PREANALYTICAL CONDITIONS OF TISSUES

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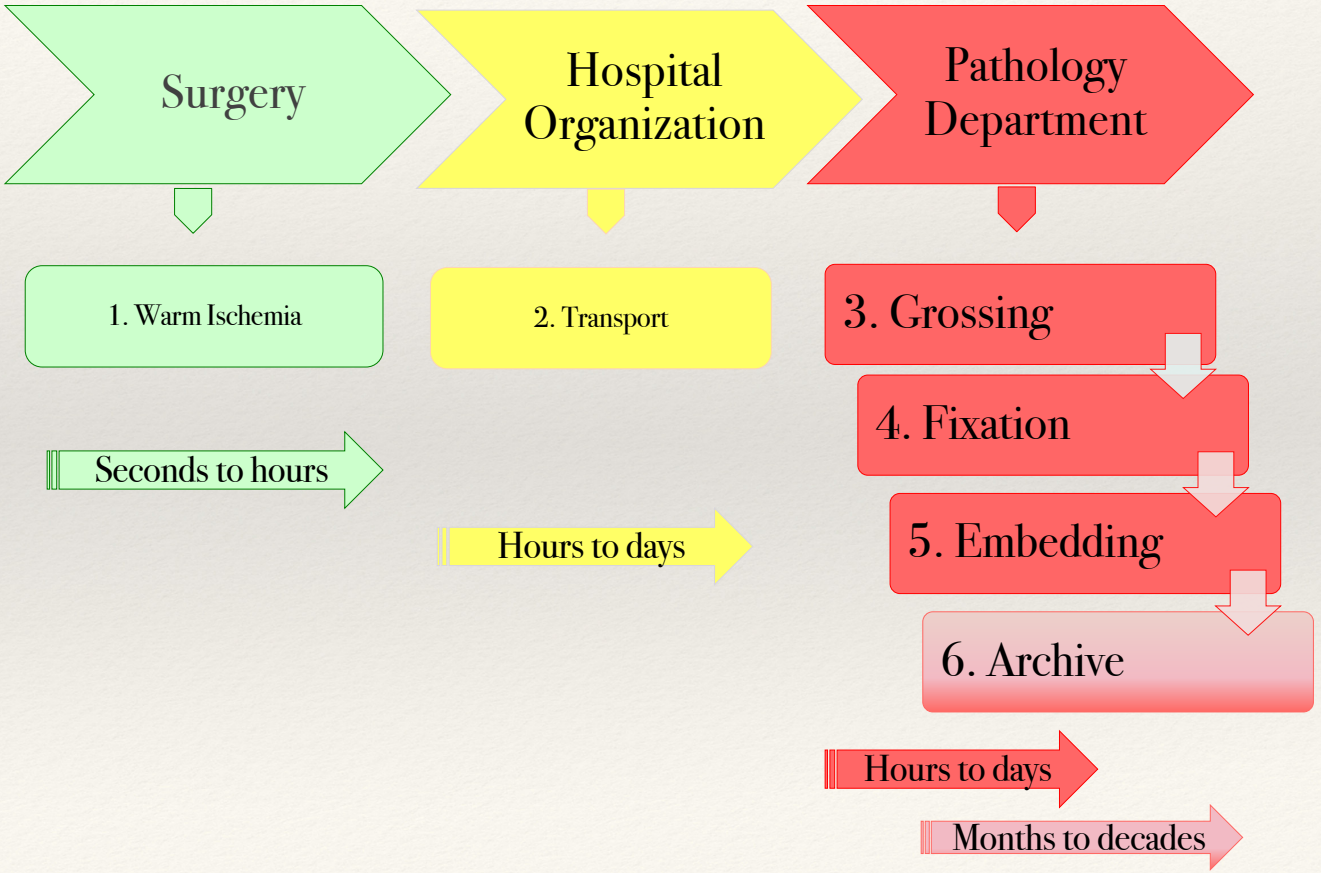
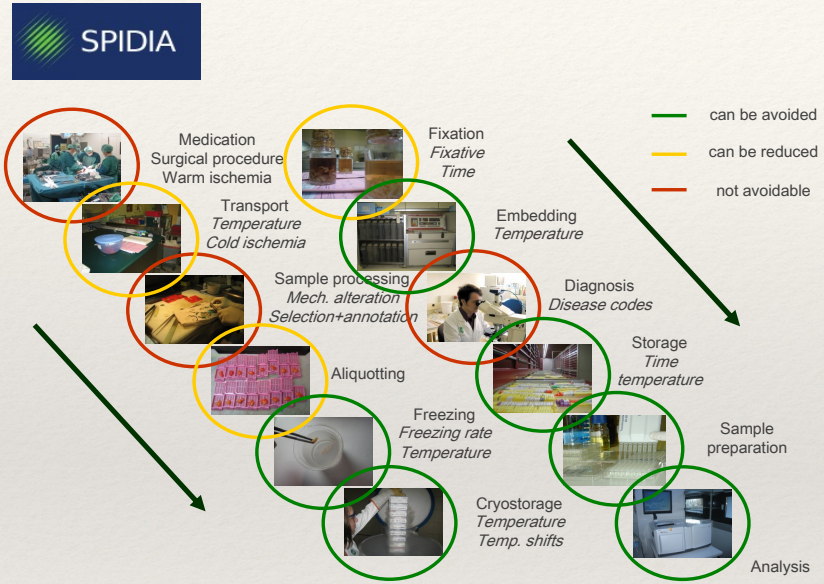
The FFPE Tissues- Pre-analytical processes

AIMS OF PRE-ANALYTICAL PROCESSES: PRESERVATION IN FFPE TISSUES OF

- MORPHOLOGICAL STRUCTURE (histological examination)
- PROTEINS (IHC+ extraction)
- DNA (ISH + extraction)
- RNA (ISH + extraction)

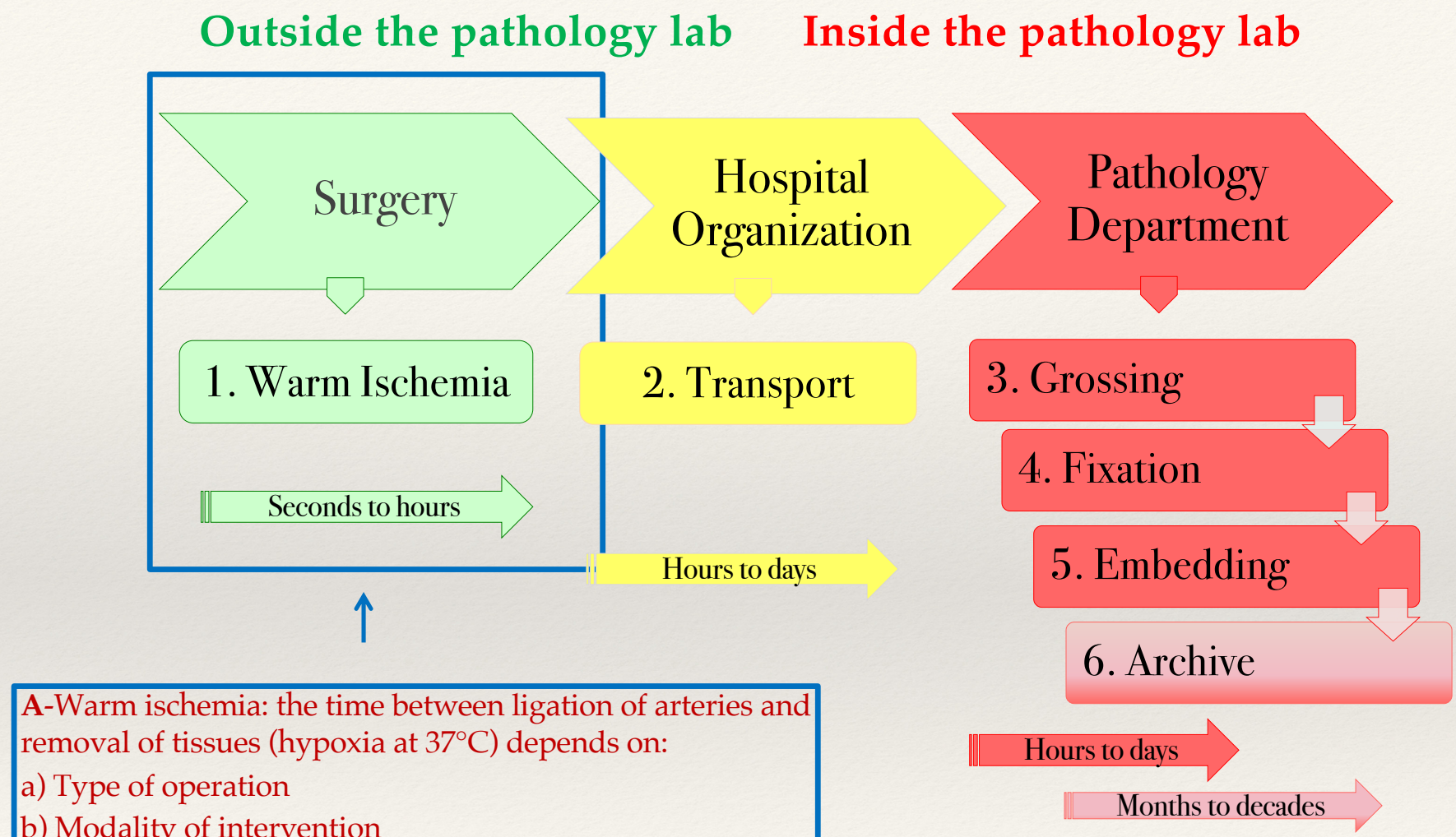


The FFPE Tissues



Original design made available by Kurt Zatloukal

Contents



A-Warm ischemia: the time between ligation of arteries and removal of tissues (hypoxia at 37°C) depends on:

a) Type of operation

b) Modality of intervention

c) Ability of the surgeon

From half to 1 hour for stomach, lung, colon, ……..

Pre-analytical Conditions

Outside the pathology lab **Inside the pathology lab**

Surgery

Hospital Organization

Pathology Department

1. Warm Ischemia

2. Transport

3. Grossing

4. Fixation

5. Embedding

6. Archive

Seconds to hours

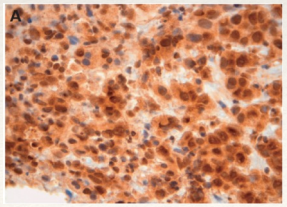
Hours to days

Hours to days

Months to decades

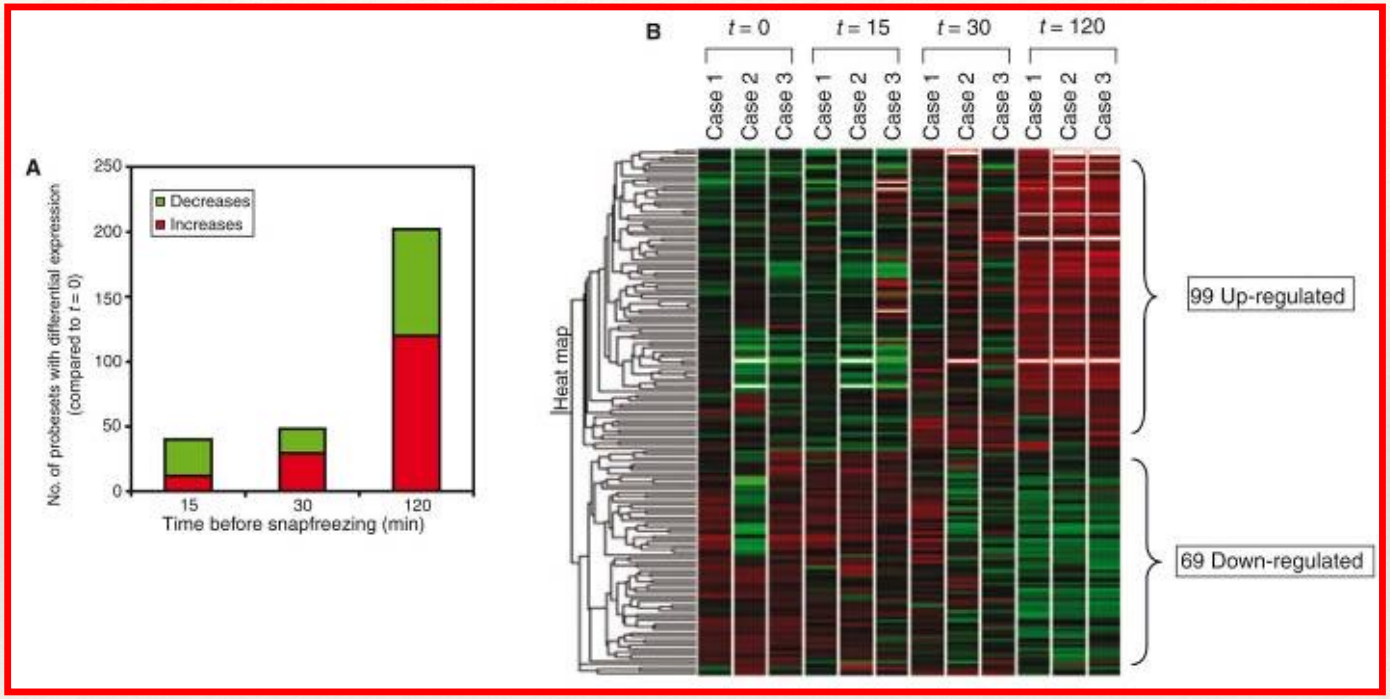
B- *In the surgical theatre the use of specific techniques (heat cutting, …) or simply the traction and distortion of tissues can influence gene expression modification, protein coagulation or their diffusion into extracellular spaces with consequences for IHC results.*

C- *IHC reliability and RNA analysis can also be affected by common pathological processes such as hemorrhagic diffusion in tissues, necrosis, inflammation or apoptosis.*



Inducible genes: warm ischemia and cold ischemia can differently influence genes with increased or decreased expression, with changes in mRNA expression but also at the protein level.

On the other hand, many genes can be totally indifferent to ischemia.



Histopathology 2010, **56**, 240–250. DOI: 10.1111/j.1365-2559.2009.03470.x

Gene expression in colorectal neoplasia: modifications induced by tissue ischaemic time and tissue handling protocol

Susan E Bray, Fiona E M Paulin, Siew Chinn Fong, Lee Baker, Frank A Carey,[1] David A Levison,[1] Robert J C Steele & Neil M Kernohan[1]

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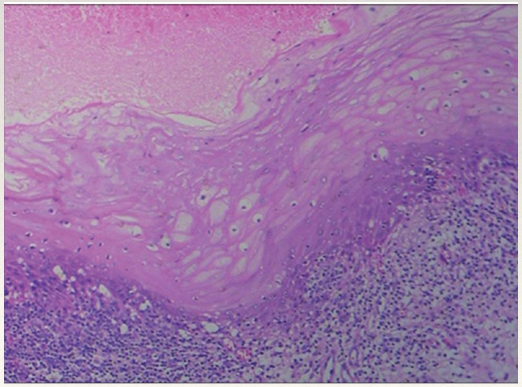


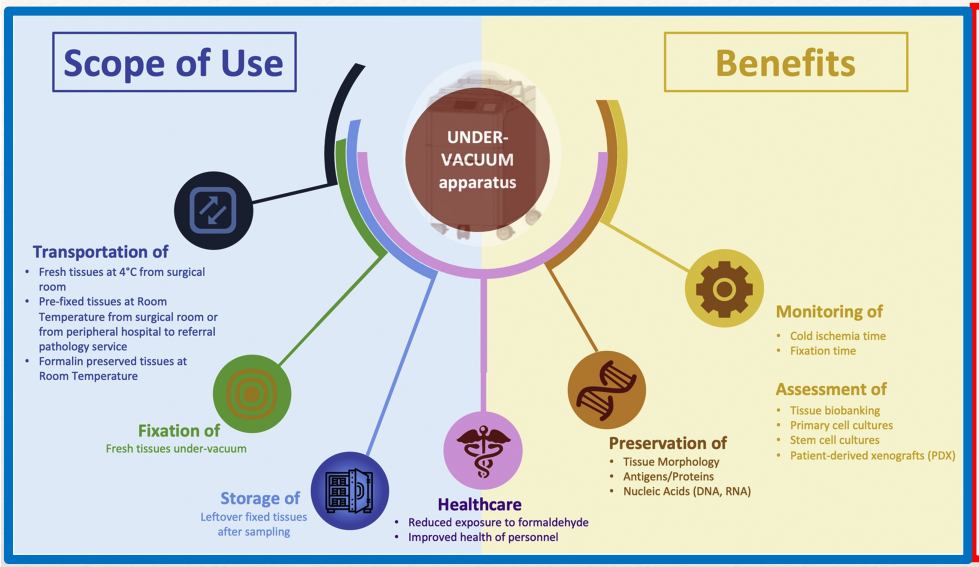
Interval from the surgical table to the pathology lab
Alternatives to shorten cold ischemia:
*a) Tissues left fresh transported at r.t. or in wet ice**
b) Tissues immersed in formalin-small biopsies-annotation time
*c) Transport at 4°C under vacuum**
Avoid to air dry the tissues

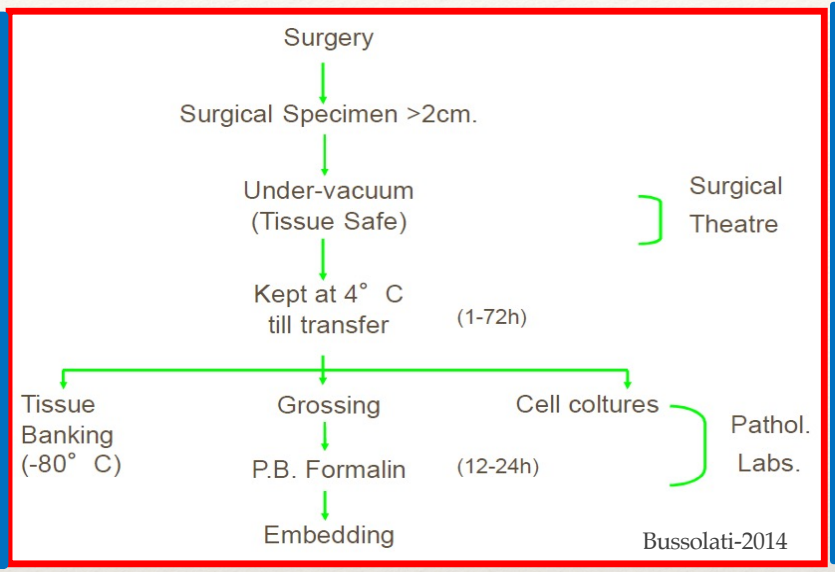
Hours to days

Months to decades

**Accidentally freezing and thawing the tissue (e.g., by using cool packs in a wrong manner) can lead to protein degradation*

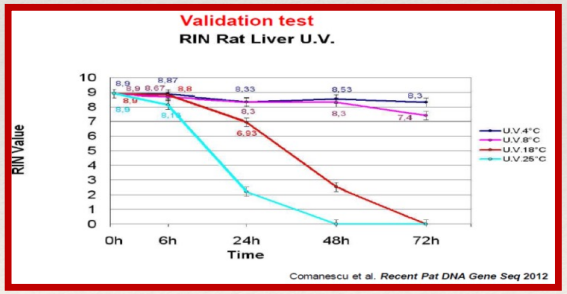
Tissue transport under vacuum

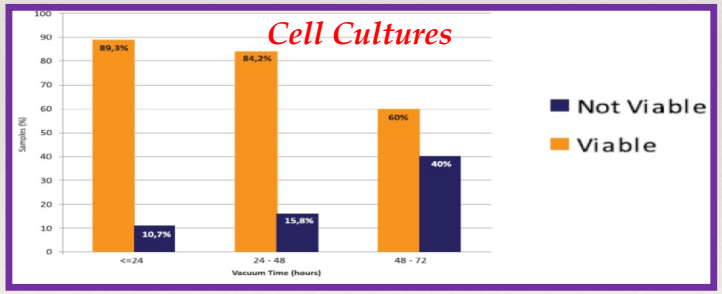


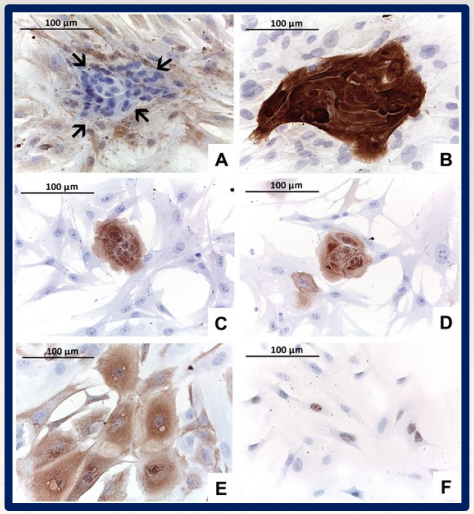


New Biotechnology 2019, 52:104-109

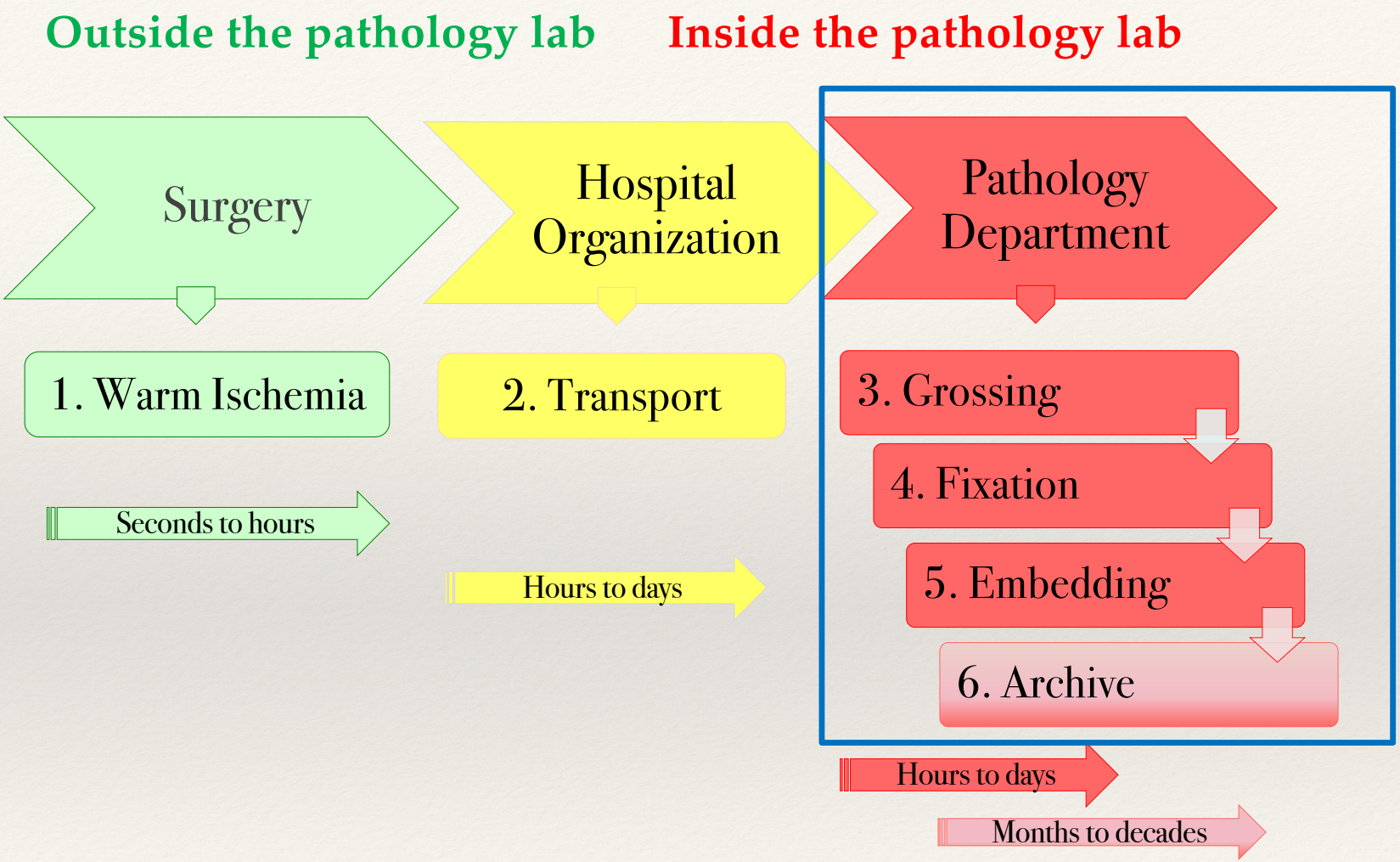
G. Bussolati Graz 2014







(2013) A Collection of Primary Tissue Cultures of Tumors from Vacuum Packed and Cooled Surgical Specimens: A Feasibility Study. PLOS ONE 8(9): e75193.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Seconds to hours
Hours to days
Hours to days
Months to decades

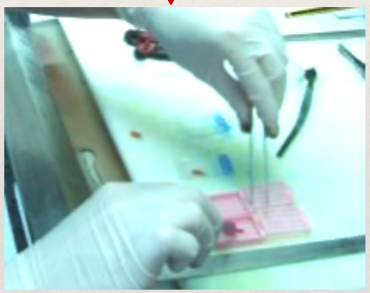
CLINICAL TISSUES – FFPE

DIAGNOSTICS

SURGERY

PATHOLOGY
Selection

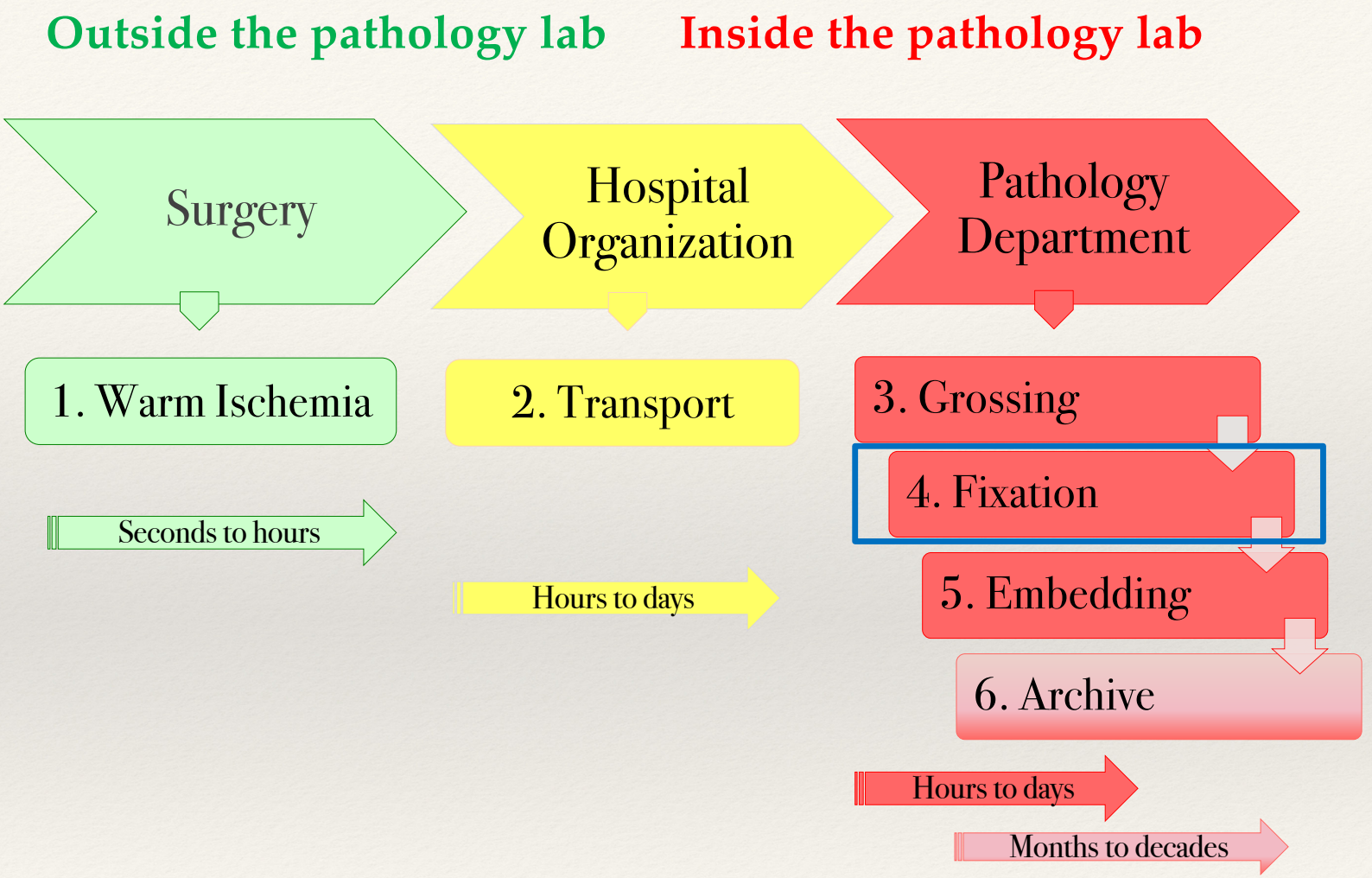
HUMAN TISSUES
DIAGNOSTIC FLOW



Fixation
Paraffin-
embedding

Slides
preparation

PATHOLOGY
ARCHIVES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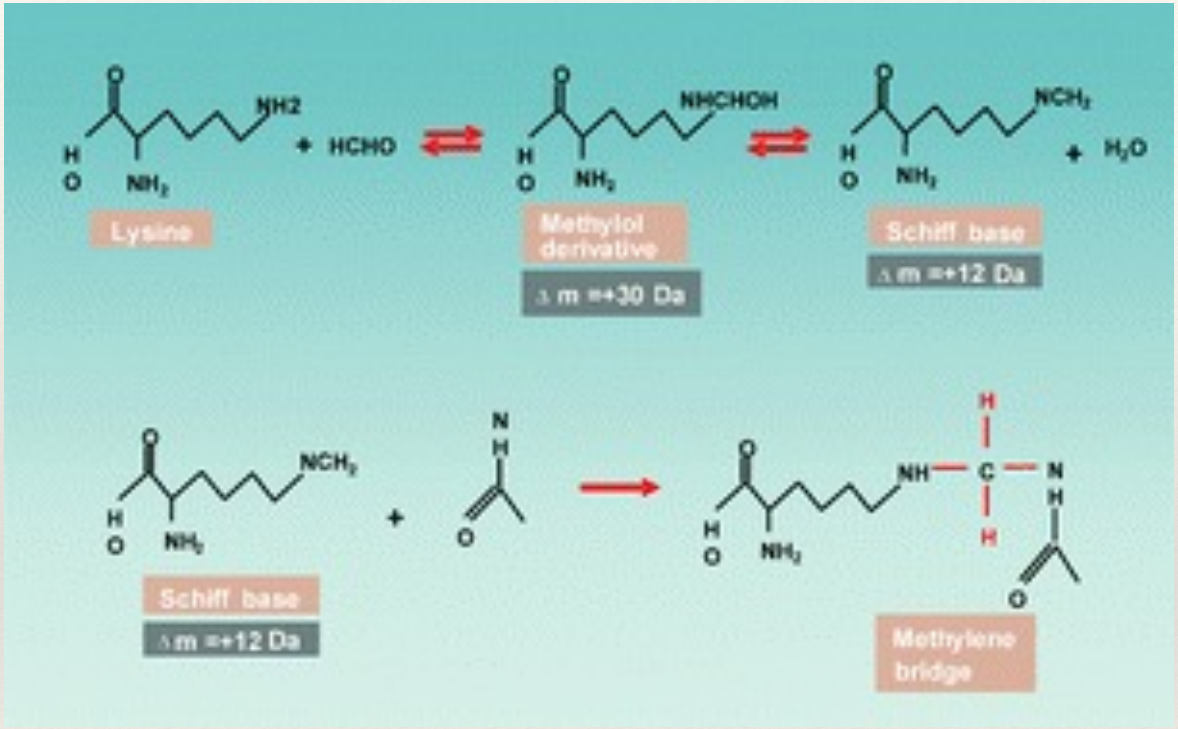
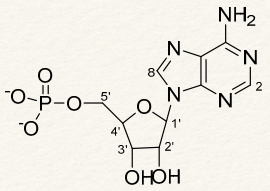
FORMALIN FIXATION

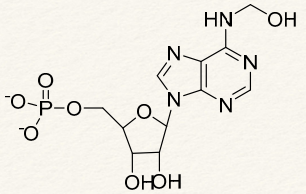


FIXATION TIME

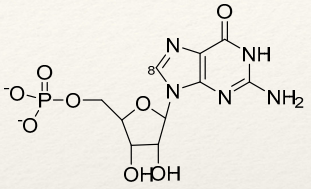
Gerard J. IN SITU MOLECULAR PATHOLOGY AND CO-EXPRESSION ANALYSES



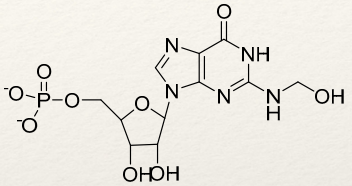
1a: AMP



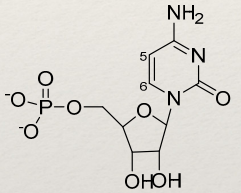
1b: Hm-AMP



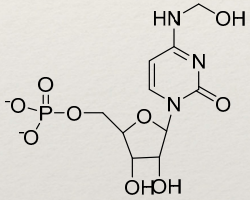
2a: GMP



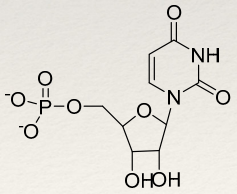
2b: Hm-GMP



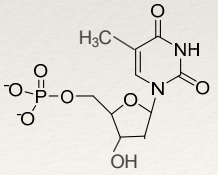
3a: CMP



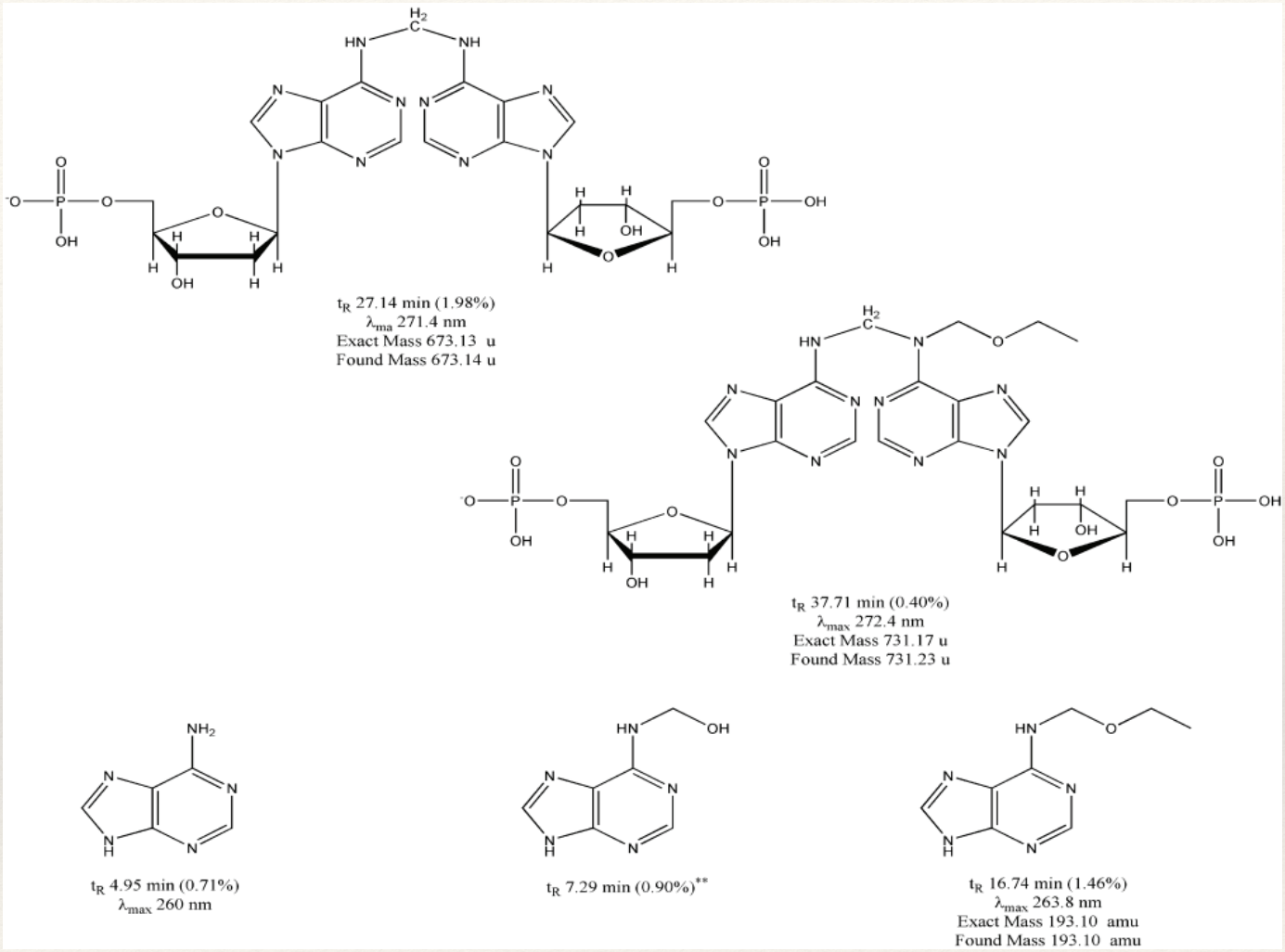
3b: Hm-CMP



4a: UMP



dTMP



Rait VK Volume 54(3): 301–310, 2006 Journal of Histochemistry & Cytochemistry

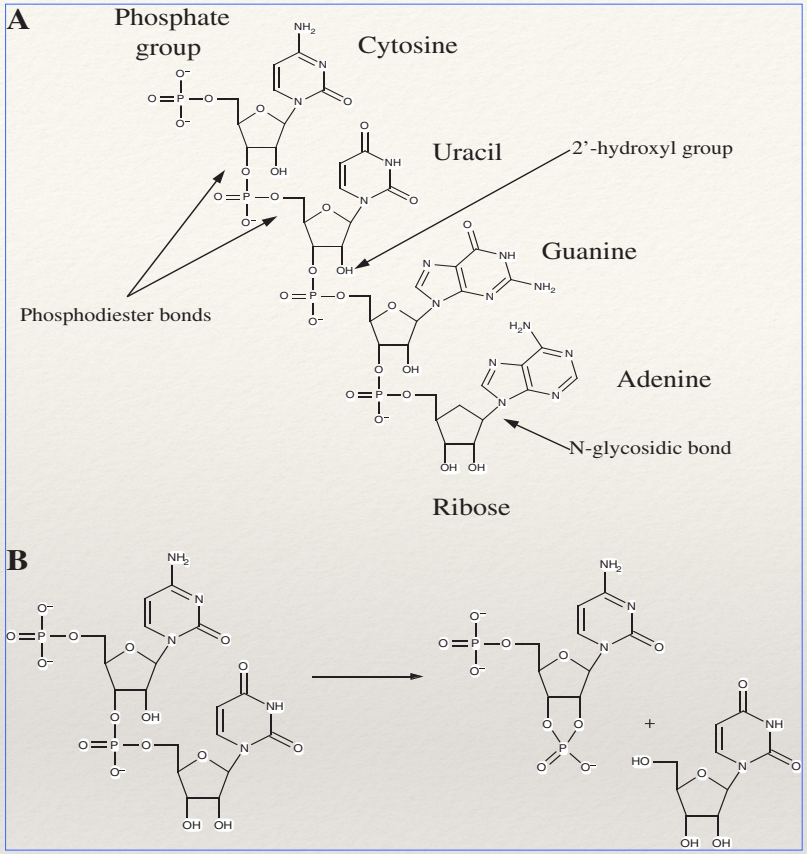
Formalin penetration rate/equilibrium RNA and protein artifacts

*# The **formalin penetration rate does not correspond to fixation**, the formaldehyde-methylene glycol equilibrium shifts towards formaldehyde raise the effective concentration of the active molecule.* $CH_2=O + H_2O \rightleftarrows OH\text{-}(CH_2O)\text{-}H$

*# Molecules are modified by fixation in formalin with **artifacts**: the formation of methylene bridges among different aminoacidic residues, RNA hydrolysis and nucleic acids mechanical rupture is due to molecule stiffening from crosslinking.*

*# Alterations are quantitatively related to **time of fixation**.*

*# **Due to the thickness of tissues, alterations are not uniform**: from over-fixation in the outer part, to hypoxia in the inner part of the tissue at the same time, alterations are complex.*



Fordyce et al; Investigative Genetics 2013

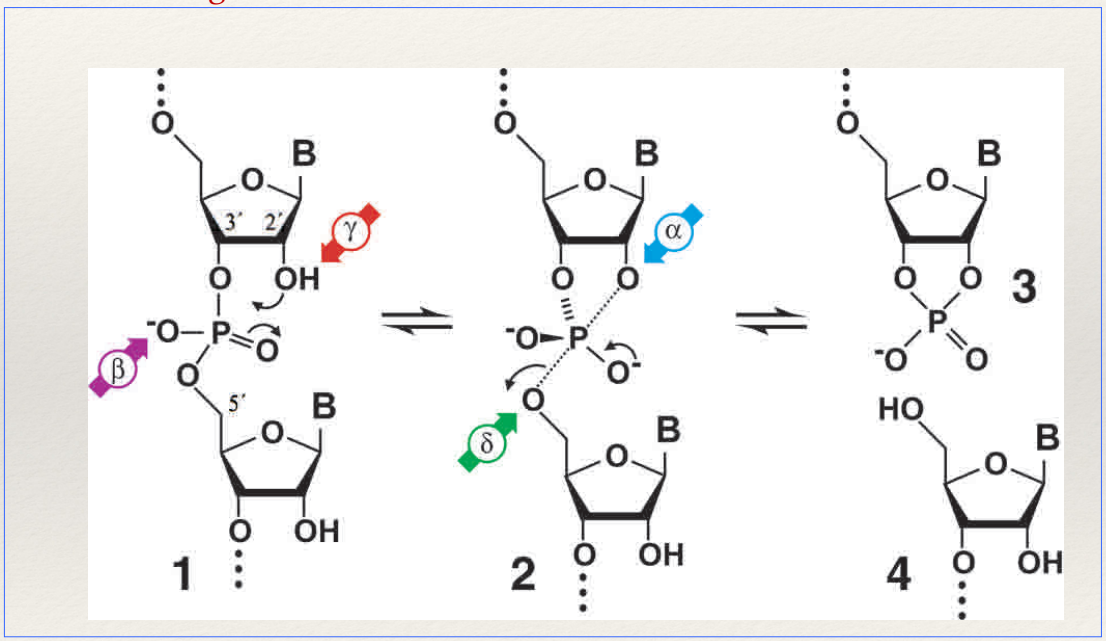
The 2′-OH allows the RNA molecule to be more easily degraded via hydrolysis than DNA.

The phosphodiester bond in RNA can be broken during hydrolysis.

The N-glycosidic bond is stronger in RNA than DNA

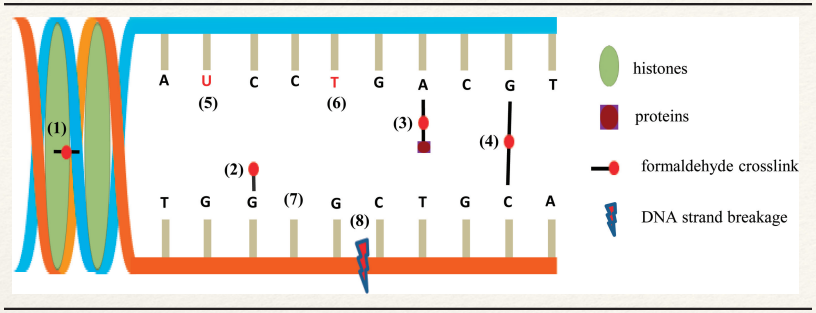
The chemical process of hydrolysis, where the 2′-hydroxyl group has attacked the adjacent phosphodiester bond, cleaving the backbone of the RNA.

Fordyce et al; Investigative Genetics 2013



RNAse A increases the rate of RNA cleavage by internal phosphoester transfer

DNA degradation and Artifacts



Types of DNA Damages in FFPE tissues

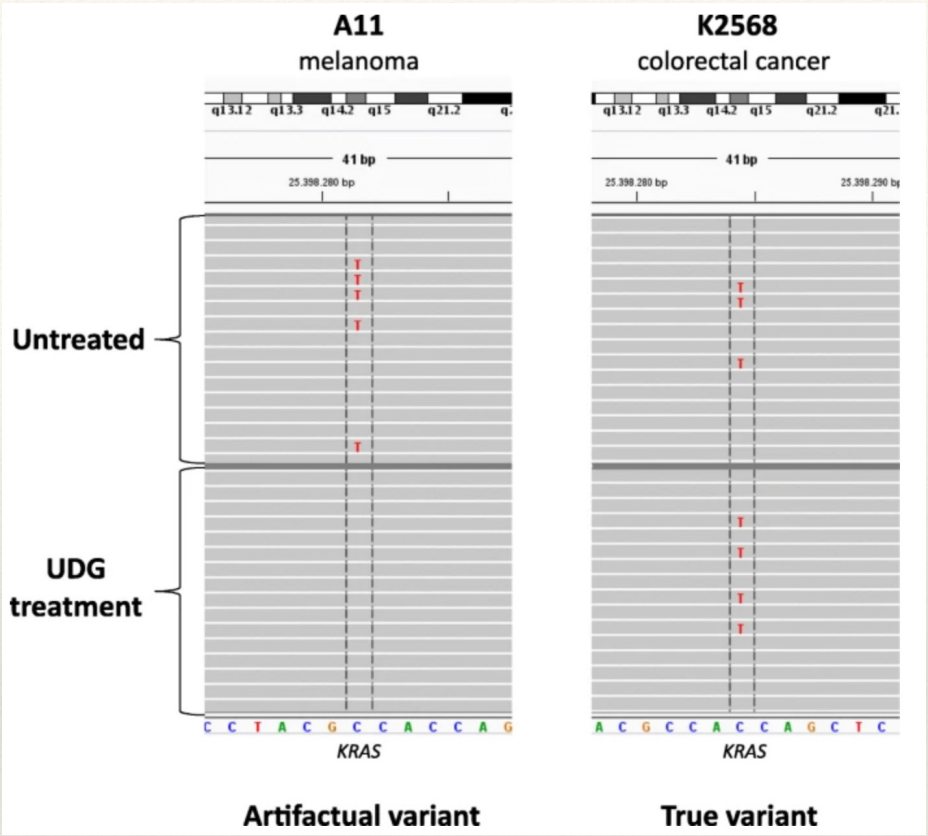
- ✓ **Crosslinks with proteins or NA**
- ✓ **Mono-methylol adducts**
- ✓ **DNA fragmentation**
- ✓ **Abasic Sites**
- ✓ **Deamination of Cytosine bases**

  C→U ☞ C:G>T:A

  5m-C →T ☞ C:G>T:A

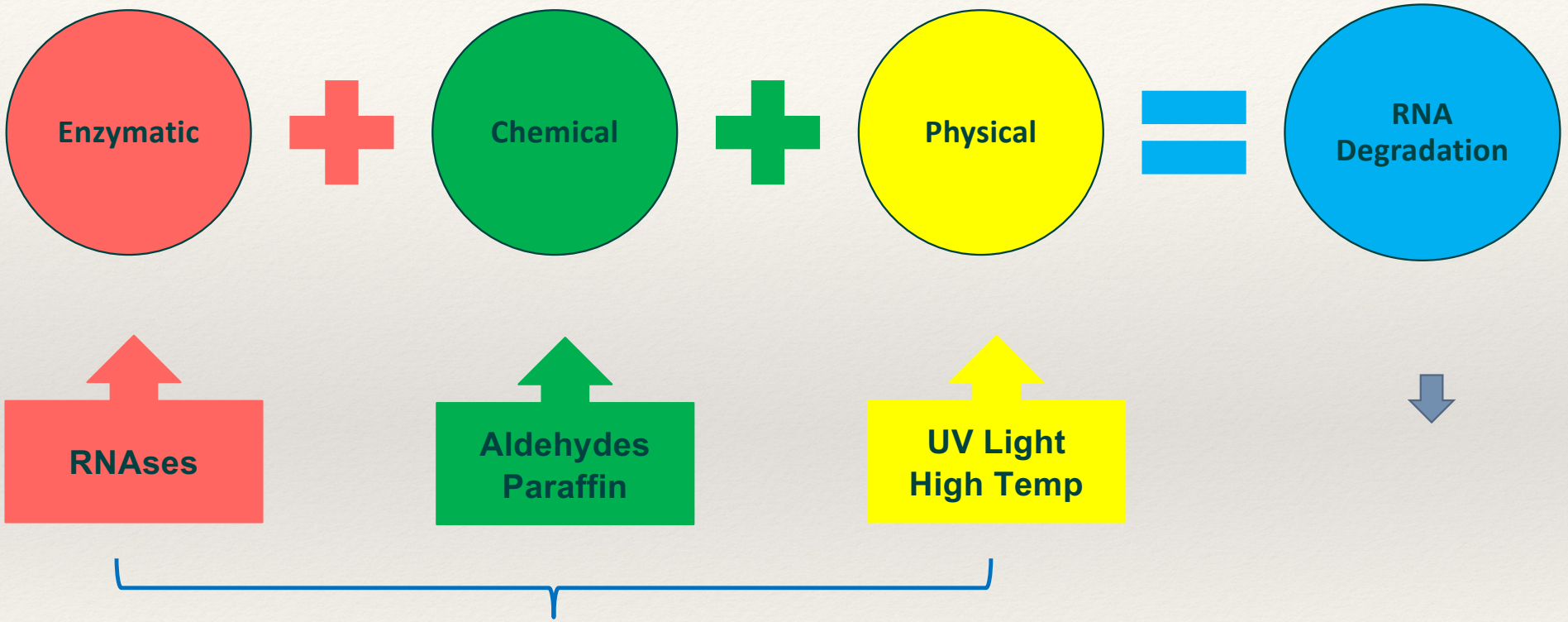
Heat de-modification during isolation

dsDNA + UDG



Applied Cancer Research **volume 39**, Article number: 7 (2019)

RNA degradation



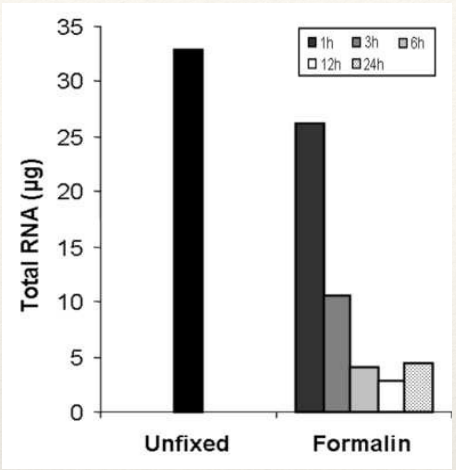
DIFFERENT EFFECTS ON rRNA AND mRNA

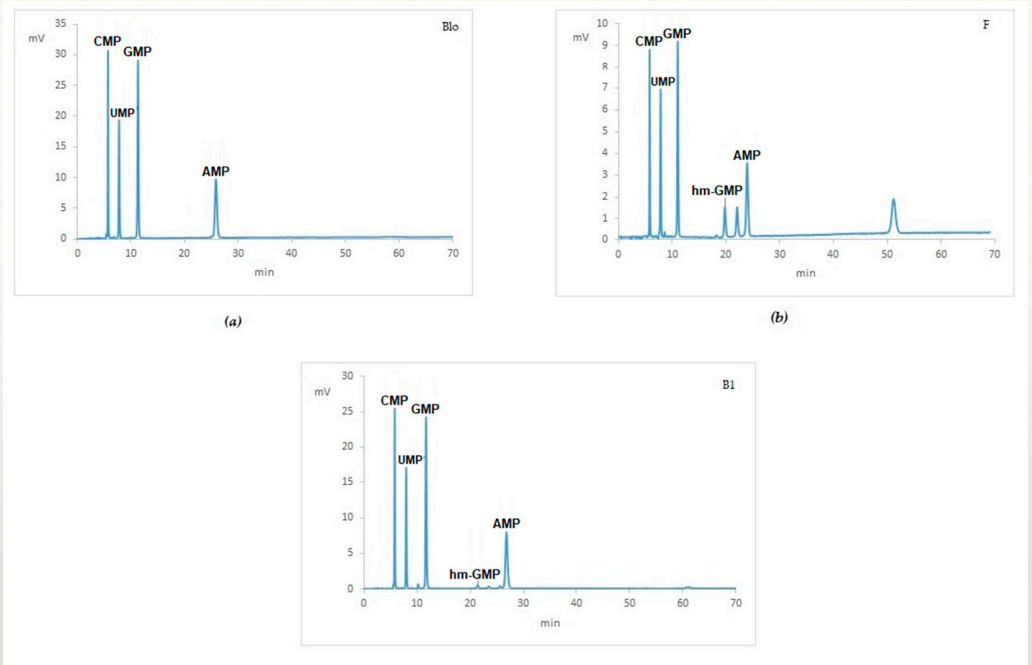
Sidova M et al. 2015 Biomolecular detection and Quantification

RNA degradation

mRNAs	rRNA	miRNA
≠ Half Lives	More resistant to RNAses	Highly conserved and resistant
Exo-RNAses	Similar to chemical	
Endo-RNAses	Similar to physical	

QUALITY AND QUANTITY OF DEGRADED RNA





RNA degradation in FFPE is a cumulative effect:

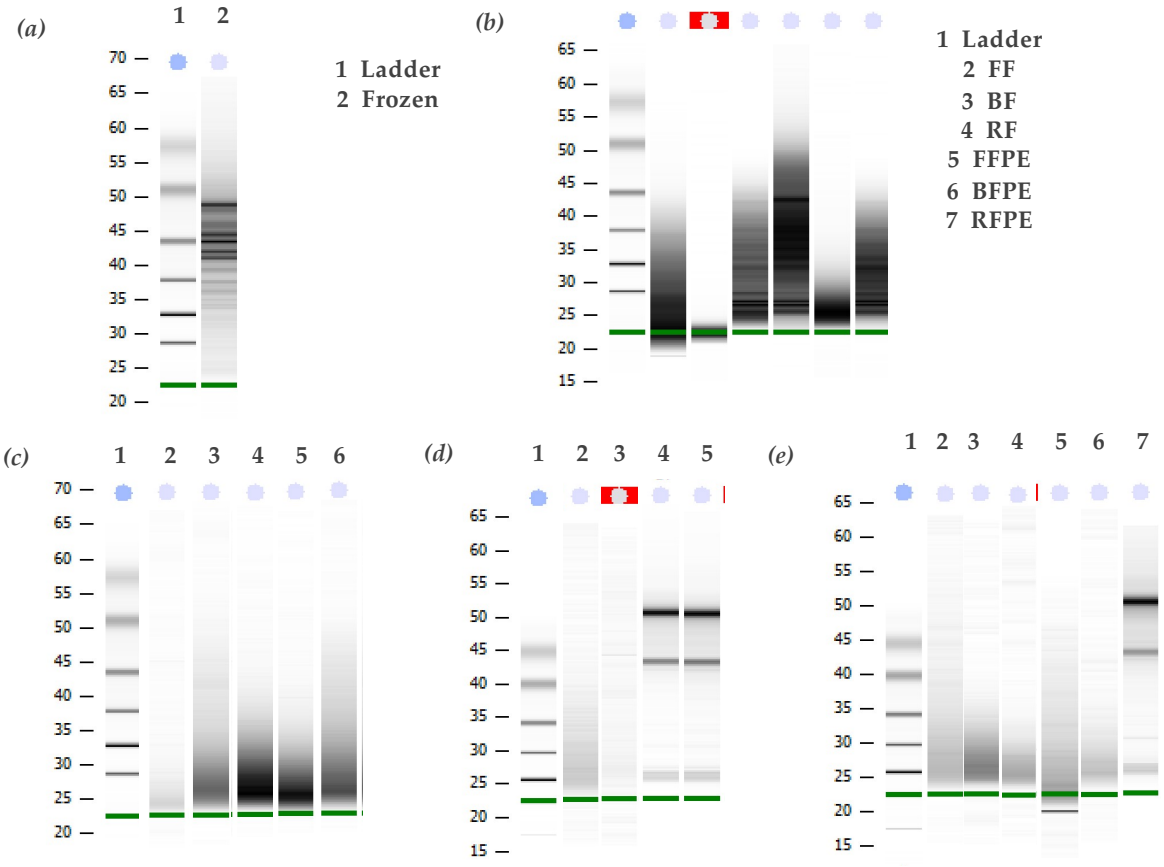
*-**RNAses activity** in pre- and during- fixation time*

*-**hypoxia** in the pre-analysis time*

*-**fixation with mechanical rupture** due to molecule stiffening from crosslinking*

*-**alkaline pH** procedures*

*-**high temperatures**: RNA is thermo-dynamically less stable than DNA because the 2′-OH group on the ribose ring promotes the hydrolytic reaction*

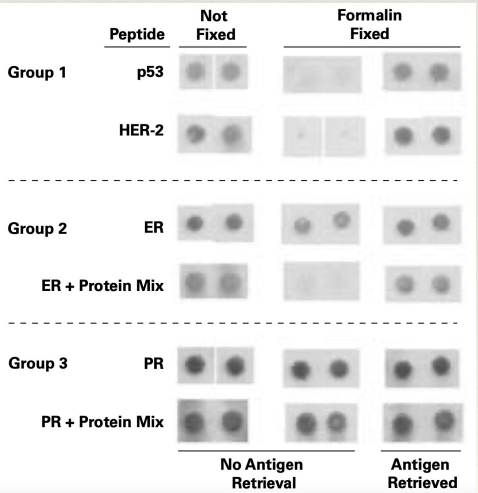

(a)
1 2
70
65
60
55
50
45
40
35
30
25
20
1 Ladder
2 Frozen
(b)
65
60
55
50
45
40
35
30
25
20
15
1 Ladder
2 FF
3 BF
4 RF
5 FFPE
6 BFPE
7 RFPE
(c)
1 2 3 4 5 6
70
65
60
55
50
45
40
35
30
25
20
(d)
1 2 3 4 5
65
60
55
50
45
40
35
30
25
20
15
(e)
1 2 3 4 5 6 7
65
60
55
50
45
40
35
30
25
20
15

PROTEINS in FFPE

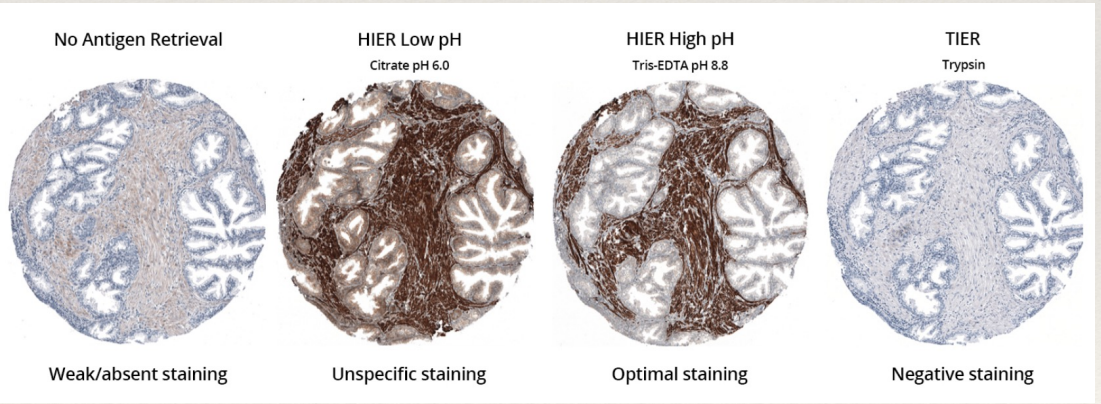
Formalin fixation → **NON SELECTIVE**

↓

CHANGES IN THE 3D CONFORMATION OF PROTEINS → **DECREMENT IN IMMUNOREACTIVITY** → **ANTIGEN RETRIEVAL**



Am J Clin Pathol 2004;121:190-199
DOI: 10.1309/BRN7CTX1E84NWWPL



Zinc finger C4H2 protein in human smooth muscle tissue. Expected membranous staining.
https://blog.atlasantibodies.com/how-to-succeed-with-your-ihc-antigen-retrieval

IS IT POSSIBLE TO IMPROVE FORMALIN FIXATION?

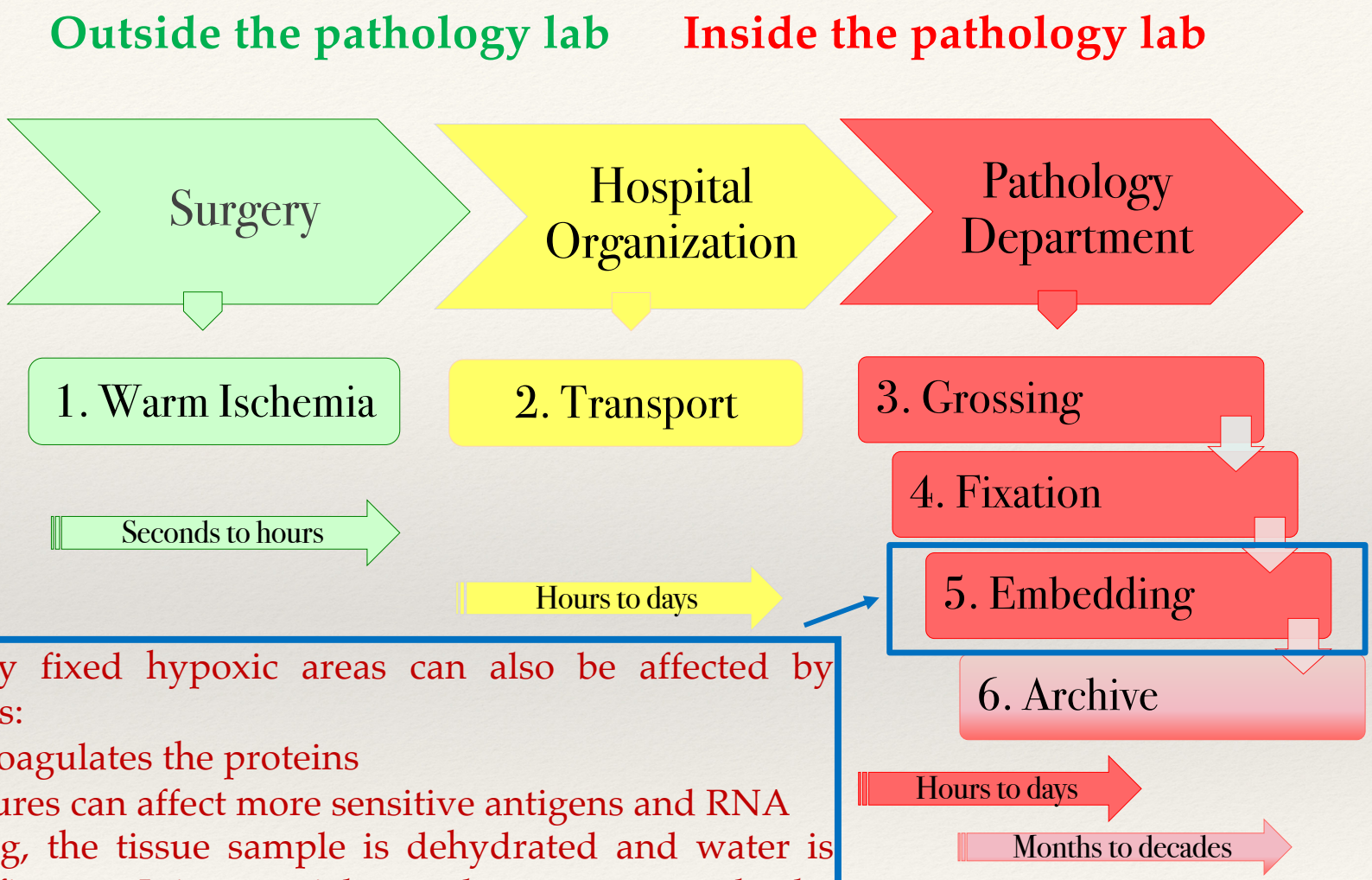
❖Controlled fixation time ➡

❖Cold fixation (longer fixation at low T)

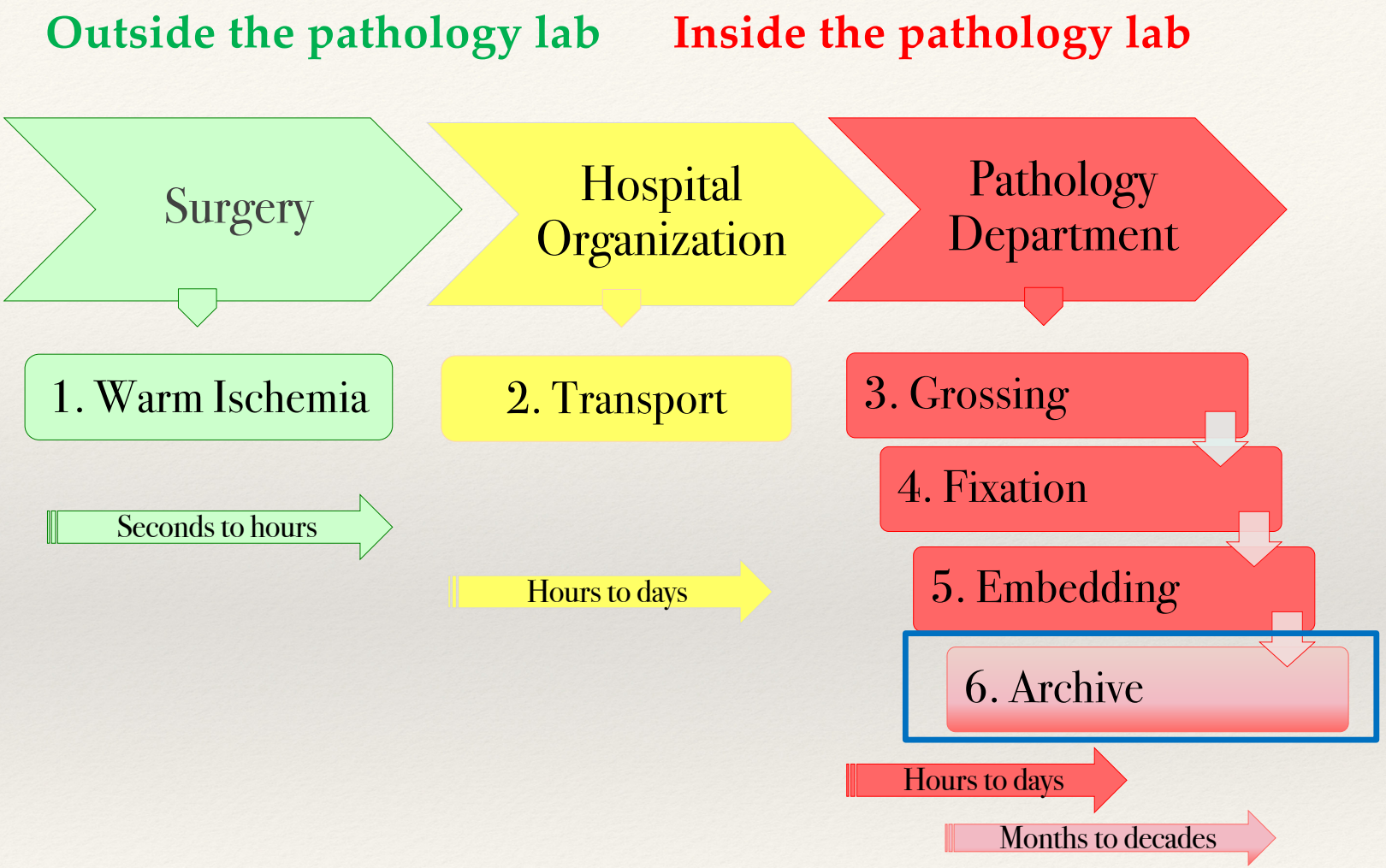
⬇

 # Protein and RNA degradation would be inhibited by maintaining low temperature throughout the fixation process.

Chafin D et al PLOS OONE 2013
Bussolati G et al. PLOS ONE 2011



The inner poorly fixed hypoxic areas can also be affected by inclusion procedures:
-alcohol treatment coagulates the proteins
-inclusion temperatures can affect more sensitive antigens and RNA
During processing, the tissue sample is dehydrated and water is replaced with paraffin wax. It is essential to replace water completely, since residual water leads to tissue degradation during storage.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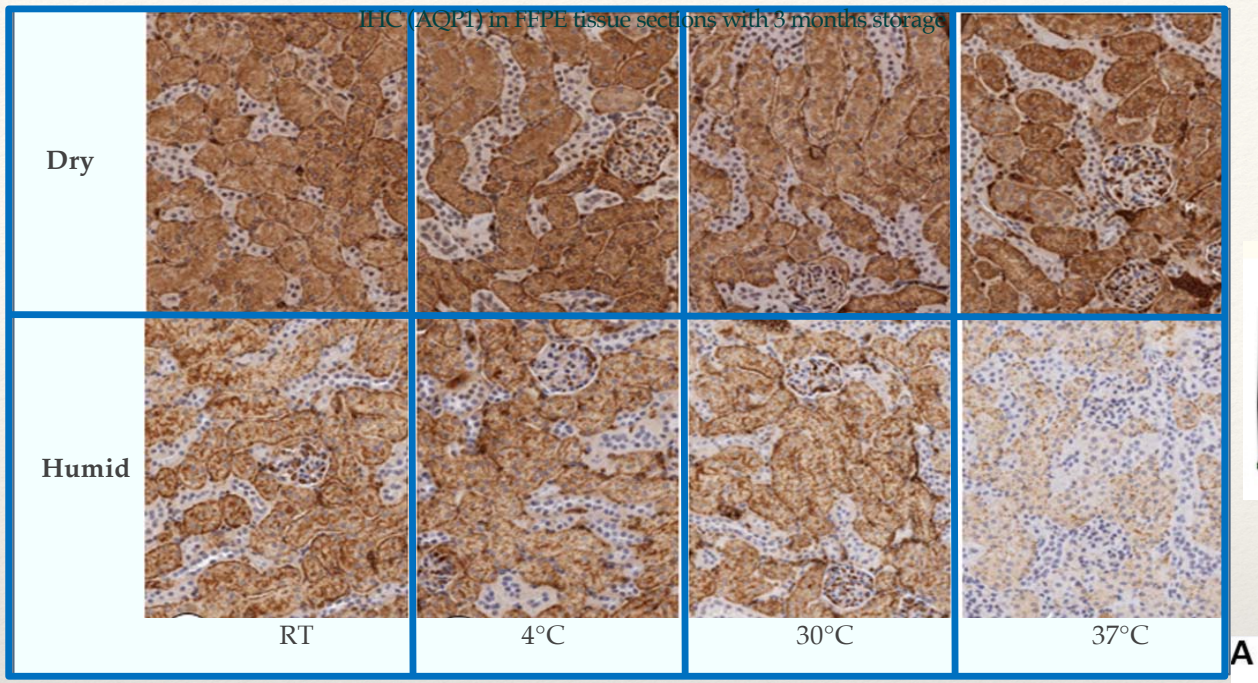
Storage Effect

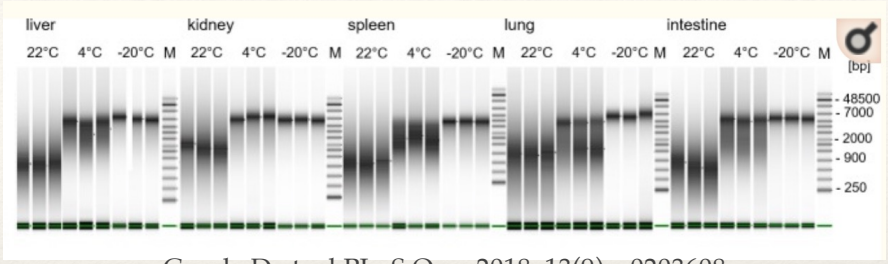


Modified from Xie et al, J Histochem Cytochem 59:356–365, 2011

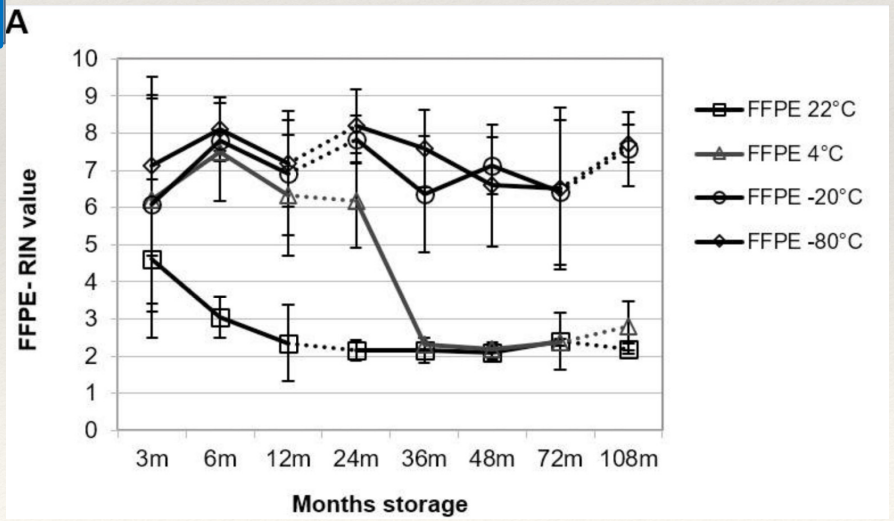
#Storage time may influence the retrieval of antigens and quality of RNA (Balgley, B. (2009) Journal of Proteome Research, 8, 917–925)

#While histology is not affected by storage, protein and RNA degradation may increase with increasing storage time, especially for long time (Wolff, C.. (2011) PloS One Journal, 6, e16353.)

#Storage conditions, such as humidity and temperature, can have an impact on protein and RNA amounts and quality (Thompson, S (2013). Proteomics - Clinical Applications, 7, 241–51



Groelz D et al PLoS One. 2018; 13(9): e0203608



Groelz D et al PLoS One. 2018; 13(9): e0203608

!!! FFPE Tissues are submitted to additional steps that alter the biomolecules' structure and promote their degradation

CEN documents and ISO standards help to define higher level of standardization in tissue processing for NA and proteins in clinical tissues

Reversal of formaldehyde adducts is possible by antigen retrieval for proteins and heat de-modification for NA

SOPs mandatory and dedicated to FFPE material (e.g. short amplicons, more HSK genes analysis, ……)

Bio-molecules (NA and proteins) degrade in FFPE blocks during storage⟹ re-evaluate HKG for subsequent analyses and/or store aliquots of NA and proteins, alternative storage conditions

THE QUALITY OF THE SAMPLE

Standardized methods and quality assurance documentation can be used as tools for:

- ✓ recognition and rejection of ‘not fit for purpose’ samples on the basis of detailed sample metadata, and
- ✓ identification of methods that contribute to irreproducibility which can be adapted or replaced.