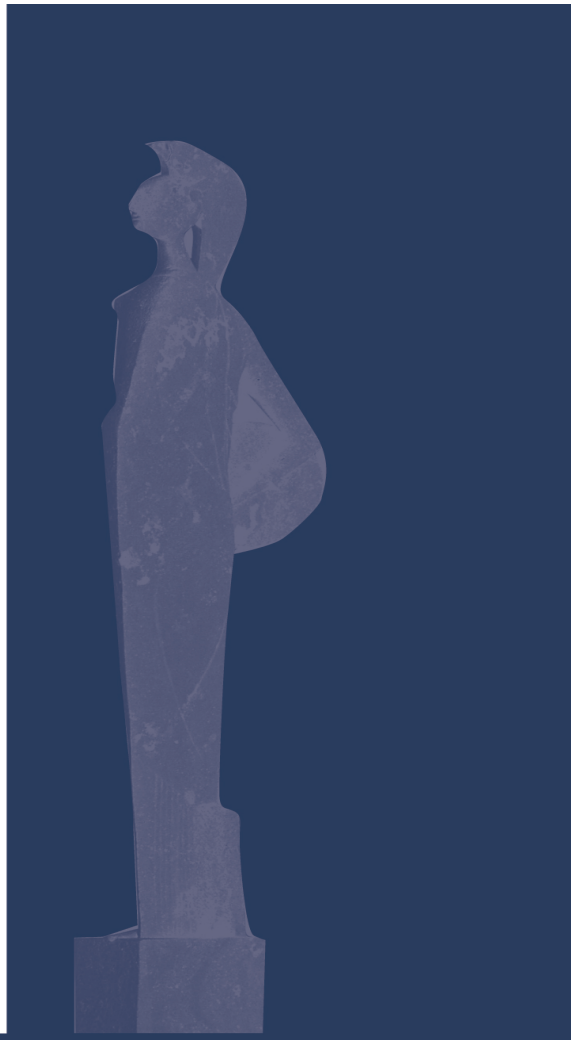
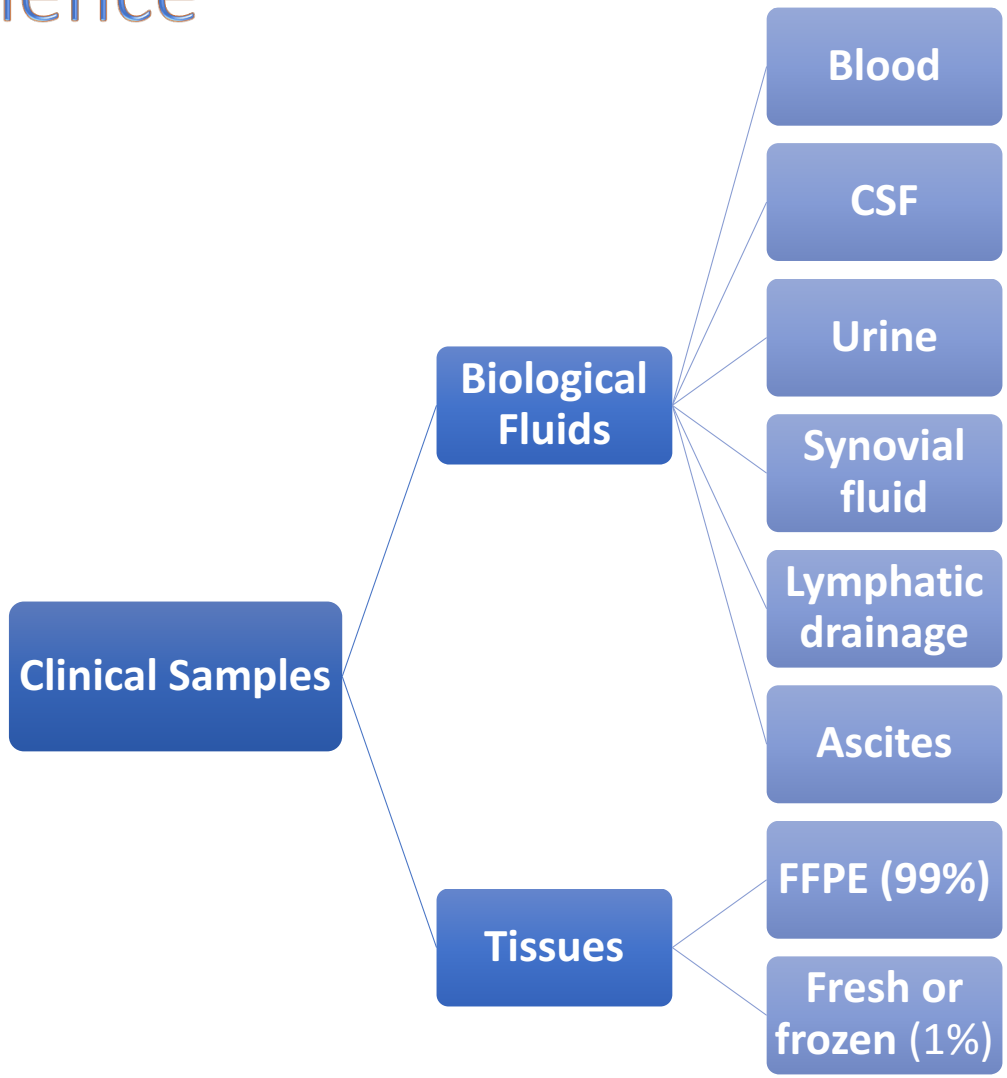
UNIVERSITÀ
DEGLI STUDI DI TRIESTE

TISSUE PROCESSING IN SURGICAL PATHOLOGY

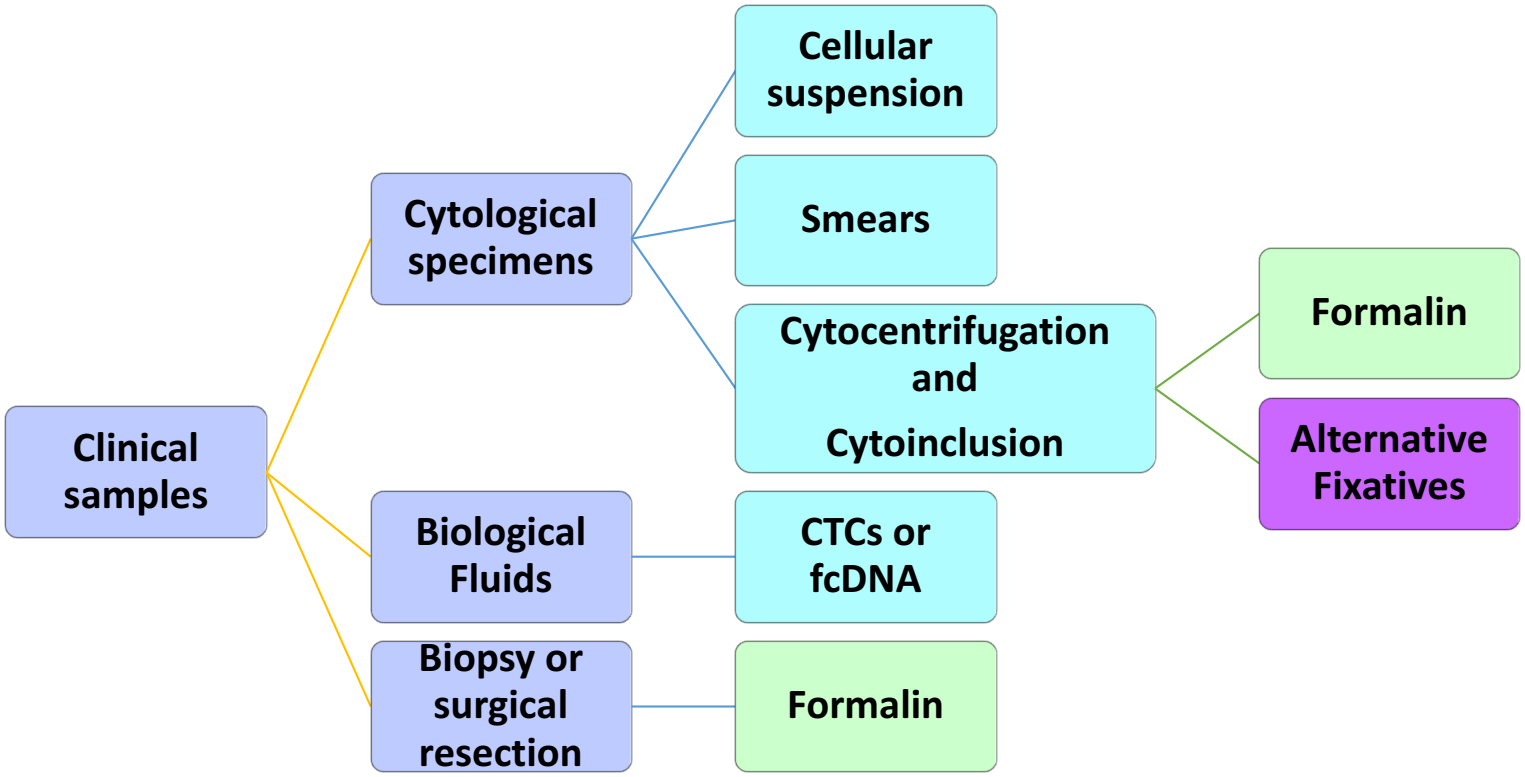
Serena Bonin
DSM-Dip. Scienze Mediche

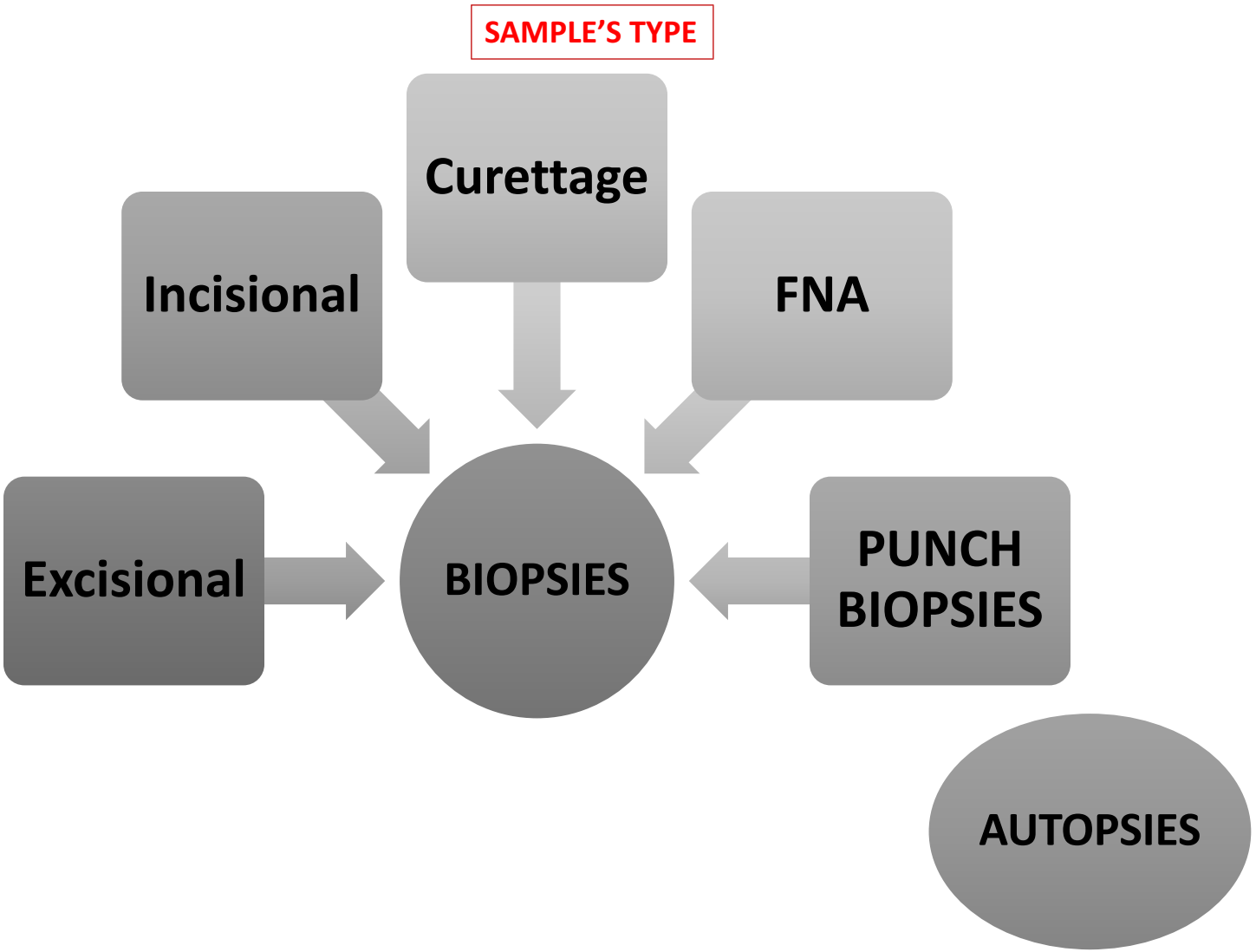


My experience

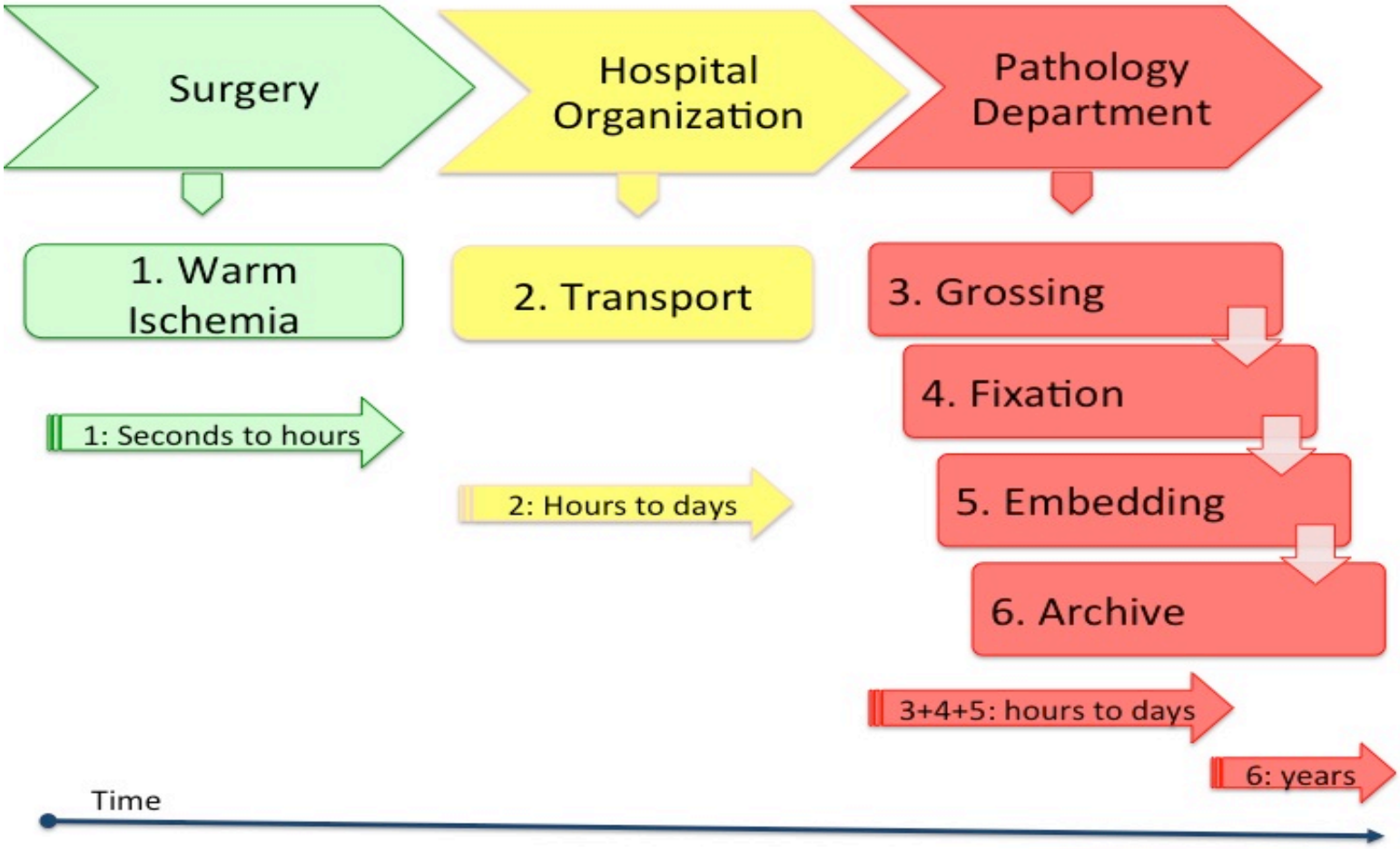


SOURCES OF CLINICAL MATERIAL




SAMPLE'S TYPE
Curettage
Incisional
FNA
Excisional
BIOPSIES
PUNCH BIOPSIES
AUTOPSIES

ALL PREANALYTICAL FACTORS AFFECT RNA INTEGRITY MORE SEVERELY THAN DNA



Bonin S , Stanta G. Nucleic acids extraction methods in fixed and paraffin-embedded tissues in cancer diagnostics. Exp Rev Mol. Diagn. 2013,13.

TissueSAFE Vacuum Unit

Dedicated vacuum unit

installed in " dirty room " adjacent to surgery suite.

Elimination of formalin in surgery theatre.

Transport biospecimens in "as fresh conditions" to the

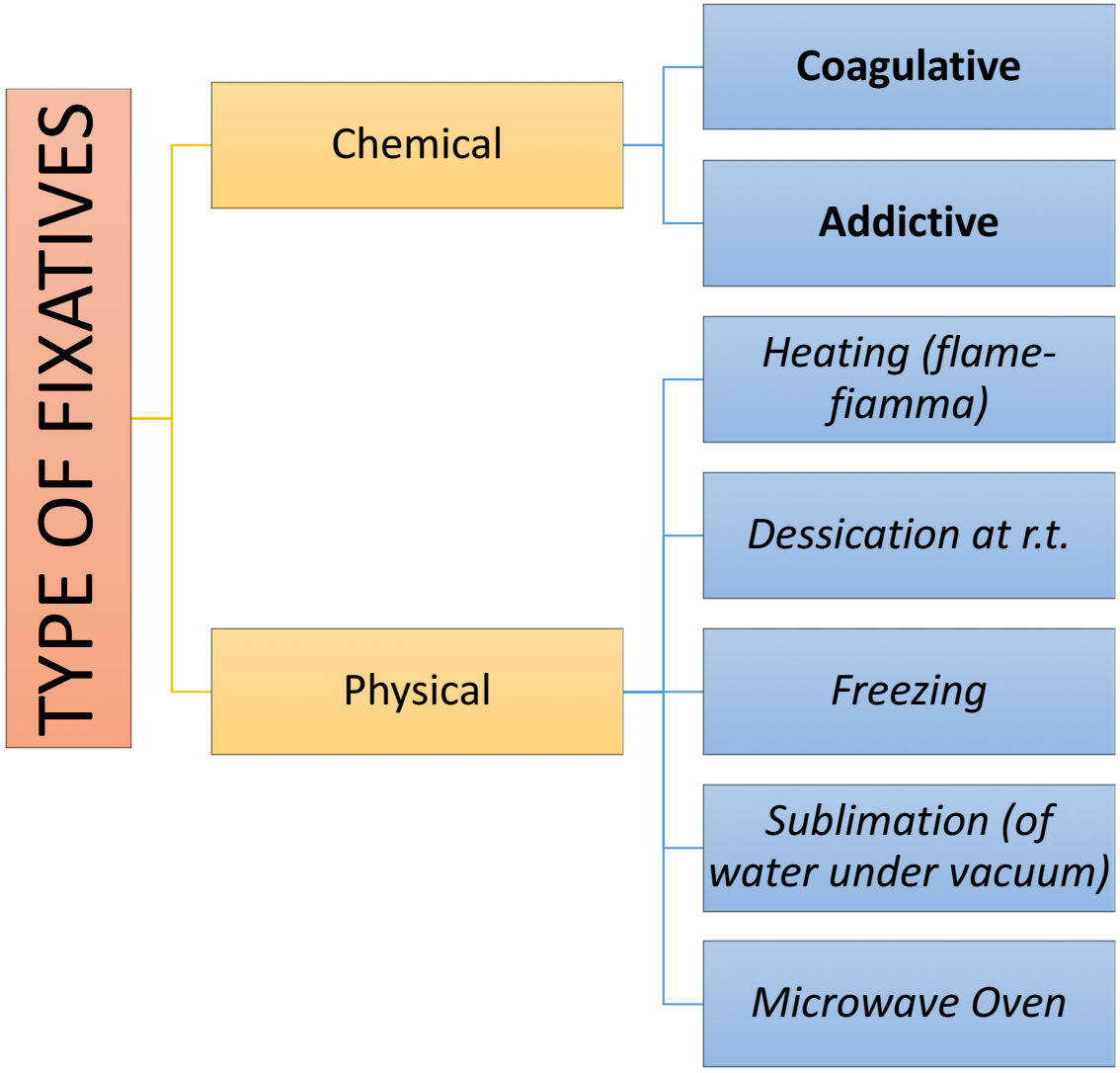
Pathology lab.

SAMPLING

- ***WASHING OF TISSUE SAMPLES*** *(in neutral buffer in physiological conditions to remove blood - with sucrose or dextran for good morphological preservation for freezing)*
- ***TISSUE TRANSPORT*** *(in fixative or under vacuum)*
- ***SHORT TIMES:*** *autolysis (type of tissue) and putrefaction*
- ***INSTRUMENTS:*** *(knives, scalpels, scissors, cork board, ...)*
- ***TECHNIQUE OF COLLECTION AND BIOLOGICAL RISKSTYPE OF MATERIAL SENT:*** *(surgical piece, biopsy, needle biopsy, autopsy, cytological material)*
- ***MACROSCOPIC DESCRIPTIONDIMENSIONS*** *(suitable for histological preparations with thickness suitable for fixation)*
- ***LOCALIZATION AND ORIENTATION*** *(anatomy, lesion)*

FIXATION

- ***AIM:*** *provide a histological image as faithful as possible to the reality or constantly reproducible (EQUIVALENT IMAGE)*
- ***FIXATION RAPIDITY****: to avoid autolysis (intracellular release of lysis enzymes) and putrefaction (saprophytic and environmental bacteria).*
- ***PENETRATION OF FIXATIVE****: rate of penetration into the tissues (depending on the temperature, which also increases degradation)*
- ***FIXATION TIME****: depends on the type of fixative, the type of fabric and the size of the sample*
- ***FIXATIVE VOLUME****: 1:20 for formalin*
- ***FIXATION CONDITION****: tissue immersion, pH 7.3-7.4, osmosis pressure 0.5 osm.*
- ***FIXATION FAILURE****: sample loss*


TYPE OF FIXATIVES
Chemical
Physical
Coagulative
Addictive
Heating (flame-fiamma)
Dessication at r.t.
Freezing
Sublimation (of water under vacuum)
Microwave Oven

Chemical Fixatives

(Acting on proteins)

PROTEIN HYDRATION :

- *COAGULATIVE FIXATIVES: the fixative replaces the hydration water of the proteins that denature and precipitate (COAGULATION)*

REACTION WITH TISSUE COMPONENTS::

- *ADDICTIVE FIXATIVES: the fixative molecules react chemically with the TISSUE components, with consumption of the fixative*

ADDICTIVE FIXATIVES

FORMALDEHYDE: *HCOH –colorless gas, water soluble. Formalin is the acqueous solution of formaldehyde (37%). In teh fixation process formalin solution is buffered with phosphate buffere at physiologic pH .*

MECHANISM:

-protein cross links (methylen bridges -CH-)

$$R\text{-}H + HCHO > R\text{-}CH_2\text{-}OH + H\text{-}R' > R\text{-}CH_2\text{-}R' + H_2O$$

PENETRATION: 0.8mm/h

USE: -SOLUTION DILUTED 10X (known as formalin 10%, real amount of formaldehyde 4%)

- Light can convert formaldehyde into formic acid (dark bottle and Calcium carbonate addiction)

-Fixation lasts from 12h to 4-5days.

-By haemoglobin degradation forma a dark brown pigment is formed. It can be eliminated adding ethanol 70% (95pp) or ammonia 5%(5pp)

-Conservation (in museums with marble fragments (Ca salts) and after bubbling with city gas, CO> methaemoglobin, to maintain colors)

-Mummification

-RESULTS:

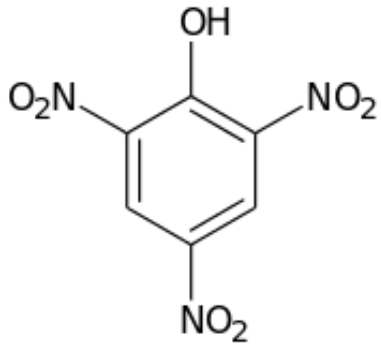
- FAT FIXATION

-poor coercion

-partially dissolves glycogen and uric acid

PARAFORMALDEHYDE: polymer of Formaldehyde

ADDICTIVE FIXATIVES



FIXATIVE MIXTURES with picric acid:

- **Bouin's Fixative**: a mixture of 15 parts of picric acid in a saturated aqueous solution, 5 parts of 40% formaldehyde and 1 part of glacial acetic acid

- **Duboscq – Brasil Fixative**: 150 ml EtOH 80%, 60 ml 40% formaldehyde, 15 ml of glacial acetic acid and 1 g picric acid

Both are very penetrating fixatives; however, the presence of picric and acetic acid is an obstacle to any retrospective investigations and DNA extraction and, moreover, it easily dissolves most of the calcifications.

COAGULATIVE FIXATIVES

ALCHOLIC FIXATIVES

- Ethanol 95°
- Ethanol 95° and ethyl ether in equal parts
- Methanol
- Methanol + acetone in equal parts

Alcohol itself, due to its low oxidation potential and moderate penetration capacity, is not a good histological fixative (but it is better than nothing); determines excessive coarctation and hardening of the tissues, denature the proteins and coagulates coarsely the cytoplasm; therefore it is preferred to use it in association with other components in order to obtain a more effective and homogeneous fixative.

Ethanol 95° or Ethanol 50° are used as pre-fixatives in cytology (equal volume of the samples).

FIXATIVES MIXTURES WITH ALCOHOL

- *Serra's Fixative*: 2 parts of **Ethanol** 95% and 1 part of 40% **formaldehyde** + sme drops of **glacial acetic acid.**
- *Carnoy and MethaCarnoy Fixatives* : 6 parts of abs Ethanol (Methanol), 3 parts of **$CHCl_3$** and 1 part of **glacial acetic acid**
- *Clarke's Fixatives*: 3 parts of abs Ethanol and 1 part of **glacial acetic acid**
- *Alcholic formalin of Lillie*: 9 parts of abs **Ethanol** and 1 part of 40% **formaldehyde**

FIXATIVE WASHING

AFTER FIXATION TISSUE FRAGMENTS MUST BE WASHED TO REMOVE THE RESIDUES OF THE FIXATIVE THAT OTHERWISE MAY IMPACT ON THE FOLLOWING PROCESSES

INCLUSION

- FIXED TISSUES, IN ORDER TO BE OBSERVED AT THE OPTICAL MICROSCOPE, MUST BE SECTIONED IN THIN SECTIONS 2-8 µm

-TO OBTAIN THIN SECTIONS TISSUES MUST BE SUFFICIENTLY HARD AND COMPACT> INCLUSION IN SEMI-SOLID SUBSTANCES

-TISSUES MUST BE EMBEDDED IN THE INCLUSION SUBSTANCE > PARAFFIN

PARAFFIN

CH_3-CH_2- - - -CH_2-CH_3 (C_nH_{2n+2})

-IT IS OBTAINED FROM THE OIL DISTILLATION RESIDUES

-THE MELTING POINT RISES WITH THE LENGTH OF THE CHAIN

-IN HISTOLOGY C22 -C28 PARAFFINS ARE USED, DIVIDING THE PARAFFINS IN LOW MWLTING (45 - 54 ° C) AND HIGH MELTING(58 - 60 ° C)

- NOWADAYS NO NATURAL PARAFFIN ARE USED (MIXTURE OF DIFFERENT LENGTH), BUT SYNTHETIC PARAFFINS, PURE AND HOMOGENEOUS (ES. PARAPLAST)

-PARAFFIN SOLVENTS:: XYLENE, $CHCL_3$, BENZENE AND TOLUENE

TISSUES' INCLUSION PROCESS

1-FIXATION

2-WASHING

-ETHANOL 70%	*- 4h*	***I***	
- ETHANOL 95%	*- 4h*	***I***	***3-DEHYDRATION***
- ETHANOL 100%	*- 4-6h*	***I***	
- ETHANOL 100%	*- 4-6h*	***I***	
-XYLENE	*- 1-2h*	***I***	***4-CLEARING***
-XYLENE	*- 1-2h*	***I***	
-PARAFFIN 2-3X	*- 3-4hX1*		***5-INCLUSION***

6-WAX CASTING *(PARAFFING BLOCK INCLUDING ORIENTERED TISSUE)*

7-COOLING *(HOMOGENEOUS PARAFFIN)*

HYSTOLOGIC SECTIONS

MICROTOMES:

-SLEDGE MICROTOMES (MOBILE OR FIX BLADE)

-ROTATORY MICROTOME (SERIAL SECTIONS)

-CRYOMICROTOME

-CRYOSTAT (ROTATOTARY MICROTOME IN REFRIGERATED CHAMBER)

MICROTOME BLADES:

- *THEY HAVE SECONDARY CUTTING FACES-*
- *INCLINATION COMPARED TO THE CUTTING SURFACE 10 - 15 °*
- *LOWER THAN 10 ° THE BLADE BRUSHES WITHOUT CUTTING, OVER 15 ° THE BLADE BREAKS THE PARAFFIN*

Microtomo a slitta
SLEDGE MICROTOME

Microtomo rotativo
ROTATORY MICROTOME

DEPARAFFINIZATION OF THE SECTION

-XYLENE - 5 min

-XYLENE - 5 min

-ETHANOL 100% - 5 min

- ETHANOL 100% - 5 min

- ETHANOL 95% - 5 min ***HYDRATION***

- ETHANOL 70% - 5 min

-DISTILLED WATER

STAINING

TISSUE DECALCIFICATION

FIRST OF ALL TISSUES SHOULD BE FIXED, OTHERWISE THEY UNDERGO MACERATION.

Decalcification is carried out by means of strong acids, to obtain soluble Ca salts.

Decalcification methods that employ acids are most widely used in pathology laboratories. Since calcium is soluble at a pH of 4.5, acids quickly and easily dissolve the calcium salts. There are two types of acids used in decalcification procedures: Strong mineral acids OR Weak organic acids. The most common acids used for decalcification are 5-10% solutions of HCl, nitric acid, and formic acid. These acids can be used alone or in combinations. The following should be considered before implementing an acid decalcifying protocol in the laboratory. Tissue must be trimmed small and fixed first. Decalcifiers with higher concentration of acids act rapidly and affect tissue staining the most. Tissue left in acid too long will lose nuclear staining. Decal. solution must be changed frequently because calcium that has leached out will become a barrier to further decalcification. Agitate tissue during decalcification to expose all surfaces to fresh decal. agent. Heat should be avoided with strong acid decalcification as swelling of tissue and possible digestion of bone collagen will occur. Tissue must be rinsed in water prior to processing, otherwise acids will continue to decalcify tissue; will also prevent possible chemical reactions with subsequent reagents and contamination of processor reagents.

$$CaCO_3 + 2\,HNO_3 > Ca(NO_3)_2 + H_2O + CO_2$$

DECALCIFIERS:

- NITRIC ACID	***5-7.5%***
-TRICLORIDE ACETIC ACID	***5%***
-FORMIC ACID	***CONC.***

Decalcification solution should be changed 2X in 24h. The process lasts some days.

A needl is used to test the decalcification reaction.