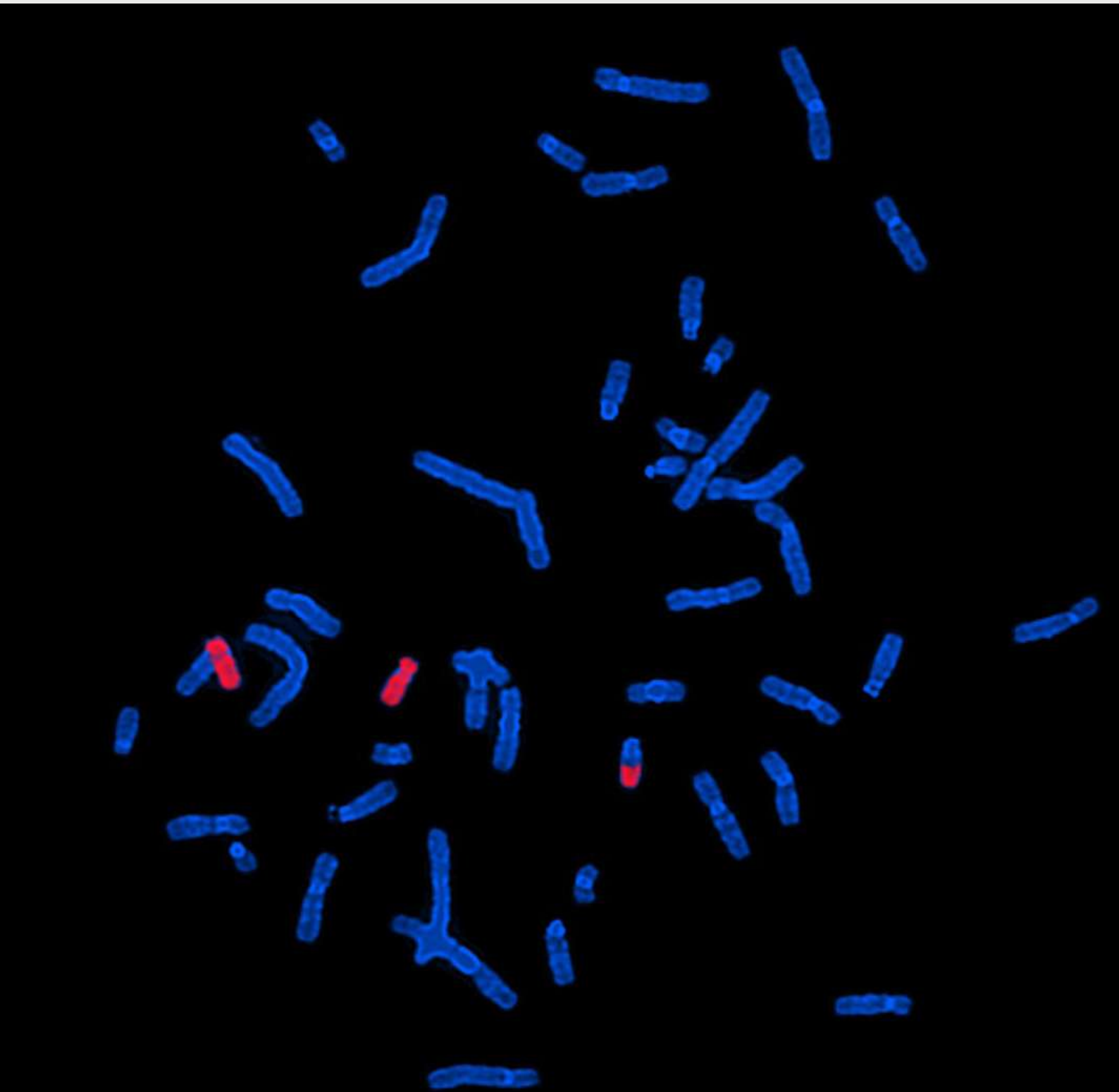
Fluorescent *In situ* Hybridization (FISH)

E-Book



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FISH:
Fluorescence *in situ* Hybridization

What is FISH?

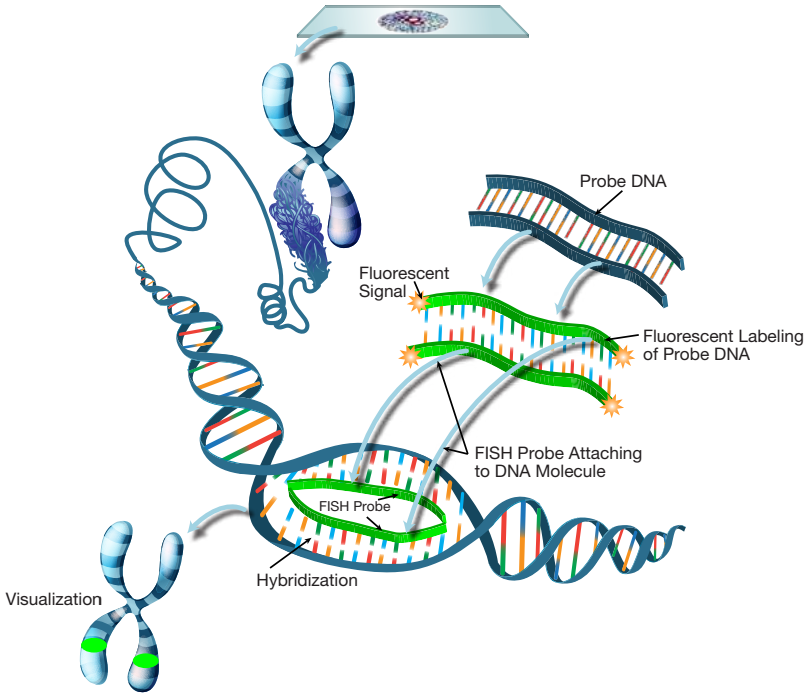
FISH, fluorescence *in situ* hybridization, is a cytogenetic technique enabling "mapping" of the genetic material of cells. It is commonly used to label DNA providing information on the location, length, and number of copies of specific genes or chromosome portions. Additionally, it can be applied to all types of RNA, and is foremost utilized to detect copy numbers and location of mRNA to visualize cellular transcription activity. FISH is based on the specific interaction between a fluorescence-labeled probe and a specific target sequence in cellular DNA or RNA.

FISH was first described in 1969 by two independent research groups. It is one of the oldest cytogenetics methods and was used as early as 1993 to determine aneuploidy for pre-implantation diagnostics.

How Does it Work?

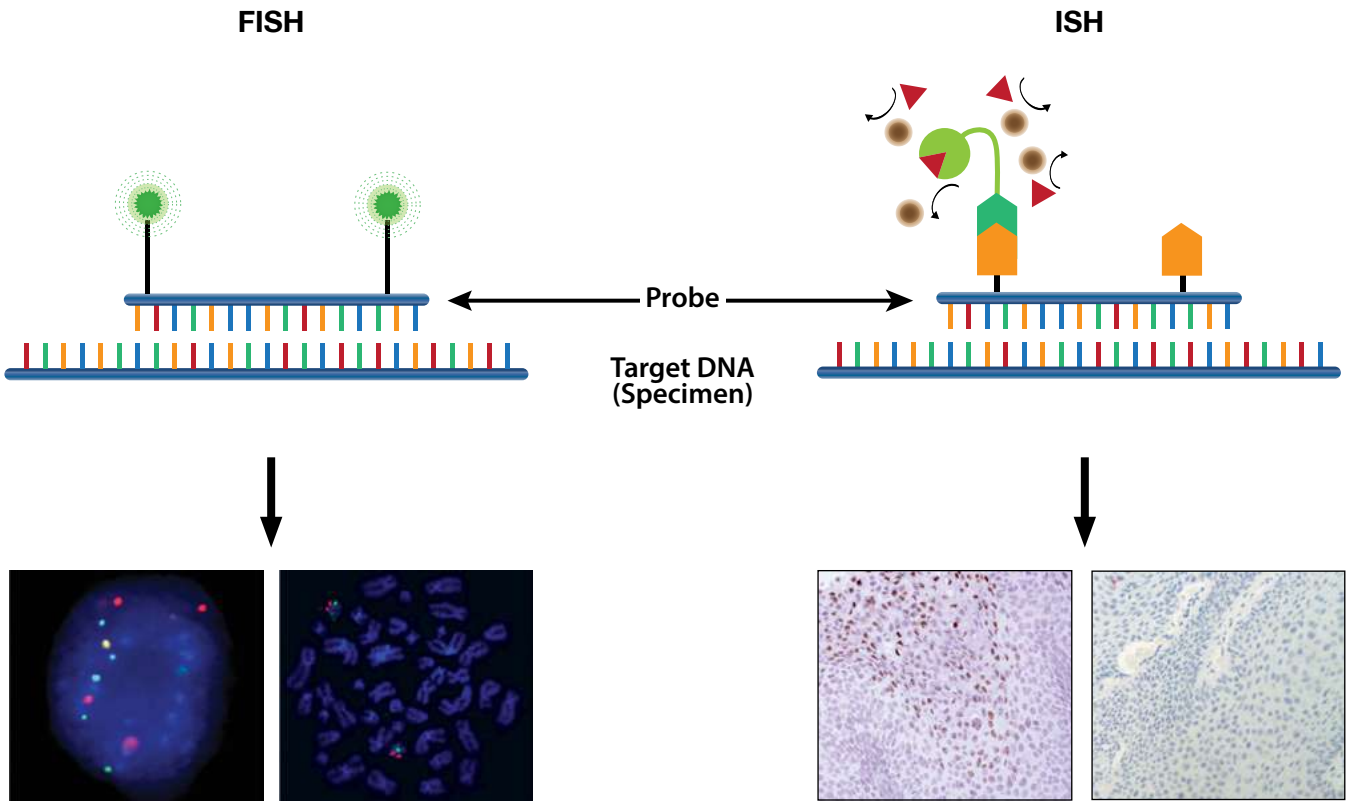
Here's the basic principle:

- A probe is generated with a complementary sequence to that of the sequence of interest. The probe is fluorescently labeled by the incorporation of nucleotides conjugated with fluorescent markers.
- The chromosomes are denatured by exposing them to heat and chemicals that break the hydrogen bonds holding the double helix structure together. Later, in proper conditions, chromosomes will go back to their original state.
- The probe is also denatured and then added to the specimen for the hybridization, that is the specific binding to its complementary sequence on the chromosome.
- The excess probe is washed off and the chromosomes are observed under a fluorescent microscope.The sites of interest bound by the probe will fluoresce!



What is the Difference Between FISH and ISH?

FISH and ISH both use the same concept of *in situ* hybridization, but FISH does so with the addition of fluorescent probes. This enables **direct detection**. Fluorescence allows the visualization of probes in combination with the surrounding cells and tissues.
Looking for a particular DNA sequence within whole chromosomes has been described as looking for a needle in a haystack. Attaching markers that cause the segments of interest to pop out with color gives a much clearer picture of where they are and can help us picture why their location on the chromosome might be significant.



FISH Detection. Fluorescent labels are attached to a probe which will hybridize to a target DNA strand. Then the fluorescent probe-target hybrids can be detected under a microscope immediately post-hybridization washes, using a fluorescent microscope.

ISH Detection. The probe is labeled with a non-fluorescent hapten like Biotin, Digoxigenin or DNP. Streptavidin or an antibody linked to an enzyme (horseradish peroxidase or alkaline phosphate) binds the non-fluorescence hapten with high specificity. Then the enzyme converts a substrate (chromogen) into an insoluble color product, which can be detected using a bright-field microscope.

What Sample Types are Used in FISH?

FISH can be used with tissue samples, chromosome spreads and cell cultures, such as:

- Blood and Bone Marrow
- Urine and Fecal Samples
- Bacterial Cultures
- Mucosal Samples
- Even Sediment!

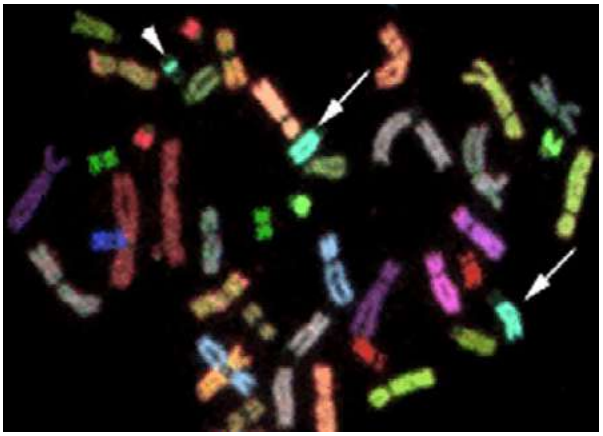
What Information does FISH Actually Provide?

FISH provides a visual, color-coded map of DNA segments of interest within chromosomes. It tells us where they are along the chromosome and approximately how many segments are present and have been bound.

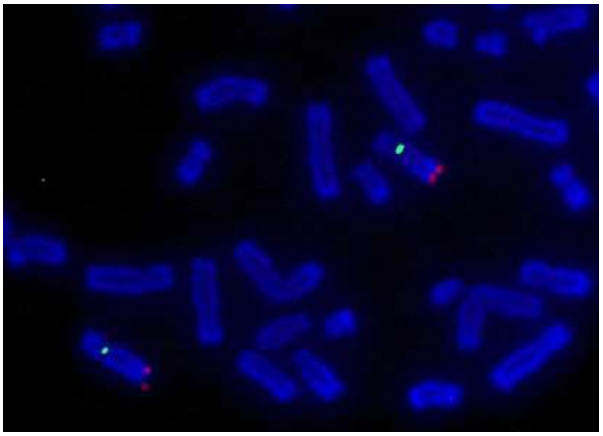
Because FISH allows for a fluorescent copy of the DNA of interest, it can be used for a multitude of different applications, including:

- **Chromosome "painting"**
  - "Paints" can be formed with hybridization probes matching sequences along the length of a particular chromosome in a process called multicolor FISH. This causes each chromosome to appear a different color, allowing rapid detection of large chromosomal changes.
- **Gene mapping on chromosomes**
  - FISH helps to identify gene locations.
- **Analyzing cells not currently undergoing mitosis**
  - Other techniques can analyze only metaphase chromosomes, but FISH can analyze both interphase and metaphase chromosomes. This means that cells don't need to be cultured far in advance before chromosomal analysis, and cells that don't divide frequently, such as solid tumor cells, may also be analyzed.
- **Detecting chromosomal abnormalities**
  - In combination with karyotyping, FISH can detect deletions, translocations, and duplications of genes.

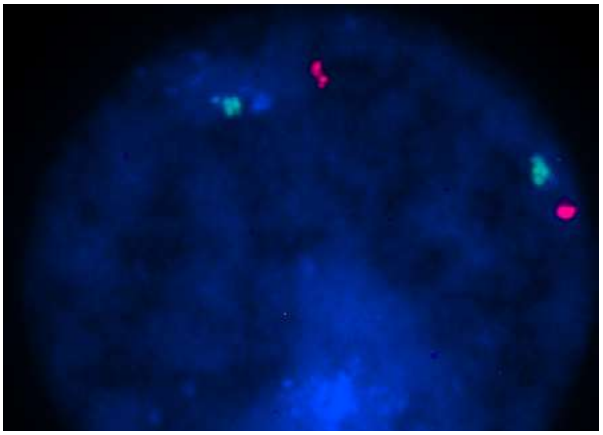
Most importantly, FISH helps us understand the organization, regulation, and function of genes, which in turn gives us valuable information on how to treat genetic diseases. Using this technique, we can examine chromosomal integrity or localize and measure DNA/RNAs within tissues, giving us the tools to catch diseases early, understand how they work, and ultimately find cures.



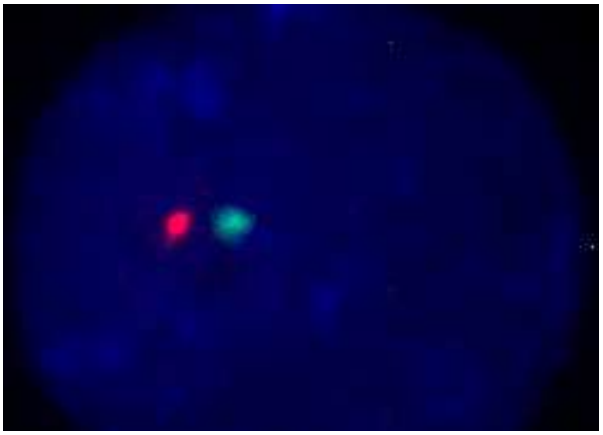
Chromosome painting. FISH probes specific to each the chromosomes hybridized to DNA in metaphase spread. The combination of colors and specific probes that hybridize to a particular chromosome allows for easy detection of deletions and translocations among chromosomes. A fragment (arrow head) can be identified as an extra piece of chromosome, since it is the same aqua color as the two normal copies of chromosome 15 (arrows). (Image from: Uhrig, S. *et al.* (1999) Am J Hum Genet. Aug; 65(2):448-62)



Gene mapping on chromosome. FISH probes hybridize to C-MYC (red signal) and to centromere 8 (green signal) in metaphase spread human chromosomes.



Normal cell not currently undergoing mitosis. FISH probe specific to the CDKN2A gene (red) and Centromere 9 (green) hybridizing to normal interphase cells as indicated by two red and two green signals in the nucleus.



Detecting chromosomal abnormalities. Acute Lymphoblastic Leukemia (ALL) tumor cell line cells hybridized with FISH probes specific to the CDKN2A gene (red) and Centromere 9 (green) showing hemizygous loss of CDKN2A as indicated by only one red CDKN2A and one green CEN 9 signal in the nucleus.

What are the Differences Between DNA and RNA Probes?

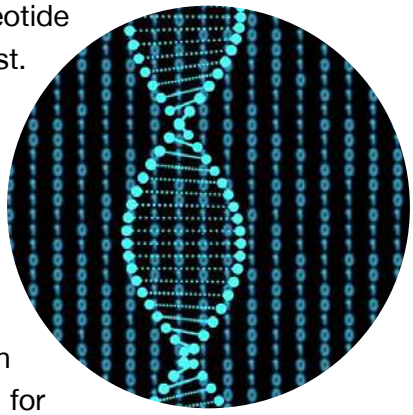
Arun Kumar, PhD
Sales Support Specialist

In the last decade, tremendous progress has been made in the field of molecular diagnostics. Many new nucleic acid-based detection tools or assays have been developed that allow analysis of DNA and RNA molecules in samples.

These assays are now routinely used for monitoring, detecting, and ultimately deciding which therapies would work best. Specific molecular probes are designed for this purpose. Molecular probes are used in various techniques for the detection of nucleic acid sequences in the food industry, and environmental, medical and veterinary applications to improve the specificity of the analyses. In medicine, they can help identify genetic and chromosomal abnormalities of infectious, acquired and inherited diseases.

Nucleic acid probes are either a single stranded DNA or RNA with a strong affinity towards specific DNA or RNA target sequence. This affinity and complementary sequence allows binding to specific regions of a target sequence of nucleotides. The degree of homology between target and probe results in stable hybridization. In developing a probe, a sequence of nucleotides must be identified, isolated, reproduced in sufficient quantity, and tagged with a label (reporter molecule) that can be detected. In theory, any nucleic acid can be used as a probe provided it can be labeled to permit identification and quantitation of the hybrid molecules formed between the probe and the sequence to be identified. Probes can be broadly categorized into DNA probes and RNA probes. At Enzo, we offer a complete set of tools for nucleic acid labeling and detection.

What are DNA Probes?



A DNA probe is a fragment of DNA that contains a nucleotide sequence specific for the gene or chromosomal region of interest. DNA probes employ nucleic acid hybridization with specifically labeled sequences to rapidly detect complementary sequences in the test sample. A variety of methodologies for labeling DNA have been described. In short, these methods are used to generate end-labeled or continuously labeled probes.

Most enzyme-mediated labeling techniques are very much dependent on polymerase activity, which is responsible for incorporation of the labeled nucleotides. Furthermore, the use of Taq or other thermostable DNA polymerases permits labeling reactions to be performed at higher temperatures via PCR, thereby reducing the incidence of enzyme-mediated point mutations during probe synthesis.

PCR is an excellent method for probe synthesis, requiring very small quantities of template material. In the presence of appropriately labeled nucleotide primers, PCR products are labeled as they are being synthesized. Alternatively, the primers themselves may be labeled non-isotopically during their own synthesis, negating the requirement for the inclusion of labeled nucleotide precursors as part of the reaction mix. Random priming is a type of primer extension in which a mixture of small oligonucleotide sequences, acting as primers, anneal to a heat-denatured double-stranded template. The annealed primers ultimately become part of the probe itself, because the Klenow fragment of DNA polymerase I extends the primers in the 3′ direction and, in so doing, incorporates the label.

Nick translation is one of the oldest probe labeling techniques. It involves randomly nicking the backbone of a double-stranded DNA with dilute concentrations of DNase I. At extremely low concentrations, this enzyme nicks a template at four or five sites, producing a free 3′-OH group that can act as a primer at each nicking location. Next, the enzyme DNA polymerase I removes the native nucleotides from the probe molecules in the 5′→3′ direction (exonuclease activity) while replacing them with labeled deoxyribonucleoside triphosphates (dNTPs) precursors by virtue of its 5′→3′ polymerase activity. Nick translation is efficient for both linear and covalently closed DNA molecules, and labeling reaction are completed in less than an hour.

What are RNA Probes?

RNA probes are stretches of single-stranded RNA used to detect the presence of complementary nucleic acid sequences (target sequences) by hybridization. RNA probes are usually labeled, for example with radioisotopes, epitopes, biotin or fluorophores to enable their detection. RNA probes as hybridization tools remain popular because of several key advantages associated with their use. These probes are synthesized by *in vitro* transcription and can be substituted for DNA probes in nearly all applications. High specific activity RNA probes or riboprobes may also be synthesized from DNA templates cloned in expression vectors such as SP 6 and T 7 systems. RNA probes are single-stranded and offer several advantages over DNA probes including improved signal or hybridization blots. Compared to the diverse methods for DNA probe synthesis, there is only one reliable method for labeling RNA probes, namely *in vitro* transcription. Because of the intrinsically labile nature of RNA and the susceptibility to RNase degradation, RNA probes must be treated with the same care as any other RNA preparations.

In vitro transcription is a reliable and economical method for generating RNA probes. Large amounts of efficiently labeled probes of uniform length can be generated by transcription of a DNA sequence ligated next to an RNA promoter. One excellent strategy is to clone the DNA to be transcribed between two promoters in opposite orientations. This allows either strand of the cloned DNA sequence to be transcribed in order to generate sense and antisense RNA for hybridization studies. One alternative method to generating continuously labeled RNA probes by *in vitro* transcription is to label the 5′ end of the molecule. This method of 5′ end-labeling is colloquially known as the kinasing reaction; it specifically involves the transfer of the γ phosphate of ATP to a 5′-OH substrate of RNA or DNA (forward reaction). The forward kinasing reaction is far more efficient than the exchange reaction which involves the substitution of 5′ phosphates.

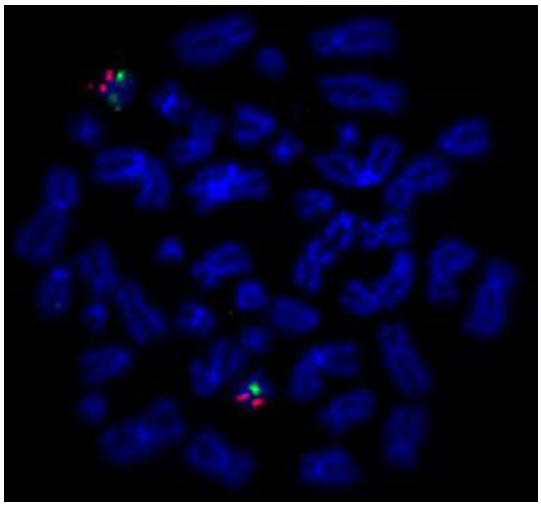
Probe synthesis by 3′ end-labeling involves the addition of nucleotides to the 3′ end of either DNA. DNA 3′ end-labeling is most often catalyzed by terminal transferase. Single- and double-stranded DNA molecules are labeled by the addition of dNTP to 3′-OH termini. RNA can also be 3′ end-labeled using the enzyme poly(A) polymerase. This enzyme, which is naturally responsible for nuclear polyadenylation of many heteronuclear RNAs, catalyzes the incorporation of Adenosine Mono Phosphate. Isotopic labeling requires α-labeled ATP precursors. In addition to its utility in RNA probe synthesis reactions, poly(A) polymerase can be used to polyadenylate naturally poly(A)–mRNA and other RNAs in order to support oligo(dT) primer-mediated synthesis of cDNA.

Methods for Generating FISH Probes

Until the early 1980's, radioisotopes were the only available reporter molecule option for labeling nucleic probes for ISH. Because radioisotopic probes have limited spatial resolution, require long exposure periods, and have a limited shelf-life depending on the half-life, the development of more optimal processes for nucleic acid probe labeling was needed. FISH, a variation of ISH, using fluorescence tagged probes, was developed in response to this need.

Because hybridization can take place between complementary deoxyribonucleotides or ribonucleotides, either DNA- or RNA-based probes (riboprobes) can be used to localize DNA or RNA in a given sample. Commercially available probes are expensive and do not always offer experimental flexibility as they come with a single label type. Therefore, many researchers still heavily rely on preparing their own FISH probes by using one of the following techniques: nick translation or random-primed labeling for generation of long, double-stranded DNA probes, terminal-labeling of oligonucleotides and *in vitro* transcription from vectors containing RNA polymerase promotors to produce riboprobes.

ISH and FISH probes for detection of nucleic acids in tissue and cell samples are usually generated from bacterial artificial chomosome (BAC) clones. They can be prepared by random-primed labeling with the use of DNA polymerase and labeled (dNTPs) to lengthen random oligonucleotides that hybridize to DNA sequences of the denatured vector. Alternatively, they may be prepared by nick translation which is often the preferred method of choice. In nick translation, the DNA to be labeled is nicked by DNase I, yielding a free 3′ hydroxyl end. DNA polymerase I then adds a new nucleotide to this end. The 5′-3′ exonuclease activity of the polymerase then moves the "nick" along the strand in the 3′ direction, and the addition of a labeled nucleotide to the reaction results in the desired probe.



Nick Translation DNA Labeling System 2.0 (ENZ-GEN111) was used to label BAC DNA probe for TP53 with SEEBRIGHT® Orange 552 dUTP (ENZ-42842) and BAC DNA probe for Centromere 17 with SEEBRIGHT® Green 496 dUTP (ENZ-42831). Labeled probes were hybridized to metaphase spreads. (Institut Universitaire du Cancer Toulouse Oncopole)

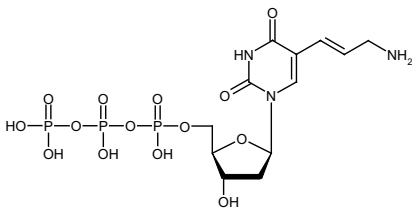
Enzo offers a Nick Translation DNA Labeling System to provide a simple, rapid, reliable, and efficient method for the end users to generate their own labeled DNA probes in just one hour. The kit can accommodate a wide range of fluorophore-labeled, biotin-labeled, and digoxigenin-labeled nucleotides. In addition to choice of label, the kit design allows the user to optimize incorporation and product size by adjusting the ratio of labeled-dUTP to dTTP. The ready-to-use nick translational Enzyme Mix is user friendly and minimizes error from pipetting. Probes labeled by nick translation can be used in many different hybridization techniques including: *in situ* hybridization (ISH), fluorescent *in situ* hybridization (FISH), screening gene banks by colony or plaque hybridization, DNA or RNA transfer hybridization, and re-association kinetic studies.

For many years, automatic DNA synthesizers have easily chemically synthesized oligonucleotide DNA probes 20 to 50 bases long that are specific to any target DNA sequence of interest. These oligonucleotides can either be directly labeled during their synthesis or labeled nucleotides may be added to their ends using terminal deoxynucleotidyl transferase or T4 polynucleotide kinase.

Although RNA probes can be problematic to work with, RNA ISH using riboprobes has been gaining traction lately. Riboprobes are prepared by either using a specially designed RNA expression vector or by attaching RNA polymerase recognition sites to vectors containing the probe sequence of choice. During *in vitro* transcription in the presence of labeled and unlabeled ribonucleotides, RNA polymerase is able to generate single-stranded riboprobes from the DNA template.

How to Use Allylamine-dUTP for FISH DNA Probe Labeling

Michael Yan
Sales Support Specialist

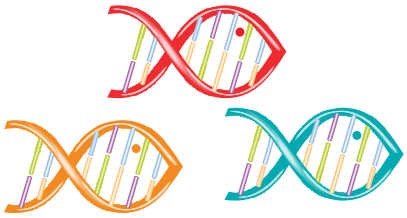


Allylamine-dUTP

Allyamine-dUTP is also known as allylamine-2' -deoxyuridine-5'-triphosphate, which signifies a modified UTP with an allylamine group attached to the 5 carbon position. Allyamine-dUTP is frequently used as a replacement for TTP in generating probes. It is usually synthesized through heck coupling, which involves uridine containing a 5 carbon halogen reacting with an allylamine. As with other indirect labeling mechanisms, this allylamine-dUTP free amine group can be reacted even further with another fluorescent dye containing an NHS (N-hydroxysuccinimide ester) group to create a stable carboxamide bond.

Using indirect labeling with allyamine-dUTPs provide great advantages for FISH probe synthesis that may not be present in direct labeling. One key benefit is that virtually any reporter molecule containing a reactive amine group such as NHS can be linked to the probe. While this of course means molecules such as biotin and other haptens can potentially bind, in the case of FISH it means that virtually any fluorescent molecule can be attached. This provides a high degree of flexibility that can be tailored to one's own preferences, the same of which cannot be said for direct labeling. While it is true that direct labeling requires fewer steps and can sometimes be cheaper, there are significant hindrances in nucleotide incorporation. Depending on bulkiness, pre-conjugated fluorescent nucleotides can induce steric effects as well as block active and binding sites in transcriptional machinery. Allylamine-dUTPs, on the other hand, do not have these same drawbacks. In various studies, allylamine-dUTP transcription has been shown to have almost identical transcriptional efficiency compared to unmodified NTPs. When conjugating allylamine-dUTP probes with amine-reactive fluorescent molecules in excess, one can expect high levels of fluorophore incorporation and consistency regardless of the chosen fluorescent dye.

Difference Between FISH BAC Probes and FISH Oligo Probes?



Historically, FISH probes were produce from bacterial artifical chromosomes (BAC) clones but more recently new technologies evolved to produce FISH probes from chemically synthetized oligonucleotides. What are the differences?

BAC Probes

The most common way to generate FISH probes is from BAC clones complementary to the region of interest followed by their labeling with fluorescent dyes. BACs are small pieces of bacterial DNA used as vectors to insert exogenous DNA fragments. The BAC can be expressed and grown in bacteria to produce colonies that will contain clones of the same fragment in each cell. Several BAC clones with different and specific DNA fragment insertions can be created and stored until needed, generating a BAC library. BAC clones were used in the initial sequencing of the human genome, reducing the entire human genome into chunks, storing the fragments for long term, and creating a BAC library. Several BAC clone libraries are available and are the basis for most FISH probes. Other similar alternatives to BAC clones are fosmid, cosmid, or yeast artificial chromosome (YAC) clones.

Despite being historically the source for generating FISH probes, there are some challenges using BAC clones. For full coverage of a gene of interest, several BAC clones will be required. Also as BAC probes have repetitive sequences, this can cause non-specific binding (hybridization) in a FISH assay. However, reagents such as Cot DNA can be used to compete out these interactions, to reduce the background. In addition, due to the large size that BAC probes can span, it can obstruct visualization of smaller DNA segments of interest that are few kilobases in size.

Oligo Probes

A more recent technology for generation of FISH probes is using chemically synthetized oligonucleotides (oligos) complementary to the region of interest. Oligo probes are short sequences of nucleotides that are synthesized to match a specific region of interest, followed by their labeling with fluorescent dyes. This approach has helped to overcome some of the challenges associated with BAC probes. FISH probes composed of oligos can be synthesized as tiles or longer sequences. As they can be smaller in size, they do not suffer from high hybridization background and non-specific signal. In addition, they can bind to the target sequence more rapidly and more efficiently compared to FISH probes produced from BAC clones, which can allow for rapid FISH assays.

However, there are also some challenges with oligo FISH probes as well. Large genes will require multiple oligos to cover the entire sequence, more than BAC probes. This requires significant amount of storage for the oligo fragments or a library of oligo fragments. It also requires the design of numerous oligos for a large target region. Additionally, for oligo FISH probe generation, a software for properly designing the oligonucleotides sequence is required. Depending on the sources available and proper design, both BACs and oligonucleotides can be used for the generation of FISH probes.

Types of FISH Probes

FISH probes can be classified by the region that is targeted.

Locus-specific Probes

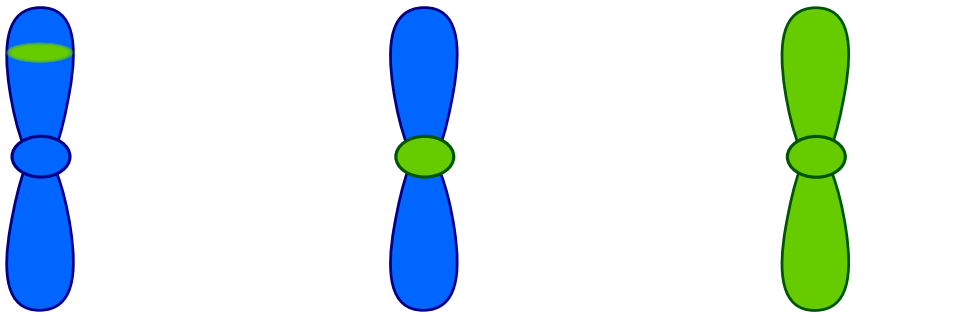
These FISH probes bind to a particular chromosome region and are often used by scientists to determine which chromosome the gene is located and on how many copies of a gene exist within a single genome.

Alphoid or Centromeric Repeat Probes

These are generated from repetitive sequences found in the middle of each chromosome and often used by researchers to determine whether an individual has the correct number of chromosomes. These probes can also be used in combination with “locus-specific probes” to determine whether an individual is missing genetic or has extra material from a particular chromosome.

Whole Chromosome Probes

These are actually collections of smaller probes, each of which binds to a different sequence in a given chromosome. Using multiple probes labeled with a mixture of different fluorescent dyes, scientists are able to label each chromosome in its own unique color. The resulting full-color map of the chromosome is known as a spectral karyotype. Whole chromosome probes are particularly useful for examining chromosomal abnormalities, for example, when a piece of one chromosome is attached to the end of another chromosome.



Locus-specific Probe

Alphoid or Centromeric Probe

Whole Chromosome Probe

Applications of FISH in Cancer Cytogenetics

Rosaria Esposito, PhD
Hartmut Pohl, PhD
Application Scientists

Cancer refers to a complex and heterogeneous group of diseases characterized by the uncontrolled and disorderly proliferation of cells, which often acquire the ability to invade other tissues. Cancer usually originates in somatic cells which, as a consequence of a series of genetic mutations, evade the mechanisms regulating tissue homeostasis, such as cell-to-cell contact inhibition, differentiation signals and cell death induction. The mutations responsible for tumor transformation concern two main group of genes, known as proto-oncogenes (stimulators of the cell cycle) and tumor suppressors (repressors of cell cycle progression). These functional alterations can occur as a consequence of single nucleotide mutations, but they can also be caused by larger modifications in genetic material, such as insertions, deletions, duplications or translocations of a chromosomic fragment. These abnormalities in cancer cells can be used as tumor biomarkers. The quantification of changes in gene copy number or gene re-arrangements is critical to our understanding of tumor biology, hence the importance of genetic tests based on molecular cytogenetics profiling.

FISH-ing Chromosome Aberrations in Cancer

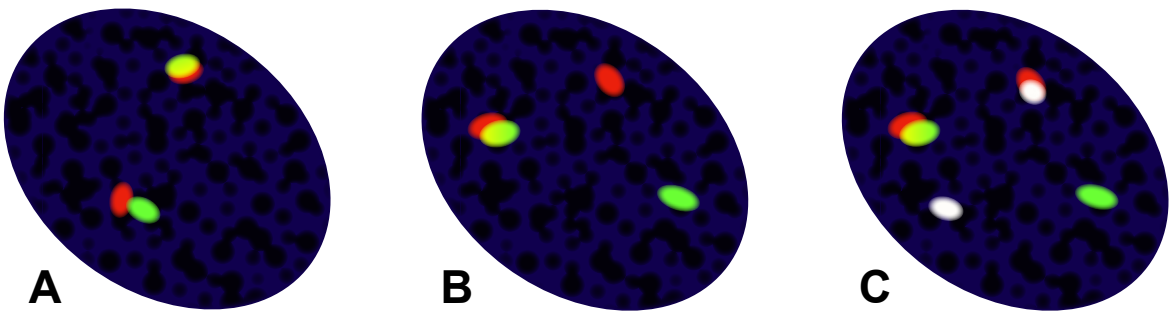
FISH has several advantages over other classical cytogenetic techniques, such as G-banding karyotyping. First, it has a higher resolution (20-150kb vs 5Mb). In addition, FISH can be applied to both metaphase and interphase chromosomes, meaning that cells do not need to be cultured for several days before chromosomes can be prepared for analysis. This also implies that FISH is suitable for the analysis of different kinds of sample types including solid tumors and formalin-fixed paraffin-embedded (FFPE) tissues. Furthermore, FISH probes can be labeled with different fluorophores, allowing for the simultaneous monitoring of multiple sites.

Thanks to its versatility, FISH can be used for the cytogenetic analysis of both solid tumors (e.g. breast cancer, non-small cell lung cancer, colorectal cancer) and hematological or cancer of the blood (e.g. leukemia, lymphomas, multiple myeloma). The detection of genetic abnormalities is useful not only for cancer, but also as a tool to analyze genetic predisposition and disease-specific information, and to predict a chemotherapeutic outcome.

FISH for Lung Cancer

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-related deaths. In particular, the non-small cell lung cancer (NSCLC) accounts for ~80-85% of all lung cancers. Somatic mutations on *EGFR* and *ALK* genes are often associated to NSCLC. *EGFR* (Epidermal Growth Factor Receptor) is a class of tyrosine kinase receptors whose activity is deregulated in different types of epithelial malignancies (including lung cancer), as they play an important role in cancer cell proliferation, angiogenesis and metastasis. For this reason, different strategies to interfere with *EGFR* function are commonly exploited for patients' therapy. Inhibitors of the tyrosine kinase activity of these receptors (e.g. erlotinib, gefitinib) are widely used in clinical NSCLC treatments. Unfortunately, because of the variety of genetic mutations underlying the *EGFR* dysfunction, some patients are resistant to this type of treatment. Different groups of patients can indeed be distinguished based on the type of alteration carried by *EGFR*, such as gene amplification, deletion or single nucleotide substitutions, which can alter its activity in different ways (i.e. not via the tyrosine kinase domain). As a result, the *EGFR* copy number determined by FISH is one of the biomarkers used to select the correct therapy.

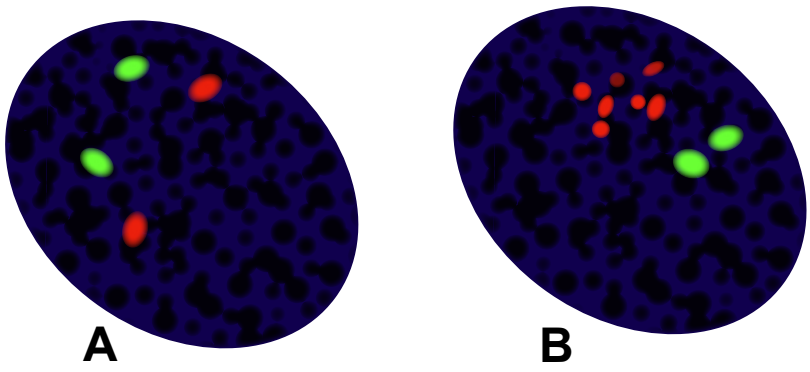
FISH is commonly used to detect inversions or translocations in the *ALK* gene. The *ALK* gene is located on the short arm of chromosome 2 (2p23) and encodes for the transmembrane tyrosine kinase receptor. *ALK* should not be expressed in the adult lung. However, under pathological conditions, the *ALK* gene breaks and fuses its 3′ (containing the tyrosine kinase domain) with the 5′ of other genes. This event can lead to the uncontrolled activation of *ALK* downstream signaling pathways. The most common fusion occurs with *EML4*, because of an inversion on the short arm of chromosome 2.



Representative view of *ALK* gene translocation detected by FISH. 5' and 3' regions of the gene are visualized with a green and a red fluorescence respectively. A. Wild type nucleus. B. Cancer cell nucleus, showing the characteristic probes split. C. Cancer cell nucleus, showing the characteristic probes split. A third probe can be used to define the chromosome rearrangement actually occurring.

FISH for Breast Cancer

This is the most common malignancy in women and the second leading cause of cancer-related death worldwide. Breast cancer is often characterized by abnormalities in receptor status, leading to an upregulation of cellular transduction pathways responsible for cell proliferation and survival. In particular, approximately 20-30% of breast cancer tumors are known to overexpress HER2/Neu, a member of the EGFR family. A common treatment in these cases is Trastuzumab, a humanized monoclonal antibody approved by the FDA in 1998 for the treatment of breast cancer. Its exact molecular mechanism remains to be elucidated, but this antibody likely prevents HER2 activation by binding to its extracellular domain. In addition, it seems to induce tumor cell lysis stimulating the antibody-dependent cellular cytotoxicity (ADCC). FISH, using an appropriate probe against HER2, can be used to identify extra copies of the gene, a sign that it is more likely respond to Trastuzumab treatment.

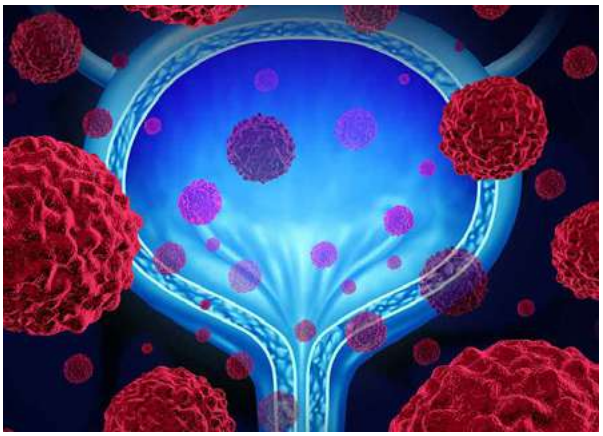


Representative view of potential breast carcinoma cells. *HER2* signal is represented in red; centromere 17 probe (green) can be used to enumerate chromosome number. A. *HER2*-non-amplified breast carcinoma: two centromeres 17 and two copy of the *HER2* gene as expected. B. *HER2*-amplified breast carcinoma multiple detection for *HER2*.

FISH for Chronic Lymphocytic Leukemia (CLL)

Analysis of hematological malignancies is one of the typical examples of the advantages of FISH for the analysis of samples characterized by a variable karyotype and a low mitotic activity. CLL is the most common leukemia in adults. It has not been associated with a specific recurring genetic alteration. Instead, similar to bladder cancer, a panel of different mutations has been associated with different severities of the disease and are used as predictive indicators of patient clinical course. FISH panels, in this case, often include probes to detect trisomy 12 and deletions 11q, 13q, and 17p. Deletion 11q, which in most cases concerns the gene *ATM*, is found in patients showing a fast progression of the cancer; trisomy 12 is associated with advanced stages of the disease, resistance to chemotherapy and shorter survival times; deletion 13q is the most commonly found and is in general associated with a more favorable prognosis; deletion 17p often includes a deletion of *TP53* gene and corresponds to advanced stage of the tumor, with a poor survival rate.

FISH for Bladder Cancer



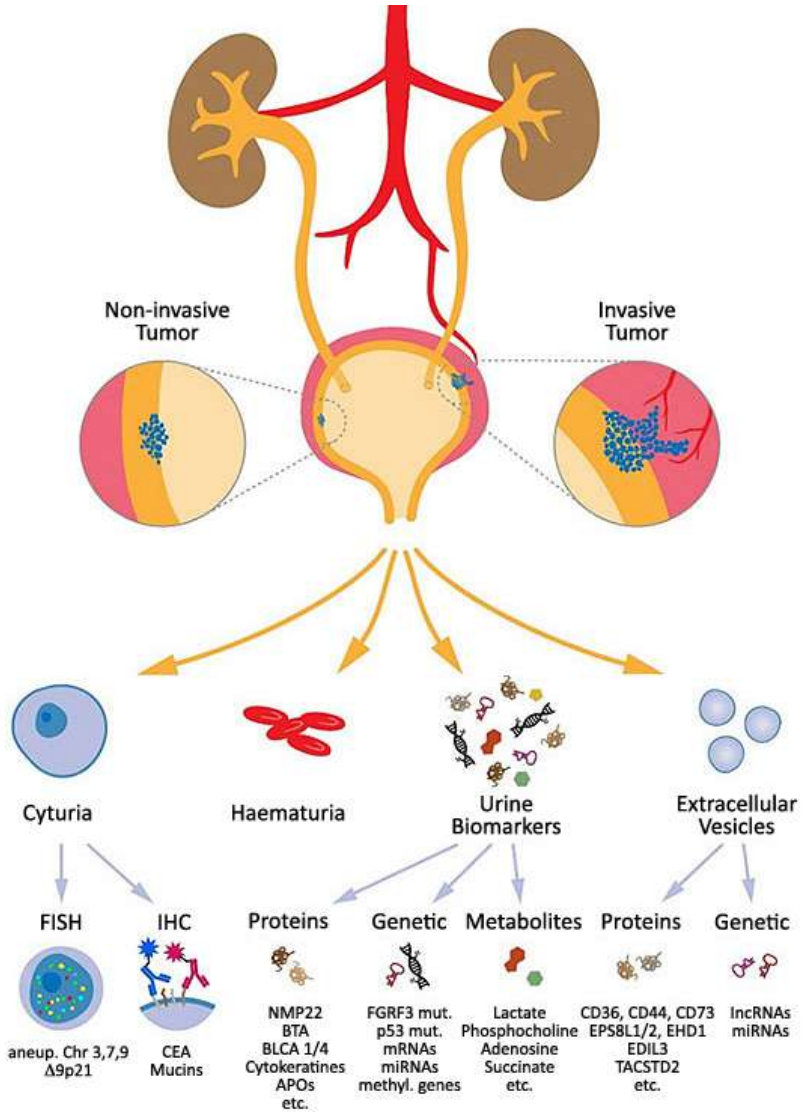
Bladder cancer is the fifth most common human malignancy and the second most frequently diagnosed genitourinary tumor after prostate cancer. It is a polygenic disease, meaning that it has been associated to multiple genetic anomalies, such as mutations in FGFR3, RB1, HRAS, TP53 or TSC1 genes. However, the initiation event is probably a mutation in the 9p21 region, containing the p16/CDKN2A gene. In addition, bladder cancer cells are characterized by an elevated degree of chromosome instability (CIN). Dysfunctional chromosome duplication or segregation during mitosis cause DNA rearrangements and translocations, gain or loss of whole chromosomes (aneuploidy) or chromosome fragments. The resulting unbalance of the genetic material worsens after each cell cycle. The consequent genomic patterns can be related to the different stages of tumor development, with the more invasive forms displaying the higher number of cytogenetic alterations.

Standard therapy for urothelial cancer consists of surgical removal of the tumor mass followed by tumor-unspecific chemotherapy. Unfortunately, the recurrence rate of non-invasive tumors is 50-70% leading to a 10-year survival rate of only ~50% and the life-long need for monitoring tumor recurrence and continued health care expense. Invasive carcinomas require much more aggressive treatments and have far worse prognosis than early stage tumors. This highlights the need for early detection and readily-available, easy-to-use and cost-efficient tools to detect and fight bladder cancer.

Standard Detection of Bladder Cancer

The current detection gold standard is a visual scanning of the bladder and ureter wall for *in-situ* cysts by endoscopy, termed cystoscopy, upon appearance of indicative symptoms, e.g. hematuria (blood in the urine), pain when urinating etc. Tumor classification and prognosis are based on subsequent histopathology of biopsies of the detected cysts. Although quite specific, cystoscopy has a number of caveats: it is expensive, time-consuming and relies heavily on the experience of the operator. Ureteral and renal cysts are often overlooked and the detection of early-stage, small cyst cancers is difficult. Additionally, whilst being minimally invasive, it often has very discomforting side-effects, such as subsequent hematuria, pain and dysuria. Non-invasive methods analyzing blood- or urine-borne factors are thus highly desirable.

Voided urine cytology, the microscopic analysis of cells from urine samples that were shed from the tumor mass, is a commonly used detection method. While being highly specific, it is impaired by low



sensitivity, especially towards small or early stage tumors. Hematuria, often a symptom accompanying larger cysts, can severely hamper cytological analysis. Specificity of cytology, especially towards smaller tumors, can be improved by immunohistochemistry based detection of carcinoembryonic antigen (CEA or CD66e) and two types of mucins on exfoliated urothelial carcinoma cells. Another method to improve cytology is fluorescent *in situ* hybridization (FISH) of the DNA of cells extracted from urine. The numeric and structural chromosomal alterations found in bladder cancer cells can be used as tumor markers.

Specifically, simultaneous detection of copy number variation of chromosomes 3, 7, and 17 and deletion of the 9p21 region (containing p16) by FISH using four distinct probes is a common practice. This method is useful for providing information about cancer progression and recurrence to detect excess (aneuploidy) of chromosomes 3, 7, and 9, and loss of the 9p21 locus, which is typical of urothelial carcinoma cells. Other methods use sandwich ELISAs or colorimetric immunoassays to detect Bladder-Tumor-Antigen (BTA) – otherwise known as Human Complement Factor H-related protein or Nuclear Matrix Protein 22 (NMP22) – contained in the urine, either as free proteins or within exfoliated cells that are being lysed prior to analysis.

How to Choose the Right Cytogenetics Technique for Your Research

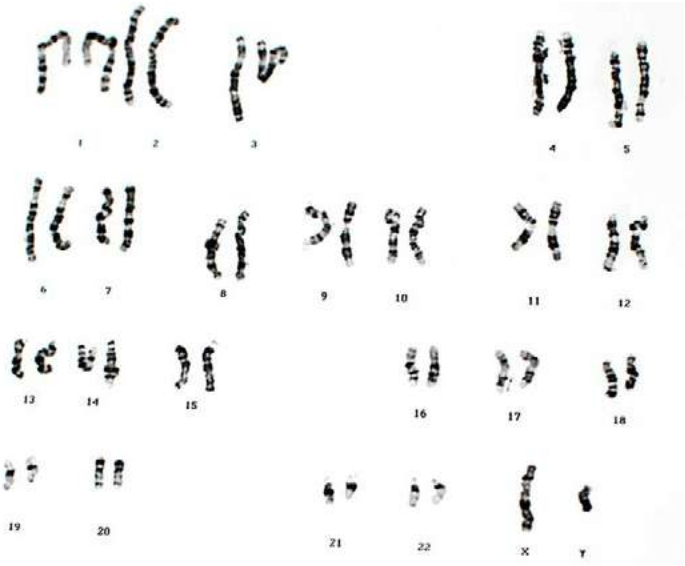
Hartmut Pohl, PhD
Application Scientist

Cytogenetics is the analysis of structure, organization and copy number of chromosomal material and how alterations in the anatomy of chromosomal DNA reflect on biological functions. This branch of genetics focuses on how structural alterations of chromosomes relate to human disease. Cytogenetics tools have driven a vital subset of both research and clinical diagnostics for decades, especially in the fields of obstetrics, gynecology, and oncology. Chromosome alterations form the basis for many diseases affecting patients – from early prenatal defects to cancer. These changes in chromosome structure and ultimately, function, can vary drastically in scale, from absence, fusion or excess of entire chromosomes to alterations at the level of individual genes and even a single nucleotide polymorphism (SNP). To detect chromosomal abnormalities, researchers and clinicians can rely on numerous methods to analyze genomic alterations, but depending on the scale and nature of these alterations, one or the other method might be beneficial. And at times, a combination of these methods might be best suited for analysis. Therefore, the analytical weapon of choice should be chosen wisely.

Karyotyping

Being one of the oldest genetic methods at a researcher's disposal, karyotyping predates even our understanding of how genetic information is encoded into the DNA by half a century. A karyotype describes the number and appearance of chromosomes. Normally, karyotyping is performed on spreads of dividing cells to obtain a karyogram (or idiogram), a depiction of condensed metaphase chromosomes rearranged in matching pairs by size. This rearranged photomicrograph is then used to analyze the phenotypic, microscopic appearance of somatic chromosomes as they appear during the mitotic division of a cell. This method allows for efficient and intuitive analysis of chromosomal alterations at the microscopic level with relatively simple means. Typically, this method is improved by additional staining, such as trypsin combined with Giemsa stain in a method called G-banding, because it will stain the

chromosomes in typical, reproducible bands. These bands have been for decades the best means to describe chromosome locations and form the basis of modern denomination of chromosomal loci. For example, a gene located on 3p22.1 means that it can be found on the p-arm –the shorter arm, with q being the longer arm – of chromosome 3, in region 2, band 2, sub-band 1. These regions and bands used to describe gene loci originate from defined G-banding of karyotypes.

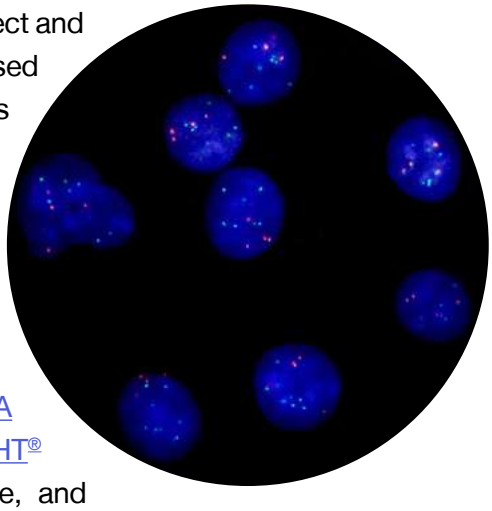


Alternative staining methods to produce band patterns on metaphase chromosomes are R-banding (i.e. a reverse- G-banding), Q-banding with the use of fluorescent quinarcine, or multicolor FISH banding with the help of a mix of defined, chromosome-specific fluorescence *in-situ* hybridization (FISH) probes. Multicolor FISH (or chromosome painting) allows for easier, more instinctive analysis of a karyotype by using a defined mixture of fluorescence probes. Each chromosome is labeled in a unique color, not only allowing to identify chromosomes very easily, but also directly highlighting translocations, where parts of a given chromosome are wrongly placed on another.

Karyotyping in combination with G banding typically offers a spatial resolution to detect chromosomal alterations bigger than 5-10Mbp. It is a cost-effective, methodologically simple screening method for larger cytogenetic alterations and can detect aneuploidy (abnormal numbers of chromosomes or chromosomal regions) as well as transpositions, deletions, duplications, and even inversions of chromosomal arms or larger chromosomal fragments. Typical examples of genetic abnormalities detected by karyotyping include Trisomy 21, Turner Syndrome (only one X chromosome, no Y chromosome) or Cri du Chat (truncated p-arm of chromosome 5). In contrast, Prader-Willi-Syndrome, a genetic disorder where ~75% of the patients show a deletion of the region 15q11-13, is at the detection limit of karyotyping and cannot be reliably diagnosed with this method.

Additionally, karyotyping relies on the availability of fresh, proliferative cell samples. Sampled cells are grown in culture and then arrested in metaphase by the use of colchicine, which blocks the formation of the mitotic spindle and allows efficient visualization of condensed metaphase chromosomes. This culture method is time-consuming and cannot be performed on growth-arrested or fixed tissue. Imaging and rearrangement of images is not only time-consuming, but also requires experienced personnel to obtain and interpret the karyograms. Nonetheless, karyotyping is a robust and highly useful diagnostic tool that is not only applied in obstetrics and gynecology, but also in cancer medicine.

Fluorescence *In Situ* Hybridization



FISH as a cytogenetics tool that offers much more than chromosome painting for spectral karyotyping. It is a powerful tool to detect and visualize known cytogenetic alterations and is frequently used as a diagnostic means. Utilizing fluorescent FISH probes against known alterations of sequences or viral sequences integrated into the host genome allows for rapid detection of common genetic alterations. Furthermore, multiplexing with several different probes labeled with different fluorophores can easily be achieved. Probes can be purchased ready-to-use or synthesized by techniques such as nick translation using Enzo's Nick Translation DNA Labeling System 2.0 Kit in combination with our SEEBRIGHT® Fluorescent Dye-dUTPs. This is a simple, rapid, reliable, and efficient method to generate custom labeled DNA probes for FISH in just one hour. However, FISH as a cytogenetic diagnosis technique is more often performed with ready-made probes against known common and uncommon genomic alterations. FISH's advantage over karyotyping is that it can be performed in interphase nuclei as well as on fixed samples, thus eliminating the need for upstream cell culture to generate test samples.

FISH allows for the rapid and easy detection of known common and uncommon alterations with standard laboratory methods. Additionally, FISH offers a resolution of alterations at the level of a few base pairs, although only within the targeted sequences. It has very limiting sample depth, allowing only for analysis of a few alterations at a time. Furthermore, it is severely hampered by being limited to known and targeted sequence alterations and does not allow for detecting random or unknown alterations. It is thus a diagnostic tool mainly applied for confirmation of suspected diagnoses.

Quantitative Fluorescence Polymerase Chain Reaction

An alternative to FISH-based RAD is Quantitative Fluorescence Polymerase Chain Reaction (QF-PCR). This method utilizes fluorescence-labeled DNA primers against chromosome-specific repeat sequences and PCR to amplify the target sequences. Differences in the number of repeats per allele will result in different length of the amplified products. Size and fluorescence intensity of the amplified segments is quantified and summarized in a graphic representation which allows for detection of copies of different alleles as well as duplication of identical alleles. However, the results will be uninformative, should the repeat sequences be homozygous and identical for all alleles present in the sample. Like FISH, QF-PCR is limited to known and targeted alterations only. However, relying on DNA samples, it is generally easier to obtain sample templates and the technical and equipment requirements are relatively simple. It is commonly used to detect trisomies 13, 18, 21, and abnormalities of the sex chromosomes. Unfortunately, initial installation costs have to be overcome with high sample numbers and frequent analyses. However, running costs are relatively low and results are easy to interpret.

Comparative Genomic Hybridization

Originally developed for the evaluation of differences between solid tumor genome and normal tissue, comparative genomic hybridization (CGH) is based on competitive binding of two DNA samples to a chromosome-wide selection of DNA templates. In CGH, two sets of whole genome DNA preparations, a healthy reference standard and the test sample, are used to generate fluorescent hybridization probes in two different colors, most commonly red and green. At Enzo, we offer excellent labeling solutions for your CGH hybridization probes. These probes are hybridized to a genome-wide template and genomic regions present in both test sample and reference standard appear yellow, while regions over or underrepresented in the test sample appear red or green. The original method of using normal metaphase spreads of chromosomes similar to karyotyping as a template to hybridize to, has meanwhile fallen into complete disregard and is widely replaced by array CGH (aCGH). Here, oligonucleotides generated from chromosomal DNA, bacterial artificial chromosomes (BACs) or by oligo-synthesis are spotted or printed to a glass slide and serve as a hybridization template to generate a microchip array. Chip-based aCGH allows for the automated detection of hundreds of thousands of known target sequences. Whole genome arrays consist of target sequences that offer genome-wide, extensive, and in regions of specific interest, often continuous coverage. Targeted arrays, in contrast, target specific regions of interest only, but offer increased detail and redundancy to improve confidence in the result. Results are comprised in a “virtual karyotype” – a graphic representation of all hybridization probes sorted in chromosomal order, and can be used to detect copy number variations (CNVs). Resolution of genome-wide CGH arrays commonly reaches 50-80Kbp and below, with targeted arrays being able to offer a fraction of this resolution. Resolution and resulting versatility in detecting CNVs make aCGH the gold standard of cytogenetics.

Array-CGH can also detect mosaicism, where a sample contains a mixture of cells with different copy numbers of alleles, as long as the sample is present in substantial amounts. Mosaicism can often be found in tumor samples that are mixed with normal cells, as well as in some genetic disorders.

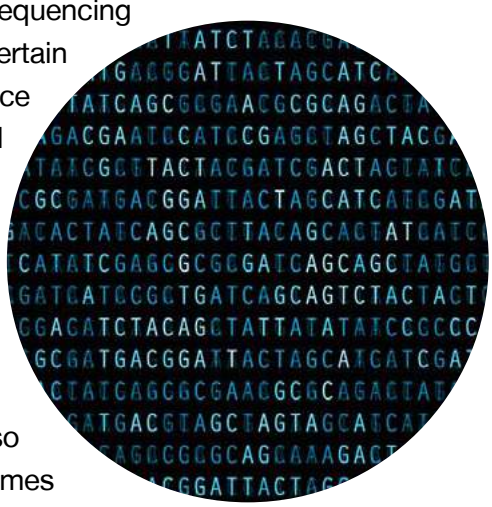
Fig 2: A virtual karyotype obtained by aCGH. Reference male DNA was compared to sample DNA using a 1x1M Human CGH SurePrint array from Agilent in combination with Enzo's CYTAG® CGH Labeling Kit. Karyotype shows a healthy male subject.

A limiting disadvantage of CGH is its inability to detect copy number neutral aberrations such as balanced chromosomal translocations and inversions. This limit can partially be overcome by complementing aCGH with array probes for known single nucleotide polymorphisms (SNPs), known allele variants that differ –often inconsequential– in a single base pair. Sequences for known variants of SNPs are spotted additionally to standard CGH probes onto the array and allow for targeted detection of specific allele variants. Thus, CGH+SNP arrays allow detection of copy-neutral haplotype variations and genomic aberrations like uniparental isodisomies, where both copies of one allele are identical due to deletion of one parental allele and replacement by the other through duplication, resulting in copy-neutral loss of heterozygosity. As this type of genetic aberration cannot be detected by conventional cytogenetics, including aCGH, SNP-based arrays are preferred for virtual karyotyping of tumors. Array-CGH+SNP also allows for determining genetic identity by descent. No matter which application or CGH method is being used, the selection of the right chip requires careful consideration to choose the right assay.

Next-Generation Sequencing

Massive parallel sequencing or next generation sequencing (NGS) is becoming a ubiquitous technology in basic biology research and starting to make its way into both diagnostic and clinical settings. While the technologies collectively known as NGS vary greatly, they are all basically more robust versions of classical Sanger sequencing. Similar to Sanger sequencing, small DNA fragments are sequenced linearly, but millions of sequences are obtained in parallel in a fully automated manner by high-throughput data acquisition. Each base pair is sequenced multiple times for accuracy. NGS allows for sequencing of the entire genome within as little as one day and thus offers a formidable tool for genome-wide individual analyses.

The process can be streamlined and sped up further if sequencing is focused on specific areas of interest only, such as certain chromosomes or regions. A common approach is to sequence only the ~22-thousand coding genes in a process called whole exome sequencing. Another method is to focus on relevant target genes only, like known oncogenes in tumor analysis. NGS is a powerful cytogenetic tool that allows detection of all types of cytogenetic aberrations, from common insertions or deletions to mosaicism and single nucleotide mutations, and even unknown inserts of pathogen DNA. Furthermore, it is extremely sensitive, so that even detection of fetal DNA from maternal blood becomes possible, and risky procedures to obtain amniotic fluid or placental cells can be omitted. These benefits make NGS an extremely powerful and attractive tool.



Although costs per reaction are relatively low, clinical application of NGS is not cost effective because it requires a high frequency of analysis and initial setup costs for obtaining the necessary machinery and infrastructure are substantial. Additionally, the requirement for experienced personnel due to the vast amount of data generated, the necessary skillful analysis, and interpretation of unknown genetic variants are an additional and lengthy caveat. Therefore, successful implementation of NGS as the cytogenetic gold standard might not be possible without supra-regional centralization.

Applications of Different Cytogenetics Methods

Due to the differences in resolution and the various benefits and limitations of each technique, great care should be taken when deciding which tests to perform. Additionally, interpretation of the results requires skillful analysis, as there might be a discordance between different methods for certain specific findings. Karyotyping remains the method of choice for common aneuploidy assessment, as in the analysis of indeterminate gender or fertility issues. FISH is a powerful tool for confirmation of well-known genetic abnormalities, as well as the analysis of oncogenetic aberrations and detection of known pathogens, and the only cytogenetics technique applicable to fixed samples. CGH and SNP is the method of choice for detection of more complex cytogenetic aberrations to explain learning difficulties, intellectual disabilities, developmental delay, behavioral problems including autism, or miscarriages. NGS offers the most comprehensive cytogenetics analysis but its limitations due to its complexity will confine it for the time being to analysis of complex cases and research settings.

Especially in cancer research, a single cytogenetics technique might not generate the needed clarity of results for clinical decisions, and cytogenetics research may continue relying on a combination of different techniques to obtain and confirm results, including techniques at the fringes between cytogenetics and molecular biology, such as chromosome conformation capture, chromatin immunoprecipitation, and others. Ultimately, individual cytogenetics techniques have their strengths and weaknesses, and the choice of the right test might further vary depending on

available expertise and familiarity of the user with the methodology.

Enzo is a global leader in DNA and RNA labeling technologies. We offer a range of products for your cytogenetics research needs. Our Array CGH labeling solutions offer outstanding label incorporation and unrivaled DLR scores. For a simple and efficient method for generating labeled DNA, please check out our Nick Translation DNA labeling kit as well as a list of our SEEBRIGHT® fluorescent dye-dUTPs.

Efficient and Convenient Method for Creating Your Own FISH Probes

Make your own precise and specific FISH probes with Enzo's one-hour labeling protocol. Choose from a wide range of high intensity fluorescent dyes to mark even the most unique target.

Nick Translation DNA Labeling System

- Ready-to-use enzyme mix, no need to dilute
- Easy one-hour labeling protocol
- Validated with fluorophore, biotin, and digoxigenin labeled dUTPs

SEEBRIGHT® Fluorescent dUTPs

- Choose from ten distinct colors, spanning the visible light spectrum
- High signal intensity and photostability
- Enhance and prolong fluorescent signals with our SEEBRIGHT® Mounting Media (with DAPI / without DAPI)

Convenient Ancillary Products

- Buy your FISH reagents from one source
- All products tested to work together
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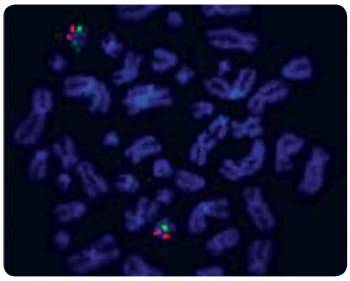
Simple, One-Hour Custom FISH Probe Labeling



Nick Translation DNA Labeling System (ENZ-GEN111) was used to label BAC DNA probe for TP53 with SEEBRIGHT® Orange 552 dUTP (ENZ-42842) and BAC DNA probe for Centromere 17 with SEEBRIGHT® Green 496 dUTP (ENZ-42831). Labeled probes were hybridized to metaphase spreads. (Institut Universitaire du Cancer Toulouse Oncopole)

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FISH Tips & Troubleshooting

Rosaria Esposito, PhD
Application Scientist

Enzo provides over 40 years of experience in the manufacturing and supply of research kits, biochemicals and biologics. As *Scientists Enabling Scientists*™, we realize the value in providing relevant information to our customers working in the fields of life sciences, drug development and clinical research. We are happy to share simple but useful hints for improving your daily tasks as well as the overall quality of your results. With this in mind, below is a list of tips for achieving high quality data by fluorescent *in situ* hybridization (FISH). This list of suggestions is based not only on Enzo's experience as a recognized pioneer in labeling and detection technologies, but also on solutions we offer regularly, in order to assist researchers in obtaining the most accurate and consistent results.

1. Select the Right Bait for FISH-ing

Ready-to-use probes are commercially available, but they can be quite expensive and do not always offer the required experimental flexibility. One option is to synthetize your own probes. In general, double-stranded DNA probes are easy to prepare, label, and work with in the laboratory; alternatively single-stranded RNA probes are uniform in size, achieve high incorporation of label, and form highly stable RNA-RNA hybrids. DNA probes are usually prepared by nick translation (from supercoiled or linear DNA) or random priming (from linearized DNA); riboprobes can be obtained by *in vitro* transcription from linearized vectors containing RNA polymerase promotors. Taking all this into account, you can decide what kind of probe is best suited for your needs (DNA- or RNA-based), depending on your starting material (type, quantity, quality) and the tools available.

2. Quality of the Input DNA

The quality of the probe is vital for successful FISH and this is in turn strictly related to the quality of the template DNA. Make sure to use good quality, purified DNA, free of RNA, protein, or other contaminants; for example, use a DNA purification procedure that selectively purifies plasmid DNA (e.g., anion exchange column-based purification). The purity of the preparation can be determined using classical gel electrophoresis, automated electrophoresis systems, or a spectrophotometric system (such as a NanoDrop).

3. Quality of the Probe

Depending on the application, you may need to purify the probe in order to remove unincorporated nucleotides. The yield, dye incorporation and fragment length should be verified. All these elements will give you a good insight on the quality of the probes. For example, if RNA probes appear as a sharp band on an agarose gel, DNA probes will most likely form a smear, generally with the majority of fragments between 100 and 250 bp. If obtaining low yield, inefficient dye incorporation or unexpected probes length, you might need to optimize the previous steps: quality of the template, amount of the starting material, reaction temperatures and time, etc.

4. Sample Preparation

The quality of test specimens is critical for obtaining reliable and consistent results. Tissues can be fixed with formalin or paraformaldehyde, whereas precipitating fixatives (e.g. alcohols) should be avoided to correctly preserve nucleic acids. In general, do not exceed 24 hours of fixation (at maximum), as this would make probe penetration more difficult and increase auto-florescence. Sections should be 3-4µm thick – thicker slices can lead to problems in probe penetration, as well as in the interpretation of the results because of different focal planes; too thin sections can instead truncate the signal and make the manipulation more difficult. For sample preparations, all tools should be treated with alcohol and/or DNAse/RNAse eliminating agents, especially if unfixed or fixed, since cryopreserved samples are processed with the same equipment.

Cells for DNA FISH, on the other hand, should be treated with a hypotonic solution (e.g. sodium citrate and BSA) and then fixed, usually with a 3:1 methanol/acetic acid solution (freshly prepared). Try to distribute cells uniformly across the slide, to avoid clumps and nuclei overlapping. Cell density can be adjusted by centrifuging and resuspending them in a higher or lower volume. Once on the slide, the cytoplasm should not be visible, as this could interfere with the hybridization. If that is the case, pepsin (or other peptidase treatments) might be used; alternatively, try to re-fix the cells in fresh solution.

> **!** Check the filters available on your fluorescent microscope in order to select the appropriate fluorophore for your labeling

5. Prepare the Slide

Pre-clean the glass slides with 70% ethanol before use. If needed, treat them in order to render their surface adhesive (e.g. with poly-lysine).

6. Pre-treatment of Specimens

Choose the appropriate pre-treatment in order to allow the subsequent hybridization. On the basis of sample type, the tools, and the time at your disposal. Evaluate the most suitable protocol for proper dewaxing (for FFPE sections), sample permeabilization (using proteases, detergents, alcohols, etc), denaturation of the probes and target (notably for dsDNA probes, using pH or heat), and slides aging in case of cell spreads, etc.

7. Hybridization

Hybridization specificity (stringency) is driven by the degree of complementarity between the probe and target sequences, as well as by the probe length. These characteristics will directly influence probe concentration, temperature and time of hybridization, and concentration of monovalent cations present in the hybridization solution needed to obtain the best results. Careful tuning of these parameters can help to remove non-specific interactions. Hybridization temperature, typically ranging between 55 and 62°C, is probably the most important variable to consider when aiming for high specificity. Among the components of the hybridization buffer, formamide allows hybridization at temperatures significantly lower than the actual melting temperature of a probe-target-hybrid and thus may assist in the conservation of the morphology of samples. In addition, Cot DNA sequences are routinely included to reduce non-specific hybridization to repetitive DNA sequences. In this phase, it is also important to keep the humidity conditions under control, in order to avoid drying out or overconcentration of the solution. This could interfere with the hybridization, lead to high fluorescent background or to altered chromosome morphology.

8. Post Hybridization

The post hybridization washes are as important as the hybridization itself to assure specificity. Gradually increasing the stringency will remove weaker (and likely less specific) interactions between the probe and the target. To set up optimal conditions, take the type of probe you are using into account: RNA-DNA hybrids are more stable than DNA-DNA hybrids.

9. Mount and Visualize

Chromosome enumeration probes (CEPs or CENs) targeting the pericentromeric regions of chromosomes can be used to enumerate them and facilitate the analysis

Use an antifade mounting medium and DAPI as a counterstaining preferentially. Do not expose the sample to high light intensity for too long, in order to reduce photobleaching. When enumerating signals in metaphase nuclei or chromosomes spreads, just take intact, clearly separated chromosomes/nuclei, with non-overlapping signals into account. Carefully evaluate doubtful cases at higher magnification. In general, split signals (e.g. small fluorescent spots close to each other) can be counted as one. Repeat counting for more accuracy in case of uncertainties.

10. Avoid Contamination

Change the solutions frequently and use preferably dedicated jars for FISH reagents; wash the material frequently. Pay attention when pipetting the probe to avoid the pipette touching the tube and remember to change pipette tips at each step. It can be beneficial to treat materials as well as the work area with DNAse/RNAse eliminating agents.

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