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REVIEW ARTICLE

Precision cytopathology: expanding opportunities for biomarker testing in cytopathology

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Precision cytopathology refers to therapeutically linked biomarker testing in cytopatology, a dynamically growing area of the discipline. This review describes basic steps to expand precision cytopathology services. Focusing exclusively on solid tumors, the review is divided into four sections: Section 1: Overview of precision pathology- opportunities and challenges; Section 2: Basic steps in establishing or expanding a precision cytopathology laboratory; Section 3: Cytopathology specimens suitable for next generation sequencing platforms; and Section 4: Summary. precision cytopathology continues to rapidly evolve in parallel with expanding targeted therapy options. Biomarker assays (companion diagnostics) comprise a multitude of test types including immunohistochemistry, in situ hybridization and molecular genetic tests such as PCR and next generation sequencing all of which are performable on cytology specimens. Best practices for precision cytopathology will incorporate traditional diagnostic approaches allied with careful specimen triage to enable successful biomarker analysis. Beyond triaging, cytopathologists knowledgeable about molecular test options and capabilities have the opportunity to refine diagnoses, prognoses and predictive information thereby assuming a lead role in precision oncology biomarker testing.

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Overview of precision cytopathology including opportunities and challenges

Precision oncology

Precision oncology, also known as personalized oncology, continues to rapidly evolve in concert with the unslacking pace of discovery of new genetic and epigenetic tumor alterations. Open access to large, high quality, whole exome and RNA sequencing databases such as the US Cancer Genome Atlas Project has sparked a surge in identification of new subtypes of tumors and corresponding potential predictive and prognostic molecular markers.[1-5] The US Food and Drug Administration approved biomarkers and their targeted therapies are typically used in advanced-stage patients, the very group of patients whose tumors are often diagnosed on cytology. For a substantial percentage of these patients, the cytologic specimen represents the sole sample of tumor. As cytopathologists, we are charged with maximizing this sample while maintaining our primary mission of rendering a clinically meaningful morphologic diagnosis. Critically, cytopathologists can advance modern cancer patient care by strategic sample triaging. This pivotal triaging role has been recognized for more

than 10 years and is becoming an increasingly routine aspect of cytopathology practice.[6]

Precision pathology

Precision pathology refers to laboratory assays and interpretations thereof providing evidence-based data that facilitate the selection of specific therapy tailored to an individual patient.[7] Precision pathology testing may be diagnostic, predictive, or prognostic or a combination. Solid tumor assays meriting the descriptor "precision pathology" encompass multiple analytes including proteins, microRNA, messenger RNA, and DNA, among others. Analyte evaluation proceeds by molecular genetic tests, epigenetic tests, protein expression tests, quantification of bright-field visual features, and others. Table 1 lists solid tumor precision pathology tests currently used in surgical tissue specimens. Because biomarkers continuously develop, Table 1 depicts a range of established oncology biomarker assays but cannot encompass all types. Many if not all these assays are amenable to validation in cytology specimens. Integrating more cytology specimens into cancer gene testing is essential for patients with surgically unresectable, high-grade, locally advanced, recurrent, or metastatic malignancies.[8] Hundreds of biotech companies market thousands of probes and assays; the Genetic Testing Registry of the National Center for Biotechnology Information (ncbi.nlm.nih.gov) lists, briefly describes, and provides links to currently offered gene tests (>55,000 registered as of October 2018).

Precision cytopathology

Cytopathology plays an increasingly vital role in precision pathology and may be termed "precision cytopathology" (Table 2). Although in its early stages regarding widespread implementation of cytology specimens for broad ancillary molecular genetic testing, many cytopathology laboratories already participate in molecular testing of 2 body sites: thyroid and lung.[9-13] As early as 2006, molecular genetic testing was proven feasible in effusions, and by 2010 multiple groups demonstrated success in endobronchial ultrasound (EBUS) samples in assessing for *EGFR* and *KRAS* mutations using fine-needle aspiration (FNA) biopsy samples for molecular analysis.[14-18] Commercial attention, however, focused on formalin-fixed paraffin-embedded (FFPE) specimens over the ensuing years despite promising results in a small but growing number of cytology-based molecular reports. This preference coincided with the relative lack of neoadjuvant targeted therapy options. Concurrently, clinical trials and national organizational recommendations endorsed the use of FFPE tissue for clinical molecular genetic testing, typically performed on tumor resections or wedge or core biopsies. Nevertheless, recognizing the necessity to use cytology samples in circumstances of no other recourse, renewed and burgeoning attention has recently led to efforts to broadly validate molecular testing in cytopathology. For example, acknowledging the recent spate of studies indicating cytology samples amply enable clinically relevant lung molecular biomarker analysis, the College of American Pathologists, the International Association for the Study of Lung Cancer, and the Association for Molecular Pathology have jointly updated guidelines that explicitly allow for the use of cytology smears for testing (although cell blocks [CBs] are still preferred).[12] In fact, as assays continue to be adapted to cytology specimens and as indications for testing grow, cytopathology should soon be acknowledged as the front line for molecular testing.[19]

Quality of cytology samples for molecular genetic testing

Cytology samples yield high-quality DNA and RNA sufficient for molecular genetic studies compared to nucleic acids extracted from FFPE tissues.[6,19-24] High nucleic acid quality prevails for at least four reasons: 1) compared with many FFPE surgical pathology specimens, cytology specimens are rapidly placed in fixative (often immediately); 2) common cytology fixatives including ethanol and methanol (or proprietary mixes thereof), yield longer length DNA fragments (>300 base pairs; a minimum size required for some assays) free of formalin-induced artifacts; 3) contrasted with tissue sections, direct smears typically preferentially contain a purer population of tumor cells amenable to microdissection for high tumor cellularity; and 4) direct smears contain whole nuclei rather than capitated nuclei as occurs when sectioning FFPE blocks.[6,19-24]

These well-recognized advantages result in higher-quality DNA and RNA than corresponding FFPE tumor blocks. Although nucleic acid quality has been confirmed in a multitude of independent cytology-molecular studies, challenges remain for seamless integration of cytology and molecular pathology testing. Additional effort is required, especially in optimizing sparse sample for multiplex assays while retaining the key capability to render a morphologic diagnosis.[24,25] Although nucleic acid *quality* derived from cytology smears, liquid-based cytology (LBC), and cytospins may exceed nucleic acid quality from a matching FFPE block, nucleic acid *quantity* can constrain assay choice or assay success. Fortunately, for many newer assays such as small targeted next generation sequencing (NGS) panels, minimal starting nucleic acid amounts/concentrations recommended by the manufacturers continue to decline. Table 3 lists test specifications for the more common ancillary tests that currently use cytology specimens or may be adapted to work with non-CB cytology specimens.

Indications and opportunities for precision cytopathology

Table 2 summarizes current and potential precision oncology uses of cytology specimens and core biopsies.

Table 1 Analytes measurable by representative precision pathology assays.

Analyte	Assay(s)	Clinical example	Example of commercial manufacturer or assay	Level of assay
Methylated DNA (hyper- or hypomethylated promoter sites, typically)	If known regulatory region/genes of interest: -Bisulfite conversion -Bead array -PCR and sequencing -Pyrosequencing -Methylation specific PCR -HRM -COLD-PCR If unknown epigenetic change: -Whole genome methylation profiling -HPLC-UV -MS-based -ELISA based -PCR of LINE-1 -LUMA -Search differentially for methylated region -Bisulfite sequencing -MSCC -Microarray or bead array	*RASSF1A*: Lung, bladder, breast cancer, and ECC (brush specimens) *PAX1*, *ZNF582*, *SOX1*, and *NKX6-1*: Cervical cancer with hrHPV+ *MLH1* and *SEPT9*: Colon cancer *RB1*: Retinoblastoma *SHOX*: Lung cancer *BRCA1*: Breast cancer *MGMT*: Glioma and colon cancer *GSTP1*: Prostate cancer	Many vendors that vary by epigenetic biomarker or assay type; for example: -EpigenDx (Qiagen, Hopkinton, MA) -PyroMark CpG Assays from Qiagen (specific equipment needed) (Qiagen, Hopkinton, MA) -Cell Biolabs (San Diego, CA) -Sigma-Aldrich (Darmstadt, Germany) -Abcam (Cambridge, MA) -Active Motif LINE1 (Carlsbad, CA) -Epigentek (Farmingdale, NY) -Illumina Technologies ThermoFisher Scientific (Waltham MA) -Human CpG Island Microarray Kit (Agilent, Santa Clara, CA) -GeneChip Human Promotor Array (Affymetrix, Santa Clara, CA) -GeneChip Human Tiling (Affymetrix, Santa Clara, CA) -Infinium HumanMethylation (Illumina, San Diego, CA)	Epigenetic
MicroRNA (miRNA)	Microarray High-throughput sequencing (RNASeq) ddPCR assay (digital droplet PCR) Targeted NGS and qRT-PCR	Potential for diagnosis and prognosis Potential for prognostic information in cancer *RAS* and *BRAF* mutations in colon cancer Thyroid nodules: FLUS/AUS and FN/SFN (Bethesda categories III and IV)	N/A ThyGenX and ThyraMIR (Interpace Diagnostics, Parsippany-Troy Hills, NJ)	Epigenetic and Gene Expression
Messenger RNA (mRNA)	Microarray Targeted NGS and microarray Targeted NGS (for RNA and DNA) NGS: transcription-mediated amplification (TMAmp) for HPV 16, 18/45 genotypes	Thyroid nodules: FLUS/AUS and FN/SFN (Bethesda categories III and IV) Thyroid nodule specifically: Bethesda III and IV nodules option on Afirma GEC; Bethesda V or V1 nodule without Afirma GEC Thyroid nodule FNAs: Bethesda III and IV Cervical specimens: Assess hrHPV 16, 18/45	Afirma Gene Expression Classifier GEC (Veracyte Inc, San Francisco, CA) Afirma Malignant Classifier AMC (Veracyte Inc, San Francisco, CA) Thyroseq V3 NGS Genomic Classifier (UPMC and CBLPath, Rye Brook, NY) Aptima HPV mRNA Assay (Quest Diagnostics, Secaucus, NJ)	Gene Expression-Transcription

(*continued on next page*)

Table 1 (*continued*)

Analyte	Assay(s)	Clinical example	Example of commercial manufacturer or assay	Level of assay
Chromosomal microarray (CGH) analysis	Array CGH: detect copy number changes SNP chromosomal microarray: detects copy number changes and copy neutral changes (LOH, UPD, ID by descent or consanguinity)	*KRAS*: Pancreas carcinoma *EGFR* amplification: NSCLC	Many available platforms and panels; for example: Platforms: Affymetrix, Agilent (Agilent Technologies Inc., Santa Clara CA), Illumina	Genetic
DNA mutation analysis/ insertions/ deletions (InDels)	Sequencing techniques Massively parallel sequencing (MPS)/Next Generation Sequencing (NGS) Mutation specific PCR Sanger sequencing Pyrosequencing	Thyroid cancer: Multiple point mutations and gene fusions Pancreas ductal adenocarcinoma *K-ras* Billiary tract: Cholangiocarcinoma *K-ras* Lung adenocarcinoma: *EGFR*, *K-ras*, *ALK*, *ROS1*, *BRAF* Lymp nodes, salivary glands, soft tissue tumors, among other malignancies	Many available platforms and panels; for example: Platforms: Illumina Sequencers: MiSeqDx system (ThermoFisher Scientific, Waltham MA) Ion Sequencers: Ion Torrent, Ion Proton (ThermoFisher Scientific, Waltham MA) Panels: Illumina Panels: TruSight RNA Pan-Cancer Panel Ion Panels: Oncomine Dx	Genetic
Chromosomal fusions, gains and losses of whole or partial chromosomes and exchange of chromosomal material between chromosomes	Karotyping	Aneuploidies, gain, or losses of whole chromosomes translocations exchange of chromosomal material between chromosomes	Many vendors for equipment, supplies, software	Genetic
	FISH	Non-small cell lung cancer: ALK/ROS1 dual BAP probe Soft tissue tumors: EWSR1 (probe): Ewing Sarcoma 22q12 rearrangement EWSR1 (probe): DSRCT t(11;22) (p13;q12) SS18 (probe): Synovial Sarcoma t(X;18) (p11;q11) *KRAS* Pancreas solid masses *EFGR* amplification: lung cancer	Vysis ALK/ROS1 dual BAP (only for formalin-fixed samples) Many available commercial probe sources; for example: -Stellaris (Biosearch, Petaluma, CA) -Agilent Technologies (Santa Clara, CA) -Zymed (ThermoFisher Scientific, Waltham, MA) -Empire Genomics (Williamsville, NY)	
	Multitarget-Multicolor FISH assay	Urothelial carcinomas: centromic probes for chromosomes 3, 7, and 17 and a locus-specific probe for 9p21, which harbors the tumor suppressor gene p16	UroVysion (Abbott Molecular, Abbott Park, IL)	

(*continued on next page*)

Table 1 (*continued*)

Analyte	Assay(s)	Clinical example	Example of commercial manufacturer or assay	Level of assay
Protein expression	Immmunohistochemistry (IHC)	*EGFR* (L858R or E746_A750del) NSCLC *ALK* rearrangement (D5F3 clone FDA approved, 5A4 clone) NSCLC	Many commercial vendors, for example: Cell Signaling Technology (Danvers, MA) Novacastra (Leica Biosystems, Wetzlar, Germany) DAKO Antibodies, Ventana Discovery automated immunostainer (Roche Diagnostics, Risch-Rotkreuz, Switzerland)	Gene Expression-Translation
Gene expression/ genomic analysis	nCounter System	Predesigned gene panels and custom panels available	NanoString Technologies (Seattle, WA) nCounter system Prosigna Breast Cancer test	Genetic, Epigenetic and Gene Expression

FISH, fluorescence in situ hybridization; CISH, chromogenic in situ hybridization; SISH, silver in situ hybridization; TILs, tumor infiltrating lymphocytes; ECC, extrahepatic cholangiocarcinoma; hrHPV+, high risk Human papillomavirus; HPLC-UV, high performance liquid chromatography-ultraviolet; MS, mass spectrometry; LINE, long interspersed nuclear elements; LUMA, luminometric methylation assay; MSCC, methyl-sensitive cut counting; HRM, high resolution melting; FNAs, fine-needle aspirations; miRNA, microRNA; FLUS, follicular lesion of undetermined significance; AUS, atypia of undetermined significance; FN, follicular neoplasm; SFN, suspicious for follicular neoplasm; ASC-US, Atypical squamous cells of undetermined significance; dPCR, digital polymerase change reaction; Pc, plasma cells; DSRCT, desmoplastic small round cell tumor; FL, follicular lymphoma; DLBCL, diffuse large B cell lymphoma; SNP, single nucleotide polymorphism; CVS, chorionic villi sampling; LOH, loss of heterozygosity; UPD, uniparental disomy; IHC, immunohistochemical testing; CGH, comparative genomic hybridization.

Improving sensitivity and/or specificity of initial diagnosis

Although microscopic examination of tumors remains the mainstay of diagnosis in cytology, cytopathologists are already accustomed to augmenting morphologic review with ancillary testing; namely, using immunohistochemistry (IHC) to narrow a differential. In some respects, performing immunohistochemical staining on a cytology specimen presents the same challenges as ancillary molecular genetic testing. IHC usually requires an adequate, FFPE section cut from a CB. Alcohol-fixed samples are appropriate if the antibody to be used has been previously validated in this fixative. In extremis, a single smear (fixed in alcohol[s] or formalin) can be further divided by a transfer technique if multiple antibodies are required. An effective cell transfer technique uses a liquid cover slip medium to absorb cells from the slide into the medium which is then peeled off the slide, snipped into desired pieces, and then re-annealed to new charged slides (Mount Quick medium; Daido Sangyo, Tokyo).[26] For IHC purposes, the Mount Quick method is most successful with Papanicolaou-stained slides rather than Diff-Quik—stained slides. For the few laboratories that prepare rehydrated, 10% neutral buffered formalin (NBF) fixed hematoxylin and eosin slides, no additional validation is necessary if using the entire smear for one antibody (Mount Quick divided slides would require validation of the transfer method). As with IHC, molecular genetic assays can be performed on CBs (essentially FFPE tissue sections), smears, cytospins, and monolayers.[27] A cell transfer technique may also be used for molecular tests when the majority of tumor cells are spread as smears on slides.[28] In such a case, the tumor cells can be microdissected and directly scraped into nucleic acid extraction buffer.

Occasionally, a more specific diagnosis may only be obtainable by using genetic/molecular assays. Some assays such as in situ hybridization are validated in smears.[27] Many other molecular assays may be performed using CBs without additional validation, although the assay limitations imposed by FFPE specimens (see CB discussion later in this review) apply.[24,27]

An example of an assay improving diagnostic specificity comprises fluorescence in situ hybridization to evaluate for

Table 2 Cytopathology roles in precision oncology = precision cytopathology.

Function	Type of specimen
Render initial diagnosis	FNA or core biopsy TPs of tumor
Determine eligibility for specific targeted therapy	FNA or core biopsy TPs of tumor
Categorize intratumoral heterogeneity	Multiple FNAs of a tumor sampling multiple sites
Define tumor immune microenvironment	FNA or core biopsy TPs of tumor or fluid sample
Monitor tumor response to ongoing treatment	FNA or core biopsy TPs of tumor
Assess genetic state of primary tumor recurrence	FNA or core biopsy TPs of recurrence
Assess genetic/epigenetic state of metastasis(es)	FNA or core biopsy TPs of metastasis(es) or positive effusion/washing

FNA, fine-needle aspiration; TP, touch preparation.

Table 3 Minimal sample requirements for representative precision cytopathology assays.

Assay	Current accepted sample types	Minimum quantity and quality required
Karotyping	Fresh viable tumor cells	Solid tumor: 1 cm cubed preferred Blood sample: at least 1 mL whole blood 20 metaphases typically assessed
Fluorescence in situ hybridization (FISH) Chromogenic in situ hybridization (CISH)	CB, FFPE, air-dried smear, touch preparation, and cytospin Morphologically preserved chromosomes, FFPE, cell preparation and circulating tumor cells	General: At least 20 cells analyzed in a typical case. Specific requirements: *ALK*, *ROS1*: at least 50 tumor nuclei Consider positive if at least 15% of tumor cells are positive for the rearrangement
Massively parallel sequencing/next-generation sequencing (MPS/NGS) of DNA	FFPE, CB, air-dried smear, cytospin, blood, BM	General rule: 200 tumor cells will yield 1.2 ng DNA (~6 pg/cell) Tumor Content: varies by assay but typically a minimum of 5% viable tumor (nuclei) cell content is required For example: Ion Torrent requires 1000 tumor cells; Illumina MySeq requires 5000 tumor cells
RNA sequencing for miRNA	FNA, FNB, frozen section, FFPE, blood/plasma other fluids	Example: 1 dedicated FNA pass with at least 50 ng of cellular material (required for ThyGenX and ThyraMIR)
RNA sequencing for mRNA	FNA, FNB, frozen section, FFPE, blood/plasma other fluids	General rule: 200 tumor cells will yield 4 ng of RNA (~20 pg/cell; ~95% is ribosomal RNA) For example, minimum of 50 ng total RNA for CTL FusionPlex Assay for Illumina platform and sequenced using MySeq instrument
Gene expression panels using oligonucleotide tags NanoString	FFPE, CB, cell suspension, smear	Minimum RNA of 10 ng (although some labs have validated down to 6.5 ng) for some multiplex assays but requires amplification step More RNA required with increasing number of targets No need for amplification with >100 ng RNA
Chromosomal microarray (CMA)	FFPE, fresh tissue (sterile salt solution medium required), blood, BM, CB (formalin-fixed)	Varies according to specimen type: Cytology specimens (including slides): 200 to 1000 ng input DNA for CGH; 250 ng of DNA for SNP - Tumor: 0.5 to 3 cm^3 - Lymph node: 1 cm^3
Immunohistochemistry (IHC)	FFPE sections of histologically non-necrotic tumor, CB, cytology smear, monolayer slide, cytospin	No specific required number of target cells. However, with only a few cells, a negative result may be non-representative. IHC is less successful in DQ-stained smears than Papanicolaou-stained smears. Antibodies must be validated in cytology fixatives, smear and monolayer specimens for clinical use.

CB, cell block; DQ, Diff-Quik; FFPE, formalin-fixed paraffin embedded tissue; CMA, chromosomal microarray; IHC, immunohistochemistry; MPS, massive parallel sequencing; NGS, next-generation sequencing; SNP, single nucleotide polymorphism.

EWSR1 rearrangement in a pediatric small round blue cell tumor. Notably, this example highlights the absolute necessity to "fuse" microscopic features with the molecular results and the clinical findings, as *EWSR1* rearrangements, although characteristic of Ewing sarcoma, are also found in other tumor types and rarely may be absent in a Ewing

sarcoma that harbors instead a *FUS* fusion.[29] Thus, a cytogenetic or molecular result is not by itself typically sufficient for diagnosis but additive to the evidence for or against a diagnosis. Microscopic examination stands as the mainstay of diagnostic cytopathology. Undoubtedly, however, with access to appropriate, validated, successful molecular genetic testing, a cytopathologist can currently render specific diagnoses that were unachievable a decade ago.[30]

Selecting neoadjuvant targeted therapy

Cytology samples provide the means to interrogate a tumor for actionable genetic alterations in a minimally invasive manner. For example, cytology samples (CBs and smears) are successfully used to test for predictive biomarkers to determine eligibility for specific tyrosine kinas inhibitors in patients with non-small cell lung cancer.[12]

Monitoring tumor response during therapy

Re-biopsy of a patient's tumor for molecular re-analysis during therapy already occurs in the setting of tyrosine kinas inhibitor resistance in non-small cell lung carcinoma. Multiple groups have reported successful molecular testing of re-biopsies (primarily comprising EBUS specimens) to identify alternate targetable genetic alterations.[31-33] Assessing the cause of acquired resistance through precision cytopathology testing is likely to become more common.[34] At least in some cases, however, it may be supplanted by measurements of circulating tumor DNA (ctDNA; one of the types of "liquid biopsy"). Molecular genetic testing of ctDNA is in an earlier phase of development than assessment of re-biopsied primary tumor.[35] In the interim, biomarker testing of ultrasound-guided FNA biopsies and positive body fluids in patients with acquired resistance will continue to grow.[36]

Assessing the tumor immune microenvironment

Immunotherapy and conventional chemotherapy show statistically significant effectiveness according to the amount and type of tumor immune/inflammatory response.[37-40] The significance of the tumor immune response as a determinant of response to therapy has been convincingly demonstrated in breast carcinoma, colon carcinoma, and lung carcinoma.[41] Optimal assessment of the tumor immune response as a predictive biomarker varies with tumor site and is undergoing refinement, but measurement methods include semiquantitation of lymphocytes by microscopic review of hematoxylin and eosin−stained sections, IHC-labeled quantitation of immune cell subpopulations in tumor sections, multiparameter flow cytometry of intermixed immune cells isolated from a tumor sample, advanced enzyme-linked immunosorbent assay testing and NGS examining the immune or inflammatory gene expression signature.[42] Most studies to date have analyzed the immune response in FFPE or fresh tissue specimens. Cytology samples could prove equally useful, however, and provide results rapidly because of the cell suspension nature of the sample (convenient for those assays requiring fluid samples) and because of advantages of intact cells accessible in non−CB cytology specimens.

Evaluating recurrent disease

Recurrent disease at the primary site is most often a clinical cytology question in thyroid carcinomas, breast carcinoma, and sarcomas. Depending on tumor type, diagnosis of recurrent disease could be accomplished with in situ hybridization or immunocytochemistry. However, best evaluation for clinically actionable genetic alterations (CAGAs) is accomplished with NGS techniques.[43,44]

Evaluating metastatic disease

Evaluating metastatic disease for new or resistant genetic alterations may be tumor-dependent in terms of the panel of specific CAGAs tested, but analysis may be most efficiently conducted with NGS such as whole exome sequencing, expression analyses, and NanoString (Seattle, Washington) methods. Analyzing one metastasis may be insufficient, as metastases may differ from each other significantly at the genetic and epigenetic levels.[45-47]

Evaluating intratumoral heterogeneity

Intratumoral heterogeneity represents a main cause of targeted and non-targeted therapy resistance due to the presence of non-sensitive subclones within the tumor.[45] A potential advantage of FNA biopsies consists of the ability to widely sample a tumor compared with large-bore core needle biopsies.[47] Advocates of single-cell sequencing to query intratumoral heterogeneity recognize that laser capture microdissection, the most common method to acquire single cells, is more easily performed with smears than tissue biopsies because of the ease of microdissection and the capture of entire rather than partial nuclei.[27]

Challenges of precision cytopathology

Challenges facing seamless expansion of precision cytopathology occur at the pre-analytical, analytical, and post-analytical phases of testing.

Few commercial NGS tests are validated for use with cytology samples (other than CBs)

Although cytology specimens are as good or superior to FFPE material for molecular genetic testing, few NGS commercial assays take advantage of cytology specimens' high-quality nucleic acids.[6] Exceptions include 3 thyroid tumor assays: Afirma (Veracyte, Inc, South San Francisco, CA), ThyroSeq v3 (CBL Path, Inc, Rye Brook, NY, and University of Pittsburgh Medical Center, Pittsburgh, PA), and ThyGenX/ThyraMIR (Interpace Diagnostics, Inc, Parsipanny NJ).[48] In the setting of a Bethesda diagnosis of "atypia of undetermined significance", "follicular lesion of undetermined significance", or "follicular neoplasm", the published validation studies report sensitivities of 74% to 90%, specificity of 52%

to 93%, positive predictive value (PPV) of 37% to 81%, and negative predictive value (NPV) of 92% to 96% with assay costs ranging from ~$3700 to $6400 (US dollars).[48]

Expense of validating assays on cytology specimens

Validating an in-house precision cytopathology assay absorbs capital and time. Analytical validation requires evaluating sensitivity, specificity, reliability, and assay robustness using known reference standards.[49,50] The product (validated assay) should be accurate, reproducible, scalable, and not cost-prohibitive—a challenging suite of objectives to meet.

Reimbursement issues

In the United States, the Centers for Medicare and Medicaid Services has finalized coverage of NGS companion diagnostics that meet specific conditions.[51] This national coverage decision, which provided immediate coverage for Foundation Medicine's FoundationOne CDx (Foundation Medicine, Inc, Cambridge, MA) and ThermoFisher's Oncomine Dx Target Test (ThermoFisher Scientific, Inc, Waltham, MA) in late 2017, will facilitate obtaining approval for and ease patient access to clinically relevant NGS assays.

Complexity of pre-analytical conditions

Fixation, collection, cell block, spins, monolayer, stains. Cytology laboratories differ from histology laboratories in the range of specimen types, fixatives, and even basic stains used to examine the microscopic features of specimens. These pre-analytical conditions contribute to the complexity of assay development. Moreover, some assays require specific fixatives (such as the Afirma test) that must be used premeditatively at the time of sample collection.[10,48]

May have to validate in both cell blocks and smears or monolayers. If a clinical biomarker is required for critical therapeutic decision-making, validation of the assay for the biomarker may be indicated in more than 1 cytology sample type, given the occasional erratic distribution of tumor cells in patient specimens. For example, one EBUS sample may contain sufficient material in an FFPE CB for testing, whereas another EBUS from another patient may have sufficient cells only in Diff-Quik (DQ; Romanowsky-type) stained smears. Thus, in this example, the same assay must be validated in 2 specimen types, or an alternative means to evaluate the sample for a CAGA must be used for the specimen type that has not been validated in-house.

Need to know local prevalence of disease

In order to accurately predict the PPV and NPV of a clinical assay, the local institutional prevalence of the disease must be known to determine the utility of a potential test.[9,48] The NPV declines with increasing prevalence, whereas the obverse pertains with PPV.

Cytology specimen may not be representative of the lesion compared with tissue biopsy assay results

a. Some samples may not be entirely representative of the tumor: Some sampling strategies, such as bile duct brushings and other cytology specimens that sample only the superficial portions of a lesion, may not be entirely representative of the molecular genetic landscape of the tumor.[52]
b. Conversely, many FNA biopsies may potentially be more representative than core needle biopsies as the sampling strategy may be more dispersed.[22,23,53]

Low cellularity of CBs

The most likely cytology specimen to fail to meet minimal assay requirements, the CB, remains the specimen most commonly approved for use in clinical trials and commercial molecular genetic assays. This preference for CBs, understandable given the similarity to FFPE tissue specimens, historical precedent, and national guidelines, is likely to change as investigators continue to report improved success with needle rinses, supernatants from CB preparation, and smears.[8,19-25]

Increased workload for technical staff, need for molecular pathologists, biostatisticians, and bioinformatics specialists

Rather than performing individually labor-intensive, sequential single-gene assays taxing of time and specimen, small NGS panels encompassing tumor-specific CAGAs should be used or developed when possible.[12,54] Collaborating with oncologists, molecular pathologists, biostatisticians, and bioinformatics experts is essential to best address local biomarker testing needs.[30]

Lack of molecular cytology proficiency testing for laboratory consistency

Proficiency testing across laboratories improves test accuracy and precision. National organizations such as the College of American Pathologists (CAP) provide clinical laboratories with this opportunity. Establishing reference standards for cytology molecular testing is in early phases, however. The first engineered set of cytology reference slides for molecular testing was deployed in a worldwide ring trial study and reported in 2017.[54] Encouragingly, this 14-institution study reported concordance for 5 mutations at 10% and 5% dilution points in all laboratories despite differences in NGS platforms and gene panels. Furthermore, there were no significant differences in mutation detection and mutant allele frequencies among unstained reference slides, non-coverslipped DQ reference slides, and coverslipped DQ reference slides.[54] This groundbreaking feasibility study represents a first step in developing standards across laboratories, platforms, and gene panels.

Unstable climate with rapid development and potential discards of methodologies and targets

Many cytology laboratories have already dipped a molecular 'toe in the water' with non-small cell lung cancer biomarker testing and indeterminate thyroid nodule molecular testing; it is time to prepare to become more fully immersed, as additional companion diagnostics are developed and approved for precision oncology-targeted therapies. However, the degree to which to expand cytology capabilities depends on local institutional needs and resources while being mindful of national organizational recommendations. Seemingly simple steps such as enhancing cellularity of CBs, often the first cytology specimen considered for testing, will significantly advance a laboratory's ability to respond to molecular genetic testing needs.

Lack of participation by a cytology laboratory could result in discordant diagnoses or inability to render a diagnosis in cases where molecular testing can augment morphologic diagnostic review either of which scenario could culminate in increased patient morbidity. For example, it is imperative to improve assay success rates with sparse cytology samples, as patients with insufficient specimens may not undergo repeat biopsy and may thus lose the ability to benefit from targeted therapy.[55] Moreover, some patients are too ill to undergo repeat biopsy, and repeat biopsies are not only costly but postpone treatment selection and initiation.

Laboratories must be nimble regarding new assays, because therapeutically actionable genetic alterations are continuously discovered and developed into clinical biomarkers. Adaptability requires collaboration with experts in other pertinent fields to evaluate local clinical utility of new assays, address in-house test feasibility and send-out options.

Basic steps in establishing or expanding a precision cytopathology laboratory

Fig. 1A-D highlight basic steps in optimizing a cytology laboratory for precision cytopathology testing. These steps are discussed further in the following sections.

Train laboratory staff and pathologists to optimally triage specimens

Improve the in-house CB technique, if necessary, to maximize sample, as many precision cytopathology assays are validated for use only with FFPE sections. Train cytopathologists or cytotechnologists to microdissect smears, CB sections, and monolayer slides (including cytospins, when used) to enrich the tumor fraction required for a specific assay—for example, a specific assay may require 20% tumor cellularity; percent tumor content in the specimen is termed tumor cellularity.[27,56]

Tumor cellularity must reach a specified threshold (the limit of detection), which varies per assay but is especially critical for mutation detection (see Table 2). Samples with initial low tumor cellularity may be enrichable by microdissection, which entails use of a standard light microscope, sterile blade or needle, and extraction buffer. Cells may be scraped directly into the buffer tube after first mixing the scraped cells with a small amount of buffer dropped on the slide; such mixing with the sticky solution enhances tumor cell capture.[8,24] Polymerase chain reaction (PCR) may also used to amplify scant initial starting nucleic acid targets. Consequently, PCR assays can improve the sensitivity and accuracy of detection.[56-58]

Analytical sensitivity of a molecular genetic assay refers to the minimal amount of the aberration that is reliably detectable, usually in the setting of abundant background wild-type normal alleles or transcripts. For instance, Sanger sequencing requires relatively high tumor cellularity to reliably determine mutation status compared with other assays that accommodate specimens with lower tumor cellularity. See Table 2 for test specifications including minimal required tumor cellularity of more common molecular genetic methods/assays. For an assay requiring high tumor cellularity, however, quality trumps quantity: an extremely small specimen with high tumor cellularity (higher fraction of tumor cells) may be superior to an abundant specimen with low tumor cellularity (many tumor cells overall but diluted by non-tumor cells). Importantly, to adequately address sparse actual patient specimens, the validation process of a cytology molecular assay must include scant samples and, furthermore, also requires establishing the minimum tumor cellularity for the assay.[57]

Store slides carefully to preserve nucleic acids and proteins for biomarker testing requested after the case has been reviewed and signed out. Adequate storage conditions for nucleic acids align with CAP recommendations for FFPE block storage.

Engage ROSE to optimize sample quantity and quality for the specific assay(s)

Rapid onsite evaluation (ROSE) at the time of FNA procedure optimizes triaging of the sample for diagnosis and ancillary studies. With pre-planning, the specimen may be specifically subdivided to increase likelihood of assay success. For example, extra smears may be prepared, and a representative slide can be maintained non-coverslipped for immediate DNA extraction.[24] If desired, fresh unfixed cells may be processed for immediate RNA extraction with optimum results. However, RNA based assays, especially interrogating short length fragments, are also successful with smears, monolayers, rinses, and even CBs.[10,24] Non-CB cytology samples offer the advantage of short acquisition time for molecular processing in comparison with surgical biopsy or resection specimens, for which the interval between tumor biopsy or removal and the initiation of processing is typically longer.

Specimen quantity presents the foremost challenge for successful testing, because currently only a limited number of

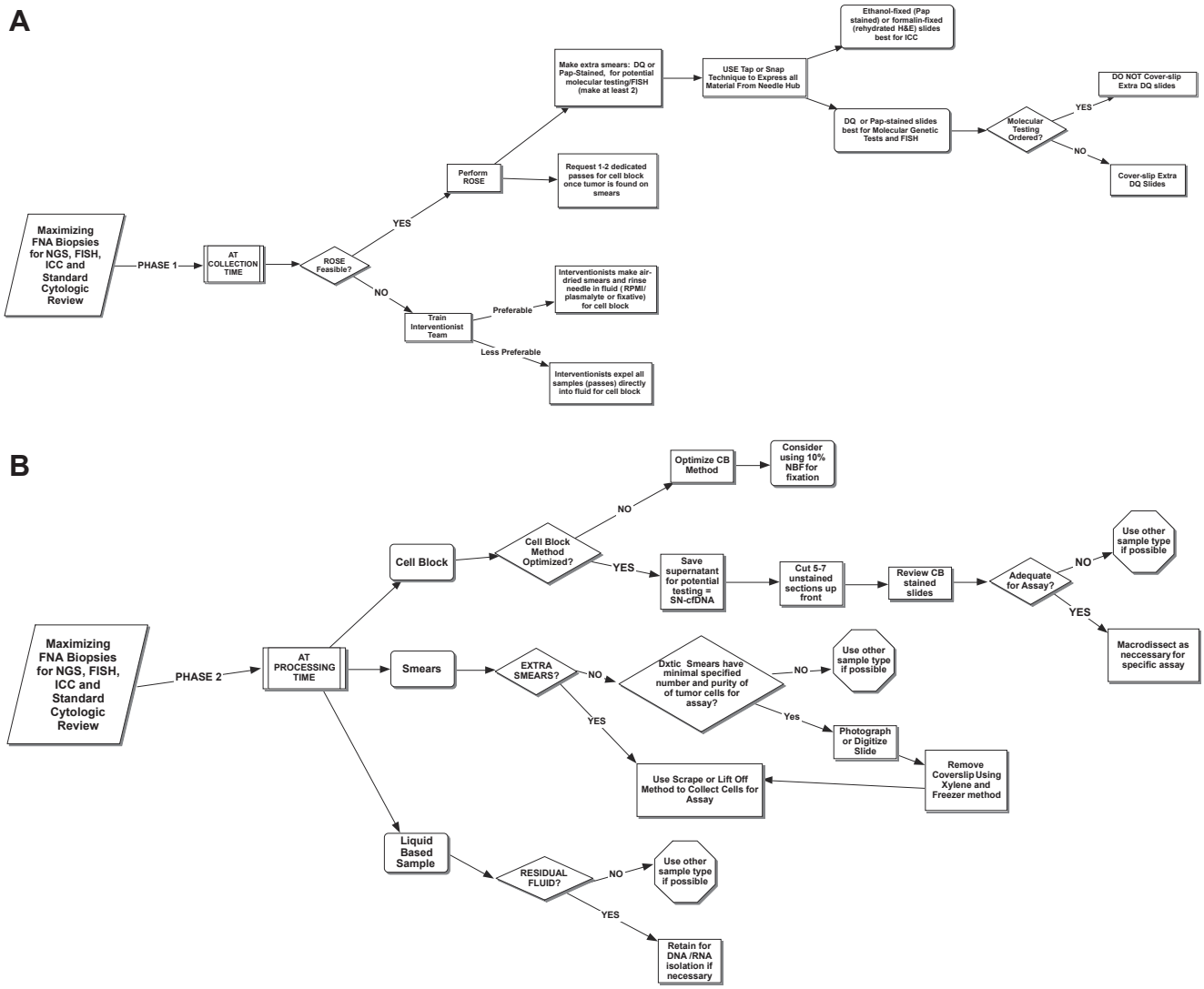


Figure 1 This diagram schematically depicts the steps involved in establishing and maintaining a precision cytopathology laboratory. Process steps are divided into four phases: (A), Collection steps; (B), Processing steps; (C), Results/reporting steps; (D), Quality control/quality assurance steps in aggregate. Dxtic, diagnostic; DQ, Diff-Quik; FISH, fluorescence in situ hybridization; FNA, fine-needle aspiration; H&E, hematoxylin and eosin; ICC, intrahepatic cholangiocellular carcinoma; NBF, neutral buffered formalin; NGS, next-generation sequencing; QC/QA, quality control/quality assurance; ROSE, rapid onsite evaluation; RPMI, Roswell Park Memorial Institute medium; SN-cfDNA, supernatant-cell-free DNA.

NGS-based molecular tests succeed with an extremely low quantity (<5 ng) of nucleic acid. See Table 3 for sample test specifications. On site cytopathology staff members (pathologist, pathology resident/fellow, or experienced cytotechnologist) assess in real time the quantity and quality of a specimen. Immediate assessment provides the opportunity to request additional passes (increase sample quantity) and to triage the sample. A test that may be performable on a cytology smear (such as in situ hybridization) may benefit from preparing additional smears, for example. Small-panel, targeted NGS testing may benefit from microdissection of extra smears to enrich tumor cellularity. If the anticipated test requires a CB, additional dedicated passes can be placed in sterile plasmalyte (Baxter, Inc, Deefield, IL), Roswell Park Memorial Institute medium (RPMI; ThermoFischer Scientific, Inc), CytoLyt (Hologic, Inc, Marlborough MA), CytoRich Red (ThermoFisher Scientific, Inc), 10% NBF, or 95% ethanol as desired or required for the assay(s). For several commercial assays, biopsy needle rinses must be placed in proprietary solution at the time of the procedure.[10,11,48] While expelling a portion of the sample can be accomplished without a cytopathology professional attending the procedure, decisions made at ROSE can improve the success rate of these commercial assays by ensuring the lesion of interest has been adequately sampled.

Plan new testing with all stakeholders

Determine the potential indication(s) for testing. Determine feasibility of using cytology samples if this is the likely or

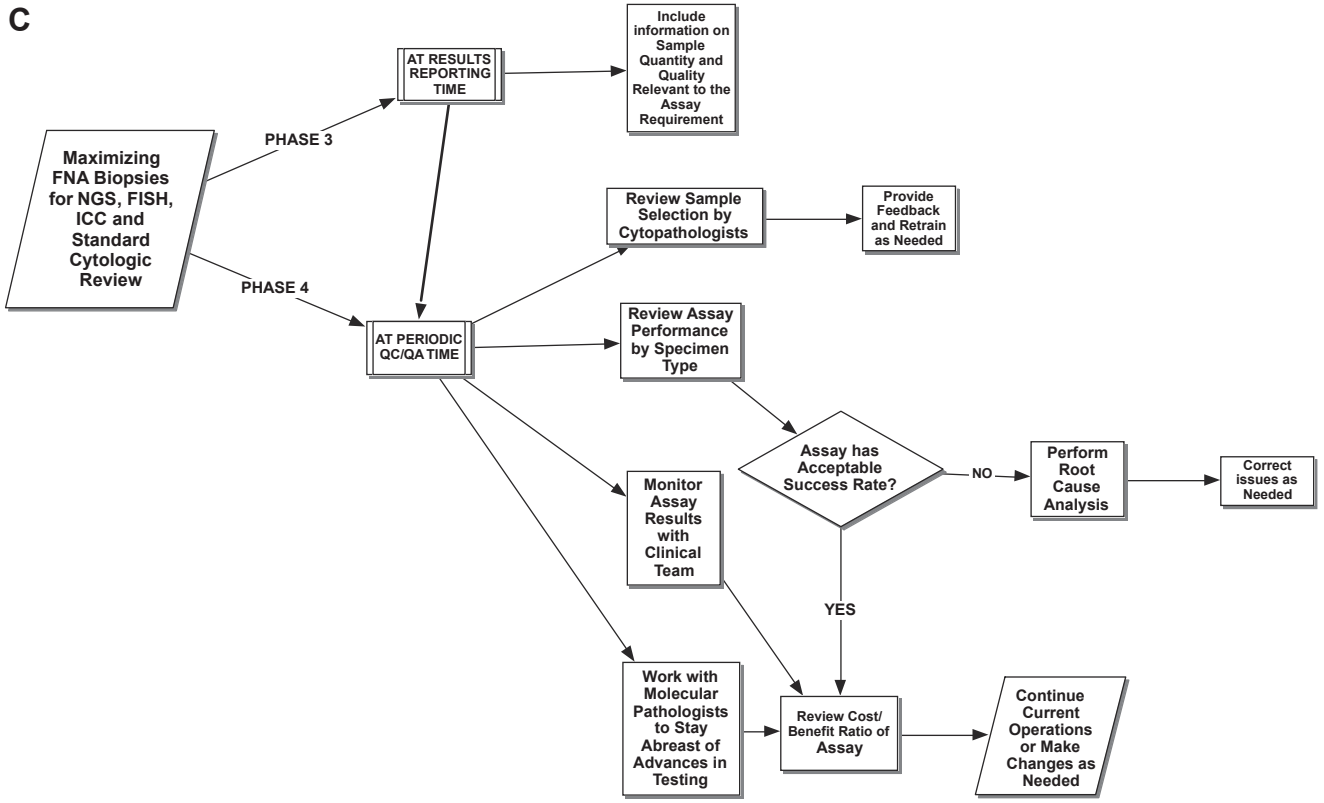


Fig. 1 (*continued*).

sole specimen for the potential assay. Conduct a cost–benefit analysis of in-house developed test ("home-brew") versus referral testing, if outside referral testing is available. External testing options may be limited, as most commercial NGS assays require FFPE samples. To accurately assess PPV and NPV, local institutional prevalence of the disease in question must be estimated.[9]

Generate a laboratory-developed test only when indicated

Align the laboratory-developed test (LDT) with relevant national organization recommendations as well as local needs. When possible, append a new LDT to current equipment and local cross-discipline expertise. Ideally, the testing platform should be capable of detection of future biomarkers to ensure a reasonable usage half-life.

Verify an unexpected or discordant result with an alternative testing method

As with the "triple test" in breast cytopathology that recommends excision of the lesion in the setting of discordant clinical, imaging, or cytology results, an unexpected or discordant molecular assay result should trigger alternative testing to confirm the result.[12] Alternative testing or repeat testing becomes particularly indicated for unexpected negative results, especially when the sample tumor content and amount of nucleic acid hover near the limit of detection.

Conduct routine periodic quality control reviews and send-out audits

As with any clinical laboratory assay performed in a CLIA-approved (or equivalent laboratory outside the US), quality-control reviews of LDTs and audits of send-out tests results must be performed.

Analyze the clinical treatment decisions based on molecular test results (perform cost–benefit analyses)

Once results are reported, the clinical consequences of the testing should be monitored. Monitoring can be accomplished by medical record reviews and Tumor Boards, for example. Perform cost–benefit analyses: does the assay provide clinically relevant and actionable results that are actually acted upon? Do the in-house tests continue to be operationally viable, when commercial assays may provide the same or expanded results at lower cost?

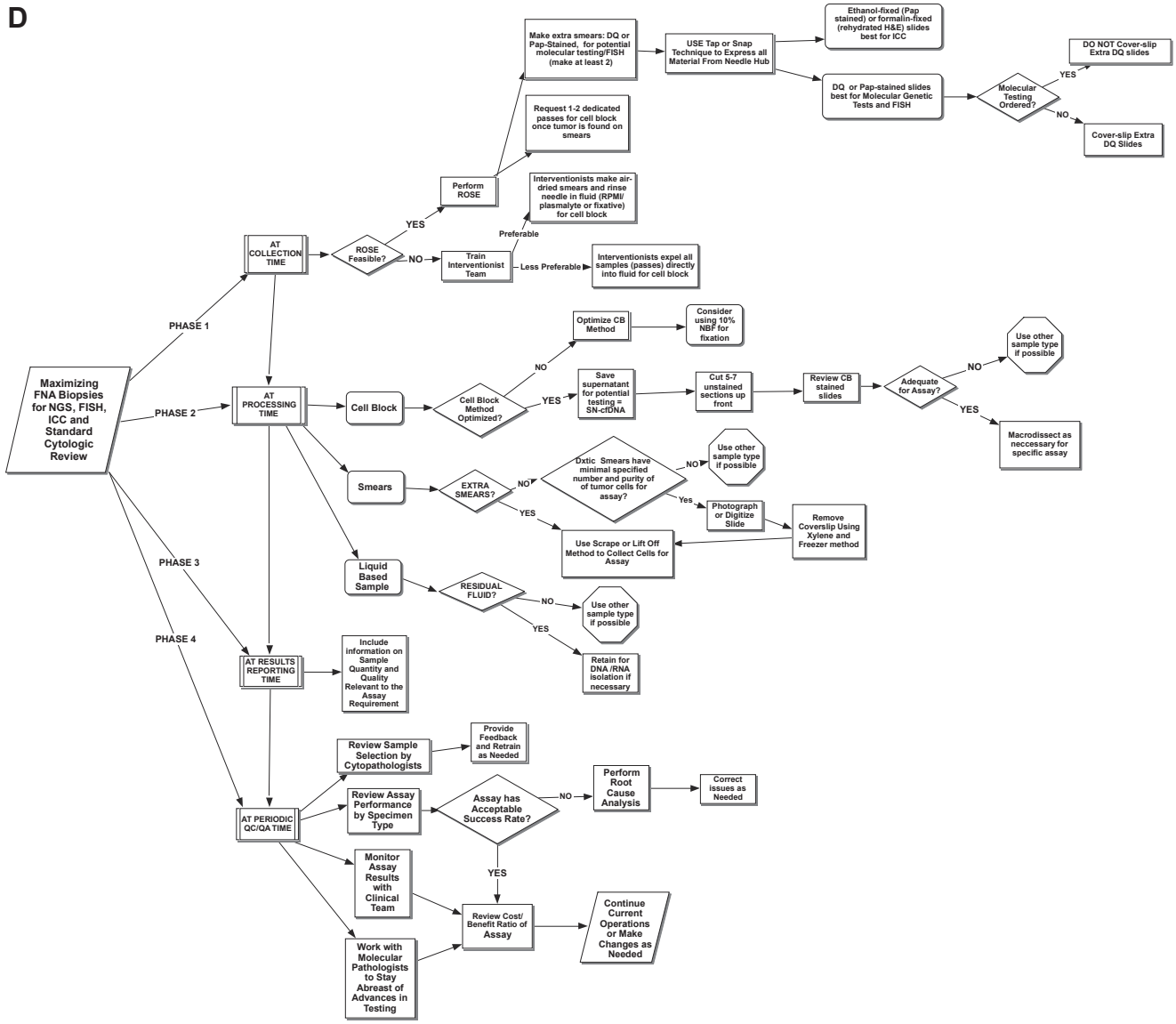


Fig. 1 (*continued*).

Stay informed in this rapidly evolving field—ease into or fully embrace the role of the "precision cytopathologist"

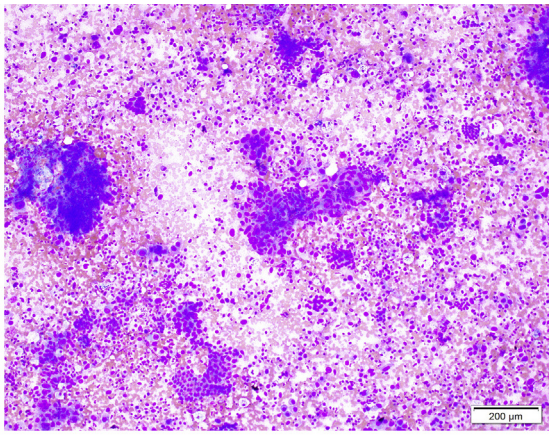
With the advent of effective targeted neoadjuvant therapy, eligibility for which depends in part on companion diagnostic molecular genetic testing, precision cytopathology becomes more an imperative than optional component of the practice of modern cytopathology. Beginning in 1990 and through the mid 1990s, investigators reported improved diagnostic sensitivity and specificity of pancreatic carcinoma by adding *KRAS* mutation analysis of FNA biopsies or duct brushings to cytologic morphologic review.[59-63] Notably, the first author of the 1990 publication now leads an international consensus effort to radically improve effectiveness of precision oncology by urging tumor assessment at the molecular genetic and epigenetic levels through space (sample the tumor widely = "eco-index") and time (sample the tumor and its metastases through time = "evo-index").[45]

Cytology specimen types in precision cytopathology

Cell block

Molecular diagnostic testing of cytology samples is performed most commonly using CBs, an integral part of the workup of malignancy in cytopathology. CBs represent a useful tissue archive for molecular testing because of the ability to generate multiple sections through the block as with traditional

A



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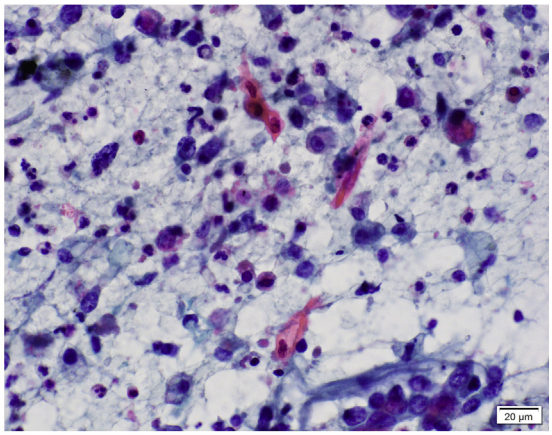

C

Squamous Carcinoma of Lung	Feature
Slide Description	1 DQ Approximately 30% Surface Area Covered by Cells Entire Slide Scraped
Tumor Cellularity	70%
Percent Necrosis	20%
DNA Concentration Total Volume (Quibit)	60 ng/microliter 50 microliters
260/280 Ratio (NanoDrop)	1.85
RNA Concentration Total Volume	160 ng/microliter 20 microliters
RIN	2.5

Figure 2 A-C: This moderately cellular Diff-Quik stained smear of a primary lung squamous cell carcinoma was scraped to obtain DNA and RNA for a targeted next-generation sequencing panel. An AllPrep DNA/RNA isolation method was used. Amount and quality of DNA and RNA was acceptable for testing with relatively abundant DNA and RNA available for additional assays if needed. (NanoDrop Spectrophotometer, ThermoFisher Scientific; Qubit Fluorometric Quantification, ThermoFisher Scientific; AllPrep DNA/RNA isolation [for formalin-fixed paraffin-embedded sample], Qiagen).

histology blocks, and in most cases do not require additional molecular assay validation. Notably, CBs, compared with core biopsies, are enriched for tumor cells with relatively little intervening stroma, at least with the smaller biopsy needles.[64] However, insufficient cellularity of CBs often represents a test limitation, in part because cellularity on a CB is evaluated by an hematoxylin and eosin—stained section; thus the percentage of tumor cells in deeper sections of the CB (the sections submitted for the assay) is an estimate.[8] If tumor cellularity meets the minimal level of detection of the assay (and ideally exceeds it) and nucleic acid amount likewise meets or exceeds minimal test specifications, paraffin scrolls (also termed curls; typically 10 microns or thicker) can be cut from the block and placed directly into a microcentrifuge tube for nucleic acid extraction. Unstained sections may be automatically cut up front to prevent the cell loss that occurs with refacing into a block.[65] Table 4 lists advantages and disadvantages of common cytology specimens.

Methods for CB preparation vary greatly among institutions and thus lack uniformity, with some practices including additional dedicated passes for cellular enrichment.[14,66] Recently, several institutions have reported improved CB quality with innovative methods.[67-72] Sufficient details are provided in these publications to trial the methods if in-house CB tumor quality could be consequently improved.

CBs are prepared with a variety of fixatives including 10% neutral formalin, 95% ethanol, CytoLyt, and CytoRich Red, among others. These fixatives feature distinct biochemical characteristics that affect performance of molecular genetic assays. Crosslinking fixatives include formalin, paraformaldehyde, and glutaraldehyde, which cause methylene bridging of bases, promote formation of crosslinks between nucleic acids and available proteins and induce random polymerase errors in nucleotide incorporation, usually C-T or G-A transitions.[8] Protein-denaturing fixatives include alcohols, Carnoy fixative, methacarn solution, acetone, and acetic acid. Both cross-linking and protein-denaturing fixatives will shorten nucleic acid fragments but to a lesser degree with alcohol(s). Moreover, over-fixation is less problematic for alcohol-based fixatives.[58]

Positive body fluids/effusions and epithelial brushings comprise other sources of cytology preparations available for testing, most often in the form of a CB specimen.[18]

Direct smears

Using enrichment methods such as microdissection, a direct smear is typically superior to the corresponding CB, because the smeared sample is more dispersed with a greater variation in the proportion of tumor to benign cells in different areas of the slide facilitating enriching tumor content to meet minimum assay specifications.[5,6,8,20,53,72-75] FNA samples typically are enriched for cancer cells, with intact whole nuclei rather than the fragments of nuclei that can occur when cutting CBs. One cellular FNA pass can contain up to 1,000,000 tumor cells; however, minimum numbers of cells on the glass slides used in current NGS tests typically

Table 4 Representative precision cytopathology assay: advantages and disadvantages.

Assay	Type	Advantages	Disadvantages
Karyotype	High Resolution Karyotype	Provides global view of entire karyotype Does not need a priori knowledge of the possible chromosomal aberrations	Requires viable living cells (non-fixed) in considerable volume Relatively low resolution (at 850 band resolution, each band contains ~3 Mb of DNA; maximum visual resolution of 5-10 Mb) Long TAT: days to weeks Risk for false negative: Normal karyotype result may represent fibroblast overgrowth and not tumor Requires well-trained technologists
In situ hybridization (ISH) variants	FISH	Flexible regarding specimen types: fixed cells, FFPE, air-dried smear, touch prep, and cytospin. Short TAT: ~24 hours in some clinical labs, especially if use cytospin sample Multiplex/multicolor FISH available. Does not require removing the target NA sequence from slide Detects: structural rearrangements including translocations, inversions, insertions and microdeletions. Enables chromosomal mapping and characterizing chromosome breakpoints	Need to know a priori the possible chromosomal abnormality Application limited to available probes If negative, result may be uninformative because the tumor may harbor an alternate site abnormality, and another rearrangement assay will be required If only a few neoplastic cells are present, FISH may give false negative result Signal fades over time Requires fluorescensce microscope Requires well-trained technologists Some probes require formalin fixed sample; otherwise internal validation is required
	CISH	Flexible regarding specimen types: fixed cells, FFPE, air-dried smear, touch prep, and cytospin. Detects: gene amplification, gene deletion, chromosomal translocation, chromosomal number changes Enables cytologic review of cells Uses low cost light microscope Staining is permanent	Limited commercially available probes Limited to single or dual color due to chromogenic detection technology Cytologic specimens not fixed in 10% NBF require internal validation
Chromosomal microarrays (CMAs)	Oligo (Oligonucleotide) SNP (single nucleotide polymorphism)	Improved resolution for detection of genomic microdeletions and micro duplications compared to conventional cytogenetics and FISH Prior knowledge of chromosomal aberrations is not required No cell culture needed Able to roughly quantify specific cellular DNA, mRNA, microRNA, ncRNA and other nucleotide entities. Multiple hybridizations can be performed in the same sample Works with small samples Provides information on NA location; combined with histopathologic data gives a complete picture of cellular changes. Does not require removing the target NA sequence from slide Uses low cost light microscopy Staining is permanent	Cannot detect point mutations Cannot detect balanced chromosomal rearrangement, including balanced inversions, translocations, insertions Low-level mosaicism may not be detected Tetraploidy cannot be detected Copy number variations of regions not represented on platform will not be detected Cannot detect epigenetic variations such as methylation

(*continued on next page*)

Table 4 (*continued*)

Assay	Type	Advantages	Disadvantages
Sequencing techniques	Sanger sequencing	Detects point mutations and small insertions/deletions Decreasing cost for testing	Detection sensitivity of ~20%-30% Long analysis time and works on one amplicon at a time
	Pyrosequencing	Detection sensitivity of 5% Decreasing costs for testing	Each run must include known positive and negative controls, as quality control and for results interpretation Short read length Sequencing error may occur (multiple consecutive runs of same nucleotide)
	Next-generation sequencing (NGS) (massively parallel sequencing)	Identifies known and unknown variants (SNPs, small indels and frameshifts) Some platforms can identify copy-number variations (CNVs) in analyzed regions of interest and translocations Assesses up to thousands of genes but many commercial panels are available to assess clinically actionable gene alterations (CAGAs)	Cannot detect large fragment-spanning indels Set-up and maintenance contracts expensive; some larger panels expensive Requires expertise in multiple disciplines (bioinformatics and biostatistics) to asssess for clinically significant alterations May require pathologist dissectiion of sample to optimize tumor cell content Requires a target enrichment step Quality control can be complex
miRNA assays	Variety of platforms including NGS	Excellent quality in a variety of specimens (FNA, FT, and in particular, FFPE due to small size) Extraction Chemical methods - High amount of RNA Column methods - High quality of RNA	General Need to enrich the fraction containing miRNA Sensitivity and specificity varies among platforms Extraction Chemical Methods - Contamination by chemical components Columns methods: - Specific kits required for different samples Detection: Not all platforms can identify novel miRNA or sequence variations
Immunohistochemistry (IHC)		Used in commonest sample type: FFPE sections Detects signal in low cellularity specimens Short TAT compared to FISH and molecular techniques Low cost Pathologists experienced in interpretation	Good quality tissue required with preserved antigenicity: variably affected by pre-analytical variables such as: ischemic time, fixative type, fixation type, antigen retrieval technique, archiving conditions. Can yield false negative results in sparse samples or be nonspecific. Formalin fixed samples required for CB unless other fixative is validated Formalin interferes with nucleic acid sequencing. Expression is not easily reliably quantifiable (interobserver reproducibility may be low) Available antibodies may not address the clinical need, eg, the relevant biomarker(s) may only be measurable via a molecular genetic or epigenetic test

(*continued on next page*)

Table 4 (*continued*)

Assay	Type	Advantages	Disadvantages
Oligonucleotide tags to tumor mRNA transcripts (a novel sequencing method)	Nanostring	Flexible regarding specimen types Relatively low sample content: as low as 4-10 ng with amplification step Multiplexing capacity from 10 s to as many as 800 different genes/targets in a single reaction Predesigned or custom panels available Has significant potential as a platform for small to medium (10 s-100 s) CAGA panels	High cost Low content mRNA sequences may be below the level of detection of the assay For cytology specimens, can only be used currently as a LDT requiring validation except for Prosigna Assay for breast carcinoma

NGS, next generation sequencing; FFPE, Formalin-fixed paraffin embedded tissue; TAT, turnaround time; mRNA, messenger RNA; ncRNA, non-coding RNA; NBF, neutral buffered formalin; NA, nucleic acids; CISH, chromogenic in situ hybridization; CAGA, clinically actionable genetic alteration.

range from 1000 to 5000 tumor cells, with a minimal tumor cellularity of 20%.[21,24,25] Notably, some investigators have reported high NGS panel success rates with as few as 50 to 200 tumor cells.[55,75]

Both alcohol-fixed and air-dried smears are generally suitable for isolation of stable high quality DNA and RNA and long-term DNA storage. Also, nucleic acids are better preserved in alcohol fixatives than in 10% NBF.[27] The common practice of fixing smears with alcohol accounts for some of the reported improved nucleic acid quality and NGS testing with smears compared with CBs.[19] Many studies using previously stained cytology smears have shown that molecular testing can be performed successfully using both DQ as well as Papanicolaou-stained slides (Fig. 2 illustrates an in-house case of DNA and RNA quantity and quality obrtained by scraping a DQ-stained immediate smear and extracting the nucleic acid using a commercial kit.). For example, Oktay et al reported successful DNA and RNA isolation from both DQ and Papanicolaou-stained slides, enabling the detection of mutations and translocations in non-small cell carcinomas and thyroid lesions.[75] They reported a 1% molecular alteration detection rate from as few as 50 cells using ultrasensitive methods.[75]

Archived smears may also be salvaged for successful molecular testing. Killian et al characterized the suitability of 57 FNAC specimens archived for more than 10 years for high-resolution genotyping, methylation, and/or comparative genomic hybridization arrays.[76] They compared DQ and Papanicolaou-stained slides from the same procedure and reported that DQ and Papanicolaou-stained slides yielded high-quality DNA even if archived for a prolonged period of time, concluding that nearly all archival FNA smear extracts were successful for all profiling assays tested showing clearly discernable genomic aberrations.[76] Thus, archival smears are a good source of material for molecular studies. Notably, the freezer technique of removing the coverslip shortens the assay turnaround time.[77] This simple, quick technique also works on recently coverslipped slides.[77] Notably, in the authors' experience, plastic coverslips are also easily removed with a 23-gauge needle after placing the slides flat in a conventional freezer (temperature about −18°C) for 10 to 20 minutes.

Liquid-based, monolayer cytology

Bellevicine et al reported that a LBC slide versus a direct smear showed minimal differences in adequacy and in mutation detection rate.[8] Molecular analysis can be performed with LBC by either scraping off the cells from the monolayer slide or using the sample suspended in the solution. Direct visualization of the material on the monolayer to determine sample adequacy may at times be preferred over submitting the vial contents directly for testing. To enrich tumor cellularity, microdissection is possible but presents more challenges in LBC compared with immediate smears because of the constricted area of cell deposition on the monolayer slide.[24]

Residual (leftover) CytoLyt fluid sample after preparation of a monolayer and/or cell block is also a source of adequate nucleic acids for molecular assays. Wei et al analyzed 17 fluid samples (13 FNAs and 4 effusions) with NGS and reported a significantly higher concentration of DNA in rinses compared with the paired CB (176 ng/μL versus 10.6 ng/(L).[78] In a large study of 597 thyroid nodule FNAs, 67% were successfully tested for clinically relevant point mutations and rearrangements using high-resolution melting PCR, multiplex quantitative PCR, and pyrosequencing methods.[79] A significantly higher success rate was achieved with sample volume of > 2 mL of residual CytoLyt (79% versus 23%).[79] Analogously, Fuller et al reported successful NGS testing of 22 of 24 residual CytoLyt fluid samples of thyroid nodules (mixture of morphologically benign to malignant cases) using both extracted DNA and RNA using the AllPrep DNA/RNA Mini preparation kit (Qiagen, Alameda, CA).[80] Two hundred targeted region in 50 genes were analyzed with the Ion sequencing kits (Life Technologies, San Francisco, CA), while a FusionPlex CTL Kit (ArcherDx, Boulder, CO) that detects 195 targets in 40 genes using extracted RNA was run on a NextSeq Instrument (Illumina, San Diego, CA).[80]

That study thus demonstrated feasibility of both DNA and RNA assays using residual CytoLyt samples.

Direct smears or LBC slides may contain the only diagnostic cells for molecular testing, in some instances entailing destruction of the diagnostic slides in order to perform the biomarker assay. Thus, scraping the entirety of a diagnostic slide can pose potential medico-legal consequences. The CAP recommends CBs for molecular testing to preserve the diagnostic material on smears and morphologically correlate the molecular diagnosis with the malignant cells. However, in cases where the diagnostic smears must be harvested for indicated ancillary testing, photographs of diagnostic material or digitization of the slides (whole slide imaging) are acceptable replacements for the original diagnostic material. Although the CAP currently recommends using CBs over cytology smears for molecular testing, the cellularity of the CBs, as discussed, may be insufficient for successful molecular testing, and in such cases use of smears is warranted and sanctioned.[81] With forethought, extra smears may be prepared at the time of ROSE, so that all diagnostic material would not be destroyed by molecular testing if only the extra smears were destroyed. As noted, smears can provide superior cellularity and tumor content compared with CBs and show improved nucleic acid quality.[8,27,53,54,73] It is prudent to prepare extra smears if molecular assays such as NGS are contemplated (see Fig. 1 for strategies to expand biomarker testing in a cytopathology laboratory).

Other specimen types

Cell-free supernatant

A promising source of high quality nucleic acids for NGS and other molecular assays comprises abundant nucleic acid found in residual supernatant leftover after centrifugation during CB preparation.[82] Because this supernatant is typically discarded, successful use of it enables preservation of the rest of the sample for morphologic assessment and IHC. Guibert et al analyzed 17 lung carcinomas and reported 100% concordance for mutations compared with the corresponding CB using droplet digital PCR and NGS methods.[82]

Other fluids

Other cytology fluid samples have proven adequate as sources of nucleic acids and proteins such a pancreatic cyst fluid, effusions, and washings.[6,8,13,22,24,78]

Cautionary reminder regarding precision cytopathology assays

For all cytology specimens subjected to biomarker analyses, the success of molecular testing depends on standardized pre-analytical protocols established prior to biomarker testing to consistently, robustly, and accurately detect molecular alterations, epigenetic changes, protein expression profiles, and other biomarkers.[19,30,44,50,54,83]

Summary: best practices for a precision cytopathology laboratory

Triage cytology samples by anticipating ancillary assay requirements

For patients to optimally benefit from advances in precision oncology, cytology samples must be made available for clinically relevant biomarker testing (these biomarkers may be diagnostic, prognostic, and/or predictive). Cytopathologists thus actively advance cancer patient care through prudential prioritization of samples and optimization of cytology sample quality. This exhortation is not new; in 2013, Troncone et al called for a "change in mentality of cytotechnologists and cytopathologists to collect and process the cytological samples not only for microscopy but also to assess clinically relevant molecular markers."[6]

Numerous studies (many more than have been cited in this review) have concluded that cytology specimens generally provide an excellent source of nucleic acids for molecular genetic and epigenetic tests. However, to achieve a high assay success rate in cytology specimens, cytopathologists must ensure optimal pre-analytical conditions. Optimizing the FNA specimen can yield high tumor content (or enables tumor content enrichment by microdissection), and may be more representative because of wider sampling of the targeted lesion. Additionally, non-CB cytology specimens offer better quality nucleic acids than FFPE tissues such as core biopsies.

ROSE optimizes precision cytopathology

Samples are triaged optimally for ancillary studies when coupled with ROSE. Triaging specimens by the real-time, actively participating cytology team maximizes the yield of minimal cytology specimens and reduces the need for rebiopsy.[84,85] A few recent studies, predominantly by endoscopists, have advocated that ROSE is no longer necessary, in part because new, larger needles (fork-tip and others) are reliably productive.[86-88] These same investigators, however, have not accounted for the complex decision-making that must occur at the time of tumor collection to adequately triage specimens.[86-88] Without an immediate assessment, optimal triaging for specific molecular assays that may depend on the initial diagnostic impression cannot be performed. Moreover, unexpected hematopoietic lesions and infections would also likely not be appropriately triaged without immediate assessment. Succinctly, we provide the RITE (Rapid [cytology] Involvement for Triaging and Evaluation) service, whereas without us, the interventionists WING IT (Without [cytology] Involvement, Nothing Gets Ideally Triaged). The Society of Pulmonary Pathology and others endorse the continuing value of onsite assessment, in part to distribute the specimen correctly for specific, anticipated ancillary assays.[89,90]

Precision cytopathology requires continuing education, co-education, and collaboration

Best-practice precision cytopathology requires an integrated understanding of ancillary molecular genetic/cytogenetic testing and other companion predictive and prognostic tests. Becoming more knowledgeable about general molecular techniques such as NGS, variants of PCR, and new approaches such as with NanoString OligoTag probe sequencing will enable cytology staff to more actively engage in decision-making regarding assay selection.[91,92] For molecular assays developed or used in-house or externally accessed, becoming well-informed about and then establishing optimal pre-analytical conditions covering specimen collection, fixation, processing, and staining parameters will significantly improve assay success rates. Cytopathologists must be aware of assay requirements for minimal tumor cell content and quantity, develop facility in enriching tumor content, and should become familiar with nucleic acid quality and quantity testing. As some assays may be required weeks to months after the cytology report has been released, application of adequate storage conditions for nucleic acid preservation is highly desirable.

With these evolving responsibilities, it is fortunate that cytopathologists will increasingly work closely with colleagues expert in molecular pathology, cytogenetics, precision oncology, immunooncology, biostatistics, and bioinformatics. Optimal cancer patient care more than ever involves a concerted team effort. In this exciting and increasingly optimistic environment for cancer patients, cytopathologists will be relied upon for foundational diagnostic skills and discerning specimen stewardship.

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