Ancillary Techniques on Direct-Smear Aspirate Slides

A Significant Evolution for Cytopathology Techniques

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Numerous cytologic techniques aimed at effectively acquiring patient material for molecular testing have been proposed. Such techniques are becoming ever more important in an age of personalized medicine. In this commentary, the authors explored some more commonly proposed techniques to aid in the molecular testing of cytologic specimens. These techniques include the use of cell blocks, direct cytologic smears, filter paper storage, frozen samples, and enriched cellular techniques such as ThinPrep and cytospin preparations. Direct-smeared slides demonstrate excellent preservation of DNA, are easy to prepare, and are amenable to immediate adequacy at the time of the fine-needle aspiration (FNA) procedure as well as effective subsequent tumor purity estimation. Cell block methods cannot be assessed at the time of FNA and often demonstrate insufficiency, whereas filter paper and frozen techniques do not allow for the direct assessment of the presence and purity of tumor cells in the sample. Direct-smeared slides are emerging as the most effective preparation and storage medium of cytologic material to be used for molecular testing. Their cost-effectiveness, ease of use, and reliability have cemented them as the optimal solution for cytopathologists to fulfill the role of providing advanced molecular testing on patient samples. Cancer (Cancer Cytopathol) 2013;121:120-28. © 2012 American Cancer Society.

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INTRODUCTION

With the increasing emphasis placed on personalized medicine in today’s modern health care systems, pathologists are experiencing steady increases in the number of requests for molecular testing on patient specimens. DNA-based mutational analyses are aimed at detecting molecular aberrations in tumors that may portend response to targeted therapeutics. Not surprisingly, such testing becomes most relevant in patients with high-stage, surgically unresectable malignant disease. In this context, smaller biopsies associated with minimal complications are desirable for the patient. Cytologic fine-needle aspirations (FNAs) are especially useful in this regard because they represent a rapid, efficient, and minimally invasive means with which to sample superficial and deep-seated lesions. Accordingly, cytopathologists are often called on to serve as liaisons between clinicians, oncologists, and molecular pathologists by facilitating molecular testing on FNA samples. Although molecular testing on FNA samples is not a novel notion, the increasing variety and complexity of tests has created both challenges and opportunities for cytopathologists.1,2 Cytopathologists are expected to implement the most optimal sample collection methods and workflow techniques and serve as advisers to clinicians in selecting appropriate molecular tests.

Additional Supporting Information may be found in the online version of this article.

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The principal goal of this commentary is to discuss the direct-smear slide as an optimal platform for preparing small patient biopsies for subsequent molecular testing. To facilitate this goal, it is first helpful to present the following 3 objectives: 1) to make the case for FNA as the optimal procedure for initial sample acquisition; 2) to briefly consider select examples of current and future molecular techniques that may be routinely requested using cytologic specimens; and 3) to discuss the advantages and disadvantages of various other platforms (e.g., cell blocks, Whatman filter paper cards, frozen specimens, and cellular enrichment techniques) for performing molecular tests.

Use of FNA to Aid in the Molecular Analysis of Patient Samples

Although FNA and core needle biopsy (CNB) represent complementary methods for obtaining tissue from both palpable and nonpalpable lesions, the use of FNA to acquire target DNA is advantageous over CNB in several respects: 1) FNA can sample a wider area of the lesion; 2) FNA allows for the acquisition of tumor cells with lower contamination by stromal tissue; and, most importantly, 3) FNA allows for rapid on-site adequacy assessments and the communication of preliminary diagnoses to the clinical care providers. Cytopathologists who provide on-site assistance during FNAs are therefore not only in a unique position to triage freshly obtained material for ancillary tests, but also to ensure that enough material has been gathered for molecular analysis while the patient is still accessible. In addition to molecular polymerase chain reaction (PCR)-based studies, other ancillary tests facilitated by on-site assessments include immunocytochemistry (ICC), conventional cytogenetics, fluorescence in situ hybridization (FISH), flow cytometry, and microbiologic culture.

Selected Existing and Emerging Molecular Studies Applied to FNA Specimens

The repertoire of molecular tests applied to nongynecologic cytology specimens is steadily growing. Salient examples include PCR-based and FISH-based assays to determine the presence of specific gene rearrangements in patients with lymphoproliferative disorders and soft tissue neoplasms, including small round blue cell tumors. In addition, FISH-based assays have served as valuable adjuncts in the diagnosis of urothelial cell carcinomas and the determination of human epidermal growth factor receptor 2 (HER2)/neu gene amplification in patients with breast cancer.

Thyroid FNAs

Thyroid FNAs play a crucial role in the preoperative assessment of thyroid nodules and the triaging of patients for surgical management. Recently, the application of ancillary molecular studies to thyroid FNAs has exploited the currently understood molecular genetic aberrations associated with thyroid follicular neoplasms. For example, an activating mutation in BRAF resulting in the V600E substitution is present in nearly one-half of papillary thyroid carcinomas, and the RET-PTC and paired box gene-8–peroxisome proliferator-activated receptor gamma (PAX8-PPARY) translocations are found in approximately 20% and 35%, respectively, of papillary thyroid carcinomas and follicular carcinomas. Point mutations in RAS proto-oncogenes are noted in a variety of thyroid neoplasms, including papillary thyroid carcinoma, follicular carcinoma, and follicular adenomas.

The application of molecular genetic assays to examine thyroid FNA material for the above mutations has shown that the presence of any one of these mutations is strongly indicative of malignancy on surgical follow-up. Therefore, molecular testing can serve an important role in these cases.

Non-Small Cell Lung Carcinoma FNAs

Specific chemotherapeutic agents for lung cancer are available for the 2 most common subtypes of non-small cell lung carcinoma (NSCLC): adenocarcinoma and squamous cell carcinoma (SQC). A recent phase 3 study demonstrated a significant improvement in overall survival in patients with pulmonary adenocarcinoma who were treated with the combination of cisplatin and pemetrexed versus those treated with cisplatin and gemcitabine. In contrast, the combination of cisplatin plus gemcitabine was found to be superior to that of cisplatin and pemetrexed for patients with SQC. FNAs of lung adenocarcinoma are triaged, at increasing frequency, for further molecular mutational analysis of the receptor tyrosine

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kinase, epidermal growth factor receptor (EGFR), and KRAS, and rearrangements involving the gene encoding the tyrosine kinase, anaplastic lymphoma kinase (ALK). These mutations are mutually exclusive. Patients with adenocarcinomas harboring EGFR mutations are candidates for treatment with tyrosine kinase inhibitors such as erlotinib and gefitinib. Recently, the tyrosine kinase inhibitor crizotinib has been approved as targeted therapy for patients with lung adenocarcinomas harboring ALK rearrangements. Hence, ensuring adequate lesional material during FNAs for the specific subclassification of NSCLC and subsequent molecular testing is paramount.

Routine cytomorphology is often sufficient to distinguish between pulmonary adenocarcinoma and SQC. Nonetheless, in a subset of cases, the distinction is difficult and ICC can be useful for the subclassification of these NSCLCs. Adenocarcinomas often express napsin A and thyroid transcription factor-1 (TTF-1) and are usually negative for cytokeratin (CK) 5/6 and p63. Conversely, SQCs are typically negative for napsin A and TTF-1, whereas they are virtually always immunoreactive for CK5/6 and p63.

**Melanoma FNAs**

FNA represents a minimally invasive means with which to establish a tissue diagnosis of metastatic melanoma, allowing for accurate staging and prompt clinical management. ICC for S-100, HMB-45, and melan-A/Melanoma Antigen Recognized by T cells (MART-1) can be applied to challenging cases to confirm the diagnosis of metastatic melanoma. Recently, investigators have found that approximately 40% to 50% of melanomas harbor activating mutations in BRAF; V600E and V600K represent the 2 most common mutations. Because vemurafenib has recently been approved for the treatment of patients with advanced stage melanomas harboring BRAF mutations, there is increased attention to molecular diagnostic assays designed to detect these mutations in FNA samples from patients with melanoma.

**Platforms Used for the Performance of Molecular Studies**

Various techniques for sample collection for molecular tests have been used, either as a routine part of the FNA procedure or as a separately collected banked sample (Table 1). Each has unique features that can be assessed for suitability with subsequent molecular testing.

**Cell Blocks**

Cell blocks are traditionally used by many laboratories for the performance of ancillary ICC and molecular studies; they have notable advantages and weaknesses. The advantages of cell blocks include: 1) formalin-fixed, paraffin-embedded tissue is treated similarly to that of traditional surgical pathology blocks; 2) multiple serial sections for ancillary studies may be prepared; and 3) a banked archive is maintained for future studies. The disadvantages of cell blocks include: 1) limited or no cellularity in a subset of cases; 2) an inability to assess cellularity and adequacy at the time of procedure; 3) the pooled nature of the sample; and 4) the possibility that deeper sections from the cell block may not contain the tumor cells observed on the originally prepared hematoxylin and eosin-stained slide. Of these, variability in cell block cellularity (ie, sparse cellularity or acellularity in a subset of cases) represents the most significant limitation. To examine this in our laboratory practice, we retrospectively analyzed the cellularity of cell blocks in 76 consecutive endobronchial ultrasound-guided FNAs performed at our

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**Table 1. Advantages and Disadvantages of Various Cytology Platforms for Molecular Testing**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Ability to Directly Visualize Sample?</th>
<th>Ability to On-Site Adequacy Assessment?</th>
<th>Ability to Assess Tumor Purity?</th>
<th>Ability to Split Sample for Multiple Ancillary Tests?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell blocks</td>
<td>Yes’</td>
<td>Yes’</td>
<td>Yes’</td>
<td>Yes</td>
</tr>
<tr>
<td>Direct smears</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes, limited</td>
</tr>
<tr>
<td>Fresh frozen sample (microcentrifuge tube)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>ThinPrep/cytospin</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Filter paper (eg. Whatman filter paper cards)</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Deeper sections of the cell block used for molecular testing are not visualized directly.*
institution that were diagnosed as being positive for malignancy. We observed that 28 (37%) cell blocks were acellular and an additional 15 (20%) cell blocks exhibited sparse/borderline cellularity (Fig. 1). Dedicated passes to enrich the cell block may help in some cases, but do not completely alleviate this limitation (Fig. 1). Compounding this issue, the ability to ensure adequate cellularity of the cell block at the time of the FNA procedure is not possible because processing is not usually complete until the following day. The third significant weakness of the cell block is that it represents a pooled specimen from multiple passes and therefore the tumor cell population from high-yield needle passes may be diluted by background benign elements in off-target needle passes. This is problematic because the analytic sensitivity of molecular diagnostic assays depends on a percentage tumor cellularity threshold, below which false-negative results will occur (ie, contaminating benign tissue will be negative for the molecular abnormality being tested). For example, in our molecular diagnostics laboratory, PCR-based assays designed to detect \textit{BRAF} and \textit{KRAS} mutations require a minimum percentage tumor cellularity of 10% and 30%, respectively. In published studies from our laboratory, we have noted 2 incidents in which a positive mutation result was obtained from tumor-enriched areas on direct smears whereas corresponding cell blocks in these 2 cases demonstrated false-negative results.²⁵,²⁶

**Filter Paper Storage or Fresh-Frozen Samples (Microcentrifuge Tube)**

One innovative approach to the storage of samples for molecular studies involves aliquoting an amount of aspirated FNA material directly onto a small piece of filter paper, which can then be stored at room temperature until needed for molecular analysis. da Cunha Santos et al applied molecular diagnostic studies to FNA material from NSCLCs stored on Whatman filter paper cards, and demonstrated that high-quality DNA could be extracted from these cards after months of storage at room temperature.²⁷ This method is advantageous because of the ease of setup and the low cost of reagents and storage conditions. However, a serious drawback still remains in that tumor purity and sample adequacy in the filter paper cards cannot be directly ascertained with certainty; there is a limited ability to determine whether adequate amounts of tumor cells were sequestered on the filter paper card because the

**FIGURE 1.** A retrospective analysis of cell block cellularity in fine-needle aspiration (FNA) specimens is shown. Cell block sections from 76 consecutive endobronchial ultrasound-guided FNA samples were stained with hematoxylin and eosin and examined and assessed in terms of overall cellularity. Acellular cell blocks were designated as exhibiting inadequate cellularity. Sparsely cellular cell blocks (those having < 100 cells) were designated as exhibiting borderline cellularity. Moderately cellular and abundantly cellular cell blocks exhibited between 100 to 300 cells and > 300 cells, respectively. (A) Representative photomicrographs for each of the 4 categories are shown (original magnification × 40). (B) Of the 76 cell blocks examined, 28 (37%), 15 (20%), 17 (22%), and 16 (21%) exhibited inadequate, borderline, moderate, and abundant cellularity, respectively. (C) Of the 76 consecutive FNA specimens examined, on-site assessments of adequacy were performed in 70 cases. In each of these 70 cases, information regarding the number of dedicated passes for the cell block was obtained; 0, 1, 2, or 3 dedicated passes were performed in 47 cases, 10 cases, 10 cases, and 3 cases, respectively. The percentage of cases with cell blocks exhibiting inadequate to borderline cellularity as a function of the number of dedicated passes for the cell block was obtained; 0, 1, 2, or 3 dedicated passes were performed in 47 cases, 10 cases, 10 cases, and 3 cases, respectively. The percentage of cases with cell blocks exhibiting moderate to abundant cellularity as a function of the number of dedicated passes is shown in the bar graph. The actual numbers of cases in each category are included in the bars.
cells cannot be observed. To determine whether adequate cells are present, one must examine a smear made from the same pass and extrapolate. Even in cases in which DNA is successfully extracted, it is not possible to determine with certainty the ratio of genetic material contributed by tumor cells and contaminating benign cellular elements because of the inability to directly observe the cells present on the filter paper cards.

Similar to the filter paper technique, an aliquot from the FNA specimen may be placed in a small tube containing cryopreservative and frozen for future use. This method helps to guarantee long-term stability, especially for RNA, which is much more vulnerable to hydrolytic degradation than DNA. Depending on the amount of cryopreserved material, an aliquot of the sample can be used, upon thawing, to prepare a Diff-Quik–stained cytospin slide. Nonetheless, if this is not performed and the frozen sample is directly submitted for molecular testing in its entirety, the similar limitations noted for the filter paper storage approach still apply (ie, the sample cannot be directly observed to ensure the presence and purity of tumor cells).

Routine Cytology Slides: Air-Dried and Alcohol-Fixed Direct Smears and Enriched Preparations

Routine cytology slides have the advantage that DNA is readily extractable and reasonably stable (ie, at least 6 months and up to and exceeding 5 years in some cases). Enriched slides such as ThinPrep or cytospin slides share some limitations with cell blocks: they are prepared from homogenized, pooled liquid-based samples derived from multiple passes and they are not available for the assessment of cellularity at the time of the procedure. Direct smears prepared on-site are not associated with these limitations. Freshly prepared, unstained direct smears may either be air-dried or fixed in alcohol and

![Diagram showing traditional and modified approaches to FNA passes](image-url)

**FIGURE 2.** Traditional and alternative, modified approaches to the triage of fine-needle aspiration (FNA) passes are shown. An example of the traditional approach to a triage of FNA passes is shown in the top panel. Here, each FNA pass leads to the creation of 2 smears: 1 air-dried smear for Diff-Quik (DQ) staining and 1 alcohol-fixed smear for Papanicolaou (Pap) staining. The needle rinse is used for cell block preparation. Dedicated passes for the needle rinse is a strategy typically used to increase the likelihood that the prepared cell block will exhibit adequate cellularity for ancillary studies. An alternative, modified approach to the triage of FNA passes is shown in the bottom panel. In this scenario, the contents of a given FNA pass are distributed over multiple smears including the routine air-dried, DQ-stained smear and the alcohol-fixed, Pap-stained smear. Extra unstained (Uns) smears can be used for immunocytochemistry or subsequently stained for tumor cell microdissection and subsequent molecular analysis. Needle rinses including dedicated passes are still used for cell block preparation.
represent effective platforms for ICC and molecular studies.\textsuperscript{25,26,30-32} To observe tumor cells to ensure adequacy for DNA extraction for molecular studies, it is easiest to use stained slides. Although both alcohol-fixed Papanicolaou-stained smears and air-dried Diff-Quik–stained smears are suitable for DNA extraction, we prefer to use Diff-Quik staining because DNA appears to be better preserved. Specifically, a recent study by Killian et al suggests that Diff-Quik–stained smears can provide high-quality DNA even if archived for a prolonged period, allowing for the performance of sophisticated molecular diagnostic studies such as high-resolution comparative genomic hybridization assays.\textsuperscript{29} In this study, Killian et al observed DNA degradation as a function of age for archived Papanicolaou-stained smears.

Traditionally, during FNA procedures, each needle pass leads to the preparation of 2 smears: 1 that is air-dried for rapid Diff-Quik staining and another that is alcohol-fixed and subsequently stained using the Papanicolaou method (Fig. 2). However, depending on the amount of material that is aspirated, it is usually possible to generate additional smears (in our experience, at least 2 additional smears) per FNA pass (Fig. 2) (see Supplemental Video). The presence of tumor cells on the Diff-Quik–stained smear, determined during the on-site assessment of adequacy, can be used as an indicator of tumor cellularity on unstained direct smears resulting from the same pass. Nonetheless, because this represents an extrapolated approach, we prefer to assess the presence of tumor cells on the unstained direct smears by light microscopy. Specifically, the size and architectural configuration of the tumor cells in the unstained slides can be directly compared with the Diff-Quik–stained slides and this can be facilitated by flipping the condenser (Fig. 3). For slides destined for molecular studies, staining of an extra, unstained smear with Diff-Quik is preferable because the tumor cells can then be directly observed and selected for

![Diff-Quik stained smear](image1)

![Unstained smear Condenser in place](image2)

![Unstained smear Condenser flipped](image3)

\textbf{FIGURE 3.} Assessment of cellularity on unstained smears is shown. In Figure 2, a modified approach to fine-needle aspiration (FNA) triage was highlighted and the material expelled from the needle was distributed over multiple smears, including unstained smears and the routine Diff-Quik–stained and Papanicolaou-stained smears. (Top) For a given pass, visualization of tumor cells on the Diff-Quik–stained smear during the on-site assessment would suggest the presence of tumor cells in the unstained smears. In addition, unstained smears can be assessed directly for cellularity under the light microscope. (Middle) Although tumor cells adopting similar configurations as seen on the Diff-Quik–stained smear can be difficult to observe while the condenser is in place, (Bottom) flipping the condenser can facilitate tumor cell visualization. The example depicted in this figure is of an FNA of a metastatic melanoma.
microdissection, DNA extraction, and molecular analysis (Fig. 4). For the purposes of tumor microdissection from smears, we routinely use the Pinpoint Slide DNA Isolation System (Zymo Research, Irvine, Calif). Regardless of the subsequent molecular test being performed, the use of smears for molecular testing is advantageous because the cellularity of the smears can be directly assessed at the time of the FNA. Areas of the smear enriched with tumor cells can be directly observed and microdissected, allowing for isolation of DNA. We and others have previously reported that EGFR and KRAS mutational analysis can be applied to stained cytologic smears of NSCLCs, and the diagnosis of metastatic melanoma, and the confirmation of metastatic Merkel cell carcinomas, malignant effusions, and Hodgkin lymphomas. Specifically, we have successfully performed ICC for a finite number of antibodies (including TTF-1, napsin A, p63, S-100, HMB-45, MART-1, CK7, CK20, cluster of differentiation [CD] 56, synaptophysin, epithelial membrane antigen, MOC-31, CDX-2, PAX8, calretinin, CD15, and CD30) on unstained direct smears using the Ventana Autostainer (Ventana Medical Systems, Tucson, Ariz). Background staining can represent a problematic issue that is associated with ICC performed on direct smears. Although we occasionally observe unacceptably high background staining in relation to the true signal, this is relatively uncommon. In our experience with the above markers, when mild background staining is observed, the signal-to-background staining ratio remains high.

Finally, cytologic smears are optimal for the performance of FISH because whole nuclei of tumor cells are being examined. Thus, direct smears can be used, in addition to cell blocks, for FISH studies.

**Conclusions**

By considering that FNA is ideally suited for obtaining patient samples for diagnostic and molecular testing, and

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**FIGURE 4.** Microdissection of tumor cells from a Diff-Quik–stained smear for DNA isolation using the Pinpoint Slide DNA Isolation System (Zymo Research, Irvine, Calif) is shown. A noncoverslipped Diff-Quik–stained smear or a previously coverslipped Diff-Quik–stained smear that has been decover-slipped in xylene can be examined to identify tumor-enriched areas by light microscopy. The area of interest is then marked on the underside of the smear. The Pinpoint solution is then applied to the area of interest and allowed to dry. A sterile scalpel subsequently is used to carefully microdissect the tumor cells embedded in the Pinpoint solution and to transfer the material to an Eppendorf tube (Eppendorf, Oldenburg, Germany) for subsequent DNA extraction. The use of the Pinpoint solution minimizes the dispersal and aerosolization of cellular material during microdissection.
reviewing some of the more common tests as well as other sample collection methods, it is easy to appreciate the usefulness of routine FNA smears in providing optimal material for the storage and harvesting of the cellular material needed for such testing. Air-dried, Diff-Quik-stained smears for use in PCR-based tests are easy to prepare, require minimal processing and storage complexity, and are less cumbersome to microdissect than cell blocks. DNA extracted from direct smears is highly reliable and provides satisfactory molecular test results. Because smears can be directly observed at the time of the FNA procedure, the pathologist can ensure that adequate material has been obtained for cytdiagnosis and necessary ancillary tests. Routine cytologic smears, when incorporated into the triage and workflow for performing ancillary molecular tests, represent a cost-effective and powerful tool to aid in effective molecular testing in the age of personalized medicine.

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