# Seminars in Histology

## From basic principles to advanced histological techniques

# “In situ hybridization”

DBM
Histology Core Facility
Dr. Diego Calabrese
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# Introduction

***In Situ Hybridization*** (ISH) is a technique that allows for precise localization of nucleic acid within a histologic section.

The underlying basis of ISH is that nucleic acids, if preserved adequately, can be detected through the application of a complementary strand of nucleic acid to which a reporter molecule is attached.

The technique was originally developed by Pardue and Gall (1969) and (independently) by John et al. (1969). At this time radioisotopes were the only labels available for nucleic acids, and autoradiography was the only means of detecting hybridized sequences.

Radioactive in situ techniques can detect low copy number mRNA molecules in individual cells (Harper et al., 1986).

The terminal fluorochrome labeling procedure of RNA probes was developed by Bauman et al. (1980, 1984), in the same year the direct enzyme labeling procedure of nucleic acids was described by Renz and Kurz (1984).

https://www.ncbi.nlm.nih.gov/probe/docs/techish/
Roche DIG Application Manual

# Why ISH

Nucleic acid localization helps in addressing questions relative to:

- Genomic DNA alterations
  - Gene amplification
  - Gene split
  - Gene translocation
  - Prenatal diagnostic
- Gene expression
  - Expression in heterogeneous tissues
  - Co-expression
- Pathogen presence and localization
  - Virus and bacteria localization

# In-situ hybridization systems

ISH experiments can be classified based on the target nucleic acid and on the detection method used.

**Target**
RNA (mRNA, lncRNA, miRNA, rRNA)
DNA

**In situ hybridization probes**
Double-stranded DNA (dsDNA) probes
Single-stranded DNA (ssDNA) probes
Labeled oligonucleotides
Synthetic oligonucleotides (PNA, LNA)
RNA probes (riboprobes)

**Labeling techniques**
Radioactive isotopes:
32P
35S
3H

Non-radioactive labels:
Biotin
Digoxigenin
Fluorescent dye

1.Genomic in-situ hybridization (GISH)
2.RNA-DNA ISH DIG system
3.b-DNA systems

https://www.ncbi.nlm.nih.gov/probe/docs/techish/

# Probe type selection

Double-stranded DNA (dsDNA) probes
Single-stranded DNA (ssDNA) probes
RNA probes (riboprobes)

**RNA Vs. DNA probes:**

- The hybridization strength decreases in the order RNA-RNA, DNA-RNA, DNA-DNA (Wetmur et al., 1981)
- With DNA-DNA in situ hybridization, the in situ renaturation of target DNA sequences cannot be prevented because in situ hybrids and renatured sequences have similar thermal stability.

In situ renaturation of target DNA can, however, be prevented with the use of single-stranded RNA probes. Since DNA-RNA hybrids are more thermally stable than DNA-DNA hybrids, hybridization conditions can be designed in which DNA-DNA hybrid formation is not favored but DNA-RNA hybrid formation is.

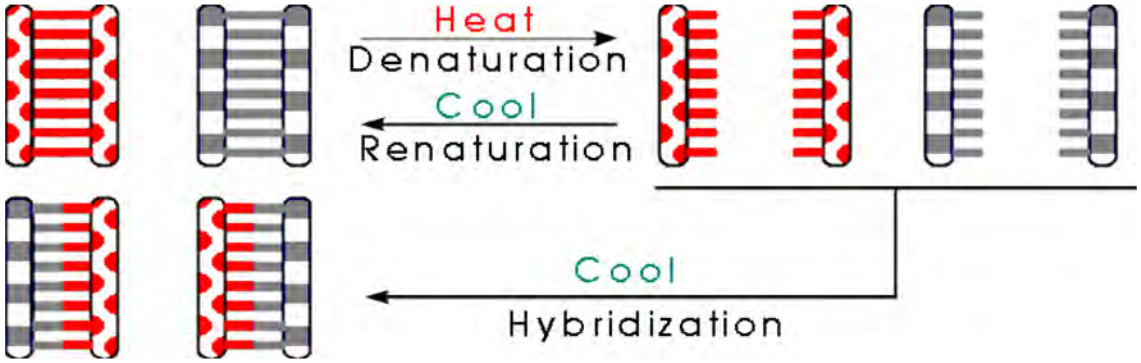
**Single stranded Vs. double stranded:**

Single-stranded probes provide the following advantages:

- The probe is not exhausted by self-annealing in solution.
- In ss probes, large concatenates are not formed in solution. Such concatenates would penetrate the section or chromosomes poorly.

# Main parameters influencing hybridization

Hybridization depends on the ability of denatured target DNA or RNA to reanneal with complementary strands in an environment just below their melting point (Tm).

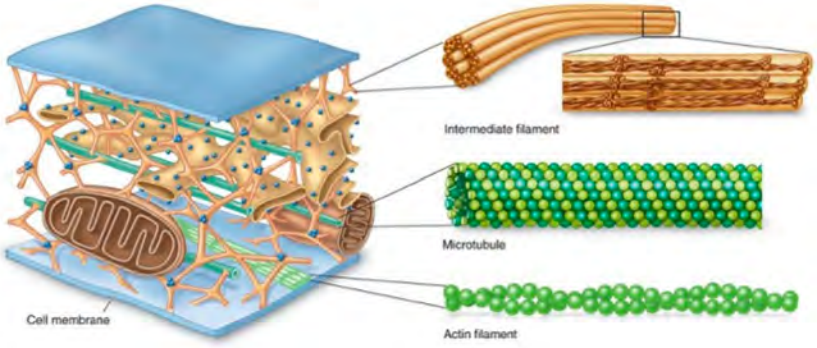



Tm and renaturation are primarily influenced by four parameters:

- Temperature
- pH
- Concentration of monovalent cations
- Presence of organic solvents (i.e. Formamide)

Access to the target sequence may be limited by:

- Cross-links derived from aldehyde fixation
- Protein associated with DNA or RNA
- Tissue/cell structures
- Probe length



# Factors influencing hybridization

**Ion concentration**

Monovalent cations (e.g. sodium ions) interact electrostatically with nucleic acids (mainly at the phosphate groups) so that the electrostatic repulsion between the two strands of the duplex decreases with increasing salt concentration

The following equation has been given for the dependence of Tm on the GC content and the salt concentration (for salt concentrations from 0.01 to 0.20 M):

$Tm = 16.6 \log M + 0.41 (GC) + 81.5$

**Organic solvents**

DNA denatures at 90°–100°C in 0.1– 0.2 M $Na^+$. For in situ hybridization this implies that microscopic preparations must be hybridized at 65°–75°C for prolonged periods.

Formamide reduces the melting temperature of DNA-DNA and DNA-RNA duplexes in a linear fashion by 0.72°C for each percent formamide.

$Tm = 16.6 \log M + 0.41 (GC) + 81.5 – 0.72 (\%\ formamide)$

# Factors influencing hybridization

**Probe length**

The rate of the renaturation of DNA in solution is proportional to the square root of the (single-stranded) fragment length. Consequently, maximal hybridization rates are obtained with long probes.

However, short probes are required for in situ hybridization because the probe has to diffuse into the dense matrix of cells or chromosomes.

**Probe concentration**

The probe concentration affects the rate at which the first few base pairs are formed (nucleation reaction). The nucleation reaction is the rate limiting step in hybridization. Therefore, the higher the concentration of the probe, the higher the reannealing rate.

**Dextran sulfate**

In aqueous solutions dextran sulfate is strongly hydrated. Thus, macromolecules have no access to the hydrating water, which causes an apparent increase in probe concentration and consequently higher hybridization rates.

# Factors influencing hybridization

**Base mismatch**

Mismatching of base pairs results in reduction of both hybridization rates and thermal stability of the resulting duplexes. On the average, the Tm decreases about 1°C per % (base mismatch) for large probes.

Mismatching in oligonucleotides greatly influences hybrid stability; this forms the basis of point mutation detection.

**Stringency**

To remove the background associated with nonspecific hybridization, wash the sample with a dilute solution of salt. The lower the salt concentration and the higher the wash temperature, the more stringent the wash.

In general, greater specificity is obtained when hybridization is performed at a high stringency and washing at similar or lower stringency, rather than hybridizing at low stringency and washing at high stringency.

# Fixation

To preserve morphology, the biological material must be fixed.

- For metaphase chromosome spreads, methanol/acetic acid fixation is usually sufficient.
- For paraffin-embedded tissue sections, use formalin fixation.
- Cryostat sections fixed for 30 min with 4% formaldehyde or with Bouin's fixative have been used successfully.

It should be noted that the DNA and RNA target sequences are surrounded by proteins and that extensive crosslinking of these proteins masks the target nucleic acid.

# Pretreatment

Protease treatment serves to increase target accessibility by digesting the protein that surrounds the target nucleic acid.

To digest the sample, incubate the preparations with up to 500 µg/ml Proteinase K (the optimal amount must be determined) in 20 mM Tris-HCl, 2 mM $CaCl_2$ , pH 7.4, for 7.5 –30 min at 37°C

The use of pepsin has been shown to give excellent results for formalin-fixed, paraffin embedded tissue sections.

Routine pepsin digestion involves incubating the preparations for 30 min at 37°C in 200 mM HCl containing 500 µg/ml pepsin.

For some applications a heat-mediated pretreatment is also necessary.

# Prehybridization

A prehybridization incubation is often necessary to prevent background staining. The prehybridization mixture contains all components of a hybridization mixture except for probe and dextran sulfate.

**For "large" DNA probes (>100 bp):**
– 50% deionized formamide
– 2X SSC (see below)
– 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 –200 ng/ml)

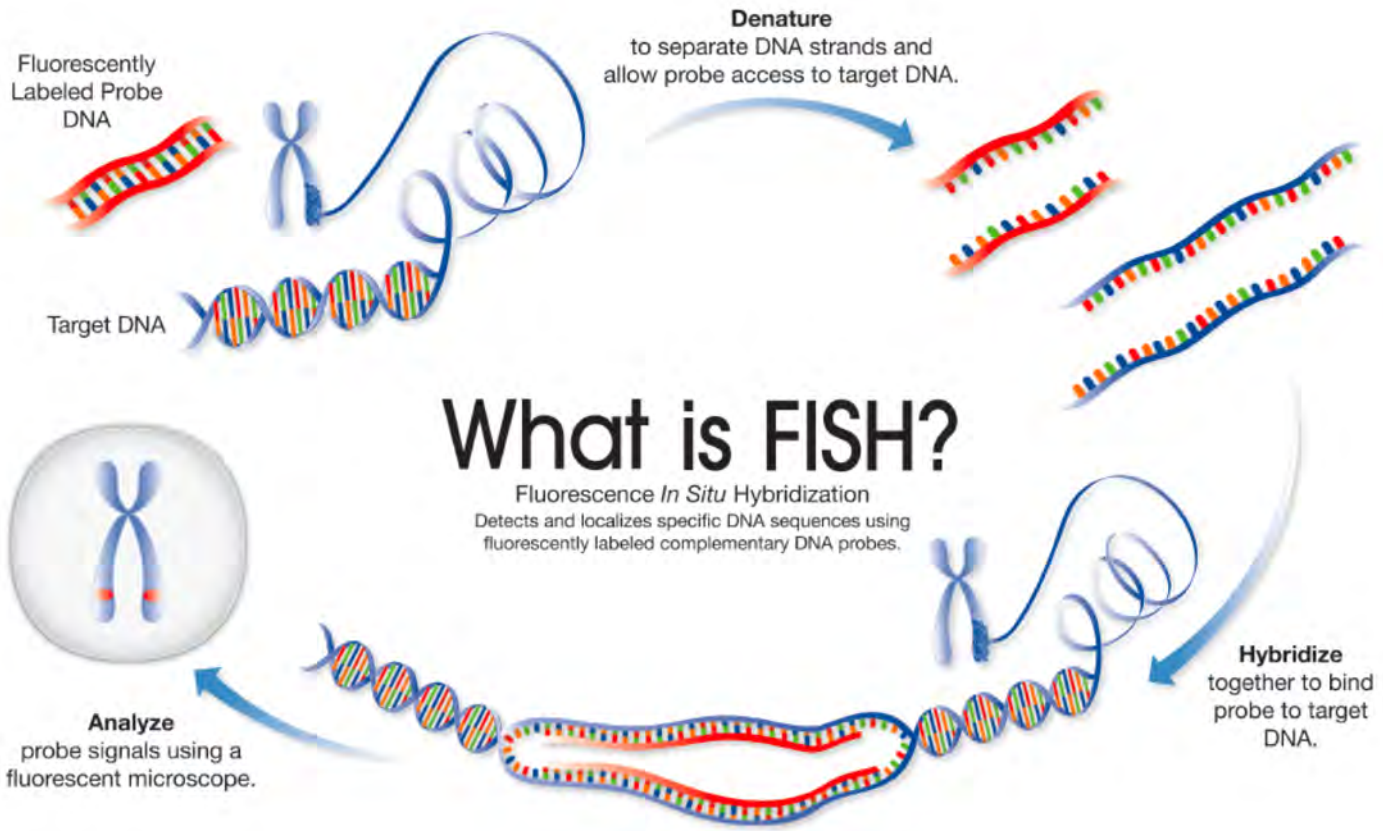
Optional components:
– 1X Denhardt's
– dextran sulfate, 5 –10%
– Temperature: 37°– 42°C
– Hybridization time: 5 min –16 h

**For synthetic oligonucleotides:**
– 25% formamide
– 4X SSC
– 50 mM $NaH_2PO_4/Na_2HPO_4$ buffer; pH 7.0
– 1 mM EDTA
– carrier DNA/RNA (1 mg/ml each)
– probe (approx. 20 –200 ng/ml)
– 5X Denhardt's
– Temperature: room temperature
– Hybridization time: 2–16 h

1X SSC: 150 mM NaCl, 15 mM sodium citrate; pH 7.0:
Make a 20x stock solution (3 M NaCl, 0.3 M sodium citrate).

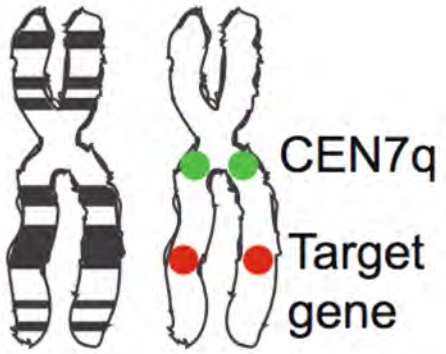
50X Denhardt's:
1% polyvinylchloride, 1% pyrrolidone, 2% BSA.

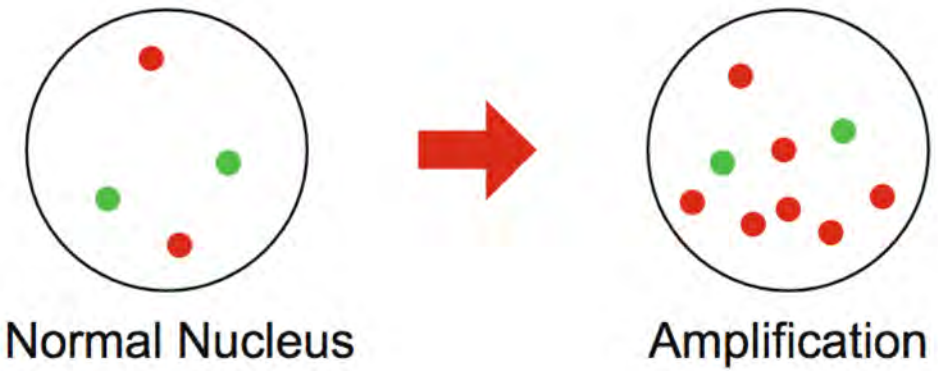
# GISH




https://www.cytocell-us.com/support/what-is-fish

# Gene amplification / gene loss

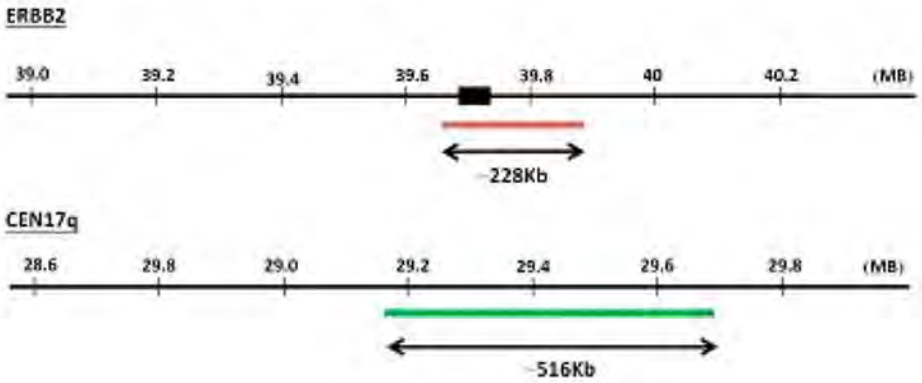


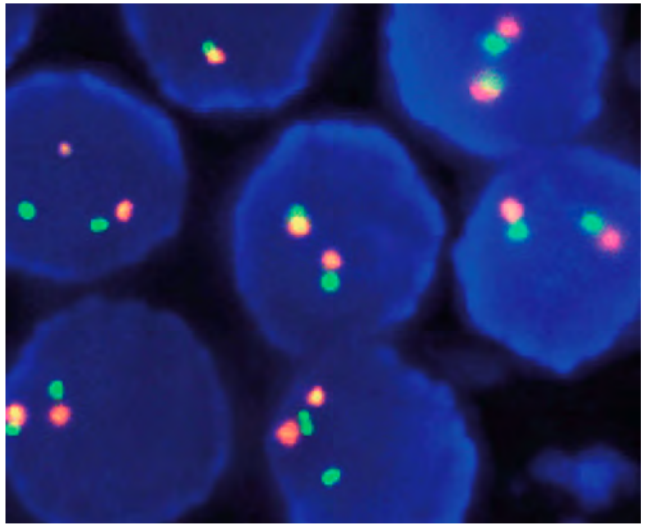




| | |
|---|---|
| FGF23/CEN12p FISH Probe | FGFR2/CEN10p FISH Probe |
| p16/CEN9q FISH Probe | PTEN/CEN10p FISH Probe |
| NF2/CEN22q FISH Probe | MDM2/CEN12p FISH Probe |
| c-met/CEN7q FISH Probe | APC/CEN5q FISH Probe |
| EGFR/CEN7q FISH Probe | ACTN4/CEN19p FISH Probe |
| HRE2/CEN17q FISH Probe | HER3/CEN12p FISH Probe |
| TOP2A/CEN17q FISH Probe | HER4/CEN2p FISH Probe |
| CX43/CEN6 FISH Probe | UBE3A/CEN15q FISH Probe |
| c-myc/CEN8p FISH Probe | TRF1/CEN8p FISH Probe |
| CCND1/CEN11p FISH Probe | 1p36.33/CEN1p FISH Probe |
| p53/CEN17q FISH Probe | 9q34/CEN9q FISH Probe |
| TYMS/CEN18q FISH Probe | VHL/CEN3q FISH Probe |
| CCNE1/CEN19p FISH Probe | CALR/CEN19q FISH Probe |
| AKT2/CEN19p FISH Probe | RUNX2/CEN6p FISH Probe |
| DYRK1A/CEN21q FISH Probe | CHGA-ITPK1/CEN14q FISH Probe |
| MLL/CEN11p FISH Probe | CDK4/CEN12q FISH Probe |
| KRAS/CEN12q FISH Probe | AIB1/CEN20q FISH Probe |

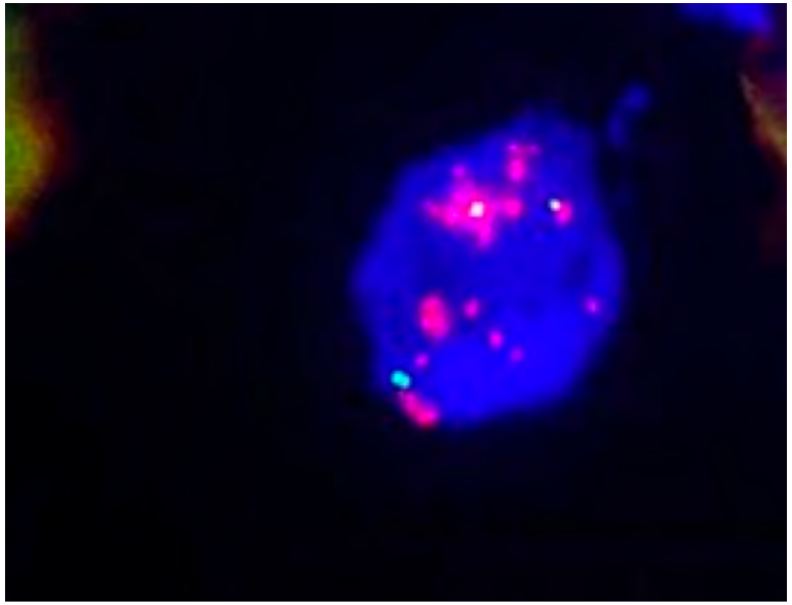
http://www.abnova.com/images/content/support/FISH.pdf

# ERBB2 (HER2)/CEN17q



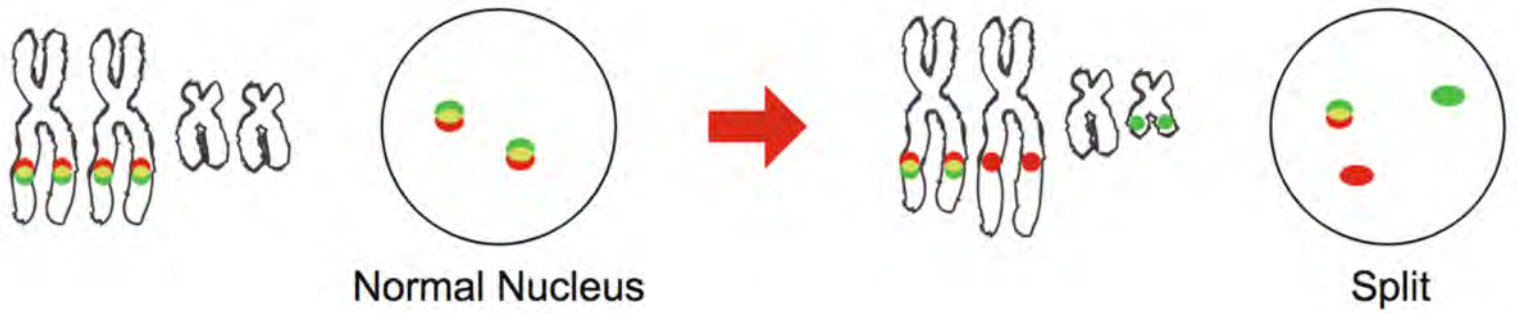



Normal cells



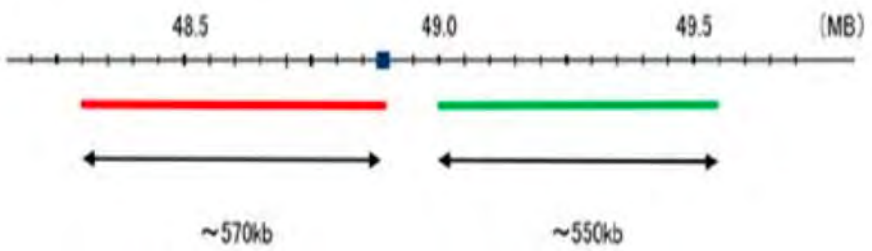
Cancer cells

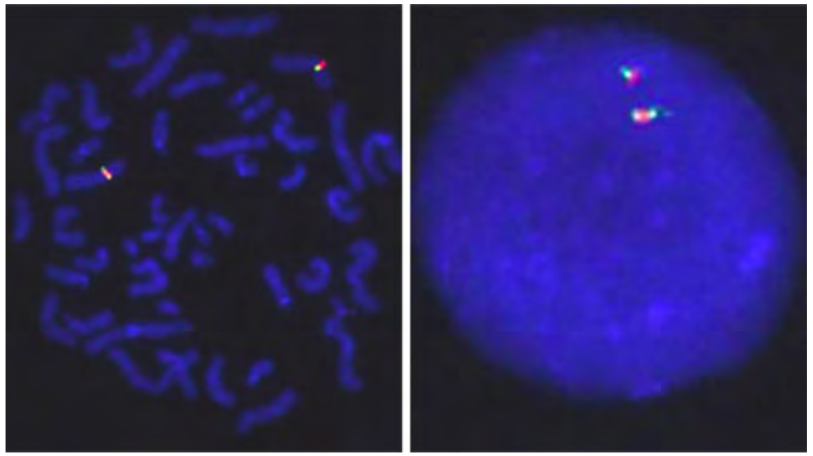
# Gene split

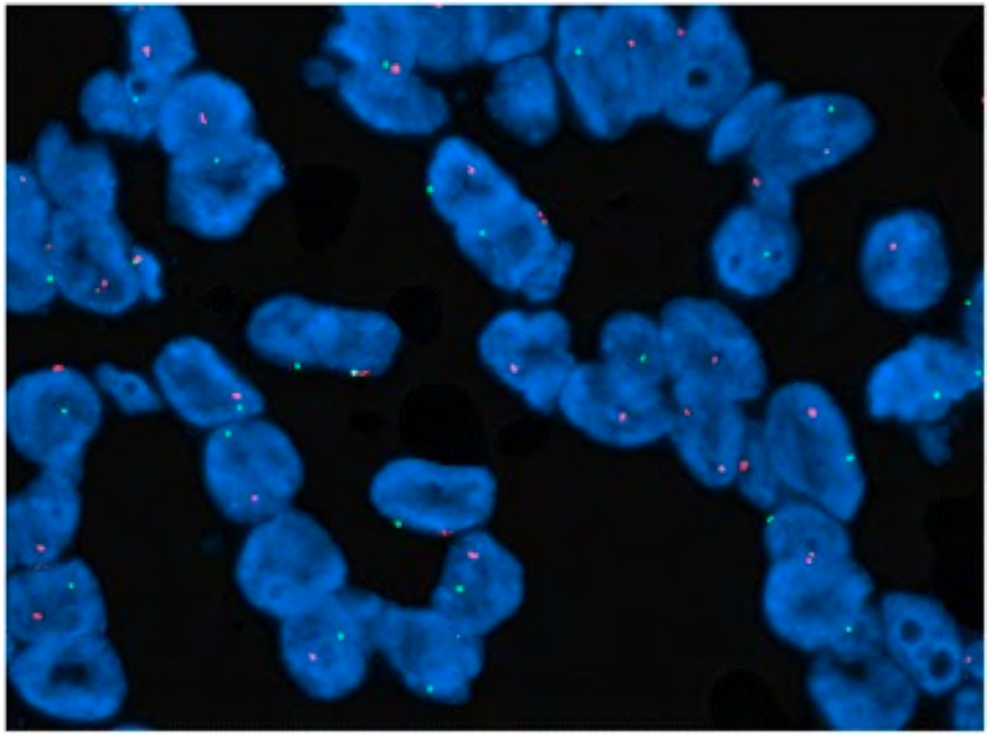



| | |
|---|---|
| ALK Split FISH Probe | RARA Split FISH Probe |
| SYT Split FISH Probe | MYC Split FISH Probe |
| EWSR1 Split FISH Probe | PDGFRB Split FISH Probe |
| TFE3 Split FISH Probe | ERG Split FISH Probe |

# TFE3 break-apart

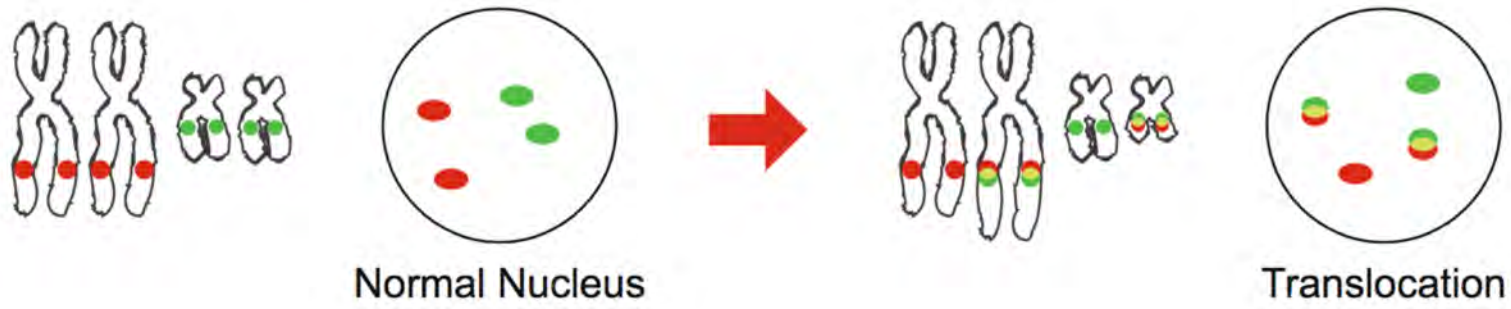




https://www.ncbi.nlm.nih.gov/pubmed/26415891

# Gene translocation



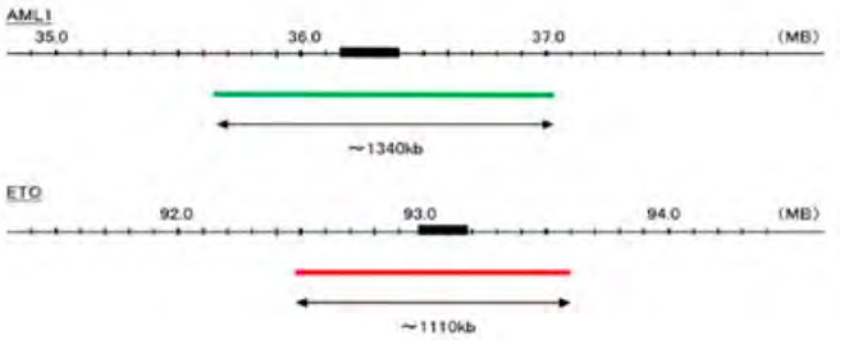

EML4/ALK DY Translocation FISH Probe

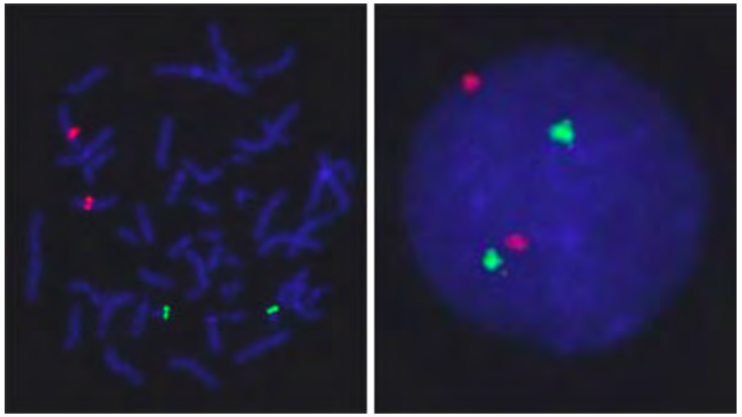
bcr/abl DY Translocation FISH Probe

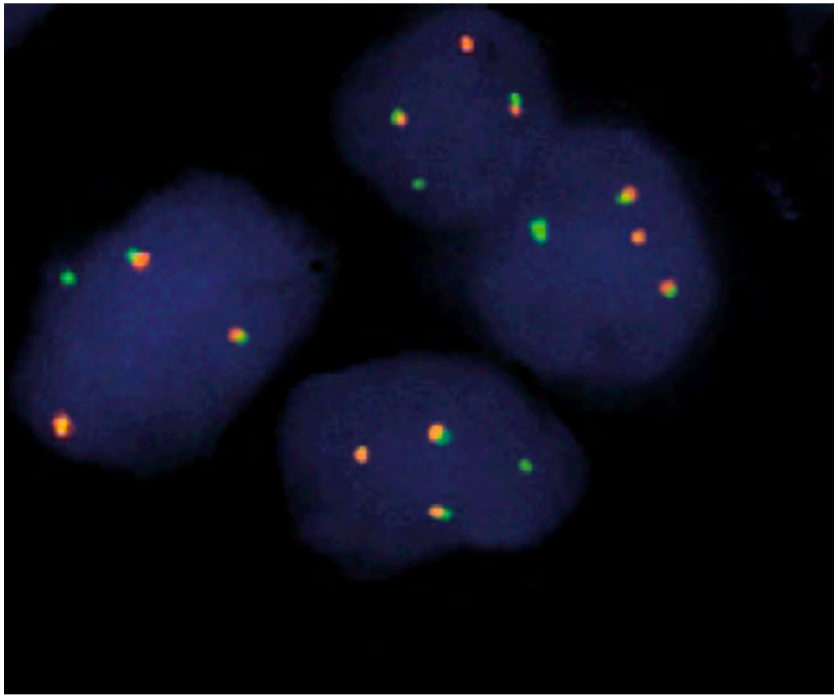
AML1/ETO DY Translocation FISH Probe

PML/RARA DY Translocation FISH Probe

# AML1/ETO DY Translocation

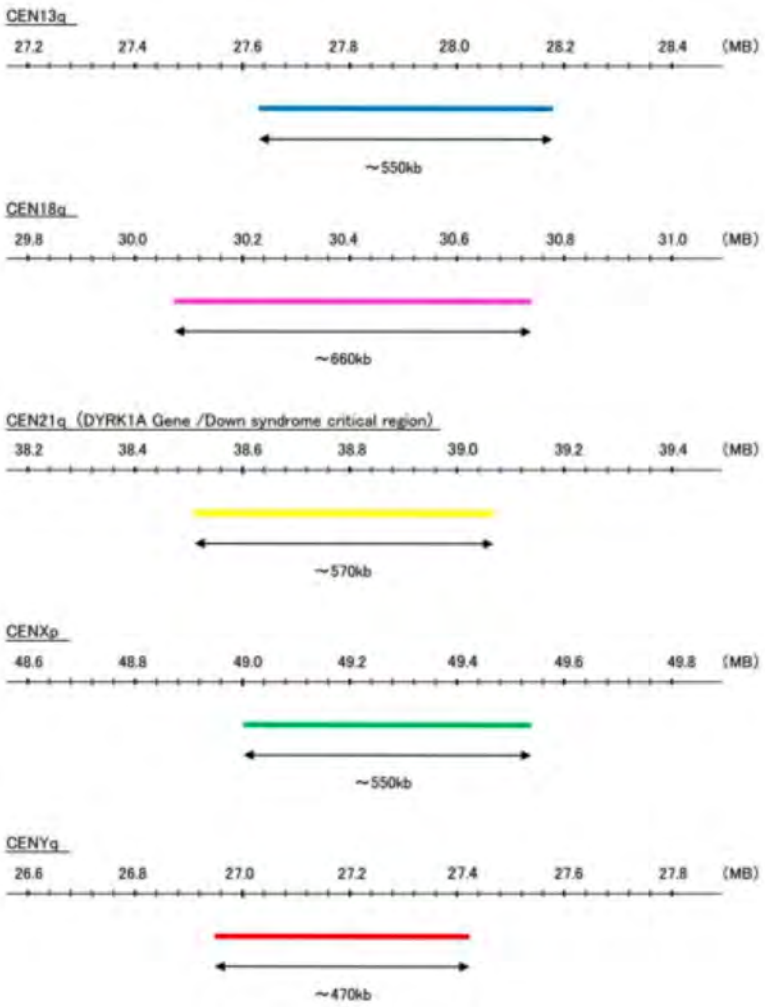


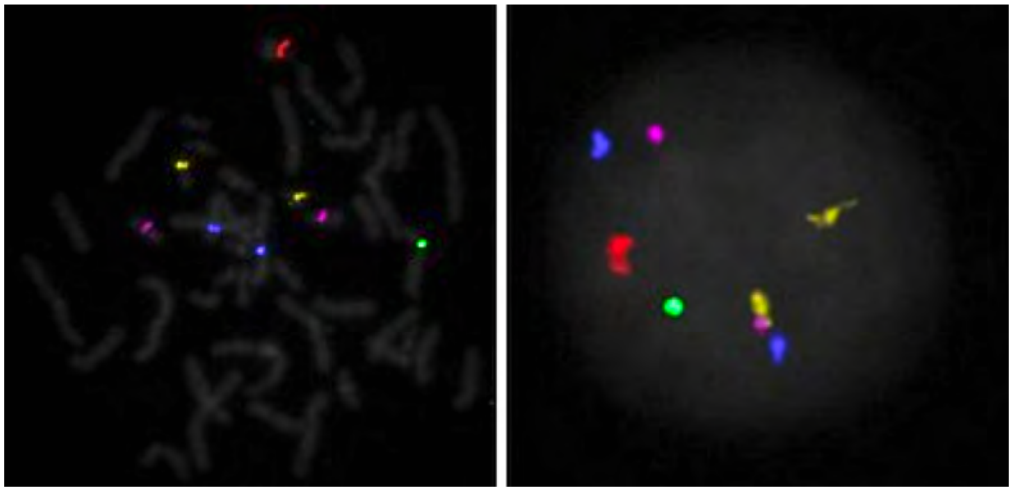





# Prenatal diagnostic

## 5-Color FISH Probe (chromosome 13, 18, 21, X & Y)

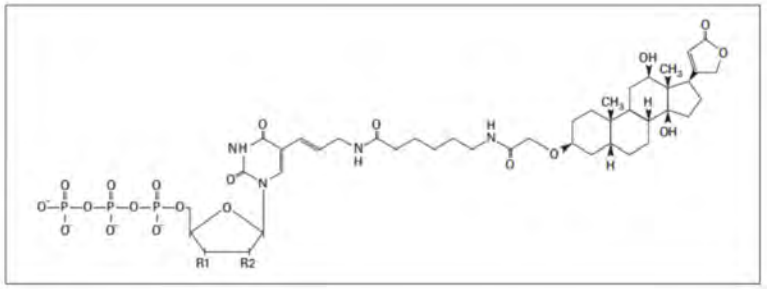


Representative images of normal human cell (lymphocyte) stain with the multi-color FISH probe. (CEN13q, blue; CEN18q, pink; CEN21q, yellow; CENXp, red; CENYq, green).

# Digoxigenin (DIG) system

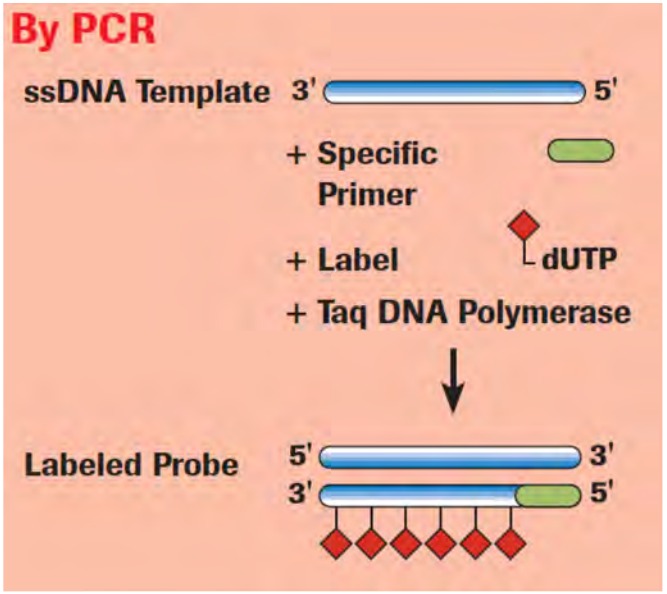
The Digoxigenin (DIG) System was developed (Kessler, 1990) and continues to be expanded by Roche

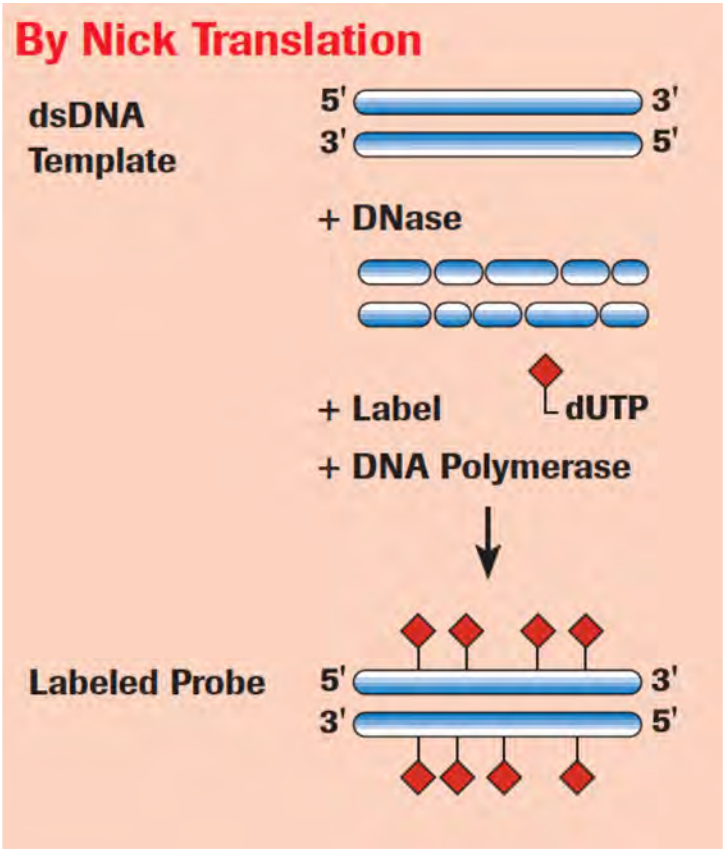


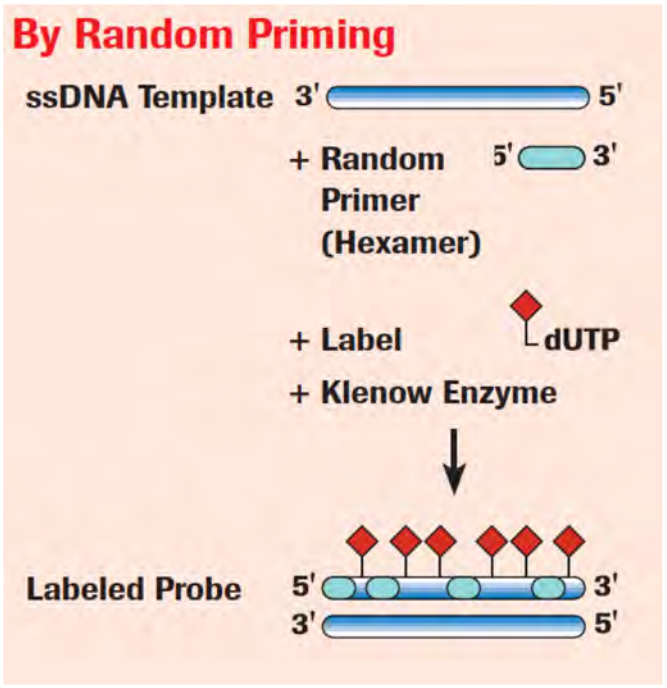
The DIG labeling method is based on a steroid isolated from digitalis plants.
As the blossoms and the leaves of these plants are the only natural source of digoxigenin, the anti-DIG antibody does not bind to other biological material.

Hybridized DIG-labeled probes may be detected with high affinity anti-digoxigenin (anti-DIG) antibodies that are conjugated to alkaline phosphatase, peroxidase, fluorescein, rhodamine, or colloidal gold.

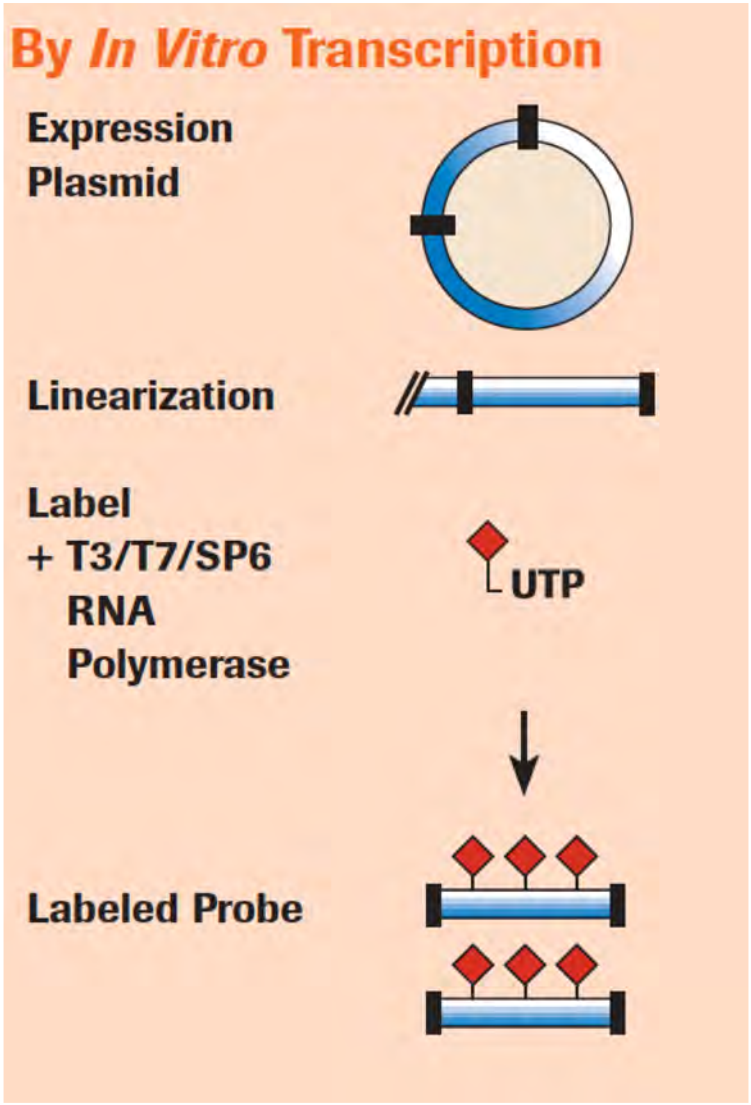
# DNA


By PCR
ssDNA Template
3'
5'
+ Specific Primer
+ Label
dUTP
+ Taq DNA Polymerase
Labeled Probe
5'
3'
3'
5'


By Nick Translation
dsDNA Template
5'
3'
3'
5'
+ DNase
+ Label
dUTP
+ DNA Polymerase
Labeled Probe
5'
3'
3'
5'


By Random Priming
ssDNA Template
3'
5'
+ Random Primer (Hexamer)
5'
3'
+ Label
dUTP
+ Klenow Enzyme
Labeled Probe
5'
3'
3'
5'

# RNA

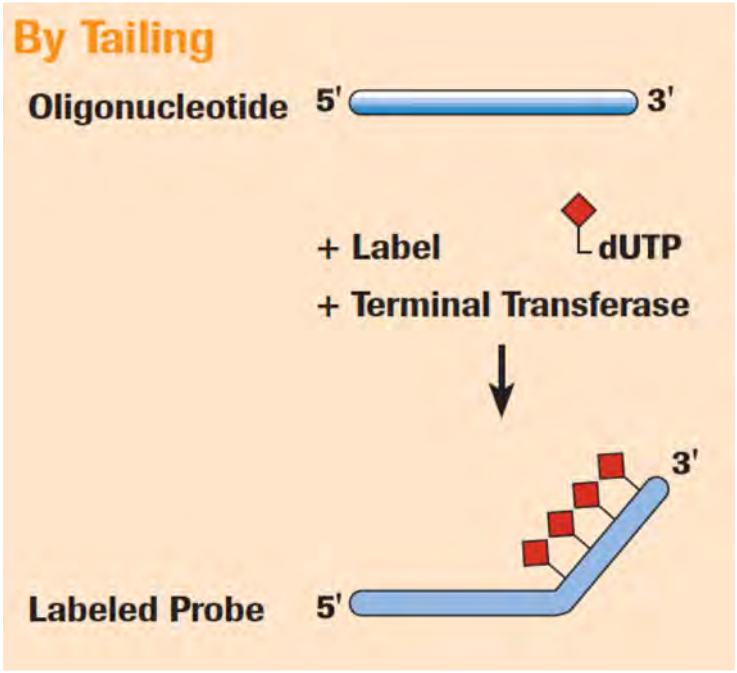


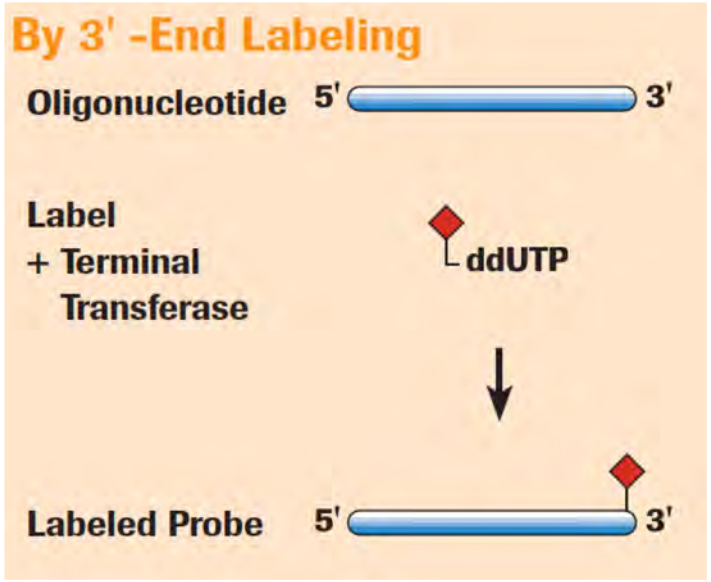

Optimal RNA probe for the localization of cellular mRMA are:

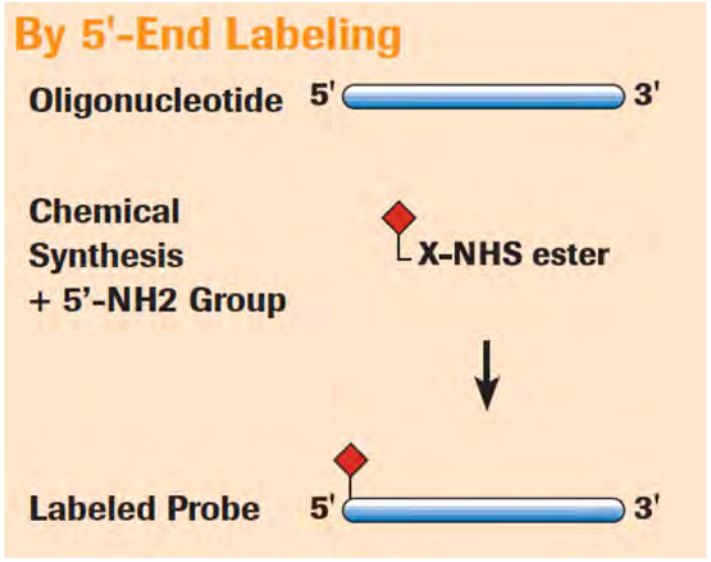
- 350-500 base long
- Targeting a transcript region including an exon-exon junction

It is fundamental to use the proper control samples.

# Oligonucleotide

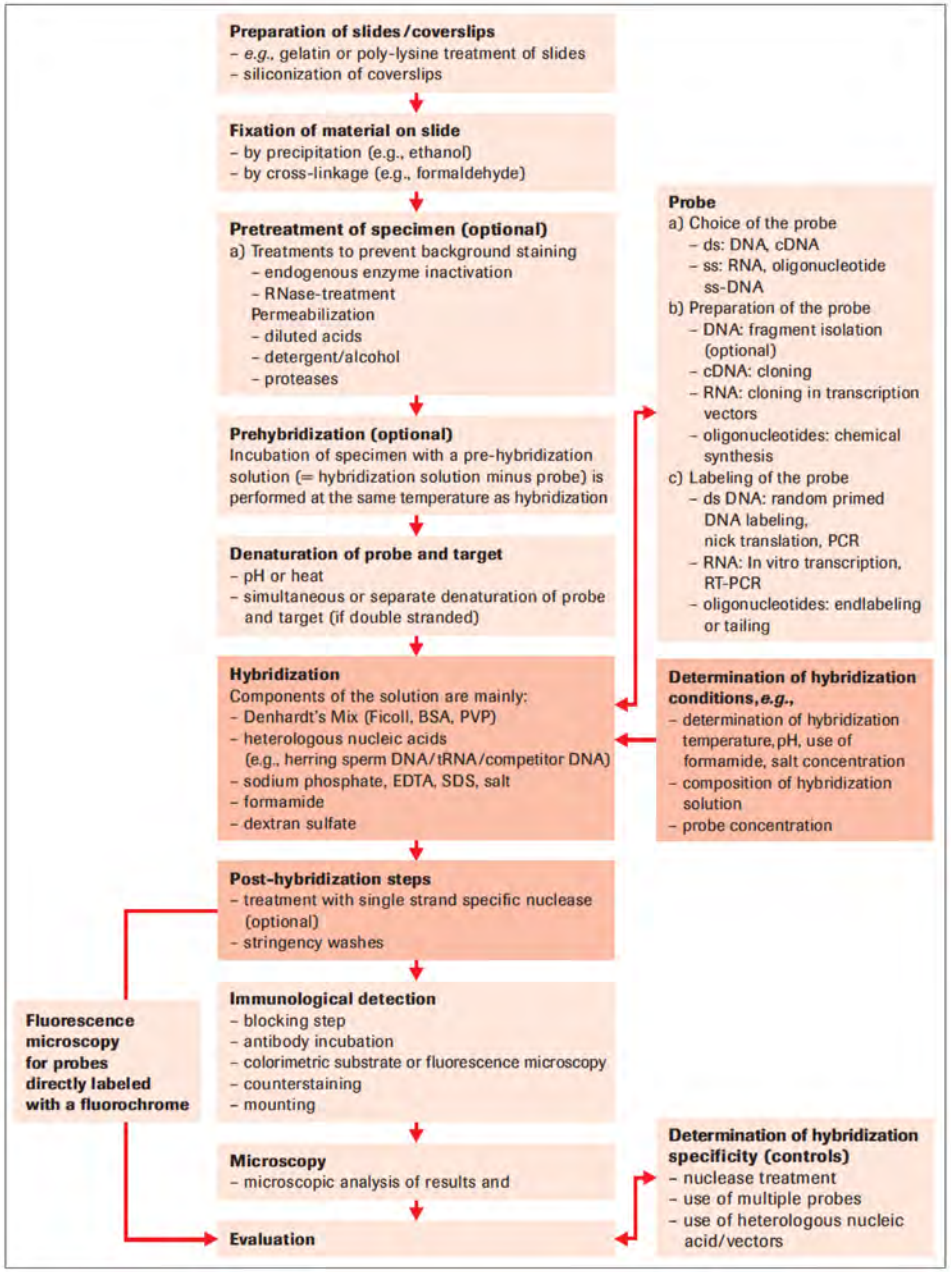


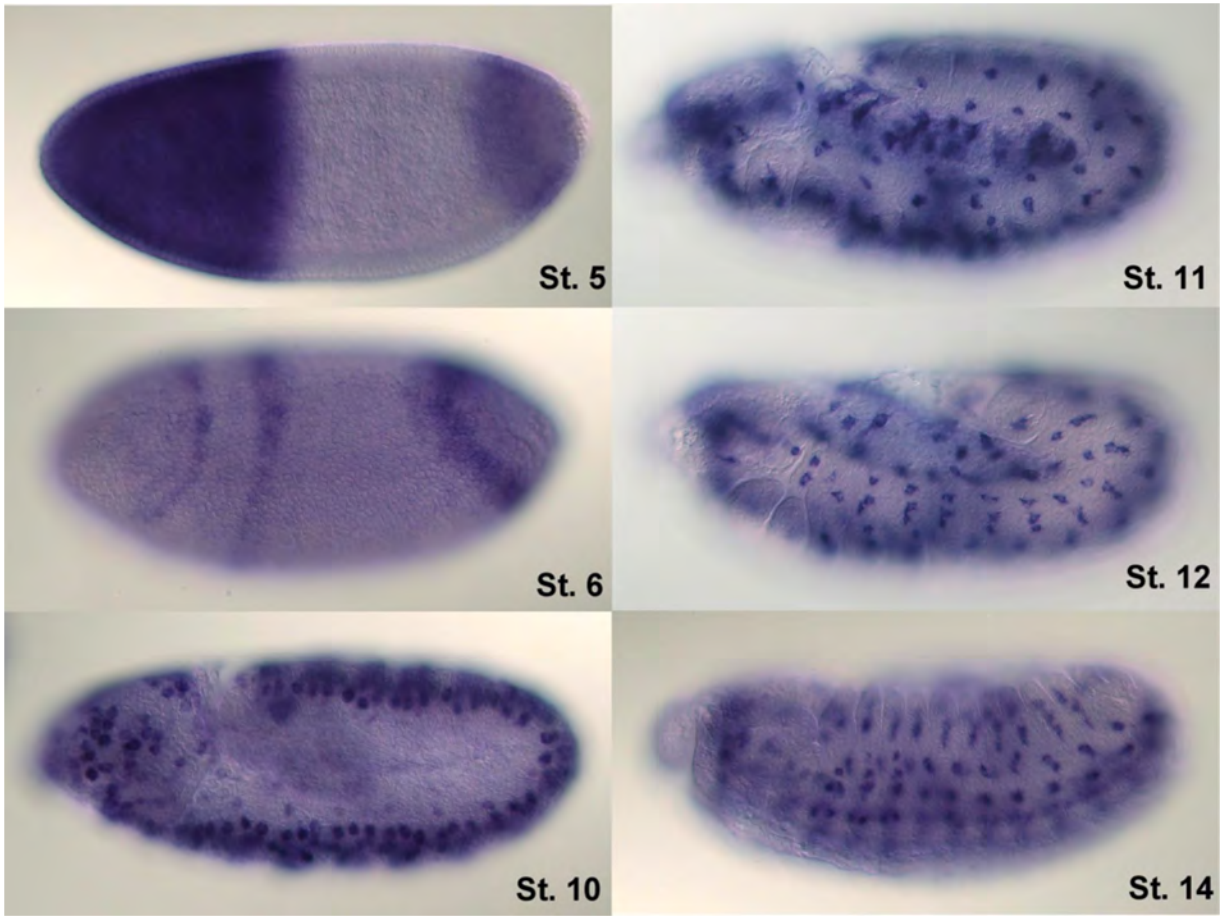




More suitable for bacterial localization (targeting rRNA)

# Experimental setup

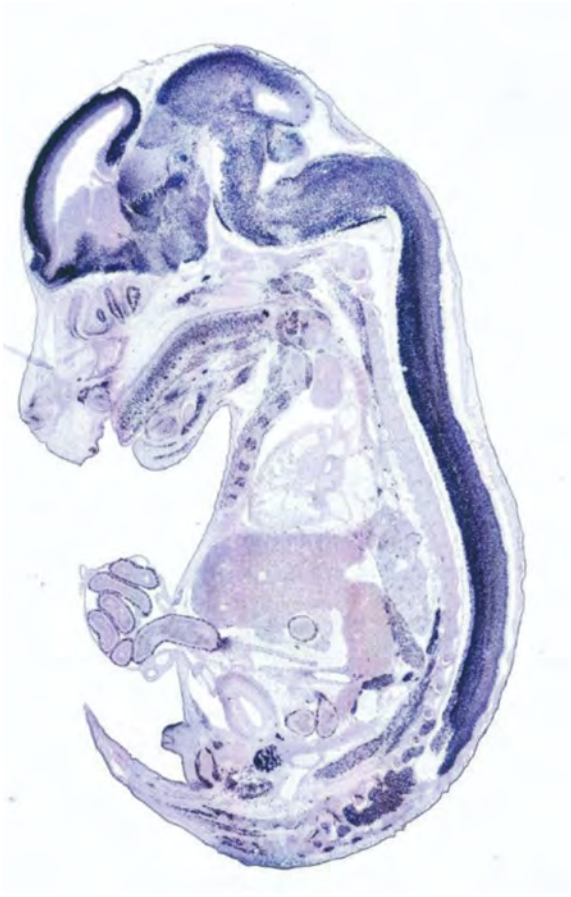


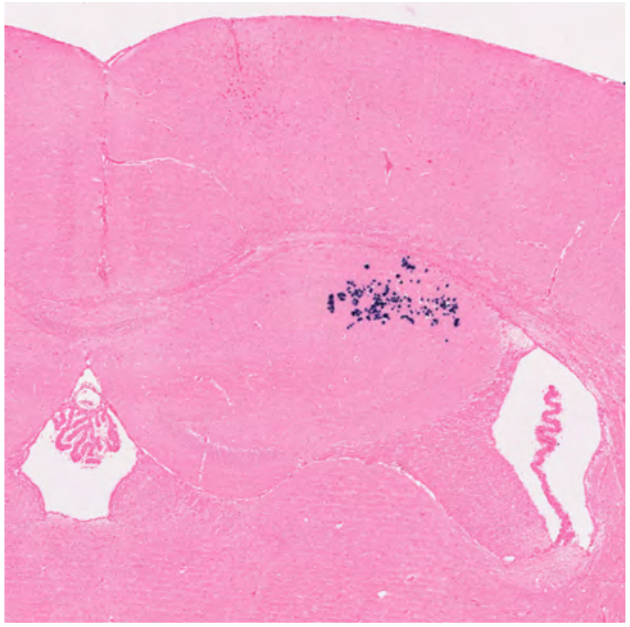
# Whole-mount DIG ISH

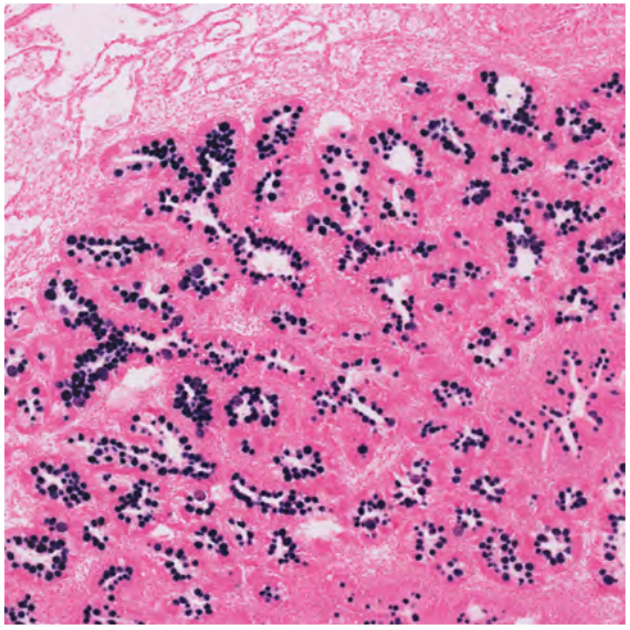



Hunchback gene in Drosophila m. embryo
Whole-mount in situ, Dig. labeled probe

# DIG ISH on tissue sections





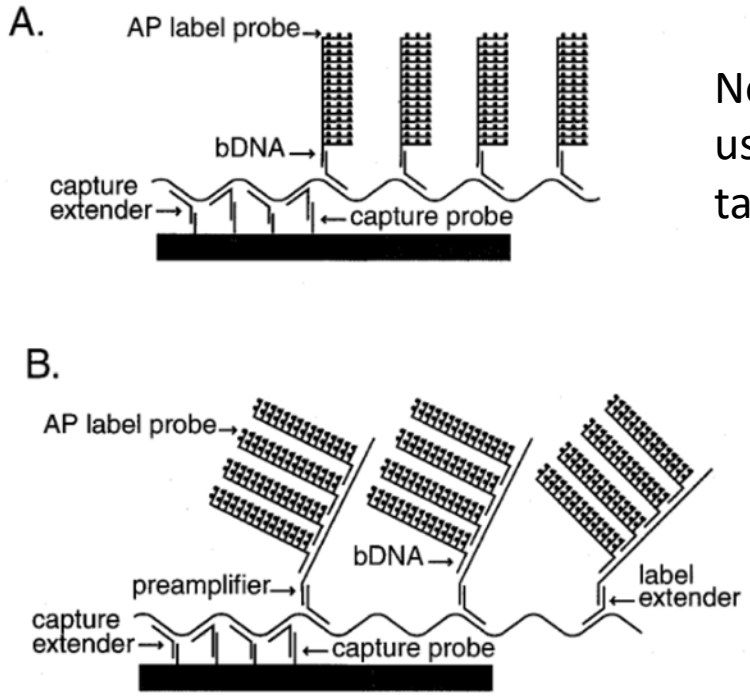


# Branched DNA (bDNA)In Situ Hybridization

Nucleic Acids Res. 1997 Aug 1;25(15):2979-84.

**A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml.**

Collins ML[1], Irvine B, Tyner D, Fine E, Zayati C, Chang C, Horn T, Ahle D, Detmer J, Shen LP, Kolberg J, Bushnell S, Urdea MS, Ho DD.




Non-natural bases, isocytidine and isoguanosine were used to reduce their hybridization potential to all non-target nucleic acids

| | |
|---|---|
| Preamplifier repeat (14) | d(TCFACGJCFCTAJGGAFAAFG) |
| Amplifier repeat (30) | d(AGTFAJCGCFGTAFCAAJTJC) |

F, isoC; J, isoG

# Branched DNA (bDNA)In Situ Hybridization

Single-copy Gene Detection Using Branched DNA (bDNA)In Situ Hybridization

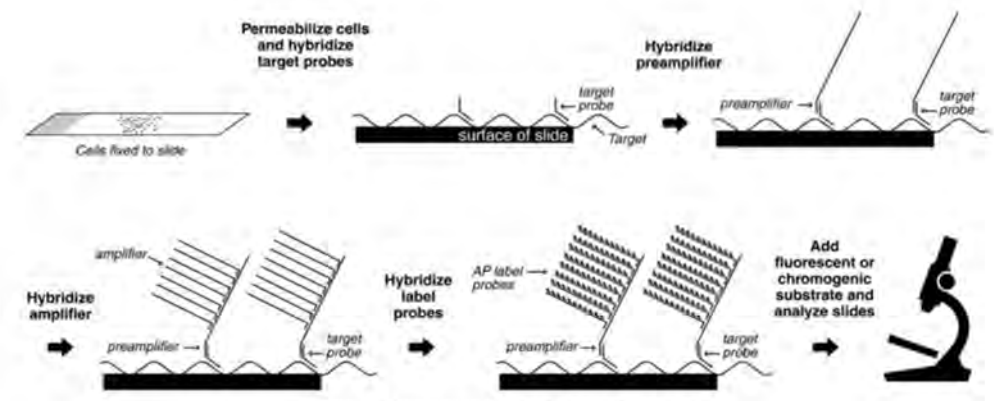
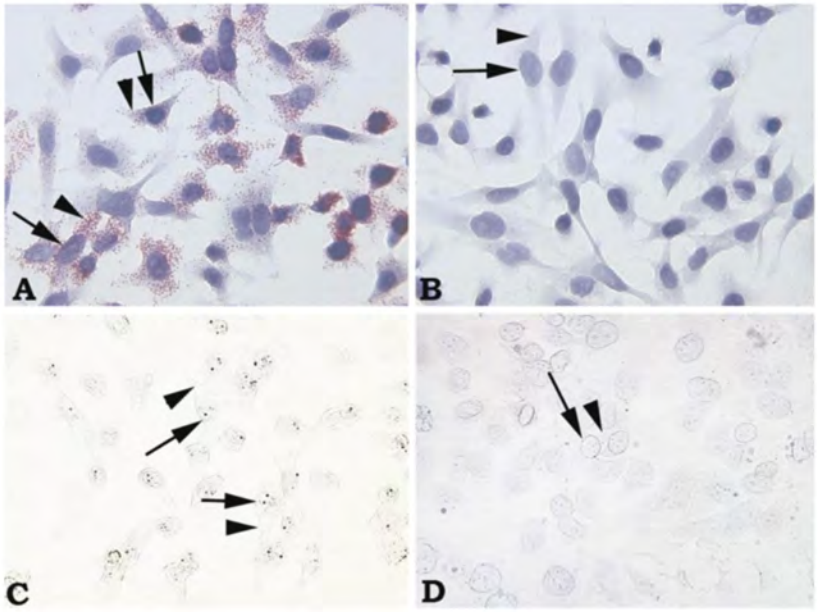



Figure 1 Schematic depiction of the bDNA ISH method.

Human papillomavirus (HPV) RNA

Human papillomavirus (HPV) DNA




https://www.ncbi.nlm.nih.gov/pubmed/11304798

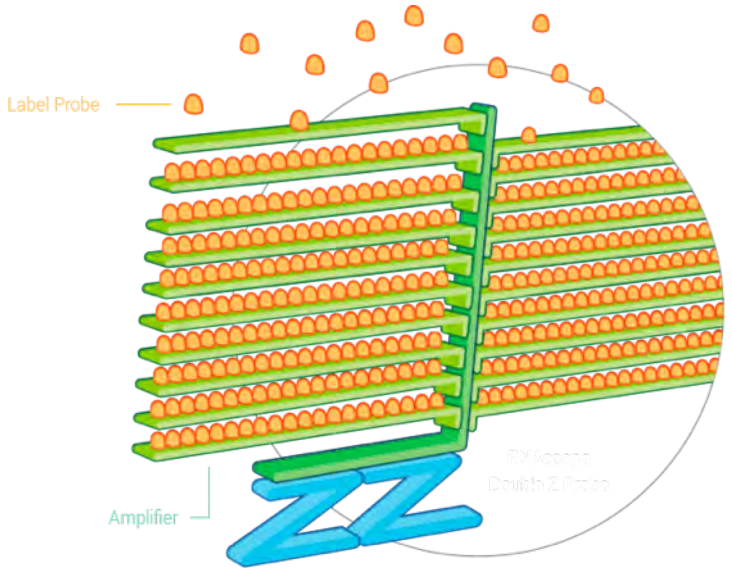
# ACD RNAscope & Panomics QuantiGene ViewRNA

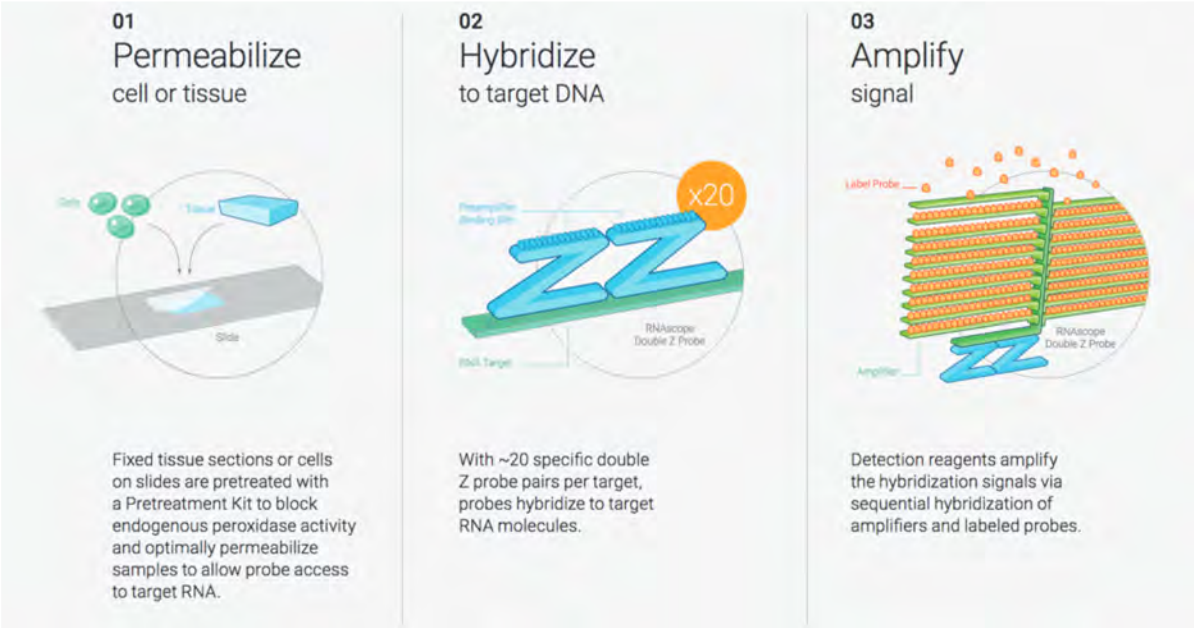
Fixed cells, frozen and FFPE tissues

Localization of:
mRNA & lncRNA
Unofficially DNA
Unofficially miRNA

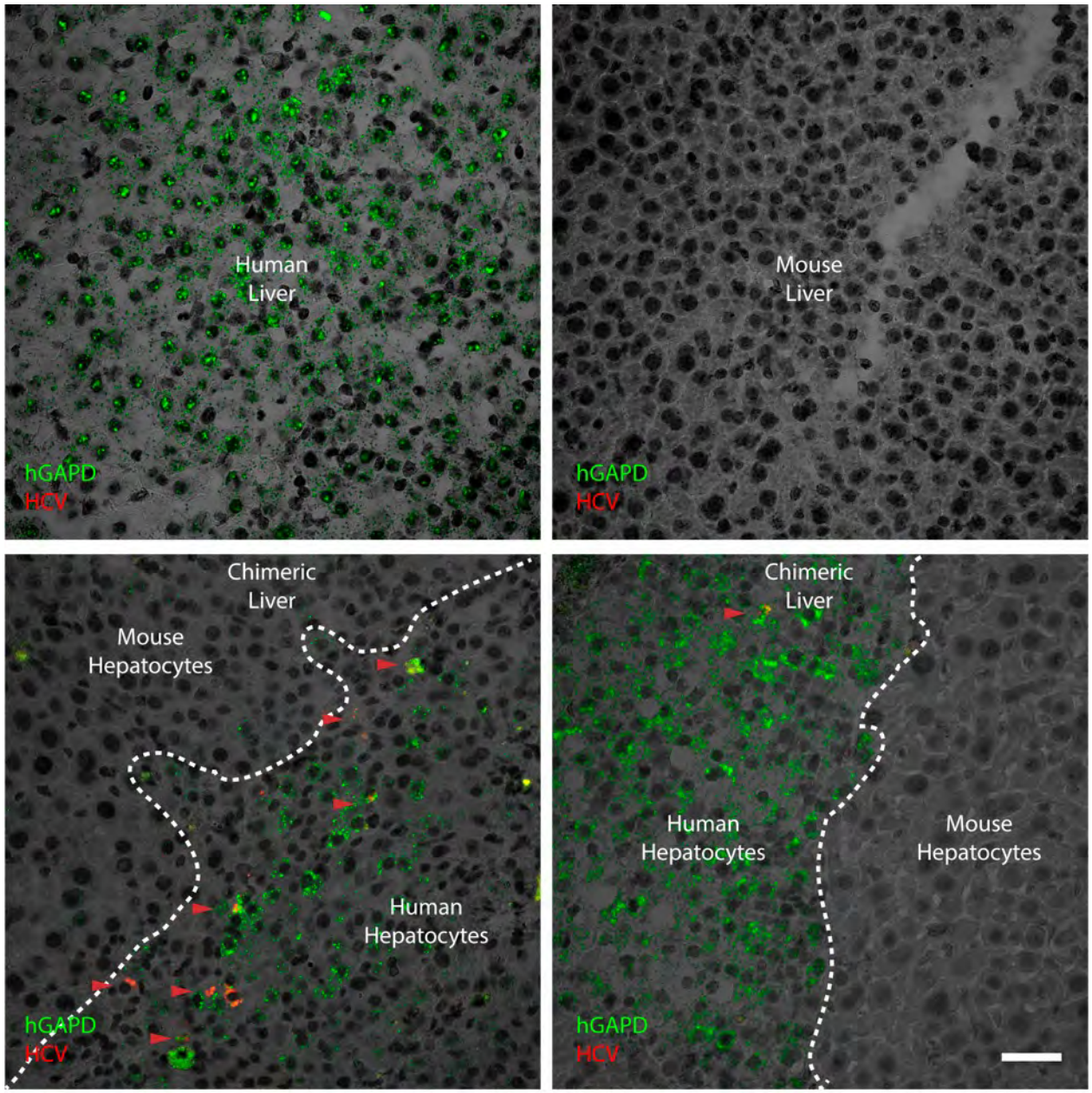
In cell:
Up to 4 colours/targets in fluorescence
Possible co-localization with proteins

In tissue:
2 colours/targets in bright and dark field



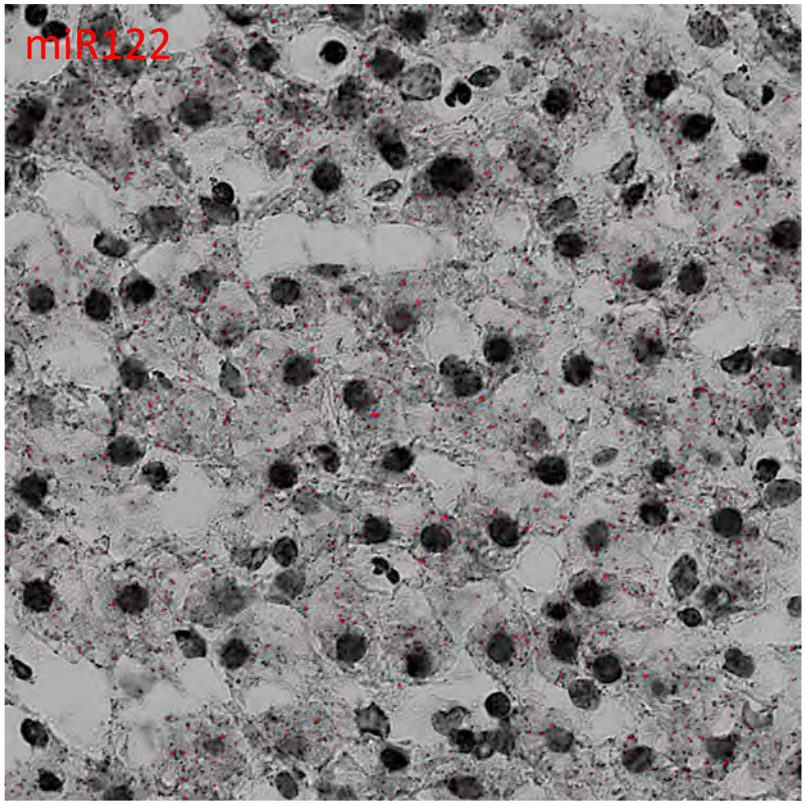



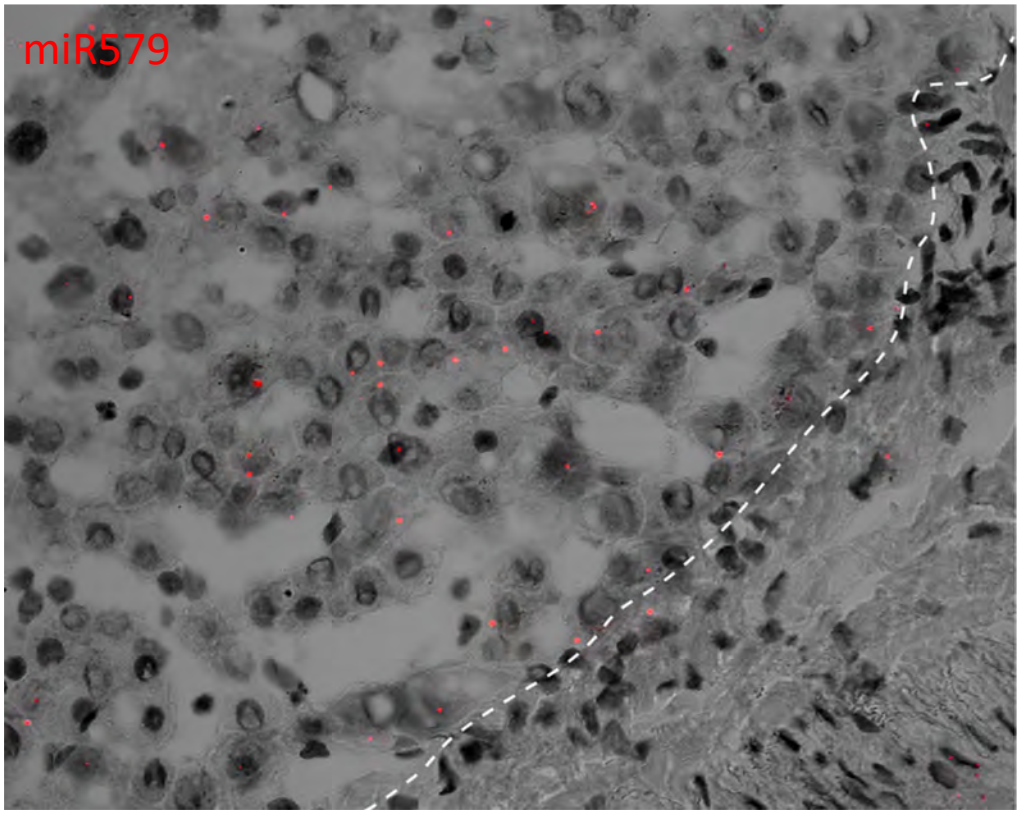
# ViewRNA ISH Tissue 2-Plex Assay



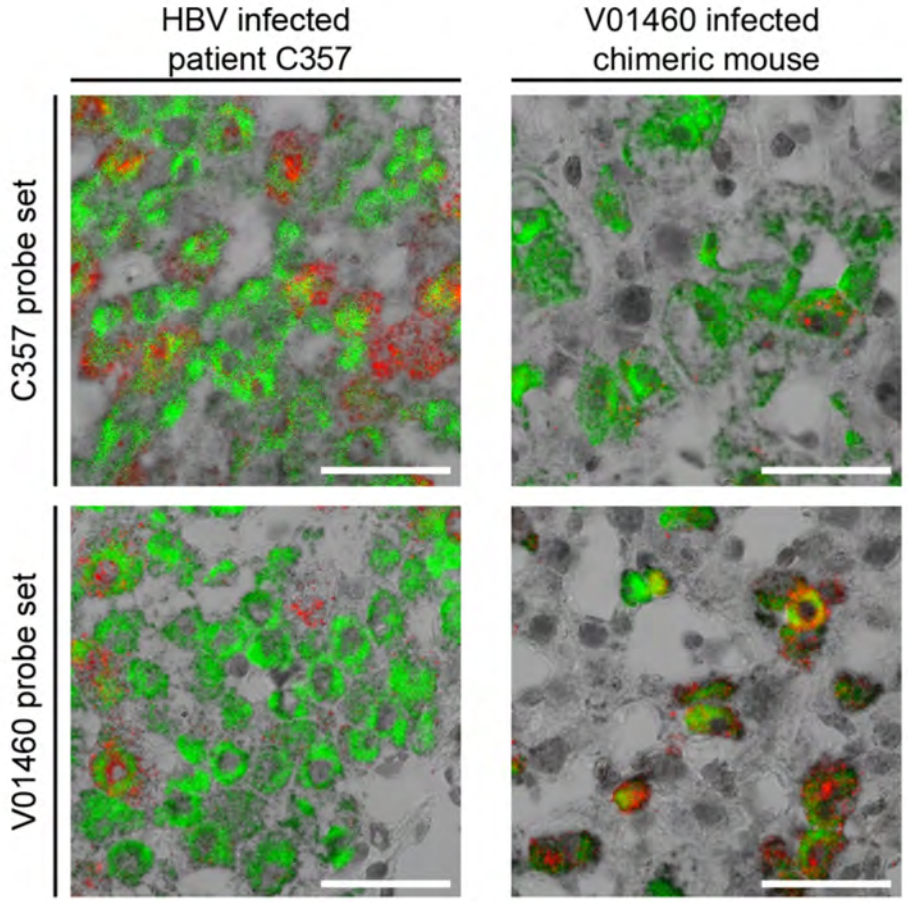

https://www.ncbi.nlm.nih.gov/pubmed/25798937

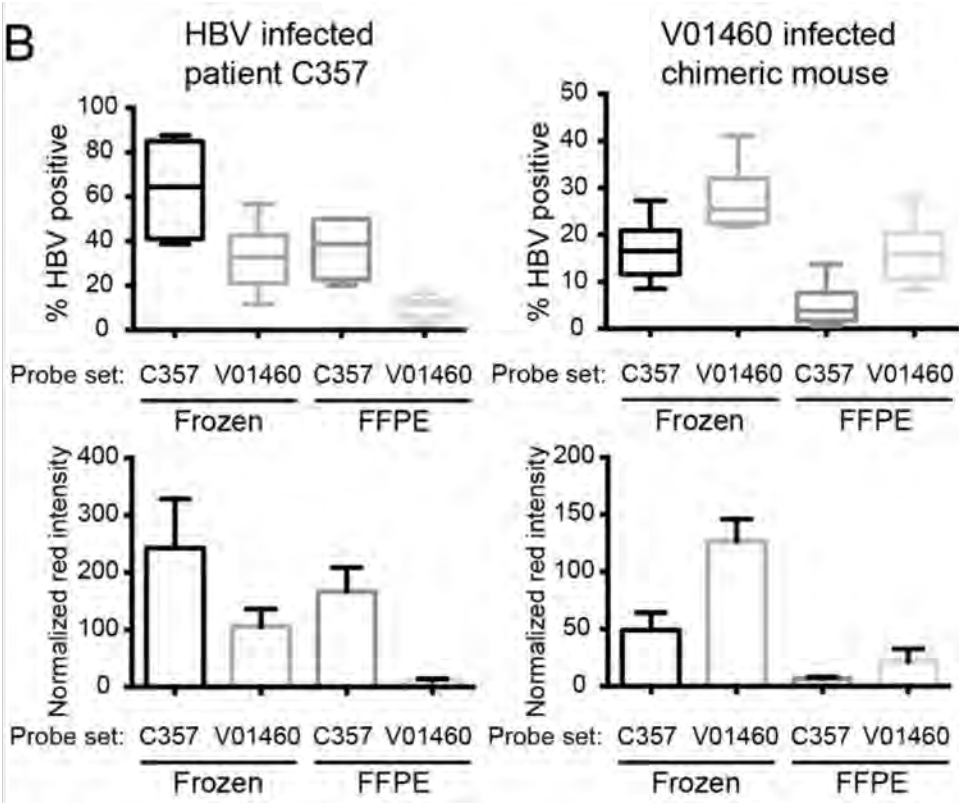
# miRNA localization



# Viral detection




https://www.ncbi.nlm.nih.gov/pubmed/27975312

# Features and limitations

- Easy to use
- More specific and sensitive than other techniques
- Suitable also in samples with partially degraded RNA
- Less sensitive to probe degradation
- Very reliable

- Very short bench life
- Very sensitive to base mismatch
- Pretreatment condition optimization
- Setup of a correct pipeline for acquisition

# Conclusions

ISH helps in answering questions about gene expression in heterogeneous tissues

GISH is nowadays a fundamental diagnostic tool

DIG system is flexible and customizable, but subject to experimental condition variations

The new techniques based on bDNA are sensitive and reliable, and perfectly suitable for most of the standard ISH experiments

Q&A