



**Hogeschool
Leiden**



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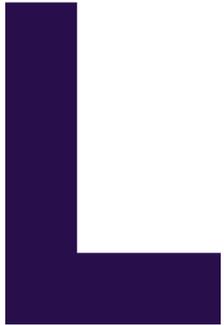
 Hogeschool
Leiden

**Centrum
Bioscience
Diagnostiek**





Fixation matters: Why it's so important?



What will we discuss this morning?

Pre-analysis

Variables in pre-fixation

Warm and cold ischemia

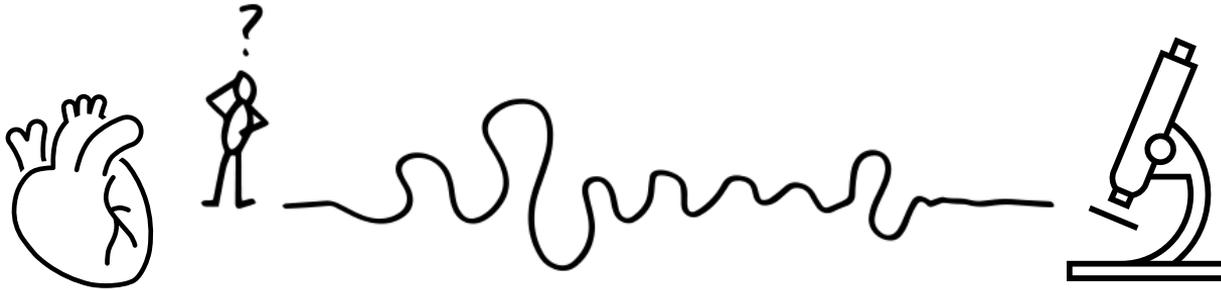
Importance of fixation

Coagulating and non-coagulating fixatives

Variables during fixation

Pre-analysis

Which steps belong to the pre-analytical phase?



Pre-analysis

Pre-fixation

Fixation

Post-fixation / decalcification

Processing (dehydration, clearing, paraffin embedding)

Sectioning

Mounting

Drying and storage

Analysis

Post-analysis

Pre-analysis

Pre-fixation

Fixation!!!

Post-fixation / decalcification

Processing (dehydration, clearing, paraffin embedding)

Sectioning

Mounting

Drying and storage

Analysis

Post-analysis

Variables in Pre-fixation

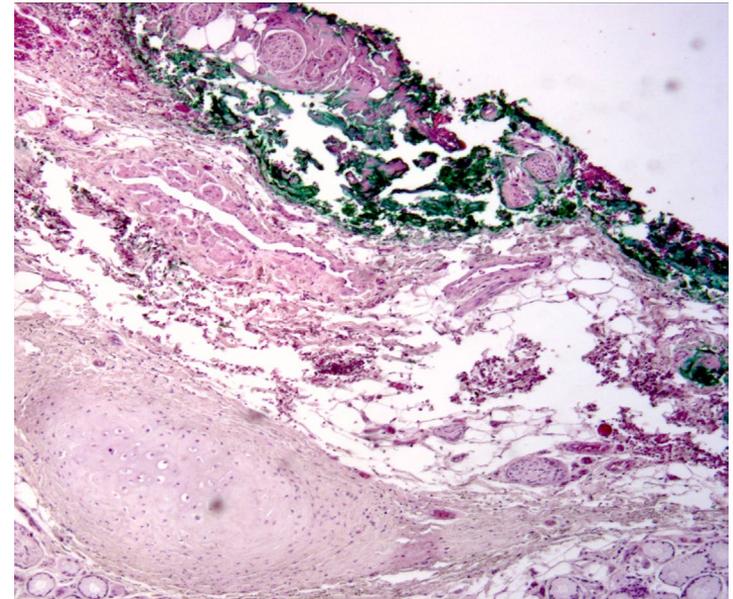
Tissue sampling

Delay before placing tissue in fixative

Time and temperature (warm vs cold ischemia)

Size and type of specimen (biopsy, resection, histology, cytology)

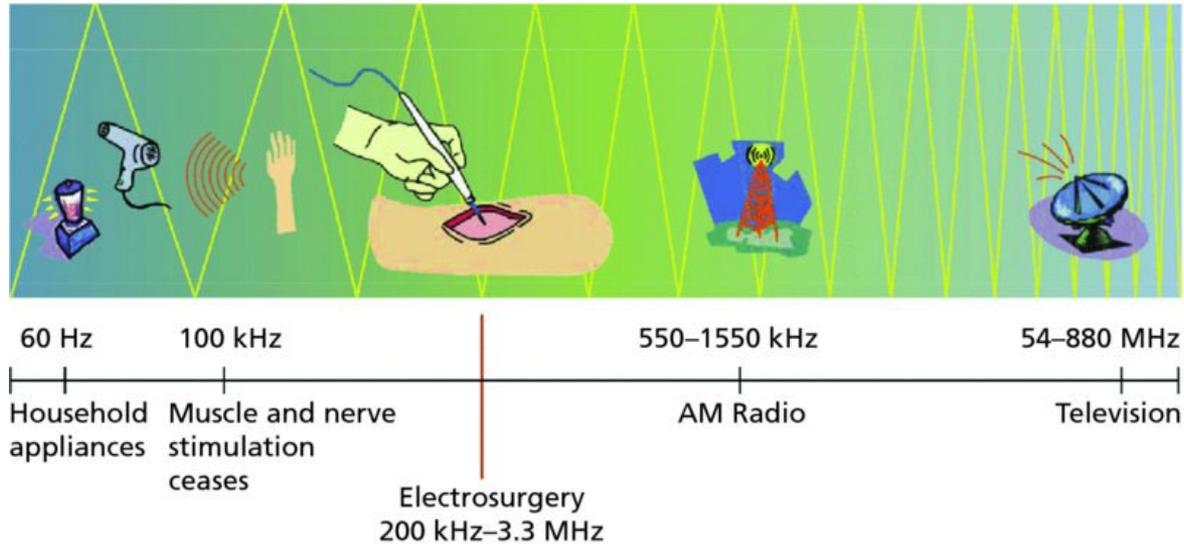
Use of marking ink



Effect of tissue removal during surgery

Electrosurgery → heat → protein denaturation

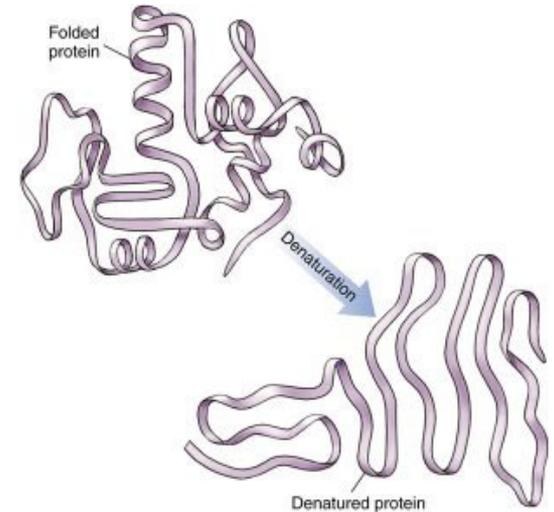
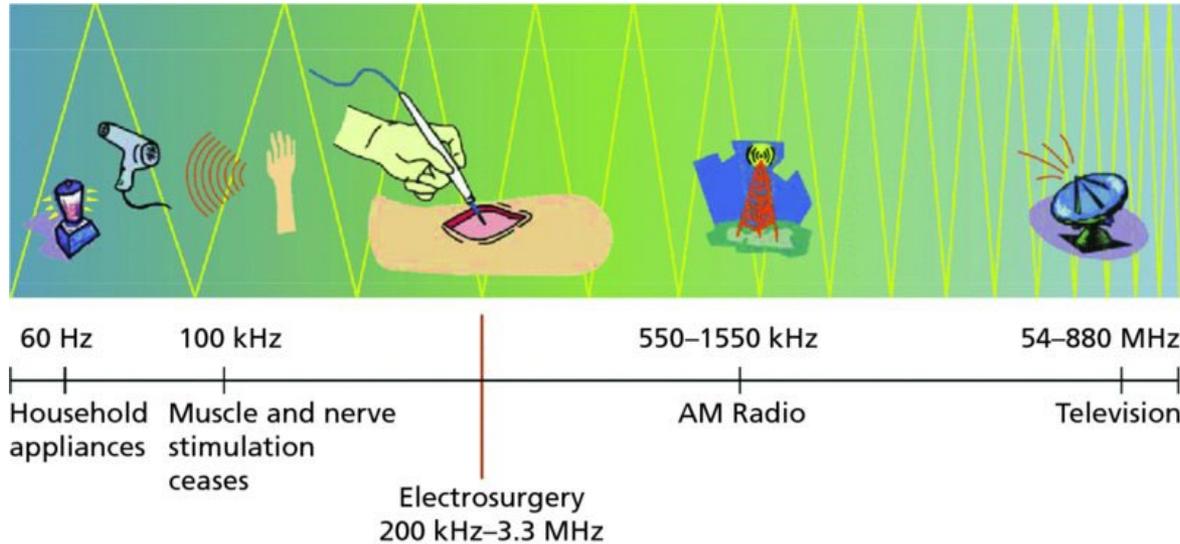
What happens to proteins under heat?



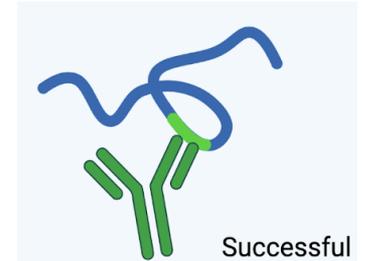
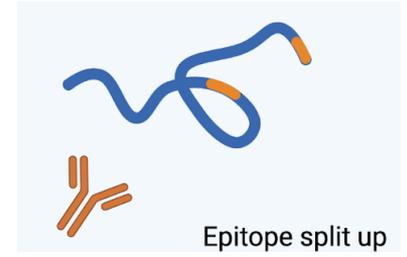
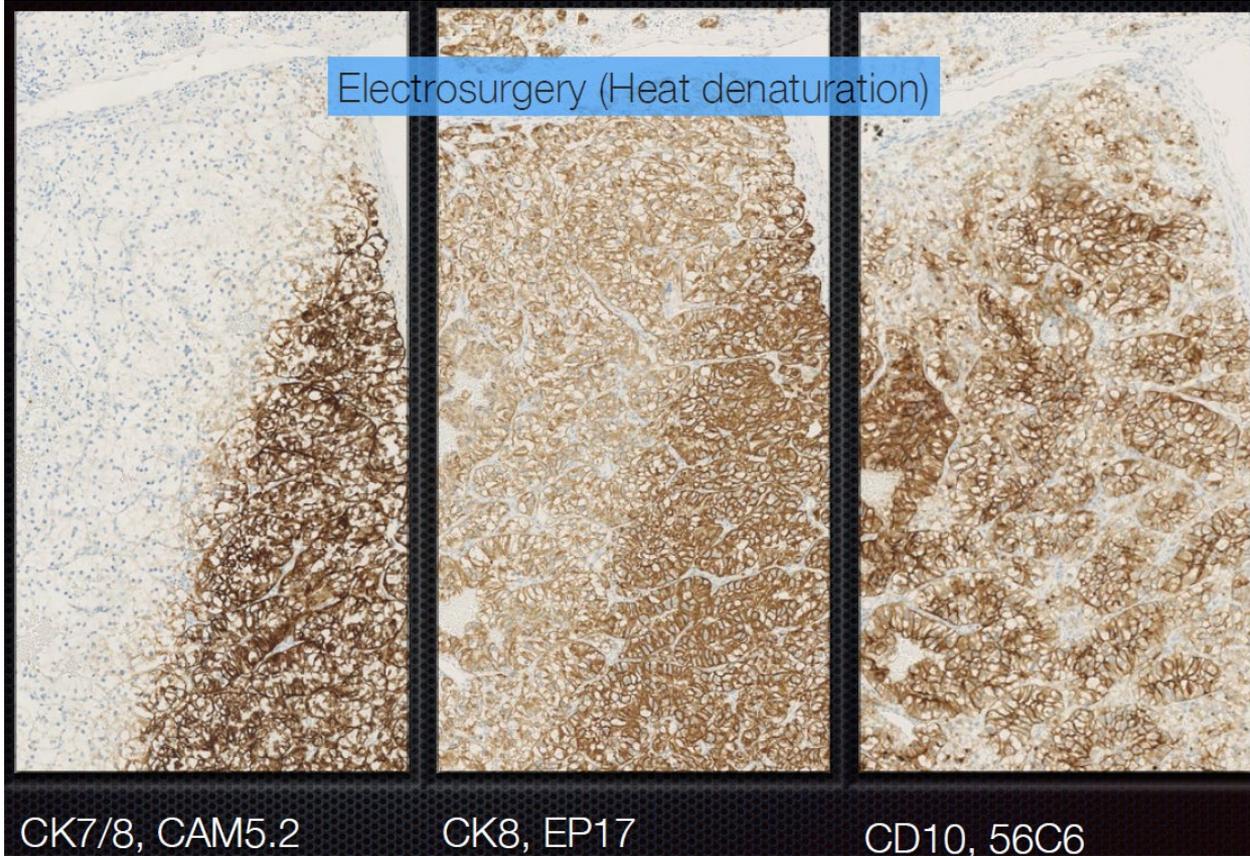
Effect of tissue removal during surgery

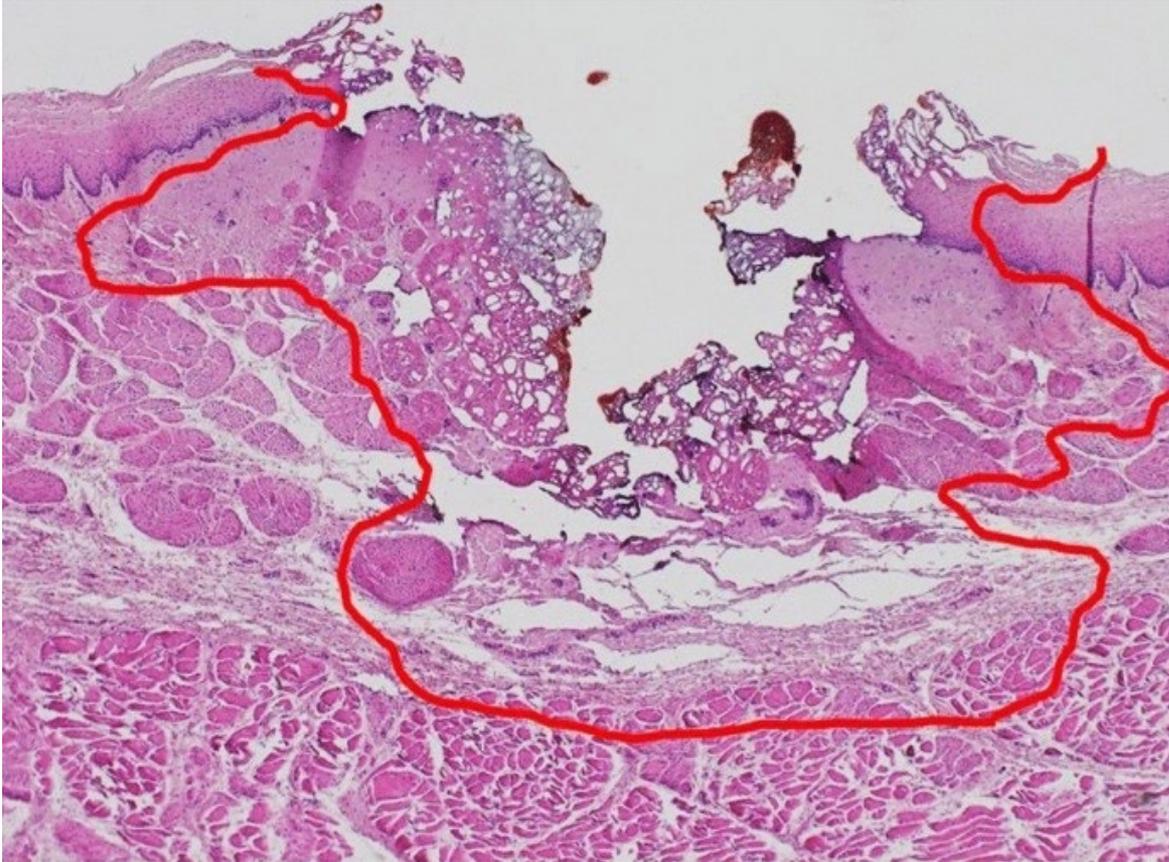
Electrosurgery → heat → protein denaturation

What happens to proteins under heat?



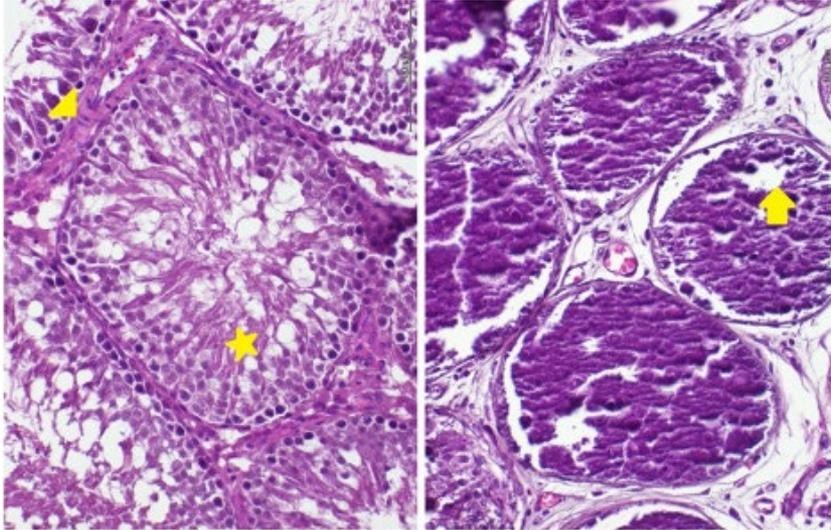
Electrosurgery (Heat denaturation)





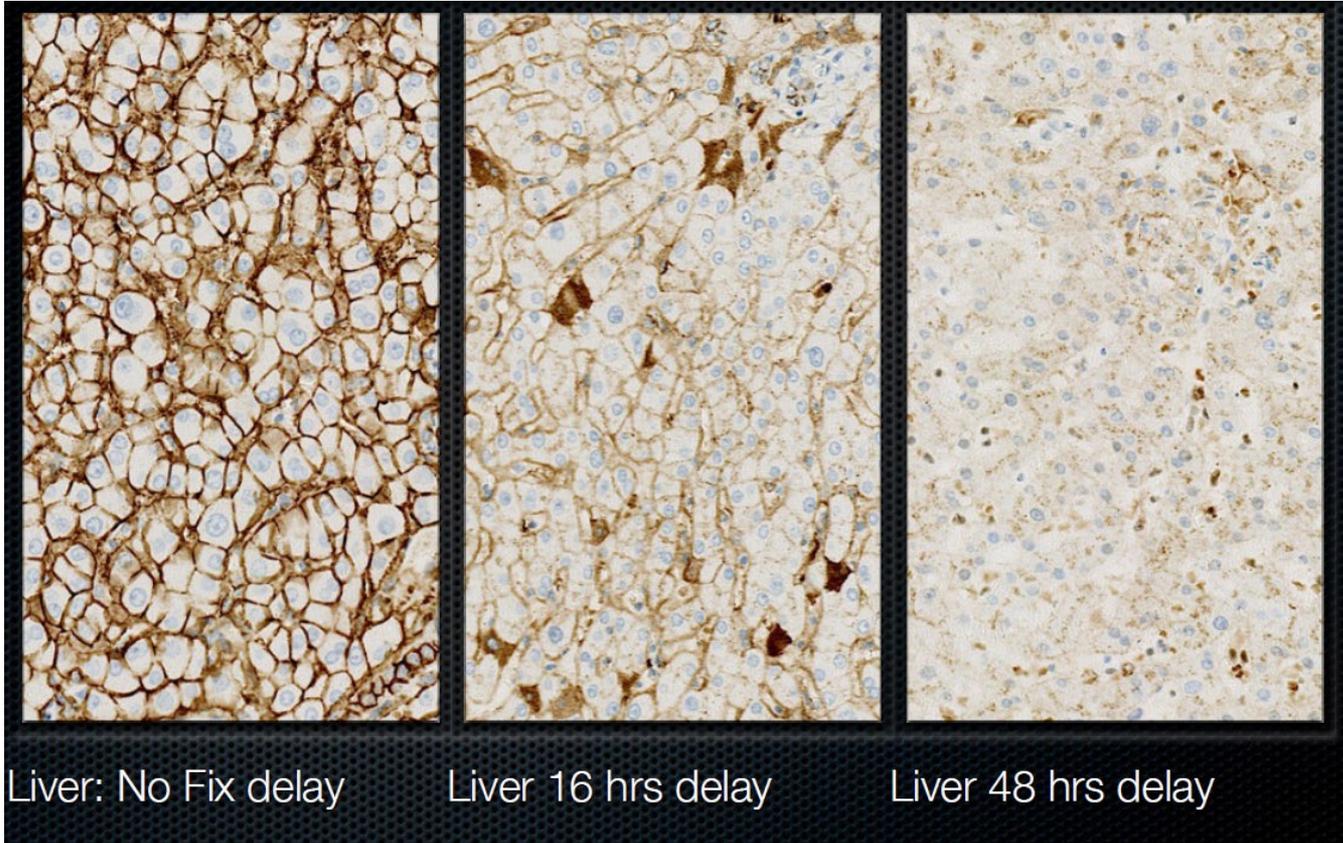
Effect of tissue removal during surgery

Organ clamping → warm ischemia
Affects tissue integrity before fixation



Variables in Pre-fixation

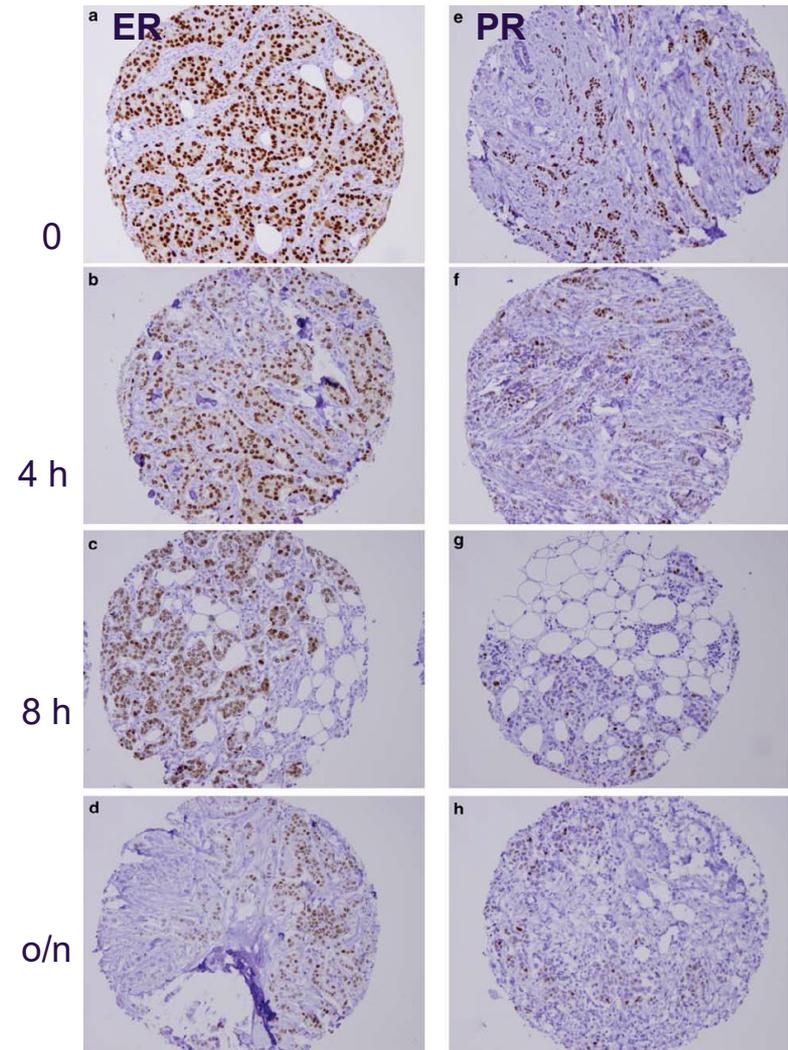




Cold ischemia

Time between tissue removal and
placement in fixative

Important for the right diagnose!



New ER Guidelines Require Recording Time Points for Tissue Handling

Require **3 time points be recorded for each specimen** and be available when you sign out report so time to fixation will be known

1. Time tissue is removed (OR staff to record)
2. Time tissue is received in grossing room
3. Time tissue was placed in fixative

 **These time point will help in trouble shooting unexpected test results**

*Standard procedure at URM Medical Center for over one year for all OR specimens

<http://www.asco.org/portal/site/ascov2>

<http://www.asco.org/ascov2/Press+Center/Latest+News+Releases/ASCO+and+the+CAP+Issue+Joint+Guideline+to+Improve+Hormone+Receptor+Testing+for+Patients+With+Brest+Cancer>

Most recent update: <https://ascopubs.org/doi/10.1200/JCO.19.02309#T2>

Cold ischemia

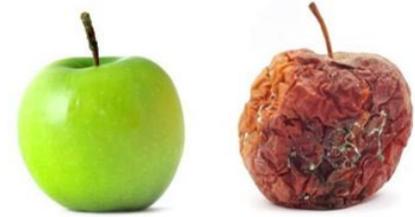
Fixation!

Should be minimized: fixation within 1 hour recommended

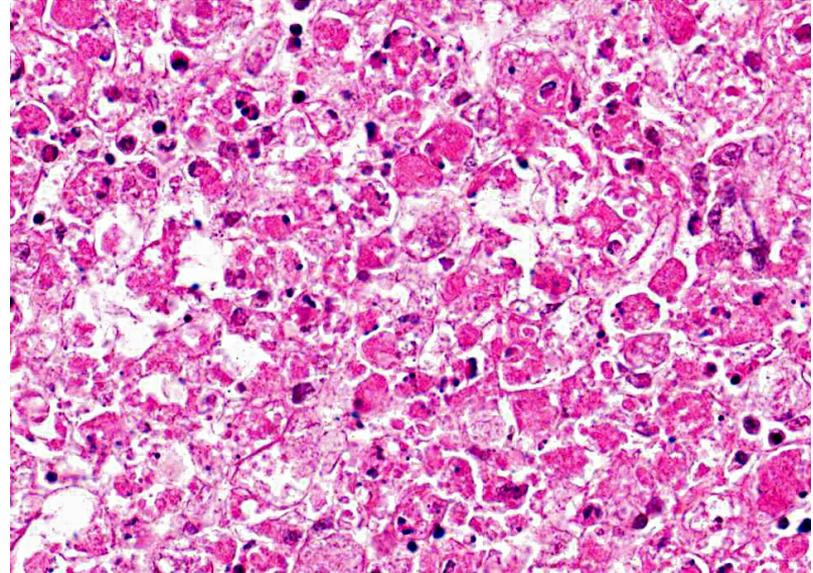
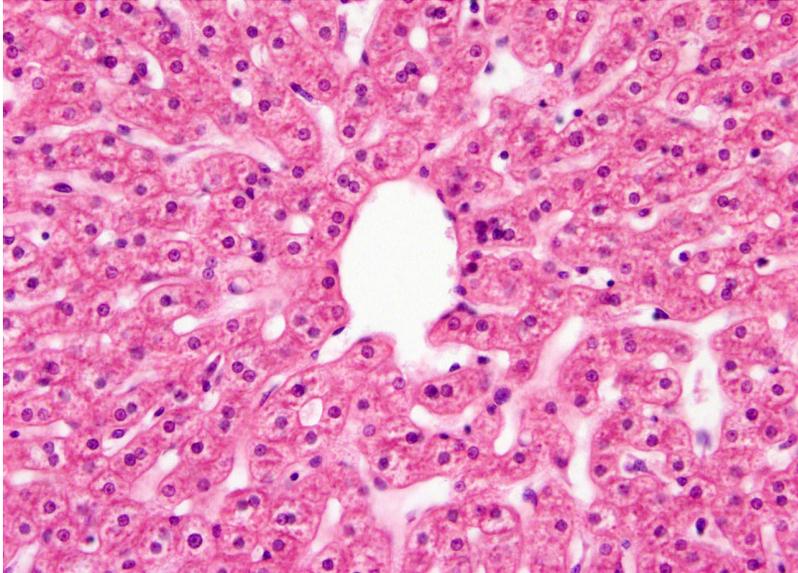
Literature-based recommendations: fixation within 12 hours maximum

*Why Is Timely Fixation
So Important?*

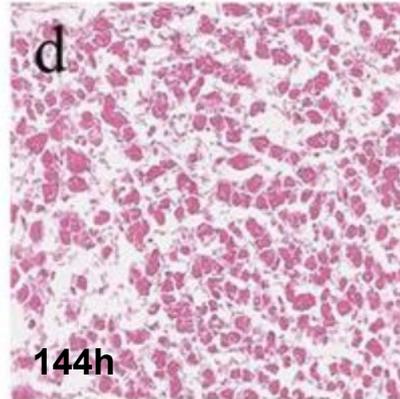
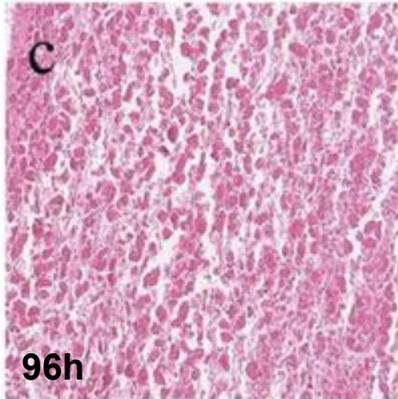
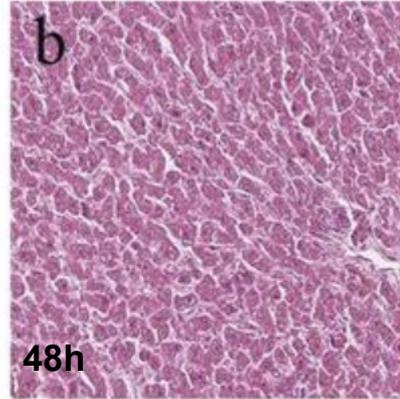
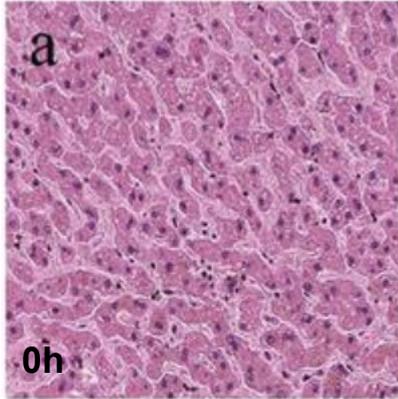




Cold ischemia – Autolysis



Do you see any difference?



Autolysis

self-degradation of cells

enzymatic activity after death

Resulting in swelling, loss of nuclear detail, and cytoplasmic fading

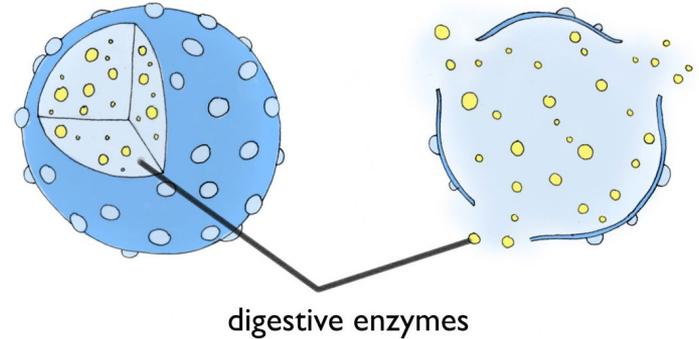
Cold ischemia – Autolysis

“Self-digestion”: degradation processes

Membrane permeability increases

Lysosomal leakage

Leads to cell swelling, lysis, faded nuclei
and cytoplasm



How to prevent or limit autolysis?

Without fixative

With fixative



How to prevent or limit autolysis?

Without fixative: keep tissue cooled

With fixative: apply as quickly as possible

Physical preservation = freezing
Chemical preservation = fixation



The importance of fixation



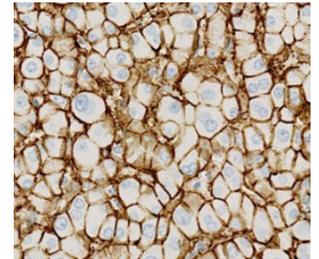
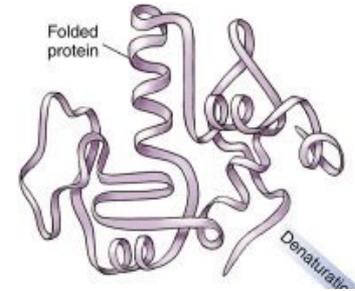
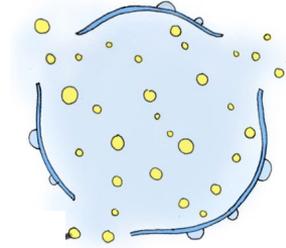
What are the main objectives of fixation?



What methods of chemical fixation are available?

Goals of fixation

- Prevent or inhibit degradation (autolysis)
- Preserve three-dimensional structure
- Stabilize tissue for histotechnical procedures
- Maintain protein configuration and antigenicity



Chemical fixation

Different mechanisms for protein preservation

Non-coagulating (cross-linking): formalin, formaldehyde

Coagulating: acetone, ethanol, methanol, acetic acid

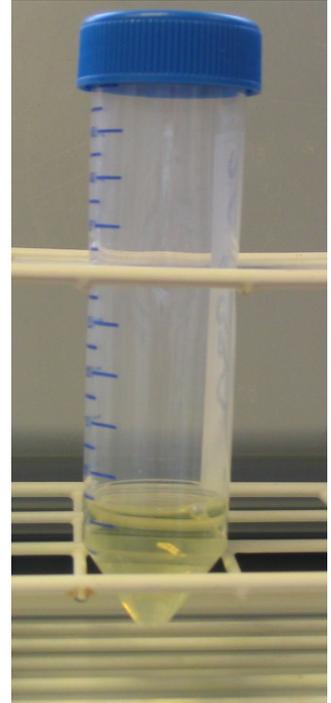
Effect of different fixatives on proteins

Two tubes each contain a BSA (protein) solution

One with added ethanol → protein coagulation

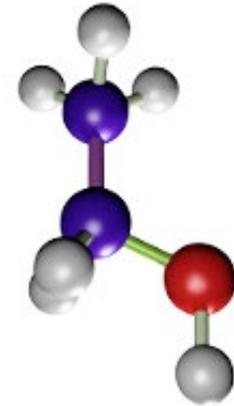
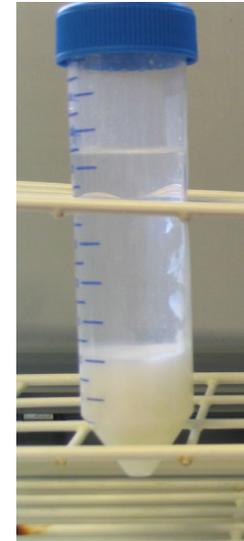
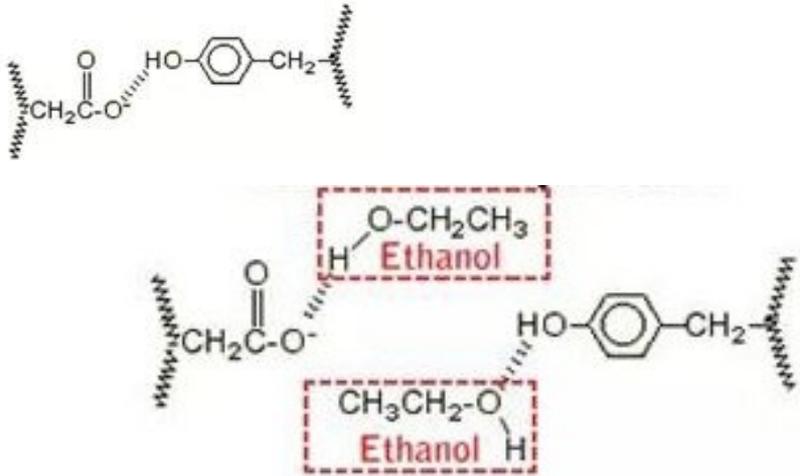
One with added formalin → cross-linking effect

What will happen?



Effect of different fixatives on proteins

One tube with added **ethanol** → protein coagulation

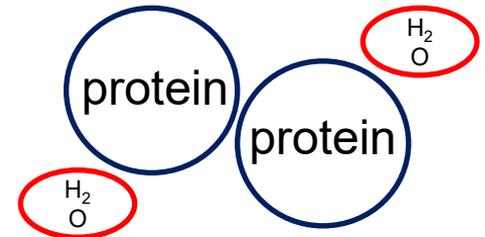
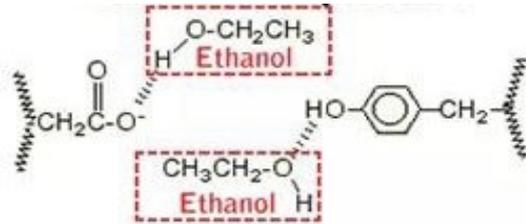
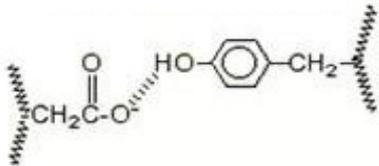
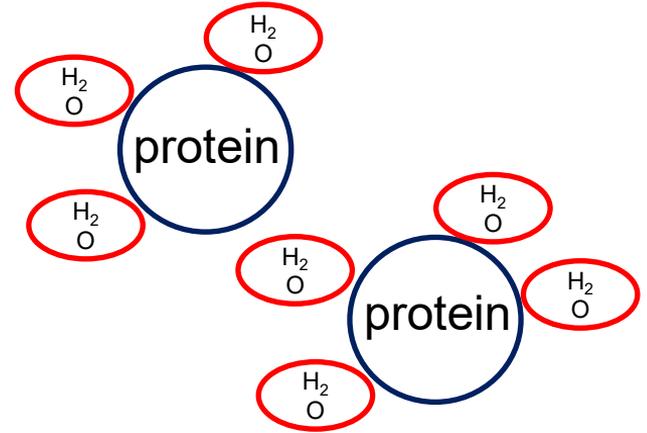


Ethanol
($\text{CH}_3\text{CH}_2\text{OH}$)

Coagulating fixative

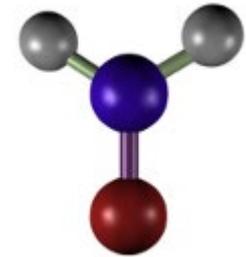
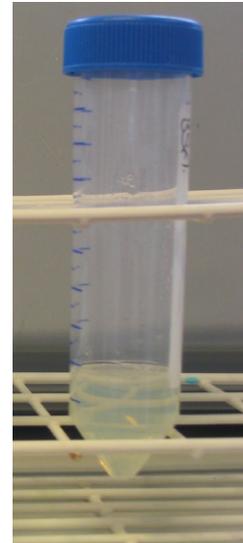
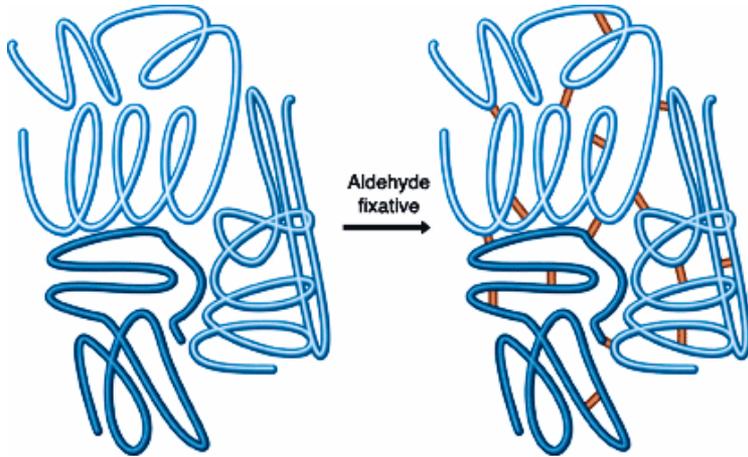
Remove the hydration shell (hydrogen bonds)

Proteins aggregate (coagulate) and precipitate

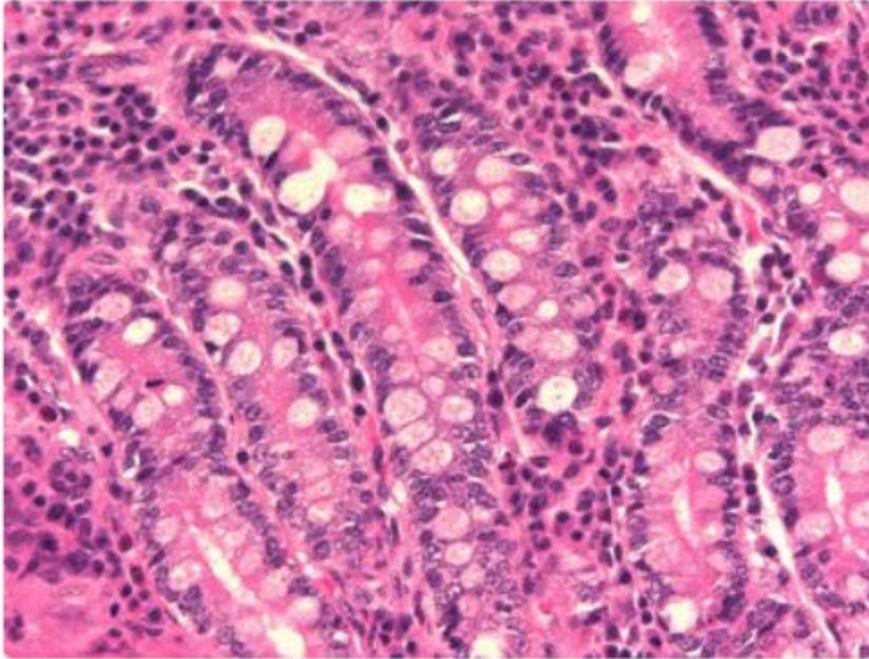


Effect of Different Fixatives on Proteins

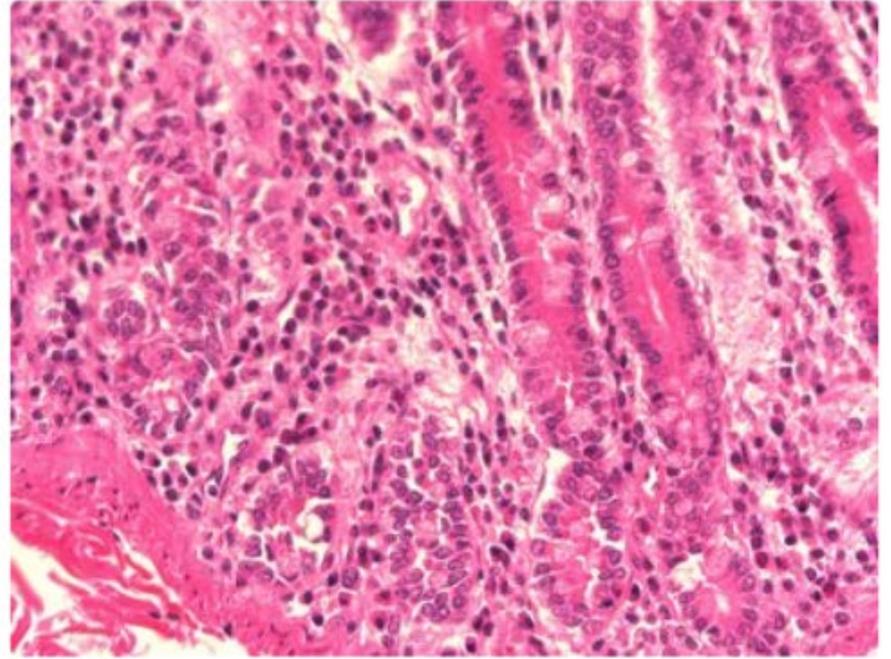
One tube with added **formalin** → cross-linking effect



Formaldehyde
(CH₂=O)



HE small intestine – NBF fixation



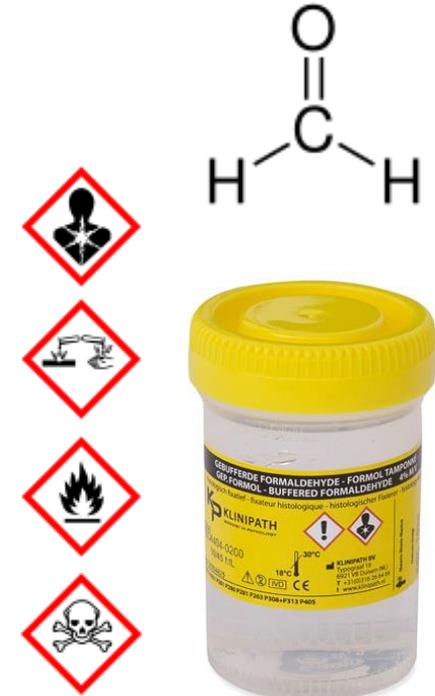
HE small intestine – 95% ethanol fixation

Non-coagulating fixatives (Cross-linking)

Aldehyde-based fixatives such as formalin and paraformaldehyde

Usually 4% buffered formaldehyde ($\approx 10\%$ formalin)

Commonly used in histopathology (NBF, FFPE)



4% buffered formaldehyde

Formaldehyde and Formalin

Formaldehyde (gas) dissolves up to 37% → 100% formalin

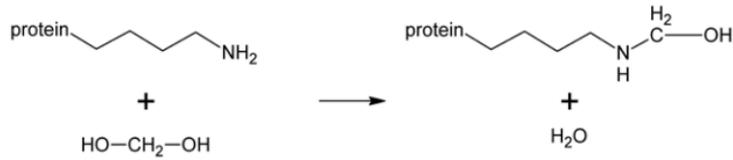
Tenfold dilution → 10% formalin

Paraformaldehyde (solid polymer of formaldehyde) → 4 g / 100 ml →

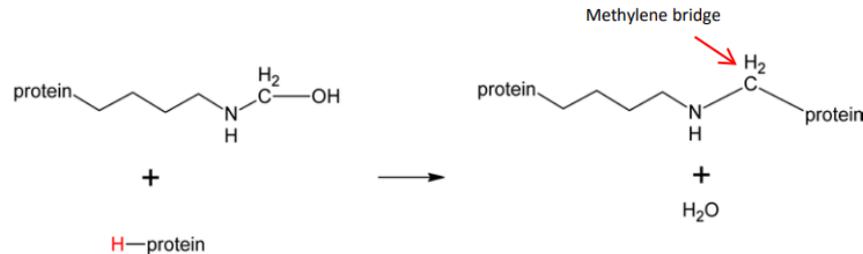
4% formaldehyde ≈ 10% formalin

Formaldehyde reaction

Addition of formaldehyde (methylene glycol) to a lysine side-chain (fast)



Formation of methylene bridge with a neighboring nitrogen atom (slow)

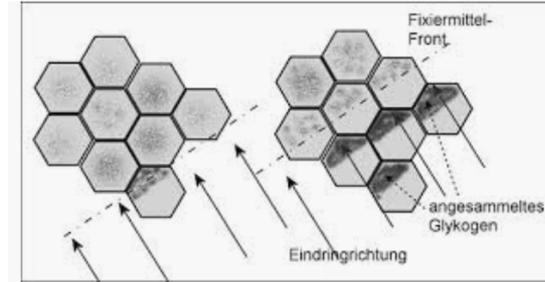
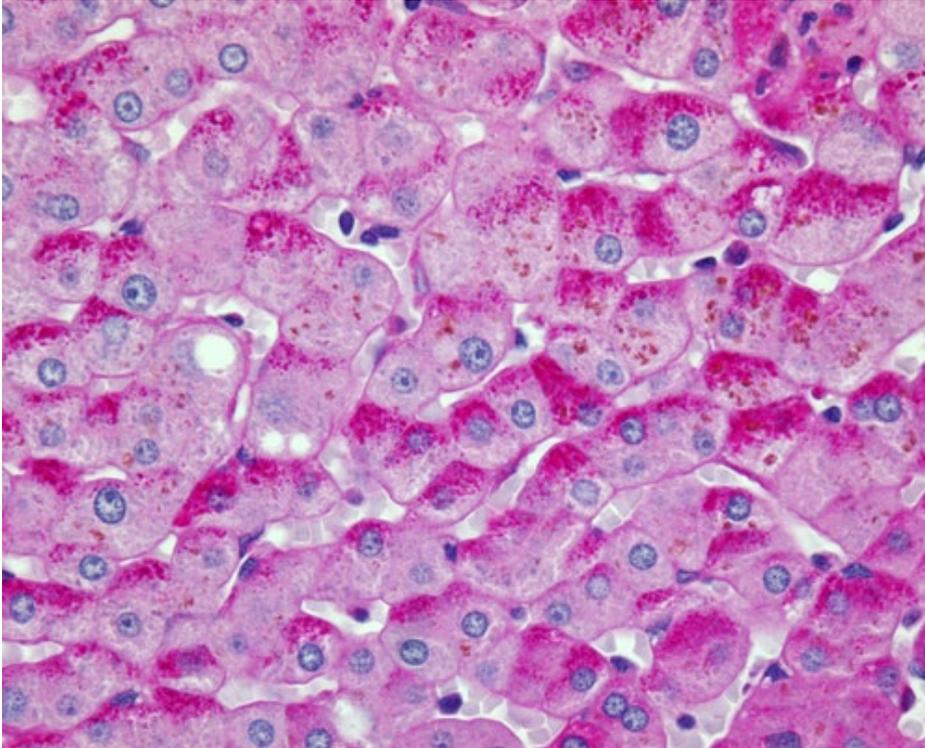


The aldehyde group can react with nitrogen $\text{-NH}_2\text{-}$ and other atoms of proteins

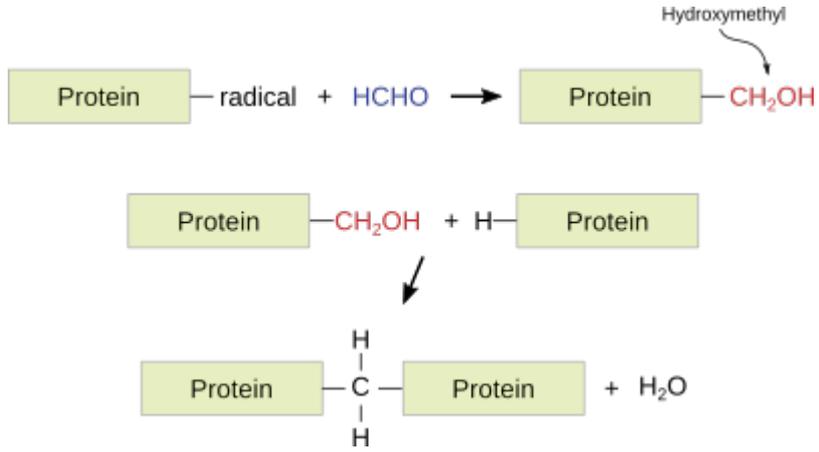
Methylene bridge $\text{-CH}_2\text{-}$ is formed between two reactive atoms in proteins that are very close together

Other molecules are though to be **trapped** in a matrix of cross-linked proteins

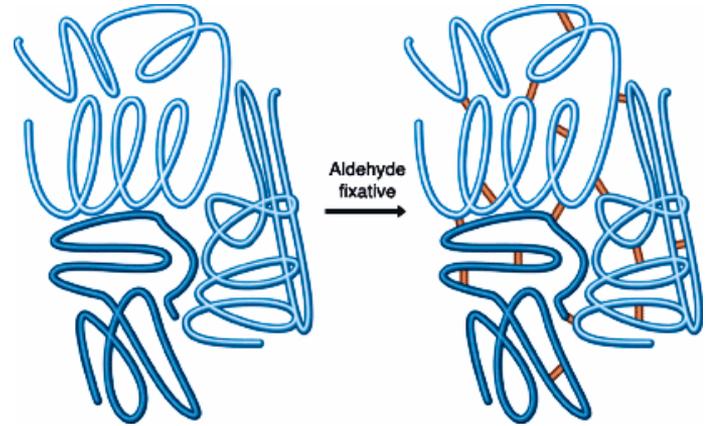
Streaming artefact



Carbohydrate (glycogen) floating



Methylene bridge

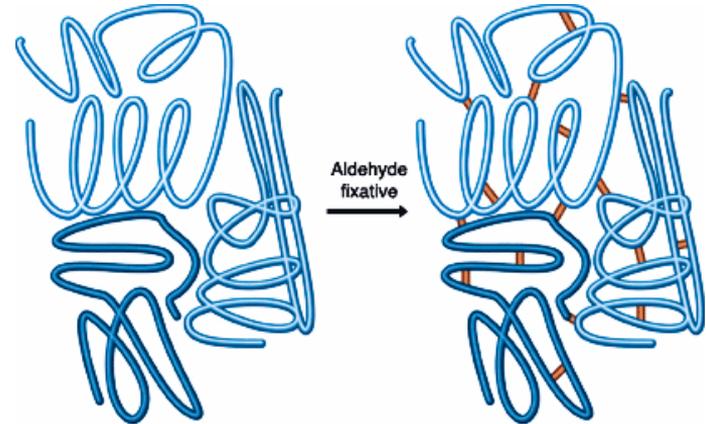
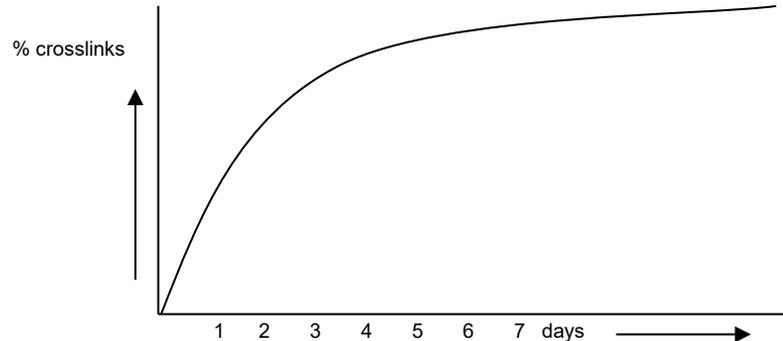


Fixation speed and duration

Fixation rate depends on diffusion and reaction kinetics

Typical diagnostic fixation: 24 hours in formalin

Cross-link formation continues over several days



Variables in fixation

Fixation time: underfixation, optimal fixation, overfixation

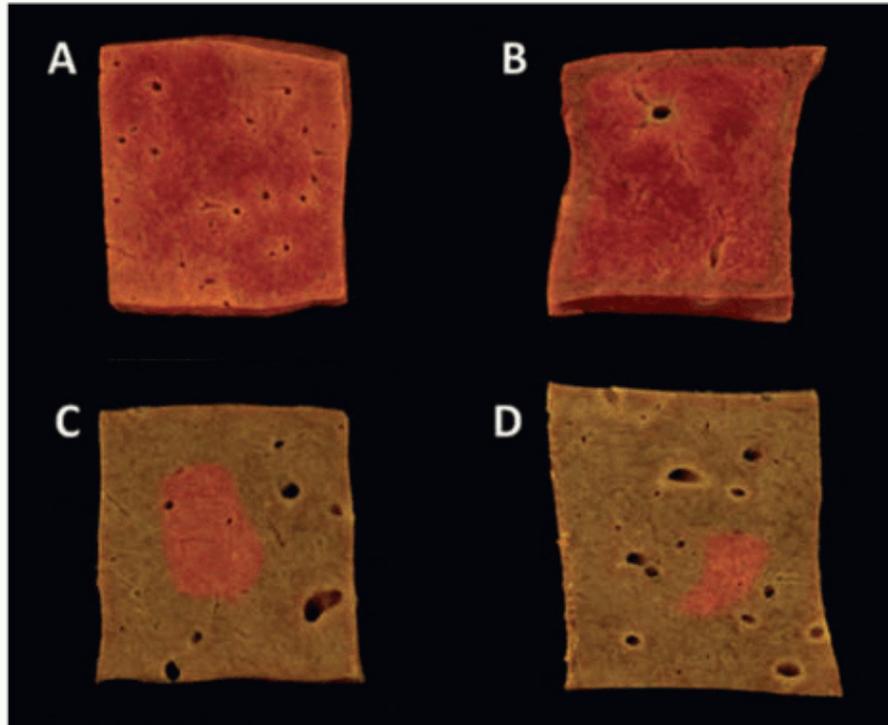
Buffer type and concentration

Fixative concentration

Temperature (room vs 4 °C)

Type of tissue (cytological vs histological)

Importance of fixation time – Macroscopy



2.5 cm

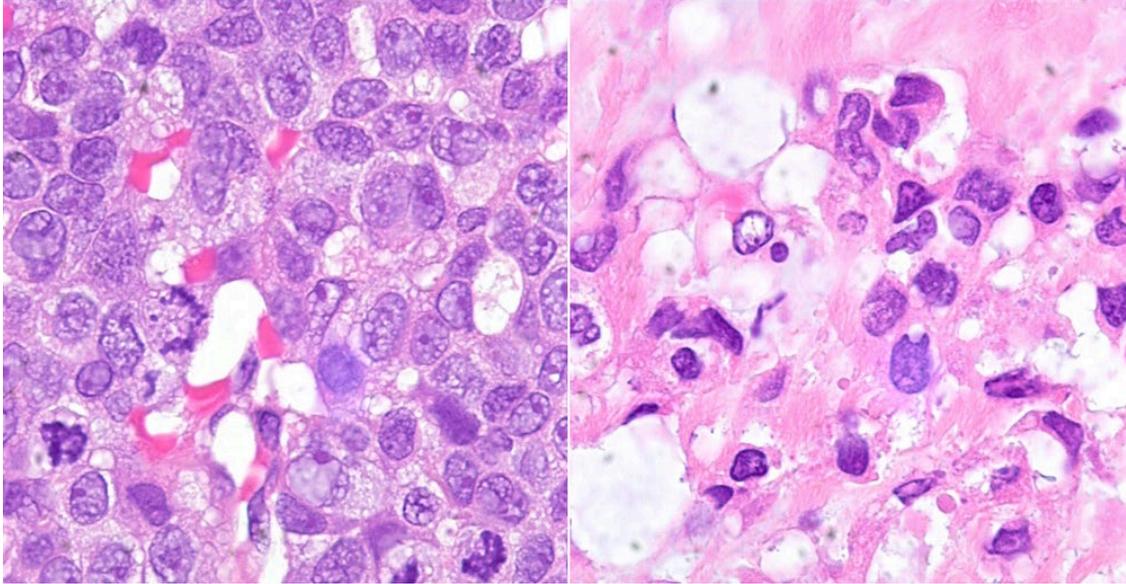
A = 1h

B = 3h

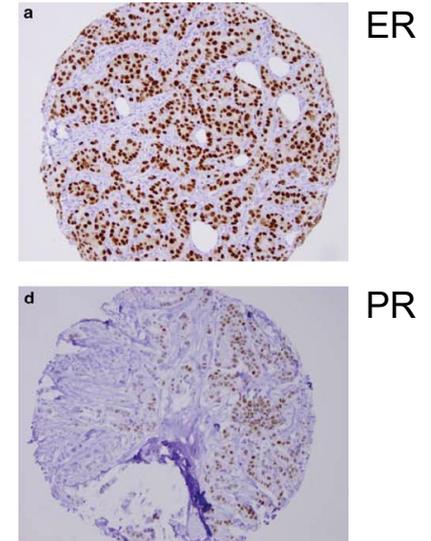
C = 12h

D = 20h

Importance of fixation time – Microscopy



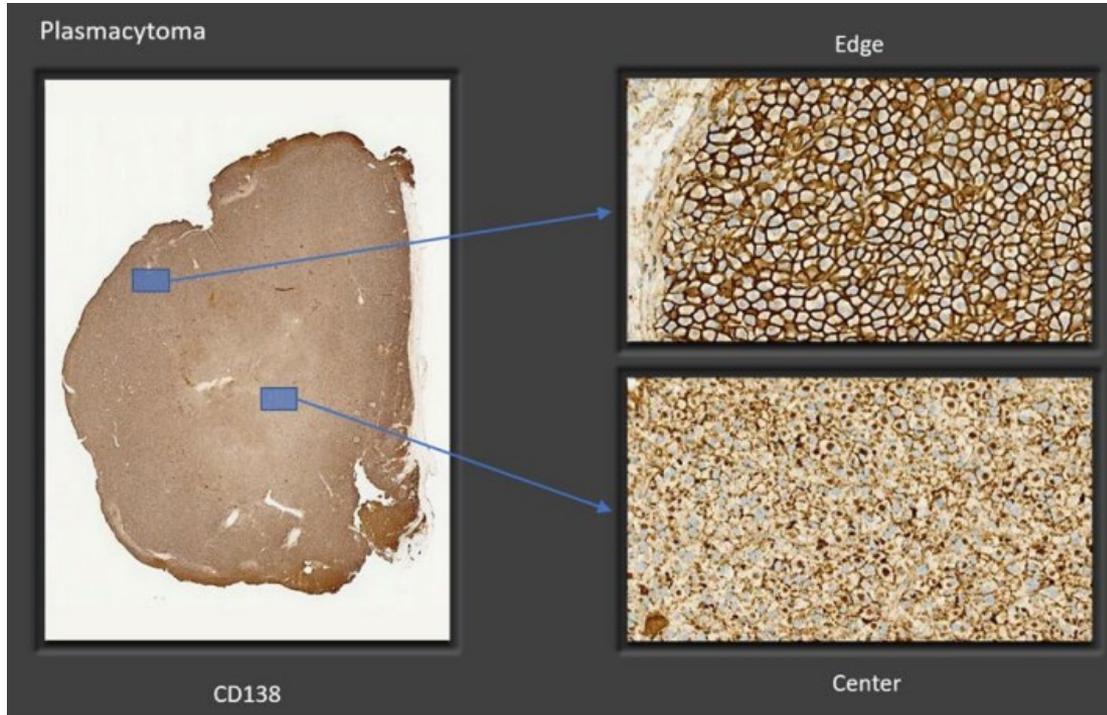
HE – Breast carcinoma



Importance of fixation time – IHC



Importance of fixation time – IHC



Diffusion laws

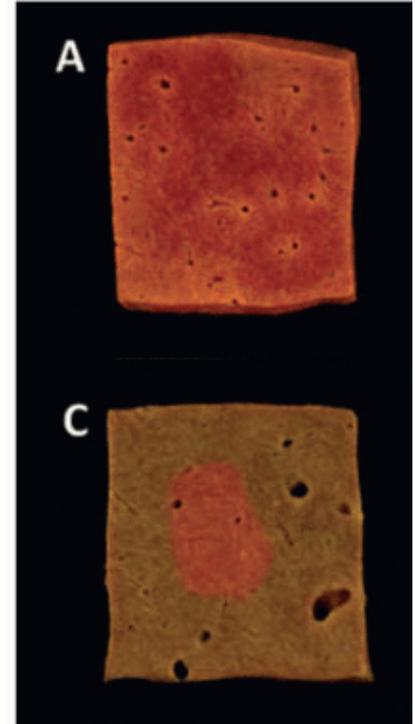
In 1941, Peter Medawar proposed that tissue fixatives follow diffusion laws and established a formula to calculate the depth of penetration:

$$d = K * \sqrt{t}$$

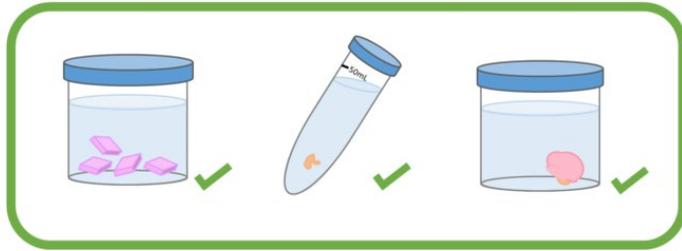
d = The depth of penetration in millimeters

K = The Medawar coefficient, specific to the fixative being used

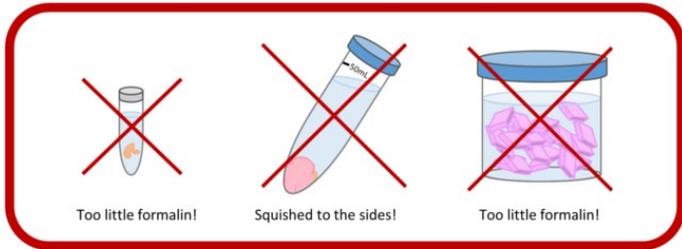
t = The time of fixation in hours



Tissue-to-fixative ratio



Yes, 20 times more formalin than your sample! Your sample should also be free to drift with lots of space around it.



Diffusion coefficient depends on tissue size and time

Minimum tissue-to-fixative ratio: 1 : 10

Larger specimens require longer fixation times

Minimum fixation time

Summary of ASCO/CAP ER and PgR Guideline Recommendations

Optimal tissue handling requirements*

*Revised per the 2011 ASCO/CAP Clinical Notice on HER2 and ER/PgR

Recommendation:

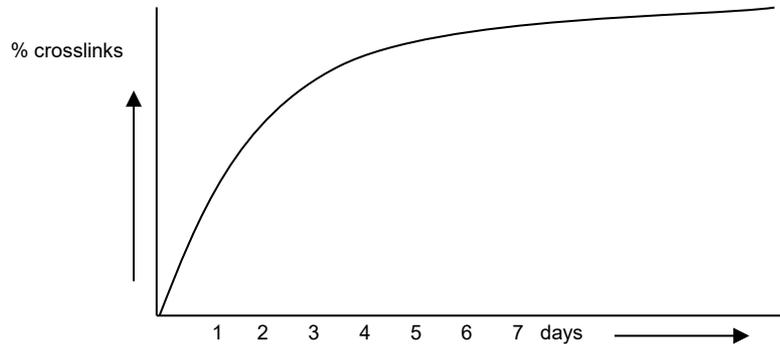
Time from tissue acquisition to fixation should be \leq one hour. Samples for ER and PgR testing are fixed in 10% NBF for 6–72 hours. Samples should be sliced at 5-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of NBF to allow adequate tissue penetration. If tumor comes from remote location, it should be bisected through the tumor on removal and sent to the laboratory immersed in a sufficient volume of NBF. Cold ischemia time, fixative type, and time the sample was placed in NBF must be recorded.

As in the ASCO/CAP HER2 guideline, storage of slides for more than 6 weeks before analysis is not recommended.

Time tissue is removed from patient, time tissue is placed in fixative, duration of fixation, and fixative type must be recorded and noted on accession slip or in report.

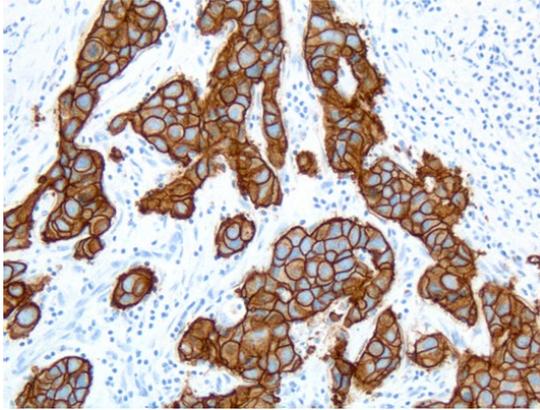
How about a maximum fixation time?

Why should there be a technical maximum?

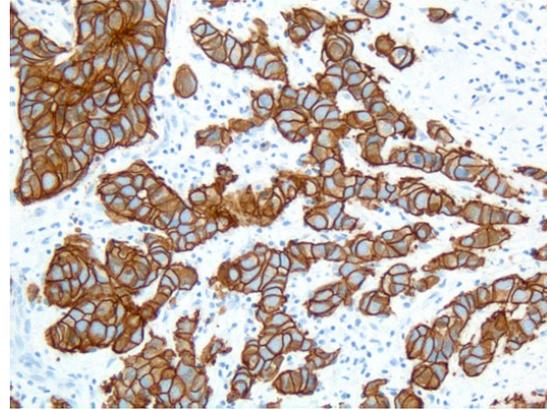


Breast carcinoma 3+, HER-2 PATHWAY, rmAb 4B5

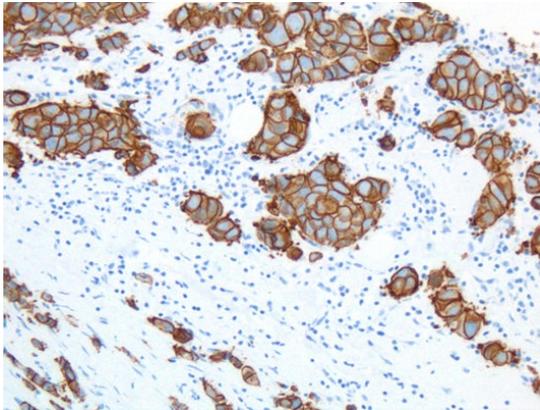
4 h



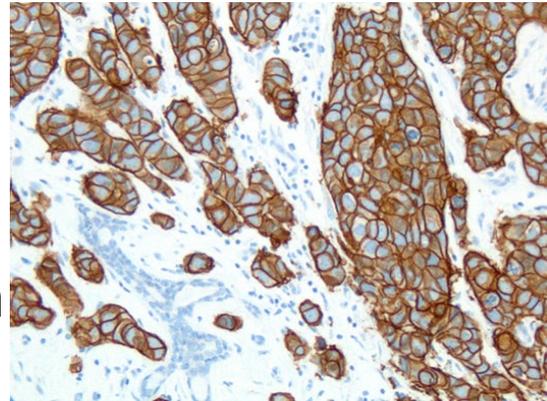
24 h



48 h

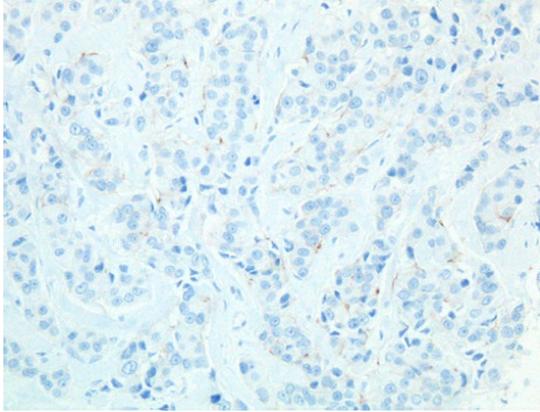


168 h

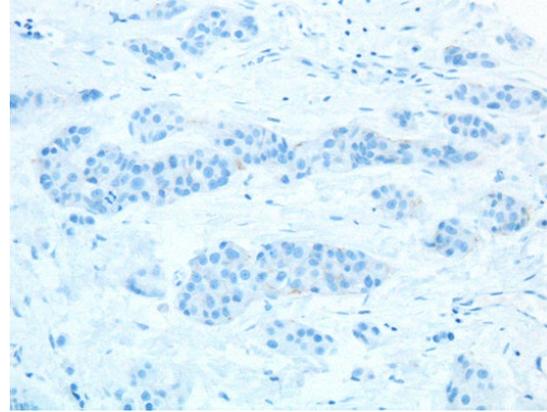


Breast carcinoma 1+, HER-2 PATHWAY, rmAb 4B5

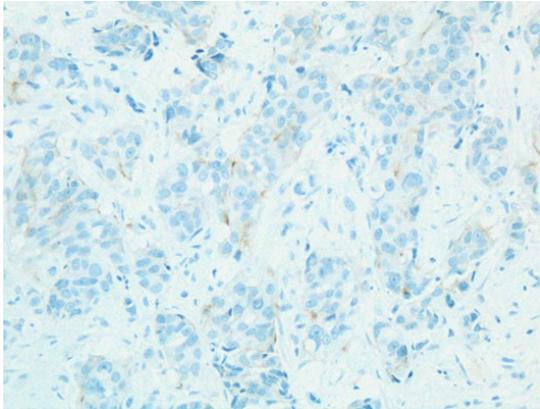
4 h



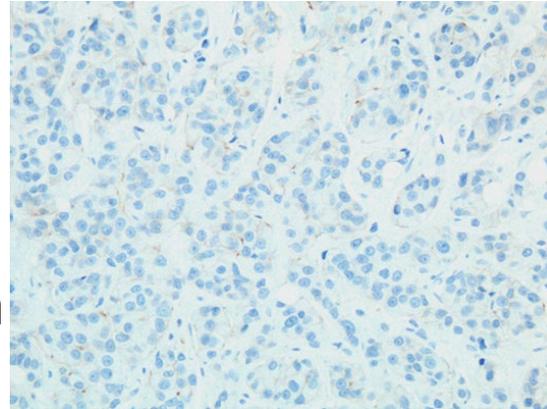
24 h



48 h

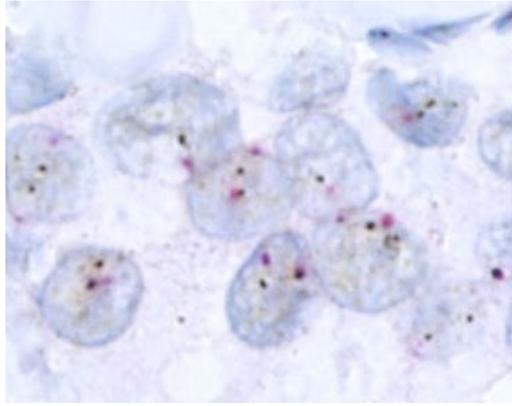


168 h

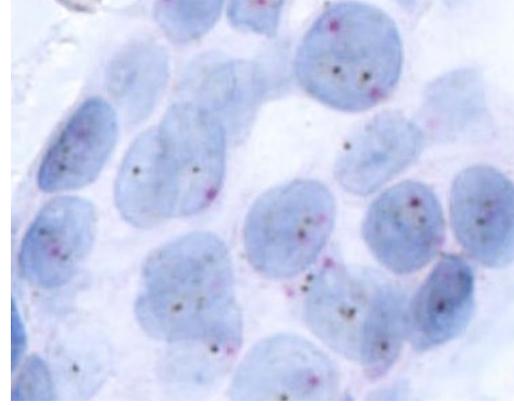


Breast carcinoma, 1+ Dual SISH CCrb ext, P3. 8 m

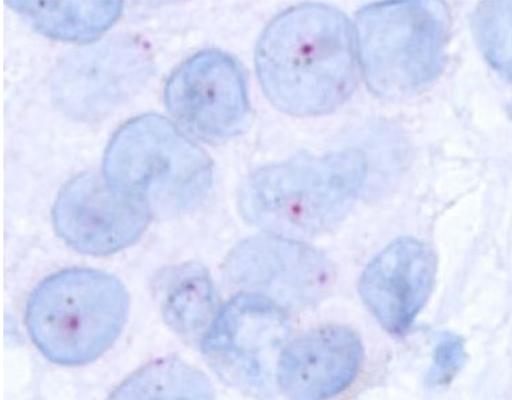
4 h



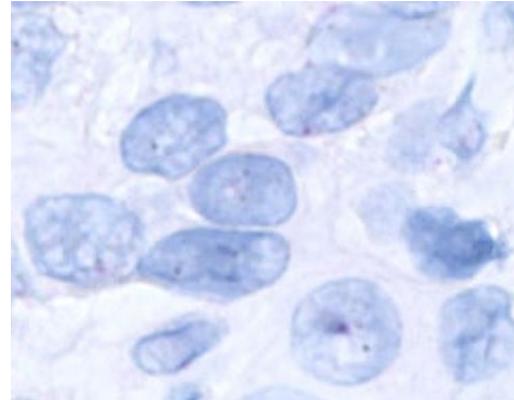
24 h



48 h



168 h



ASCO CAP guideline HER-2 test

	2007	2013	Most recent update 2023
Optimal tissue handling requirements	<p>Time from tissue acquisition to fixation should be as short as possible; samples for HER2 testing are fixed in 10% neutral buffered formalin for 6–48 hours; cytology specimens must be fixed in formalin.</p> <p>Samples should be sliced at 5-mm to 10-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of neutral buffered formalin</p>		<p>Duration of fixation has been changed from 6–48 hours to 6–72 hours. Any exceptions to this process must be included in report.</p>

Guidelines fixation

Time tissue is removed and received in grossing room

Time tissue was placed in fixative

Used fixative

Time of tissue fixation

Guidelines fixation

Time tissue is removed and received in grossing room

Time tissue was placed in fixative

Used fixative

Time of tissue fixation



What did we discuss this morning?

Pre-analysis

Variables in pre-fixation

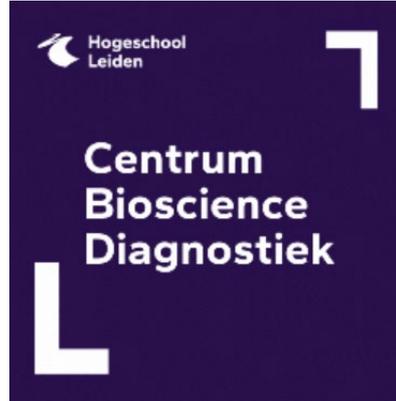
Warm and cold ischemia

Importance of fixation

Coagulating and non-coagulating fixatives

Variables during fixation

Do you want to learn more?



Histotechnology the basics

Online course for those starting in a pathology lab

Special stains

Online course about purpose, principle and trouble shooting

Immunohistochemistry

Online course about principle, design of assay, validation, quality control, trouble shooting

Control tissues used for IHC

Online course about the histology of common control tissues



With thanks to Anke Tiggelman
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What do you think?



True

False

What do you think?

Fixation is a very important part of the pre-analytical phase

True – Stand up

False – Remain seated

What do you think?

A concentration of 37% formalin is routinely used for fixation

True – Stand up

False – Remain seated

What do you think?

The ratio between fixative and tissue volume must be at least 1:10

True – Stand up

False – Remain seated

What do you think?

Cross-linking fixatives remove the hydration shell surrounding proteins

True – Stand up

False – Remain seated

What do you think?

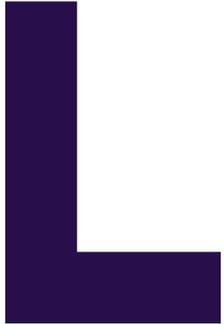
The recommended maximum delay before tissue fixation is 1 hour

True – Stand up

False – Remain seated



Fixation matters!





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Thank you!



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