# Chapter 1
# Cytology Specimen Collection, Preparation, and Stains

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## Specimen Collection Techniques [1]

- Slides preparation:
- The appropriate sample should be collected sufficiently for any cytology specimen to prepare slides (Table 1.1).
- Each slide should be labeled with at least two patient identifiers [2].
- A small amount of sample should be gently expelled onto a slide with the needle tip pointing down towards the slide near the frosted edge to give the most space for subsequent preparation [3].
- "Two-Slide Pull" method: a new clean slide can be used to spread the sample across the first slide to create a thin, evenly distributed smear with constant gentle pressure, or place the specimen close to the midpoint of the slide and place another slide on the top of the specimen and gently pull both slides on the opposite direction. This will generate two slides containing the specimen. One slide can be used for rapid onsite evaluation (ROSE) by airdrying first and then staining with Diff-Quik stain. In contrast, the other slide should rapidly be fixed using 95% ethyl alcohol or cytology spray fixative.
- For staining details, refer to *the specimen preparation section.*

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S. M. Gilani, G. Cai (eds.), *Non-Neoplastic Cytology*,
https://doi.org/10.1007/978-3-031-44289-6_1

**Table 1.1** Specimen collection techniques

| Cytology technique | Procedure | Indications/uses | Preparation | Adequacy criteria | Contaminants |
|---|---|---|---|---|---|
| Fine needle aspiration (FNA) | Image guided: Endobronchial ultrasound (EBUS) | • Diagnosis, staging lung cancer<br>• Mediastinal lymphadenopathy<br>• Mediastinal mass<br>• Parenchymal pulmonary nodules, endobronchial lesions | Direct smear, ThinPrep, cell block | Presence of lesional material | Bronchial cells, cartilage, upper respiratory tract epithelial cells |
| | Image guided: Endoscopic ultrasound (EUS) | • Primary diagnosis or staging of lesions of gastroesophageal, pancreaticobiliary, ampullary, intestinal or rectal area<br>• Mass in pancreas, gallbladder, biliary tree, liver<br>• Some retroperitoneal lesions | | Presence of lesional cells | Oral and GI contaminants |
| | Image guided: Endoscopic retrograde cholangiopancreatography (ERCP) | • Strictures<br>• Ampullary lesion | | Epithelial cells or lesional cells | GI contaminants |
| | Thyroid | Thyroid lesion or nodule | | ≥6 groups of follicular cells with ≥10 cells/cluster with certain exception [4] | Gel material, skeletal muscle |
| | Salivary gland | Salivary gland lesion or nodule | | Lesional cells<br>Only normal salivary gland elements in context of mass may not represent lesional cells [5] | Skin |
| | Breast | Cystic or mass forming lesion | | Presence of lesional cells (in correlation with clinical and imaging features) | Skin |
| | Superficial: Fat pad FNA | Amyloidosis | | Fibroadipose tissue fragments and associated vessels | Skin, muscle |

| | | | | | |
|---|---|---|---|---|---|
| Exfoliative cytology | Cervical pap, anal pap | Screening for dysplasia or cancer infection | Liquid-based preparations (LBP: ThinPrep SurePath): Pap | Squamous cells [6]<br>• Conventional: 8000–12,000<br>• LBP: ≥5000<br>Anal<br>• Conventional: 2000–3000 nucleated squamous cells (NSC) | – |
| | Urine | Screening, infection, neoplasia | | Voided urine >30 mL [7] | – |
| | Cerebrospinal fluid | Infection, neoplasia/malignancy | | – | Cartilage, blood elements |
| | Bronchial lavage | Infectious (pneumonia, TB, fungi) Malignancy | | >10 alveolar macrophages or any abnormality [8] | Oral contaminants, upper respiratory epithelial cells |
| | Sputum | TB, *pneumocystis jirovecii* (PJP) | | Preferably macrophages [8] | Oral contaminants (squamous epithelial cells) |
| | Brushings | Bronchial, biliary, esophageal | | Lesional cells or epithelial cells | |
| | Washings | Bronchial, gastric, colonic, bladder, ureteral | | Lesional cells or epithelial cells | |
| | Body cavities | Pleural, pericardial, peritoneal | LBP, cell block | Preferably >50–75 mL of fluid [9] | Skin, muscle |
| | Synovial fluid | Gout, pseudogout infection | | – | – |
| | Vitreous fluid | Infection neoplasia | ThinPrep or direct smear | – | – |
| | Cyst fluid | Malignancy | LBP | – | – |
| | Nipple discharge | Malignancy | Direct smear | – | – |

(continued)

**Table 1.1** (continued)

| Cytology technique | Procedure | Indications/uses | Preparation | Adequacy criteria | Contaminants |
|---|---|---|---|---|---|
| Touch prep or FNA procedure | Intraoperative consultation<br>Intraoperative consultation and rapid on-site evaluation: Aims not only to improve diagnostic yield of the procedure but to also help ensure that adequate material is collected and allocated for ancillary tests based on the preliminary cytologic impression [10] | Infection, nonneoplastic, or neoplastic processes | Direct smear | Presence of lesional cells | – |

- All specimens received directly into the cytopathology lab should be tightly secured in their respective containers, labeled, and accompanied by an appropriately completed patient requisition form.
- Fixation is in 95% alcohol (ethyl alcohol) for the majority of cases. Alcohol shrinks the cells making nuclear details clearer. 100% alcohol may be used in place of 95% ethyl alcohol.

## Fine Needle Aspiration (FNA)

- This procedure is used to evaluate either superficial or deep-seated lesions. FNA of superficial lesions is with or without image guidance, while FNA of deep-seated lesions is performed under image guidance.
- Pathologists often perform FNA of superficial palpable lesions or fat-pad FNA.
- Superficial palpable lesions are generally aspirated with a 22–25 G needle attached to a 10–20 cc syringe.
- Some aspirated contents (2–3 drops) are placed onto a glass slide, and two direct smears are usually prepared using another clear slide. One slide is used for ROSE, while the other is fixed in alcohol for further evaluation.
- Needles should be rinsed in an appropriate solution or formalin to prepare a cell block that can be used for ancillary testing.

## Exfoliative Cytology

- *Gynecologic cytology*: Liquid-based gynecological pathology specimens are collected using SurePath or Cytyc ThinPrep preparations for Pap tests.
- *Voided urine*: Discard first early morning urine; patient voids into specimen container; collect urine starting at midstream. Specimens must be submitted immediately to the lab to avoid cellular degeneration. Specimens can be submitted fresh or in 70% alcohol fixative for possible ancillary testing (FISH analysis). Cytology preparations of samples are examined for infections or malignancy.
- *CSF*: Aspirated CSF is evaluated for infection or malignancy.
- *Sputum*: Specimen is evaluated for infection or neoplastic process; complete series: early morning specimens each day for 3 days (if suspected tuberculosis).
- *Brushings*: Specimens are cellular, and mostly CytoLyt solution is used for preservation, but direct smears can also be prepared. The specimen is examined to evaluate for infection, nonneoplastic, or neoplastic processes.
- *Washings*: Bronchial washing is mostly used to evaluate for infection or malignancy. Peritoneal washing is to assess for a neoplastic process.
- *Body cavity fluids*: ThinPrep or cytospin preparations are commonly used. They are examined to determine the cause of fluid accumulation (nonneoplastic versus neoplastic).
  - Direct scrapings: Herpes virus detection: Tzanck test.

- Touch imprint: Touch imprint cytology is mostly used either for ROSE or during intraoperative frozen sections. The biopsy material is touched or scraped on the glass slide and is immediately fixed in alcohol with subsequent staining (Papanicolaou or rapid H&E) or air-dried and then stained with Diff-Quik stain. Squash preparations are also used (mostly in brain-frozen sections).

## Specimen Preparation

- Cytology specimen received in the lab is processed based on the specimen site and volume. Direct smears from an FNA procedure usually have an air-dried preparation (Diff-Quik stained) and an alcohol-fixed preparation (Papanicolaou stain).
- Specimens may be concentrated by centrifugation to obtain a cell pellet.
- Papanicolaou (Pap) stain.
  - This staining technique is helpful for optimal visualization of cancer cells exfoliated from epithelial surfaces of the body.
  - The polychrome staining reaction is helpful in highlighting cytologic features, chromatin and cytoplasmic details.
  - Procedure.

    Fixation: Coplin jar with 95% ethyl alcohol (5–15 min).
    Hydration: running water.
    Nuclear staining: Harris hematoxylin (1–5 min).
    Rinse: running water.
    Scott's tap water/distilled water.
    Rinse: running water.
    Rinse: 95% ethyl alcohol, two changes (10 dips).
    Cytoplasmic staining: OG-6 for 30 s to a min.
    Rinse solution: 95% ethyl alcohol, three changes (10 dips).
    Cytoplasmic staining: EA-50 for 5 min.
    Rinse: 95% ethyl alcohol, three changes (10 dips).
    Final dehydration: 100% ethyl alcohol (10 dips).
    Clearing: xylene, three changes.
    Coverslip.

- Diff-Quik stain.
  - Use for ROSE, evaluate the cytoplasmic details, infectious organisms, and hematological elements.
  - Procedure.

    Fixative solution (methanol) for 30 s to 1 min.
    Solution I (eosinophilic) 10 dips.
    Solution II (basophilic) 10 dips.
    Rinse slides in running water.
    Coverslip.

- Rapid H&E stain.
  - The method is used mostly for intraoperative frozen sections and ROSE.
  - Nuclei stain blue; overstaining with hematoxylin results in dark nuclear staining and vice versa.

    Cytoplasm/other tissue elements, including RBCs, stain pink; overstaining with eosin results in dark cytoplasmic staining.
- Cell block.
  - A cell block may be prepared from any fluid, washing, brushing, or FNA, with the added benefit that this procedure obtains any tissue which may be present in the specimen. Cell block material can be used for ancillary testing if required.
  - Procedure.

    Centrifuge the specimen to obtain a pellet which includes the following procedure: Add 10 mL of absolute alcohol and centrifuge it, and gradually decant the supernatant. Add 15–20 mL formalin, again centrifuge it and pour supernatant. Add HistoGel drops to the pellet and then refrigerate it for a few minutes. Put the pellet in the cassette and submit it for processing.

## Special Stains

| Workup | Stain | Uses | Positive interpretation |
|---|---|---|---|
| Infectious organisms | Ziehl-Neelsen | Acid fast organisms | Red rod-shaped organisms on a blue background |
| | Modified Ziehl-Neelsen | Acid fast organisms | |
| | Kinyoun | Acid fast organisms | Red organisms on a blue-green background |
| | Fite | *Mycobacteria leprae* | Red organisms on a blue background |
| | Auramine-rhodamine | Acid fast organisms | Yellow-red fluorescence |
| | Grocott's methenamine silver (GMS) | Fungi especially PJP | Black organisms on pale green background |
| | Periodic acid Schiff (PAS) | Fungi; polysaccharides; neutral mucosubstances, basement membrane | Magenta-red organisms on blue-green background |
| | Brown-Hopps | Bacteria | Gram+: Blue<br>Gram–: Red |
| | Brown-Brenn | Bacteria | Gram+: Purple-blue<br>Gram–: Pink-red |
| | Warthin-starry | Spirochetes, *helicobacter pylori* | Dark brown-black organisms on pale yellow background |

| Workup | Stain | Uses | Positive interpretation |
|---|---|---|---|
| Other | Alcian blue | Acid mucopolysaccharides; for Cryptococcus spp. | Acid mucins: Blue |
| | Cresyl violet | Neurons (Nissl substance) | Granular purple-blue neuropil |
| | Fontana Masson | Melanin, argentaffin cell granules (of carcinoid tumor) | Melanin: Brown-black; on pale pink background |
| | PAS with diastase digestion | To differentiate mucin from glycogen, fungal infections, Whipple's disease | Light pink (replaces deep magenta of PAS only) |
| | Masson's trichrome | Connective tissue/vasculature, collagen, fibrosis | Collagen: Blue; nuclei: Brown/black; muscle: Red; cytoplasm: Pink |
| | Von Kossa | Calcium deposits | Gray-black calcium deposits on light pink background |
| | Reticulin | Reticulin fibers (tumor architecture) | Black fibers on gray-pink background |
| | Luxol fast blue | Myelin in nerves | Myelin: Blue-green Neuropil: Pink |
| | Verhoeff's elastic stain | Elastic fibers | Elastic fibers: Black Collagen: Red |
| | Bielschowsky silver stain | Nerve fibers (neurofibrillary tangles in Alzheimer's disease) | Black nerve fibers |
| | Oil red O | Lipid, lipid laden macrophages | Red |
| | Prussian blue | Iron (hemosiderin) | Bright blue on pink background |
| | Congo red | Amyloid | Salmon-pink and green birefringence under polarized light |
| | Thioflavin-T | Amyloid | Bright yellow-green fluorescence on black background |
| | Mayer Mucicarmine | Acid mucopolysaccharides; epithelial mucin; for *Cryptococcus* spp. | Red on yellow background |
| | Bile | Bilirubin | Emerald green |
| | Rhodanine | Copper | Bright red |
| | Myeloperoxidase (MPO) | Myeloid lineage, MPO deficiency | Blue-green or brown, if present |
| | Sudan black B | Neutral lipids | Black granular |
| | Leukocyte alkaline phosphatase | Alkaline phosphatase activity | Brown-black granules |
| | Toluidine blue | Mast cells | Dark rose-violet on a blue background |
| | Movat pentachrome | Collagen, elastic fibers, muscle, and mucin | Elastic fibers-black Collagen-yellow Mucin- blue Muscle-red |

## Immunohistochemistry

- An important development in cytopathology is the application of immunohistochemical staining (IHC) techniques on direct smears and ThinPrep slides, along with the traditional use on cell block material.
- Specimens with limited material or with no cell block can further be evaluated by performing stain on additional ThinPrep slide (such as PTH stain for parathyroid, calcitonin stain for medullary thyroid carcinoma).
- On cytology preparation (direct smear or ThinPrep), interpretation of IHCs with nuclear staining pattern is more straightforward.

### *Limitation*

- Polyclonal antibodies are more sensitive and bind with more epitopes. Titration should be done cautiously to avoid overstaining (false positives).
- Monoclonal antibodies are more homogeneous and specific against a single epitope.
- Cytoplasmic or membranous staining interpretation on ThinPrep or direct smear slides is often challenging.
- Fixative-related issues: Alcohol fixative versus formalin fixative.

**Acknowledgments** None.

**Conflict of Interest** None.

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