papanicolaou stain, for nuclear detail, and the Romanowsky-type stains (Giemsa/Diff-Quik), for cytoplasmic detail (Table 1). Some pathologists may have a personal preference for one of these stains, but they highlight different cellular features [4]. The reason that we advised that one FNA smear be air-dried and the other wet-fixed is the Romanowsky-type stains require an air-dried smear and the Papanicolaou stain a wet-fixed smear. Providing both smears allows the pathologist to use both stains and the best opportunity to assess all aspects of the specimen.

Table 1 What pathologists can do with Romanowsky-type and Papanicolaou stains of FNA smears

Features emphasised by Giemsa/Diff-Quik stain	Features emphasised by Papanicolaou stain
Cytoplasmic detail and stromal components well demonstrated	Nuclear detail is excellent
Ideal for visualising: Colloid (thyroid) Mucin (salivary gland tumours) Basement membrane globules (adenoid cystic carcinoma) Lymphoglandular bodies (lymphomas)	Ideal for visualising: Squamous differentiation/keratinization (squamous carcinomas) Nuclear chromatin (e.g. salt and pepper Chromatin of neuroendocrine tumours) Oncocytes (salivary gland tumours)
 Adenoid cystic carcinoma (Giemsa stain 400× magnification) The aspirate shows the presence of large globules of extracellular matrix material which stain purple (blue arrow). There are surrounding basaloid cells (red arrow)	 Squamous cell carcinoma (Papanicolaou stain 400× magnification) The cytoplasm of squamous cells stains bright orange. The nuclei appear almost black due to condensation of the nuclear chromatin
 Lymphoma (Giemsa stain 400× magnification) Small blue lymphoglandular bodies are visible in the background (blue arrows). These are small cytoplasmic fragments often seen in aspirates of lymphomas	 Merkel cell carcinoma (Papanicolaou stain 400× magnification) This stain highlights the typical salt and pepper chromatin seen in the nuclei of neuroendocrine tumours. Merkel cell carcinoma is a highly aggressive neuroendocrine carcinoma of the skin and is a prevalent cancer of the head and neck in elderly and immunosuppressed individuals

3.4 Artefacts: What Can Go Wrong in FNA Smears and How to Avoid It

3.4.1 Drying Artefact

The thyroid follicle cells show artefactual degenerative changes (Fig. 5). The nuclei are pale and have lost the nuclear detail, making assessment for lesions like papillary carcinoma challenging for the pathologist.

Problem: The material has dried or has been fixed too slowly.

Solution: Dry with a fan or hair dryer or fix material immediately after smearing.

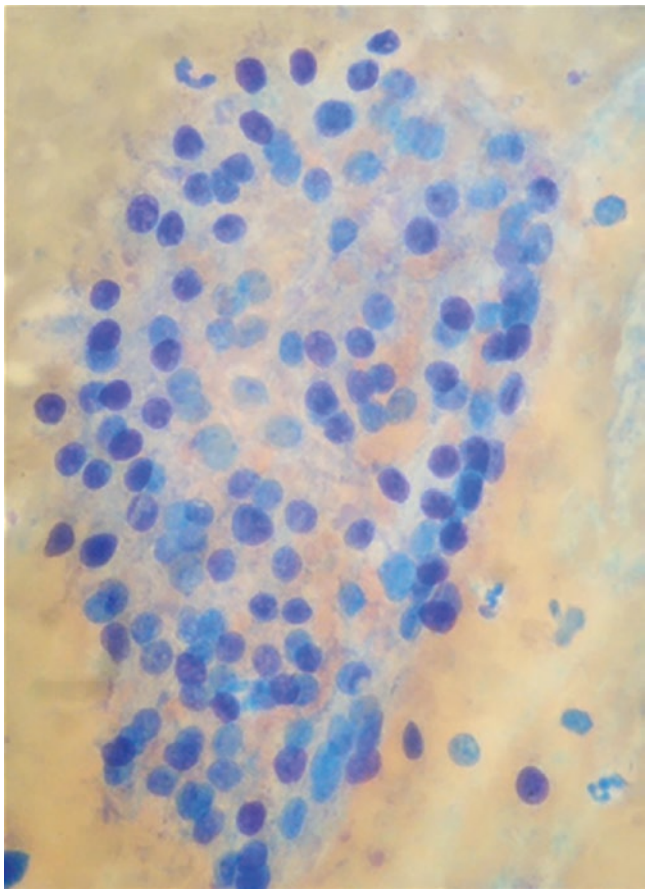


Fig. 5 Thyroid aspirate (Giemsa stain 200× magnification)

3.4.2 Crush Artefact

Problem: Too much force was used whilst smearing the slide (Fig. 6).

Solution: Use no more force than the weight of the top slide when preparing a smear.

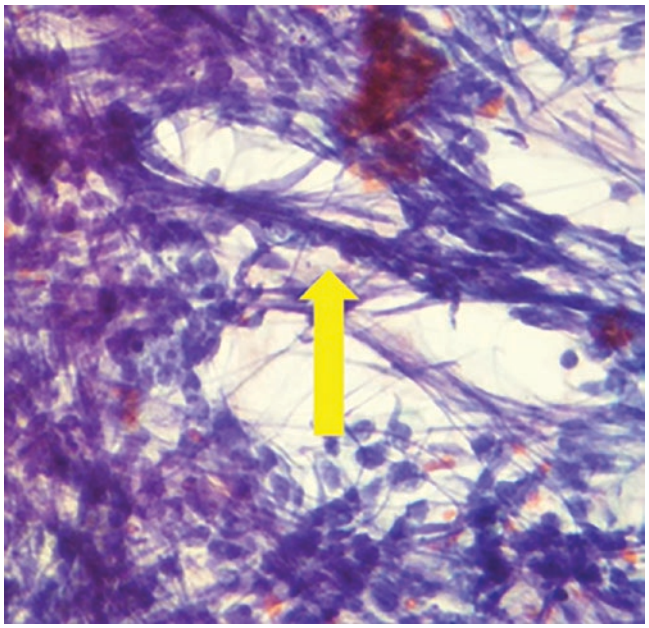


Fig. 6 Lymph node aspirate for metastatic Merkel cell carcinoma (Giemsa stain 200× magnification). In this slide the nuclei have been crushed leaving long blue strands of nuclear material (yellow arrow)

3.4.3 Too Thick

This aspirate from the lymph node is so thick that individual cell characteristics have been lost and it is difficult for the microscope to focus on the group of cells (Fig. 7).

Problem: Too much material in a localised area means individual cells cannot be seen.

Solution: Limit the amount of material and smear evenly. If you aspirate abundant material, it is better to prepare more slides than put a large drop on one slide.

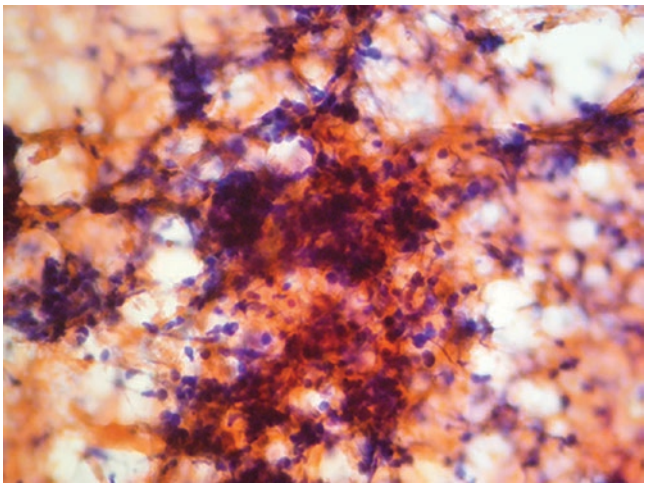


Fig. 7 Lymph node aspirate (Papanicolaou stain 200× magnification)

3.5 Preparing FNA Specimens for Ancillary Studies

Harvesting as much of the sample as possible allows for smears to be supplemented with additional procedures (see "Ancillary histological studies"). The remaining material can be placed in liquid medium after smears have been prepared or primarily if a designated FNA pass is performed for this purpose. A small amount of the liquid should be drawn into the syringe and then expelled back into the medium container. This process may be repeated several times. In this way, a sample may be provided for flow cytometry, microbiology, a cell block, immunocytochemistry or molecular studies [5]. Whilst specific media are ideal, if they are not available, saline may be used, as long as the specimen is transported quickly to the laboratory. Because processing in the laboratory needs to be immediate if the specimen is in saline, let your pathologist know it is on its way. Try to avoid sending FNA specimens in saline late in the day, or before a weekend, because of the risk of deterioration of valuable cellular material.

4 Frozen Sections

Frozen sections are used when pathological analysis is required immediately, during surgery with the patient remaining anaesthetized. A specimen taken from the patient is frozen, and a thin section is cut on a cryostat machine (Fig. 8), which is a microtome with a freezing stage. The thin section is placed on a glass slide where it melts and is dried, stained and examined under the microscope. Pathologists can report on a histological examination by frozen section within 15–20 min of receiving the specimen.

Frozen section histological analyses usually are performed for one of three reasons [6]:

1. To guide intra-operative management, e.g. examination of margins of excisions.
2. For triaging of tissue for later special studies.
3. To confirm that lesional tissue is present in the specimen.

Specimens for frozen sections need to be provided fresh (not in formalin or other fixative) and should be accompanied by patient details including relevant clinical history and information about the tissue site and the reason for requesting frozen section. If the clinician is aware of, or suspects, an infection like tuberculosis, hepatitis B or HIV, this information also should be communicated to the pathologist so that the necessary protective and cleaning protocols can be instituted. Orientation of the specimen is vital, and a discussion with the pathologist about which area on the specimen should be sampled, and what information is being sought, will improve the information the pathologist will be able to provide.

Frozen section histological examination is not a rapid substitute for what the pathologist can do with a permanent section. Table 2 summarises the differences.

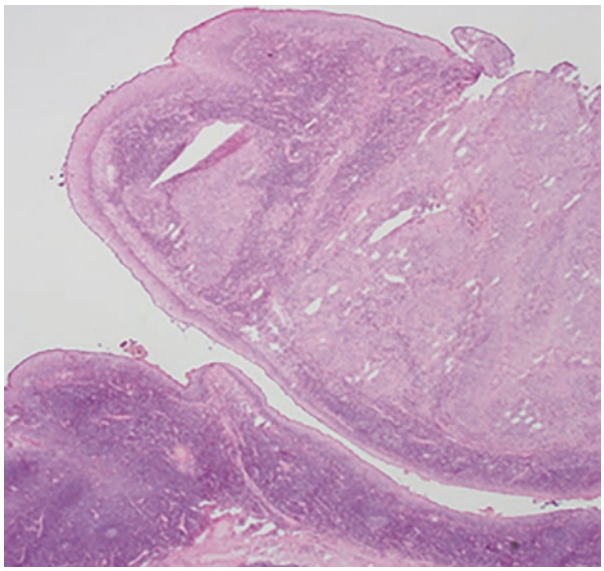
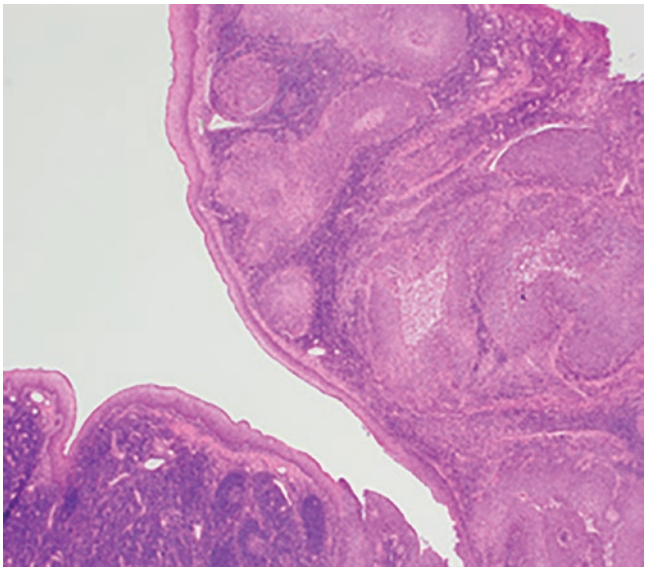
Requesting a frozen section in theatre because it would be "nice to know" the diagnosis but when knowing the diagnosis immediately actually does not impact on patient management is a waste of resources. Frozen sections are labour intensive and time-consuming. They require committed and concurrent time (often including travel time) from a pathologist and from a skilled technician. There are some frozen sections that would be deemed inappropriate, and even detrimental to the patient, including small melanocytic lesions where freezing the entire lesion may remove the possibility of better histological examination by permanent section.

Frozen section histology has a sensitivity of 89% and a specificity of 99% for the evaluation of margin status for head and neck squamous carcinomas [7]. The specificity and sensitivity of frozen section histology for some other lesions, like follicular lesions of the thyroid, are much lower, and frozen section histology then is inappropriate. Even in ideal circumstances, a discordance rate between the frozen section histology and permanent section histology of 3% has been reported [7].

Discrepancies between frozen section histology and permanent section histology may be due to sampling error or to interpretation error. A sampling error may occur, for example, if tumour tissue is not in the section that was stained at the time of the frozen section but is present on the deeper levels that can be examined in permanent sections. An interpretation error may occur, for example, if a pathologist, faced with the poorer resolution of a frozen section, interprets tissue on the slide as being tumour when it is not or interprets tumour tissue as benign. Interpretation errors can be reduced if pathologists specialise in an organ system and develop expertise, just as surgeons do.

Fig. 8 Leica™ CM1860UV cryostat machine, used for cutting frozen sections either in theatre or in the laboratory

Table 2 What pathologists can do with frozen section and permanent section histology

Frozen section	Permanent section
Done rapidly in theatre or laboratory	Usually processed over hours, in a laboratory
Creates freezing artefact which may impact diagnosis and later ancillary testing	No freezing artefact
Examines a targeted small proportion of the specimen	Multiple sections can examine the entire specimen
Ancillary testing is usually not performed	Ancillary testing, like special staining, immunohistochemistry and molecular markers, can be performed
Usually no opportunity for consultation with pathologist colleagues, for challenging cases	Consultation with pathologist colleagues is routine for challenging cases
 Frozen section p16-positive squamous carcinoma (200× magnification haematoxylin and eosin) Image resolution adequate but not ideal	 Permanent section p16-positive squamous carcinoma (200× magnification haematoxylin and eosin) Superior image resolution

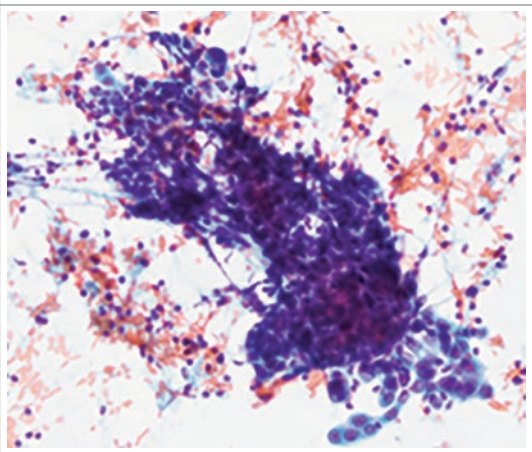
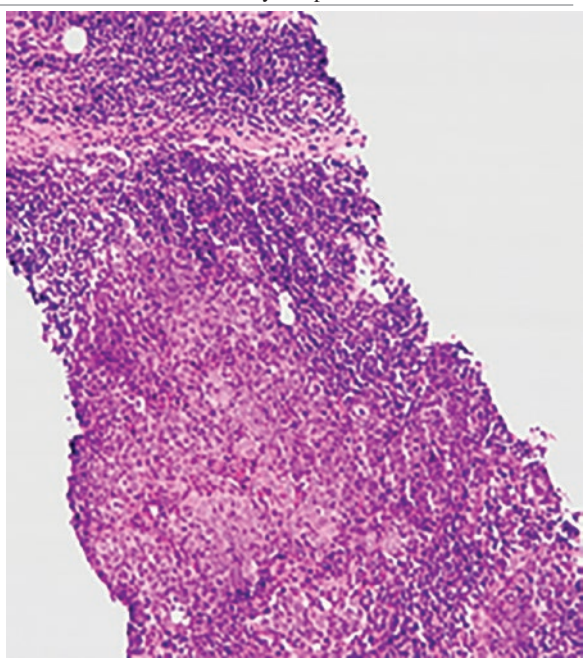
5 Biopsy/Excision or Incision

The majority of the work undertaken by pathologists is excisional or incisional biopsies. Routine management of biopsy samples usually at least entails overnight processing. It is sometimes possible to accelerate the management, for example, with small samples that do not require lengthy fixation. Accelerating the management is labour intensive and requires the technical staff to bypass usual processes, so is best reserved for the exceptional case where an urgent result is of the utmost importance to the patient. Accelerated management also requires communication with the pathologist so that every step of the process can be streamlined. Depending on the individual laboratory, it is possible to have slides prepared for the pathologist to examine within 3 1/2 h of a specimen arriving in the laboratory.

5.1 Core Needle Biopsy

Core needle biopsy usually is performed with a larger-gauge needle, ranging from outer diameter of 2.1 mm (14 gauge) to 0.91 mm (20 gauge), than is FNA [8]. The larger diameter of the core biopsy in theory translates to a larger sample of target cells acquired and often with intact tumour architecture. We cannot over-emphasise the value of preserved tumour architecture in the slides used for cancer diagnosis. Indeed, tumour architecture preservation is the main advantage that core needle biopsy (or excisional biopsy for the same matter) has over fine needle aspiration. That said, core needle biopsy (and excisional biopsy) is not without flaws. The advantages and disadvantages of core needle biopsy and FNA are outlined in Table 3.

Table 3 Advantages and disadvantages, for the pathologist, of core needle biopsies and FNAs

	Advantages	Disadvantages
Fine needle aspiration	1. Rapid procedure 2. Can be done anywhere, including remote settings 3. No anaesthetic required, usually 4. Less traumatic and usually less bleeding due to smaller needle gauge 5. Specimen amenable to rapid on-site evaluation	1. FNA skills variable from operator to operator 2. Deeper lesions require needle guidance, e.g. by ultrasound 3. Less preservation of tissue architecture 4. Low yield for fibrotic lesions 5. Pathologist not always available for rapid on site evaluation
Core needle biopsy or excisional or incisional biopsy	1. Produces more tissue allowing more histological investigation 2. Better preserves tissue architecture for higher accuracy assessments 3. Processed as routine histology which is familiar to all pathologists (training in cytology not required)	1. May require a more elaborate set up with involvement of interventional radiologist and nurse. Cannot be done in a bedside setting 2. More costly 3. Relatively more traumatic; normally local anaesthetic will be given 4. Single trajectory of needle may limit the extent of sampling 5. Tissue requires fixing and longer processing. Rapid on-site assessment usually not possible
Left cervical lymph node with metastatic p16-positive squamous carcinoma FNA (Papanicolaou stain 200× magnification). Core biopsy (haematoxylin and eosin 100× magnification)		

5.2 The Complex Resection Specimen

There is no doubt that extreme facial surgery results in large complex histology specimens. Whilst we have general guidelines for the management of such samples when they get to the laboratory, pathologists benefit, and the outcomes for clinicians and patients are better, from attention to multiple steps before that, some of which begin before surgery has been completed.

5.2.1 Submitting Pathology Specimens

The requirement for correct patient identification, identification of the requesting surgeon/ clinician and date of the procedure goes without saying, and these details invariably are provided. Adequate clinical history, sometimes provided less reliably, is of equal importance and has a direct impact on the ability of the pathologist to interpret the pathological features meaningfully. Whilst the vote of confidence is flattering, pathologists cannot accurately derive that clinical information from information on glass slides alone [9].

Clinical information beneficial to pathologists includes relevant prior diagnoses and, if histology has been carried out previously, a copy of the original report, or at least an indication as to which laboratory performed that earlier histology. Tell your pathologist the type of specimen and what you are trying to achieve by the procedure. For instance, does the specimen represent a debulking procedure or an incisional biopsy, or is it an attempt at a complete excision. The former requires little attention from the pathologist with respect to margin status, but establishing margin status is imperative in the latter. The locations and nature of some lesions that are clearly evident in vivo may become less obvious after excision and cessation of blood flow (e.g. vascular lesions and cystic lesions). Small lesions may be difficult for the pathologist to pinpoint, and specimens from lesions treated with neoadjuvant therapy may no longer be grossly identifiable [7].

If there is a specific request, or a specific question that needs to be answered, include that request/question on the form that accompanies the specimen. Pathologists will be able to respond better if they know about special needs before the specimen is processed as they may alter markedly the way in which a specimen is processed. For example, if the question is about clearance of a specific margin, the specimen needs to be sectioned to display that margin well. If the question is whether an infection is present, a sample of non-fixed tissue may be submitted for culture and sensitivity. If it is whether a lymphoma is present, fresh tissue can be sent for flow cytometry.

5.2.2 Orientation of Pathology Specimens

Preserving the three-dimensional orientation of tissue after resection can be difficult. When complex anatomy and formalin fixation compound the issue, the orientation of the specimen that arrives in the laboratory may be even more obscure. To assist the pathologist, please consider the following:

1. Mark the designated borders/points of orientation with sutures, clips or ink during or immediately after resection [6]. Sutures of variable composition, length or number may be used. Sutures and clips also are useful to mark the resection margins of pertinent nerves or to identify a sentinel point, for example, 12 o'clock using a clock face template [10] for orientating the specimen (Fig. 9).
2. Pinning the specimen to a cork board (Fig. 10) has multiple benefits. The tissue is fixed in a non-rotated plane which is easier to section. Tissue shrinkage and tissue curling at the edges are reduced by pinning. Pins of different colours (e.g. indicating different anatomic locations) may be used to attach the tissue to the board and can be referenced in a specimen diagram (*see* Fig. 12).
3. Alternatively, the clinician may wish to define the orientation of the specimen by writing or drawing directly on the cork board. If so, the specific pen and ink need to withstand exposure to formalin. Some ink washes away almost entirely in formalin or smudges so badly that the writing/drawing becomes illegible. The images in Fig. 11 show the consequences for cork board drawings with different inks of being submerged in formalin overnight. This test is an easy one for clinicians to replicate when selecting a pen for this purpose.

   Once the specimen is attached, the cork board should be inverted in the specimen container to ensure that the tissue is submerged in formalin.
4. Drawing a diagram (Fig. 12) is an excellent way of orientating a specimen. It gives the pathologist a clear indication of the surgeon's view of the sample and provides a platform for specific landmarks and margins to be identified. Diagrams also are convenient for identifying specific issues regarding the specimen for the pathologist's attention, for example, identifying a margin of concern.
5. Inking of the specimen to delineate margins or other landmarks usually takes place in the pathology laboratory. However, it also can be a valuable tool for the surgeon [11]. The surgeon may apply ink to orientate the specimen, to indicate a specific margin of concern or, conversely, to identify an area that does not represent a true surgical margin (Fig. 13). The ink can be applied with a cotton swab, patted dry and then dabbed with acetic acid (vinegar) as a mordant that combines with the ink to fix it to the material.

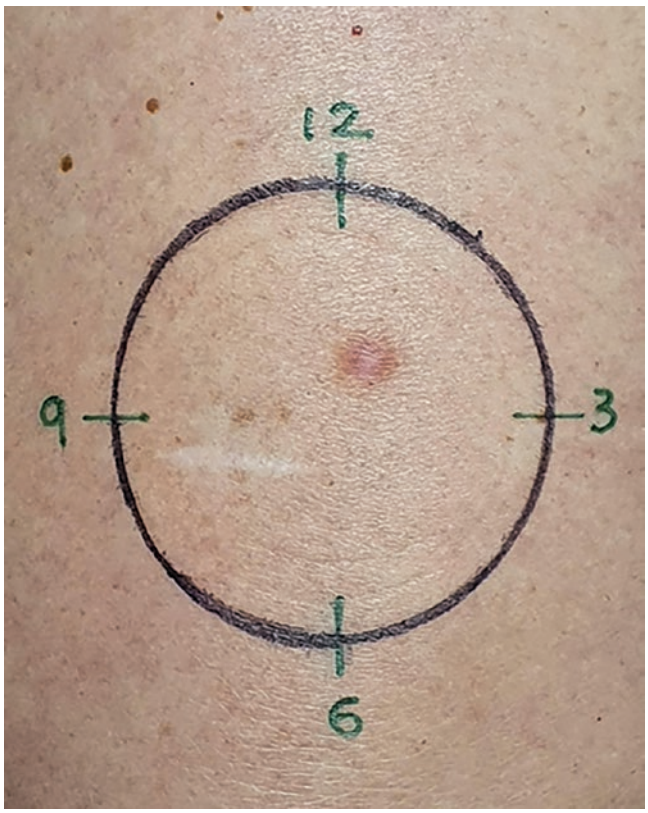


Fig. 9 Orientation of a specimen in situ using the clock face template. Orientation of a specimen is important especially if there is a positive margin on histology and the lesion requires re-excision

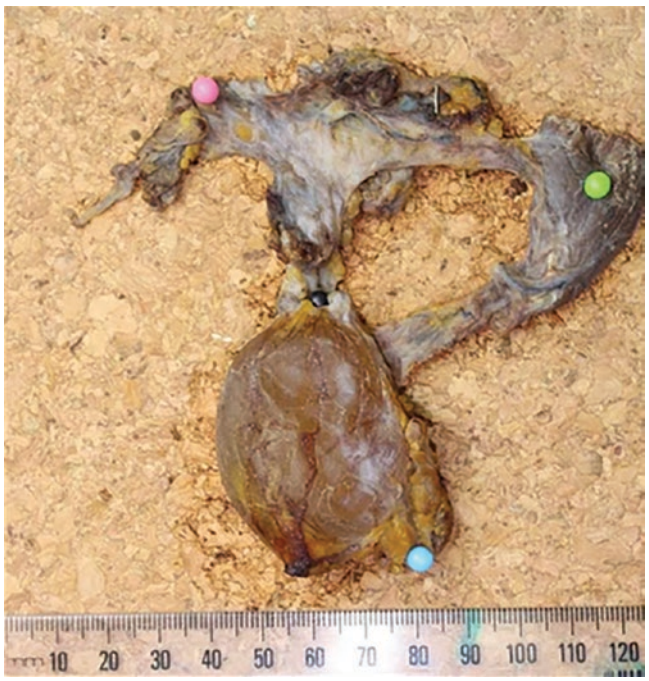


Fig. 10 A neck dissection specimen, including a level III cystic mass, pinned on a cork board and orientated with coloured pins

Fig. 11 (**a**) Drawings made with four different marking pens on cork boards (BIC vivid™, Premier™ ballpoint, Artline 210™ medium, Sharpie finepoint™ from left to right). The cork boards were then submerged in 10% neutral buffered formalin for 24 h. (**b**) The same drawings after 24 h. Some pens resist formalin but are more difficult to use. Use a convenient pen in theatre but test its formalin resistance in advance

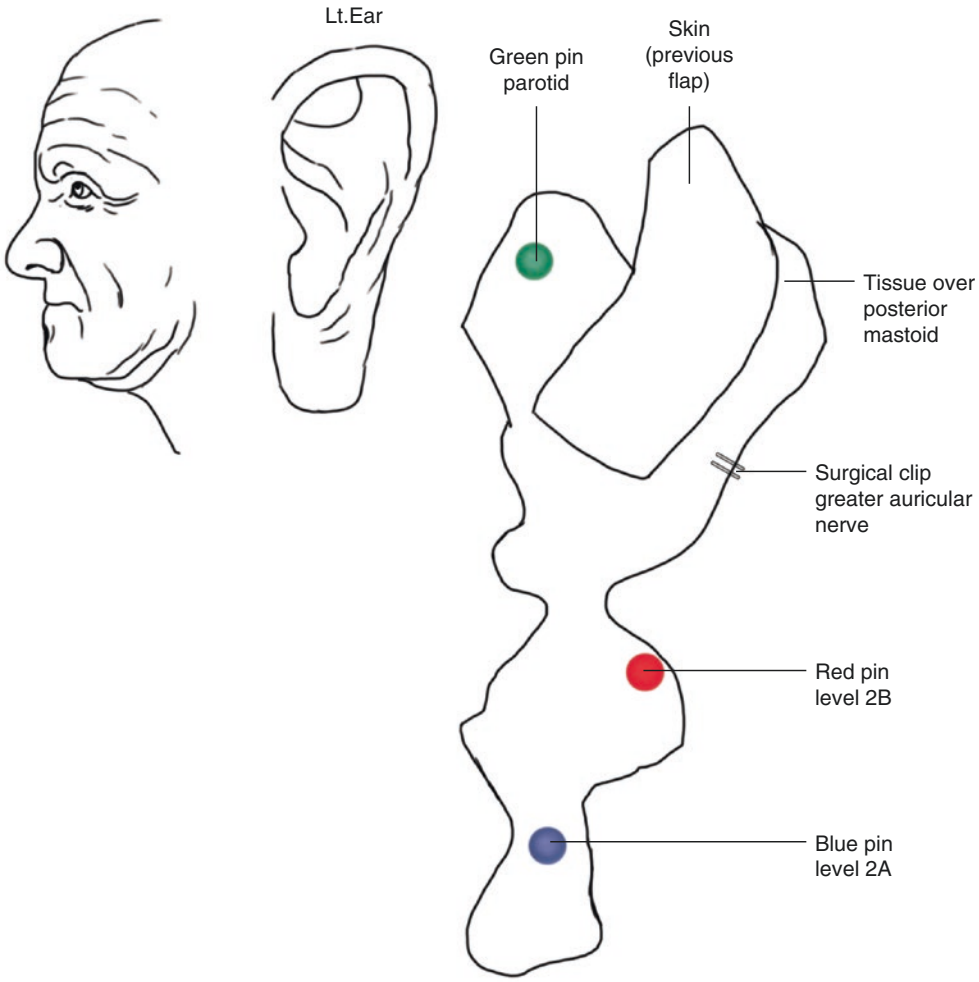


Fig. 12 Example of a drawing for which your pathologist would be very grateful

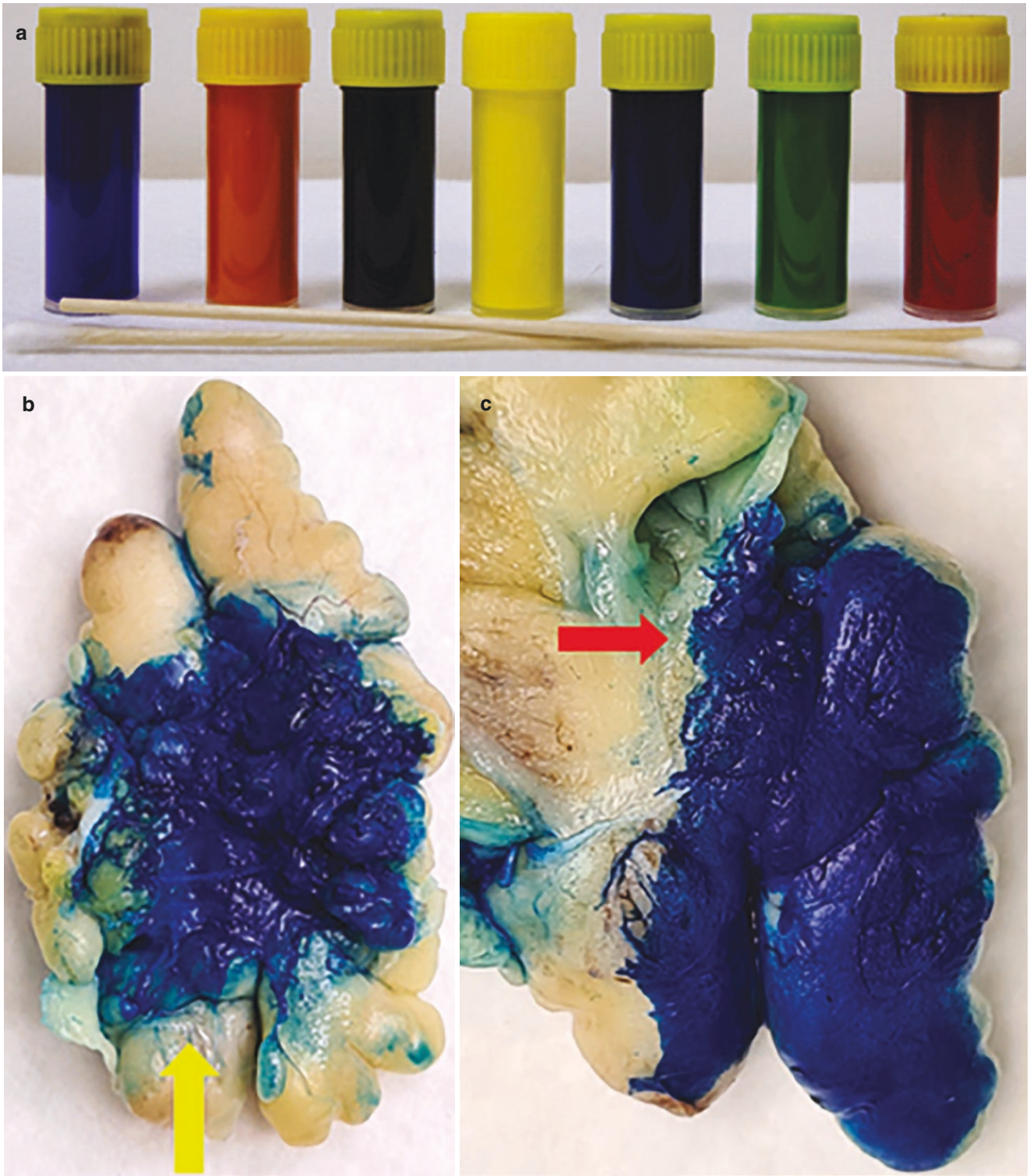


Fig. 13 (**a**) A range of ink colours (Aero Color Professional finest acrylic ink™) is available and can be used to orientate specimens. The ink can be applied with cotton swabs. Be sure to annotate which ink colour corresponds to which margin or other anatomical point of interest. (**b** and **c**) A lipoma inked with and without a mordant. (**b**) The lipoma has been inked with blue ink without a mordant. The inked margin denoted by the yellow arrow is fuzzy, and the ink has smudged and run into all the adjacent cracks. This problem may result in a pathology report with a false-positive margin. (**c**) The same lipoma has been inked with blue ink which has then been set with acetic acid (vinegar). The margin denoted by the red arrow is much sharper

6 Transporting a Specimen

Tissue undergoes cytolytic degeneration very quickly once extracted from the body. The smaller the volume of tissue, the more rapidly this degeneration occurs. Therefore it is crucial to prepare slides and to place any remaining material into an appropriate transport medium swiftly, for immediate preservation. The volume of transport medium should be at least ten times the volume of the specimen. Neutral buffered formalin is perhaps the best-known medium. There are situations, however, where alternative mediums are preferred. Table 4 below gives advice about which transport medium to use in different situations.

1. 10% neutral buffered formalin

   Formalin is formaldehyde gas dissolved in water. Formaldehyde stabilises ("fixes") tissue by creating crosslinks in macromolecules like proteins. Raw formalin is an acid solution, which reacts with haemoglobin to give a black precipitate, hampering subsequent histological assessment. To avoid this problem, a buffer such as sodium phosphate is added to the solution, to create "neutral buffered formalin".

   As long as formalin can penetrate all parts of the specimen, tissue preservation usually is consistent and reliable. For most core biopsies, punch or small open biopsies where the working diagnosis does not include lymphoma or require identification of microorganisms by culture, formalin remains the default option as the transport medium. Formalin-fixed paraffin-embedded tissue can be processed for routine haematoxylin and eosin staining and for immunohistochemistry and other molecular studies.
2. Alcohol-based transport medium

   Alcohol stabilises tissue by coagulation. Alcohol-based preserving transport medium usually is supplied commercially in individual vials. Two products used commonly in Australasia are ThinPrep Cytolyt™ (by Hologic) and CytoRich™ (by BD). They are similar in the way that they render cells as monolayer films on the slides. Alcohol-based preservatives have become more popular over the years due to the consistency they produce, the ease of use and good level of cell preservation. Cells preserved in alcohol-based medium will be suitable for routine cytomorphology (either in addition to a direct smear or without), haematoxylin and eosin histology, immunohistochemistry and molecular studies [12].
3. Roswell Park Memorial Institute Medium (RPMI)

   RPMI (also known as RPMI 1640) is a nutrient-rich medium containing 19 different amino acids, 11 vitamins, sodium bicarbonate and various salts. Iscove's Modified Dulbecco's Medium (IMDM) is a similar medium but with a higher calcium concentration. RPMI and IMDM are not fixatives. They do not preserve cells but maintain viability of adherent cells. A nutrient-rich medium is the preferred medium for preserving and transporting cells destined for flow cytometry and mutational studies, including karyotyping. These nutrient-packed media need to be stored in a dark chilled environment (recommended storage temperature is 2–8 °C). They contain a pH-sensitive indicator to monitor possible degradation. Too long elapsed time and failure to store the medium in an appropriate manner will lead to degradation which is recognised by a change in colour from salmon orange to dark pink (Fig. 14).
4. Normal saline

   Normal saline is the simplest transport medium. Without any cell fixing property or nutrient, normal saline is not a good choice if the sample is expected to be in anything but urgent transit. However, if a specimen in saline can be sent to the laboratory sufficiently quickly, the specimen can be used for routine histology (cell block); flow cytometry; biochemical studies, e.g. thyroglobulin level for metastatic thyroid carcinoma; microorganism culture; and potentially molecular studies.

   If a wide differential diagnosis has to be considered, more material than usual is required, which may require multiple specimens. Preparing multiple samples can be a complex exercise. If a pathologist or a trained lab technician can attend for rapid on-site evaluation, specimen triaging can be done immediately, and the differential diagnosis may be narrowed, reducing the number of specimens required.

Fig. 14 The bottle on the left shows the normal colour of IMDM. The bottle on the right shows a degraded IMDM which is unsuitable for specimen preservation

Table 4 Recommended transport mediums for different clinical scenarios and tests potentially to be done

Clinical scenario	Recommended medium		Tests to be done
	FNA	Biopsy	
Almost certainly carcinoma (including nodal metastasis) Non-nodal lesion, uncertain whether benign or malignant (lymphoma not being considered)	Alcohol-based medium or saline	Formalin	Histology with or without immunohistochemistry
Carcinoma and lymphoma both being considered	Preferably one pot with alcohol-based medium and a second pot with saline	One pot with formalin and a separate pot with either RPMI or saline	Histology with or without immunohistochemistry Flow cytometry
Carcinoma, lymphoma and infection all being considered	Same as above plus an extra pot with saline	Same as above plus an extra pot with saline	Histology with or without immunohistochemistry. Flow cytometry Bacterial and mycobacterial culture/sensitivity

7 Ancillary Studies

Only decades ago, a simple distinction between benign and malignant tumours was enough for cancer management. Up until the 1990s, classifying lung cancer as small cell carcinoma versus non-small cell carcinoma was all that was required for treatment. Tumour classification, including for head and neck tumours, has become much more complex and beyond the capacity of simple light microscopy alone. So-called ancillary studies have become essential components of diagnostic anatomical pathology. Special stains, immunohistochemistry, cell markers and molecular studies are amongst the techniques that a modern pathologist uses.

8 Special Stains

Use of special stains probably was the first step away from simple light microscopy and haematoxylin and eosin. The advent of more sophisticated ways of marking tissue has resulted in a decline in the use of special stains in diagnostic pathology. The few stains that still are used fairly commonly today include the mucin stains (e.g. mucicarmine) used to identify glandular differentiation, the Perl's stain (Perl's Prussian blue) to aid in the distinction between haemosiderin and melanin, silver stains (e.g. Grocott-Gömöri methenamine silver stain) to identify fungal elements and Congo red stain for amyloid.

9 Immunohistochemistry

Immunohistochemistry has revolutionised diagnostic surgical pathology. There is a growing catalogue of immunohistochemical markers for histology. Whilst the interpretation of immunohistochemical markers can be challenging, the basic mechanism of action of immunohistochemical marking is quite simple. Immunohistochemical markers contain antibodies that bind to specific antigens and a coloured histochemical tag that makes the antigen-antibody binding visible. Most pathology laboratories tend to use a brown tag (chromogen) like 3,3′-diaminobenzidine tetrachloride [13].

Every tumour type has a unique antigen profile. Pathologists rely on those profiles to aid in a diagnosis. The catalogue of immunohistochemical markers already is extensive. In Table 5 we list some that one may encounter in pathologists' reports concerning the histological diagnosis of head and neck cancer.

Table 5 Immunohistochemical markers used commonly in histological diagnosis of head and neck cancers

Pan cytokeratin (Pan CK)	General epithelial marker. Positive in most if not all carcinomas regardless of origin/primary site
CK5, CK14	More specific cytokeratins. Positive in most squamous carcinomas. Highlight cell cytoplasm
CK20	Specific cytokeratin used typically to diagnose carcinoma of the intestinal tract, but in the context of head and neck skin cancer, positive in Merkel cell carcinoma
p40, p63	Squamous as well as myoepithelial markers. Highlight cell nuclei
BerEP4	An epithelial cell adhesion molecule. In skin cancer it is most useful in the distinction between poorly differentiated squamous carcinoma (negative) and basal cell carcinoma (positive)
Calponin	Myoepithelial cell marker
S100	Positive in most melanomas but also marks neural tissue
SOX10	Melanoma marker but also can mark myoepithelial cells
MelanA	Another melanoma marker
PRAME (preferentially expressed antigen in melanoma)	A relatively new marker. Positive in melanoma. Extremely useful in distinguishing between metastatic melanoma in lymph node (positive) and benign melanocytic nevus in lymph node (negative)
Synaptophysin, chromogranin	Neuroendocrine markers. Positive in Merkel cell carcinoma
DOG1	Positive in normal salivary serous acini and also in acinic cell carcinoma
Androgen receptor	Positive in salivary duct carcinoma
HER2	80% of salivary duct carcinomas show HER2 overexpression. HER2 overexpression is associated with a poorer prognosis, but these tumours may respond to a HER2 inhibitor [15]
Mammaglobin and GCDFP (gross cystic disease fluid protein)	Both are well known as breast markers. Positive in secretory carcinoma of salivary gland (previously known as mammary analogue secretory carcinoma)
p16	Surrogate marker for high-risk human papillomavirus. Positive in HPV-mediated non-keratinising squamous cell carcinoma of oropharynx
Ki67	Proliferation marker. The more nuclei this marker highlights, the more active or fast-growing the tumour is
BRAF V600e	This BRAF immunomarker for melanoma is a cheaper and more rapid substitute for the more comprehensive BRAF molecular test (Oncofocus™) and can be used in urgent cases to guide treatment options. The Oncofocus™ test is a next-generation DNA sequencing study which targets more than 250 different mutations affecting major oncogenes such as BRAF, EGFR, KIT, KRAS and NRAS

10 Flow Cytometry

Flow cytometry is a technique for detecting and measuring properties of cells from their diffraction patterns as they transit through a laser beam [14]. Flow cytometry is a highly efficient way of identifying a cell population of interest in a large volume of tissue. In head and neck cancer, it is particularly helpful in determining if there is clonality of lymphoid cells, indicating likely lymphoma. Flow cytometry is hardly ever used in the diagnosis of carcinoma. Flow cytometry requires fresh tissue so specimens should be submitted in RPMI/IMDM (preferred) or normal saline (see "transporting a specimen"). The specimen cannot be submitted in formalin or alcohol-based fixative.

11 Molecular Studies

Fluorescence in situ hybridization (FISH) has become an integral part of diagnostic pathology. In head and neck pathology, it plays an important role in the diagnosis of salivary gland tumours and sarcomas (Table 6). FISH is a cytogenetic technology based on the complementary nature of DNA and RNA double strands. FISH probes are single-strand fragments of DNA or RNA that bind to complementary nucleotide sequences in target cells, including tumour cells [16]. The probes are tagged with fluorescent nucleotides.

Table 6 Some of the common nucleotide abnormalities that can be revealed by FISH in head and neck pathology [22]

PLAG1 fusion	Pleomorphic adenoma
MAML2 translocation	Mucoepidermoid carcinoma
MYB fusion	Adenoid cystic carcinoma
ETV6-NTRK3 translocation	Secretory carcinoma
SS18 translocation	Synovial sarcoma
FUS or EWSR1 translocation	Myxoid/round cell liposarcoma
FUS translocation	Low grade fibromyxoid sarcoma
USP6 translocation	Nodular fasciitis

Two common types of FISH probes used in diagnostic pathology are break-apart and fusion probes. The probes are used to detect translocations, the most frequent genetic defects in cancer cells. Break-apart probes reveal nucleotide sequence separations that occur typically in a cancer cell. Two tags of different fluorescent colours are placed at opposite ends of a probe with a nucleotide sequence complementary to a targeted gene breakpoint. In normal cells, there is no break in the gene,

so under fluorescence microscopy one observes the two tags (usually one red and one green) joined as a pair. When there is a translocation at that gene location, there is a break between the two tags, so they separate into individual colours. Fusion probes work in the opposite way and are used to reveal a specific rearrangement of nucleotides. Markers of different colours are placed on two different gene locations. In normal cells, the red and green signals remain separate, but when there is an abnormal fusion of those gene locations, the two colours emerge as a pair. FISH cytogenetics can be done on formalin-fixed paraffin-embedded tissue.

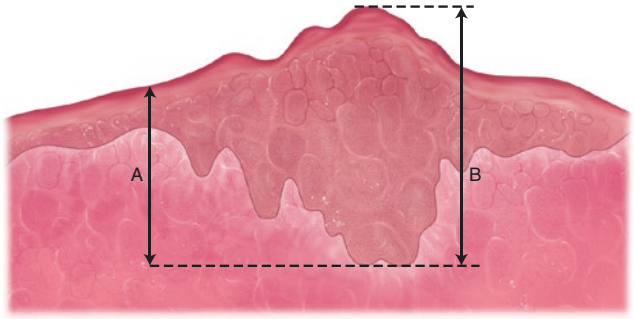


Fig. 15 Illustration of tumour thickness versus depth of invasion. (**a**) Depth of invasion is measured from the top of the granular layer of adjacent uninvolved skin to the base of the tumour (AJCC eighth edition). (**b**) Tumour thickness is measured from the granular at the apex of the tumour to its deepest base

12 Making Sense of the Pathologist's Report

In the past pathology reports were entombed in long-winded text, but often important information was omitted. In recent times pathologists submitting reports on most malignancies rather use synoptic reports constructed on a prescribed format that ensures that all the relevant information is covered and succinctly. They frequently employ the TNM staging system of the American Joint Commission on Cancer (AJCC), which assigns T codes for the extent of the tumour, N codes for lymph node involvement and M codes reporting whether metastases are present or not. The benefits of synoptic reports include completeness of pathologists' reports for surgeons or other clinicians, easier readability and digestibility of information and providing a useful framework for research. Currently the synoptic reports tend to be paper-based, but in the future they undoubtedly will move to web-based systems, which, amongst other benefits, will allow for greater accuracy through the body of the report [17].

To help clinicians make sense of pathologists' reports, we comment on concepts that appear routinely in synoptic reports, in the context of squamous carcinomas, melanomas, basal cell carcinomas and salivary gland carcinomas, the most frequent tumours of extreme facial malignancies.

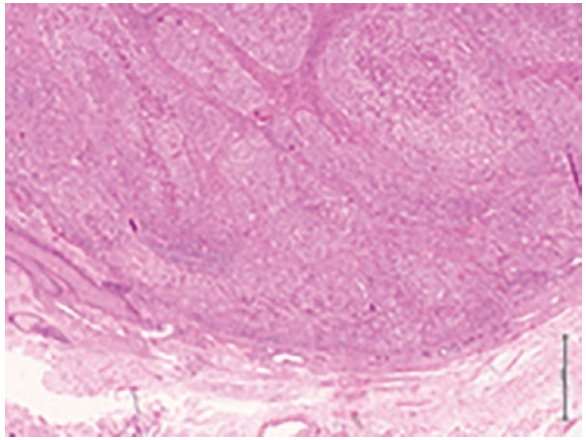
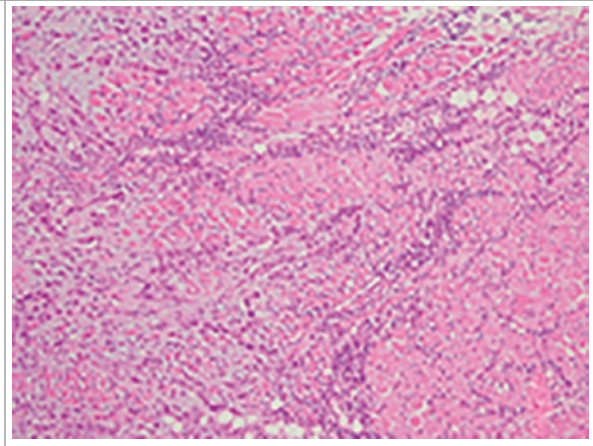
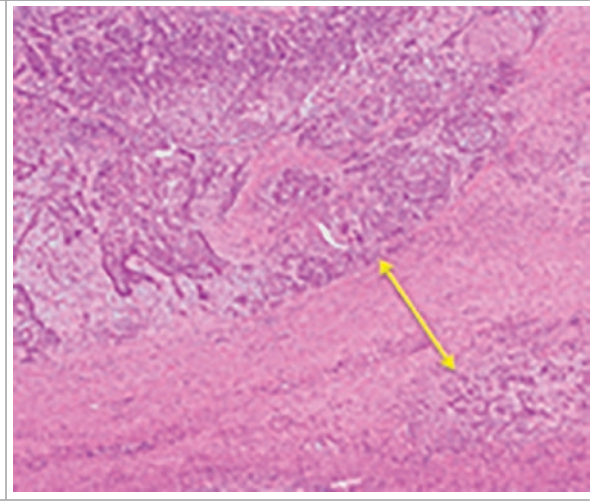
12.1 Tumour Thickness and Depth of Invasion

Tumour thickness and depth of invasion are not synonymous terms (Fig. 15). Tumour thickness refers to the largest vertical dimension of the tumour, whilst depth of invasion gives an indication of how far a tumour has invaded into normal tissue. Tumour thickness/depth of a primary tumour is associated with adverse biological outcomes like risk of recurrence, metastasis or death [18]. The thicknesses of melanomas and depth of invasion of squamous cell carcinomas are measured differently. Melanomas are measured from the granular layer of the epidermis to the deepest level of invasion in the area of the tumour, whereas squamous carcinomas are measured from the granular layer of the nearest adjacent normal epidermis to the deepest part of the tumour. This method for squamous carcinomas adjusts for exophytic lesions. Ulcerated melanomas may have their thickness underestimated. Oral cavity carcinomas are measured differently.

12.2 Pattern of Invasion at the Tumour Front

Table 7 shows the three main patterns of tumour growth at the infiltrative front.

Table 7 The three main patterns of tumour growth at the infiltrative front

Cohesive	Non-cohesive	Dispersed
Large islands of cells	Small nests and single cell infiltration	Widely separated satellite foci
Pushing border	Infiltrative border	Cells more than 1 mm (yellow arrow) from the main tumour
Slow-growing tumour	Aggressive tumour	Most aggressive tumour
		

12.3 Perineural Invasion

Perineural invasion (Fig. 16) occurs when a tumour has invaded into the nerve sheath [19]. Squamous carcinomas of the head and neck often affect nerves and those that do tend to have poor outcomes, as the tumour may track the cranial nerves back to the brain. AJCC eighth Edition recognises the importance of perineural invasion in the staging for primary cutaneous carcinoma; involvement of a named nerve, an unnamed nerve of at least 0.1 mm diameter or an unnamed nerve deeper than dermis will upstage a tumour from a pT2 to pT3. For some of the salivary gland tumours, the presence of perineural invasion aids with the diagnosis of malignancy. This feature is particularly prominent in adenoid cystic carcinomas. Perineural invasion far away from the invasive front portends a poor prognosis.

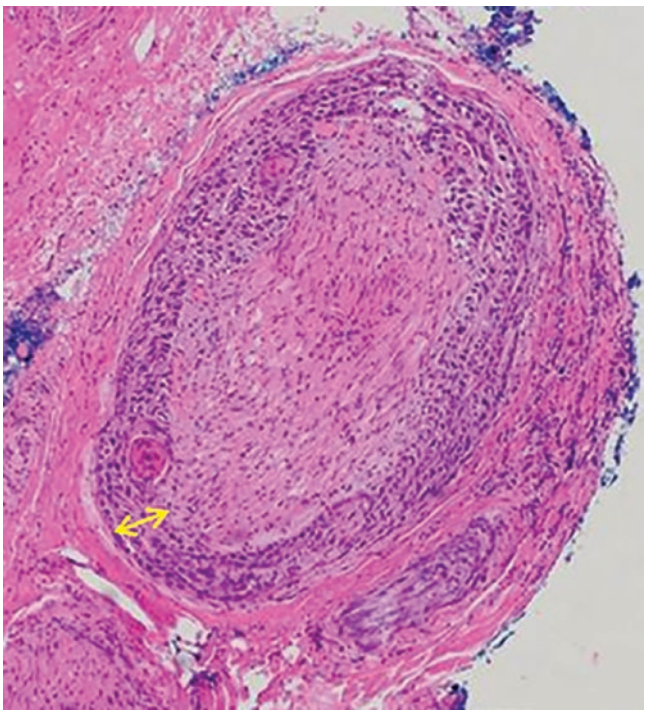


Fig. 16 Perineural invasion by moderately differentiated keratinising squamous carcinoma (haematoxylin and eosin 100× magnification). The nerve is surrounded by squamous carcinoma that is depicted by the yellow arrow

12.4 Margin Status

Pathologists pay a lot of attention to the margins of surgical specimens, especially resections for malignancy, because it is the condition of the margins that determines the completeness of the resection (Fig. 17). Sections can be taken either perpendicularly to the margin or *en face* at the margin. Perpendicular sections allow the pathologist to visualise the distance between the tumour and the surface of the specimen (the surgical margin) as both tumour and margin appear in the same section and so to advise the surgeon, if the margin is negative, how close to the resection surface the malignant tissue was. If the tumour is close to the margin, the pathologist can measure and report its separation from the margin using the graticule within the microscope's eyepiece or the scale on the microscope's stage. An *en face* section allows the pathologist to decide only whether the specimen is positive or negative for malignancy, so whether the margin from which it was taken was healthy or diseased.

What do pathologists report as a positive margin? If a specimen has been inked and tumour extends to the inked surgical margin, that margin is reported as positive. If tumour extends to a cautery artefact, that too is reported as positive. In some situations, for instance, in the case of a basal cell carcinoma, if the characteristic stroma of the tumour extends to the margin, even though the actual epithelial tumour islands are not at the margin, the margin also is reported as positive.

An adequate excision depends on the type of tumour but also on the anatomical site of the tumour. If a tumour has been excised inadequately, does the anatomy allow room for additional surgery? If not, is the tumour type amenable to treatment with modalities other than surgery? Again communication between the surgeon and the pathologist is beneficial to arrive at the decision best for the patient, given the evaluation of the status of the margins, especially if the specimen is fragmented.

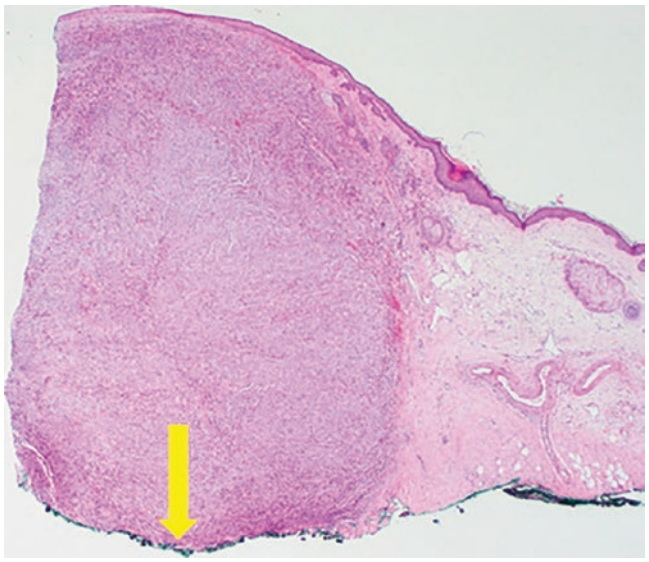


Fig. 17 Atypical fibroxanthoma extends to the deep margin (yellow arrow) (haematoxylin and eosin 100×). The differential diagnosis of an atypical fibroxanthoma includes a pleomorphic dermal sarcoma. One of the distinguishing features is extension into subcutaneous tissue. In this biopsy where the tumour is extending to the deep margin and the subcutaneous tissue is not visualised, a pleomorphic dermal sarcoma cannot be entirely excluded

12.5 Nodal Status and Pathological Staging

To deliver a good service, pathologists would like a selective neck dissection to yield at least 10 lymph nodes and a comprehensive neck dissection to yield 15 or more lymph nodes. We consider the proportion of nodes that are positive, the maximum dimension of the largest node and that of the largest metastatic deposit as important elements in the pathological assessment, though the UICC and AJCC staging systems base their pN categories on the size of the involved node, rather than the size of the tumour deposit. In some cases there may be a considerable difference in the dimensions of the largest metastatic deposit and the largest involved node, and, when both sizes are recorded, the oncologist will have extra information upon which to plan treatment.

Another element that we consider, and report, is extranodal extension, which is the spread of tumour outside the capsule of the lymph node into the perinodal soft tissue (Fig. 18). It is measured perpendicular to the external aspect of the node capsule and may be categorised as microscopic (≤2 mm in extent) or macroscopic (>2 mm in extent). Extranodal extension is a risk factor for a poor prognosis in cervical node positive carcinoma, except in HPV-mediated oropharyngeal cancer, where its significance has yet to be established. The presence of extranodal extension in head and neck cancers correlates with a risk of regional local recurrence and distant metastasis and may be an indication for adjuvant combined chemotherapy and radiotherapy. The risk of regional recurrence and distant metastasis is higher with macroscopic rather than microscopic extranodal extension. Tumours with extranodal extension should be excised with a clear margin. If a margin is positive, the risk of local recurrence is increased and is an indication for radiotherapy to that site [20].

We draw attention to changes relevant to interactions between clinicians and pathologists that have been introduced in the eighth edition of the AJCC [21] and also in the International Union for Cancer Control (UICC) cancer staging manuals. These changes have resulted in changes to the synoptic reports and staging of tumours. The changes include:

1. Separating p16-positive HPV-related carcinomas from p16-negative carcinomas.
2. Including extranodal extension in the pN categorisation for p16 negative oropharyngeal, hypopharyngeal, oral cavity, laryngeal, skin, major salivary gland, nasal cavity, paranasal sinus and unknown primary cancers.
3. Introducing a separate category for occult primary tumours of the head and neck with p16 and Epstein-Barr virus testing recommended.
4. Introducing a separate chapter for cutaneous squamous cell and other carcinomas (with the exception of Merkel cell carcinoma).

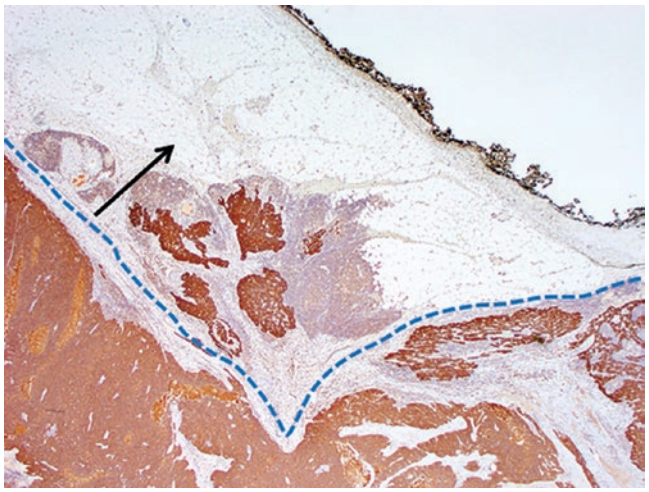


Fig. 18 Extranodal extension (Pan cytokeratin immunohistochemical stain 40× magnification). The black arrow perpendicular to the node capsule (indicated by dashed blue line) shows how extranodal extension is measured. The extranodal extension is clear of the surgical margin, inked black

13 A Take-Home Message from Pathologists

We have belaboured the theme of cultivating the clinician-pathologist relationship and amalgamating the diverse wealth of information to benefit patient care. A small consideration by clinicians about what they are going to biopsy and what they want to achieve with that biopsy, before taking the specimen, can make all the difference in the value to the clinician of what the pathologist can do subsequently. We cannot stress enough the importance of providing pathologists with all the relevant clinical and radiological details; our own experience is that important information is omitted regrettably often. Recognising, and making sense of, those features in a pathology report that may influence treatment will impact your patient's prognosis. We also submit that cultivating the clinician-pathologist relationship is personally stimulating, educational and fulfilling. Take time to ponder. We look forward to hearing from you soon.

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