# Hybridization techniques and their practical applications


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**Department of Pathology**



Basic Molecular Pathology Course
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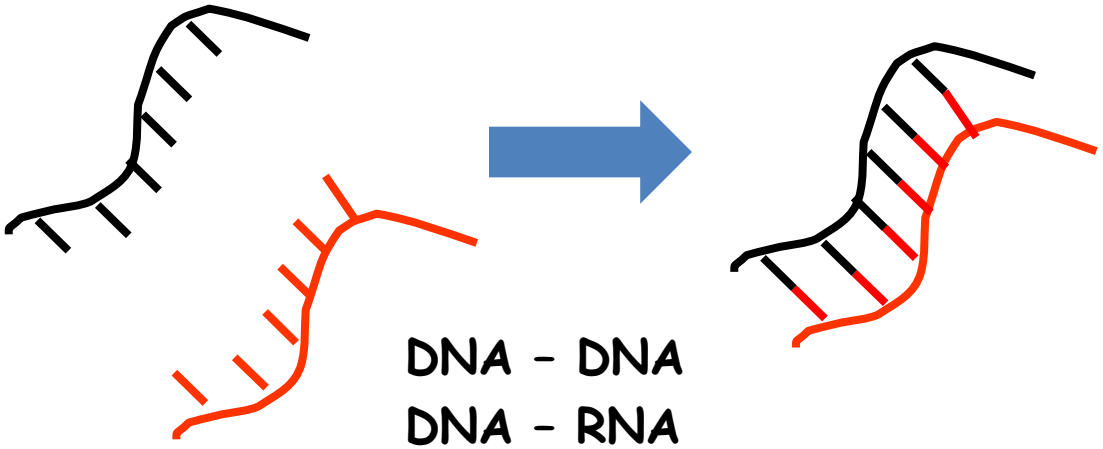
# Hybridization techniques and their practical applications

- Definition and terminology
- Explanation of the technique
- Interpretation
- Applications for diagnostic / research Pathology.

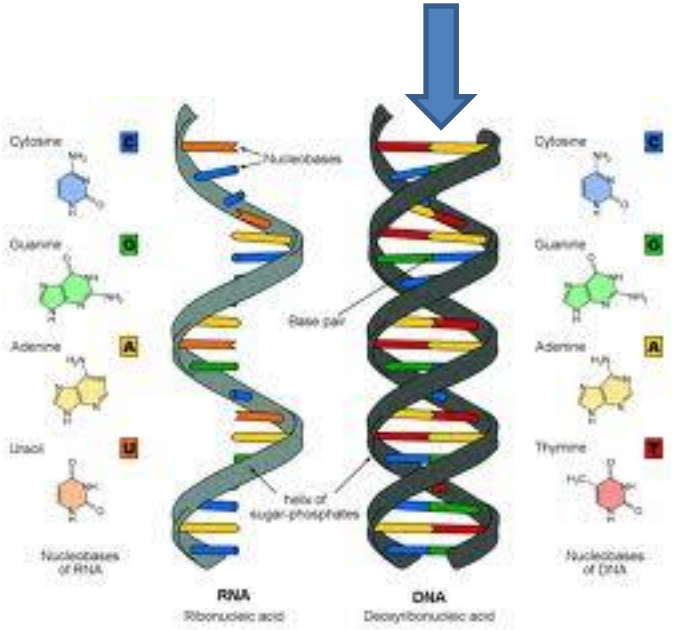
# TERMINOLOGY

## Hybridization

Process of establishing a non-covalent, sequence-specific interaction between two complementary strands of nucleic acids into a single complex



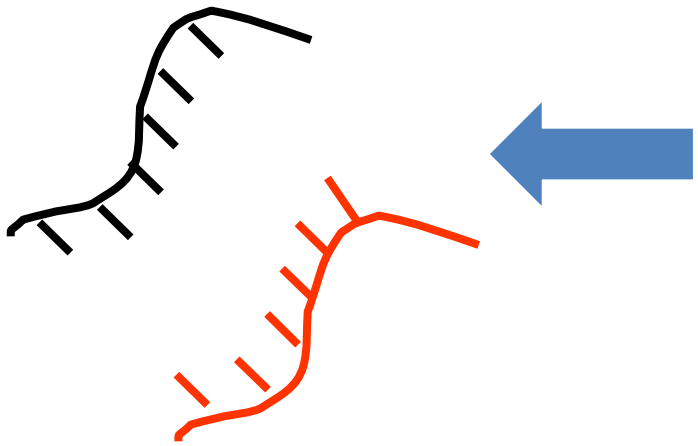
**DNA – DNA**
**DNA – RNA**
**RNA – RNA**

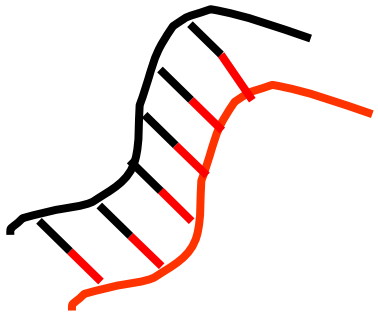


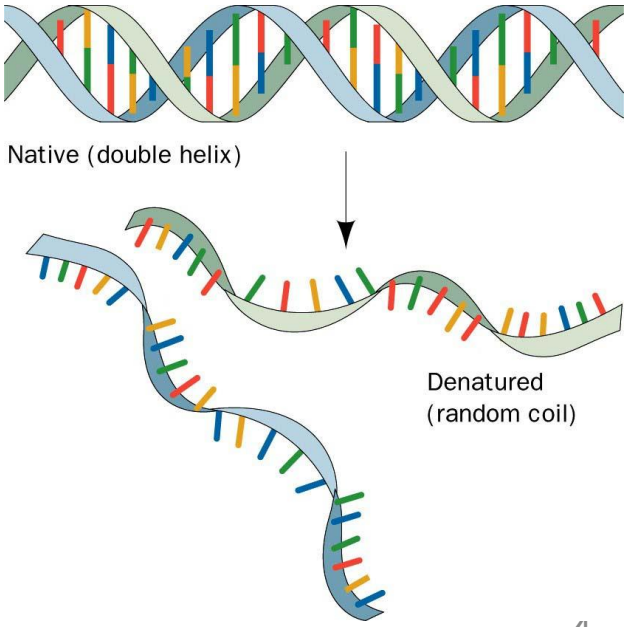
# TERMINOLOGY

## Denaturation

DNA melting, is the process by which double-stranded nucleic acid separates into single-stranded strands through the breaking of hydrophobic stacking attractions between the bases.





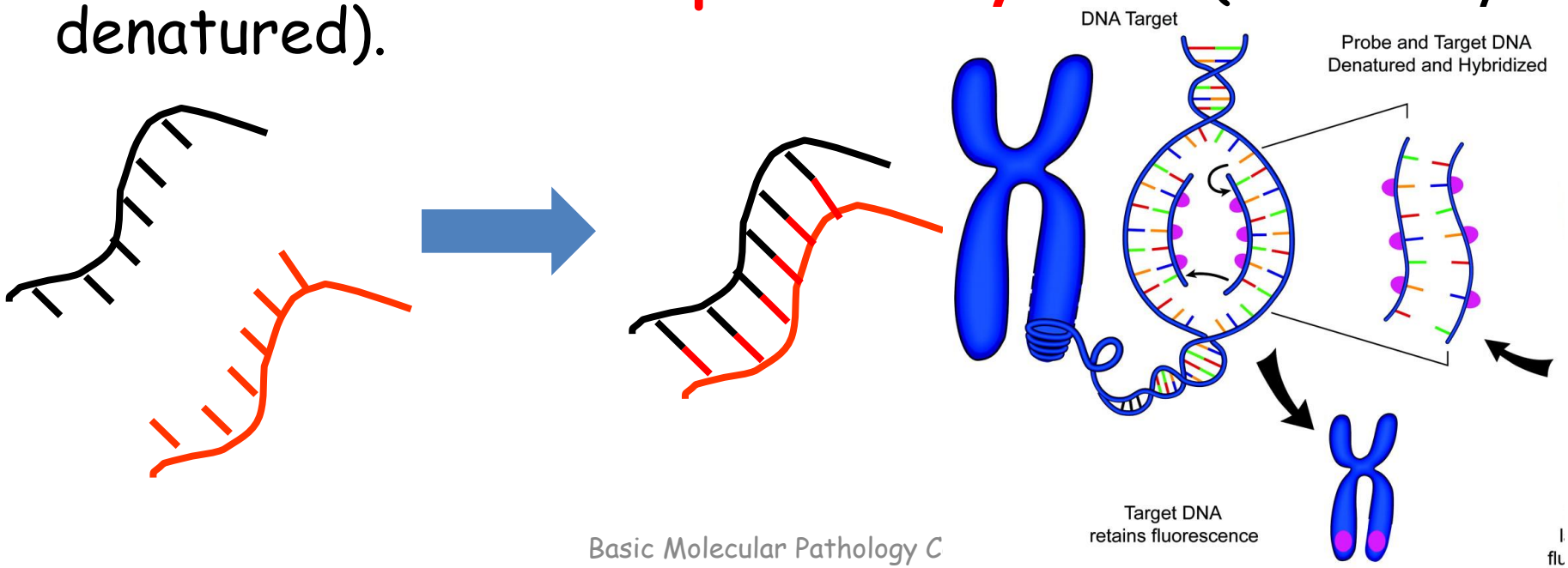


# TERMINOLOGY

## Annealing

For complementary sequences of single-stranded DNA or RNA to pair by hydrogen bonds to form a double-stranded nucleotide complex.

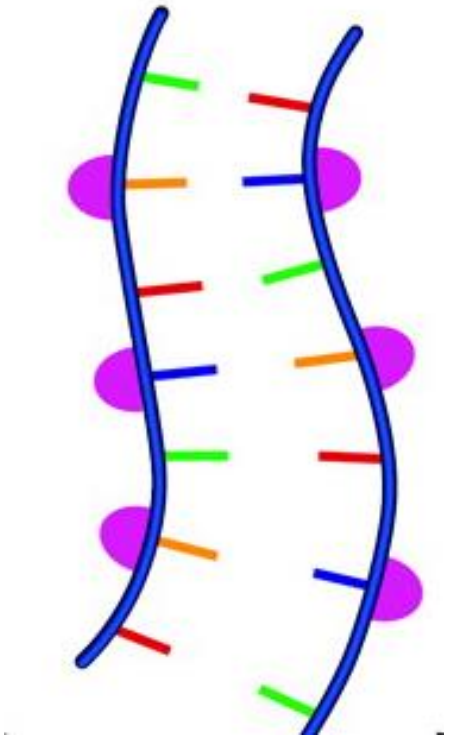
Binding of a probe. The term is also often used to describe the **renaturation of complementary strands that were separated by heat** (thermally denatured).



# TERMINOLOGY

## Probe

A fragment of labelled DNA or RNA of variable length (usually 100-1000 bases long) which is used in DNA or RNA samples to detect the presence of nucleotid sequences that are complementary to the target sequence.



# HYBRIDISATION

DNA – DNA
DNA – RNA
RNA – RNA

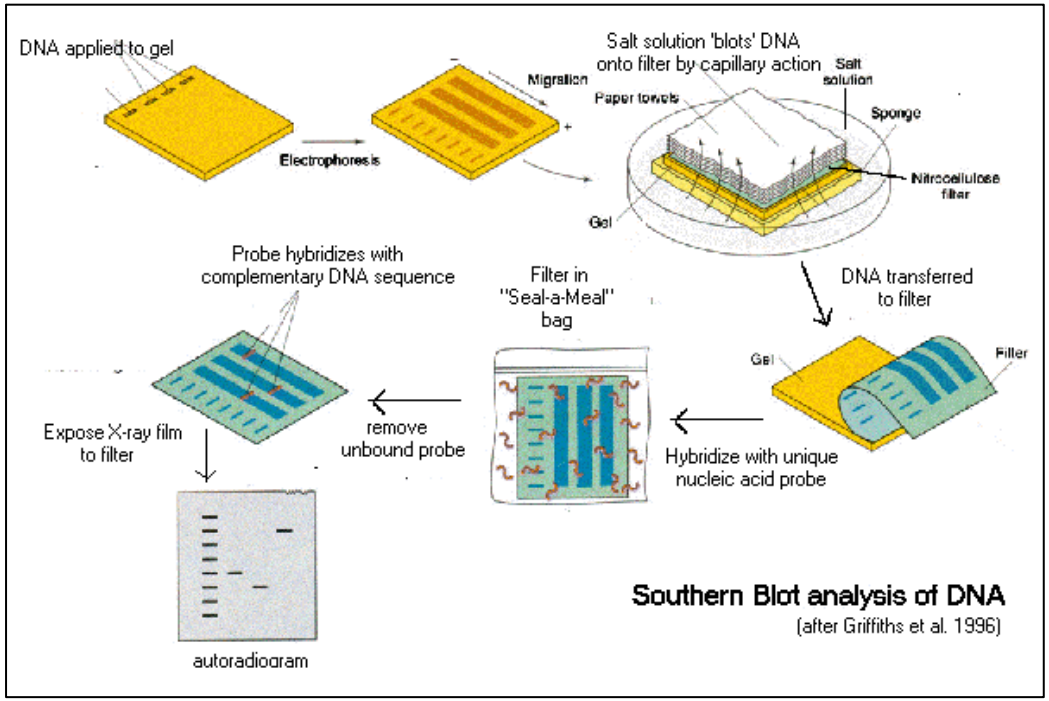
## THE MAIN PRINCIPLE OF "HYBRIDIZATION TECHNIQUES" ARE TO VISIULIZE THIS REACTION

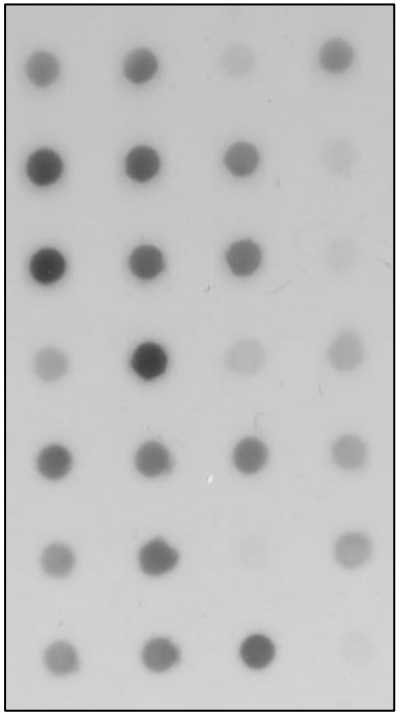
# HYBRIDIZATION TECHNIQUES

1- Blotting
2-Array technologies
3- **In situ hybridization**
4- Comperative Genomic Hybridisation

# HYBRIDIZATION TECHNIQUES

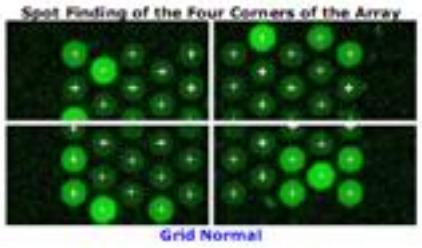
## 1- Blotting :Southern Blot - Northern Blot, Dot blot



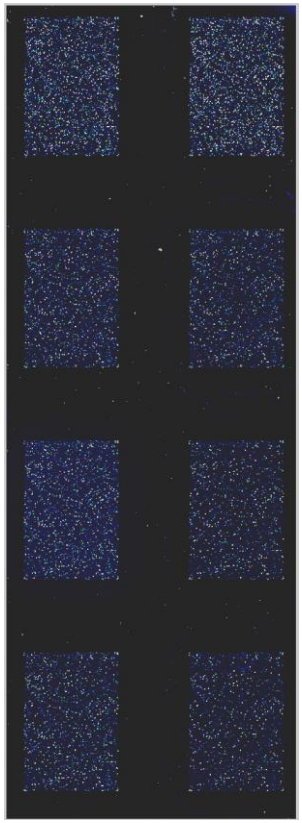



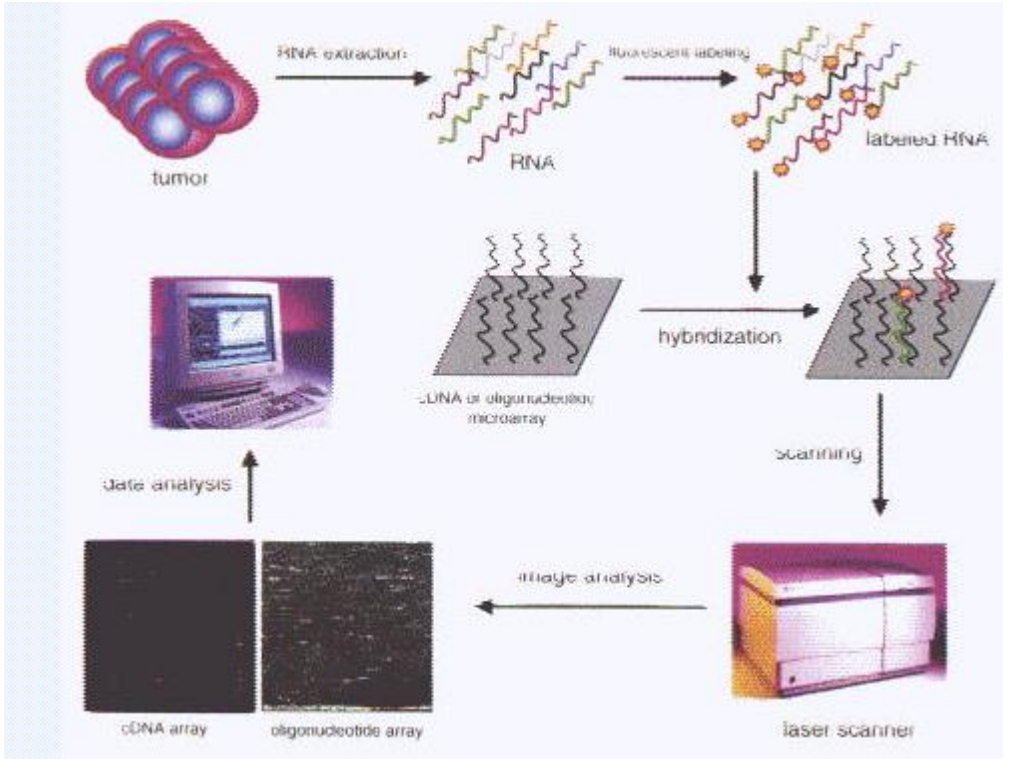
# HYBRIDIZATION TECHNIQUES

2-Array technologies : (macroarray ve microarray, gene expression profile detection).



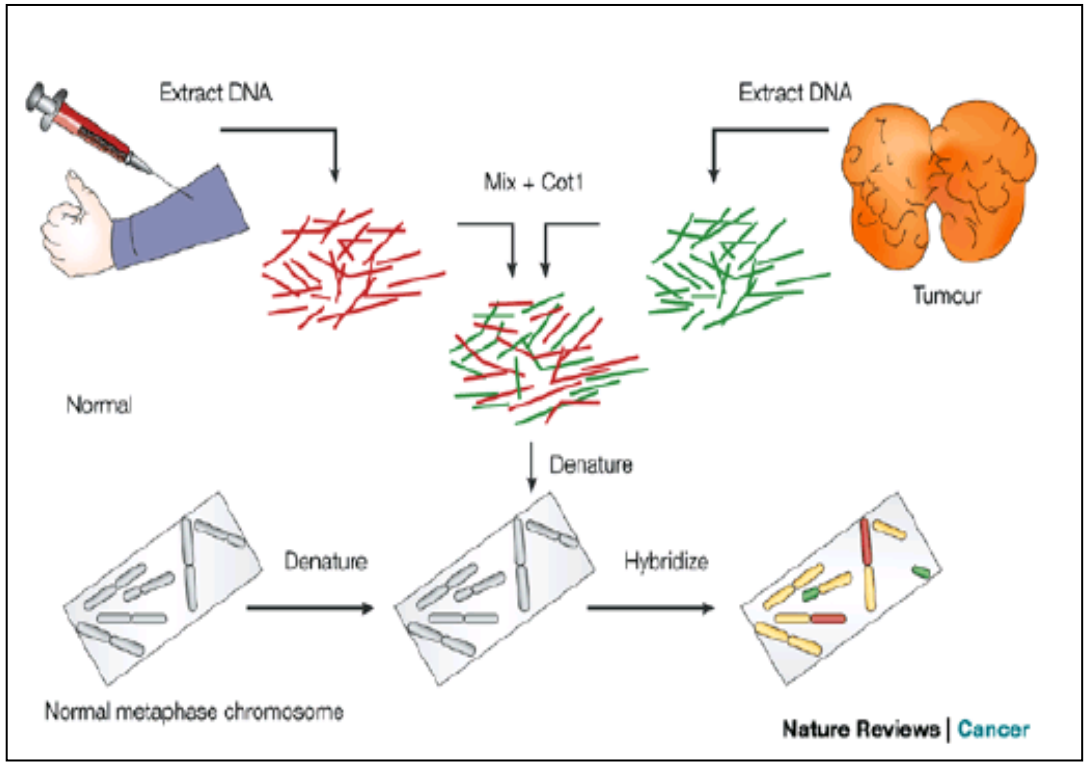


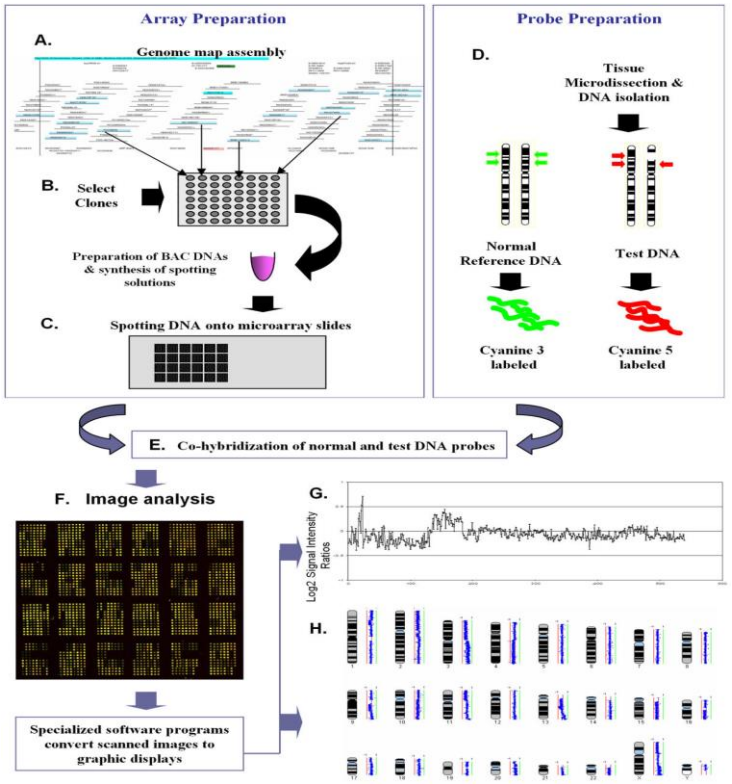




# HYBRIDIZATION TECHNIQUES

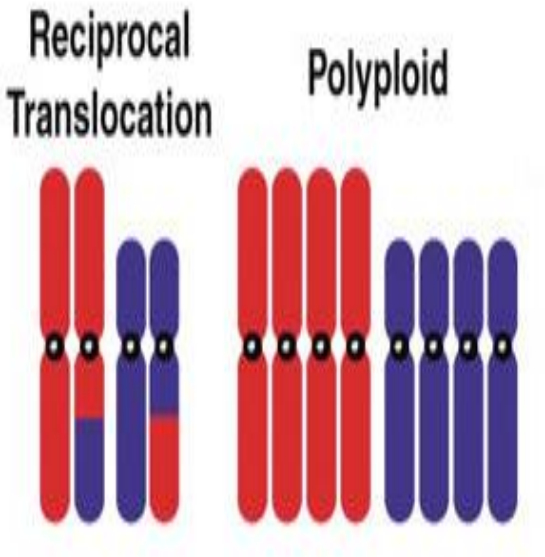
## 4- Comperative Genomic Hybridisation (CGH, ARRAY -CGH)



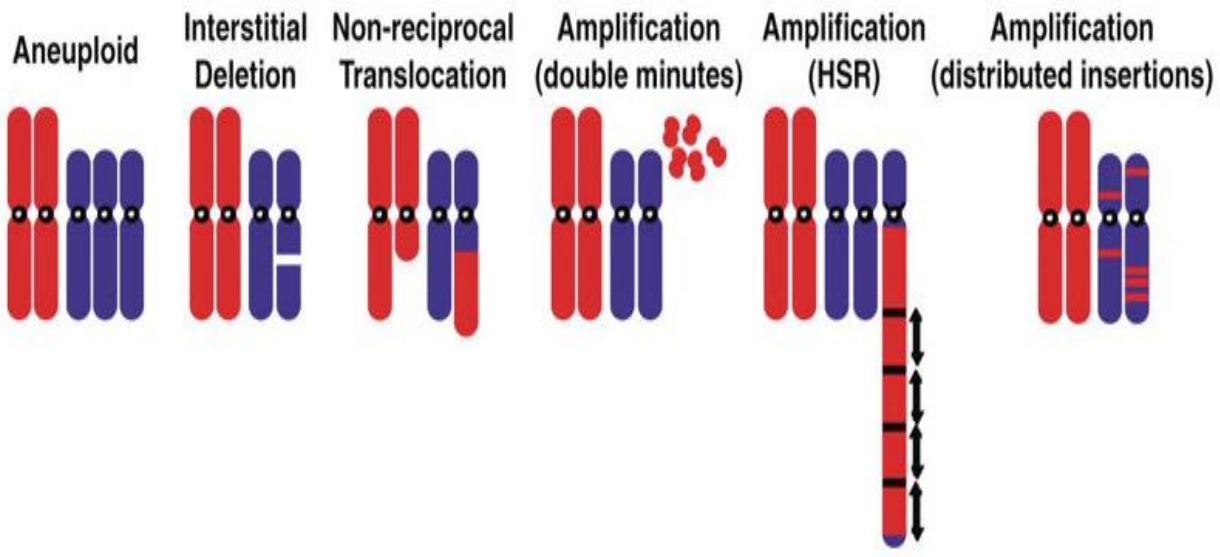



# LIMITATIONS OF CGH

**Can not be detectable**
**Balanced chromosomal abnormalities**




**Can be detected**
**Unbalanced chromosomal abnormalities**



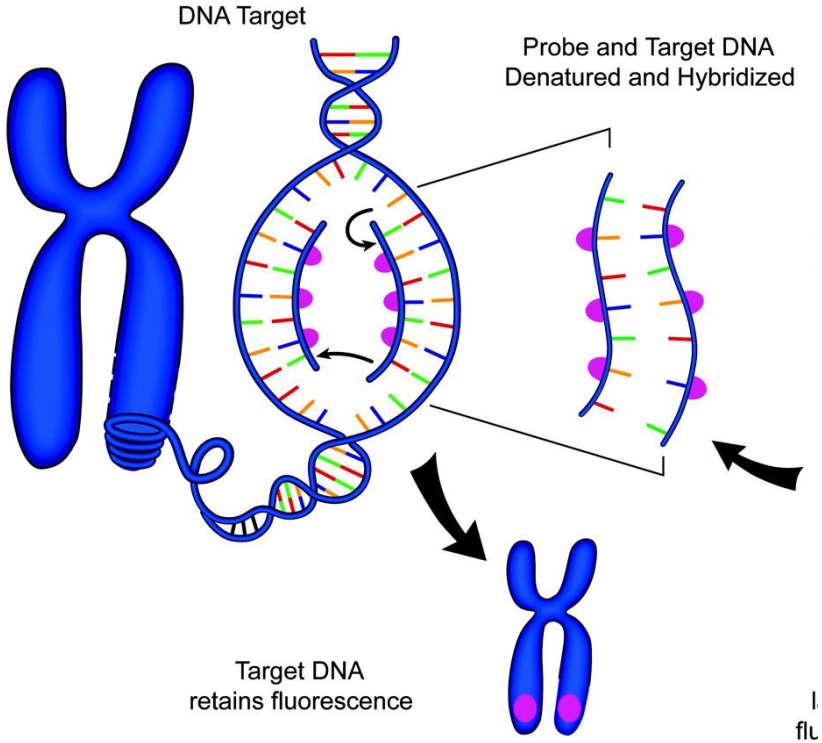



Albertson *et al.*, 2003

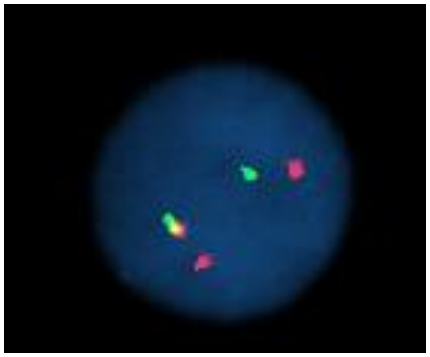
# IN SITU HYBRIDIZATION

## The principle of ISH

1- The **annealing of a labelled** probe to its complementary strand within the chromosomes of fixed cells or tissues

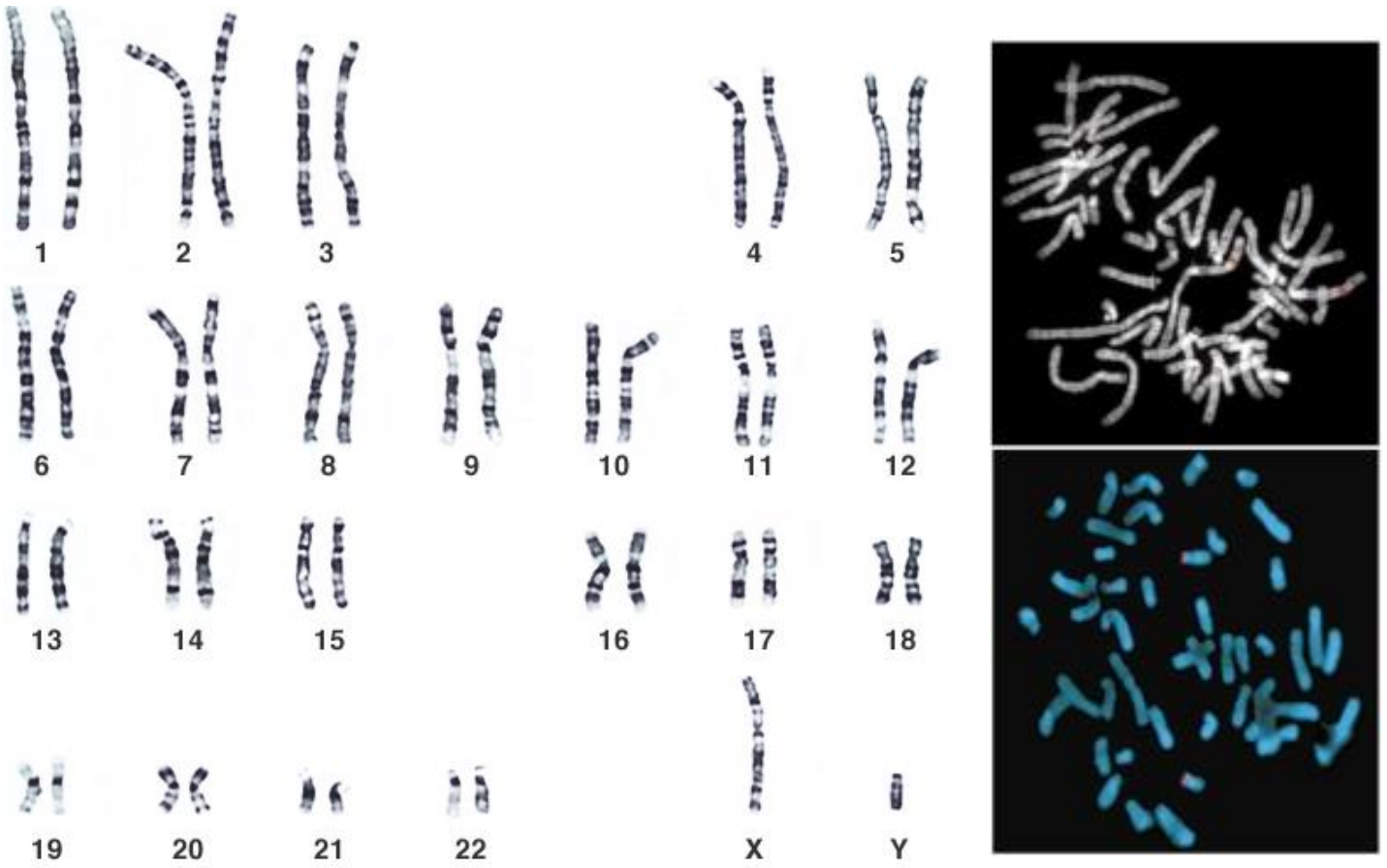



2- The **detection of the label**.



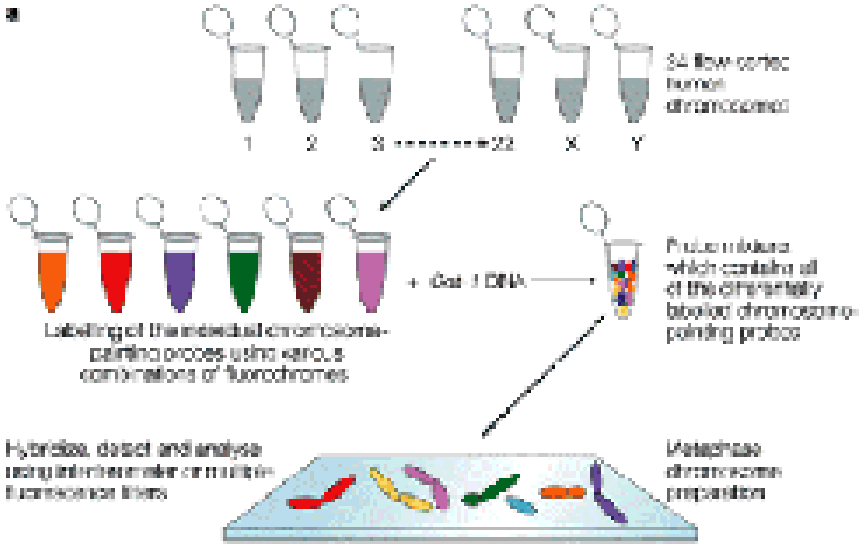
# IN SITU HYBRIDIZATION

## important tool for cytogenetic analysis

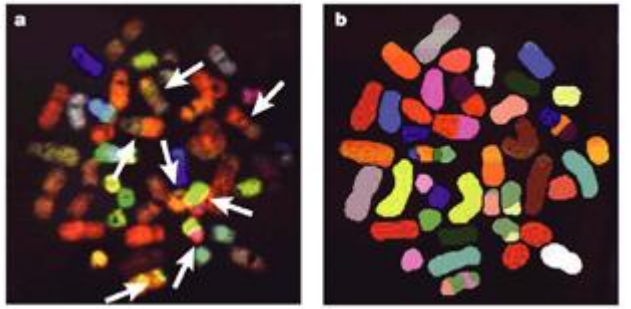


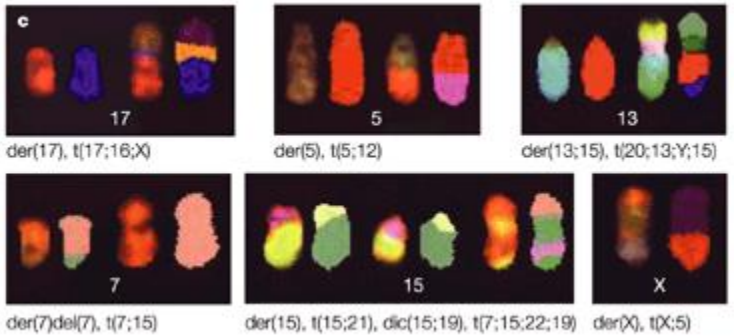

HUMAN CHROMOSOMES IN METAPHASE

# SPECTRAL CARYOTYPING

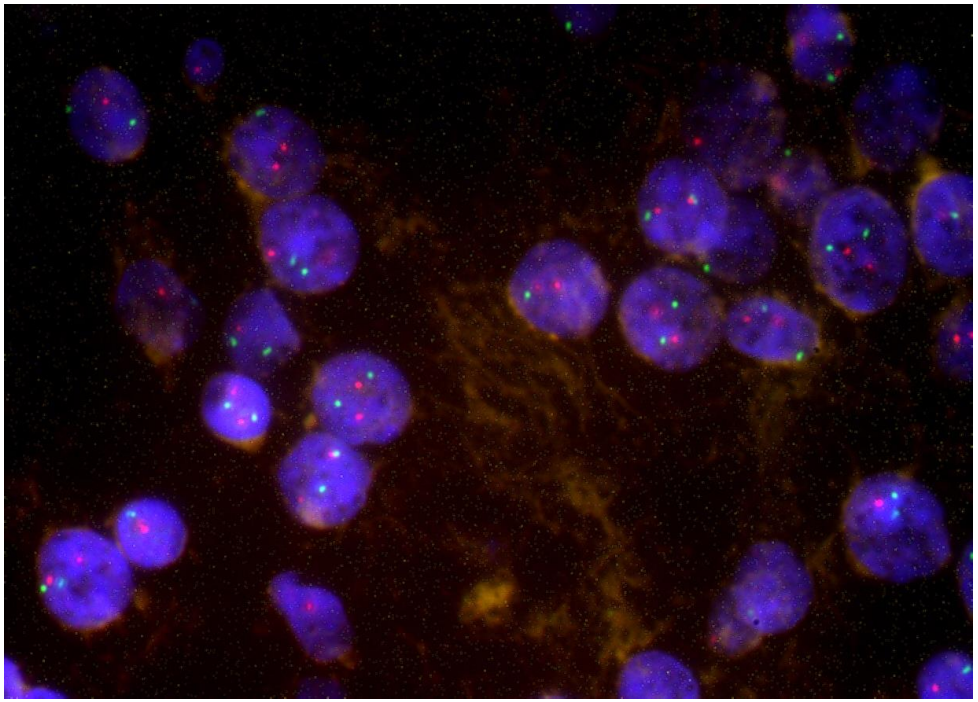


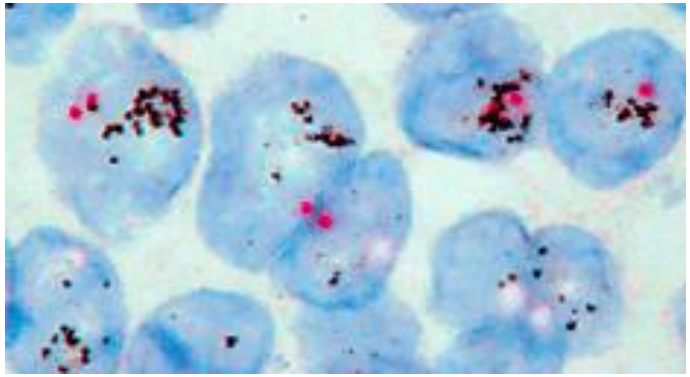

Nature Reviews | Genetics



# ISH FOR PATHOLOGY

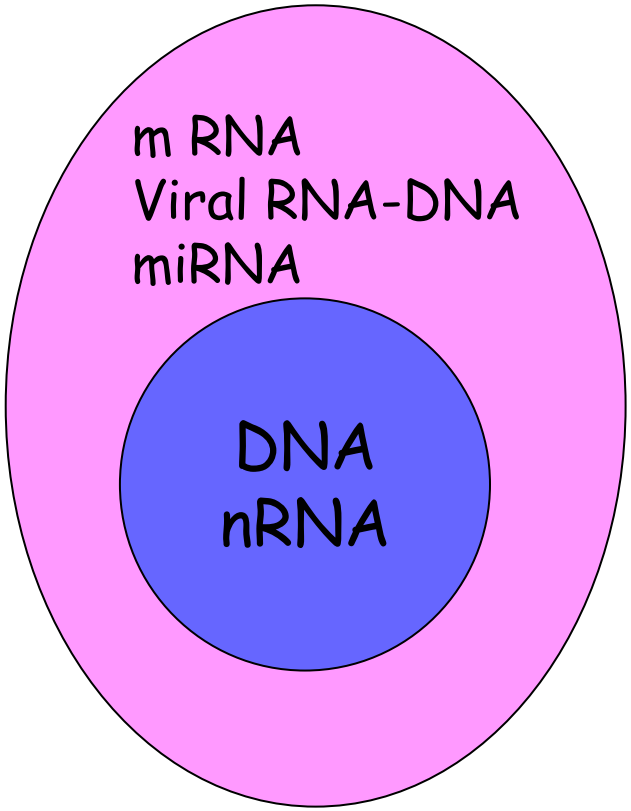




**Chromosomes on interphase**

# WHAT COULD BE SEEN BY USING ISH?

GENE COPY NUMBER INCREASE (AMPLIFICATIONS)
GENE OR CHROMOSOME LOSS (DELETIONS)
GENE REARRANGEMENTS (TRANSLOCATIONS)
INFECTIOUS AGENTS
PROTEIN EXPRESSION (mRNA)
CHIMERISM (X, Y)
miRNA's



# PROBE TYPES

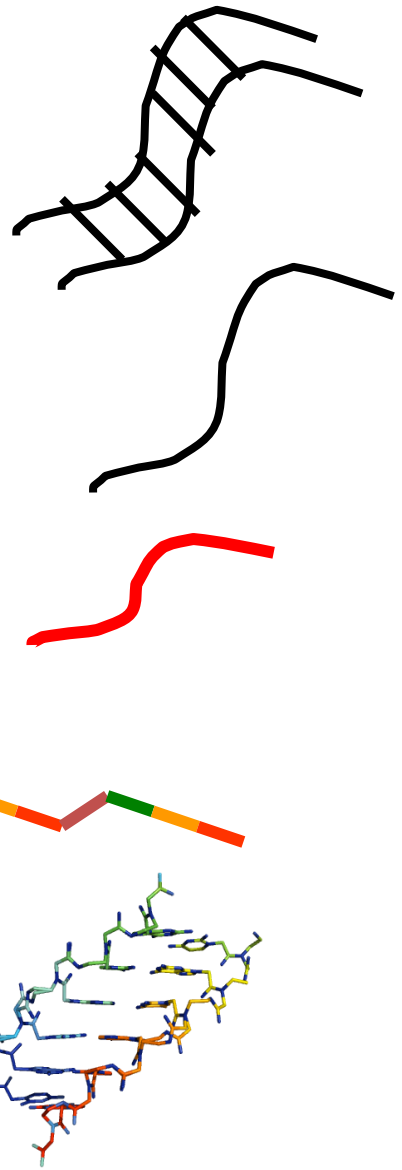
Double stranded DNA

Single stranded DNA

RNA

Oligonucleotide

PNA (pepetide nucleic acid)



# PROBE LABELS

1- Radioactive labeled probes

2- Non radioactive labeled probes

**Biyotin-Avidin-Fluorophore conjugated**

**Biyotin-Avidin-Enzyme** (Alkalin phophatase / HRP)

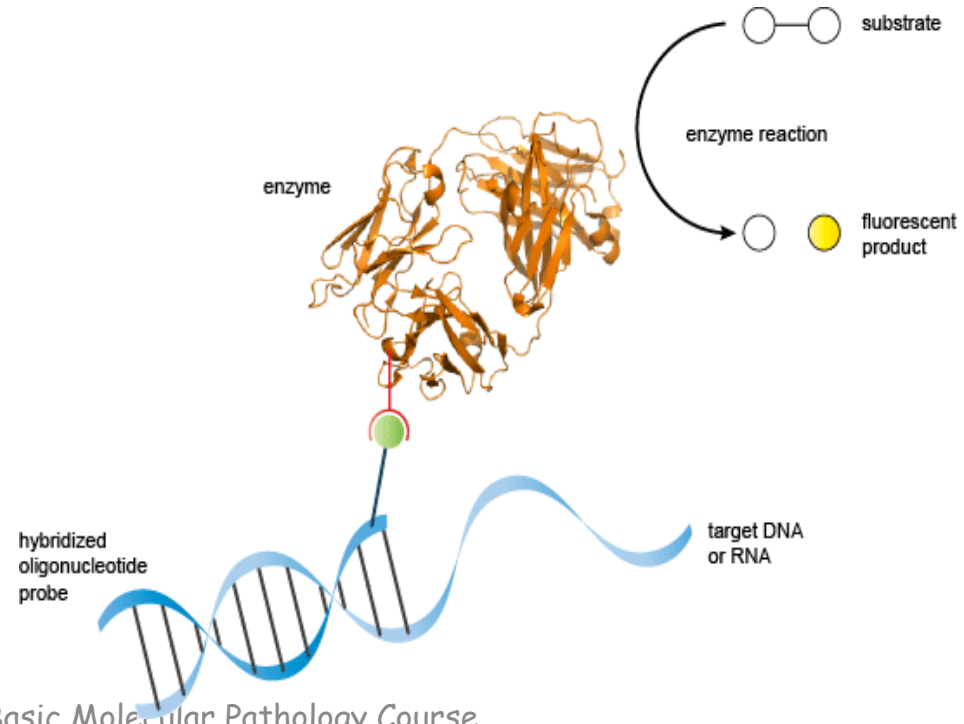
**Hapten** (Digoxigenin (DIG) , Dinitrophenyl (DNP))

**Fluorophore**

Enzyme
+
Substrate

⇩

Colourimetric,
Chemiluminescent
Fluorescent
product



# PROBE SYNTHESIS

The probes (DNA or RNA) usually prepared by polymerase enzyme-based methods

**Nick translation,**
**Random priming**
**PCR**

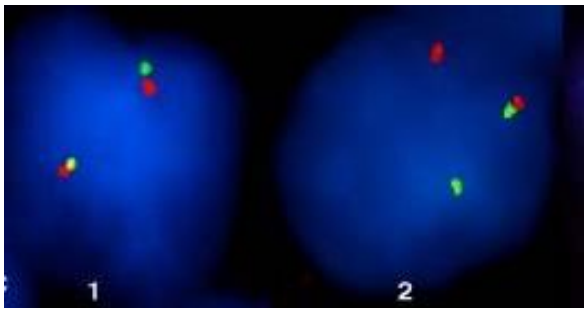
} incorporation of fluorescently-labelled deoxynucleotide triphosphates

The length of a DNA probe can be between 100 bp and 1000 bp.

**Longer probes** increase non-specific background fluorescence

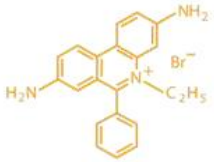
**Shorter probes** difficult to detect - low levels of labelling.

# Fluorescence in situ hybridization



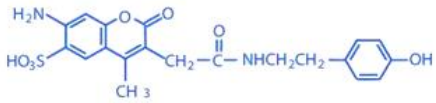

# (FISH)

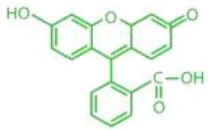
## Definition of Fluorescence



Ethidium bromide

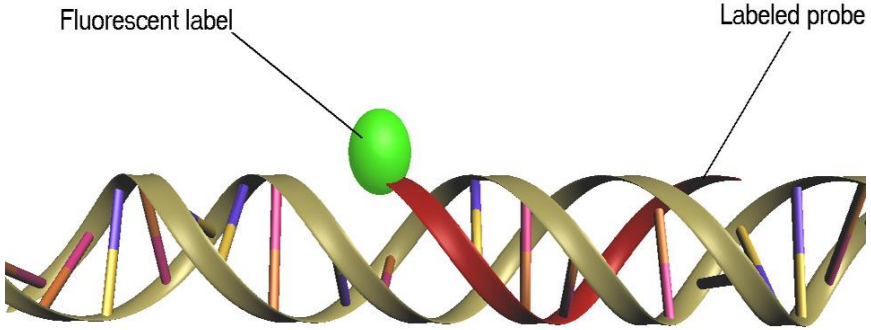
Alexa Fluor 350

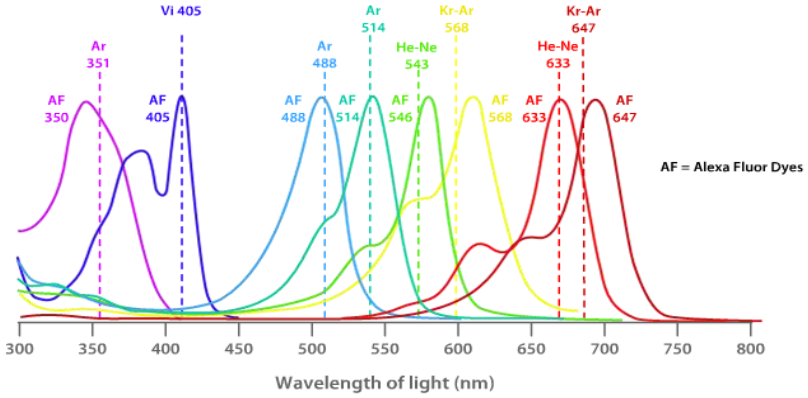




Fluorescein

How and why do these dyes and stains emit different colors of light?



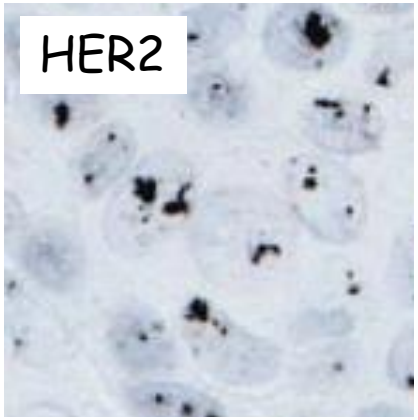





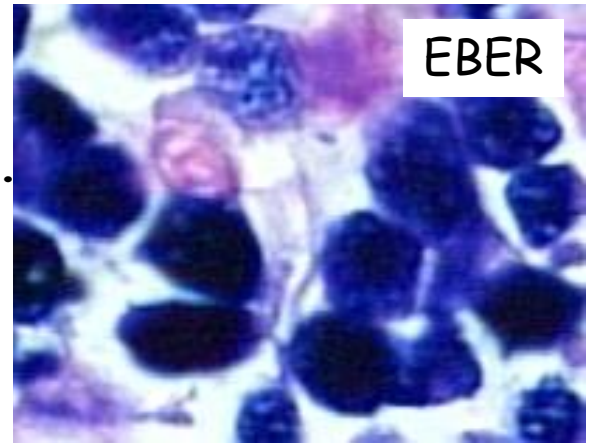

http://www.lifetechnologies.com/tr/en/home/support/tutorials.html

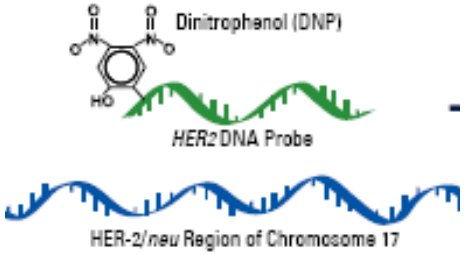
http://www.histalim.com/pages/en/in-situ-hybridization.php

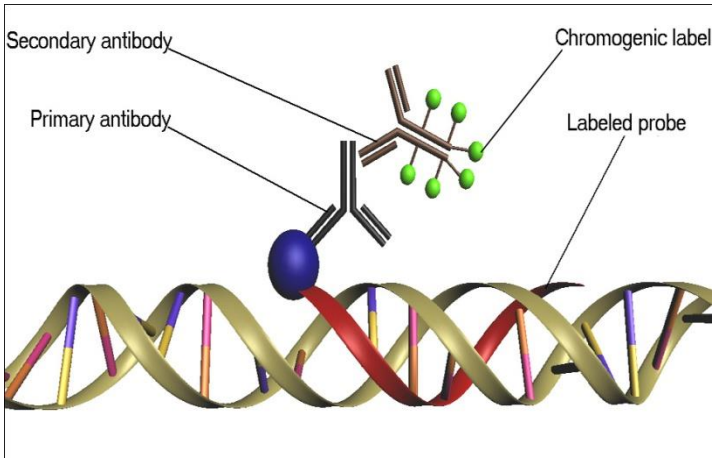
# Chromogenic in situ hybridization (CISH)

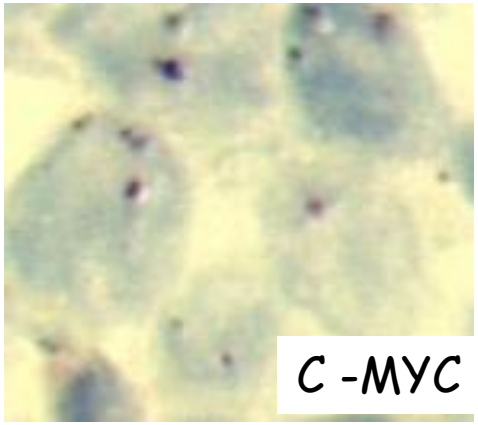


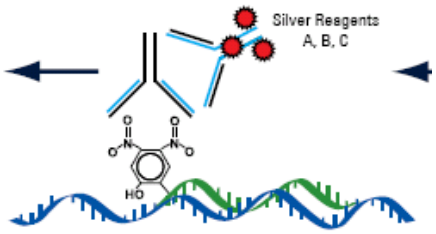

**Less sensitive than FISH.**
**Better for seaching amplification signals**



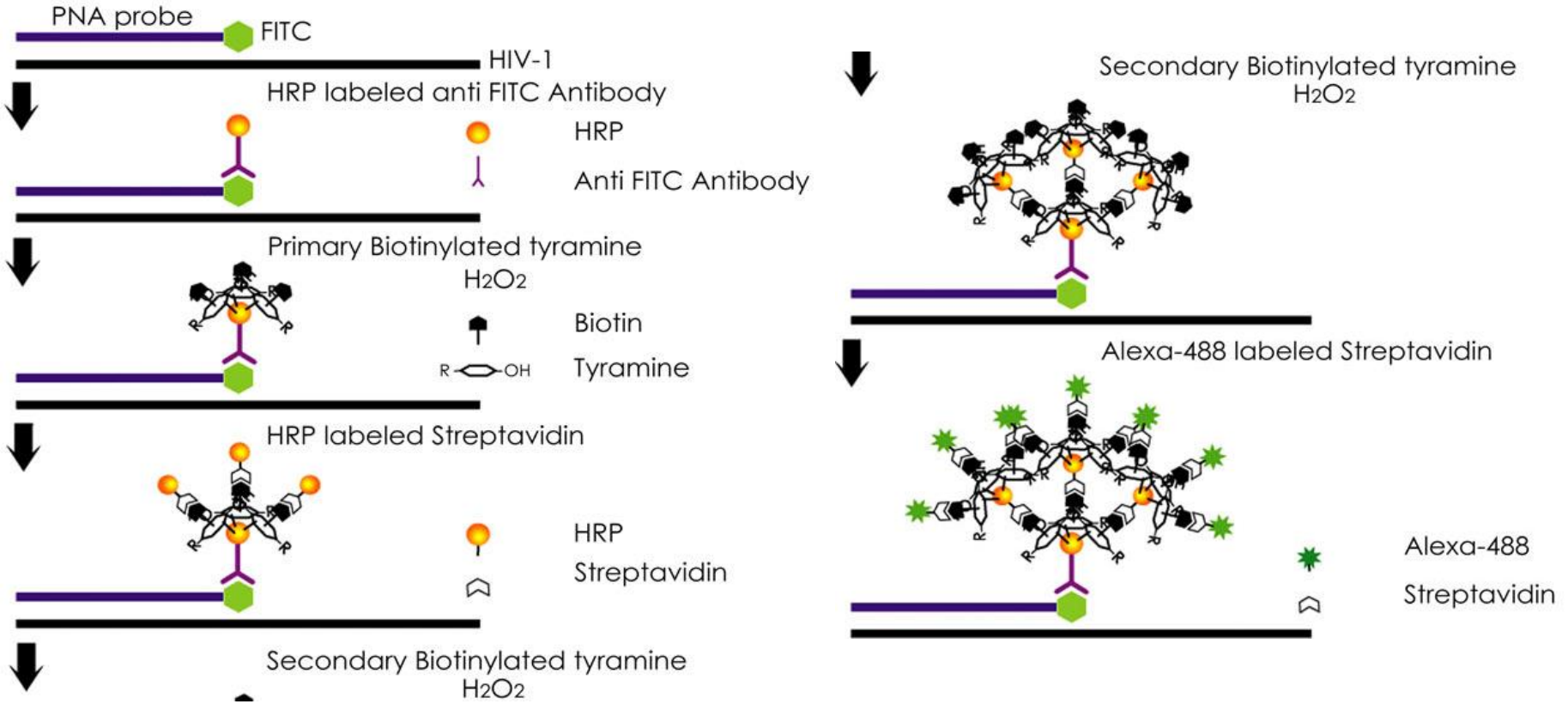




http://www.histalim.com/pages/en/in-situ-hybridization.php

# PROBE SIGNAL AMPLIFICATION




Hagiwara T, 2006,Methods in Mol Biol 326 Ch 9, ED: Darby IJ

# VISUALIZATION OF THE HYBRIDISATION

The target must be accessible by the probe

The target must retain in situ, not degraded by nuclease enzymes.

DNA is more stable than RNA

# MOST FREQUENTLY USED MATERIALS FOR ISH APPLICATIONS

- CULTURED CELLS
- ASPIRATION SMEARS
- FRESH FROZEN TISSUE SECTIONS
- PARAFFIN SECTIONS

# IN SITU HYBRIDISATION PROCEDURE ON PARAFFIN TISSUE

- TISSUE PRETREATMENT
- PROBE PREPERATION
- PROBE INCUBATION
- WASHING
- MOUNTING
- EXAMINATION

# SPACE NEEDED FOR THE PROCEDURE

# Full automated systems


# TISSUE FIXATION

- **Probe penetration**
- **RNA damage**
- **DNA damage**
- **Preservation of the tissue architecture**

**Most frequently used fixatives**

Alcohol–acetic acid : allows probe penetration / RNA damage

Gluteraldehide : poor probe penetration / RNA preservation

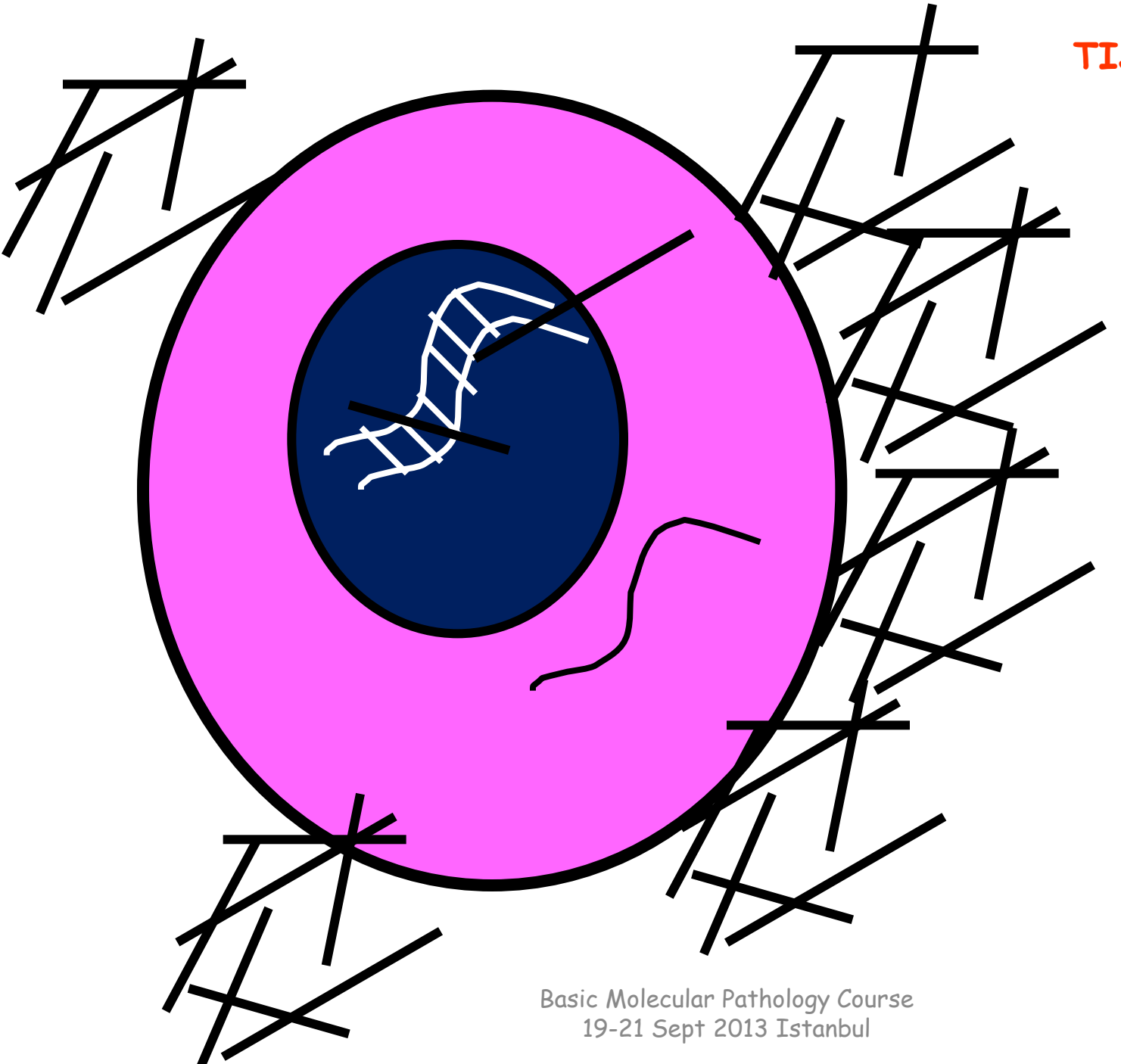
**Paraformaldehyde (%4) Formalin (%10)** 

# PRETREATMENT OF THE TISSUE BEFORE ISH

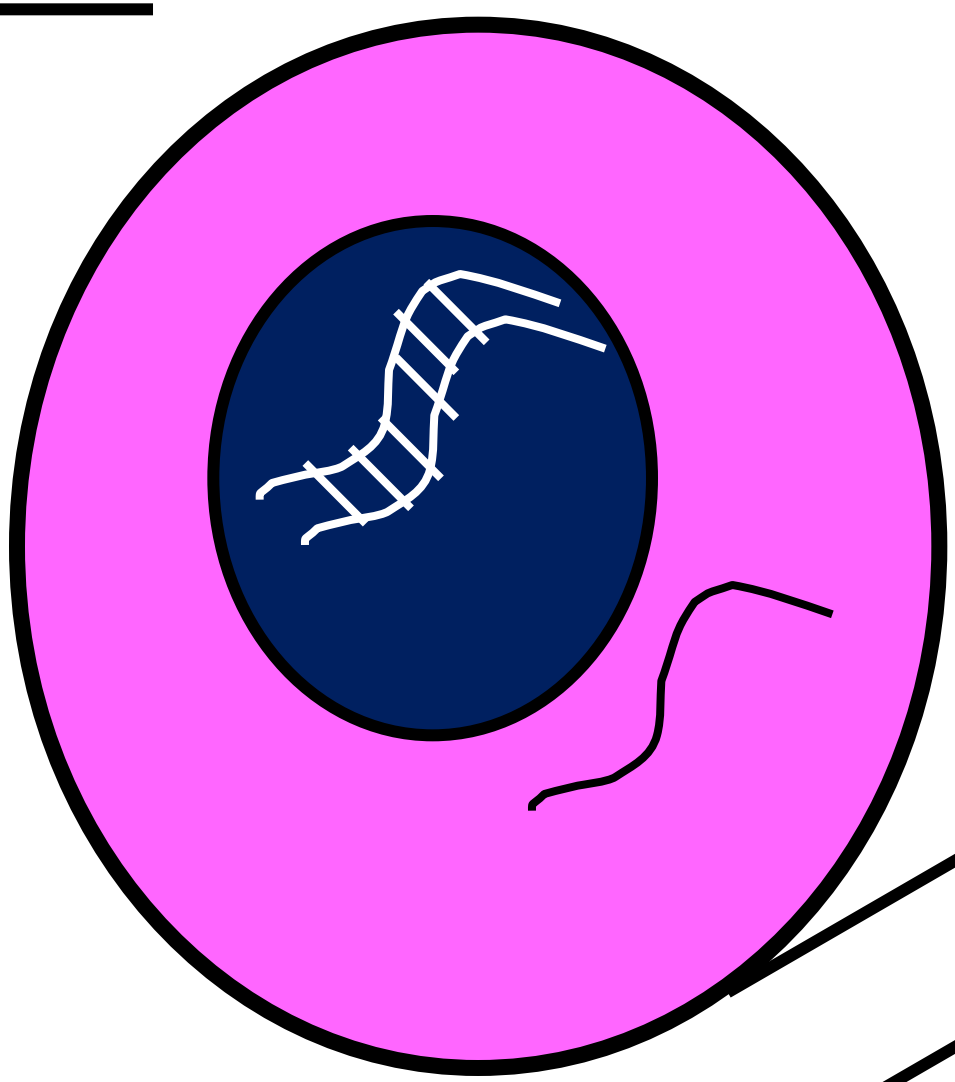
- DEPARAFFINIZATION
- INCREASING PROBE PENETRATION (HCl, enzyme, heat)
- DENATURATION
- STABILIZATION OF DENATURED DNA

# DEPARAFFINIZATION

# TISSUE



# INCREASING PROBE PENETRATION

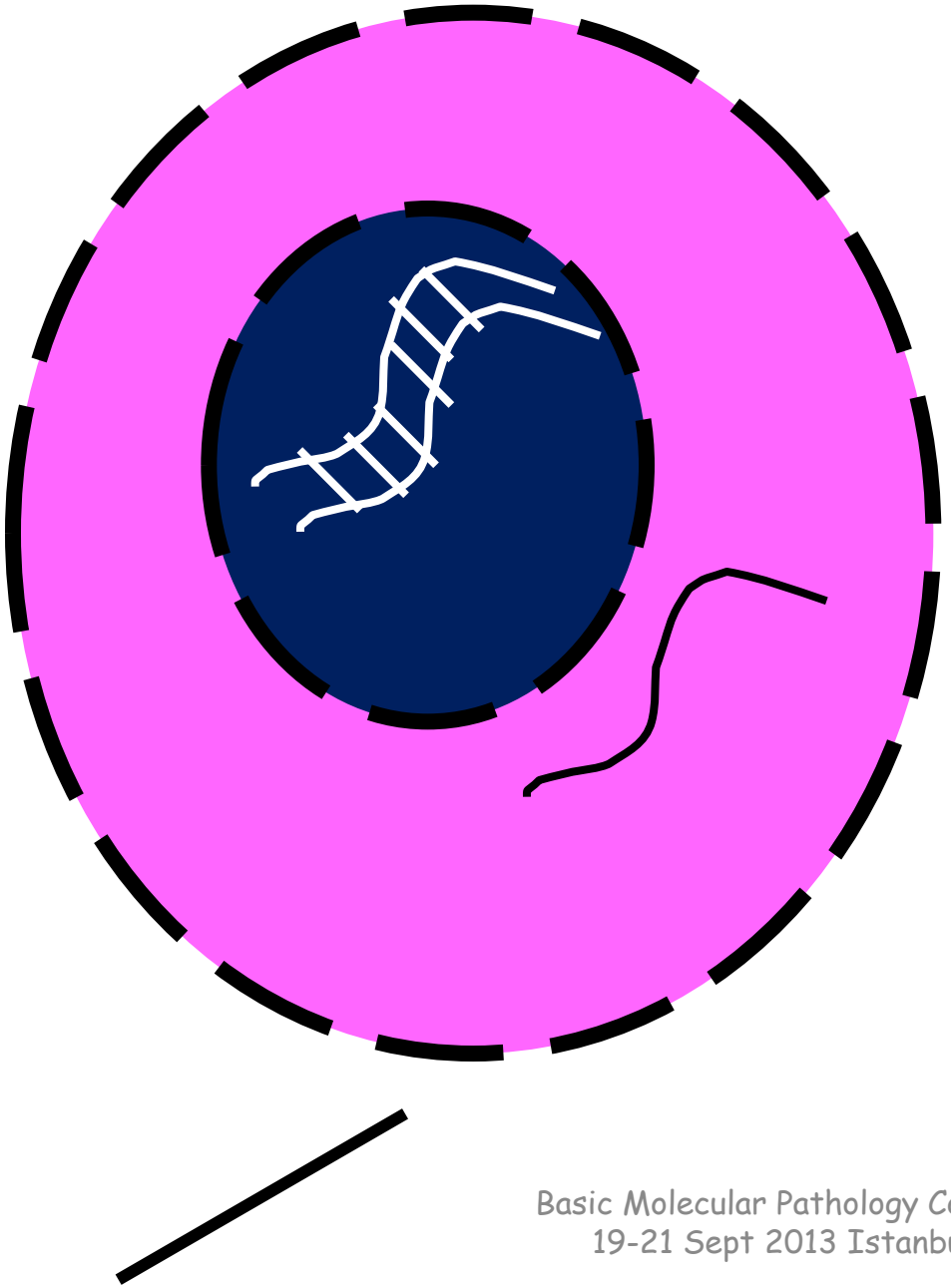


## TISSUE PRETREATMENT

Removing lipids and some other matrix matters

**Detergents & HCl**

# INCREASE PROBE PENETRATION

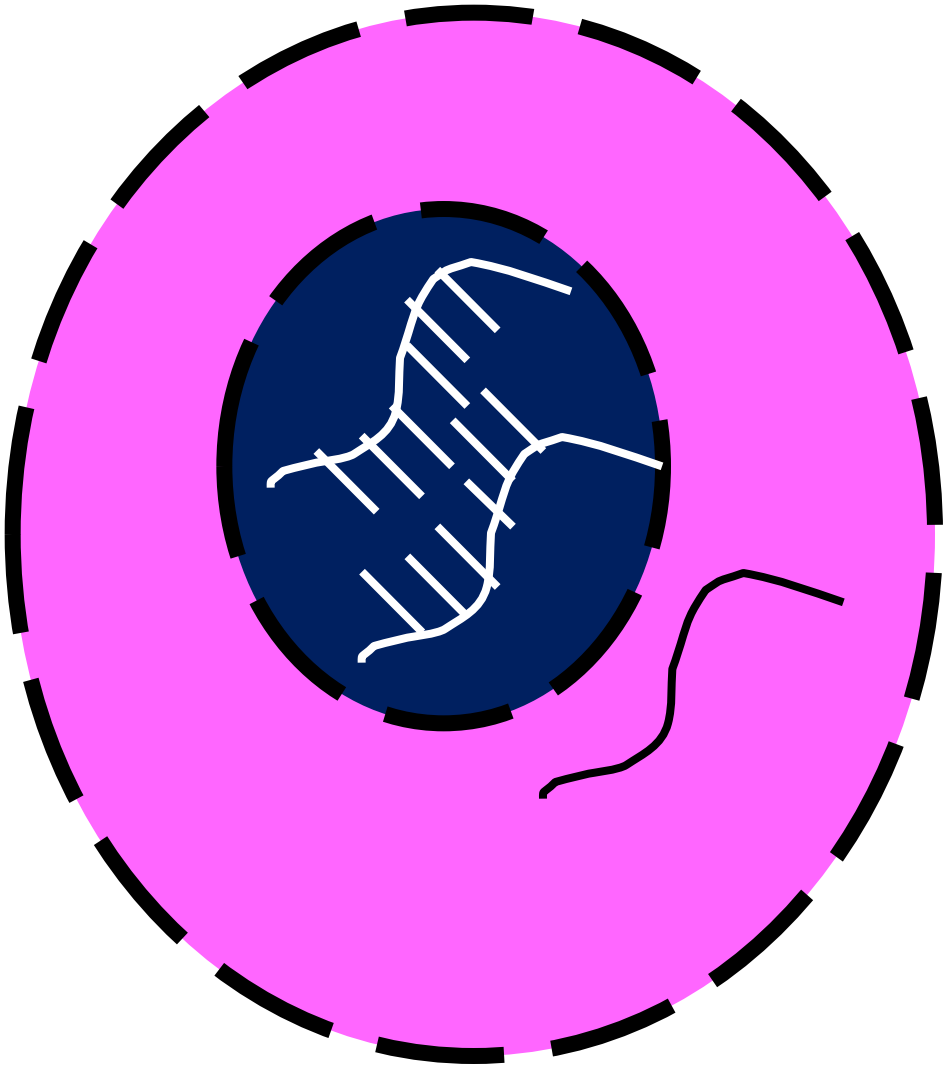


## TISSUE PRETREATMENT

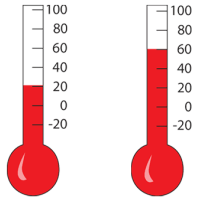
*Removing matrix proteins
*Making pores on cytoplasmic and nuclear membranes

**Detergents & Enzyme**

# DENATURATION & STABILIZATION OF GENOMIC DNA



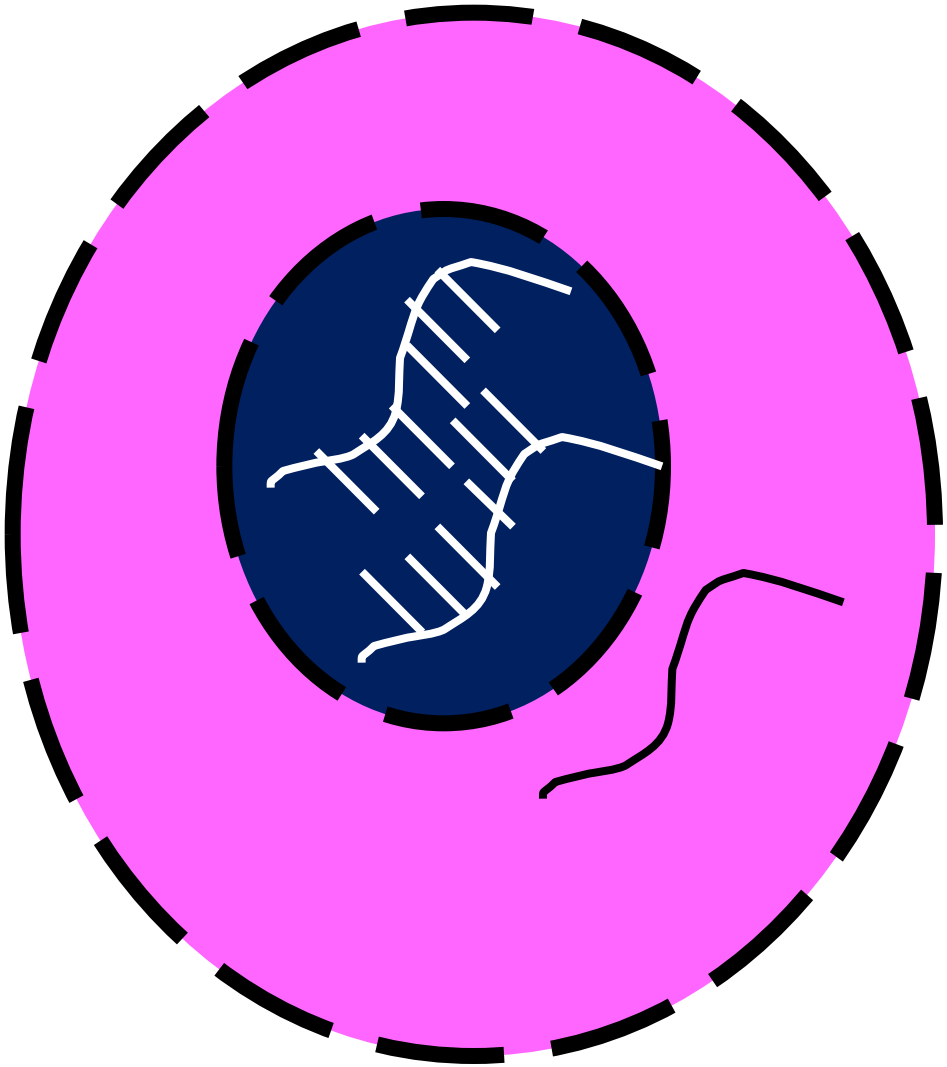
-HEAT




+

- Buffers containing Formamide

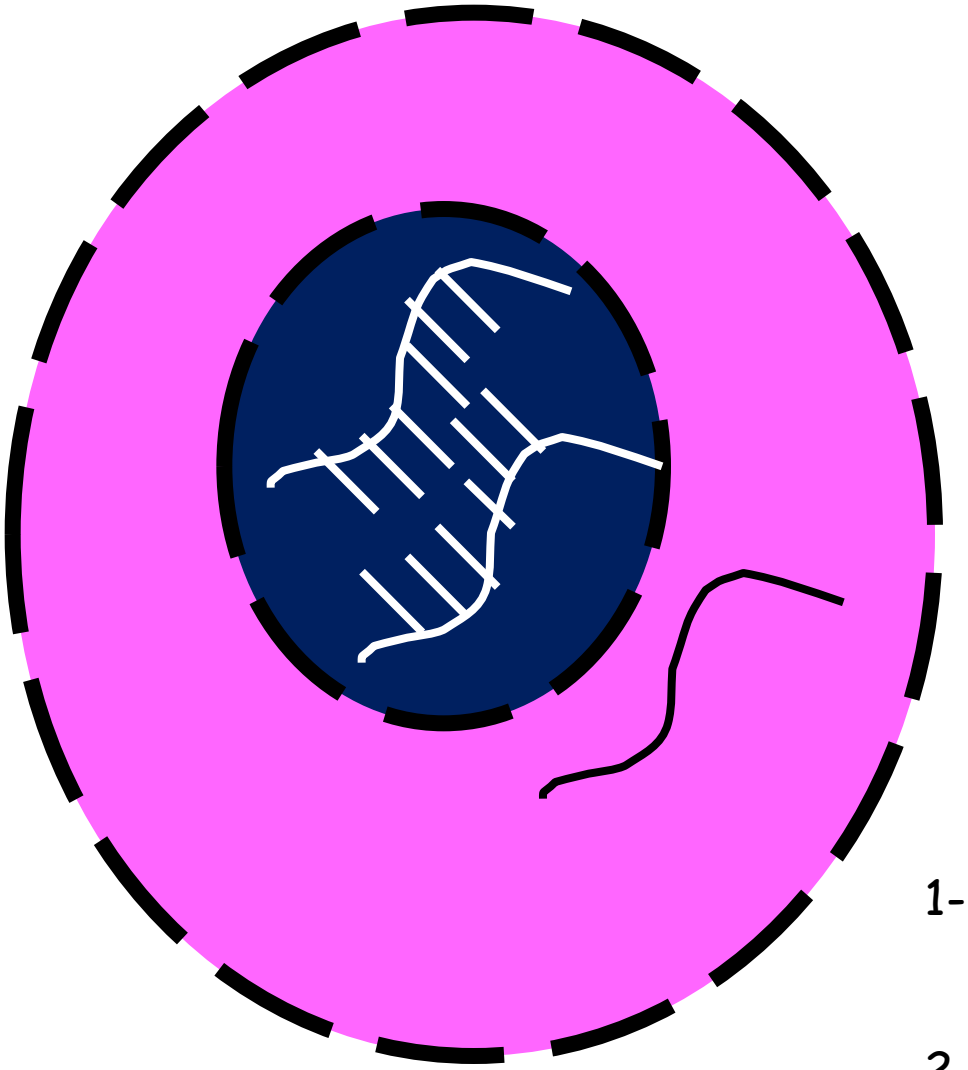
# PROBE INCUBATION

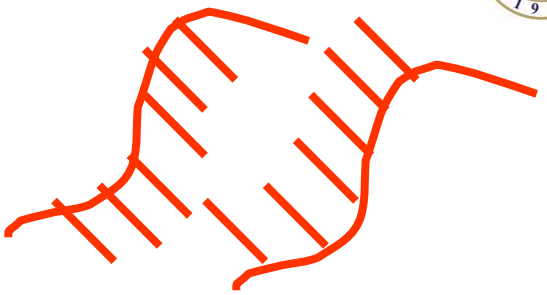


1. SINGLE STRANDED PROBE

2. DOUBLE STRANDED PROBE

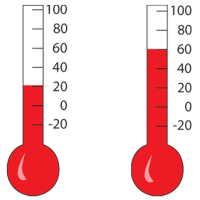
# DENATURATION & STABILIZATION OF THE DOUBLE STRANDED PROBES





-HEAT

+

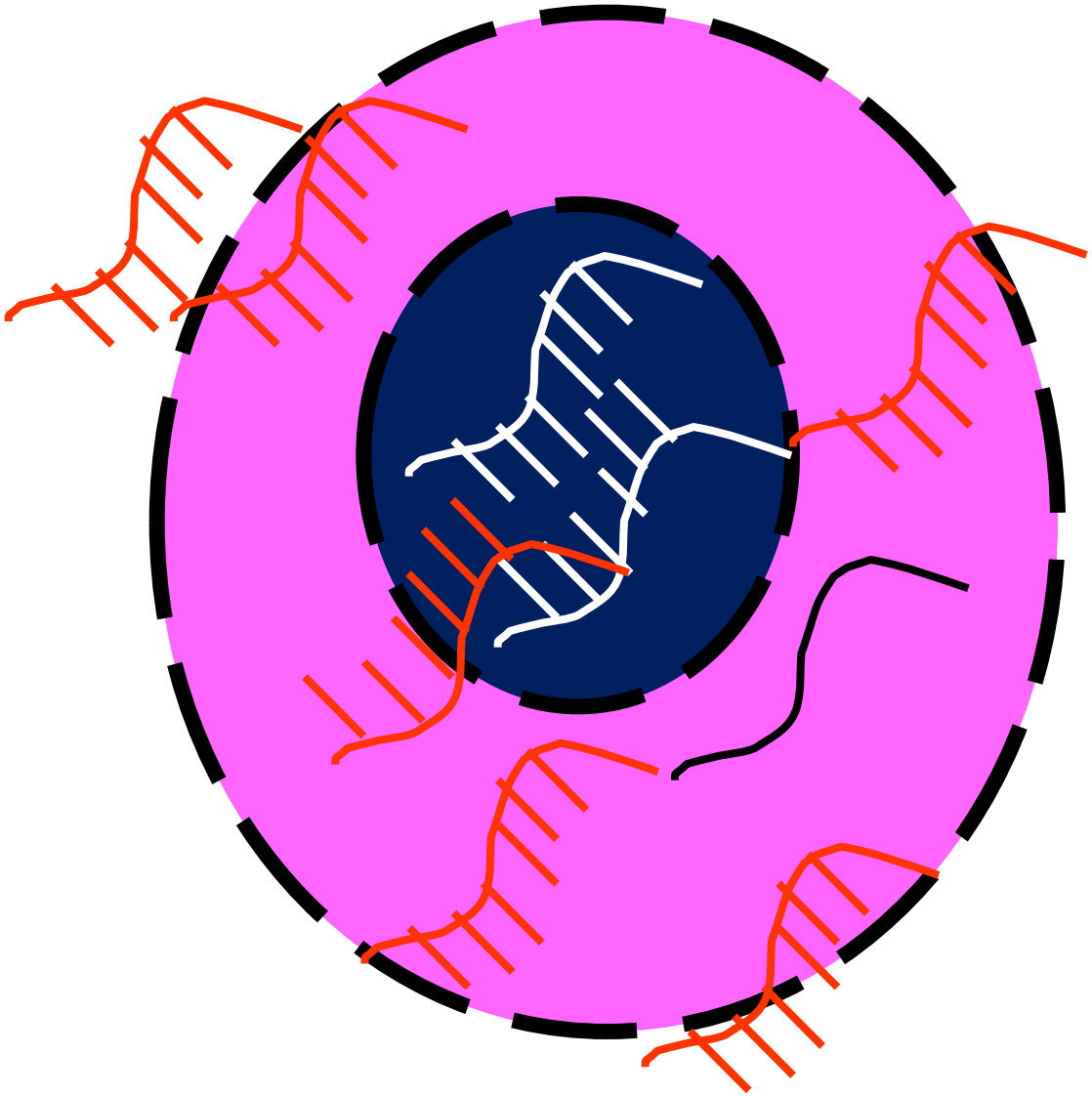


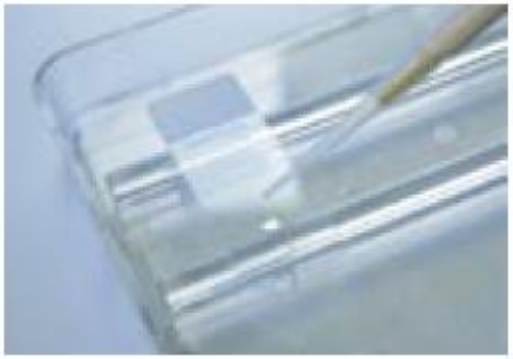

- Buffers containing **Formamide**

1-SEPERATE

2- IN SITU ON THE TISSUE

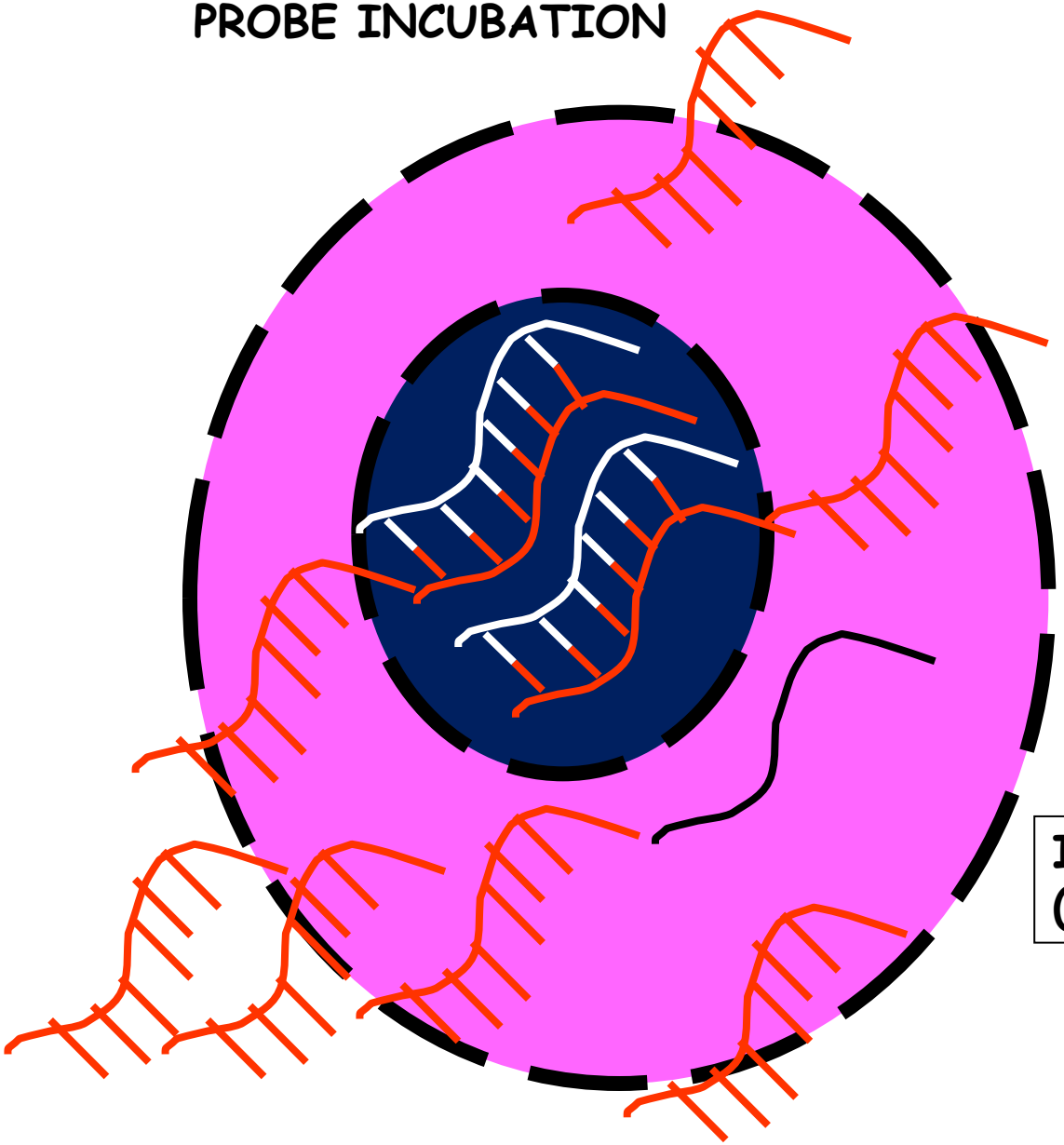
# PROBE INCUBATION



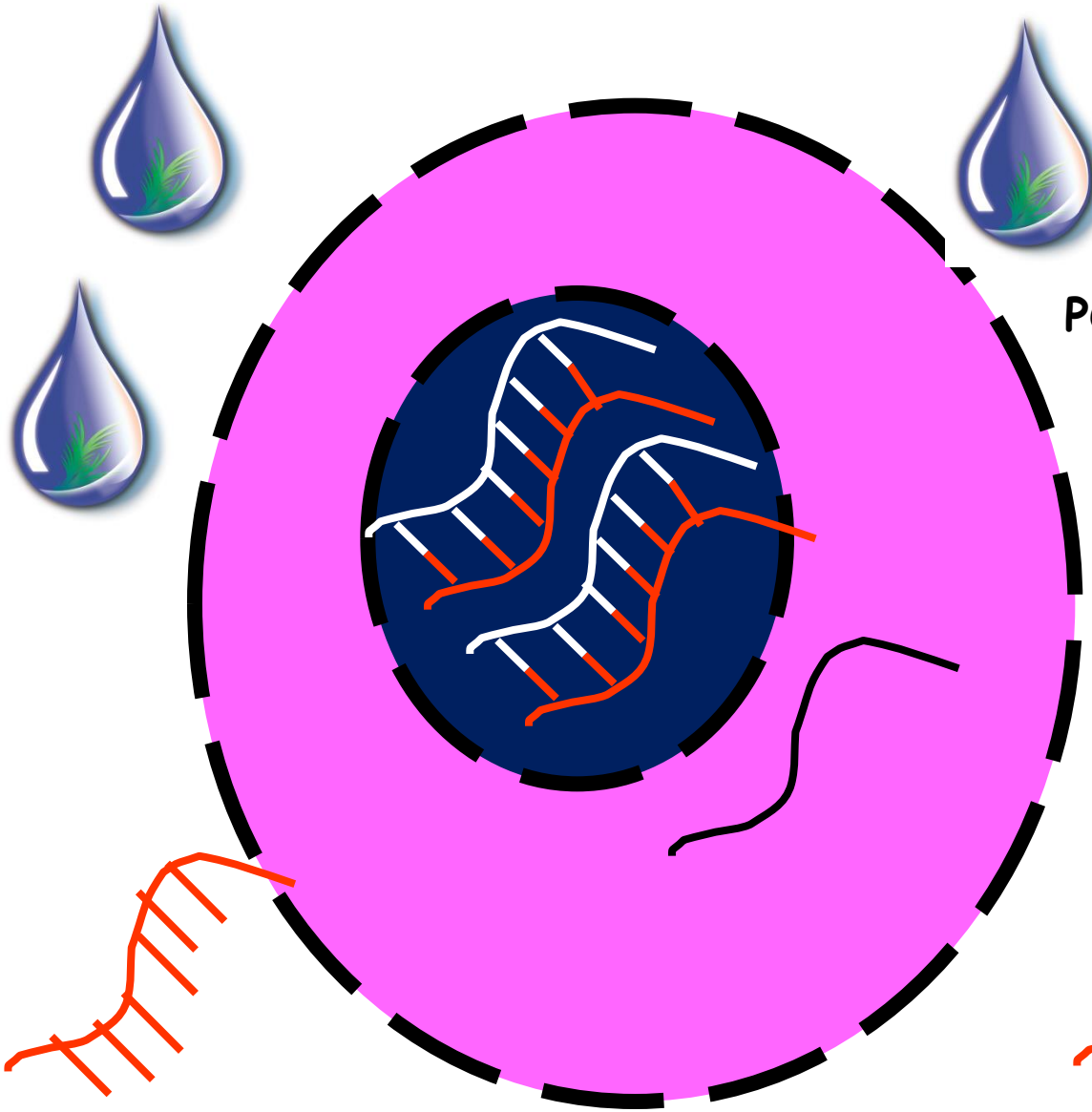


TIME (4-24 hr)
TEMPERATURE
HUMIDITY

# PROBE INCUBATION



**INCUBATION TEMPERATURE (37-42°C)**

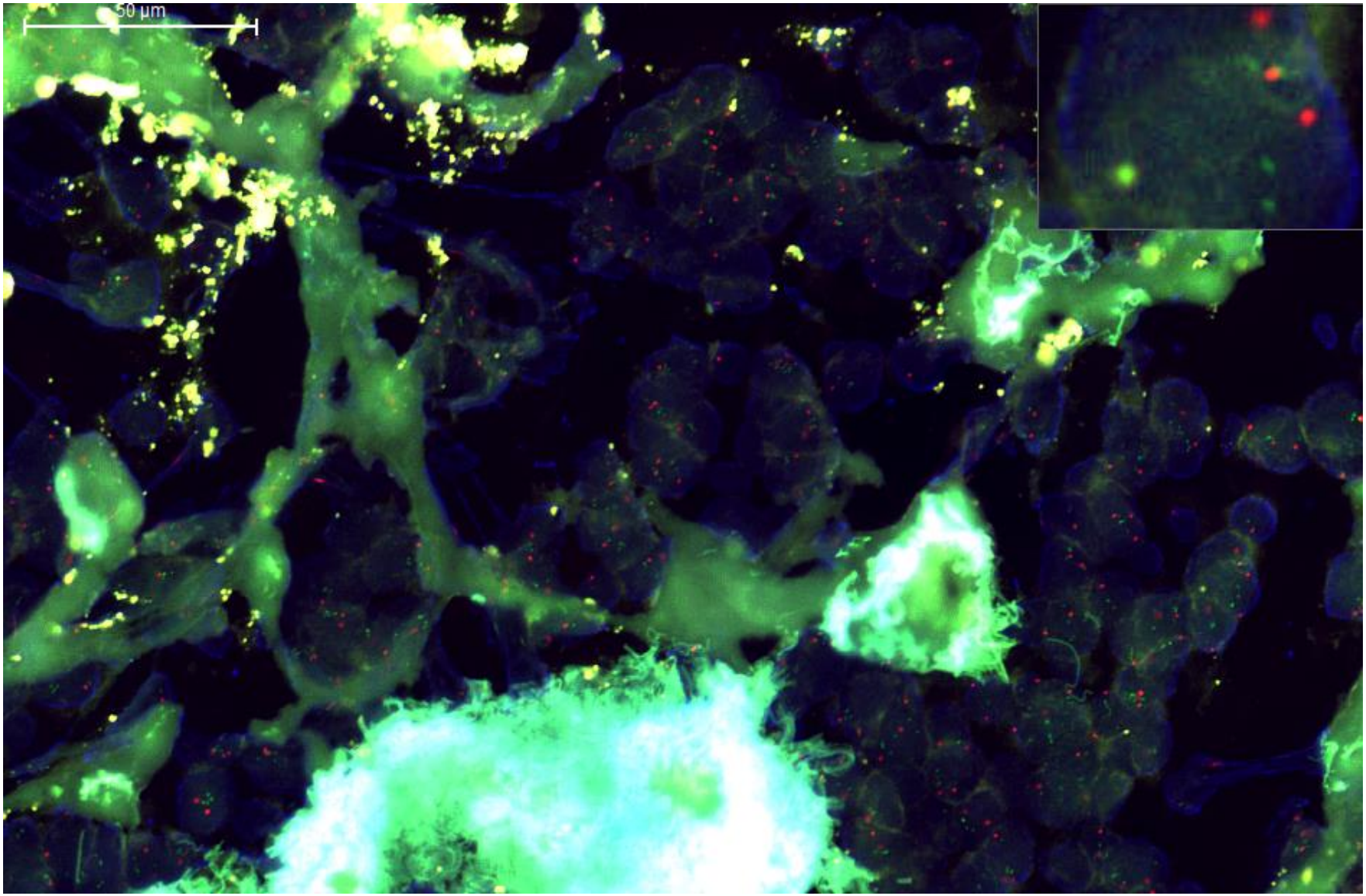


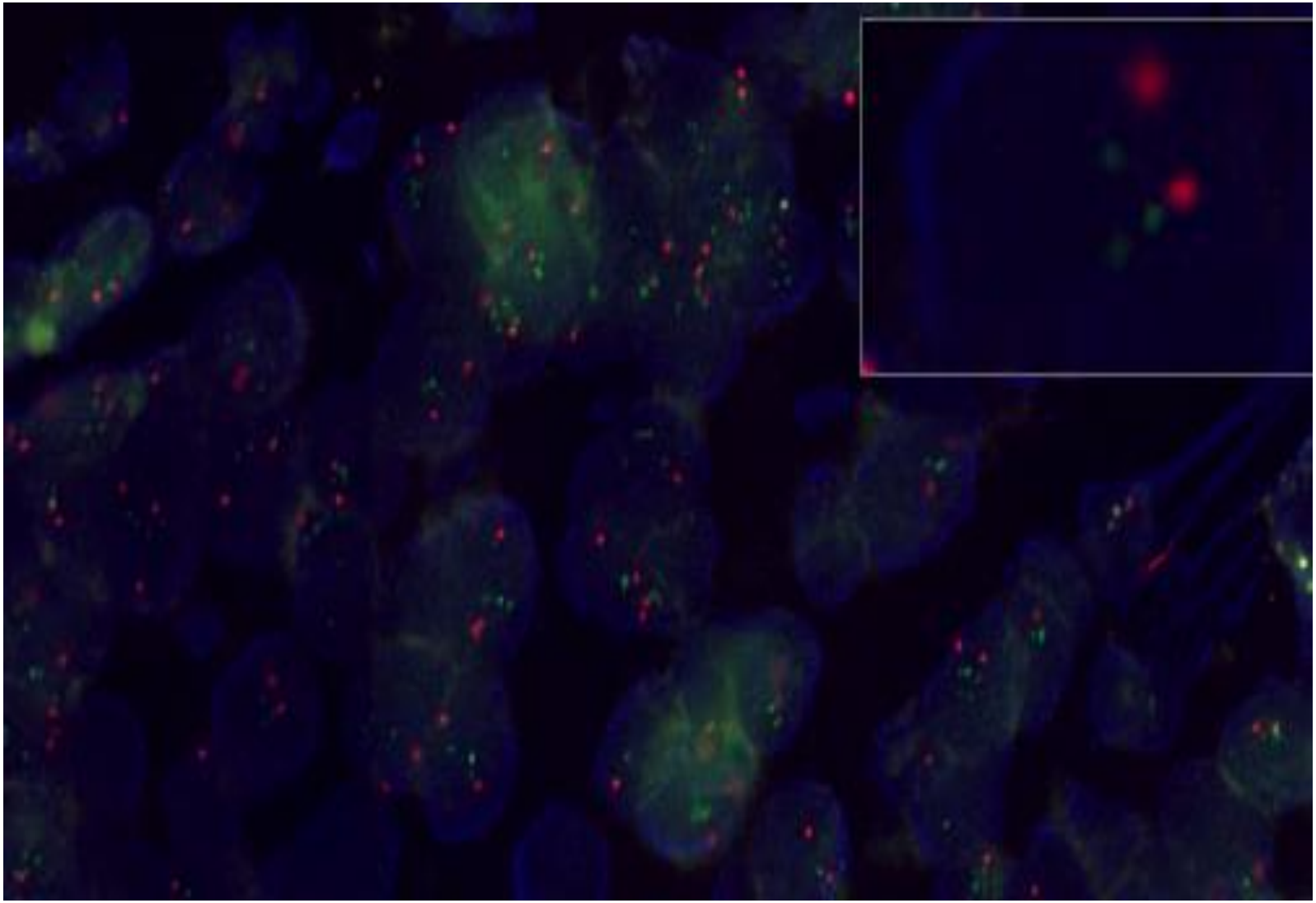
## POST INCUBATION WASHING

**MOUNTING**

**+**

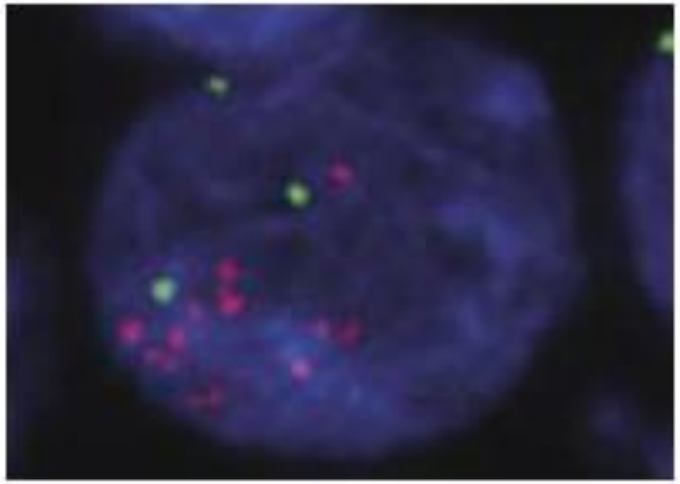
**SEALING**


50 µm

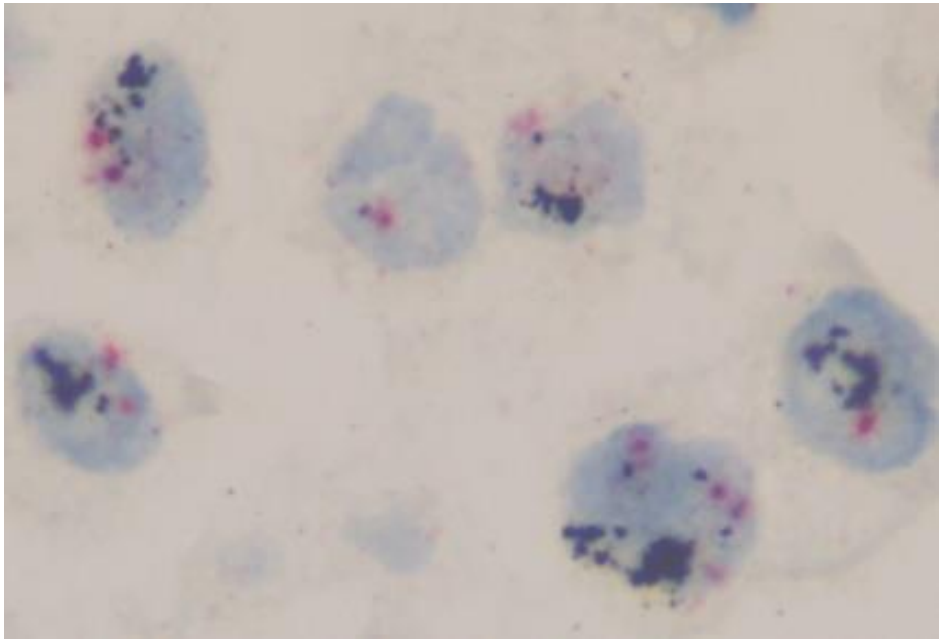


# EXAMINATION

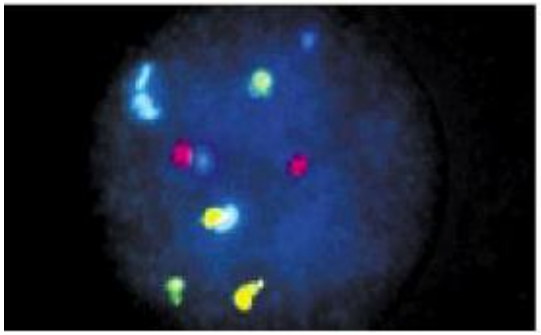
DARK FIELD



BRIGHT FIELD

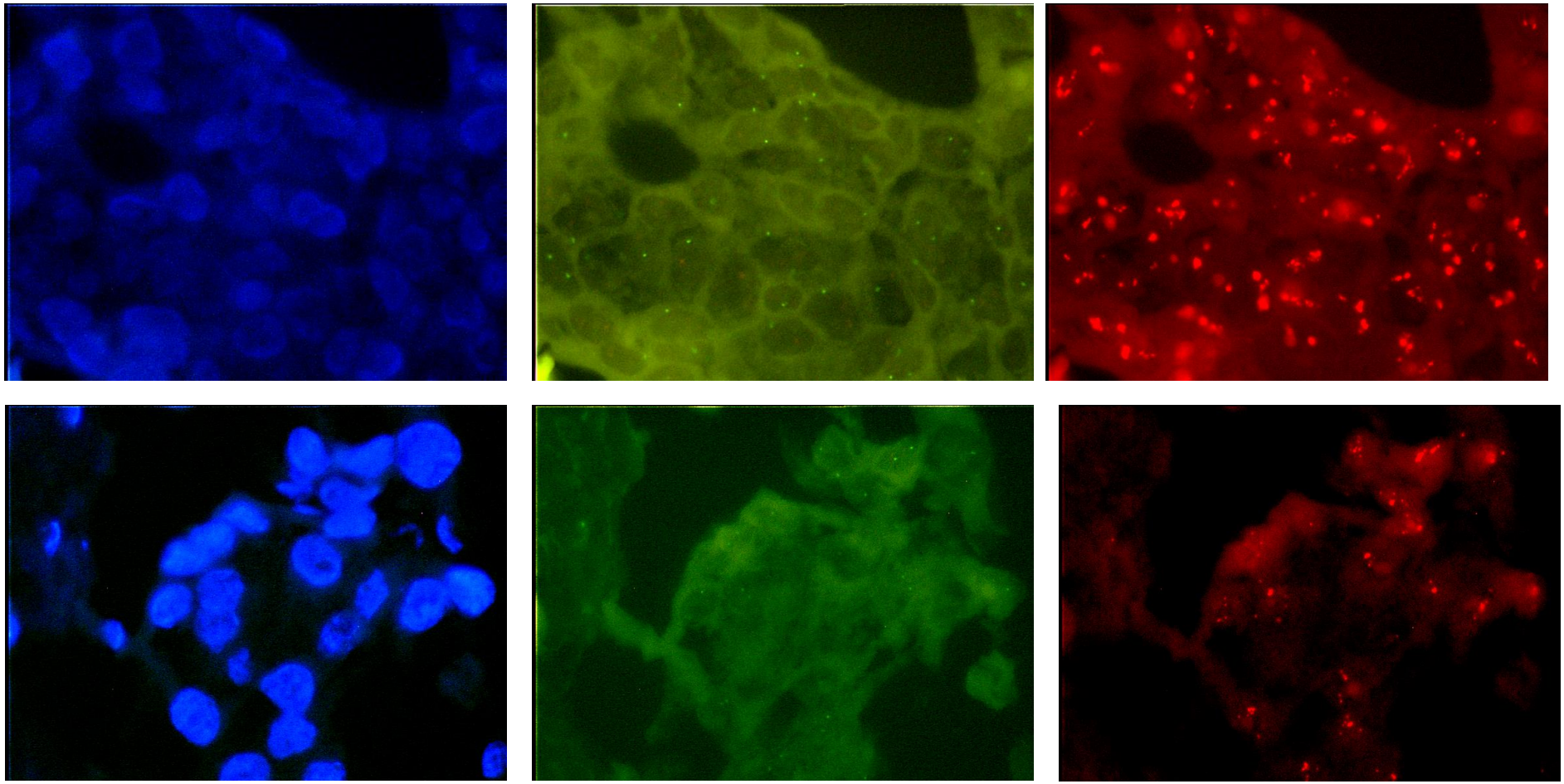


# MICROSCOPE FILTERS FOR FISH EXAMINATION



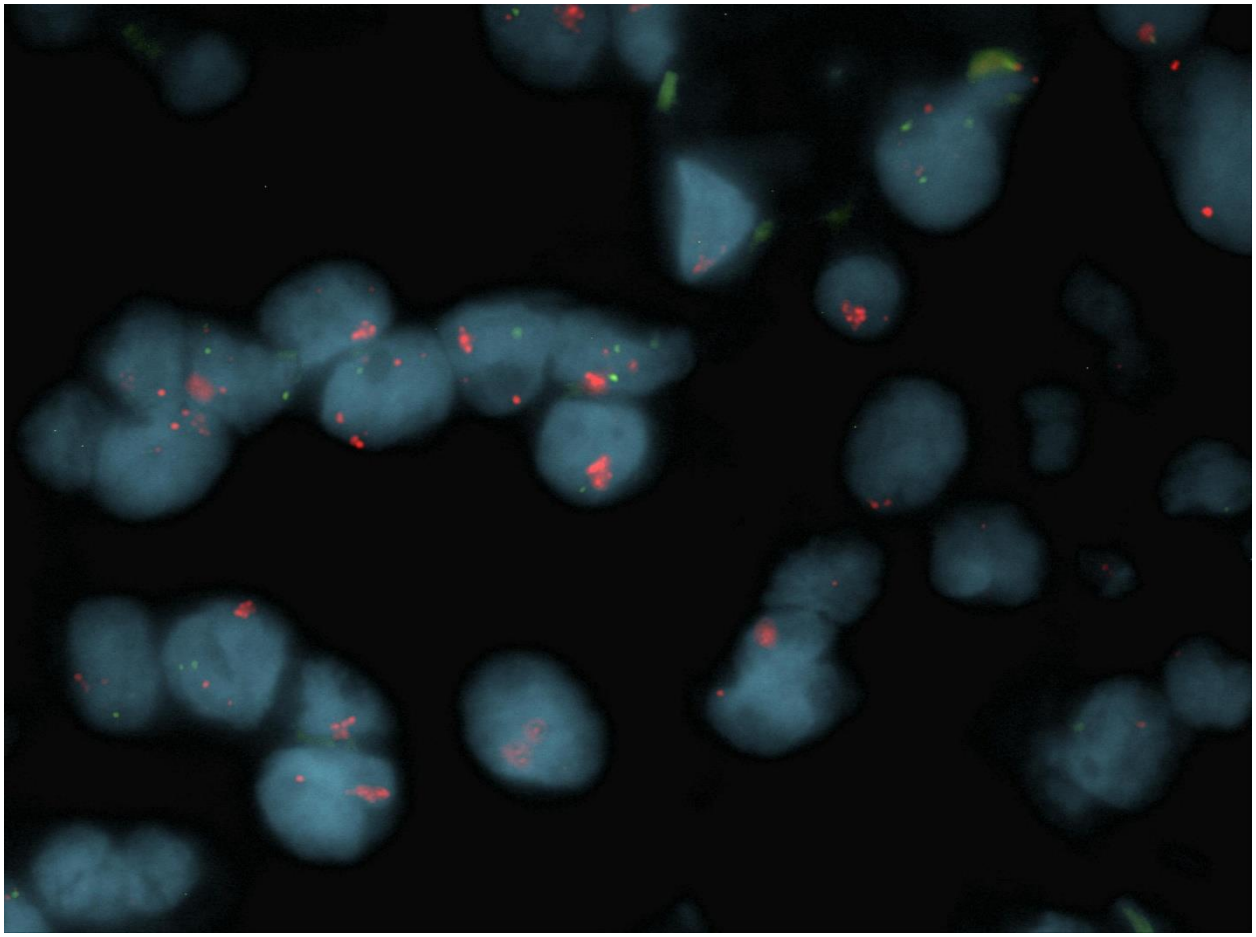
| | Exitation wave lenght | Emission wave lenght |
|---|---|---|
| FITC | 495nm | 520nm |
| TR | 596nm | 615nm |

# FISH (HER2)



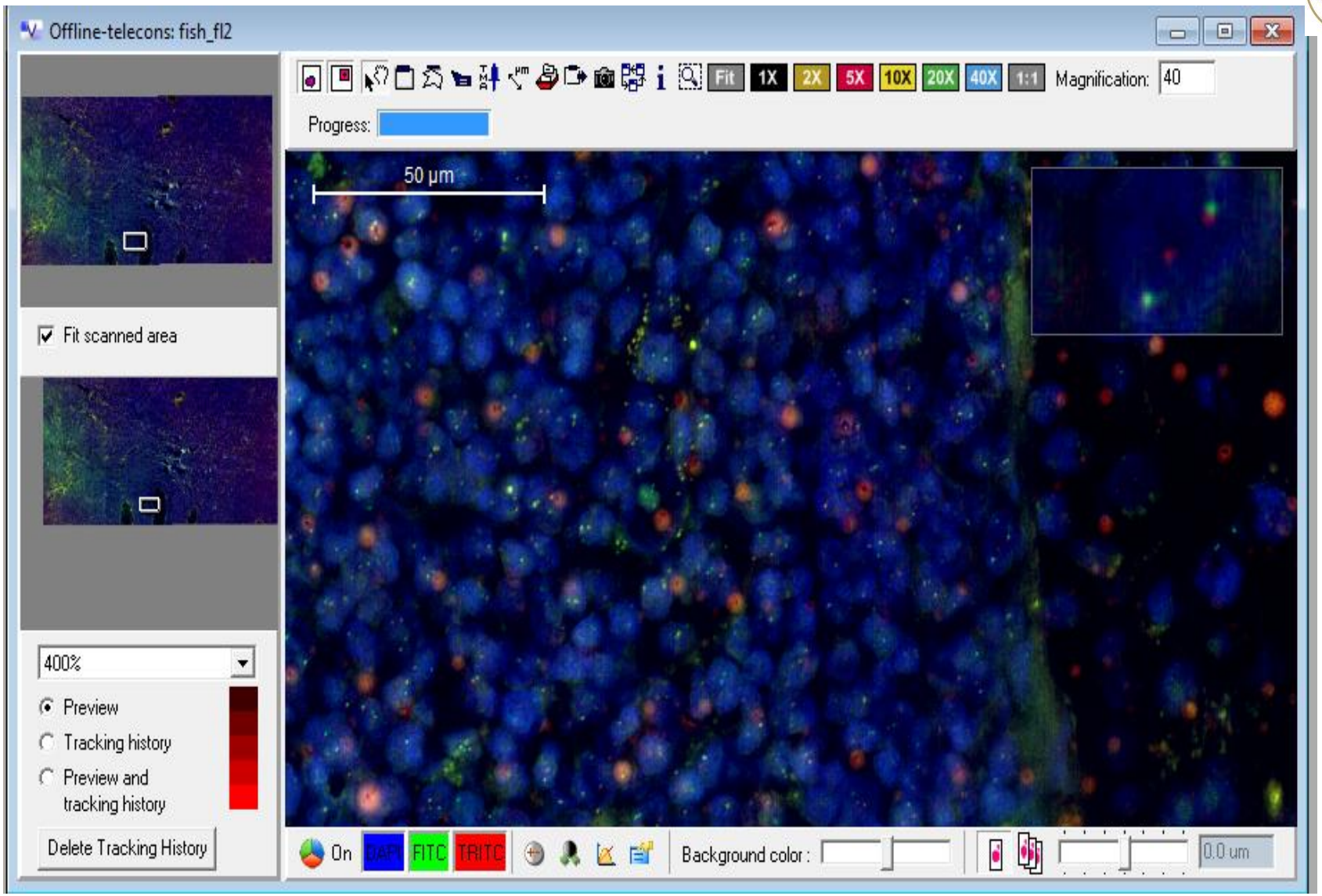
DAPI DAPİ FITC CEP17 TR HER2

# FISH (HER2)

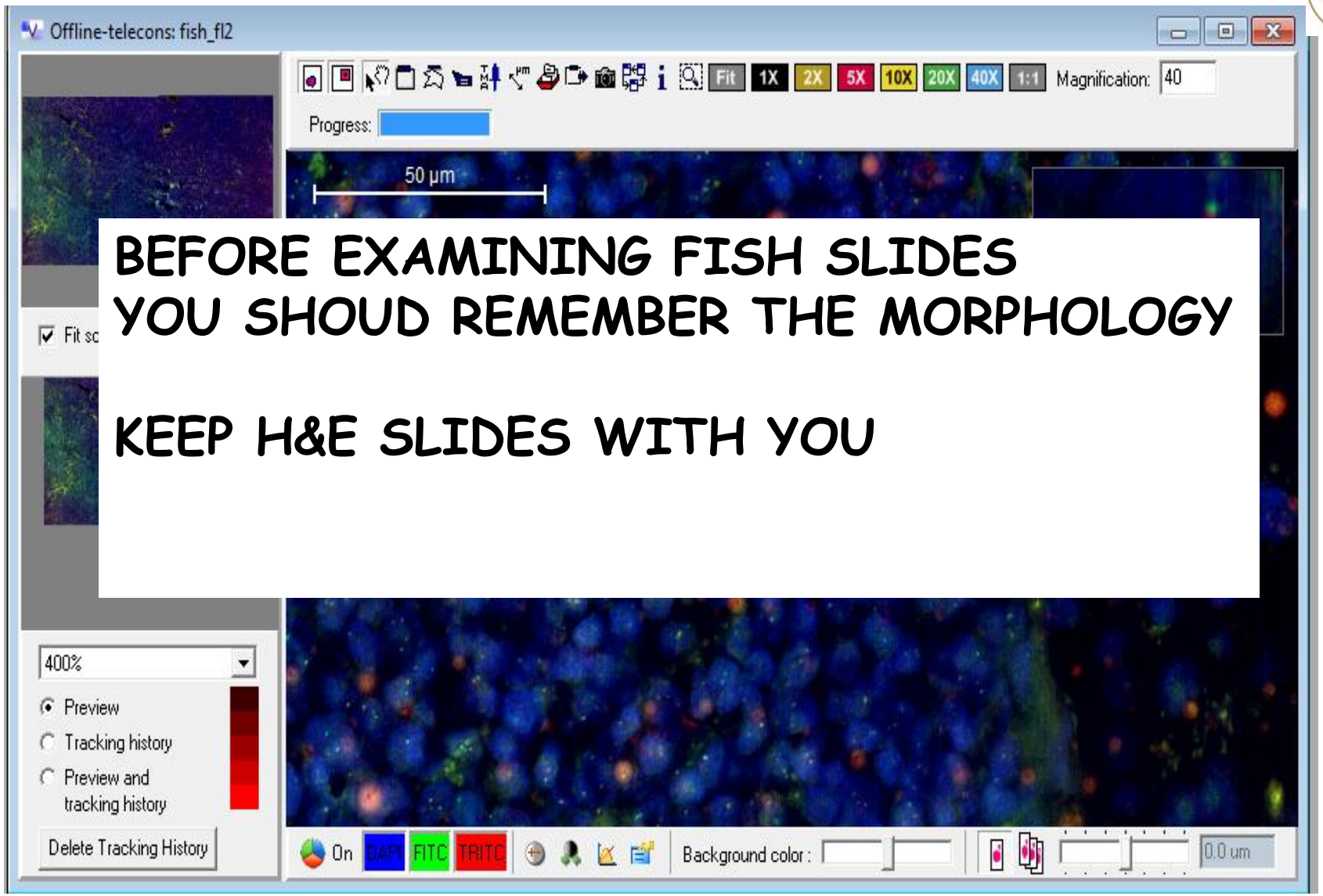


**DAPI / FITC/TR Triple filter image**

# EXAMINATION



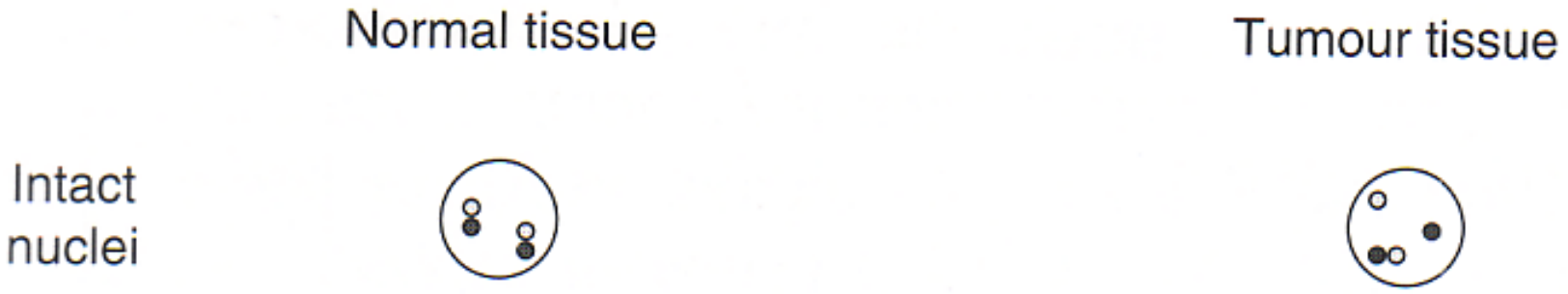
# EXAMINATION




BEFORE EXAMINING FISH SLIDES
YOU SHOUD REMEMBER THE MORPHOLOGY

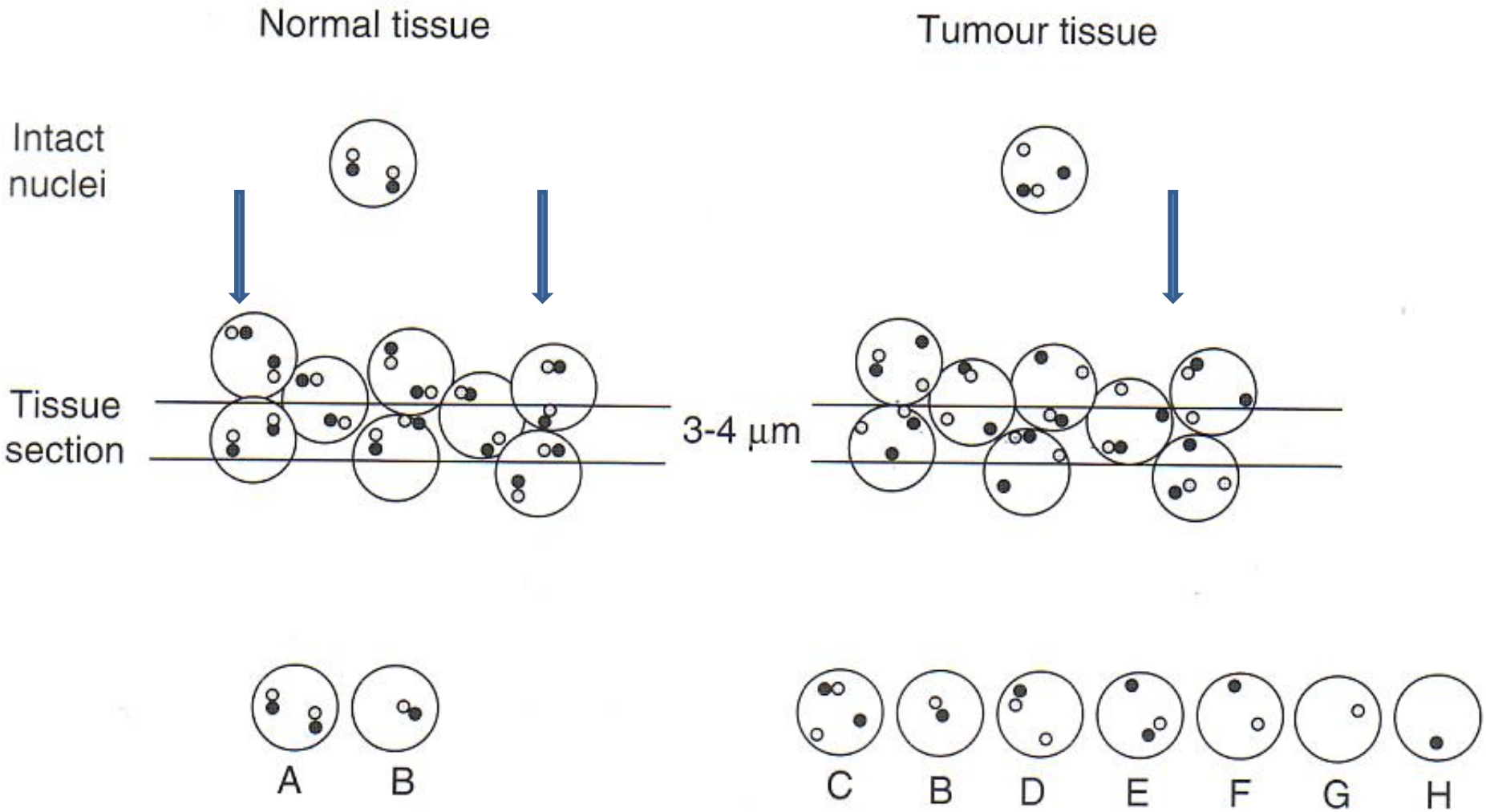
KEEP H&E SLIDES WITH YOU

# INTERPHASE FISH ON PARAFFIN TISSUE SECTIONS




**Journal of Pathology 2002,198 (2):163-170**

# INTERPHASE FISH ON PARAFFIN TISSUE SECTIONS

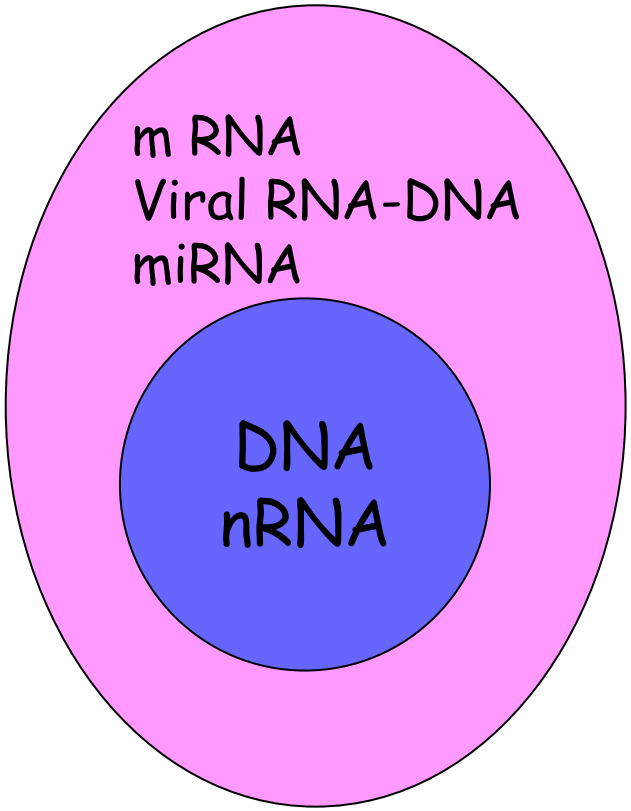




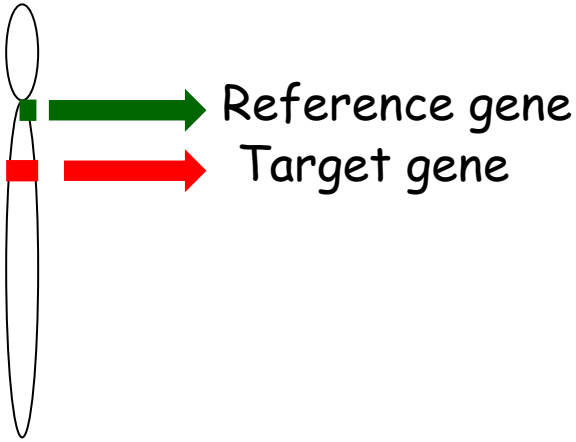

Journal of Pathology 2002,198 (2):163-170

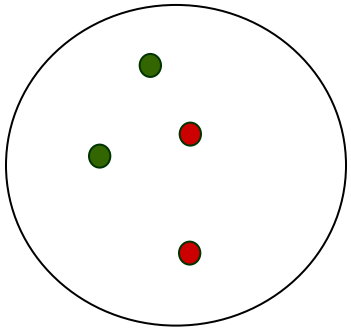
# WHAT COULD BE SEEN BY USING ISH?

GENE COPY NUMBER INCREASE (AMPLIFICATIONS)
GENE OR CHROMOSOME LOSS (DELETIONS)
GENE REARRANGEMENTS (TRANSLOCATIONS)
INFECTIOUS AGENTS
PROTEIN EXPRESSION (mRNA)
CHIMERISM (X, Y)
miRNA's

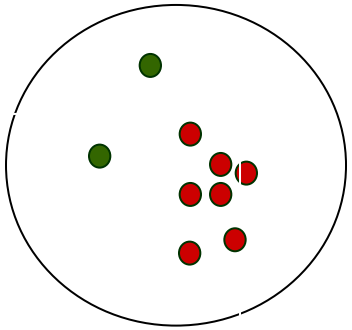


# AMPLIFICATION SIGNALS



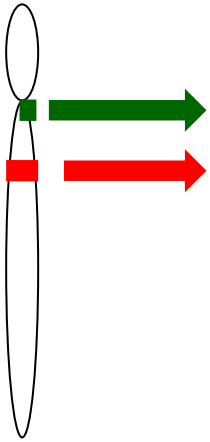



NORMAL



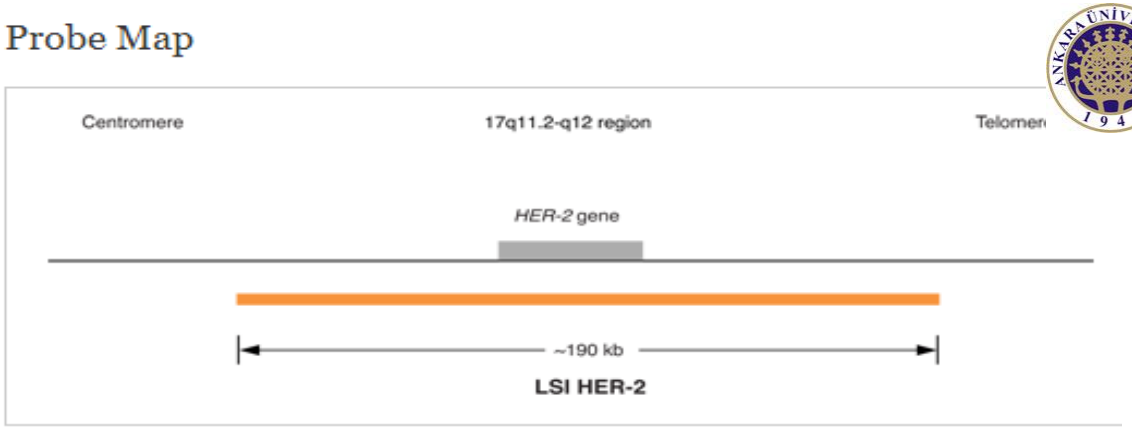
AMPLIFICATION

# AMPLIFI

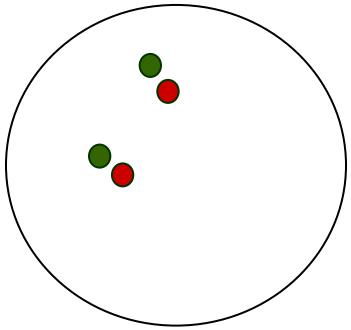


Reference gene

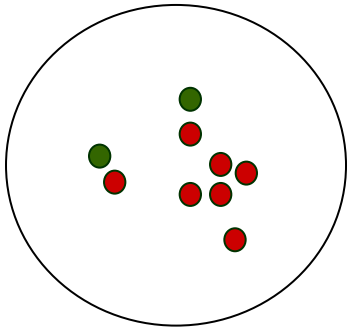
Target gene





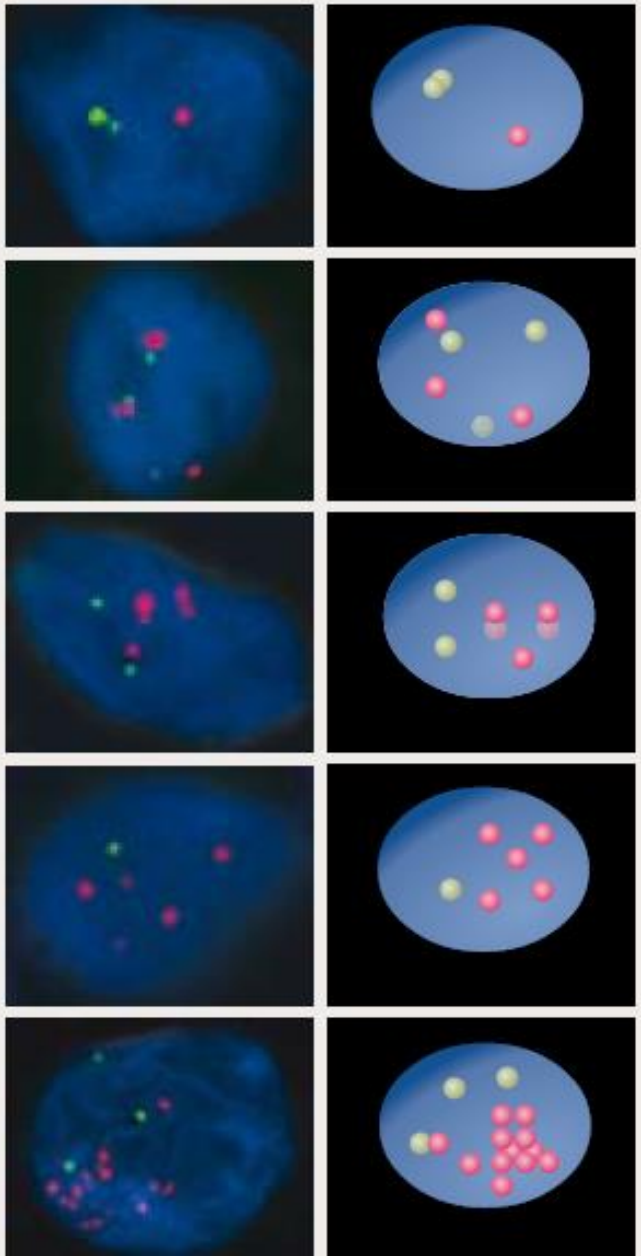


NORMAL



AMPLIFICATION

# Counting Guide



One green signal (split) indicates the presence of one copy of chromosome 17*. One red signal indicates the presence of one copy of the *HER2* gene.

**The ratio of *HER2* to CEN-17 is 1/1 = 1; non-amplified.**

Three green signals (one out of focus) indicate the presence of three copies of chromosome 17. Three red signals indicate the presence of three copies of the *HER2* gene.

**The ratio of *HER2* to CEN-17 is 3/3 = 1; non-amplified.**

Two green signals indicate the presence of two copies of chromosome 17. Three red signals (two split signals) indicate the presence of three copies of the *HER2* gene*.

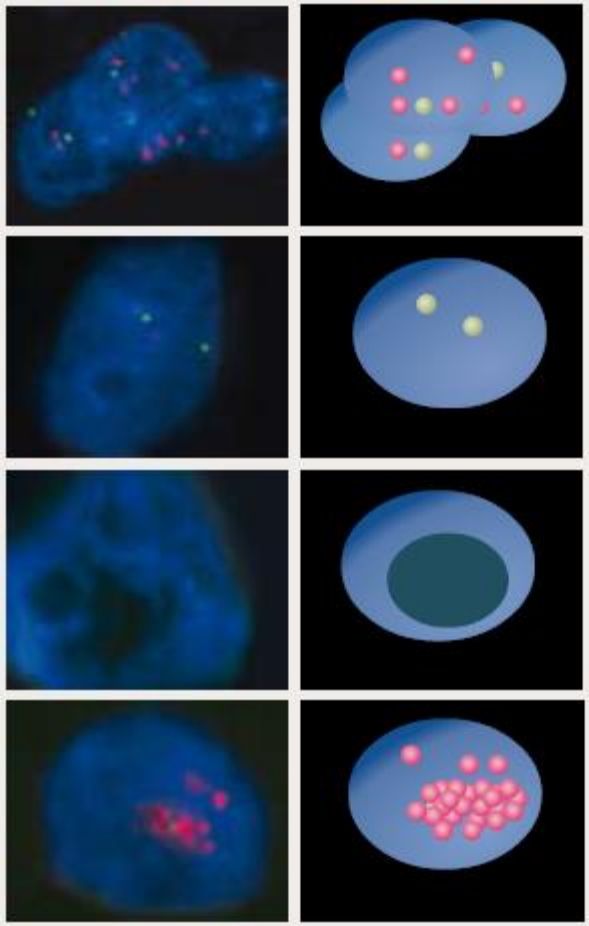
**The ratio of *HER2* to CEN-17 is 3/2 = 1.5; non-amplified.**

One green signal indicates the presence of one copy of chromosome 17. Five red signals indicate the presence of five copies of the *HER2* gene.

**The ratio of *HER2* to CEN-17 is 5/1 = 5; amplified.**

Three green signals indicate the presence of three copies of chromosome 17. Approximately 12 red signals indicate the presence of 12 copies of the *HER2* gene (cluster estimation).

**The ratio of *HER2* to CEN-17 is 12/3 = 4; amplified.**



Do not score (nuclei are overlapping, not all areas of nuclei are visible).

Do not score nuclei with signals of only one color (two green signals).

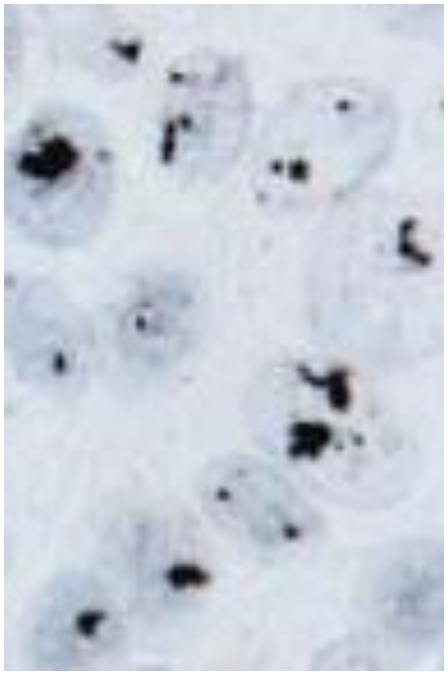
Do not score (overdigested nuclei).

Cluster of red signals hiding green signals. Check the green signals with a specific FITC filter, or do not score.

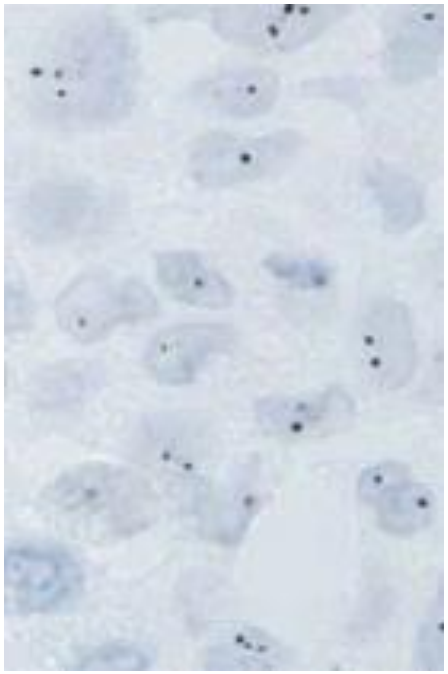
*Two signals of the same size, separated by a distance equal to or less than the diameter of one signal, are counted as one signal.

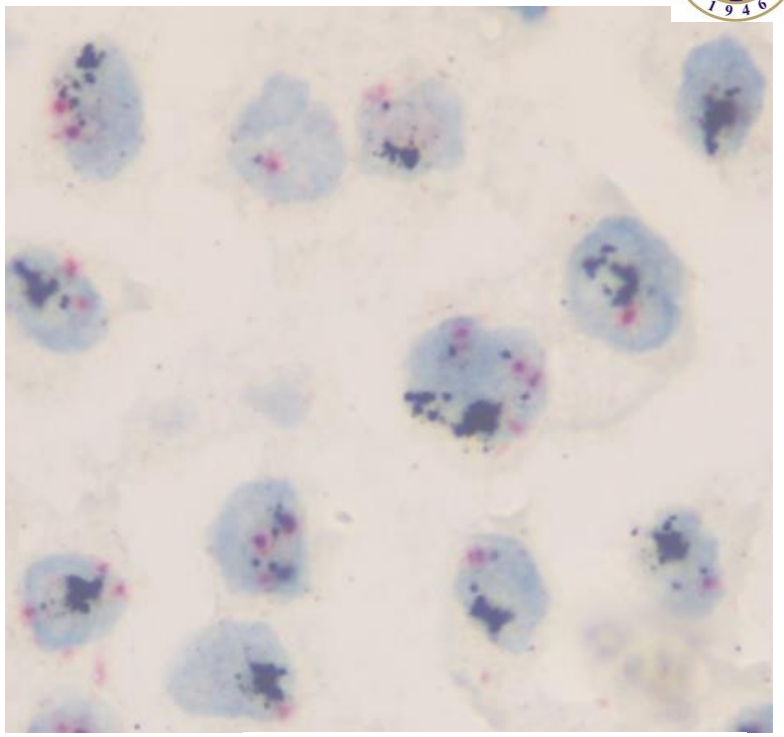
# SISH

Her 2 neu

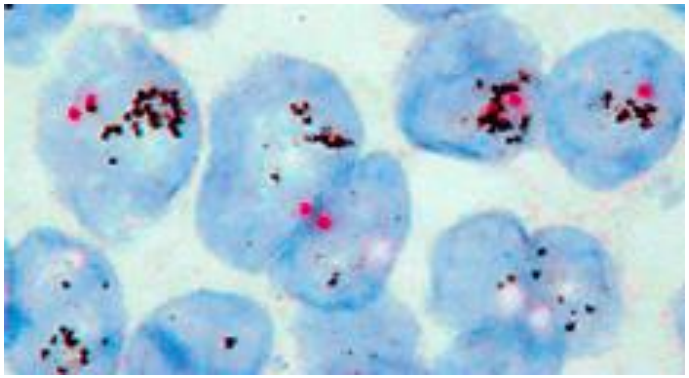


Chr 17

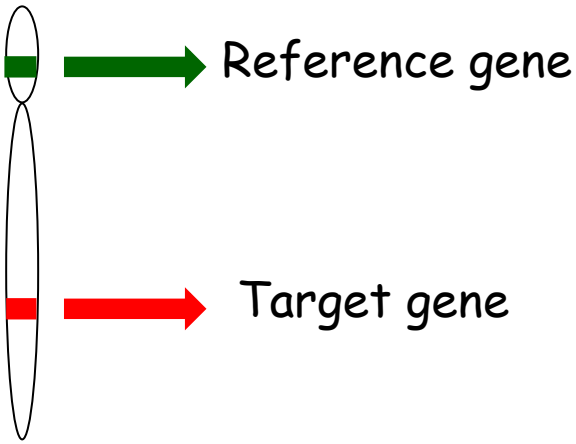


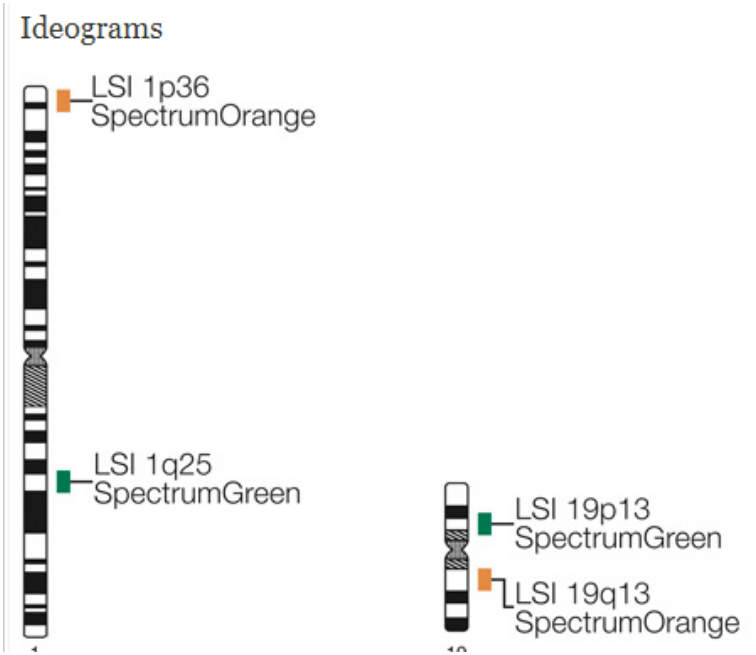


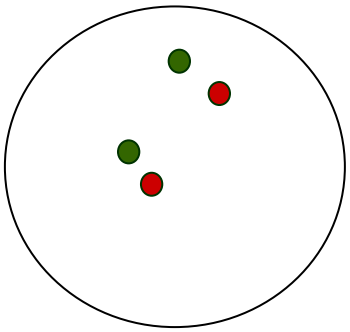
Her 2 neu / Chr 17



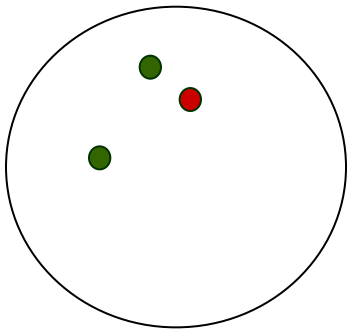
# DELETION SIGNALS



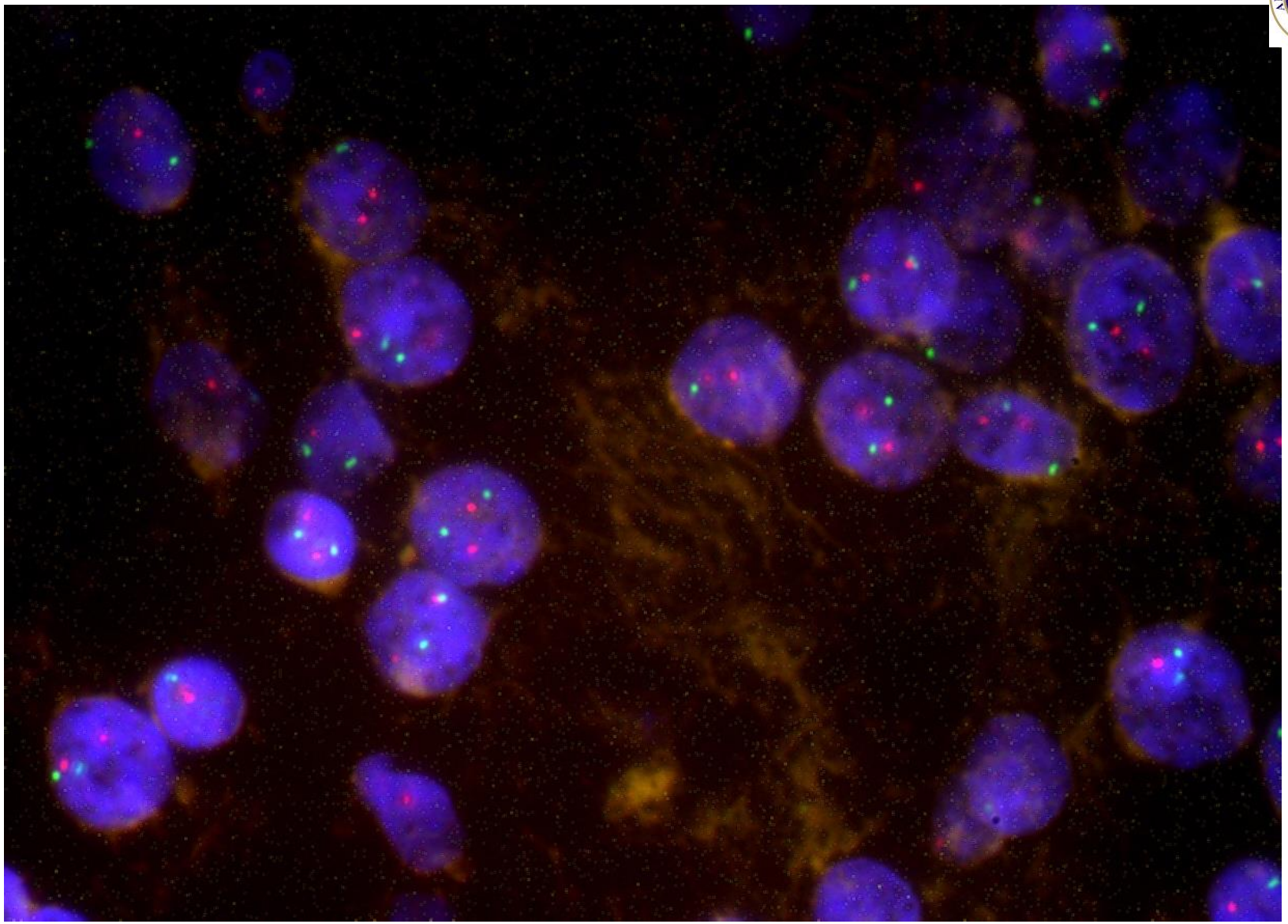



NORMAL

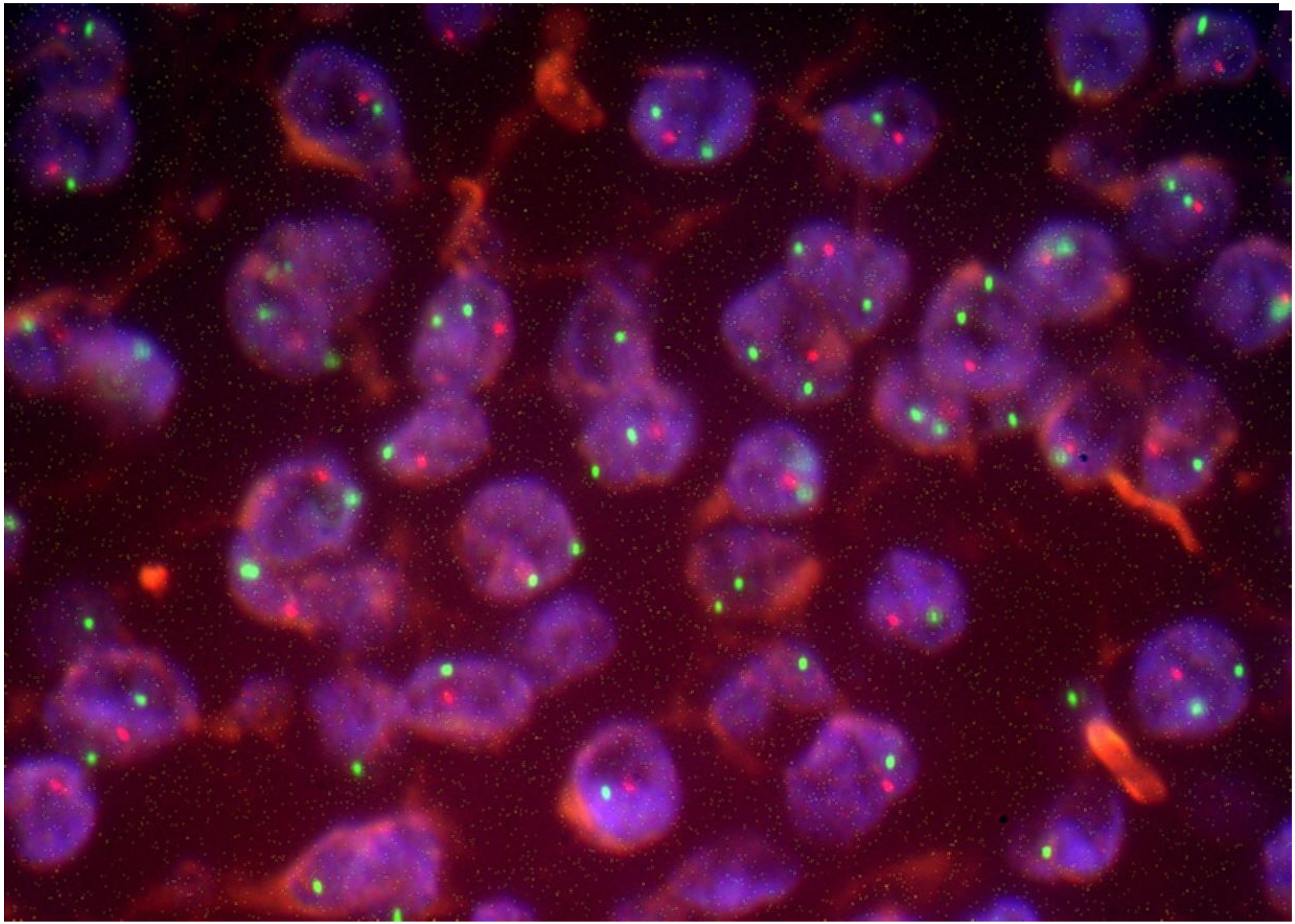


DELETED



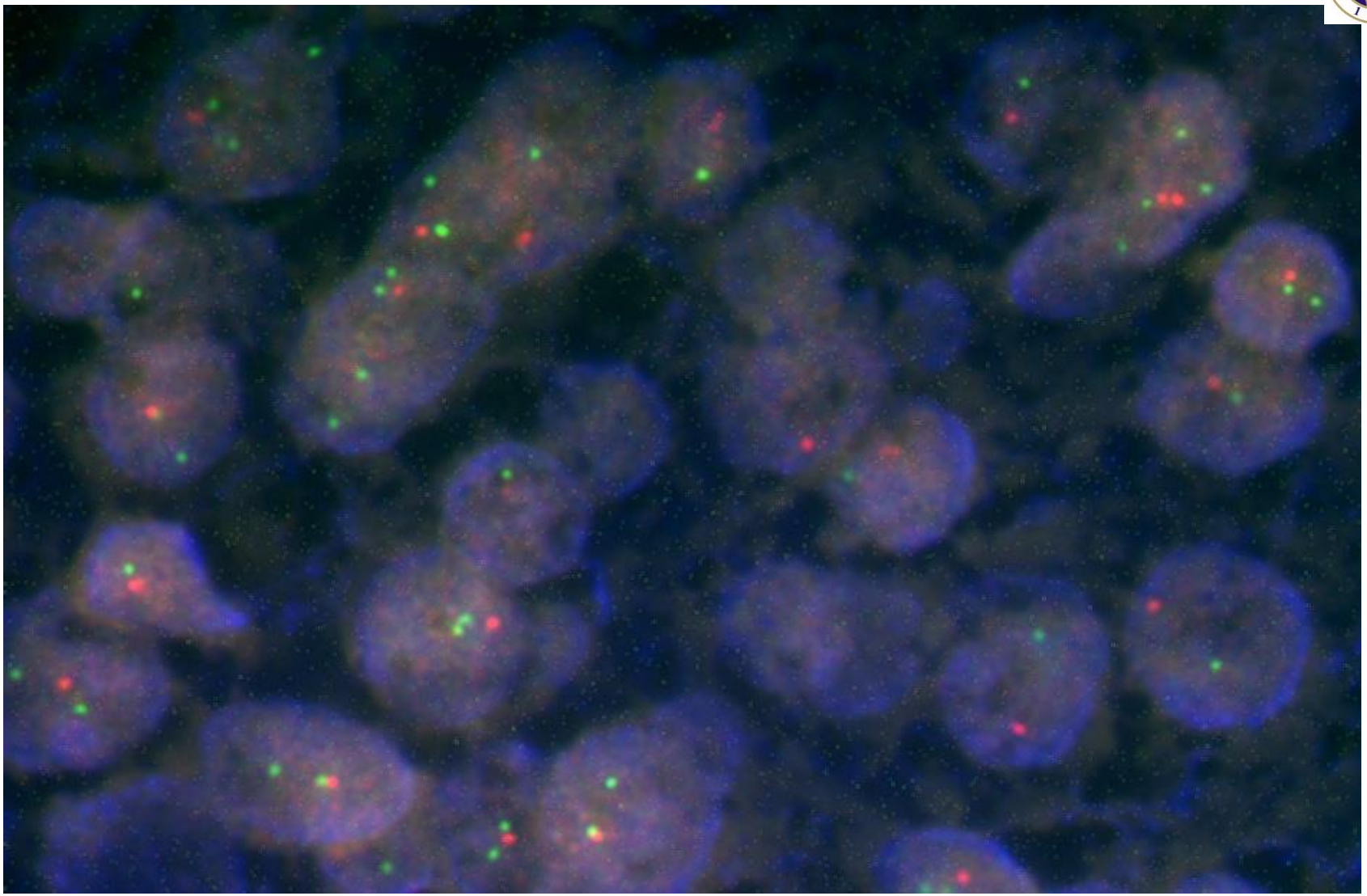
Chr 1P

Normal signals



Chr 1P

Deletion Signals

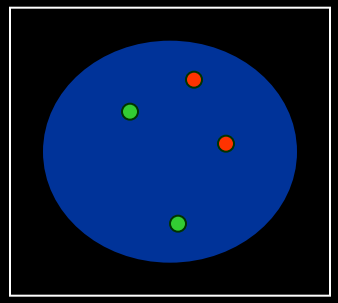


Chr 19q

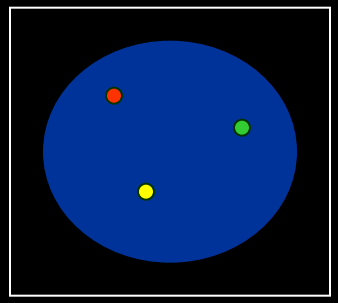
Deletion signals

# TRANSLOCATION (REARRANGEMENT) SIGNALS

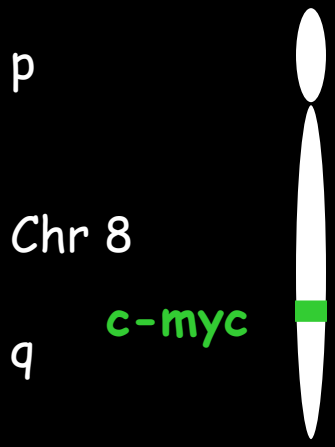
# FUSION PROBE

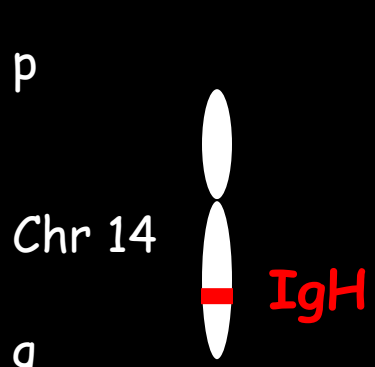


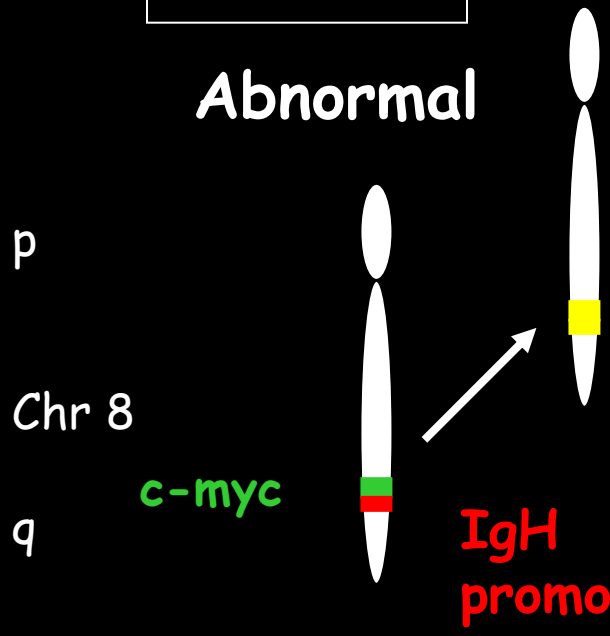
c-myc; IgH t(8;14)(q24;q32)



**Normal** **Abnormal**



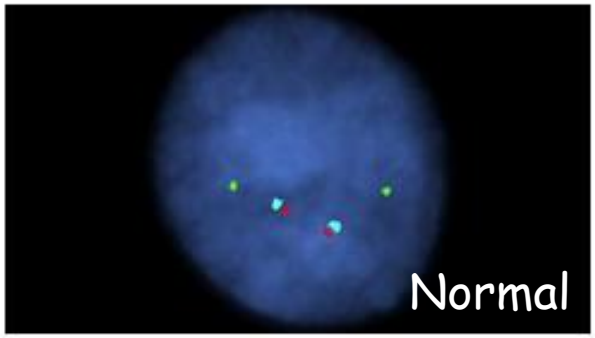

Pitfals:

Does not recognize translocations between alternative partner genes

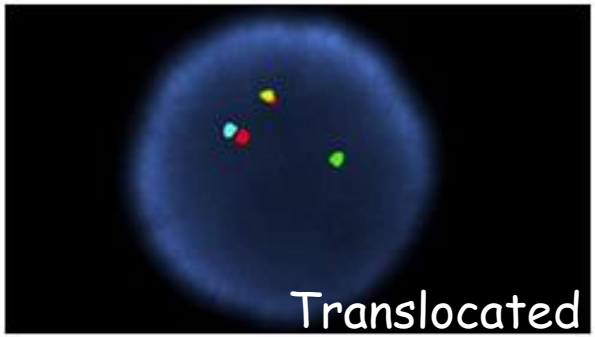
%5-10 false positive fusion signals due to the cellular superposition on cell smears

False positive signals are incerease on tissue sections.

# Triple signal fusion probes



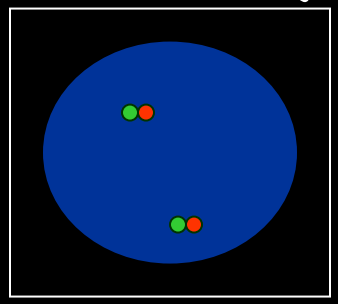

Nucleus showing the two aqua/orange and two green signal pattern.



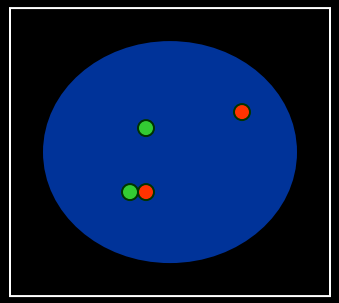

Nucleus showing the one aqua/orange, one green, and one orange/green fusion (yellow) signal pattern.

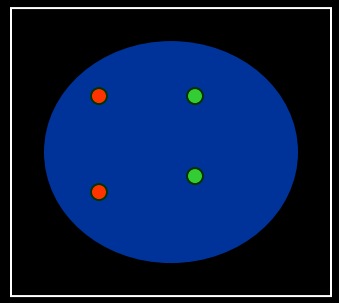
Bcr-Abl


# SPLIT PROBE (SEGREGETION ASSAY)



Normal





Abnormal

p

Chr 8

q

c-myc-c
c-myc
c-myc-t

c-myc-c (FITC)
c-myc-t (TR)

(> 500kb)
8q24

Probe sensitivity spectrum

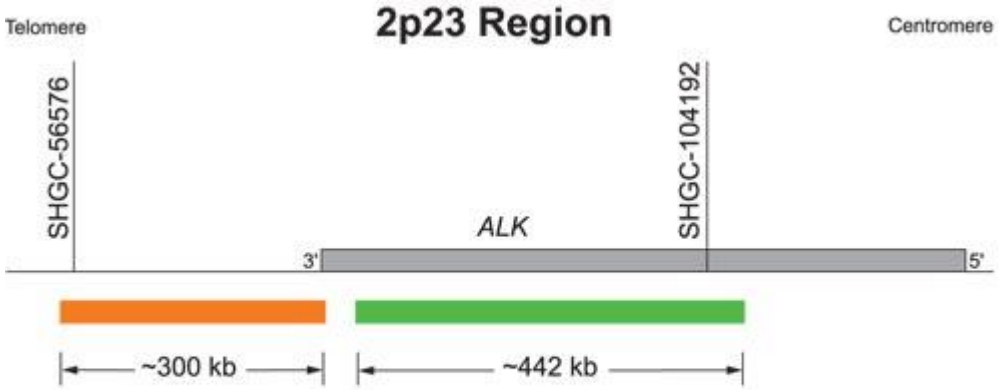
| | |
|---|---|
| c-myc; IgH | t(8;14)(q24;q32) |
| c-myc: Igk | t(2;8)(p12;q24) |
| c-myc: Igl | t(8;22)(q24;q11) |
| c-myc: TCR-a | t(8;14)(q24;q11) |

**Adventages:**

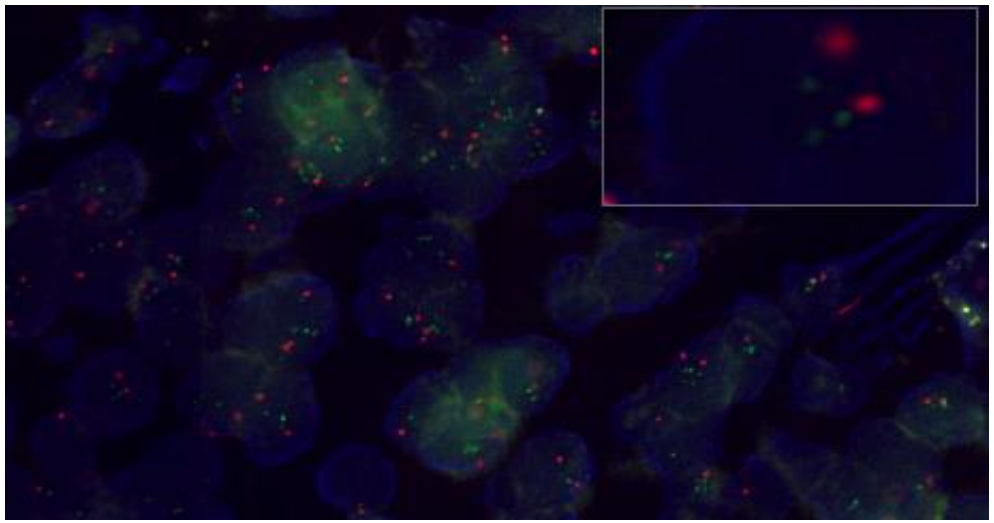
**All the alternative translocatiins independent from the partner genes can be detected**

**Decreases the false positive signals due to the superposition**

# Split signal probes


**LSI ALK Dual Color, Break Apart Rearrangement Probe**

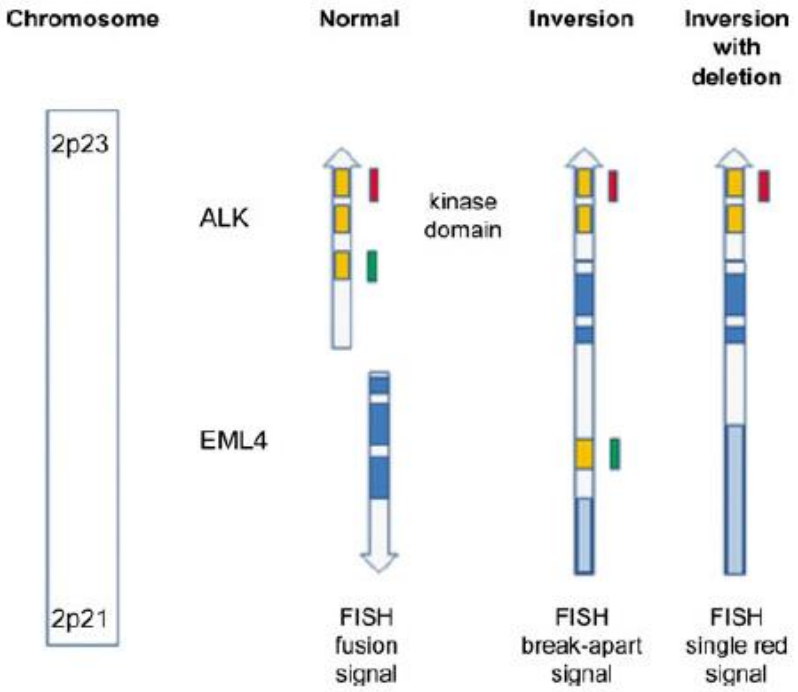


Virchows Arch (2012) 461:245–257
DOI 10.1007/s00428-012-1281-4

REVIEW AND PERSPECTIVES

# EML4-ALK testing in non-small cell carcinomas of the lung: a review with recommendations

Erik Thunnissen · Lukas Bubendorf · Manfred Dietel · Göran Elmberger · Keith Kerr · Fernando Lopez-Rios · Holger Moch · Wlodzimierz Olszewski · Patrick Pauwels · Frédérique Penault-Llorca · Giulio Rossi



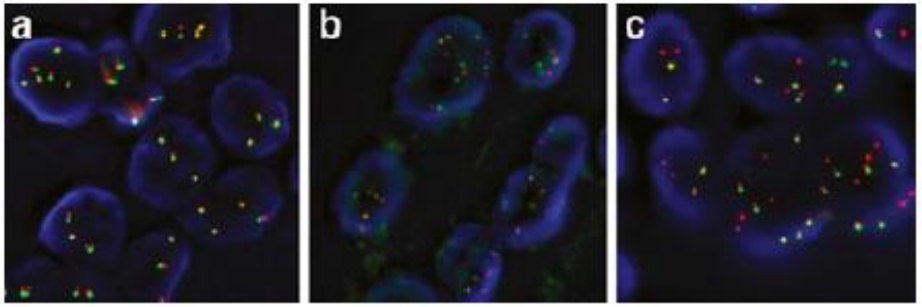
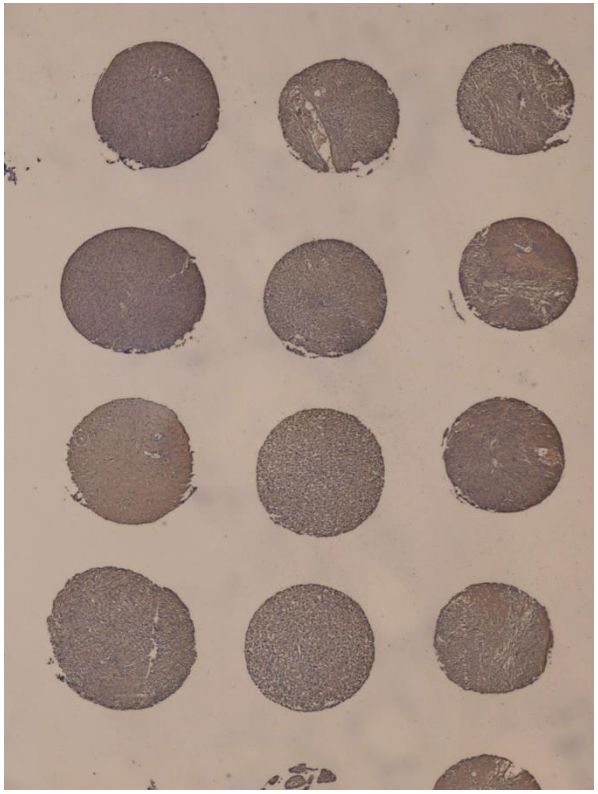
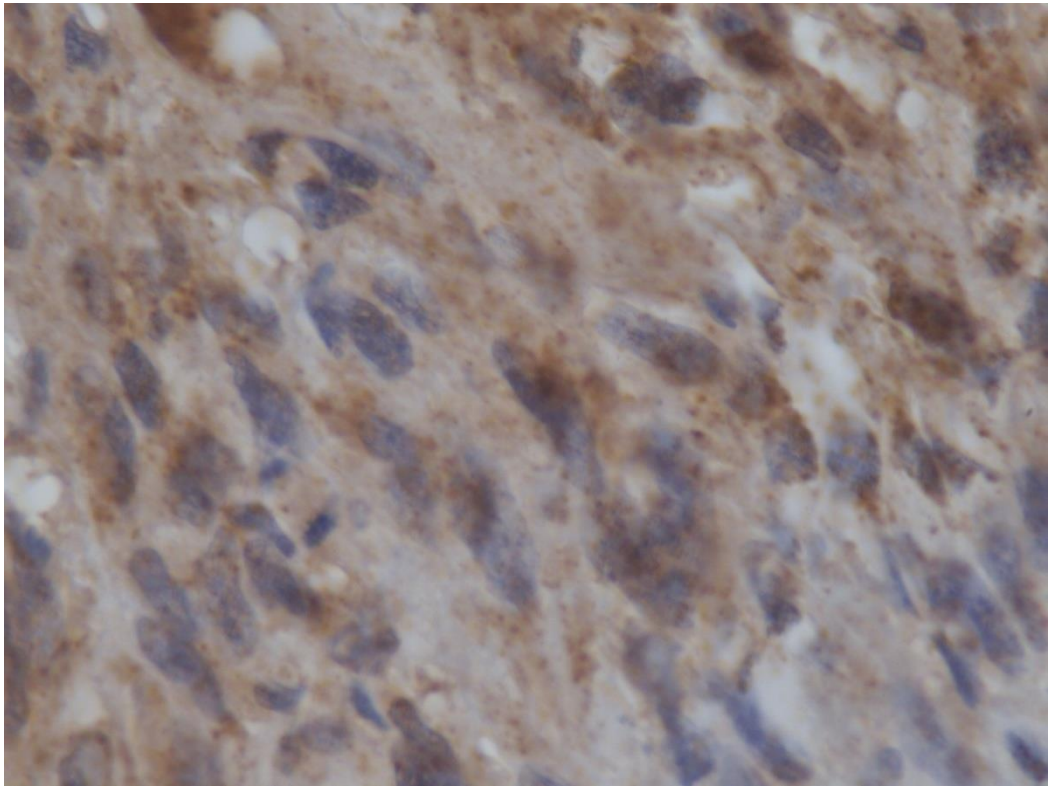




Fig. 2 Representative examples of ALK FISH findings in three pulmonary adenocarcinomas (Vysis ALK Break Apart FISH probe). All three carcinomas show increased ALK copy number. **a** Normal signals, no rearrangement. Note that some of the signals are fused and produce a yellow signal, while others have *green* and *red* signals in close proximity. **b** One or two break apart signals per nucleus, indicative of inversion. **c** Single red signals, indicative of inversion and deletion. Note that the cancer cells in **b** and **c** contain both rearranged and normal ALK signals. Cells are considered ALK FISH positive when there is: (1) ≥1 set of red and green signals that are ≥2 signal diameters apart, or (2) a single red signal without a corresponding green signal in addition to fused (normal) signals. A sample is considered negative if <5 cells (<10 %) are positive and positive if >25 cells (>50 %) are positive. A sample is considered equivocal if 5–25 cells (10–50 %) are positive

# RNA ISH for PDGFRA expression





J Hematopathol
DOI 10.1007/s12308-012-0148-6

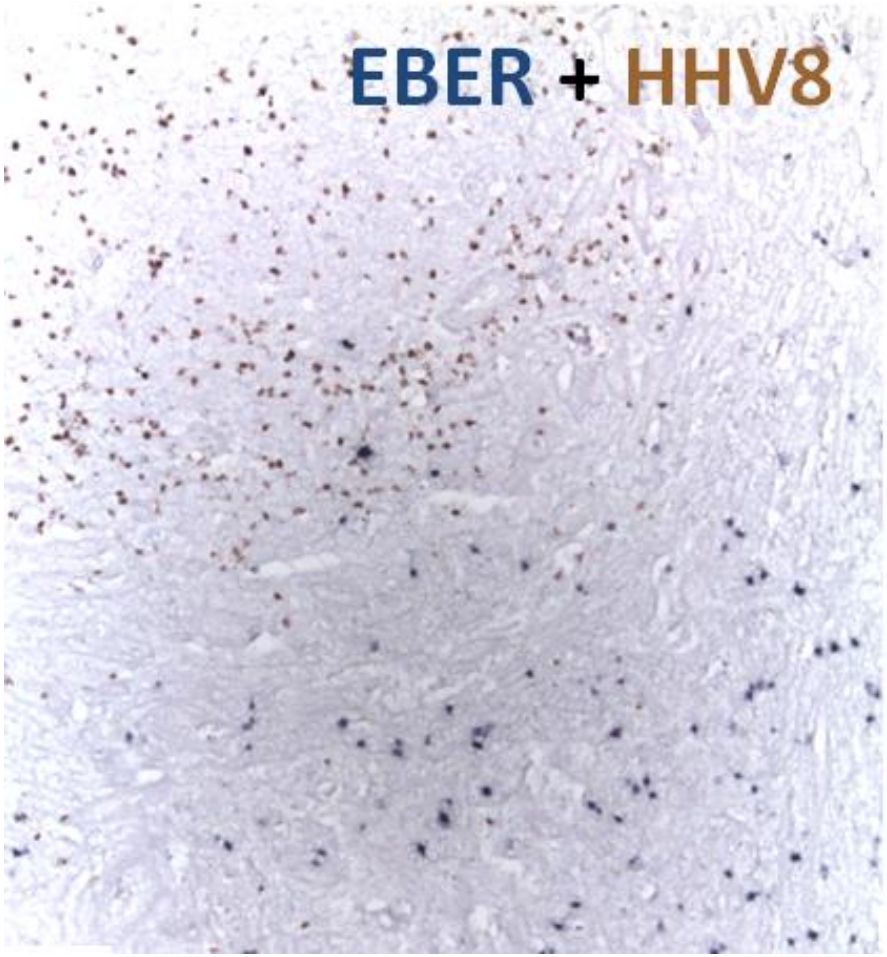
ORIGINAL ARTICLE

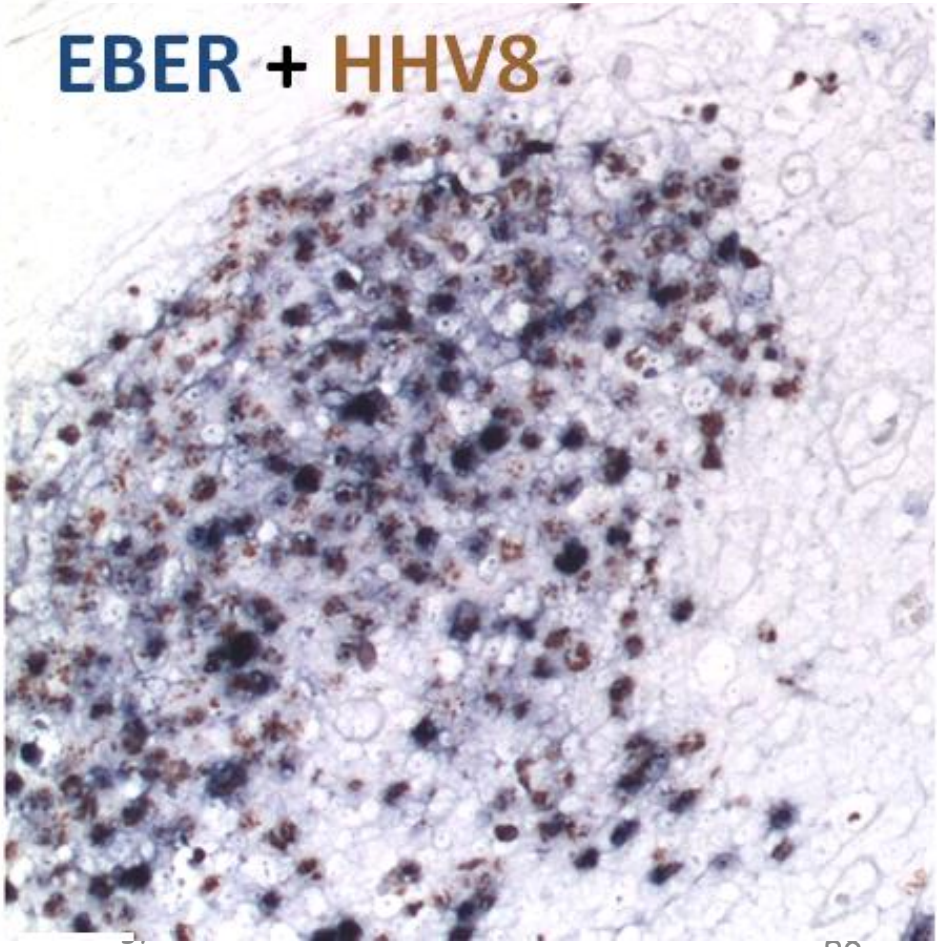
## Early lesions in lymphoid neoplasia

**Conclusions based on the Workshop of the XV. Meeting of the European Association of Hematopathology and the Society of Hematopathology in Uppsala, Sweden**

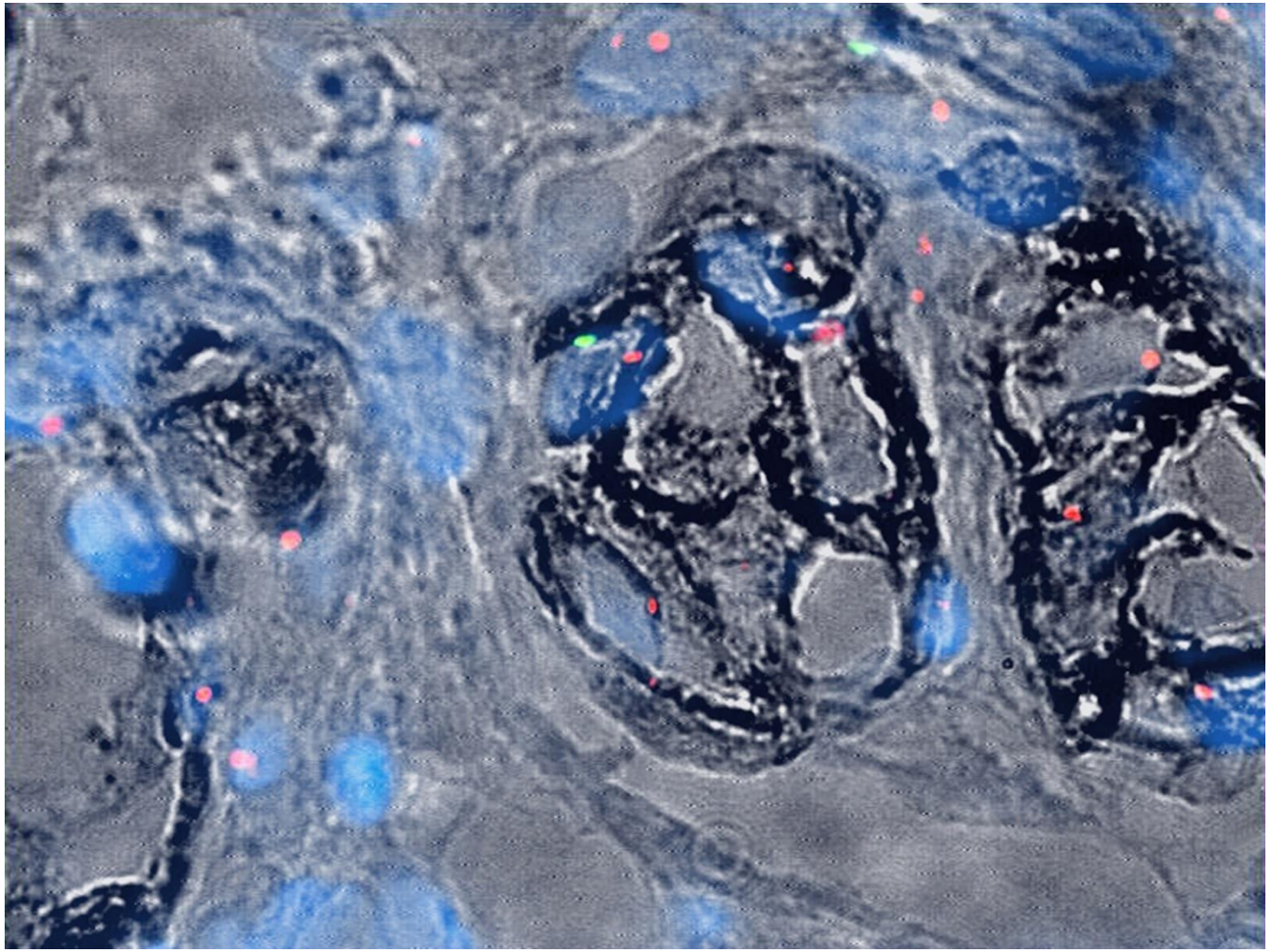
Falko Fend · José Cabecadas · Philippe Gaulard · Elaine S. Jaffe · Philip Kluin · Isinsu Kuzu · LoAnn Peterson · Andrew Wotherspoon · Christer Sundström

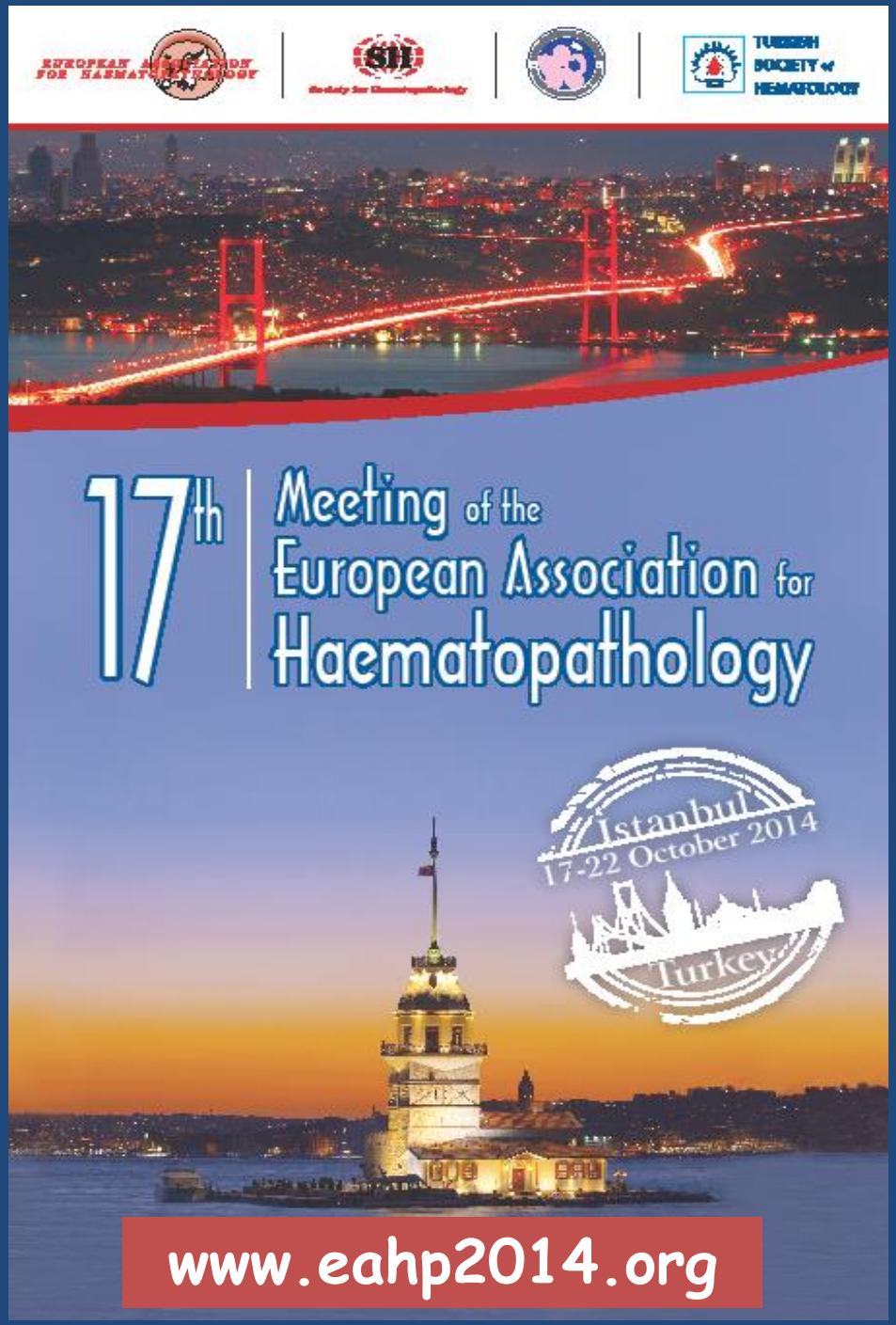
# DOUBLE LABELLING
# IMUNOHISTOCHEMISTRY
# +
# ISH



# DOUBLE LABELLING
# IMUNOHISTOCHEMISTRY + FISH






# 17-22 October 2014
# Istanbul
# Hilton Convention Center

**TOPIC**

**"Redefining the spectrum of small B-cell lymphomas in light of current technology".**

**GOAL**

To integrate modern bio-technological developments and morphological pathology to better understand small B cell lymphomas.

EUROPEAN ASSOCIATION FOR HAEMATOPATHOLOGY

Society for Hematopathology

FEDERATION OF PATHOLOGY SOCIETIES OF TURKEY

TURKISH SOCIETY of HEMATOLOGY

# THANK YOU FOR YOUR ATTENTION