Utility of UroVysion® FISH Testing for Bladder Cancer Detection

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Overview Fluorescence in situ hybridization (FISH), as an adjunct to cytology, is an ancillary technique that promotes early disease detection for patients. Recognizably, the responsibilities that cytotechnologists and cytopathologists engage in are significantly changing according to test platforms and will continue to do so. In reality, conventional cytology is, and will continue to be, essential. Yet, the skills required by cytotechnologists and cytopathologists will likely be adapted to new modalities, many of which are morphology-based molecular techniques, similar to FISH.	▪ FISH utilizes fluorescently-labeled nucleic acid (DNA or RNA) probes to assess intact cells for various types of genetic alterations. ▪ FISH has revolutionized anatomic & cytopathology laboratories as they aid clinicians & oncologists in the diagnosis & treatment of patients. ▪ Common applications of FISH include: o detection of cancer cells in cytologic specimens, o identification of chromosomal alterations in resected tumor specimens that predict prognosis, o response of certain cancer types to therapy and the detection of microorganisms in various specimen types. ▪ Routine cytology specimens that FISH can be performed on include: o urine, o bronchial brushes and washes, o biliary tract brushes, o esophageal brushes o gynecologic specimens o other specimen types, (e.g., breast core needle biopsies)
Advantages of FISH technique; relationship to cytopathology FISH offers several advantages over other ancillary, molecular techniques	▪ Provides an opportunity for cytotechnologists and pathologists to utilize their morphologic skills in providing diagnostic and prognostic information. ▪ Assessing intact cells offer the benefit of identifying specific cells that have chromosomal anomalies. ▪ The sensitivity of FISH, in comparison to both cytology and other genetic or molecular techniques, has been well documented in several published studies.[1-5] ▪ The sensitivity of FISH rests on the ability to identify specific cells for analysis, for which a very small subset of abnormal cells (in some cases as few as 4-5 cells) can provide diagnostic feedback. ▪ During FISH microscopic analysis, nuclei are examined for morphologic features indicative of neoplasia (e.g. nuclear enlargement, nuclear membrane irregularity and irregular chromatin patterns) as well as for chromosomal abnormalities.[6] o Since most tumors do have chromosomal anomalies, FISH is a reliable technique to identify these malignant cells
General Principles of the FISH Technique	FISH utilizes fluorescently-labeled DNA probes that hybridize (anneal or "stick to") specific chromosomal loci, which can be enumerated for abnormalities at the cytogenetic level. DAPI (4',6-diamidino-2-phenylindole) is the counterstain that binds strongly to DNA and is essential for the microscopic identification of abnormal nuclear morphologic features.
Overview of Steps Involved in Performing FISH	The required steps involved in performing FISH include: 1) specimen collection, 2) slide preparation and pre-hybridization, 3) denaturation and hybridization to target DNA, 4) removal of non-specifically bound probe, and 5) microscopic assessment of nuclear features and chromosomal signal patterns

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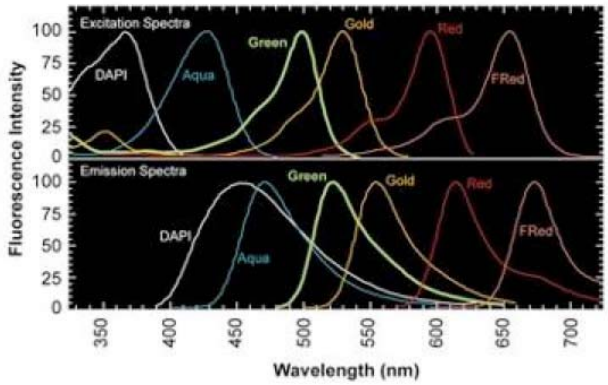
Specimen Collection FISH can be performed on a wide range of specimens including cytology specimens, peripheral blood and paraffin-embedded tissue.	Specific to cytology specimens, a wide range of specimen types can be utilized. In consideration of urine cytology, the following samples are acceptable: o voided urine (FDA approved for use with Abbott Molecular UroVysion™ probe set), o catheterized urine, o bladder washings, o upper tract washings (to include cell sampling from the ureters and renal pelvis), o stoma specimens and o previously stained ThinPrep slides. ▪ Urinary tract specimens can be prepared and analyzed with the FISH technique as fresh specimens(i.e. no preservative) or fixed with either ethanol or methanol of variable concentrations, PreservCyt® or CytoLyt®.
Slide Preparation and Pre-Hybridization Slide preparation for the FISH technique can vary widely from the use of cell suspension sediments, use of ThinPrep™ technology or cytospins.	▪ Cellularity is a key facet of this preparatory step, with the goal being to capture as many cells on the slide while ensuring minimal cell overlap. ▪ This is imperative so that the FISH probe cocktail can enter the cell and efficiently hybridize to the nuclear DNA target but do so without significantly disrupting the morphology of the cells. ▪ For more detailed protocols on pre-hybridization please reference Van Stedum and King (2002).[7] ▪ Pepsin increases the accessibility of the probe DNA to the nuclear DNA target. o This protease needs to be carefully controlled so as not to over- or under-digest the cell components. o Over-digestion may lead to a decrease in signal intensity and destroy nuclear morphology. o Underdigestion can lead to auto-fluorescence and an underestimation of signal copy number may result.
Probe Cocktails Probes are designed to hybridize to specific target sequences of interest, such as genes that have been implicated in probe selection studies based on the chromosomal makeup of body-site specific cancer cells. DNA probes that are commonly used for cytology applications have been categorized as chromosome enumeration probes (CEP) and locus specific indicator (LSI) probes.	CEP probes hybridize to repetitive DNA sequences found near the centromeres of chromosomes which are referred to as alpha-satellite DNA. o These regions are composed of ~171 bp sequences that are tandemly repeated hundreds to thousands of times and span approximately 250,000 to 5,000,000 bases.[8] ▪ CEP probes are used to enumerate the number of copies of a given chromosome in a cell. ▪ CEP probes represent chromosome copy number because if the centromere of a chromosome is lost, the whole chromosome is generally lost. ▪ One advantage of CEP probes is that because these probes hybridize to sequences that have high copy number, they provide strong (i.e. bright) signals. ▪ In addition, because these regions are tightly compacted in the heterochromatic regions of the chromosome, the signals provided with CEP probes are generally tight (“crisp”) rather than diffuse. Locus specific indicator (LSI) probes hybridize to unique sequences (i.e. non-repetitive DNA sequences) and are generally used to determine if specific genes are amplified (e.g. *HER2*), deleted (e.g. *p53* or *p16*), or translocated (e.g. *BCR*/*ABL* translocation). ▪ These probes typically hybridize to a region that ranges from 40 to 500 kilobases (kb).[9] ▪ Probes that hybridize to regions smaller than 40kb often produce weak signals that can be difficult to see. ▪ Probes larger than this can produce diffuse signals that are difficult to distinguish as single signals.

Denaturation and Hybridization Denaturation (relaxation) of both the probe and cellular DNA occurs with subsequent re-annealing of the probe to the cellular DNA target. The probe cocktail contains both probe DNA and other proprietary components. The proprietary probe components are intended to hybridize specifically to highly repetitive DNA sequences that are present throughout the genome. This helps to reduce non-specific binding and in turn, non-specific signal generation.	A reliable method for co-denaturation of the probe and target DNA takes place at ~73ºC for about 3 minutes. ▪ Formamide may be added to lower the temperature at which the probe and cellular DNA melt. ▪ Higher temperatures can destroy the cellular morphology that is attempting to be maintained at this step. ▪ The temperature is then lowered to about 37ºC, which allows the probe DNA to fastidiously hybridize to its specific target. ▪ Hybridization generally takes 4-12 hours to complete. Some factors that may influence the efficiency and specificity of the hybridization include: ▪ The probe sequence and hybridization temperature. ▪ The probe sequence must be unique so that additional signals are not generated. ▪ Non-specific hybridization may confound the recognition of the probe signals/hybridization. ▪ If the hybridization temperature is too high the generated signals may appear indistinct, whereas if the hybridization temperature is too low non-specific background signals may be generated again causing issues for microscopic analysis.
Removal of Non-specifically Bound Probe After hybridization, slides are washed in a solution to remove excess probe that is not specifically bound to the appropriate target. Failure to complete this step will result in non-specific background signals during fluorescent microscopy. Conversely, it is important that this step does not remove bound probe from the desired target sequence.	A typical preparatory sequence is to wash the slide in a 73ºC solution containing 0.4XSSC/0.3%NP40 for cytology specimens. Finally, a fluorescent staining solution that contains DAPI and other chemicals (which inhibit photobleaching or fading of the nuclear and chromosomal stains) is placed on the slide, followed by coverslipping. ▪ DAPI stains the nuclei and fluoresces a blue-grey hue when viewed with a fluorescent microscope. ▪ DAPI allows one to visualize intact nuclei and recognize morphologic features. ▪ Without DAPI, the FISH signals would appear to be free-floating in a dark background.
Fluorescence Microscopy It is at this stage, following hybridization, that the nuclei are assessed for chromosomal abnormalities (FISH signals). Fluorescence microscopy typically takes place in a dark room, in order to maintain stability of the DAPI and chromosomal probes.	As part of best practices for fluorescent microscopy, it is important to remove the slide from the path of light by closing the condenser while not viewing a FISH case. Should the staining be squelched, slides can be restored to their original fluorescent quality by re-hybridization as target DNA is still intact. An in-depth review of the specifics of fluorescence microscopy is provided by Tanke, HJ (1999).[10] A few of the basic, yet important aspects of how fluorescence microscopy works are provided here. ▪ The goal of fluorescent microscopy is to cause the fluorophore-labeled DNA probe to fluoresce and highlight the target of interest in the specimen. ▪ A fluorophore is a molecule that absorbs light in a certain range of wavelengths and then re-emits that energy as light of longer wavelengths.

Fluorescence Microscopy, con't.	For example, as shown in ***Figure 1*** the fluorophore that is referred to as Spectrum Green™ absorbs light that is lower in wavelength (470-510 nanometers) and then re-emits the light, inducing fluorescence, in higher wavelengths (500-550 nanometers). To do this, the fluorescence microscope must have a light source that is capable of exciting the fluorophore. Mercury or xenon arc lamps emit light of wavelengths that can be absorbed by the fluorophores.  **Figure 1:** *Absorption and emission spectra. The goal of fluorescent microscopy is to cause the fluorophore-labeled DNA probe to fluoresce and highlight the target of interest in the specimen. A fluorophore is a molecule that absorbs light in a certain range of wavelengths and then re-emits that energy as light of longer wavelengths and is exemplified in Figure 1 as absorption (shorter wavelength) and emission (longer wavelength) spectra.* A combination of filters (excitation, beam splitting interference, and barrier) select an appropriate wavelength of light to excite the fluorophore and ensure that the main light that reaches the observer's eye is the desired fluorescence wavelength as part of the filter cubes. Filter cubes are available for most fluorophores and should be equipped with appropriate filters to visualize the fluorescent signals of the nuclei (typically red, green, aqua and gold fluorophores, along with the DAPI counterstain). Composite images of DAPI-stained nuclei and associated fluorescent signals are generated as part of the FISH process. This requires the use of imaging systems and software that enable one to capture the different colored probe signals in a single image. Z-stack cameras, or other intuitive imaging systems, are preferred to adequately demonstrate signals that are located in unique planes of focus as part of the nuclear image.
FISH Case Microscopic Analysis Microscopic canning and recognition of target cells is a skill that cytotechnologists and cytopathologists are especially adept at performing. Screening patterns, similar to those for routine cytology, are followed during the FISH analysis.	Nuclear features, as identified with the DAPI stain and fluorescent microscope, are relied upon to determine which cells are examined for chromosomal anomalies and which cells are not. In reference to ***Figure 2***, (next page)it should be noted that all normal appearing cells need not be assessed or scored at the chromosomal level. However, any abnormal-appearing cell must be assessed for all identified chromosomes as part of the case analysis. This is to determine if there is a gain or loss of a chromosome and/or gene. DAPI screening is critical because it is unrealistic to evaluate every cell, while the cytomorphologist who is analyzing the case must identify all nuclei likely to be abnormal.

FISH Case Microscopic Analysis, con't.

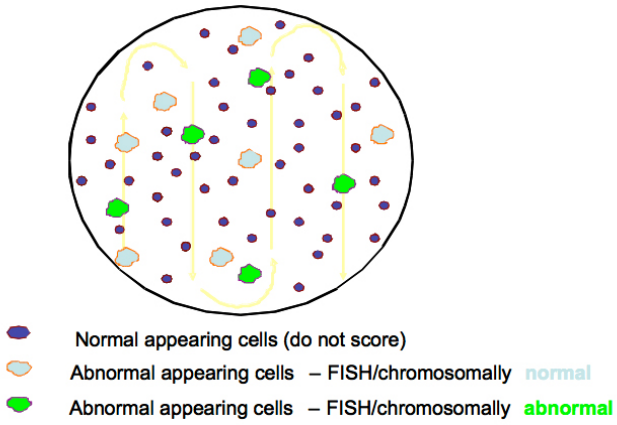


Figure 2: *Screening for DAPI – abnormal cells. Very similar to the screening approach taken for routine cytology, scanning for DAPI-abnormal cells takes place in a consistent pattern with appropriate overlap identifying and assessing abnormal appearing cells, which require further assessment at the chromosomal level.*

Pre-neoplastic and neoplastic nuclei generally have the following features when visualized with the DAPI stain: nuclear enlargement, nuclear membrane irregularity, weak-DAPI or mottled staining nuclei.[6] The unique chromatin patterns that are distinguished by DAPI staining are identified in ***Figure 3***.

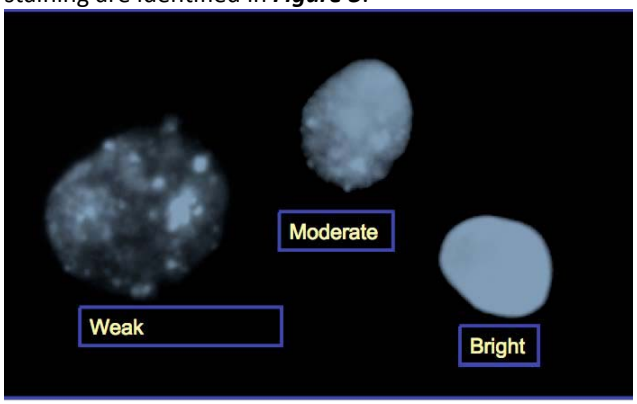


Figure 3: *Chromatin patterns exemplified by DAPI-staining during fluorescence microscopic analysis. The chromatin patterns of a mottled appearance (moderate-to weak-DAPI staining) are more consistent with parachromatinic clearing, associated with neoplasia.*

Analysis of Chromosomal Makeup

Normal, or disomic cells, reliably have two copies of each chromosome that are identified with the FISH assay of choice. In comparison, the most frequent cytogenetic anomaly of neoplastic tumor cells constitutes an interpretation of polysomy. Polysomy is defined as gains of two or more of the four probes that are typically utilized in a FISH probe cocktail.

A comparison of the cytogenetic features of disomy and polysomy, by FISH, are represented in ***Figure 4***.

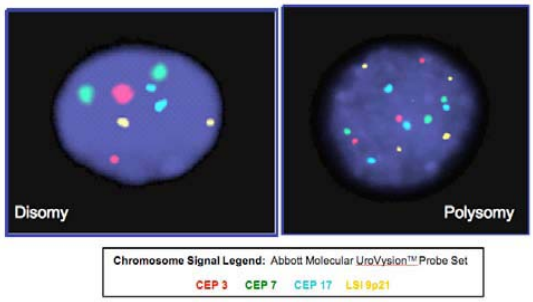


Figure 4: *Normal vs. abnormal chromosomal makeup. This figure represents the chromosome signal pattern for normal (disomic) nuclei with two copies of each chromosome in comparison to an abnormal, likely neoplastic, polysomic nucleus with gains in two (or more) of the four highlighted chromosomes.*

Polysomic cells are rarely if ever seen in normal value studies. In urine FISH assays, the finding of polysomic cells (even in small numbers) is virtually diagnostic for the presence of a tumor.[11] Other chromosomal alterations include tetrasomy, trisomy and gene deletions. These comprise a relatively small percentage (<5%) of abnormal urine cases in our experience.[12]

Training for FISH Analysis

In our experience, cytotechnologists are well-suited for the analysis of FISH cases thanks to their astute screening skills and outstanding ability to identify subtle nuclear changes which may be consistent with chromosomal anomalies. At Mayo Clinic, we provide an education-based training program for which instruction, guidance, knowledge-building and skill development are integral to the process.

While it is critical that an individual performing this assay fully understand the genetic mechanisms and preparatory steps involved in the process, it is also important to ensure that the theories and principles are understood well enough to facilitate high-quality microscopic analysis of FISH cases. In full, a minimum of 150 cases are reviewed as part of the training protocol, with thresholds set for competency ahead of independent, clinical case screening and analysis.[12]

Caveats of FISH analysis are included in the training protocol and may include such artifacts as cell overlap, split signals, background signal assessment, and inadequate hybridization, among others. An example of split signal patterns, which should not be inappropriately assessed as a chromosomal anomaly, is shown in ***Figure 5***.

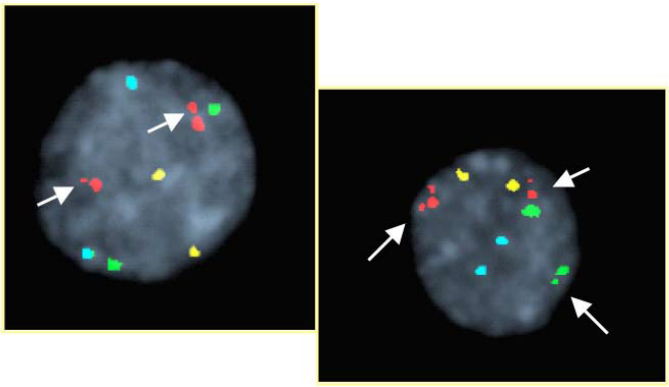


Figure 5: *Figure 5 – Split signal patterns. These composite images of disomic nuclei, may exhibit features of polysomy to the untrained eye due to the presence of split signal patterns. Split signals (identified by arrows) comprise only one represented chromosome (rather than multiple signals of unique chromosomes). Fine focus microscopic assessment of nuclei such as these will reveal that the seemingly disparate chromosome signals, in fact represent one chromosome and are typically 'linked' by a fine thread of chromatin.*

Recommendations on Introducing the FISH Assay to a Cytopathology Laboratory

A FISH assay should be considered for incorporation into a cytopathology laboratory's test mix based primarily on the benefit provided to patients. FISH cytology assays offer the potential to reduce cancer mortality by improving our ability to accurately detect early tumors.

It is essential to educate and train according to the needs of your staff, including correlation studies to clinical outcomes.

- A rigorous review of genetics may be required for some staff members. All members in the FISH "pool" (cytotechnologists and pathologists, alike) need to be trained on the utility of a fluorescence microscope, having the opportunity to analyze an adequate number of cases to assess competencies and understand the troubleshooting aspects of FISH analyses.
- Partnering with an established laboratory to compare FISH results may be appropriate for validation of the assay in our lab.
- Investigating the best practices of an experienced FISH cytology lab may allow for fewer issues on the preparatory and analysis work, alike. The experienced lab could provide clinical reporting for cases, while a split sample assessment could be utilized for the determination of concordant results.
- Even after implementation of the FISH assay in your own laboratory setting, continue to investigate your discordance rates from the FISH cytology results vs. clinical follow up.
- The high level of sensitivity offered by this assay may identify early tumors that require close clinical follow up.
- It is advisable to use discretion when rendering a positive FISH result in "borderline" cases due to potentially aggressive treatment decisions. While FISH assays are highly sensitive in the detection of malignant cells, it is also imperative to maintain high specificity.

Summary

Fluorescence *in situ* hybridization is a well-recognized ancillary technique that melds very well with the morphology- based skills of cytologists. The benefits that this molecular assay offers to patients, with increased sensitivity and detection of early disease, makes FISH an appropriate test choice for the ever evolving cytopathology laboratory.

The above is an excerpt from a previously published article in the American Society of Cytopathology's *Bulletin,* as written by Amy J. Wendel, Volume XLV, No. 5; September, 2008.

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Other related resources

Halling KC, Wendel AJ. In Situ Hybridization: Principles and Applications for Pulmonary Medicine. In Cagle PT (ed.), Molecular Pathology Library: Molecular Pathology of Lung Diseases, pp. 117-129. New York, New York, Springer Press, 2008.