

## Opinion Paper

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# Pre-analytical issues in effusion cytology

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**Abstract:** Effusions or body cavity fluids are amongst the most commonly submitted samples to the cytology laboratory. Knowledge of proper collection, storage, preservation and processing techniques is essential to ensure proper handling and successful analysis of the sample. This article describes how the effusions should be collected and proper conditions for submission. The different processing techniques to extract the cellular material and prepare slides satisfactory for microscopic evaluation are described such as direct smears, cytospins, liquid based preparations and cell blocks. The article further elaborates on handling the specimens for additional ancillary testing such as immunostaining and molecular tests, including predictive ones, as well as future research approaches.

**Keywords:** body cavity fluids, effusions, FISH, immunostaining, molecular, processing, storage, techniques.

## Introduction

The serosal cavities are normally lined with a single layer of small mesothelial cells and contain a scant amount of fluid for lubrication. Serous effusions are pathologic body fluids collected from the serosal cavities such as the pleural, peritoneal and pericardial cavities that have accumulated due to a wide range of underlying diseases both benign and malignant. Examination of the exfoliated and exudated cells can shed a light on many conditions besides the diagnosis of cancer such as inflammatory and autoimmune conditions of the serous membranes, as well as infections. Serous effusions and pelvic washes are among the most commonly encountered specimens in the cytology laboratory. Fluids can be

collected by inserting a wide-bore needle through the body wall into the fluid cavity and aspirating the fluid. Pelvic and peritoneal washes are collected intra-operatively by flushing the peritoneal wall by a small amount of balanced salt solution and collecting the fluid. The proper collection, storage and processing of such fluids can make the difference between an accurate and definitive diagnosis versus an uninterpretable specimen.

This article provides a review of the literature and the recommendations of the authors regarding the pre-analytical phase of body fluids for the cytology laboratory including the collection and handling, laboratory triage of specimen, specimen processing for routine examination and for ancillary testing. A literature search utilizing the Ovid Medline and PubMed search engines was performed using the following words singly and in combination: cytology, effusion, pleural fluids, peritoneal fluids, pericardial fluids, storage, technique, processing, smears, cell block, immunostaining, molecular, and FISH. A total of 659 records and 2 text books were identified. Duplicate and irrelevant records were excluded resulting in 93 relevant records.

## Fluid collection

The clinicians throughout the health care facility should be properly educated on the preferred methods for the collection of fluids and their transfer to the laboratory. An instruction manual periodically updated by the laboratory should be available as a reference. The idea is to have the fresh fluid collected and transferred to the laboratory without degeneration or compromise of the cellular viability. Fluids are natural cell suspensions and a fresh fluid, i. e. collected in its natural state and without added preservatives is best suited for cytological processing and further triage for additional testing [1].

Body fluids are usually divided among several laboratories for testing depending on the clinical differential diagnosis. In general a portion of the fluid is submitted to the cytology laboratory for morphological analysis of cells, identification of malignancies and when appropriate performing additional ancillary tests. Additional aliquots may be submitted to the hematology

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laboratory for cell count (lymphocytes and other inflammatory cells) or for flow cytometry in cases suspected of lymphoma; chemistry for establishing characteristics of the fluid such as pH, albumin levels, etc. to classify the fluid as a transudate versus an exudate or for serological testing especially in the setting of autoimmune diseases; and microbiology testing and cultures, etc.

Body cavity fluids also have higher concentration of thrombin and fibrinogen than blood. Consequently in aspirated bloody fluids the thrombin can act on the fibrinogen and convert it to fibrin which tend to entrap the diagnostic cells. This clotting tendency can be counteracted by using pre-heparinized glass bottles using 3 units of heparin per 1 mL of the bottle capacity. Currently, blood collection bags are used instead and pre-heparinized hypodermic needles can be used to prevent clotting during aspiration. It is important to know that heparin added after the collection of a fluid in a glass bottle will not inhibit clotting [1, 2]. However, many laboratories may not require the use of anticoagulant and prefer the fluid in its natural state [3]. While the addition of heparin does not impact the morphologic features it can negatively affect other findings e. g. causes lower pH [4].

Individual laboratories have their unique requirements for the containers that would be accepted by the laboratory e. g. glass versus plastic. A minimum volume of approximately 100 mL is recommended to ensure adequacy of sample for proper cytological processing and evaluation. Studies evaluating minimum fluid volume required indicated that at least 60 mL of fluid is necessary for adequate cytological diagnosis of malignancy in pericardial effusions [5] and a minimum of 75 mL for pleural fluids [6].

## Key facts

1. Fresh fluids are natural cell suspension.
2. Adding few drops of heparin may inhibit clotting of bloody samples and does not interfere with cytological diagnosis but may interfere with other tests.
3. A minimum of approximately 100 mL is recommended for adequate evaluation

## Fluid preservation

The natural fluid is rich in its own proteins that serve as nutrient for the cells while in storage and the fresh status allows for better cell spread and adhesion on the microscopic glass slides [1]. It is recommended that fresh fluids are refrigerated at 4 °C until processing. Studies evaluated the effect of storage on fluid morphology, immunostaining

(IMS) and serologic markers. Guzman et al evaluated the number of cells, morphology and IMS with HEA-125 from day 0 to day 4 in fluids collected in EDTA-coated tubes and stored either at 4 °C or room temperature and found that IMS staining was consistent until day 4, however the storage had a negative effect on some lymphocyte markers [7, 8]. Similarly, Masosca et al evaluated aliquots of fluids refrigerated at 4 °C between day 0 and day 14 and reported that both the morphology, IMS profiles and amplifiable DNA were preserved [9]. Antonangelo et al. evaluated the effect of storage at either 4 °C or –20 °C on the determination of adenosine deaminase in suspected tuberculous effusions and found that the enzyme could be measured from either conditions up to 28 days of storage without significant change [10].

While it is ideal to send the fluid fresh, rare situations such as shipping to a centralized laboratory may necessitate preservation. Because fluids are rich in nutrients, there is no need to add solutions such as normal saline. However in specimens such as pelvic and gutter washes, it is recommended to collect them in a small volume of balanced electrolyte solution (BES) also known as physiological solution. BES is sterile and pyrogen free and therefore can be used in vivo or in vitro. It should be noted however that balanced salt solution (BSS) is not pyrogen free and therefore should only be used in vitro. BSS is more expensive, sold in small amounts and would only be required if cell cultures will be pursued [1]. For cytological evaluation an aliquot of the fluid can be submitted in alcohol based collection media. The specimen can be added to an equal volume of alcohol based collection media that is specified by the laboratory. Currently, two such media are commercially available and commonly used for liquid based preparations (LBP); CytoLyt™ a methanol based media used for ThinPrep™ (TP; Hologic, Marlborough, Mass., USA) and CytoRich™ an ethanol based media for SurePath (SP; BD. TriPath, Burlington, N.C., USA). Other alcohol based preservatives such as Saccomanno can also be used. It is important to realize however, that once fixed, these specimens cannot be used to prepare direct smears and no air dried preps regardless of the technique used can be prepared with acceptable Romanowsky stain results.

## Key facts

1. Fluids are rich in nutrients
2. When needed BES and BSS are suitable as collecting media
3. Can collect in specified alcohol based solutions for LBP

## Laboratory processing

Once the specimen is received in the laboratory, it is entered in the laboratory data system and provided an accession number. The fluid is grossly evaluated and the volume and gross characteristics are documented, e. g. color, clarity and any unusual features such as viscosity. The fluid is then shaken to ensure equal distribution of cells and aliquots are collected in 50 mL tubes for centrifugation and further processing depending on the techniques used by the laboratories. Effusions are unique specimens and can vary widely in their cellularity, protein or blood and clot content and consequently a wide range of techniques to process fluids have been described in the literature. To harvest the cells, the fluid is usually concentrated first. In the past prior to the introduction of centrifugation and LBP, the fluids were filtered and the cells were captured on the filter surface and stained along with the filter and then mounted on a slide for examination (Millipore or Nuclepore). This method is rarely used now and instead the fluids are centrifuged and additional preps are performed from the cell pellet. A variety of methods have been described to handle bloody fluids including flotation technique, blood hemolysis before sample preparation e. g. Saponin technique [1] or after sample preparation and even after the slide is prepared e. g. rehydration of air-dried smears with normal saline [11].

The easiest and most efficient and cost effective method is preparing a direct smear from a drop of the sediment. Many laboratories also include a concentration technique to harvest the cells such as cytopins, Millipore filters and LBP as well as a cell block (CB). These preps can be either air dried and stained by the Romanowsky stain, or ethanol fixed and stained by the Papanicolaou method. Many studies evaluated these processing techniques seeking to find the best methodology and combination. Based on these studies, it is recommended to use a combination of techniques such as air dried and fixed smears along with a concentration method to achieve highest sensitivity [12, 13]. Starr and Sherman evaluated the performance of 5 different preparation techniques in the diagnosis of 108 malignant effusions. Malignant cells were detected in 68 % of the ethanol fixed Papanicolaou stained smears, 68 % of the air dried Romanowsky stained smears, 83 % of the cytopins and 85 % of the CB and Millipore filters. Cancer cells were detected in 90 % of cases when a combination of one fixed, one air dried and one of the three concentration techniques were used. Of interest is their finding that 5 of the 30

hematologic neoplasms would have been missed if the combination did not include a CB [12].

Recently LBP emerged as a methodology that provides numerous advantages. TP is based on sample filtration (Figure 1) while SP is based on sedimentation through a Ficoll gradient tube. The methodology is automated and the preps produce thin-layer with minimal cell overlap and reduced contamination by blood or other obscuring factors. A single prep is considered representative of the sample thus eliminating the need for preparing several slides. In the first author's experience additional slides prepared from the same vial for teaching or research are usually reproducible and consistent with the original slide [14]. It is recommended that a cell block is prepared in conjunction with LBP (reports for TP) this is particularly important because fluids may contain large fragments that may not pass through the filter and are only noticed on the CB [15]. Other reports echoed similar findings in transbronchial aspirates and washes [16–18]. This has also been the first author's experience especially in hemorrhagic effusions. Data is not sufficient to comment on the SP method.

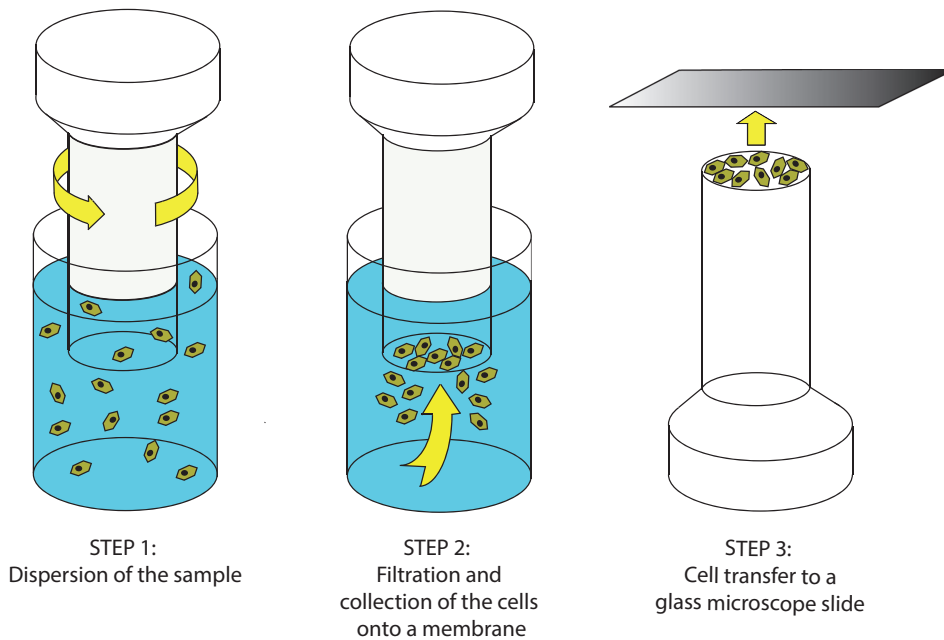
## Key facts

1. Need to check for the special processing technique used by your laboratory
2. Specimen is subjected to vigorous shaking to redistribute the cells, centrifugation or other enriching process and cell deposition on the slides according to selected method.
3. Slides are prepared in various ways: Direct smears, filters, cytopins, or LBPs
4. Slides may be air dried and stained with Romanowsky stain or fixed and stained with Papanicolaou stain. However LBP are always fixed.
5. Cell block is frequently prepared as a complement in many laboratories

## To screen or not to screen

Prior to further processing, many laboratories screen the fluid by preparing a toluidine blue test (wet film) which involves placing a drop of the fluid sediment and a drop of toluidine blue dye on a slide, mixing the two drops with the corner of a coverslip and covering the slide by the cover slip. The slide can be immediately screened by the cytotechnologists to assess the cellularity and the

### Cytology: Liquid-based preparation(LBP)



**Figure 1:** In ThinPrep, one example of LBP, the sample is gently agitated to disperse mucus and other debris and promote random distribution of cells (step 1). A vacuum is then applied to force the broken erythrocytes and debris to pass through a polycarbonate thin filter attached to the end of the cylinder while the cells of interest adhere to the surface of the filter (step 2). The cylinder is finally removed, inverted and pressed against a slide while a gently positive air pressure is applied to ensure transfer and adherence of cells to the slide.

presence of malignant cells. Based on the results, the cellularity can be adjusted e.g. low cellular specimens can be concentrated while highly cellular specimens can be diluted to ensure proper assessment. In addition, many laboratories will hold the malignant cases to be stained at the end before cleaning the staining circuit thus avoid cross contamination of other slides. This is based on the notion that clusters of malignant cells particularly in the super positive fluids can shed in the staining solutions and deposit on other slides [3, 19] (Figure 2). While this represents an additional step, it is well worth it in the authors experience particularly when using direct smears. Laboratories using liquid based preparations tend to consider this step unnecessary. However, in the first author's experience, while contamination from super positive highly cellular cases is very rare with LBP, doing wet film is still well worth the trouble since such incident can pose serious diagnostic problems and bring the entire laboratory to a halt while cleaning the staining circuit. Additional individual specimen triage for immediate interpretation and recommendations for further processing can be done on wet film or air dried Romanowsky stained smears. However, it was shown that such process may not provide practical

advantage in the turnaround time or diagnostic accuracy particularly in light of the resources used [20, 21].

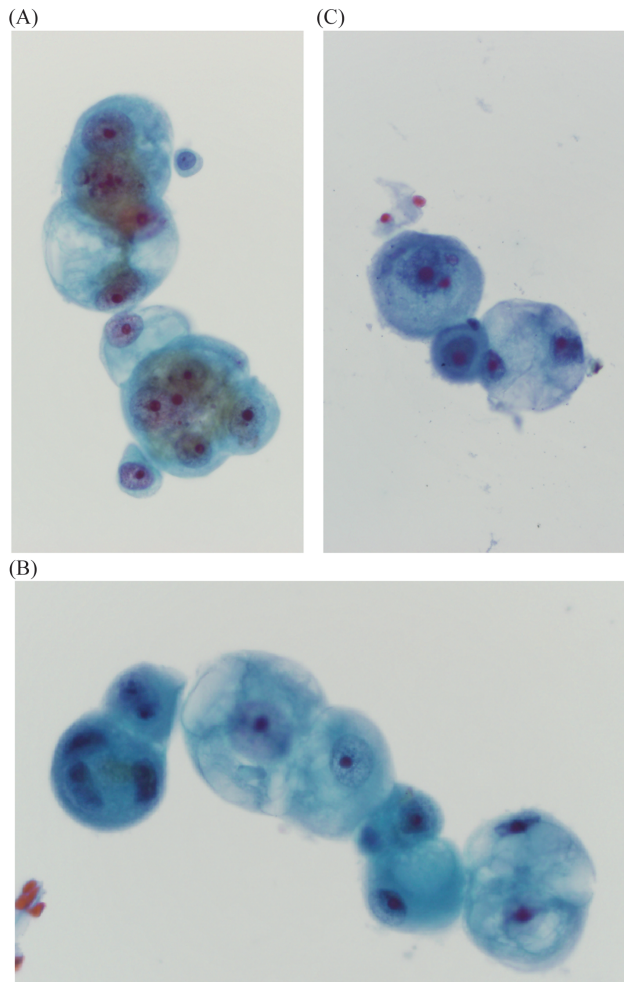
### Key facts

1. Wet film is used to screen for:
  - a. Cellularity-adjust for processing
  - b. Super positive cases-stain at the end to prevent contamination

### Cell blocks

The cell block is a process in which the cellular pellet in its entirety can be fixed and embedded in paraffin blocks from which 4–5  $\mu\text{m}$  sections are cut and stained with hematoxylin and eosin (H&E) or additional stains as the case requires e.g. special stains for microorganisms, IMS to classify malignant cases etc.

A variety of techniques have been described for cell blocks but the main principle is the same. The fluid is centrifuged, the supernatant is discarded and the pellet is fixed in situ and removed en bloc to a cassette that will



**Figure 2:** Example of contamination caused by a positive pleural effusion. Upon comparison of all cases it was determined that malignant clusters in B and C are due to contamination. (A) A direct smear prepared from a fluid with metastatic breast carcinoma was erroneously stained in the same circuit with other non-gynecologic specimens. (B) Slide from a negative pancreatic FNA contained a cluster of adenocarcinoma at the periphery. (C) Slide from a negative thyroid FNA contained a similar cluster of malignant cells.

be formalin fixed and paraffin embedded (FFPE) and processed as any histology specimen. Pellets can be clotted through the thrombin clot technique by adding 4 drops of fresh plasma and 1–2 drops of thrombin, mix well and allow to clot for 1–5 min [1]. The pellet can also be solidified by adding a gelling agent such as melted agar or HistoGel [22, 23] and allowing it to cool at room temperature. For both these techniques, the pellet can still be fragile and so many labs resort to wrapping it in tea bags or tissue wrappings prior to placing it in the cassette to avoid loss of tissue (Figure 3). The Shandon Cytoblock system [24] offers an alternative cell block preparation that eliminates the need for pellet wrapping.

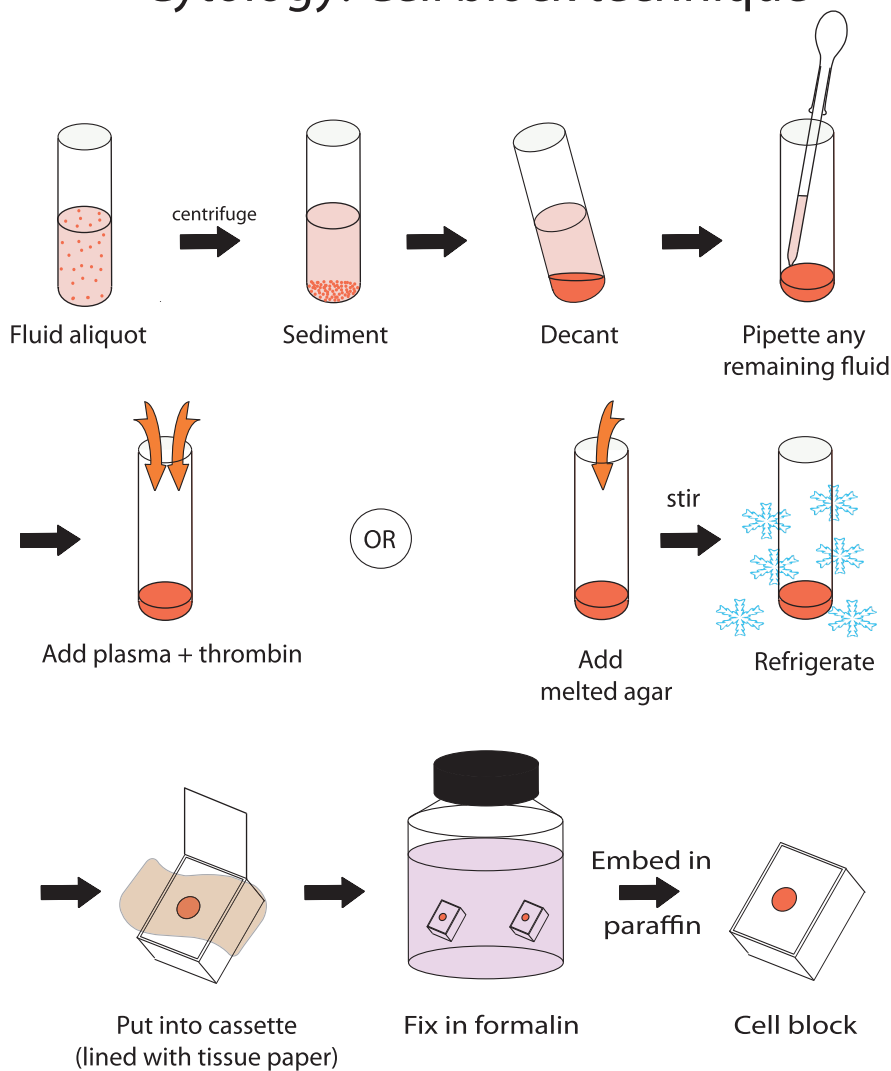
Hologic Cellient system is an alternative fully automated cell block preparation that is currently available [25].

Although the cell block methodology was described and practiced as early as the introduction of the cervical smears, there is still controversy on the necessity to use it. Some consider that the routine use of CB is not cost effective as the detection of few malignant cases that could be missed do not justify the cost involved. They recommend that it can be done on selective cases as needed after the slide review [26]. This however would delay the diagnosis by at least 24 h since the CB will require formalin fixation for several hours in addition to the time needed for further processing in the histology laboratory. Others also argue that additional cytopsins are more cost effective, can be prepared faster and can be used for ancillary testing. Starr et al. evaluated the comparable cost of the different cytological techniques accounting for the cost of disposables, preparation, staining and screening. They reported that the material cost of cytopsins when the slides sold by the manufacturer were used exceeded the cost of CB. However, the cost was comparable if regular slides were used for the cytopsins [12]. On the other hand, there are many advocates for the use of CB routinely in conjunction with smears or other preps. Several reports recommended the use of CB to enhance the diagnostic accuracy. As previously mentioned, its use in combination with smears improved the detection of cancer cells in the malignant fluids from 66% and 68% for fixed and air dried smears respectively to 90% [12]. Also as previously mentioned it is highly recommended to use CB in conjunction with LBP [15]. The CB is particularly valuable in providing a high concentration of cells in a small area of the slide, a histologic correlate of the cytology material and sometimes shed the light on the architecture of micro-fragments, additional slides for further ancillary testing to classify malignant cases and even molecular testing, and archival material for future use [27, 28].

## Key facts

1. Cell block is prepared by solidifying a pellet either by forming a thrombin clot or adding a gelling agent
2. It allows for the presentation of a large number of cells concentrated on a small area of the slide
3. Provides a histological correlate to the cytology sample and better visualization of micro-fragments
4. Provides additional slides for ancillary testing
5. Can be easily archived for future testing or research

## Cytology: Cell block technique



**Figure 3:** Cytology: cell block technique.

Preparing a cell block requires centrifugation of the sample aliquot to concentrate the cells. The supernatant is then discarded and the cell pellet is solidified either by using the thrombin clot technique or by adding a gelling agent. The solidified pellet is put in a cassette lined by either a sponge or tissue paper, closed and fixed in neutral formalin for several hours. The fixed pellet is then embedded in paraffin and sectioned at 4–5  $\mu\text{m}$  slices that are deposited on glass slides and stained by H&E or other IMS.

## Preparations for ancillary testing

### Immunostaining (IMS)

Different preps and conditions have been tested particularly for IMS use. While there are advocates on both sides i. e. use of smears/cytospins versus CB, there is a general agreement that when properly prepared and validated all techniques produce satisfactory results. Ueda evaluated the immunoreactivity in reactive and malignant effusion samples to a panel of IMS using ethanol fixed smears,

ethanol fixed CBs, and formalin-fixed CBs and reported that the immunoreactivity of both CB types were significantly lower than that of the ethanol fixed smears [29]. Fetch et al. evaluated immunoreactivity using three commonly utilized preparations: air dried cytopspins post fixed in ethanol, FFPE CBs prepared by the thrombin clot method, and TP. They reported that cytopspins and TP performed in a similar manner. Those preps tended to have a high background staining encountered in 66 % of cases, most evident in three-dimensional cell clusters. They also noted difficulty in interpreting membranous stains. The CB provided the best milieu with high

background staining in only 17 % of cases and the results were closely similar to those reported in the surgical pathology literature [30].

Shidham et al first evaluated the impact of different smear fixation methods on the immunoreactivity for anticytokeratin AE1/AE3 using scrapes from fresh specimens and compared the results with the correlating paraffin embedded tissue sections (FETS). The fixation methods included: smears directly fixed in 95 % ethanol, air dried then rehydrated in saline or directly fixed without rehydration by one of three fixatives (alcoholic formalin, 95 % ethanol with 5 % acetic acid or 95 % ethanol) and found that air-dried, saline rehydrated smears post fixed in alcoholic formalin had both higher intensity and proportion scores compared to those without rehydration and had comparable immunoreactivity to those with wet fixed smears and FETS [31]. In an extended study, they evaluated scrapes prepared from 34 fresh specimens fixed with the above methods and stained with a larger panel. The immunoreactivity was compared with that seen on the correlating PETS. Again their results showed that except for vimentin, all IM stains showed the best results when either directly fixed in 95 % ethanol or air-dried-rehydrated and post fixed in alcoholic formalin [32]. Additional studies confirmed success using direct smears air dried and post fixed in formalin [33, 34].

Gong et al. evaluated the immunoreactivity on TP versus CB and reported that both techniques performed equally, except for nuclear stains (Ki67, PCNA and p53), where both the frequency and intensity were significantly reduced on TP compared to CB [35]. Jing et al evaluated the cellular morphology as well as frequency and intensity of IMS in CB prepared directly by thrombin clot, HistoGel, or fixed in CytoLyt (methanol based) then prepared by HistoGel. They concluded that methanol pre-fixation did not negatively impact the morphology or IMS and all cell blocks performed equally. The background staining was highest in the HistoGel CBs and least in the CytoLyt collected samples [36].

## Key facts

1. IMS can be successfully employed on all types of preparations.
2. Cell blocks are easiest to validate and assess
3. Specimens collected in alcohol based media are usually not affected but individual antibodies should be first validated since some have been reported to have reduced signaling

## Other ancillary testing

In addition to IHC, a wide range of analyses may be applied to serous effusions, including diagnostic assays and predictive tests, as well as research approaches which may yet find future application within the first two categories. These assays are based on analysis of both the effusion supernatant and the cells pellets. The last author's lab requests clinicians to submit fresh non-fixed specimens of maximal volume immediately after tapping, thereby guaranteeing high-quality material for these analyses.

Effusion supernatants may be analyzed in the fresh state, the more common situation in the diagnostic setting, or following freezing at  $-80^{\circ}\text{C}$ . As discussed above, agents which prevent coagulation may be used, particularly when dealing with hemorrhagic effusions, but are generally not needed for non-sanguineous specimens.

The oldest approach assessing the liquid fraction of the effusion is the measurement of tumor markers, e.g. CA 125, CA 19–9, CA 15–3 and the cytokeratin fragment CYFRA 21–1 as evidence of malignancy using ELISA-based techniques [37]. More recently, markers whose measurement may be more tumor-specific have been identified, such as mesothelin and hyaluronan in the diagnosis of malignant mesothelioma [38].

Other authors have studied effusion supernatants for microRNA (miRNA) signatures that allow for the differentiation of benign from malignant effusions. miRNAs are short non-coding RNAs which negatively or positively regulate gene expression in physiological and pathological conditions by binding to the 3'-UTR of their target mRNA, thereby inhibiting target gene translation into proteins. Effusion supernatants from lung, gastric and ovarian carcinoma have been shown to differ from benign specimens [39–41]. The latter study, in which miRNA content was analyzed in exosomes, small secreted vesicles involved in tumor signaling, was performed on archived material frozen at  $-80^{\circ}\text{C}$ , including specimens stored for over 10 years [40].

Long-term freezing of effusion supernatants also preserves the metabolic profile of tumor cells, as a recent comparative analysis of ovarian carcinoma, breast carcinoma and malignant mesothelioma specimens demonstrated [42].

Another diagnostic approach utilizing effusion supernatants is through analysis of secreted DNA fragments, as shown for *CCNE1*, encoding cyclin E. Archival material was used in this study too [43]. Analysis of the mRNA levels of *BIRC5*, encoding the anti-apoptotic marker

Survivin, by qPCR, showed overexpression of this mRNA in supernatants from malignant compared to benign effusions [44].

As discussed above, cells in effusions may be preserved and analyzed in different forms, including smears, cytopsins and cell blocks. This is true for both immunohistochemistry and molecular analyses.

FISH was successfully applied to fresh effusions using protocols including fixation in Carnoy's solution (methanol: acetic acid, 3:1 ratio) to detect malignancy-associated aberrations in chromosomes 11 and 17 [45] or 7, 11 and 17 [46]. The same solution was applied in analysis of *CDKN2A*, the gene coding for p16 at chromosome 9p21, which is deleted in different cancers, including malignant mesothelioma [47, 48]. De-staining of Diff-Quik-stained slides was applied in another study in which changes in chromosomes 7 and 9 were assessed [49].

The commercial FISH test UroVysion™, which includes probes for chromosomes 3, 7 and 17, as well as chromosome 9p21, was applied to effusion specimens fixed in CytoLyt® solution and re-suspended in methanol-based fixative [50]. Changes in the same chromosomes were assessed in another study, in which smears fixed in Delaunay solution, containing ethanol and acetone, and PAP-stained smears were used [51].

FISH, chromogenic ISH (CISH) or silver ISH (SISH) are also applied to assessment of *HER2* status. Studies investigating *HER2* status successfully used cytopsins, including air-dried specimens frozen at  $-80^{\circ}\text{C}$  [52] or  $-20^{\circ}\text{C}$  [53] prior to analysis, specimens fixed in Carnoy's solution [54] and cell blocks [55]. FISH analysis of pleural effusion specimens fixed in Carnoy's solution was also performed in a study of TTF1 in lung carcinoma [56]. Colorimetric ISH has been applied to cell block sections in analyses of expression of cancer-associated mRNAs, e.g. angiogenic molecules, transcription factors of the ETS family and the epithelial-to-mesenchymal transition (EMT) mediator Snail1 [57–59].

PCR has been widely applied to analysis of serous effusions in the routine laboratory setting and in research.

PCR-based methodology has been widely used in testing of epidermal growth factor (*EGFR*) mutation status in advanced non-small cell lung carcinoma (NSCLC). Analyses have been successfully performed on both cells directly isolated from fresh effusion specimens [60] or on material frozen at  $-80^{\circ}\text{C}$  [61]. Both cell blocks and archival smears were used for assessment of *EGFR* mutation status in 2 other studies [62, 63], while cell blocks were used exclusively in the report by Yeo et al. [64]. Both cell blocks and supernatants from pleural effusion

specimens, as well as plasma and tissue specimens, were studied in another report [65]. New generation sequencing (NGS) was already shown to be highly sensitive in assessment of *EGFR* mutation status [66] and is likely to assume a central role in this setting in the future. The latter study was performed on methanol-fixed PAP-stained smears.

Cytological lung carcinoma specimens frozen at  $-80^{\circ}\text{C}$  were informative for fusion between the echinoderm microtubule-associated protein-like 4 (*EML4*) and the anaplastic lymphoma kinase (*ALK*) genes [67]. Specimens were mixed with a buffer preventing RNA degradation before freezing in the latter study. RNA was directly isolated from pleural effusion cell pellets in another study in which tumors with wild-type *EGFR* were analyzed for *EML4-ALK* fusion gene by RT-PCR [68].

In a recent study of RET rearrangements, another molecular event in NSCLC, cell pellets frozen in RNAlater™ were used [69].

Freezing of cell pellets at  $-80^{\circ}\text{C}$  or in liquid nitrogen prior to PCR analysis has been used by several groups, i.e. in analysis of effusions for prepro-gastrin releasing peptide (prepro-GRP) in small cell lung carcinoma [70], *hMAM* and *hMAMB*, coding for the breast carcinoma markers mammaglobin and mammaglobin B [71], and in analysis of different epithelial markers, including *CLDN1*, *CLDN4*, *CLDN18*, *CEA*, *EPCAM*, *CK19*, *CK20*, *MUC1* and *MUC16* using quantitative PCR (qPCR) [72].

The last author's group freezes effusion cell pellets at  $-70^{\circ}\text{C}$  in equal amounts of RPMI 1640 medium containing 50 % fetal calf serum and 20 % dimethylsulfoxide, retaining cell viability and high DNA, RNA and protein quality over long periods of time. This material has been extensively used for both gene expression array analyses of different cancers affecting the serosal cavities [73–75] and validation studies of the array data using qPCR [76–82].

Cells from effusions may also be cultured prior to molecular analysis, as performed in a study of the melanoma-associated antigen (MAGE) family genes *MAGE1* and *MAGE3* and the related genes *BAGE* and *GAGE1-2* in ovarian carcinoma [83].

Effusion specimens studied for DNA methylation have been frozen at  $-80^{\circ}\text{C}$  prior to analysis in the majority of studies [84–87], although freezing at  $-20^{\circ}\text{C}$  has also been used [88]. Studies of effusion specimens applying proteomics used fresh samples, as well as samples frozen at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  [89–93].

In Conclusion it is important to remember that effusions lend themselves to a wide range of diagnostic and molecular testing. It is best to submit the fluids fresh and

fast to the laboratory which allows proper triage of the sample.

## Key facts

1. Both effusion supernatants and cell pellets may have a diagnostic, predictive and prognostic relevance
2. Both cell blocks and fresh-frozen material may be used in most analyses
3. All molecular methods may be applied to effusion specimens with robust results, provided specimens are rapidly and adequately handled
4. Fixation, if any, depends on the molecular test applied

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