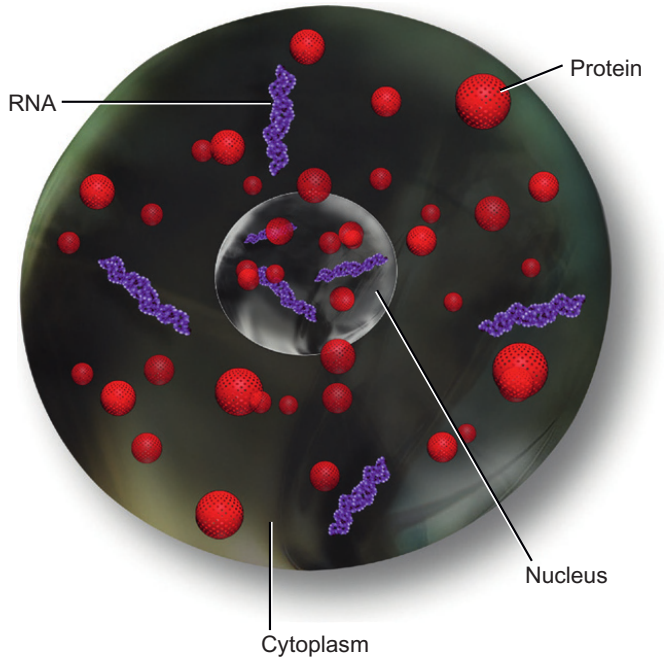
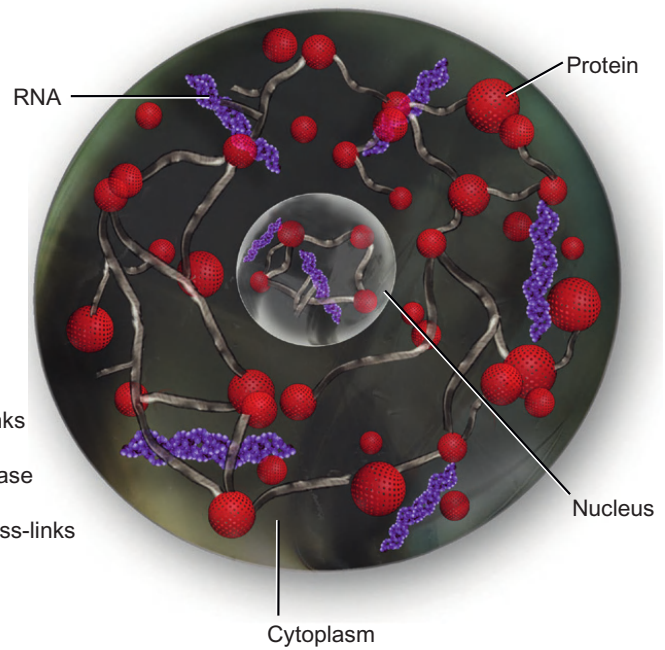
A



UNFIXED CELL

FORMALIN ADDED

B



FIXED CELL

Formalin fixation gives more resistance to cells, but nucleic acids become fragile

Consequences:

1) Extensive Protein to Protein cross-links

2) Inactivation of RNase, DNase, Protease

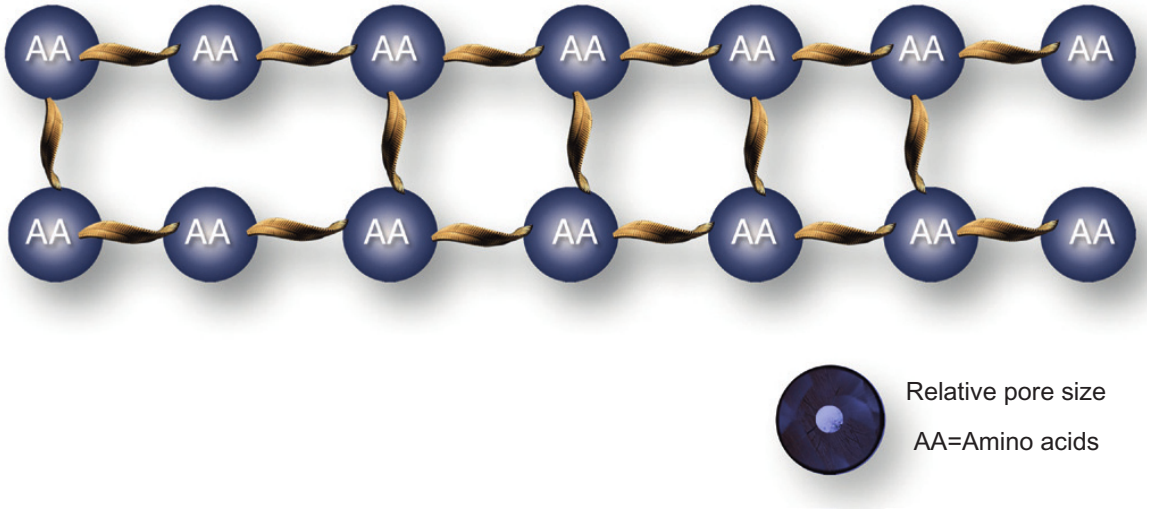
3) Extensive Protein to DNA + RNA cross-links

In Situ Molecular Pathology and Co-Expression Analyses

Gerard J. Nuovo

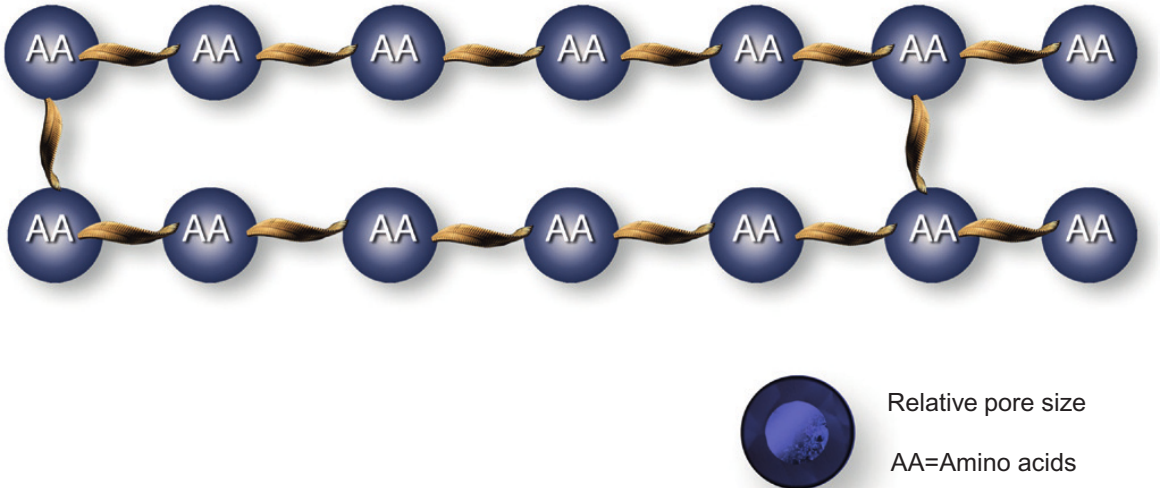
A

Many cross-links
Small pore size



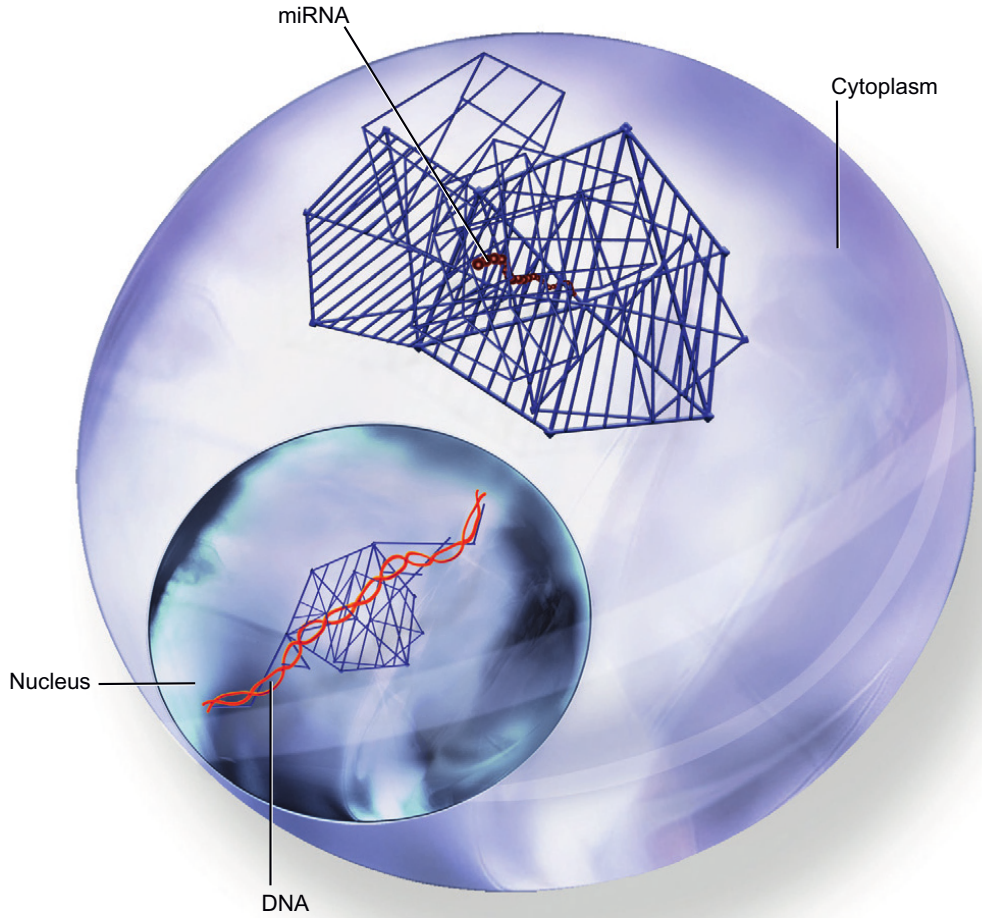
B

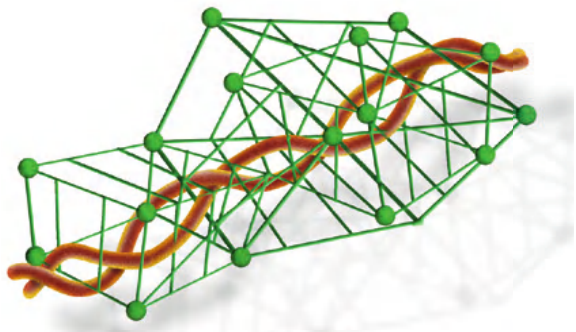
Few cross-links
Large pore size



The longer is fixation the higher is number of crosslinks

VARIABLE SIZED CAGES FOR DIFFERENT TARGETS IN THE SAME CELL





- The “cages” would represent the different protein density that surrounds any DNA, RNA, or protein epitope in the living state after variable cross-linking with formalin.
- This explain the marked differences in optimal pretreatment conditions for different targets in the same cell. It is to hypothesize that they are surrounded by variably sized “cages.”

In situ hybridization-ISH

Reaction between 2 single-strand nucleic acid sequences complementary to each other ⇒ interaction: H bonds

Detection of Hybrids in tissue sections or cells ⇒ morphology

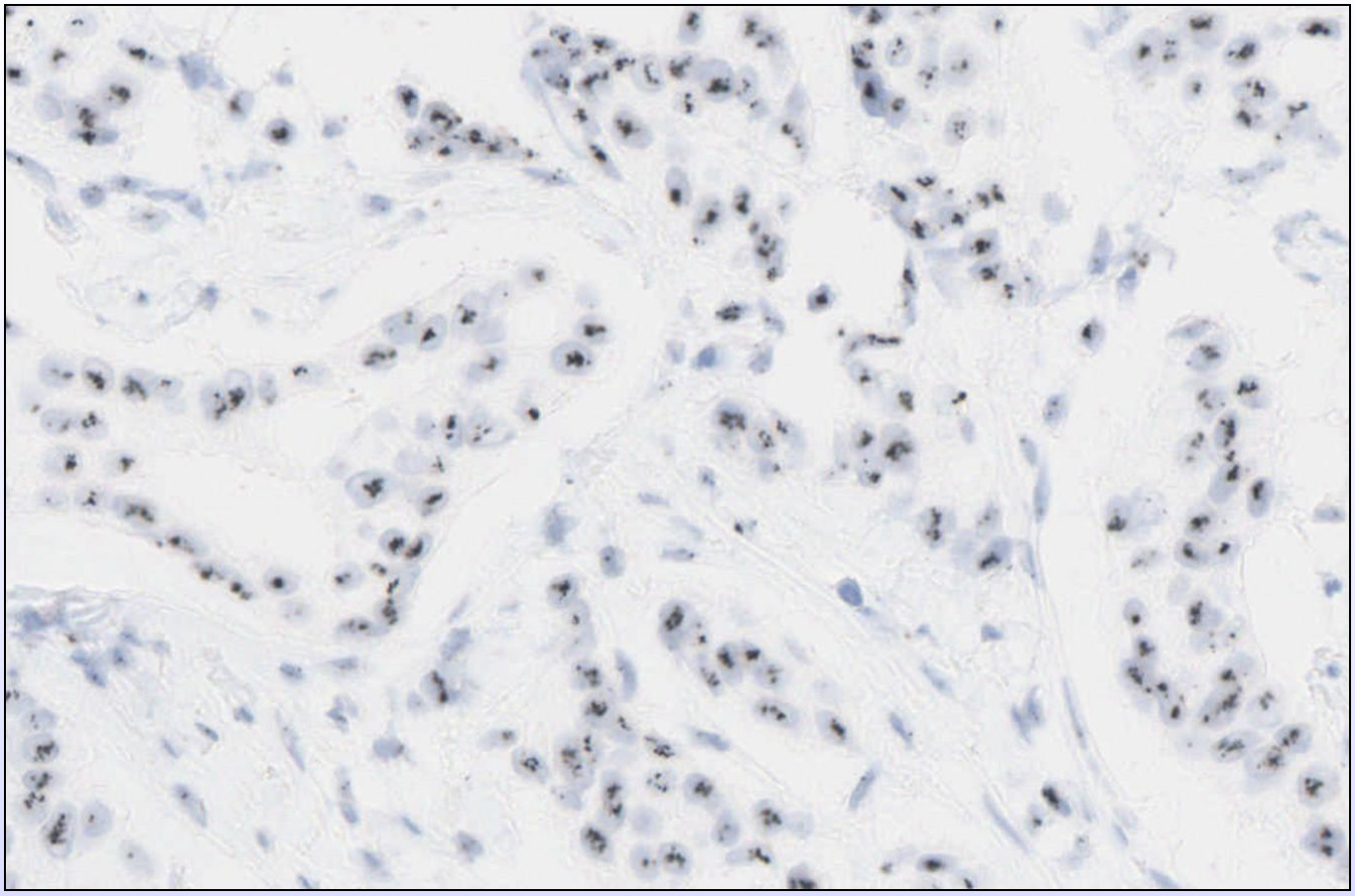
Used the first time for the detection of DNA sequences or amplified genes (RNA) in cellular nuclei.

FISH: highlights specific sites on nuclei or chromosomes by using fluorescent probes.ISH: for cytoplasmic RNA used the first time to detect viral sequences in infected tissues.

FISH

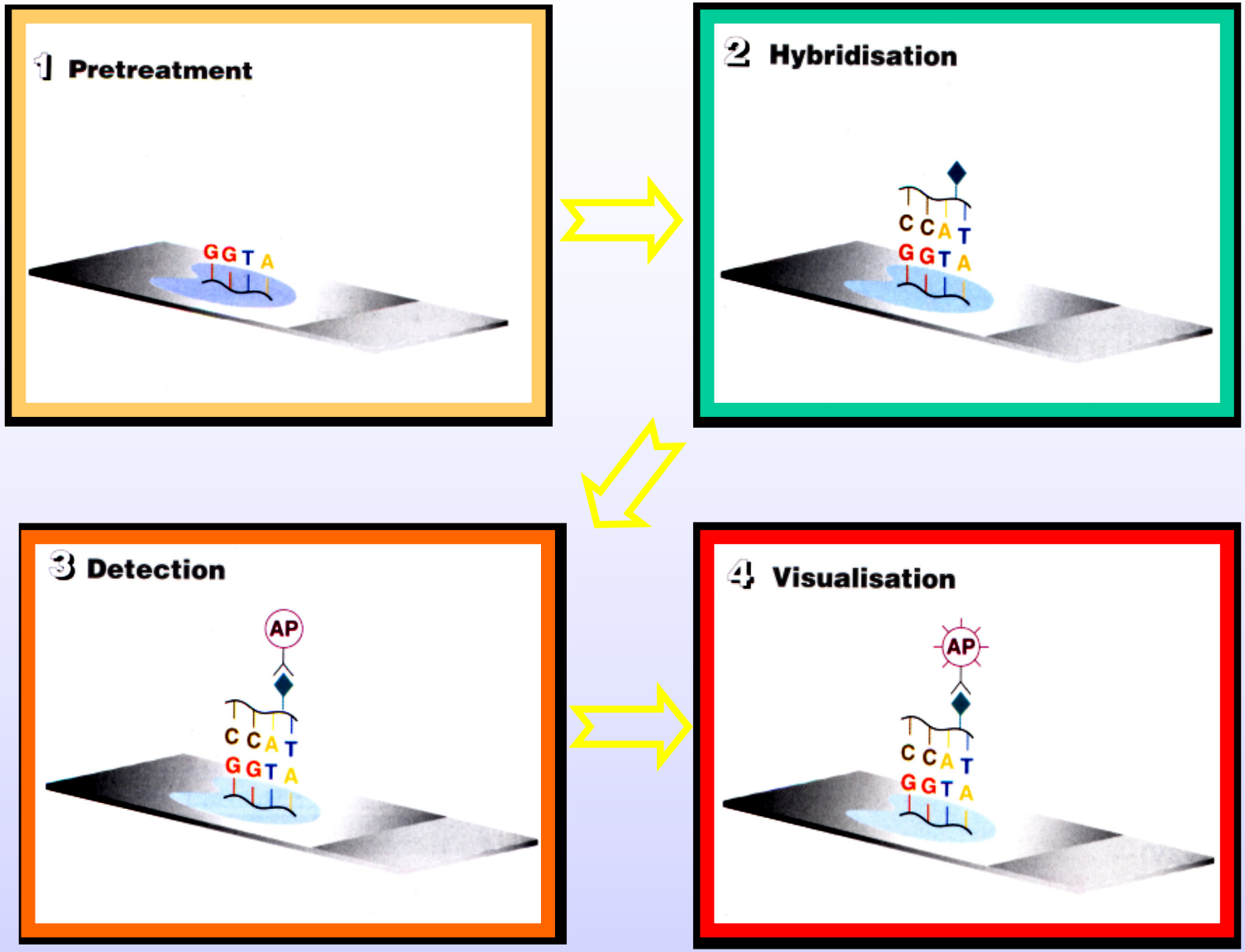
CISH

SISH



SISH for HER 2 gene in breast K

IN SITU HYBRIDIZATION



From Novocastra laboratories Ltd product catalogue 1997

In situ Hybridization (non radioactive)

Direct Method

In the direct method, the detectable molecule (reporter) is bound directly to the nucleic acid probe so that probe-target hybrids can be visualized under a micro-scope immediately after the hybridization reaction.

Indirect Method

Indirect procedures require the probe to contain a reporter molecule, introduced chemically or enzymatically, that can be detected by affinity cytochemistry. One of the most popular is the biotin-streptavidin system and the Digoxigenin

GENERAL OUTLINES

- ✓ Preparation of slides and fixation of material
- ✓ Pretreatments of material on slides, (e.g., permeabilization of cells and tissues)
- ✓ Denaturation of *in situ* target DNA (not necessary for mRNA target)
- ✓ Preparation of probe
- ✓ *In situ* hybridization
- ✓ Posthybridization washes
- ✓ Immunocytochemistry
- ✓ Microscopy

In situ Hybridization

✓ **SLIDE PREPARTAION**: Dewaxing and rehydration in EtOH

✓ **Permeabilization:** Incubate the sections with a Proteinase K solution (100 mM Tris, pH 7.5; 50mM EDTA; 2 µg/ml Proteinase K) for 30 min at 37° C. The activity of proteinase K can be blocked by slide immersion in a Glycine solution 0.1M in PBS

✓Post-Fixation of nucleic acids: para-formaldehyde 4% 3'. Rinse with PBS 2x to eliminate PFA (crosslinking fixative).

✓ Acetilation: tri-ethanolamine 0.1M acetic anidride 0.25%, 10' wash with ddH2O and dry @ 37° C. Acetilation is facultative an is used to decrease background (OH block).

✓Probe resuspension in buffer 10–50 µg/ml.

✓Prepare hybridization solution containing:

In situ Hybridization

✓50% deionized formamide, dextran sulfate, 5x Denhart's sol, salmon sperm DNA denaturato 100 µg/ml di soluz, SDS 2%.

✓**Hybridization**: Cover each section with 250–500 µl of hybridization mixture (depending on the size of the section) and incubate in a humidified box at 42° C overnight (T can vaty depending on the probe).

✓**Washing:** After hybridization, wash the slides as follows:

5 min at room temperature with 3xSSC.

5 min at room temperature with NTE (500 mM NaCl, 10 mM Tris-HCl, 1mM EDTA;pH 7.5).

In situ Hybridization

For RNA probes-

To remove unhybridized single-stranded RNA probe, put slides into a humidified box and cover each section with 500 µl of NTE buffer containing 50 µg/ ml RNase A. Incubate for 30 min at 37° C.

After RNase treatment, wash the slides 3 x 5 min at room temperature with NTE.

To remove nonspecifically hybridized probe, wash the slides as follows:

30 min at room temperature with 2xSSC.

1 h at 57° C with 0.1xSSC.

PROCEED WITH DIG DETECTION

In situ Hybridization

✓ Incubate the slides first with blocking solution, then with blocking solution containing 1.25 units/ml of alkaline phosphatase-conjugated anti-DIG Fab fragments 2h a RT

✓ After the antibody incubation, wash the slides to remove unbound antibody

✓ Prepare the BCIP-NBT-PVA color development solution

✓place the slides in 30 ml of BCIP-N BTP-VA color development

✓ Incubate the slides in the color development solution

✓ Monitor color formation visually.

✓When the color on each slide is optimal, stop the color reaction by washing theslide 3 x 5 min in distilled water.

✓ Dehydrate the sections and mount them for microscopy

In situ Hybridization

Advantages:

- *Detection of expression in heterogeneous systems*
- *Morphology*

Disadvantages:

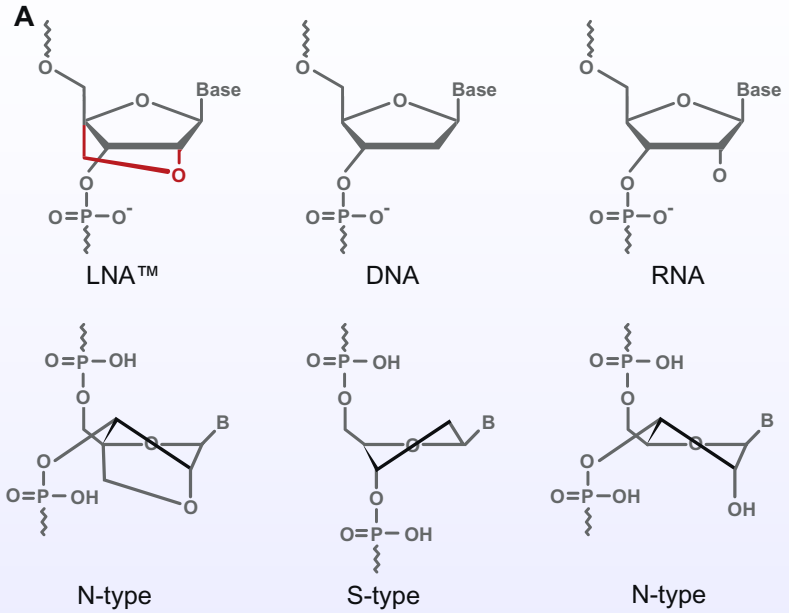
- *Time consuming*
- *Low swnsitivity*
- *Limited quantitative analysis*

Why LNA?

1. High affinity RNA
2. Ribose ring is “locked” in the ideal conformation for Watson–Crick binding.
3. As a result, LNA™ oligonucleotides exhibit thermal stability when hybridized to a complementary DNA or RNA strand.
4. For each incorporated LNA monomer, the melting temperature (Tm) of the duplex increases by 2° C–8° C (Figure A-2).
5. LNA oligonucleotides can be made shorter

Graz, 1st April 2014

A



B

Probe \ Target	Perfect match 3′-acgaccac-5′	Single mismatch 3′-acggccac-5′	Δ T_m
DNA 8-mer 5′-tgctggtg-3′	T_m = 35° C	T_m = 25° C	10° C
LNA™ 8-mer 5′-TGCTGGTG-3′	T_m = 71° C	T_m = 45° C	26° C

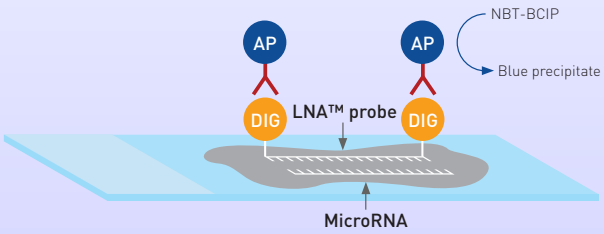
Figure A-1 Structure and conformations of nucleotides (A) and the effect of LNA™ on melting temperature of duplexes (B).

From: G.J. Nuovo (Eds) In Situ Molecular Pathology and Co-expression analyses (2013)

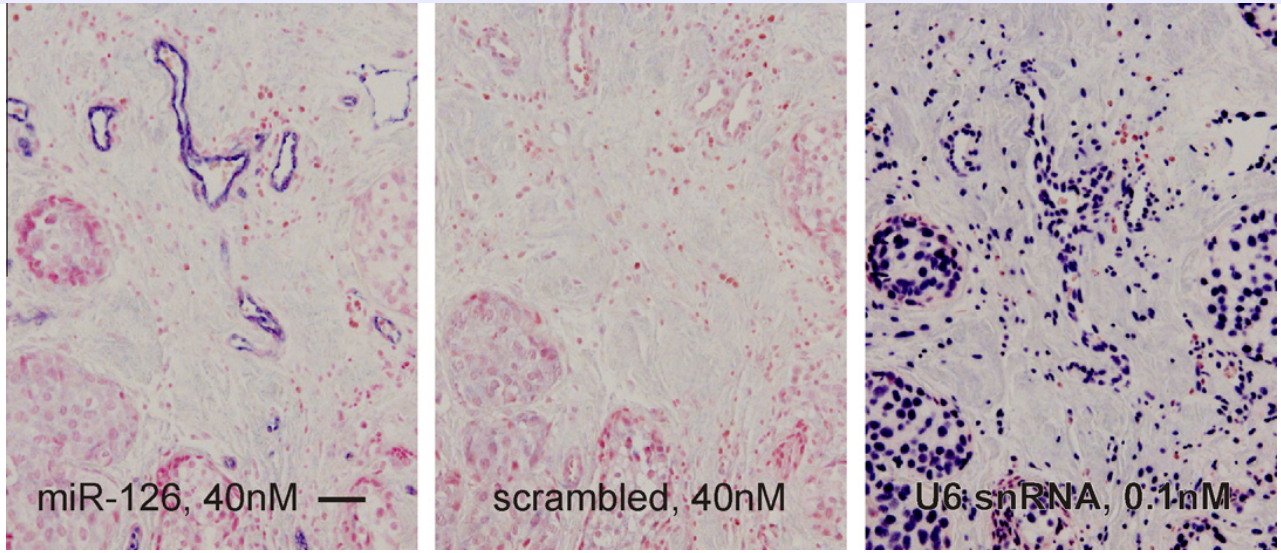
ISH for mRNA and miR detection

- ✓ Morphology
- ✓ Sensitivity
- ✓ miRs have high degree of similarity between the sequences. Some micro- RNA family members vary by a single nucleotide.
- ✓ Use LNA-enhanced oligonucleotides.
- ✓ The use of LNA™ in probes enables highly sensitive detection and analysis of the short miRNA sequences, but also mRNA.
- ✓ The use of LNA probe allows a better control of Tm even in AT rich sequences and for short stretches (miR)

Methods 52 (2010) 375–381



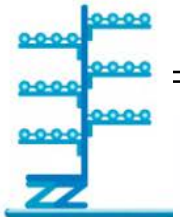
miRCURY LNA™ microRNA ISH
Exiqon



RNAscope

Fay Wang, et al *The Journal of Molecular Diagnostics* Volume 14, Issue 1, Pages 22-29 (January 2012) DOI: 10.1016/j.jmoldx.2011.08.002

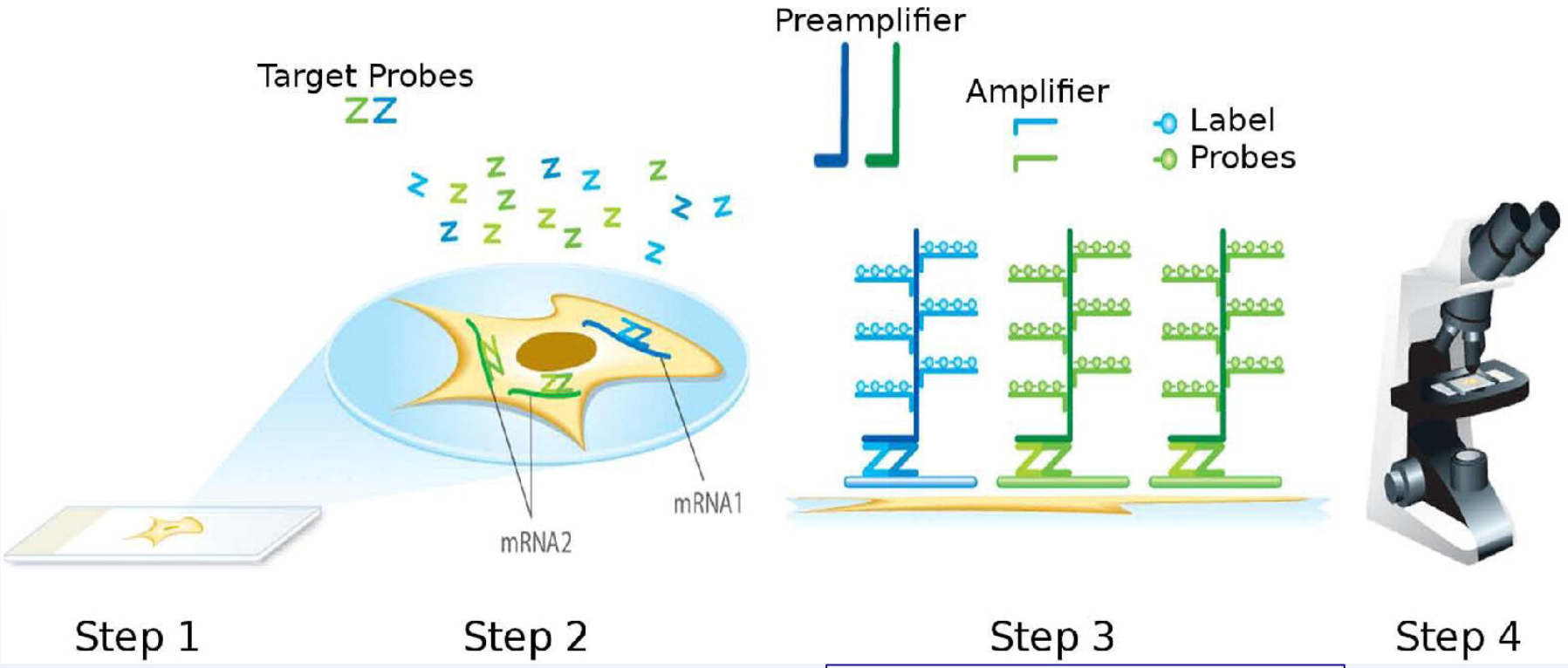
- ✓ Single-molecule visualization in individual cells
- ✓ Novel probe design strategy and a hybridization-based signal amplification system to simultaneously amplify signals and suppress back- ground.
- ✓ multiplex detection for up to 4 target genes (number of spectrally discernible fluorescent dyes)
- ✓ A series of target probes are designed to hybridize to the target RNA molecule.
- ✓ Each target probe contains an 18- to 25-base region complementary to the target RNA, a spacer sequence, and a 14-base tail sequence (conceptualized as Z). A pair of target probes (double Z), each possessing a different type of tail sequence, hybridize contiguously to a target region (☐ 50 bases).



RNAscope

Wang, et al *The Journal of Molecular Diagnostics* Volume 14, Issue 1, Pages 22-29 (January 2012) DOI: 10.1016/j.jmoldx.2011.08.002

- ✓ The two tail sequences together form a 28-base hybridization site for the preamplifier, which contains 20 binding sites for the amplifier, which, in turn, contains 20 binding sites for the label probe.
- ✓ Typically, 1-kb region on the RNA molecule is targeted by 20 probe pairs; thus, sequential hybridizations with the preamplifier, amplifier, and label probe can theoretically yield up to 8000 labels for each target RNA molecule.
- ✓ The probe can be either fluorescently labelled for direct visualization or conjugated to an alkaline phosphatase or horseradish peroxidase (HRP) molecule for chromogenic reactions
- ✓ Multiple RNA species can be measured simultaneously in two ways: the target probes for different genes can have the same tail sequence recognized by the same signal amplification system, generating a pooled signal; alternatively, multiple signal amplification systems with different label probes can be used to detect each RNA species, allowing for multiplex detection of multiple target RNAs.



Step 1: fixed cells or tissues are permeabilised to allow for target probe access.

Step 2: target RNA-specific oligonucleotide probes (Z) are hybridized in pairs (ZZ) to multiple RNA targets

Step 3: multiple signal amplification molecules are hybridized, each recognizing a specific target probe, and each unique label probe is conjugated to a different fluorophore or enzyme

Step 4: signals are detection with a epifluorescent microscope or standard bright-field microscope.

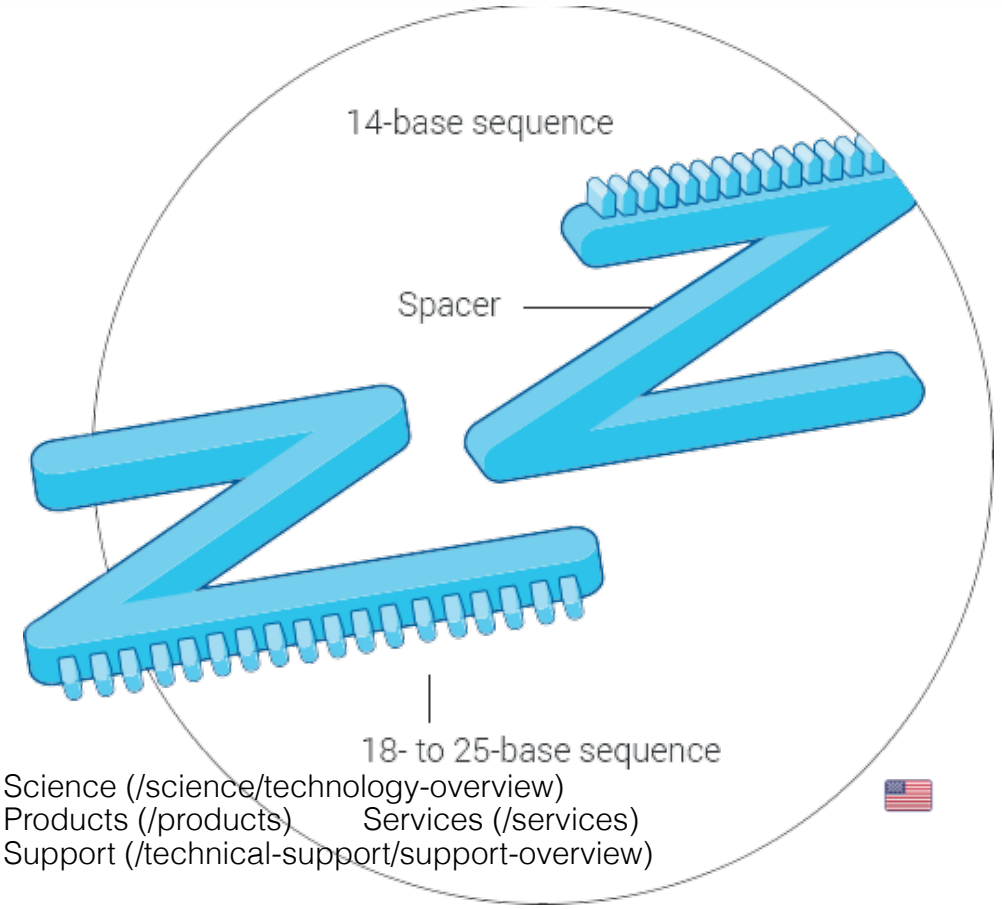
The Journal of Molecular Diagnostics 2012 14, 22-29DOI: (10.1016/j.jmoldx.2011.08.002)

Contains Three Elements

The lower region of the Z is an 18-to 25-base region that is complementary to the target RNA. This sequence is selected for target specific hybridization and uniform hybridization properties.

A spacer sequence that links the two components of the probe. The upper region of the Z is a 14-base tail sequence.

The two tails from a double Z probe pair forms a 28 base binding site for the pre-amplifier.



Signal Amplification is Achieved by a Cascade of Hybridization Events

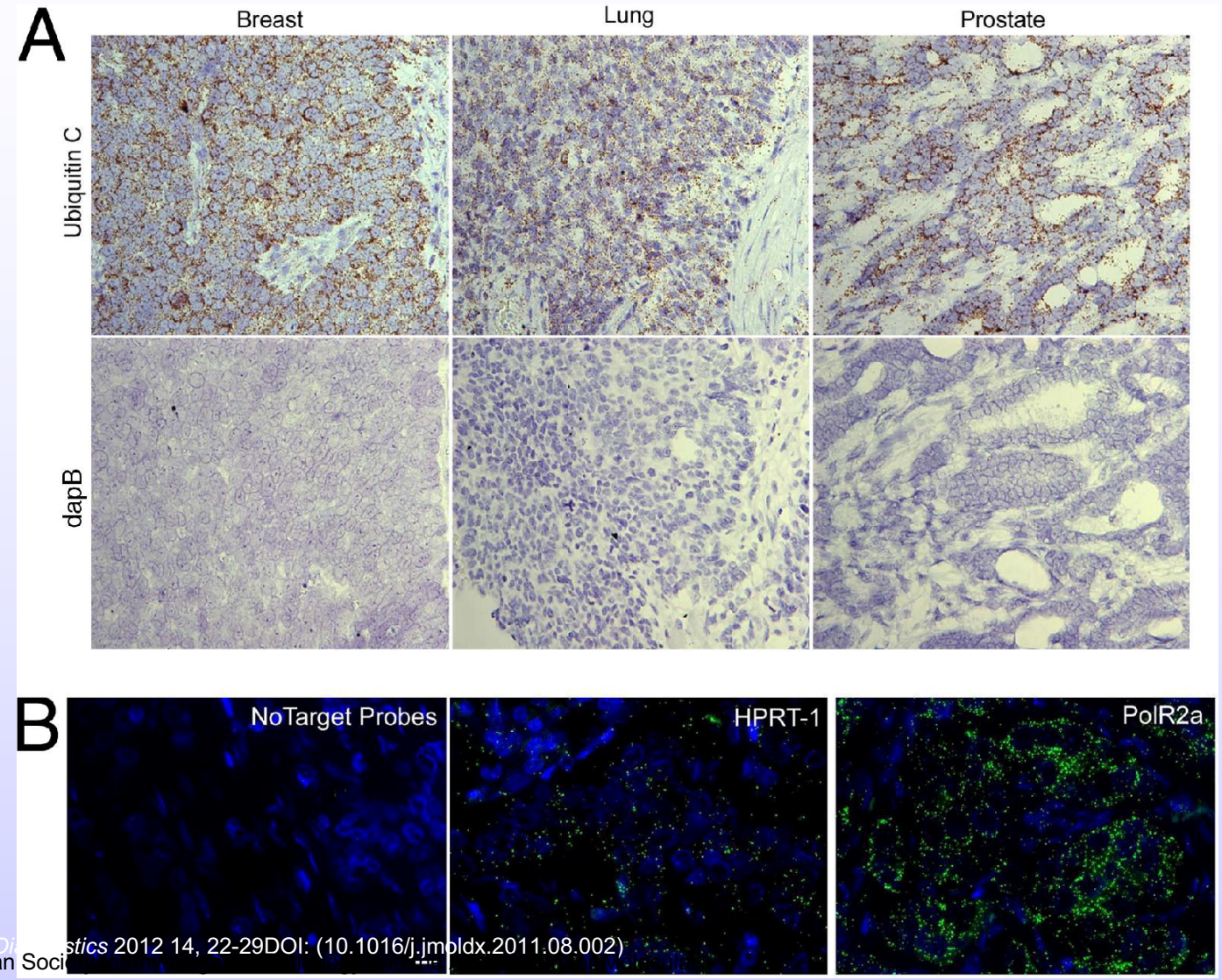
Step 1. Double Z target probes hybridize to the RNA target (~1kb)

Step 2. Pre-amplifiers hybridize to the 28-base binding site formed by each double Z probe

Step 3: Amplifiers are then binding to the multiple binding sites on each preamplifier.

Step 4: Labeled probes, containing a fluorescent molecule or chromogenic enzyme, bind to the numerous binding sites on each amplifier.

Detection of RNA in FFPE tumor tissues. **A:** Chromogenic staining (DAB) hybridized with either probes to ubiquitin C (*UBC*) or probes against the bacterial gene *dapB* as negative control. Nuclei were counterstained with hematoxylin. **B:** Fluorescent detection of low-copy transcripts in FFPE samples. Breast tumor tissue section was hybridized with either no probes or with Alexa Fluor 488-labeled probe sets (green) to HPRT1 or POLR2A. Nuclei were counterstained with DAPI (blue). Scale bar = 10 μm.



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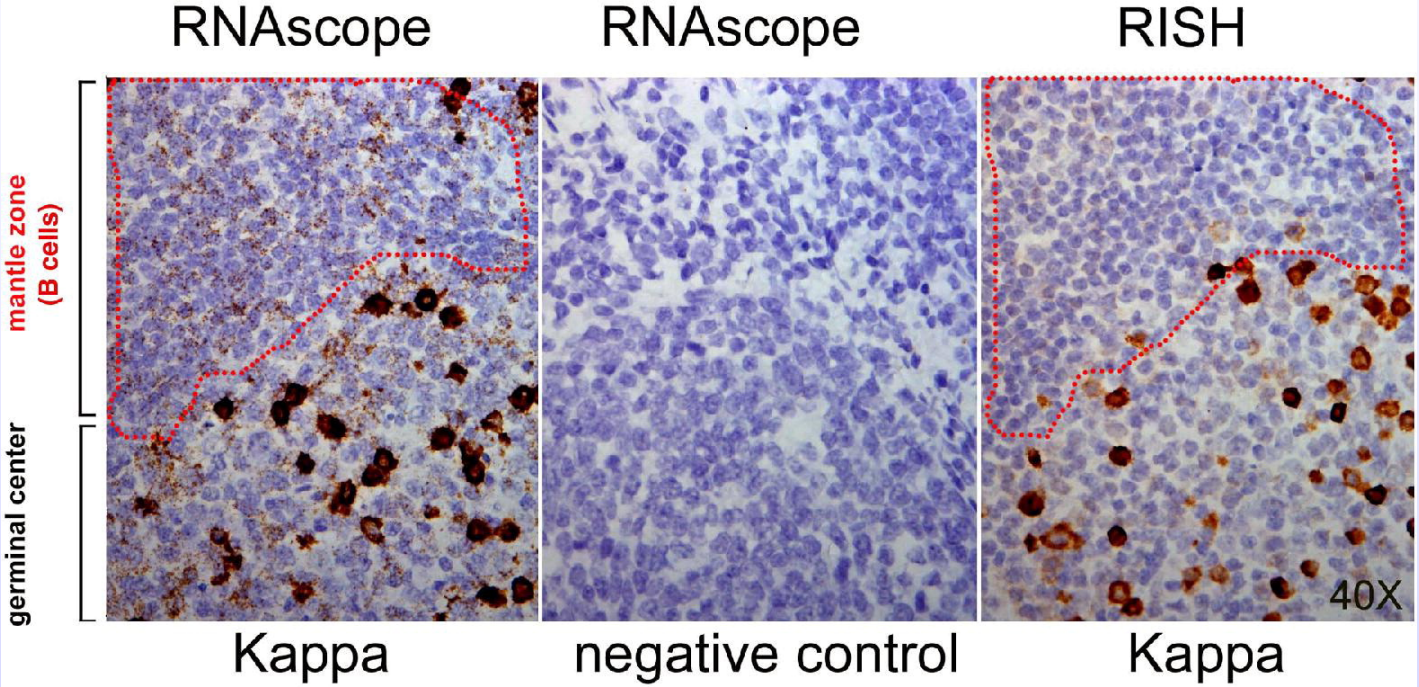
Detection of Ig κ chain expression in B lymphocytes in FFPE human tonsil tissue. κ light chain mRNA transcripts were stained using RNAscope or a commercial non-radioisotopic RNA ISH kit. For RNAscope, a negative control (bacterial gene *dapB*) was also included. The **dotted line** outlines the mantle zone. Original magnification, ×40.



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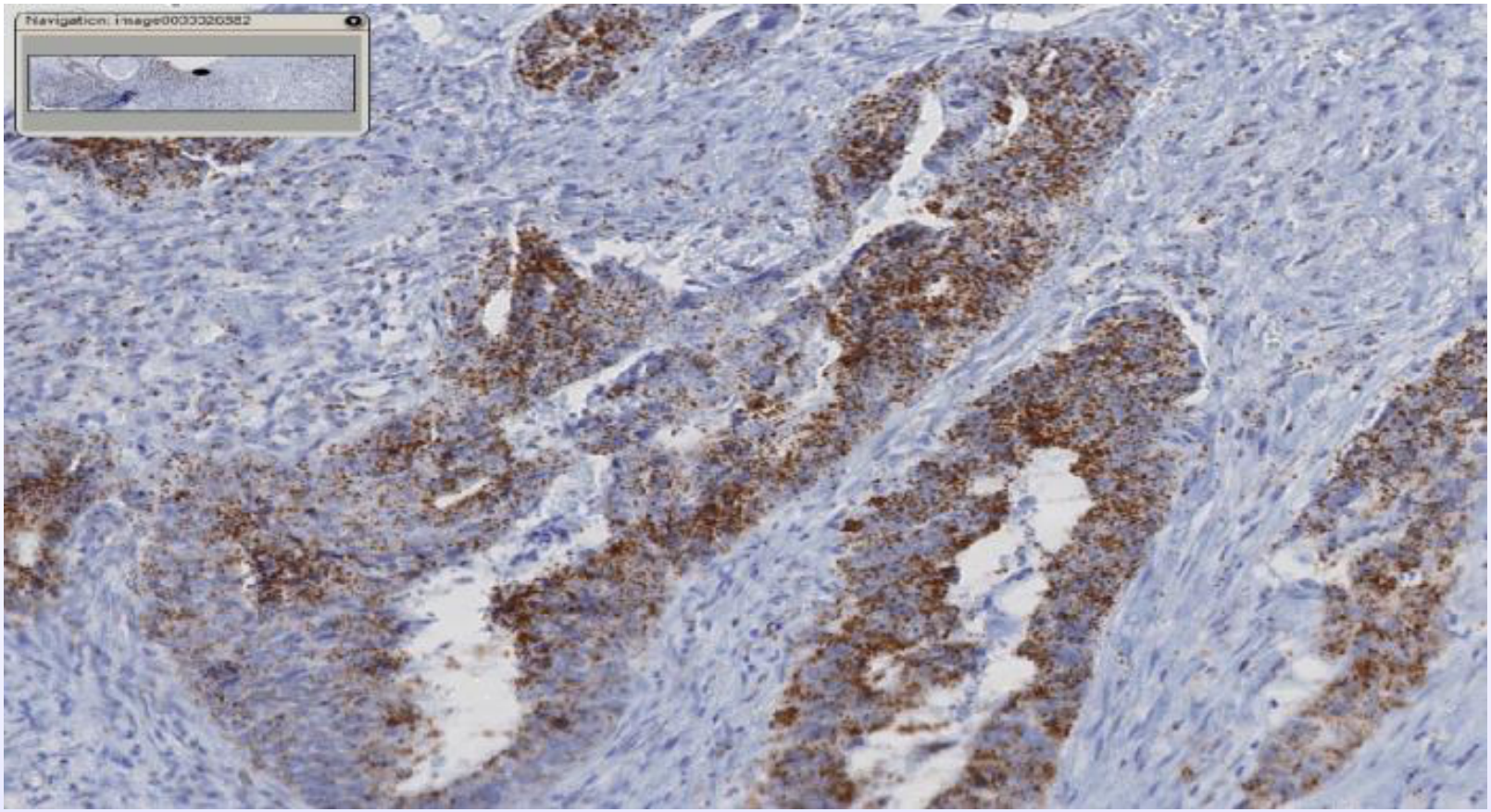
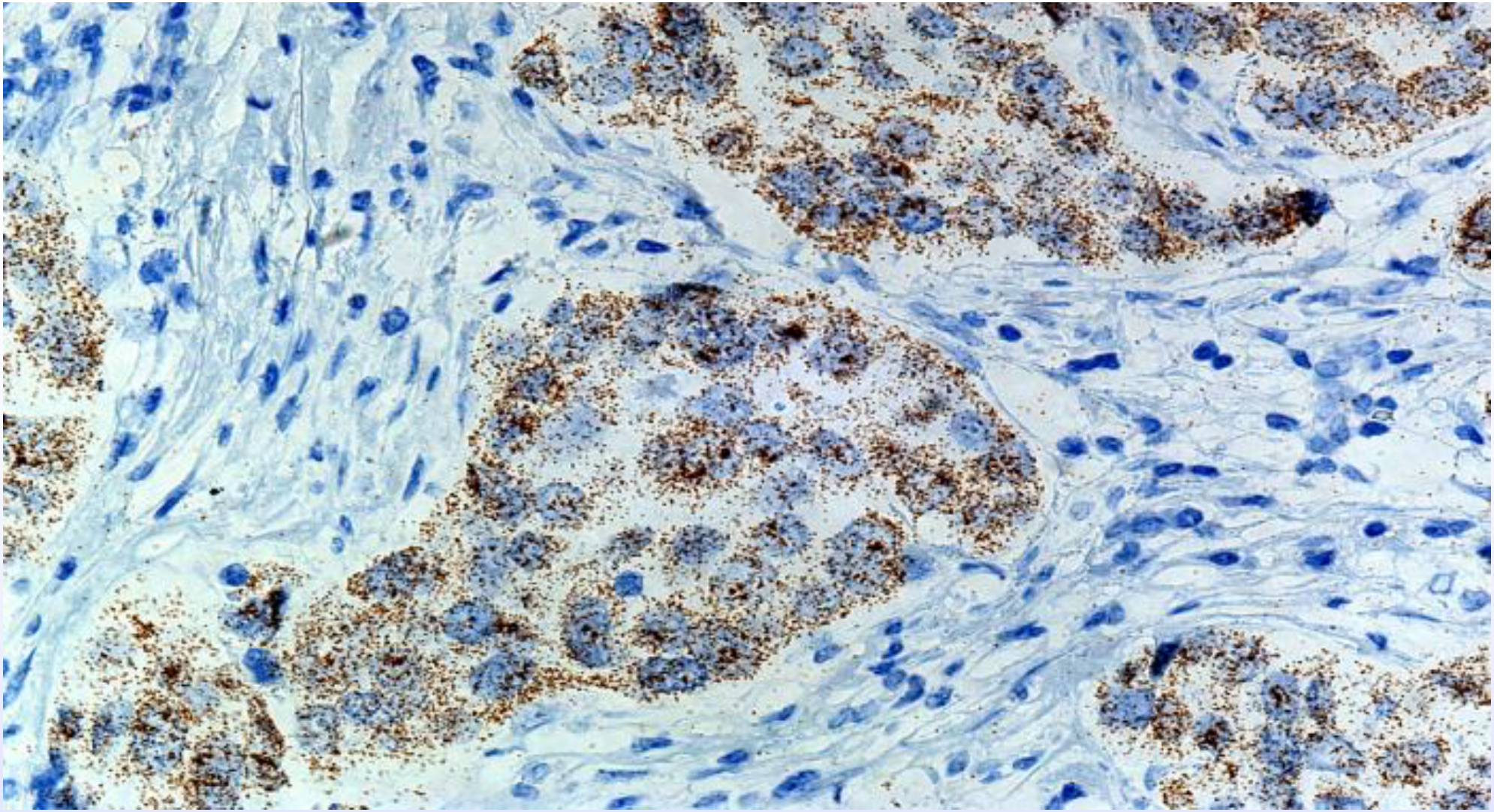
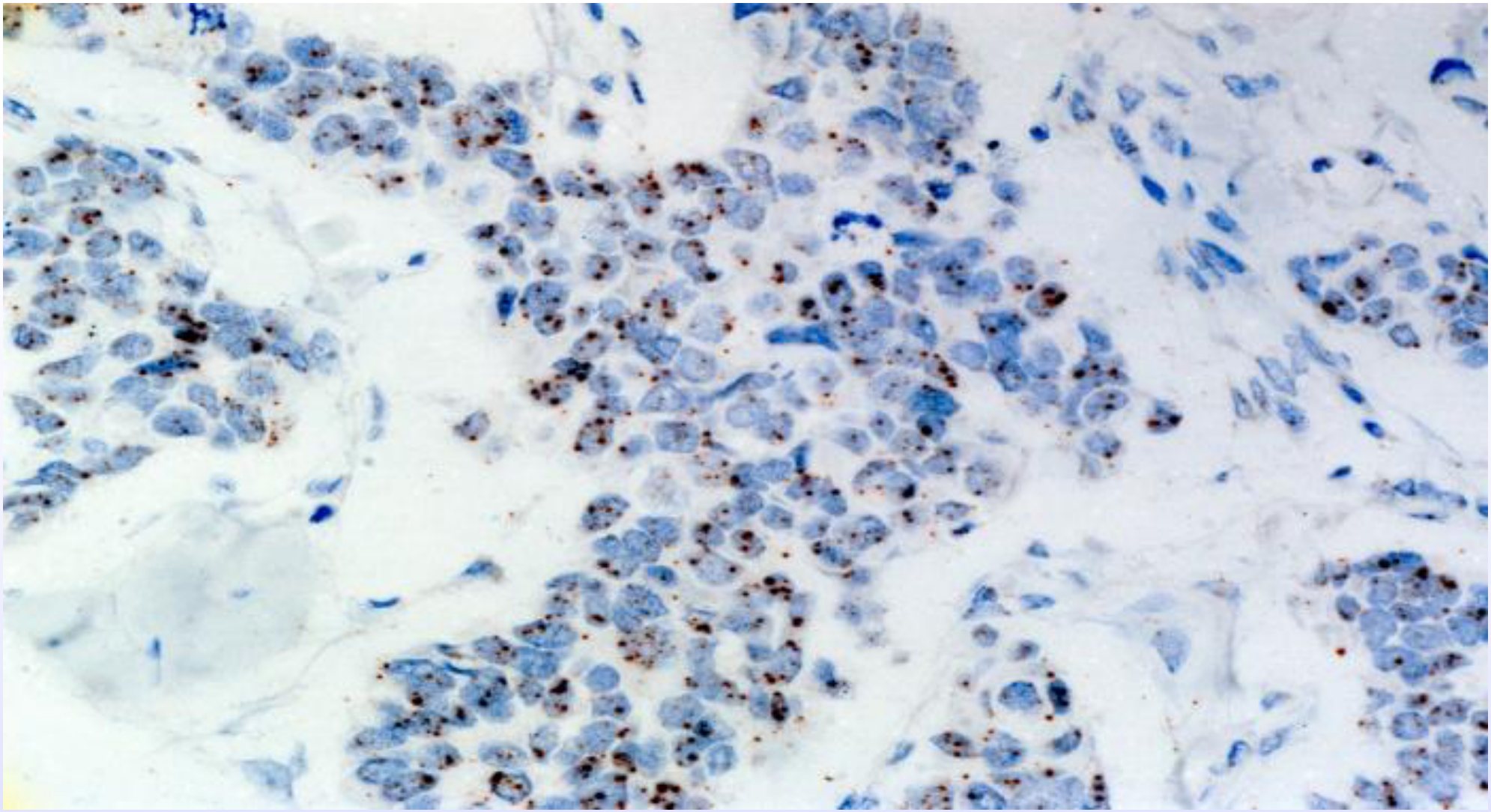


TP53 mRNA expression in human colon FFPE tissue with Automated RNAscope® VS Brown

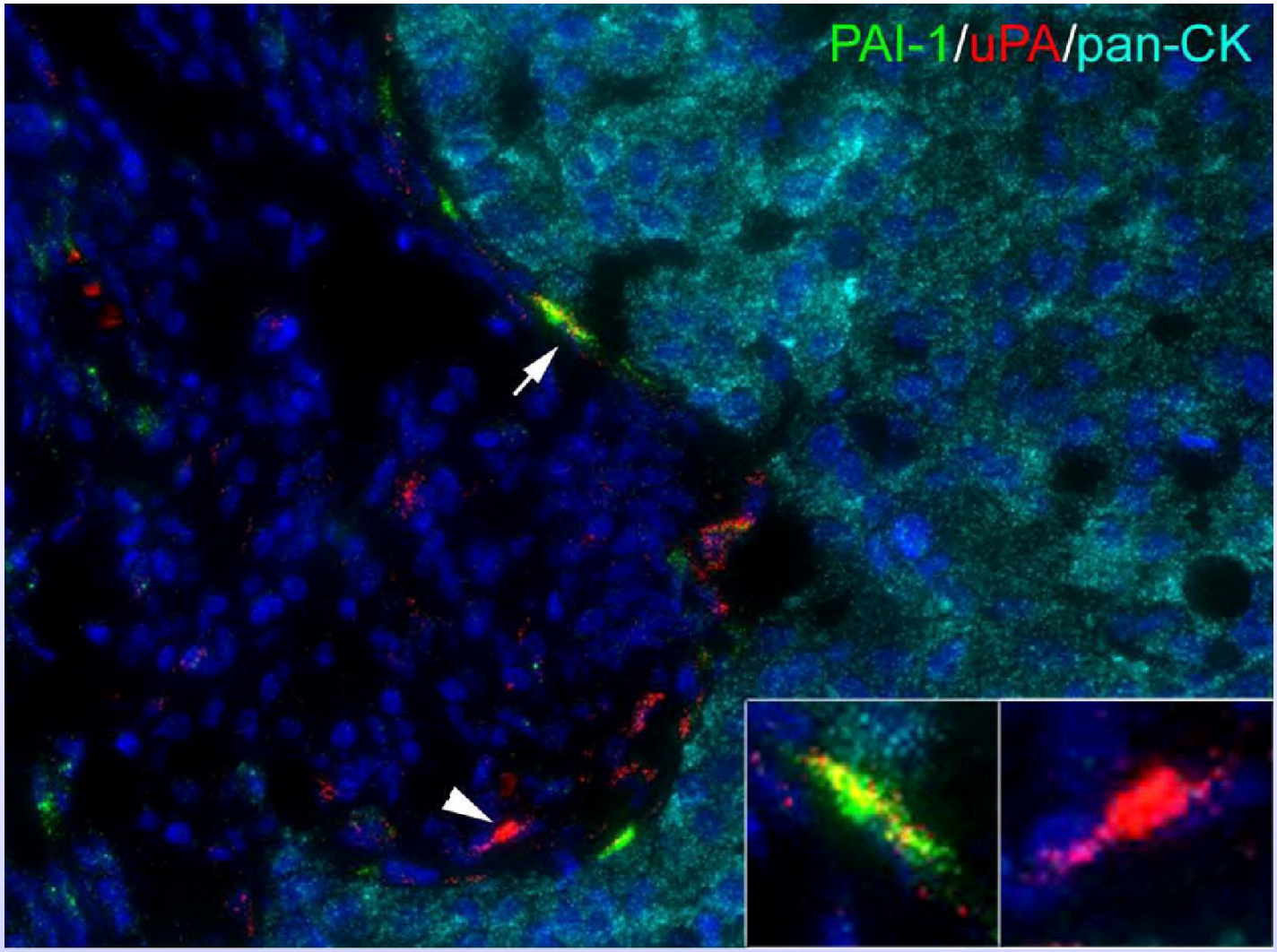


Human breast cancer FFPE tissue: HER2 expression using RNAscope® 2.0 HD Reagent Kit-BROWN



Expression of HOTAIR RNA (brown dots) in human breast cancer FFPE tissue, RNA in situ hybridization (ISH) using RNAscope® 2.0 HD Reagent Kit-BROWN

Multiplex fluorescence detection of uPA and PAI mRNAs in breast cancer. Merged pseudo-colored image of a metastatic breast cancer tissue section stained with probes specific to cytokeratins [PanCK (CK-8, CK-18, and CK-19), labeled with Alexa Fluor 647], uPA (labeled with Alexa Fluor 546), and PAI-1 (labeled with Alexa Fluor 488).



Both uPA expression (**arrowhead** and **right inset**) and coexpression with PAI-1 (**arrow** and **left inset**) were detected. Nuclei were counterstained with DAPI (blue). Original magnification, ×40.

The Journal of Molecular Diagnostics 2012 14, 22-29DOI: (10.1016/j.jmoldx.2011.08.002)