

Manuals for Training in Cancer Control

Manual for Cytology



Directorate General of Health Services
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FOREWORD

India is one of the few countries in the world to have a National Cancer Control Programme. The programme was conceived with the objectives of providing preventive and curative services through public education and enhancement of treatment facilities.

We have been able to develop 23 Regional Cancer Centres and several Oncology Wings in India, which provide comprehensive cancer care services. One of the major limitations of the programme is the late stage at presentation of common cancers thus reducing the chances of survival. There is a need to increase awareness among the community regarding prevention and early detection of cancers. The programme is developing IEC materials for the same. Once the population is armed with the necessary information, it is expected that the health system should be geared to tackle the increased demand for care. There have to be trained health care professionals to support the needs of the community. This can be addressed by proper training and sensitisation of general practitioners and health care providers.

These manuals are developed for training health professionals and specific modules have been prepared for Cytology, Palliative care and Tobacco cessation. The facilitator's manual will assist the trainers to conduct the programmes. The manuals are self-explanatory and the health professionals will be able to use them on their own.

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PREFACE

Demographic and epidemiological transitions and changes in lifestyle are leading to the emergence of cancer and other chronic diseases as public health problems in India. Cancer pattern in India reveals the predominance of tobacco related cancers, which are amenable to primary prevention. Cancer Registries in different parts of the country reveal that majority of cancer cases present in an advanced stage and makes treatment options prolonged and expensive. Therefore, the National Cancer Control Programme has placed its emphasis on prevention, early detection, enhancement of therapy facilities and provision of pain and palliative care. Comprehensive legislation on tobacco by the Government of India will help to control the tobacco related cancers. The programme has been able to augment the treatment capacity and to address the geographical gaps in cancer care services. Awareness and early detection programmes are undertaken through District Cancer Control Programmes.

Health care personnel have a major role in providing awareness, promoting early detection, prompt referral to a cancer treatment facility and in providing pain relief and palliative care. The knowledge and skills in the above areas have to be enhanced and these manuals have been developed in response to this need. This set of manuals, which consists of a facilitators' manual and separate manuals for health professionals, cytology, tobacco cessation and palliative care, is an attempt at providing the minimum required capacity. The manuals are self explanatory and will help the trainers, who will be from Regional Cancer Centres and other cancer treatment centres.

The manuals and the compact disc will be widely disseminated and same will be available on the website of the Ministry of Health and Welfare. The National Cancer Control Programme will urge that these may be used in cancer control training programmes in various settings.


(K. RAAMAMOORTHY)
22.8.2005

Role of Diagnostic Cytology

Diagnostic cytology is the science of interpretation of cells that are either exfoliated from epithelial surfaces or removed from various tissues. George N Papanicolou introduced cytology as a tool to detect cancer and pre-cancer in 1928. It is now a widely accepted method for mass screening in asymptomatic population. Many European countries have achieved reduction in incidence of cervical cancer by systematic pap smear screening of the population.

The advantages of diagnostic cytology are that it is a non-invasive, simple procedure, helps in faster reporting, is relatively inexpensive, has high population acceptance and facilitates cancer screening in the field. Diagnostic cytology can be carried out by different methods, which includes collection and examination of exfoliated cells such as vaginal scrapes, sputum, urine, body fluids etc. Collection of cells by brushing, scraping or abrasive techniques is usually employed to confirm or exclude malignancy. Fibreoptic endoscopes and other procedures can be used for collecting samples directly from the internal organs.

Fine-Needle Aspiration Cytology/ Biopsy (FNAC/FNAB) is now a widely accepted diagnostic procedure, which has largely replaced open biopsy. This method is applicable to lesions that are easily palpable, for example swellings in Thyroid, Breast, superficial Lymph node etc. Imaging techniques, mainly ultra-sonography and computed tomography, offer an opportunity for guided FNAC of deeper structures.

The practice of diagnostic cytology needs proper training of the laboratory personnel including cytopathologist, cytotechnologist and cytotechnician. The role of cytotechnician is very important in cancer control programmes where large numbers of asymptomatic population have to be screened.

The accuracy of the cytologic examination from any body site depends greatly on the quality of collection, preparation, staining and interpretation of the material. Inadequacy in any of these steps will adversely affect the quality of diagnostic cytology.

Diagnostic accuracy and reliability are major issues in cytology practice. Over the years many quality control measures have been introduced for ensuring high standards in cytology, Among them the most important are regular continuing education of medical and technical personnel, certification and accreditation of laboratory to national authorities such as Indian Academy of Cytopathologists (IAC), introduction of quality assurance and quality control measures, computerization, introduction of internationally accepted terminology, improvement of sample preparation techniques, quantitative and analytical cytology techniques and advanced technologies including automation.

Collection and Preparation of Material for Cytodiagnosis

Accurate interpretation of cellular material is dependent on the following factors:

- Methods of specimen collection.
- Fixation and fixatives.
- Preservation of fluid specimens prior to processing.
- Preparation of material for microscopic examination.
- Staining and mounting of the cell sample.

Methods of specimen collection

Individual cells may be studied in many ways.

A. Exfoliative Cytology: It is the study of cells that have been shed or removed from the epithelial surface of various organs. Cells from all organs, which communicate with the exterior of the body, are suitable for study. These cells can be recovered either from natural secretions such as urine, sputum and vaginal or prostate fluids or by artificial means such as paracentesis or lavage. The cells can be collected from the epithelial surfaces by lightly scraping the surface, by swabbing, aspirating or washing the surfaces.

Normal cells are cohesive in nature but exfoliated when they attain maturation. During malignant conditions or during infection, the exfoliation becomes exaggerated and the epithelial cells show variation in morphology. Such exfoliated cells, when collected and appropriately stained, give information on the living epithelium from which they are derived. These characteristic cellular and nuclear appearances in cells thrown off from healthy epithelium, differ distinctly from those, derived from inflamed or malignant lesions. Thus by studying the alterations in morphology of the exfoliated cells and their pattern, the diagnosis of various pathologic conditions can be made.

B. Fine Needle Aspiration Cytology (FNAC): This is a technique used to obtain material from organs that do not shed cells spontaneously. It is valuable in diagnosis of lesions of the breast, thyroid, lymph nodes, liver, lungs, skin, soft tissues and bones.

C. Body Fluids: Body fluids like Urine, Pleural fluid, Pericardial fluid, Cerebrospinal fluid, Synovial fluid and Ascitic fluid can be studied by cytology.

A. EXFOLIATIVE CYTOLOGY

Female Genital Tract (FGT)

The cytological specimens collected from FGT include cervical smear, vaginal smear, aspiration from posterior fornix of vagina (vaginal pool smear) and endometrial smear.

Cervical smear: Cancer of the uterine cervix is the commonest cancer in the FGT. Almost all invasive cancers of the cervix are preceded by a phase of preinvasive disease, which demonstrates microscopically a continuing spectrum of events progressing from cervical intraepithelial neoplasia (CIN) grade I to III including carcinoma in-situ before progressing to squamous cell carcinoma. This progressive course takes about 10 to 20 years. Early detection even at the preinvasive stage is possible by doing cervical smear (Pap Smear Test). This can identify patients who are likely to develop cancer and appropriate interventions may be carried out.

Advantages of Pap Smear:

- It is painless and simple
- Does not cause bleeding
- Does not need anesthesia
- Can detect cancer and precancer
- Can identify non-specific and specific inflammations
- Can be carried out as an outpatient procedure

Patient Preparation: Proper patient preparation is the beginning of good cervical cytology. The patient should be instructed before coming for smear collection, that she should not douche the vagina for at least a day before the examination. No intravaginal drugs or preparations should be used for at least one week before the examination and the patient should abstain from coitus for one day before the examination. Smear should not be taken during menstrual bleeding, because of contamination with blood, endometrial component, debris and histiocytes.

Sampling: A cervical cytological sample is considered satisfactory for cytological diagnosis when their composition reflects the mucosal lining of the cervix, encompassing ectocervical, squamous metaplastic cells and endocervical columnar cells in fair numbers. It is generally agreed that majority of epithelial abnormalities that eventually lead to an invasive cancer originate in the squamo-columnar junction (transformation zone). As stated by the British Society for Clinical Cytology (BSCC), a cervical smear if properly taken should contain cells from the whole transformation zone (TZ). The sample should contain a sufficient quantity of epithelial cells, and both metaplastic and columnar cells should be present. According to the Bethesda System, an adequate smear contains an adequate endocervical/transformation zone component. Lubricant should not be used while examining, as it can obscure the cells during smear examination.

Factors affecting specimen collection:

The experience of the person who is taking the smear is very important in getting smears with adequate cellular composition. Clinicians must receive appropriate training in taking cervical scrape samples and slide preparation. The cervix must be clearly visualized and the entire transformation-zone is scraped. It is also the responsibility of sample takers and quality assurance programmes to monitor the quality of specimens, so as to minimize / avoid inadequate samples and preparation / fixation artifacts. Periodic feed back to clinicians regarding the quality of their samples is important in this regard.

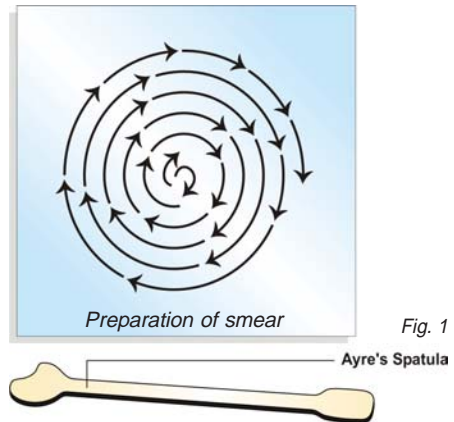


Fig. 1

Sampling Devices: The collection device may play an important role in sample adequacy. The shape, surface, texture and material of the device may determine how much of the scraped material is deposited on to the glass slide and is available for screening and analysis. Several methods of obtaining cytologic material from the uterine cervix are available. However, use of cotton swab for collection of cervical smear is to be discouraged, in view of the drying artifacts and loss of cells, which are caused by this method.

- Smears obtained with original Ayre's spatula are often easier to screen. Wooden spatula is preferable to plastic spatula, because of its mildly rough surface that can collect more material. The disadvantages are that the method may occasionally be traumatic to the patient, and the tip of spatula that does not fit the external os may fail to remove some of the valuable material from the squamo-columnar junction. (Figure 1)
- Based on the original wooden Ayre's spatula, many devices of different shapes and sizes have been introduced to improve sampling. This includes Endo-cervical Brush, Cervex, Cytobrush, etc.
 - The pointed Aylesbury version of cervical spatula was designed to sample cells from both endocervix and the transformation zone (TZ) of the cervix.
 - The Cervex brush device is a flexible plastic brush, which follows the shape of the endocervix, transformation zone and ectocervix as well and is suitable for every cervix shape. (Figure 2)



Fig. 2 : Cervex brush

- Endo-cervical brush is a small bottlebrush like device with one end having fine bristles made up of nylons. This device is strictly for taking materials from endocervix. Gently insert the brush in endocervix and rotate one turn pressing in the upper and lower wall. (Figure 3)



Fig. 3 : Endo-cervical brush

- The cytobrush is similar to that of endocervical brush except that the projected tip is without bristles. This can be used for obtaining cells from the whole cervix.

Single sampling devices and methods have their limitations in obtaining adequate smears from the cervix. A combination of two devices, usually spatula and endocervical brush, give better results. Triple smear or the vaginal-cervical-endocervical (VCE) technique can provide the best results. However, feasibility and cost factor need to be taken into consideration.

In postmenopausal women, the squamo-columnar junction recedes making it difficult to obtain good amount of endocervical cells and cells from TZ. Hence a combination of two devices, spatula plus endocervical brush is preferred. In those with a prolapsed uterus, the cervix is first soaked with normal saline and scrape is collected with cytobrush. To obtain a satisfactory smear from a bleeding cervix, the blood is wiped with wet cotton and smear is obtained by wooden spatula.

There has been some concern that the use of the endocervical brush can result in the appearance of a much greater number of endocervical cells in a smear and that their arrangement in large sheets might mimic malignancy. To avoid this problem clinicians should inform the laboratory when an endocervical brush is used for collecting the smear.

Preparation of Smear: After smear collection, the cellular sample is evenly smeared on to the centre of the non-frosted area of the glass slide, by rotating both sides of the scrape end of the spatula in multiple clockwise swirls in contact with the slide and fixing it immediately. Excessively thin or thick smears can result in false-negative reports. The smear should be visually inspected after fixation. If it does not appear satisfactory, repeat it during the same examination and submit both slides for cytological examination.

Some studies have shown that two-slide cervical smears detect more abnormalities than a one-slide smear. Two smears do increase screening costs over a single-slide smear, but those costs are not double that of a single-slide examination. A two-instrument collection on a single slide increases screening time only minimally over a single instrument.

Vaginal smear: Introduce an unlubricated speculum, scrape the lateral vaginal wall at the level of cervix with a spatula. The broad and flat end of Ayre's spatula is used for this purpose. The cellular material is rapidly but gently smeared on a clean glass slide and the smears are fixed immediately. If no spatula is available a cotton swab dipped in normal saline can be used.

Vaginal pool smear: The aspiration can be performed after the introduction of unlubricated speculum. The technique allows collection of cells under direct vision from posterior fornix pool. When a speculum is not employed the pipette is gently introduced into the vagina until resistance is encountered. It is important to compress the suction bulb during the introduction of the pipette to avoid collecting the cellular material of the lower vaginal origin. The cellular material is spread on a clean glass slide and fixed immediately.

Endometrial aspiration smear: After preliminary visualization and cleaning of cervix a sterile cannula is introduced into the uterine cavity and aspiration is then carried out with a syringe. The specimen is squirted on a clean glass slide, gently spread and rapidly fixed.

Respiratory Tract

Respiratory tract malignancies can be detected mainly by sputum cytology or by bronchoscopic material.

Sputum Cytology: Sputum specimen can be obtained from the patient either spontaneously or by aerosol – induced method. Morning specimen resulting from overnight accumulation of secretion yields best results. Three to five consecutive days' sputum samples should be examined to ensure maximum diagnostic accuracy. Fresh unfixed specimens are better than prefixed specimens in 70% ethyl alcohol or coating fixative such as carbowax or saccomano fixative. (*Fixation of slides is discussed in a separate chapter*)

The sputum must be carefully inspected by pouring the specimen into a petri dish and examining on a dark background. Select any bloody, discolored or solid particles, if present, place a small portion of each particle on a micro slide, spread evenly and fix it immediately. Prefixed specimens should be smeared on albumen or polylysine coated slides.

Bronchoscopic Specimens: Specimens that are obtained by bronchoscopy are secretions (bronchio-alveolar lavage), direct needle aspirate from suspicious area and bronchial brushing and washings. Post bronchoscopic sputum is one of the most valuable specimens for the detection of pulmonary lesions.

Other Sites

Oral lesions: Scrape the lesion with a tongue depressor, spread material on a clean slide and fix immediately.

Nasopharynx: Cotton tipped applicator is used to obtain material for cytological examination.

Larynx: A cotton swab smear of larynx may be a useful adjunct to clinical diagnosis if biopsy is not contemplated.

Oesophagus

Oesophageal washing and brushing are usually recommended for collecting cytology sample from oesophagus. To collect a good specimen for cytology one should first localize the suspicious lesion by oesophagoscopy .

Stomach

Cytology specimen can be collected from the surface of the lesion by scraping (abrasion) under direct vision of a flexible endoscope. The cells collected can be directly smeared on a glass slide. Gastric lavage is also recommended for cytological investigations.

Discharge from nipple of the breast

Spontaneous nipple discharge and discharge produced by breast massage are collected by applying the slide directly to the nipple followed by immediate fixation.

B. FINE NEEDLE ASPIRATION CYTOLOGY (FNAC)

Procedure, Preparation and Preservation:

FNAC is the study of cellular samples obtained through a fine needle under negative pressure. The technique is relatively painless and inexpensive. When performed by well-trained pathologists / surgeons / clinicians and reported by experienced pathologists, it can provide unequivocal diagnosis in most of the situations.

It is useful in lesions that are easily palpable, like growth of skin, subcutaneous soft tissue tumours, thyroid, lymph nodes, salivary glands and breast. Guided aspiration by internal imaging techniques like C.T or ultrasonography allows FNA of lesions of internal organs like lung, mediastinum, abdominal and retroperitoneal organs, prostate etc. The low risk of complications allows it to be performed as an out-patient procedure. It is highly suitable in debilitated patients, multiple lesions and easily repeatable.

The three pre-requisites for a meaningful diagnosis on FNAC are:

1. Proper technique - procedure, preparation of smears, fixation, staining.
2. Microscopic evaluation of smears.
3. Correlation of morphology with the clinical picture (history, clinical features, radiological and laboratory findings).

The Technique: Attention to technique is necessary to optimize the yield of the sample, making its interpretation easier and more reliable. Expertise regarding the technique comes from constant practice and correlation of the smear technique with the results (feedback).

Equipment: The success or failure of the aspiration procedure depends to some extent on the organization of the set up. Some institutions set aside appropriately equipped areas dedicated to the procedure. Otherwise, the materials can be arranged on movable carts or even in portable containers. Thus FNA can be performed as an outpatient procedure or at the patient's bedside.

Needles: Standard disposable 22-24 gauge 1-1½-inch needles are used for plain FNAC. The length and caliber of the needle should fit the size, depth, location and the consistency of the target. For small subcutaneous lesions, one-inch 23-gauge needle is ideal while for a deep-seated breast lesion, longer and larger needle is required. Finer needles are also recommended for children, and for vascular organs like thyroid.

Syringes: Standard disposable plastic syringes of 10ml are used. Syringe should be of good quality and should produce good negative pressure. 5cc syringes can be used for vascular organs like thyroid. One important factor is to check the tight fit of the needle on the syringe tip. A loosely fitting needle can render the procedure useless and may injure the patient. Syringe holder: A syringe piston handle can be used, leaving one hand free to immobilize the lesion. This is not absolutely essential and is a matter of choice of the aspirator.

Slides: Plain glass slides of good quality are used. Slides should be clean, dry, transparent and grease free.

Fixative: 95% ethyl alcohol is recommended. Fixative is kept ready in Coplin jars.

Other supplies: Test tubes, pencil for marking, alcohol, swabs for skin, watchglass, saline, adhesive dressing, gloves etc. are needed. All the materials required are assembled in advance before starting the procedure. This is extremely important as delay in fixation can make interpretation of smears difficult.

Aspiration Procedure (Figure 4)

Steps to be followed before performing the aspiration

1. Relevant history and clinical details, radiological findings, provisional diagnosis etc. must be entered in the requisition form. Site of FNA must be clearly stated.
2. Lesion to be aspirated is palpated and its suitability for aspiration assessed. The appropriate needle is selected accordingly.
3. The procedure must be clearly explained to the patient and consent and co-operation ensured. Patient may be anxious which needs to be allayed. Ignoring this simple but crucial step can result in failure.
4. Before starting the procedure, ensure that all the required equipment, instruments and supplies are available.
5. All universal precautions should be followed during the procedure.

Steps to be followed in the actual performance of the aspiration:

- **Positioning the patient:** Any comfortable position can be chosen depending on the convenience to palpate the lesion and the comfort of the patient. FNA is usually carried out with the patient lying supine on an examination couch.
- **Immobilization of the lesion:** Skin is cleansed firmly with an alcohol swab (as used for routine injection). Local anesthetic may not be necessary. Apprehensive patients must be reassured about the procedure.

The lesion is fixed between the thumb and index finger of the left hand, with the skin stretched. Try to avoid significant muscle mass eg. sternocleidomastoid, while fixing the lesion because it is not only painful, but also muscle tends to plug the needle tip, preventing further material from entering the needle.

- **Penetrating the lesion:** Fixing the lesion with one hand, grasp the syringe with the needle attached (with or without syringe holder) by the dominant hand and introduce through the skin into the lesion, carefully and swiftly. The angle and depth of entry varies with the type of lesion. For small lesions, aspiration of central portion is indicated. For larger lesions that may have necrosis, cystic change or hemorrhage in the center, aspiration may be done from the periphery. If pus or necrotic material alone is aspirated from larger lesions, FNA can be repeated immediately from the periphery. With experience, a change in tissue consistency will be felt as the needle enters the lesion. If the needle goes tangentially missing a small slippery lesion or if penetrates beyond the lesion, representative material will not be obtained.

Note: If the site of FNA is located near the thoracic cage e.g. axillary or supraclavicular swellings, aspiration is better performed in a plane parallel to the thoracic cage to avoid pneumothrax. In thyroid FNA, patient should be instructed not to swallow or talk when the needle is inside the nodule.

- **Creation of a vacuum and obtaining the material:** Suction is applied after entering the lesion and while maintaining the suction, needle is moved vigorously back and forth in a sawing or cutting motion, changing the direction a few times, ensuring that the needle is inside the mass throughout; the whole procedure taking only 4-8 seconds. Do not rotate the needle or pump the plunger in the syringe in and out. Purpose of suction is to pull the tissue against the cutting edge of the needle and to pull the dislodged tissue fragments and cells into the lumen of the needle. Material is procured by cutting motion of the needle and not by suction. This is evident in the non-aspiration technique in which the needle alone is moved back and forth in the lesion and withdrawn. Admixture with blood is less with this technique and is useful in thyroid aspiration.

When the needle is moved in different directions, it samples a much wider area than a core biopsy (FNA is thus more representative than a core biopsy). The to and fro movements and changing the direction of the needle, while it is still inside the lesion are the two crucial steps in procuring an adequate representative sample.

Movement of the needle is adjusted according to the type of lesion. A sclerotic lesion will require more force than a soft tumor. A cyst will almost aspirate by itself. When fluid is aspirated, its color, consistency and amount should be recorded in the requisition form, which allows the lesion to be recognized as cystic. Fluid can be sent in a bottle for centrifugation and preparation of smear. In cystic lesions, especially of breast and salivary gland, a large cyst may obscure a small malignant tumor. Hence cysts should be completely aspirated (fluid is sent for centrifugation) and residual lump if any, should be re-aspirated and labeled separately.

In sclerotic / fibrotic lesions e.g. Breast, little or no material will be obtained and the aspiration should not be continued indefinitely. There is no use trying a wider bore needle; in fact a finer needle may succeed in obtaining more material.

Vascular organs like thyroid must be sampled rapidly with minimal movement of the needle. If blood appears in the barrel of the syringe, the procedure is discontinued as blood will dilute the sample and render it diagnostically useless. Except for cystic lesions or vascular organs, nothing should be seen in the barrel of the syringe. Thus the purpose of syringe is not to collect material, but to provide suction facilitating entry of cells into the needle and then to expel them from the needle, while making smears.

Observations while doing the aspiration regarding site, size, and consistency (solid / cystic / soft / sclerotic / vascular) must be correlated while interpreting the smears later. The clinician / pathologist should record all these relevant observations in the requisition form.

- **Release of vacuum and withdrawal of the needle:** When material is seen in the hub of the needle, procedure is discontinued. Before withdrawing the needle, suction is released and needle pulled straight out. The piston is just allowed to slowly fall back by itself (never push). Failure to release negative pressure within the lesion will cause the aspirated material to enter the syringe, which is difficult to recover. In desperate situations, syringe and the needle can be rinsed with saline or fixative and then centrifuged to prepare a smear. Immediately after withdrawing the needle, firm local pressure is applied at the site for sometime, preferably by an assistant. This is to prevent bruising or haematoma formation especially in thyroid, breast etc.

Note: If a cork of tissue is obtained during FNA or if the sample clots quickly, entrapping the cells, the clot or tissue can be fixed in formalin and processed as for histology.

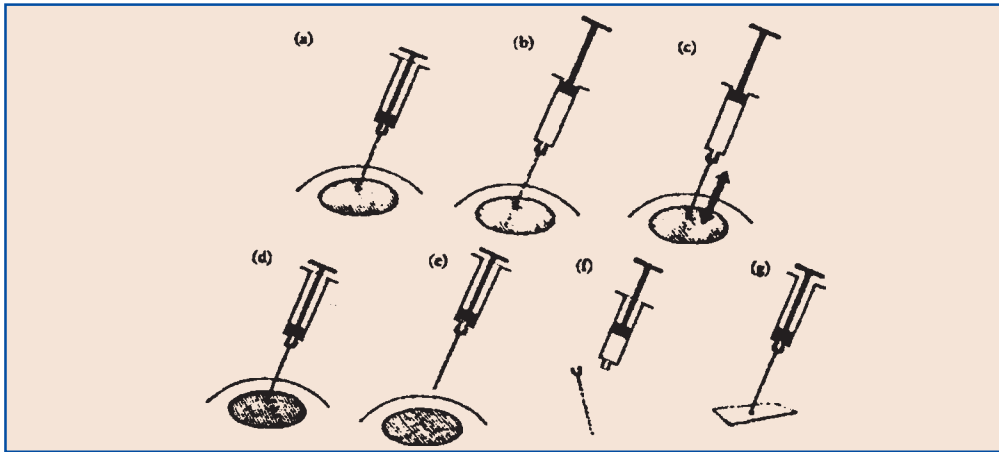


Fig. 4 —Aspiration procedure

(a) needle positioned within target tissue (b) plunger pulled to apply negative pressure (c) needle moved back and forth within target tissue (d) suction released while needle remains in target tissue (e) needle withdrawn (f) needle detached (g) aspirate blown onto slide.

Preservation and processing of Smears

There are two fundamental methods of processing smears obtained by FNA. Smears are prepared and fixed according to the requirements of the stain to be used.

1. *Air-drying followed by hematological stains like May – Grunwald –Giemsa (MGG), Diff Quik, Giemsa etc.:* In this method, smears are intentionally air dried, but if smears are not correctly made and dried quickly artifacts will result. One advantage is the speed with which smears can be stained especially with use of rapid stains like Diff Quik (2-3 minutes). Rapid stains are particularly useful in preliminary assessment of adequacy of the sample before the patient is released. Colloid, mucin, endocrine cytoplasmic granules etc are better brought out in air-dried preparations. It is also useful in patients with hematological malignancies like lymphoma or leukemia.
2. *Alcohol fixation followed by Papanicolaou (pap) or hematoxylin and eosin (H&E) staining:* Rapid fixation in alcohol (wet fixation) is essential for pap staining, which brings out nuclear details clearly, allowing better identification of malignant cells. It also allows better comparison with histology and hence is favored by majority of pathologists. But if the smears are not quickly made and fixed, drying artifact can occur in which case, the cytoplasm takes up more eosin (red color) and nuclear details are less clear. A cellular sample can be unfit for diagnosis if there is significant drying. Hence with pap staining, air-drying is avoided as much as possible especially by dropping the slides into the fixative immediately after the smears are made. Poor quality of preparation, fixation or staining can all make a cellular sample unsatisfactory for evaluation. Hence great care must be taken in preparation and fixation of smears.

Preparation and fixation for pap staining

Immediately after withdrawing, detach the needle, draw air into the syringe, reattach the needle and express the material in the needle onto a slide. Needle tip is brought into light contact with the slide and the aspirate is carefully expressed without spraying into the air, which can cause air-drying and also can form aerosols, which are potentially infectious.

Preparing proper smears is critical for the end results. No matter how expertly the aspiration is performed, if the slides are not interpretable, the procedure is totally worthless for cytologic diagnosis. (*Smearing technique is better demonstrated than described*).

An ideal aspirate is of creamy consistency with numerous cells suspended in a small amount of tissue fluid without admixture with blood. Such aspirates are smeared immediately using another slide or cover slip or with the needle itself and dropped into the fixative.

At the beginning of the smearing process, while the material is still in a drop on the slide, the surface area for evaporation is relatively small and hence a short delay will not cause significant air-drying. Once the smear has been made, the surface areas are greatly increased and the thickness of the smear is greatly diminished. Thus from the instant the smear is made, air-drying proceeds extremely rapidly; hence the urgency for fixation.

Material diluted with blood can be spread like a peripheral smear, where particles tend to come to the edge of the smear. Larger particles can be crushed gently by firm flat pressure. Undue pressure can result in crush / smearing artifact. It can also be spread in a circular motion with the needle itself, when particles and cells tend to distribute around the periphery of the smear. In either case, smear should not go to the edges of the slides, where particles can be lost over the edges. Outline of a perfect smear is completely contained on the slide without going to any of the edges.

The cells must be delicately and thinly smeared with minimal distortion and fixed according to the stain to be used. However, spreading the cells too thinly as well as preparing too many smears is an error because of cellular distortion or dilution. Thus the smears must be of adequate thickness. Obtaining optimal smear is a fine balance between too thick and too thin smears (*or fixation and crush artifacts*) and comes with experience. If a large amount of material is aspirated, multiple smears can be made, both air-dried and wet fixed which are complementary to each other. Extra smears can be used for special stains or other supplementary techniques. (*While taking multiple smears, do not prepare all the smears and then go back and fix them. Fix the smears as soon as they are made to avoid drying*).

Smears can also be prepared indirectly by centrifugation, filtration etc. In lymph node aspiration, a cell suspension can be prepared in addition to direct smears. Other systems like "cell print" are now available for cell collection and preparation. In some centres, needles

and syringe rinse preparations are routinely done and smears prepared by centrifugation or filtration. These types of indirect smears provide thin film of concentrated cells in a clear background from samples of low / high cellularity. This is ideal for special stains and immunocytochemistry.

Note: In guided FNA, once the needle is in the mass, the procedure and smear preparation is similar to plain FNA, including the cutting motion with the needle as well as preparation and fixation of smears.

Causes of unsatisfactory smears

Unsatisfactory smears can be due to non-representative / inadequate samples or due to poor quality of preparation (thick smears, extreme admixture with blood, delayed fixation, over staining etc). Attention to matters of technique regarding the procedure and preparation of smears will considerably reduce the number of unsatisfactory smears received in a cytology lab.

Clinical correlation and final interpretation by the pathologist

Final diagnosis on FNA is based on clinical assessment prior to the aspiration procedure, observations during the procedure as well as microscopic evaluation. Optimal diagnosis is obtained when the same pathologist correlates the clinical features, performs the aspiration and evaluates the smears. When this is not possible, close communication between clinician and pathologist helps to maintain high quality of diagnosis and safeguards against errors. Inaccurate, misleading, incomplete or absent clinical information can be important sources of error. Clinical information is critical and is a part of FNA diagnosis as the morphological features may vary with the site of FNA and have to be correlated with the site of aspiration and other investigations for a meaningful diagnosis. Thus, systematic inclusion of clinical and lab data should be considered as part of the procedure. *The technique (aspirator), morphological interpretation (pathologist) and clinical information (clinician) constitute a diagnostic triad on which the FNA diagnosis rests.*

It is preferable not to report on technically poor slides or give a definite diagnosis without adequate clinical information and correlation. Clinical data serves as a safeguard in avoiding errors.

Other Quality control Measures

In addition to details of technique (procedure, preparation, quality of materials used) and clinical correlation; other routine quality control practices regarding specimen reception (checking patient details, identification of slides, number of slides from each patient, labeling the slides), preparation and maintenance of stains, staining procedure, mounting, record keeping etc. are applicable to FNA also for optimal quality of diagnosis.

Imprint Cytology Smears

This is indicated in the case of tumours especially of lymph nodes. Soon after an excision biopsy of lymph node, the specimen is cut using a sharp scalpel blade. If there is blood oozing from the outer surface, touch the surface with a cotton ball soaked in normal saline. Then take imprint smears by touching the cut surface with a clean microslide and fix immediately.

C. BODY FLUIDS

Urine: For cytological evaluation of bladder, three morning samples of urine (each of 50 - 100 ml) obtained on consecutive days are recommended. Centrifuge the urine for 10 minutes and place one or two drops of sediment on a glass slide, spread the material and fix immediately. Catheterised samples are also acceptable.

Cerebrospinal Fluid (CSF): CSF and other fluids of small volume have considerable bearing on diagnostic accuracy, the larger the sample the better the results. If several samples are obtained the second or third should be used for cytology. The addition of an equal amount of ethyl alcohol to the CSF is recommended if a delay in processing is anticipated. Considering the low volume and cellularity, CSF specimen should be processed by cytocentrifugation.

Cytocentrifugation: The fluid samples with low cell content such as CSF and urine are centrifuged in Cytospin where the cells are sedimented directly on the microslides. *Other serous effusions are dealt with in a separate chapter.*

Preservation of Fluid Specimens Prior to Processing

Preservation of cellular morphology until the sample can be processed is essential for accurate cytologic interpretation. Specimens may be sent to the laboratory without preservatives / prefixatives, if facilities for immediate processing are available. The duration between collection and preparation of the sample before cellular damages occur depends on pH, protein content, enzymatic activity and the presence or absence of bacteria. It is not possible to predict these variables even in specimens from the same anatomic site. The following guidelines are useful to get acceptable results.

- a. **Specimens with high mucus content** such as sputum, bronchial aspirates, mucocele fluid can be preserved for 12 to 24 hours if refrigerated. Refrigeration slows down the bacterial growth, which causes cellular damage. Mucus apparently coats the cells, protecting them against rapid degeneration. The cells in specimens diluted with saliva are not as well protected and may deteriorate more rapidly.

- b. Specimens with high protein content** such as pleural, peritoneal or pericardial fluids can be preserved for 24 to 48 hours with refrigeration. The protein-rich fluid in which the cells are bathed acts as a tissue culture medium in preserving cellular morphology.
- c. Specimens with low mucus or protein content** such as urine or CSF will be preserved for only 1-2 hours even if refrigerated. The fluid medium in which these cells are bathed contains enzymatic agents capable of causing cell destruction. Refrigeration may inhibit bacterial growth but does not protect the cells.
- d. Specimens with low pH**, such as gastric material, must be collected on ice and be processed within minutes of collection to prevent cellular destruction by HCl.

Cytopreparatory Techniques of Serous Effusions

The term serous effusion refers to the fluid accumulated in the three serous cavities namely pleural, pericardial and peritoneal. It forms an important source of useful diagnostic information in clinical practice. Certain benign processes like florid tuberculosis or rheumatoid pleurisy can be recognized cytologically but the most important goal of effusion cytology is the recognition of malignant cells. For an accurate cytologic diagnosis of serous effusions, attention to proper technique is of paramount importance. The essential requirements are:

- Freshly tapped specimen
- Immediate processing
- Rapid fixation of slides

Collection and preservation

Pleural, pericardial and peritoneal fluids can be collected in tubes or syringes that may be either plain or pre-heparinised, to prevent coagulation. Cells in heparinised fluids do not deteriorate rapidly and there are some advantages in the processing of these fluids like layering of many malignant cells in the buffy-coat of the centrifuged sample and better adherence of the cells to the slides.

Freshly tapped specimens are preferred for cytology, if facilities for immediate processing are available. If immediate processing is not possible, it can be preserved in the refrigerator for a period of 24-48 hours. Preservation of cells by pre-fixation in 50% ethanol is also possible. Pre-fixation and spray fixatives are recommended when sample has to be sent to a distant laboratory. Albuminized slides should be used to prepare smears from prefixed sample. 20–30 ml fluid is generally sufficient to get enough cells for cytological evaluation. If the entire specimen tapped cannot be sent to the laboratory, a representative sample from the bottom part of the fluid should be sent to the laboratory.

Gross Examination

When the specimen is received in the laboratory, the gross appearance and the amount of fluid received are noted down. The fluid may be clear, transparent, straw coloured, yellow, brown, red, chylous, purulent, mucoid or hemorrhagic. The appearance of the fluid also helps in diagnosis.

Processing

Routine processing

The fluid received is stirred briskly to disperse the suspended cells. A representative volume of the fluid (10 -15 ml) is centrifuged at 2500 rpm for 5 minutes. If possible glass tube should be avoided because of the disadvantages like tendency for cells to adhere to glass and possible breakage. The centrifuge tube must be meticulously cleaned to ensure a perfectly clean inner surface. If the quantity of fluid is too little for centrifugation, an equal amount of normal saline can be added before centrifugation. If fibrin clot has already formed, the clot may be smashed against the sides of the tube by using an applicator and if large clot remains, may be processed as cellblock. Place one to two drops of the sediment on the slide and allow it to spread evenly by placing another slide over it. Gently pull slides apart with an easy sliding motion to get alternate thick and thin area.

Sparsely cellular Fluid

Clear, sparsely cellular fluids yield scanty or no sediment after centrifugation at 2000 rpm for ten minutes. Cyto centrifugation should be used for such cases. Cyto centrifuge concentrates small number of cells suspended in fluid specimens. Spinning samples at 2000 rpm for 2 minutes sediments cells directly to slides. The blotter or filter card simultaneously absorbs the fluid medium. The result is a mono layer of well preserved cells with in an area of 6 mm. Major objection to the use of cyto centrifuge is the distortion of cellular morphology due to air drying artifacts, which can be avoided by immediate fixation or by using an equal volume of polyethylene glycol. The fluid is first concentrated by routine centrifugation at 2000 rpm for 10 minutes. Large portion of the supernatant is discarded leaving behind a few drops in the bottom of the centrifuge tube. This portion is stirred well and 2-5 drops (optimum 3 drops) are used for cyto centrifugation.

Haemorrhagic Fluids

Carnoy's fixative is used to lyse RBCs in haemorrhagic fluids. Alternatively glacial acetic acid alone or saline re-hydration technique can be used in which the smears are rapidly dried at 37°C for 5 minutes and re-hydrated in normal saline for 30 seconds and then fixed in alcohol fixative.

Cell Block Preparation

There are different methods of cellblock preparation like bacterial agar method, plasma thrombin clot method etc. An alternative method of cellblock preparation is a modified technique using AAF fixative (95% ethyl alcohol 34 ml + formalin 4 ml +Glacial acetic acid 2 ml).

Technique of Cell Block Preparation

- The cell pellet remaining after preparing smears is mixed with thrice the volume of AAF fixative and one or two drops of the supernatant fluid and centrifuged for 10 minutes at 2000 rpm.
- Re-suspend the cell button in AAF fixative and centrifuge for 10 minutes at 3000 rpm
- Set aside the centrifuge tube for 4 — 6 hours
- Scrape out the cell button and wrap in lens paper and process in tissue processor.

Staining

Pap stain or MGG is recommended for routine diagnosis. Cell loss and cell crowding is found to be very high in Pap method as compared to air-dried method. Cytoplasmic details are well preserved in Giemsa than in Pap stain. Crisp chromatin granularity is preserved in Pap stain, whereas, nuclear chromatin transparency is less in Giemsa, and thus the limitation of one method can be counterbalanced in the other method.

Artifacts due to faulty techniques

- Delay in processing may lead to degenerating smear picture with loss of cell morphology and plenty of bacteria in the smear background.
- Delay in fixation may lead to Air-drying artifacts - pale stained nuclei, lack of differential cytoplasmic staining, cytoplasmic and nuclear eosinophilia.
- Contamination from other smears and cell from effusion smears to other slides should be avoided. All the alcohol and xylene solutions should be filtered every day using Whatman No.1 filter paper. The fixative should be filtered after each use.

Disposal of Effusion Samples

Proper care should be taken in handling the effusion samples. After preparing the smears a disinfectant should be added to the sample bottle before it is discarded. Never discard the excess sample into the sink.

Fixation of Cytology Specimens

Rapid fixation of smears is necessary to preserve cytologic details of cells spread on a glass slide. Fixation means prevention of degeneration of cells and tissue by the autolytic enzymes present in the cells and preservation of cells as close as possible to the living state. To achieve this smears are placed in the fixative solutions for specific periods of time before the staining procedure is started. Fixation changes the physical and chemical state of the cells and determines the subsequent staining reactions that could be carried out on the smears.

Properties of Cytologic Fixatives

- Do not excessively shrink or swell cells.
- Do not distort or dissolve cellular components.
- Inactivate enzymes and preserve nuclear details.
- Kill microbes.
- Improve optical differentiation and enhance staining properties of the tissues and cell components.

Cytological Fixatives

Wet Fixation:

A. Routine Fixatives

The process of submerging of freshly prepared smears immediately in a liquid fixative is called wet fixation. This is the ideal method for fixing all gynecological and non-gynecological smears and any of the following alcohols can be used. All alcohol fixatives should be discarded or filtered (Whatman No: 1 filter paper) after each use.

- 1. 95% Ethyl Alcohol (Ethanol):** The ideal fixative recommended in most of the laboratories for cytological specimen is 95% ethanol alone. It produces the characteristic effect desired on nucleus. It is a dehydrating agent and causes cell shrinkage as it replaces water. But it causes only the desired amount of cell contraction to yield optimal chromatin detail characteristics of cytological preparations. Absolute (100%) ethanol produces a similar effect on cells, but is much more expensive.
- 2. Ether alcohol mixture:** This fixative was originally recommended by Papanicolaou. It consists of equal parts of ether and 95% ethyl alcohol. It is an excellent fixative, but ether is not used in most of the laboratories because of its safety hazards, odour and hygroscopic nature.

3. **100% Methanol:** 100% methanol is an acceptable substitute for 95% ethanol. Methanol produces less shrinkage than ethanol, but it is more expensive than ethanol.
4. **80% Propanol and Isopropanol:** Propanol and Isopropanol cause slightly more cell shrinkage than ether-ethanol or methanol. By using lower percentage of these alcohols the shrinkage is balanced by the swelling effect of water on cells. Hence 80% propanol is a substitute for 95% ethanol.
5. **Denatured alcohol:** It is ethanol that has been changed by the addition of additives in order to render it unsuitable for human consumption. There are many different formulae for denatured alcohol; all of them contain ethanol as the main ingredient, and hence this can be used at a concentration of 95% or 100%. One formula is 90 parts of 95% ethanol + 5 parts of 100% methanol + 5 parts of 100% isopropanol.

Time of Fixation: Minimum 15 minutes fixation prior to staining is essential. Prolonged fixation for several days or even few weeks will not affect the morphology of cells. If smears are to be preserved over a long period of time in alcohol, it is better to store them in capped containers in the refrigerator.

B. Coating Fixative

Coating fixatives are substitutes for wet fixatives. They are either aerosols applied by spraying the cellular samples or a liquid base, which is dropped onto the slide. They are composed of an alcohol base, which fixes the cells and wax like substance, which forms a thin protective coating over the cells e.g. Carbowax (Polyethylene Glycol) fixative. Diaphine fixative Spray coating fixative (Hairspray) with high alcohol content and a minimum of lanolin or oil is also an effective fixative.

Most of these agents have a dual action in that they fix the cells and, when dry, form a thin protective coating over the smear. These fixatives have practical value in situations where smears have to be mailed to a distant cytology laboratory for evaluation. This method is not recommended for smears prepared from fluid within the laboratory as in any good method of fixation the coating fixative should be applied immediately on fresh smears. The distance from which the slides are sprayed with an aerosol fixative affects the cytology details. 10 to 12 inches (25-30 cm) is the optimum distance recommended for aerosol fixative. Aerosol sprays are not recommended for bloody smears, because they cause clumping of erythrocytes. Waxes and oils from hair spray fixative alter staining reactions if they are not adequately removed. Prior to staining, the slides have to be kept overnight in 95% alcohol for removal of the coating fixative.

C. Special Purpose Fixative

- **Carnoy's fixative:** This is a special purpose fixative for haemorrhagic samples. The acetic acid in the fixative haemolyses the red blood cells. It is an excellent nuclear fixative as well as preservative for glycogen but results in considerable shrinkage of cells and tends to produce over staining in hematoxylin. Overfixing in Carnoy's also results in loss of chromatin material.

Carnoy's fixative must be prepared fresh when needed and discarded after each use. It loses its effectiveness on long standing, and chloroform can react with acetic acid to form hydrochloric acid

- **AAF Fixative:** This is the ideal fixative used for cellblock preparation of fluid specimens

Mailing of unstained smears

Glycerine method for mailing slides: Smears are first fixed in 95% ethanol for 12 minutes and removed. Two drops of glycerine are placed on smears and covered with a clean glass slide. This may be wrapped in wax paper and mailed to the laboratory in a suitable container. Coating fixative such as carbowax fixative and spray coating fixative can be used primarily to facilitate transport of smears, mailing etc.

Prefixation of cytologic material

Prefixation may preserve some specimens for days without deterioration of cells. Some of the disadvantages of pre-fixation are precipitation/coagulation of proteins, hardening of cells in spherical shapes and condensation of chromatin. The coagulation of proteins may interfere with the adherence of cells to glass slides. It also 'rounds up' the cells - causes the cells to gather together into tight clusters making stain absorption and interpretation difficult. Albuminized slides should be used to prepare smears from prefixed sample. The most common solutions used for this purpose are:

- Ethyl alcohol (50% solution)
- Sacomanno's fixative (50% alcohol with 2% Carbovax 1540)
- Mucollex (A commercial mucoliquifying preservative for the collection of mucoid and fluid specimens)

Many other preservatives have been developed for use with automated cytology systems.

Rehydration of Air Dried Smears

Unfixed, air-dried gynaecological smears received from peripheral areas can be used for Papanicolaou staining by rehydration method. The simplest rehydration technique is to place air dried cytological specimens in 50% aqueous solution of glycerine for three minutes followed by two rinses in 95% ethyl alcohol, and then stained by the routine Papanicolaou method.

Staining Methods in Cytology

Papanicolaou Staining Method

Papanicolaou staining method is the routine staining procedure used in cytopathology laboratory. This technique is named after Dr. George N. Papanicolaou, the father of exfoliative cytology and is devised for the optimal visualization of cells exfoliated from epithelial surfaces of the body. It is a polychrome staining reaction designed to display the many variations of cellular morphology showing degree of cellular maturity and metabolic activity. The use of the Papanicolaou stain results in well stained nuclear chromatin, differential cytoplasmic counterstaining and cytoplasmic transparency.

Steps of staining procedure

a. Fixation

The cytology smears are fixed in 95% ethyl alcohol or in other substitutes for a minimum of 15 minutes.

b. Nuclear staining

It is done by using haematoxylin stain. Harris haematoxylin or its modified form is used in Papanicolaou staining in regressive method, in which we deliberately over stain with haematoxylin and remove the excess stain by using a differentiating solution such as acid alcohol (0.05% HCl in 70% ethyl alcohol) or 0.05% aqueous solution of HCl alone. As haematoxylin is used in an acid pH, a pink colour will form and it is not stable. In order to make it stable, the compound is brought to alkaline pH (bluing) by treating with a weak alkaline solution. Running tap water which is slightly alkaline (pH 8) is used as bluing solution in small laboratories. Ammonium hydroxide solution (15 ml of ammonium hydroxide 28-30% weight/volume to 985 ml of 70% ethanol) can also be used.

c. Cytoplasmic staining

Cytoplasmic stains are OG-6 and EA-36. Both are synthetic stains and OG-6 is a monochrome stain while EA-36 is a polychrome stain.

d. Dehydration

Rinse the smears in absolute alcohol for two or three changes for the removal of water. Smears left in rinses for long will lose too much stain. Alternative to 100% ethanol are 100% isopropanol and 100% denatured alcohol. Rectified spirit affects the cytoplasmic staining and hence is not recommended.

e. Clearing

Cells are not transparent while the smear is in the staining or alcohol solutions. During clearing, alcohol is being replaced with Xylene, which is also miscible in mounting medium. Xylene has a refractive index as that of glass and mounting medium and it prevents cellular distortion.

f. Mounting of slide

The mounting media must be miscible with the clearing agent to prevent fading of the stains. Practice is essential to achieve well-mounted slides, free of air bubbles and artifacts. A minimum of mounting medium should be used. Too much mounting medium interferes with microscopic detail, making the cell film appear hazy or milky when examined under the high power objective. If the mounting medium and cover slip are applied too slowly, a common artifact appears as a brown refractile pigment like substance on the surface of the cell when xylene evaporates. If this artifact occurs, the slide must be soaked in xylene, absolute alcohol and 95% alcohol, rinsed in running tap water and restained in OG and EA. A possible means of preventing the “brown artifact” is to coverslip slide behind a transparent chemical splash shield set at the front edge of the fume hood. The shield diverts air around the local workspace and reduces the rate of xylene evaporation. The usual size of the coverslip for a cervical smear is 22x30mm. If the smear spread is beyond the coverslip area, ideally use another small coverslip or put a drop of DPX and spread evenly with the same coverslip without affecting the focus.

Precautions

1. Immediate fixation of smears is essential.
2. Smears should never be allowed to dry before placing the coverslip.
3. Haematoxylin is filtered everyday before use.
4. All solutions and other stains are filtered daily after use, to keep them free of sediment.
5. Avoid contamination from one smear to another.
6. Keep stains and solutions covered when not in use.
7. All dishes are washed daily.
8. Stains are discarded and replaced as the quality of the stain deteriorates.
9. Avoid contamination during placing of the coverslip, with the dropper used to dispense the mounting medium.
10. Place the coverslip on the microslide slowly without trapping air bubbles.

Maintenance of stains and solutions

- ▶ Solutions may be used for longer period of time, if the slide carrier is rested on several layers of tissue paper (paper toweling) for a few seconds before transferring to the solutions.
- ▶ Stains keep longer if they are stored in dark coloured, stoppered bottles.
- ▶ Haematoxylin keeps relatively constant staining characteristics and do not require frequent discarding if small amounts of fresh stain are added to replace stain loss due to evaporation.
- ▶ Use of coating or spray fixatives may cause contamination making frequent changes necessary.
- ▶ OG and EA stains lose strength more rapidly than haematoxylin and should be replaced each week or as soon as the cells appear without crisp staining colours.
- ▶ Bluing solution and HCl should be replaced at least once daily.
- ▶ Water rinses should be changed after each use.
- ▶ Alcohol used for the process of dehydration prior to the cytoplasmic stains may be replaced weekly. The alcohol rinses following the cytoplasmic stains are usually changed on a rotating basis after each use. The alcohol rinse immediately following the stain is discarded and the other two rinses are moved to the first and second position and fresh unused alcohol is replaced in the third position. Ideally this rotation must continue after each staining run. The absolute alcohols should be changed weekly and can be kept water free by adding silica gel pellets.
- ▶ Xylene should be changed as soon as it becomes tinted with any of the cytoplasmic stains. Xylene becomes slightly milky if water is present in it and if so the clearing process may be disturbed. Tiny drops of water may be seen microscopically on a plane above the cell on a slide. Addition of silica gel pellets to the absolute alcohol will minimize water contamination of xylene.
- ▶ Agitation of the slides by occasional dipping is necessary to remove excess dye. Dipping should be done gently to avoid cell loss and the slide carrier should not hit the bottom of the staining dish.
- ▶ The quality of the stained slide is dependent on timing, solubility and percentage of dye concentration.

Papanicolaou Staining Procedure

1.	90% Ethanol (fixation)	-	15 minutes(mt)
2.	80% Ethanol	-	2 mt.
3.	60% Ethanol	-	2 mt.
4.	Distilled water	-	5 dips
5.	Distilled water	-	5 dips
6.	Haematoxylin stain	-	2 mt.
7.	0.05% HCl solution	-	2 mt.
8.	Running tap water (Bluing)	-	10 mt.
9.	60 % Ethanol	-	2 mt.
10.	80% Ethanol	-	2 mt.
11.	80% Ethanol	-	2 mt.
12.	95% Ethanol	-	2 mt.
13.	OG-6 stain	-	2 mt.
14.	95% Etanol	-	2 mt.
15.	95% Ethanol	-	2 mt.
16.	95% Ethanol	-	2 mt.
17.	EA-36 Stain	-	2 mt.
18.	95% Ethanol	-	2 mt.
19.	95% Ethanol	-	2 mt.
20.	95%Etanol	-	2 mt.
21.	95% Ethanol	-	2 mt.
22.	Absolute Ethanol	-	2mt.
23.	Absolute Ethanol	-	2 mt.
24.	Absolute Ethanol	-	2 mt.
25.	Absolute Ethanol+ Xylene (1:1)	-	2mt.
26.	Xylene	-	5 mt.
27.	Xylene	-	5 mt.
28.	Xylene	-	till clear
29.	Mounting in D.P.X		

Rapid Papanicolaou Staining

The purpose is to save staining time and money by combining OG and EA and reducing the number of rinses. This procedure needs to be done only for emergency situations and not for routine use.

Contamination Control

All stains, Haematoxylin, OG-6 and EA-36 should be filtered at least once daily and after staining any slides containing known cancer cells. The alcohols used for rehydration, dehydration, absolute alcohols and xylene must be filtered or replaced daily. Gynaecological and non-gynaecological materials may be stained separately. Specimens notorious for shedding cells like sputum and specimens suspected to have large number of cancer cells should be stained at the end of the day using separate rack. Even with all these precautions, gross contaminations may occur and if this happens with malignant cells all solutions and stains must be immediately filtered or discarded.

Haematoxylin and Eosin (H&E) staining method

Some laboratories use routine H&E stain for non-gynecological smears. The benefits of using Papanicolaou stains are clear definition of nuclear details and differential counter staining giving cytoplasmic transparency. H&E stain does not satisfy these criteria and hence unacceptable for cervical smears.

May-Grunwald-Giemsa (MGG) Staining method

Many laboratories use MGG (Romanowski type stain) staining method for cytological diagnosis of non-gynaecological specimens in addition to Pap and H&E stains. Combination of all these stains increases the efficiency of microscopical interpretations. MGG stain is performed in air-dried aspirates or fluids.

Stock solutions of May-Grunwald Reagent and Giemsa Stain are available commercially.

Staining procedure

1	May-Grunwald solution	-	5 mt.
2.	Running water	-	1 mt.
3.	Geimsa solution	-	15 mt.
4.	Running water	-	1 - 2 mt.
5.	Air-dry (No mounting necessary)		

Labeling of slides

After the slides have been cleaned, they are ready for labeling. Place a small square label on the edge of the slide on the same side as the cover slip. Use water proof ink and record the institution, the number, the year, the nature of specimen etc. on it.

Filing the slides

The slides must be protected from breakage, light, moisture and dust. After microscopical interpretation, the slides must be filed in slide filing cabinets in serial order, in numbered slots. They are kept for a minimum of 5 years and are retrieved when necessary.

Appendix

Organisation of Cytopathology Laboratory

The organization of a smoothly functioning cytopathology laboratory requires knowledge of community needs, available professional and man power support, financial limitations, physical service facilities, and record keeping systems.

Laboratory Personnel

The personnel needs of a laboratory depends on overall work load and the different types of cytology materials to be processed.

The Chief of the Laboratory

He / she should be a cytopathologist / pathologist or a gynecologist / medical officer trained in cancer related cytology.

Cytotechnologist

Screening of smears should be performed by well trained and certified cytotechnologists. The IAC recommendation is that cytotechnologist should have undergone one year cytology training from a recognized, accredited centre or should have passed National Examination for cytotechnologists conducted by IAC, after graduation/post-graduation in any of the life science subjects. There are very few institutions in India accredited for conducting training in cytology.

The duties of the cytotechnologist include supervision of cytopreparation, preparation of stains and maintenance of its quality, screening of smears and formulation of preliminary diagnosis, and formulation of final diagnosis in certain well defined circumstances. They are also responsible for supervising the record keeping, analysis of data and slide filing system.

Cytotechnician

Cytotechnicians will have a diploma in medical laboratory technology from a recognized institution who have also undergone 6 months training course for cytotechnician from an accredited laboratory or passed the National Examination for cytotechnicians conducted by IAC. They are employed in the support segment of the laboratory operation viz specimen collection, preparation and staining areas. They perform highly skilled repetitive procedures and can be involved in the preliminary screening of cytology samples received from the population based cancer control programmes. All such procedures are under direct supervision of cytotechnologist. After 5 years of experience in a good cytology laboratory

with facilities for continuing education programmes, they are eligible to write the National Examination for cytotechnologist or to join for the one year cytotechnologist training course.

Support Staff

Clerical and secretarial work in the laboratory may be performed by specialized support personnel or in small multipurpose laboratories by technical personnel. It is better to avoid utilization of technical staff for clerical jobs compromising their time on technical work. Appointment of well-trained clerical personnel is preferred.

Physical Infrastructure

The laboratory must be well designed and conveniently located to enable the professional and support personnel to perform their duties effectively. It must contain four definitely separated areas:

- Reception.
- Specimen collection room.
- Processing and staining area.
- Reporting room.

The area of the laboratory may be preferably 20ft. x 12ft. The work bench with 2.5 feet width and 3 feet height from the floor may be at two adjacent sides of the laboratory at the side where there is enough ventilation. The bottom of the work bench can be made as cupboards with one or two racks for storage of materials. The reagent shelf with size of 3 feet height and $\frac{3}{4}$ feet width may be fitted over the workbench. Laboratory sink must be fitted at one end of the work bench. Four Power plugs, 2 with a capacity of 15 amps and the other two with a capacity of 5 amps must be made available at the other end of the work bench.

It must be well ventilated and must have a powerful exhaust fan. The chemicals and volatile substances used in the specimen processing area must be stored in separate rooms adjacent to the laboratory. The laboratory should have separate space for specimen collection, smear preparation, staining and screening purposes. Screening areas should be well lighted and ventilated. Distracting noise of nearby traffic or equipment should be minimized. Cytoscreener should have sufficient space and comfortable seating arrangement to permit easy performance of microscopic examination.

Clerical and record keeping system should be located near the screening area for rapid retrieval of data. Health and fire regulations of the state and local authorities must be observed for the personal safety of laboratory personnel.

The following criteria are to be applied while planning a cytology laboratory:

- Provision for getting all relevant clinical information.
- Collection of specimens, preparation of smears, proper fixation and staining.
- Provision for complete screening of all specimens.
- Reporting of specimens, enabling clinical management of patient.
- Provision for quality control and laboratory safety measures.

Receiving of specimens

Identification and absolute specimen integrity are to be maintained throughout the entire processing and reporting of all cytology samples. The following protocols are followed during specimen reception:

- Ensure that the specimen is properly labelled and submitted along with the specific requisition form.
- Match all slides with the requisition form.
- Check names and verify mismatches, if any, and report it to the referring hospital / doctor.
- Verify patient's history including Last Menstrual period, Last Child Birth along with previous Cytology / Histopathology reports, if any.
- It is mandatory to specify the site from where the specimen has been collected in order to avoid confusion between a non-gynaecological specimen and a cervical smear.
- The number of slides received from each site should be mentioned in the requisition form.
- Nature and method of sample collection are to be mentioned in the requisition form. (Cytobrush / Spatula / Swab / for gynaecological smears and plain/guided FNAC for aspiration smears)
- Check whether the fixation is proper. (Type of fixation: alcohol / spray fixative / prefixed / air dried)
- Separate registers may be maintained for gynaecological, non gynaecological and sputum (if not computerized.)
- Enter patients name, age, sex, address, brief clinical details, and name of referring hospital / doctor in the register.

- A unique sequential accession number that will be preceded by the last two digits of the current year should identify each specimen.
- Mention date and time of receipt of specimen.
- Address to which report is to be dispatched should be mentioned in the requisition form.

Guidelines to be followed for laboratory safety

- Laboratory request forms accompanying specimen should not remain wrapped around the specimen container.
- Prepare specimens in a room or area away from other work place.
- It is ideal to process all specimens under a laminar flow hood.
- The technical person must wear disposable gloves, gowns, aprons, face paper etc.
- Inspect centrifuge tubes for cracks.
- Never pipette samples with mouth.
- Keep hands away from mouth, nose, eye and face during specimen processing.
- Discard needles into a needle disposal container and residual fluids into autoclavable, splash proof container.
- Before disposing, contaminated utensils must be autoclaved.
- Areas where infectious materials are handled may be disinfected.
- Keep a properly functioning fire-fighting system in the laboratory.
- Avoid eatables in the working area.
- Skilled professional should carry out maintenance / servicing of all equipments including microscope at regular intervals.

Record keeping and follow up

Record keeping is very important to enable proper data retrieval for various purposes. A system for proper maintenance of registers or use of computer for maintaining data is advisable. Smears must be preserved for 3-5 years, in year wise accession number after reporting. This is important as a quality control measure and also for follow-up.

Minimum Requirements for Setting up a Small Cytology Laboratory

Space

- Reception
- Laboratory (20 ft x 12 ft)
- Examination Room (10 ft x 10 ft)
- Reporting /Office Room (10 ft x10 ft)
- Toilet. (Separate for males and females)

Furniture		Number
Work bench with reagent rack with cupboards & drawers on both ends (8 ft x 4 ft height-2.5ft)		1
Small Table (4ft x3ft)		1
Office table		1
Revolving Chair		1
Plastic Chair		3
Plastic Stool		2
Laboratory Equipments		Number
Microscope (Binocular)		1
Centrifuge (4 tubes capacity)		1
Chemical Balance		1
Refrigerator		1
Stainless Steel Electrical Sterilizer		1
Distilled Water Unit		1
Slide Filing Cabinet (10,000 Capacity)		1
Speculum Cuscos: Large		2
Medium		6
Small		2
Bivalve Vaginal Speculum Sims; Medium		2
Kidney Tray (Stainless Steel)		2
Metal Tray (Stainless Steel)		2
Punch holding forceps (Stainless Steel)		1
Forceps (Stainless Steel)		2
Gynecological couch (With step and focusing lamp)		1
Slide Tray (MetallicTray,20 slides capacity)		5
Electric Heater (Hot coil)		1

Chemicals	Quantity
Haematoxylin powder	25gms
OG 6 Certified	25gms
Eosin Yellow Water soluble Certified	25gms
Light Green SF (Yellowish) Certified	25gms
Phloxine B Certified	25gms
Phosphotungstic acid pure	100gms
Aluminium Ammonium Sulphate AR	2x500gms
Sodium Iodate (Extra pure)	100gms
Xylene Extra pure	4x2.5lit
DPX Mountant	500ml
HCl	500ml
Isopropyl Alcohol	10x2.5lit
Consumables	Number
Ayre's Spatula (Wooden)	500
Disposable tongue depressor	100
Disposable syringe 10ml	250
Disposable needle 22 G	250
Gloves (Disposable)	100
Koplin jar (Plastic)	30
Glass Staining jar with lid (20 slides carrier)	30
Slide carrier (20 slides capacity Stain less steel)	5
Bottle brush	2
Microslides	25 boxes
Micro cover glass (22mm x 30mm)	20x10gm
Slide Boxes (Plastic 100 slides)	3
Rubber Sheet (For gynaec couch)	1
Diamond glass marking pencil	3
Filter paper Whatmann No 1	5 sheets
Blotting paper	50 sheets

Chemicals		Quantity
Glass Wares		Number
Centrifuge tube		1 dozen
Test tube		3 dozen
Conical flask 250ml		1
Conical flask 500ml		1
Beaker 500ml		1
Round bottom flask 1000ml		1
Measuring Cylinder 500ml		1
Measuring cylinder 100ml		1
Pipette 10ml		1
Funnel (Medium size)		2

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