29 The Preparation of Biopsy Specimens for Routine and Molecular Cytology: Technical Steps, Pearls, and Pitfalls

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29.1 Introduction

The importance of optimal specimen preparation cannot be over emphasized. Poor specimen preparation hinders the optimal cytopathologic evaluation. Cytopathology specimens have numerous methods of collection, preparation, fixation, and staining.

29.2 Collection

One method for thyroid fine-needle aspiration (FNA) collection is as a rinse for liquid based fixation samples. The rinse collection method may be used for a liquid based cytology preparation such as ThinPrep®. This liquid based cytology preparation allows for the FNA samples to be rinsed directly into a liquid fixative. Each pass from the same nodule is rinsed into the same vial. It is important to repeat the aspirating, rinsing, and expressing of the needle in the liquid fixative to elute all of the sample from the needle hub.

Liquid based collection can be used to prepare a cell block. In this collection method, the aspirated specimens are expressed into a formalin fixative cup. Although not necessary for thyroid FNA, this method allows the sample to be processed as a paraffin-embedded block, which may be helpful when immunohistochemical stains are needed to exclude metastatic disease.

For techniques using slides, prepared as classic smears or bookends, the FNA specimen is procured in the same fashion. However the specimen is placed directly on the glass slides instead of into a liquid. This method allows for multiple slides to be prepared for different types of fixation and stains. Since the slides can be made at the bedside and stained rapidly, it allows for immediate assessment of adequacy or ROSE (Rapid on Site Evaluation).

29.3 Preparation

To prepare the liquid based slide, the vial containing the sample is sent to the lab and placed on an instrument to process the sample. The vial is centrifuged to form a concentrated pellet. The pellet is then resuspended and an aliquot placed into the PreservCyt solution vial. This solution is left to stand for 15 min and then processed on the ThinPrep ® machine using the non-gyn sequence. From this vial, generally a single slide is made that is a representative cellular collection from all the FNA passes rinsed into the container. This resulting slide is a monolayer of the aspirated

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cells. The fact that this method typically produces only one slide may be considered as a drawback. Since only one slide is produced, only one staining method can be utilized, so the ability to evaluate the specimen with two stains, both a Papanicolaou and Romanowsky, is lost [1]. In this setting, the single slide is stained with a Papanicolaou stain (Fig. 29.1). This method has been gaining in popularity; however, its utilization has been controversial particularly in thyroid where extracellular matrix (colloid or amyloid) and architectural features are important. From the collection perspective, it is very simple to rinse the entire specimen into the vial and lack of smearing eliminates possible crush artifact. From the diagnostic perspective, the mechanical distribution results in an even monolayer of specimen to be evaluated in a smaller area of the slide. The downside is that background elements, such as blood and colloid, are decreased, resulting in a higher concentration of the cellular elements; this makes the diagnosis of hyperplasia versus neoplasia more challenging. Another concern with liquid based preparations is the lack of optimal cytologic features needed for the diagnosis of papillary thyroid carcinoma. This lack of crisp nuclear diagnostic features led to a false negative diagnosis in 7% of liquid based cases compared to smear methods [2].

Some liquid specimens can be used for a cytospin preparation. This method concentrates the liquid sample and a portion is placed in a cytofunnel on a cyto-centrifuge machine. This method produces a monolayer of cells within a well-defined area of the slide. Depending on the amount of the sample, multiple slides can be made for different types of stains, though the Papanicolaou stain is the most common. This method is most commonly used when the cellularity of a sample is scant (Fig. 29.2).

Cell blocks are made from the formalin-fixed collection sample. The cell block can be prepared via several different methods, based on the techniques available to the specific lab. The sediment, clot, or tissue fragments are processed and embedded in paraffin. The paraffin block is cut on a microtome that makes thin tissue sections subsequently placed on glass slides. The end product is

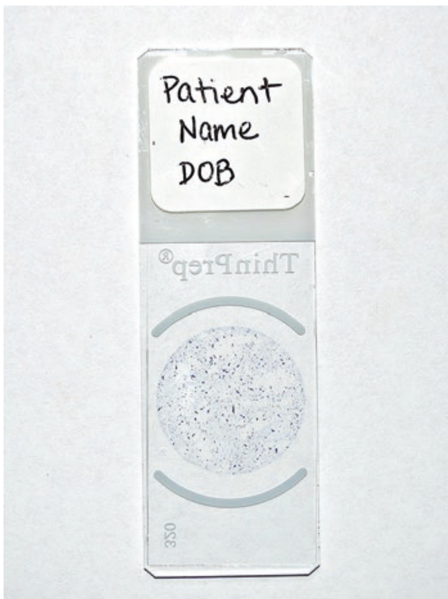

Fig. 29.1 Liquid-based slide prepared on Thinprep machine and stained with Papanicolaou stain. The single slide provides a representative monolayer of the aspirated material

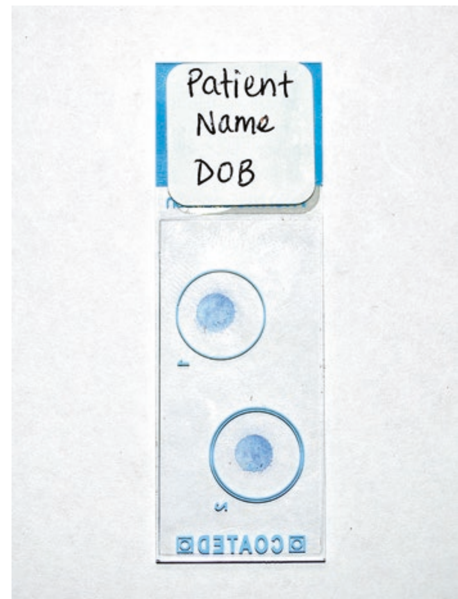

Fig. 29.2 A Cytospin preparation stained with Papanicolaou stain after liquid fixative collection method. This method is often performed when the sample collected is small

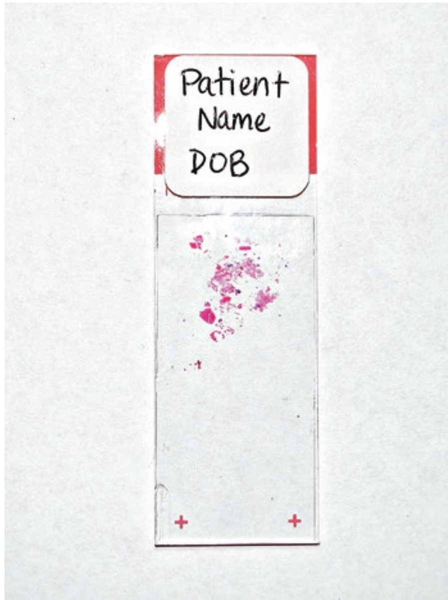

Fig. 29.3 A representative slide of hematoxylin and eosin stained cell block specimen. These samples are collected in a liquid fixative and paraffin embedded for cutting sections to place on slides

the traditionally hematoxylin and eosin (H&E) stained slides. The author's laboratory does not routinely use cell blocks. In our experience this method does not add additional information unless metastatic carcinoma needs to be confirmed with immunohistochemical stains (Fig 29.3).

There are numerous methods for direct slide preparation. To prepare smears the aspirate is expelled onto the slide with the needle tip bevel down touching the glass to minimize spraying of the sample and possible air-dry artifact for any alcohol-fixed slides.

29.3.1 Classic Smear Technique

1. Label frosted end with two patient identifiers (name, date of birth, medical record, etc.) using a #2 pencil
2. Place a small drop of the aspirate a few millimeters from the frosted end
3. Place another slide at the edge of the drop at a 45° angle and allow the sample drop to spread along the edge of the second slide
4. Decrease the angle, then gently and quickly, push the specimen evenly away from the frosted end down the slide, trying to keep the smear at least 1 mm from the edges of the glass
5. Immediately alcohol or spray fix one slide for Papanicolaou stain to be performed in the lab. The other slide can be air-dried and stained with a modified Romanowsky for ROSE or in the lab (Fig. 29.4a, b).

Depending on the amount of material aspirated, multiple slides can be made. Optimally, one air-dried and one alcohol-fixed slide should be made from each pass. This allows for ROSE to be performed if desired and allows for evaluation of the specimen using both stains.

29.3.2 Bookend Smear Technique

1. Label two slides at the frosted end with two patient identifiers (name, date of birth, medical record, etc.) using a #2 pencil.
2. Place a small drop of the aspirate in the middle of one slide.
3. Place the labeled, frosted side of the second slide face down onto the slide with the sample drop.
4. Allow the sample to spread by capillary action, do not apply pressure.
5. When the sample stops spreading, open the slides like a book, or pop them open. DO NOT SLIDE THEM ACROSS EACH OTHER.
6. This will produce two mirror image slides. Immediately alcohol or spray fix one slide for Papanicolaou stain to be performed in the lab. The other slide can be air-dried and stained with a modified Romanowsky for ROSE or in the lab (Fig. 29.5a, b).

This method ensures that each FNA pass results in one slide for an air-dried and one slide for an alcohol fixation method. ROSE can be performed if desired on the air-dried slide. Bookend smears render slides as mirror images of each other allowing mates for the Papanicolaou and Romanowsky stained slides from each pass. This unique feature provides the ability to evaluate atypical cells seen on the Romanowsky slide again on the Papanicolaou

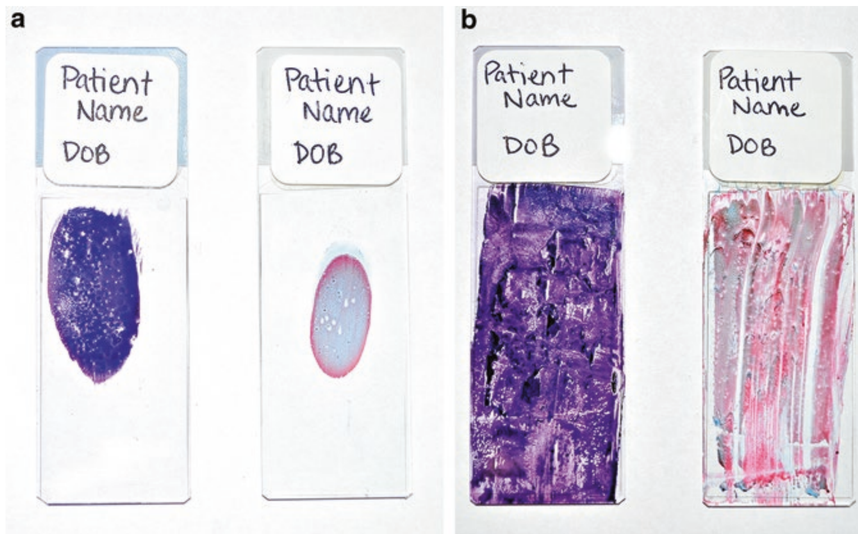

Fig. 29.4 (**a**) Properly prepared direct slide preparation with classic smears. The left slide is a Romanowsky stain stained smear and the right is a Papanicolaou stained smear that has the proper amount of specimen and keeps the smear edges at least 1 mm from the edges of the glass slide. (**b**) Improperly prepared direct slide preparation with classic smears. The left slide is a Romanowsky stain stained smear and the right is a Papanicolaou stained smear. Both slides have too much specimen causing the sample to cover the entire slide. The smearing pressure was uneven causing irregularities in the smear as well

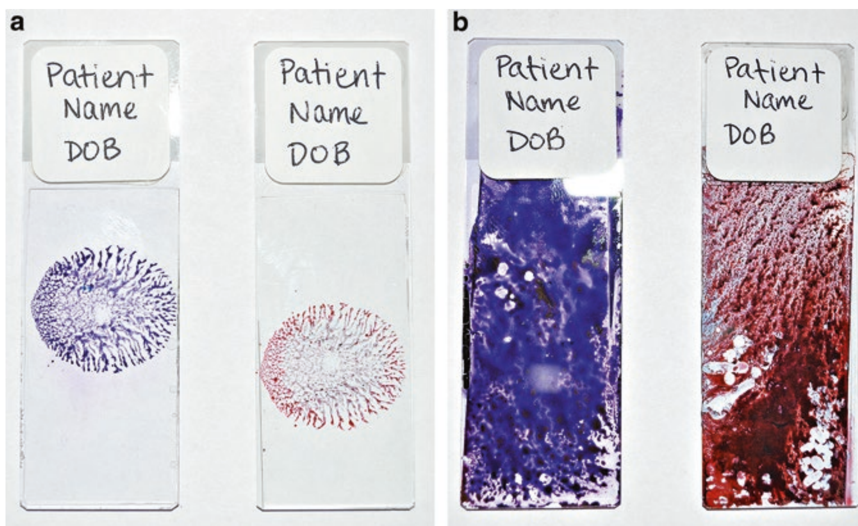

Fig. 29.5 (**a**) Properly prepared direct slide preparation with bookends method. The left slide is a Romanowsky stain stained smear and the right is a Papanicolaou stained smear that has the proper amount of specimen and creates mirror image slides from one pass. (**b**) Improperly prepared direct slide preparation with bookends. The left slide is a Romanowsky stain stained smear and the right is a Papanicolaou stained smear. Both slides have too much specimen causing the sample to cover the entire slide. The cover slip does not sit well on the specimen due to the increased thickness of the sample. The thickness issue could have been fixed by applying an additional slide to remove the excess sample, thus providing additional better prepared slides

Table 29.1 Comparison of different specimen preparation and fixation techniques

Differences in thyroid FNA preparations		
Prep and fixation		
Liquid based	Classic smears	Bookends
Liquid vial collection	Slide collection	Slide collection
Wet fixation only	Wet and/or dry fixation	Wet and/or dry fixation
Papanicolaou stain only	Pap and/or Romanowsky stain	Pap and/or Romanowsky stain
Single slide to evaluate	multiple individual slides	Multiple slides (mirror images for Pap and Romanowsky)
Consistent slide quality	Quality dependent on practice	Quality less dependent on practice
Even monolayer	Uneven with larger area to screen	Uneven with moderate area to screen
ROSE[a] not eligible	ROSE eligible	ROSE eligible
Diagnostic features		
Decreased colloid	Colloid visible on Romanowsky	Colloid visible on Romanowsky
Decreased background blood	Some blood lysis on wet fixed slide	Some blood lysis on wet fixed slide
Large fragments are broken	Large papillae retained	Large papillae retained
Intranuclear inclusions reduced	Intranuclear inclusions retained	Intranuclear inclusions retained
Loss of psammoma bodies	Psammoma bodies visible	Psammoma bodies visible

[a]*ROSE* rapid onsite evaluation

slide. The area of atypia on the Romanowsky slide should be matched to the area on the mirror image Papanicolaou slide to try to clarify the atypia. While this method causes some increase in three dimensional features it has minimal overall effect on the cytologic features used for thyroid FNA diagnoses.

For either of the smear methods, more sample does not yield more diagnostic material or better slides. For the non-pathologist, this may seem counterintuitive but only a small quantity of appropriately obtained sample is required to make adequate slides for evaluation. Either preparation method may result in too thick a sample if too much is placed on the slide. It is harder to correct a poorly made classic smear than a bookend smear. If the bookend smear is too thick, an additional labeled slide can be touched to the original slide to remove excess sample and all slides can be stained as desired (See Table 29.1).

29.4 Fixation

Cytopathologists' preferences for fixation methods differ depending on the type of stain they prefer to use for specimen interpretation. The author prefers to use both alcohol wet fixed slides and air-dried slides because some cytologic features are more readily appreciated from one stain over the other. Certain specimen preparation methods allow for a single aspiration to be split into two or more slides allowing at least one slide to be fixed wet and one to be fixed dry.

Once smears or bookends have been made, it is imperative that the slides to be wet fixed are processed rapidly. There are several methods for wet fixation of the specimen. Spray fixative for the slides that will undergo Papanicolaou staining is quick and easy to perform; however, it requires additional prep time in the lab. The spray fixative must be soaked off in 95 % ethyl alcohol prior to the staining of the slides. If this is not done, poor staining quality is noticed. Spray fixative does not lyse any of the blood in the slide, which can be detrimental if the specimen is bloody. The slide should be held 12 in. from the spray, and the spray should be applied without delay, well coating the specimen. Any delay will cause air-dry artifact when performing the Papanicolaou stain. Well coated follicular cells will be well preserved and well visualized if the smear in not too bloody and thick.

Fixation in 95 % ethyl alcohol is quick and easy. Since the slide is placed directly into the 95 % alcohol container, the process is faster and

the specimen has a more even appearance on the slide vs the droplet appearance of the spray fixative. The slides do not have to be sent in the alcohol as long as they have been soaked for at least 15 min [3]. Since some FNA specimens are bloody, the fact that alcohol lyses some of the background blood can be beneficial. Cell sizes are similar to that seen in tissue specimens; however, smaller than the enlarged cells of air-dried slides.

A less commonly used wet fixative is saccomannos. It is composed of 2 % carbowax or polyethylene glycol in ethyl alcohol. Cell block samples and slides can be fixed with this method.

Whichever of the wet fixative methods used, it is imperative that it happens rapidly. When cells dry, they swell and appear larger and the nuclear detail is lost. The edges of well-fixed smears can even become air-dried causing these cells to be unsuitable for interpretation.

The last technique in slide fixation is the air-dry method. While wet fixed slides provide better nuclear detail, air-dried slides provide better visualization of cytoplasm and extracellular components such as colloid or amyloid. Air-dried slides also allow for ROSE to be performed in the clinic or at the bedside. Once the smear or bookend slides are made, the sample can be left to dry or can be fanned dry. It is critical that the slide is completely dry before staining.

29.5 Staining

Wet fixed specimens are most often stained with a Papanicolaou stain. The Papanicolaou stain is a polychromatic stain with multiple dyes that stain various parts of the cell differently [4]. The hematoxylin component stains the nucleus a dark blue and the three acidic components of the dye stain the cytoplasm giving it a paler cyanophilic type appearance. The Papanicolaou stain is a vital component of thyroid FNA diagnosis because it effectively highlights the nuclear alterations of papillary thyroid cancer, such as grooves and inclusions. It also aids in the diagnosis for Hurthle cell and C-cell lesions because nuclear changes can be more easily appreciated.

Table 29.2 Differences in Papanicolaou and Romanowsky stains for thyroid aspiration

Papanicolaou stain	Romanowsky stain
Wet fix preparation	Air-dried preparation
Air-dry artifact possible	No fixation artifacts
Cell size shrinkage	Cell size enlargement
Some cellularity loss possible in fixation	No cell loss in fixation
Crisp nuclear grooves and inclusions	Poor nuclear grooves and inclusions
Poor intracytoplasmic features	Cytoplasmic features (granules) preserved
Squamoid cytoplasmic features prominent	Squamoid cytoplasmic features noticeable
Extracellular material (colloid) not well visualized	Extracellular material easily visualized

The air-dried slides are stained with one of the many Romanowsky stains. The two components of the dye include methylene blue and eosin Y [4]. The combination of these two dyes causes the nucleus and nucleolus to stain shades of deep purple and the cytoplasm to stain pale purple-blue. The metachromatic nature of this stain allows for a spectrum of reddish-purple color that is beneficial in evaluating some secretory granules and extracellular components, such as colloid and amyloid, which do not stain as well with the Papanicolaou stain. This stain is simple and rapid making it the stain of choice for ROSE. See Table 29.2.

29.6 Ancillary Studies

On rare occasion, additional testing is needed to characterize the nodule. This can require different types of specimen collection techniques depending on the ancillary tests. Immunohistochemical studies can be performed depending on the cellularity of the specimen. From the cell block, additional sections can be cut from the block. Liquid based and cytospin slides can sometimes have an additional prepared slide. If using previously stained slides, the slide must be destained prior to the immunohistochemistry being performed.

When the differential diagnosis includes lymphoid lesions, flow cytometry can be of benefit. This specimen should be a rinse of the multiple passes made into the nodule or dedicated passes rinsed into a dedicated flow cytometry media such as RPMI (Roswell Park Memorial Institute medium). This can be used for thyroid nodules as well as lymph nodes. If being shipped overnight to a lab, flow cytometry specimens should be sent cold.

If the thyroid FNA diagnosis is one of the indeterminate categories, thyroid molecular tests may be of use according to American Thyroid Association Guidelines [5]. These tests have different collection and/or shipping requirements depending on the test used and the reader is referred to the manufacturer's instructions.

At times non-thyroid specimens are biopsied. To evaluate a parathyroid lesion, FNA slides should be prepared in the usual fashion and the remainder of the sample can be rinsed into a sterile red top chemistry tube containing 1 cc of normal saline for measurement of PTH level. This is referred to as a PTH washout. This same procedure is used to evaluate neck lymph nodes suspected of metastatic papillary thyroid carcinoma involvement. After preparation of the slides for cytologic evaluation, the remainder of the specimen is rinsed into 1 cc of normal saline for a thyroglobulin analysis.

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