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Fixation matters:

Why it’s so important

Nynke Hahn
Hahn.n@hsleiden.nl
Met dank aan Anke Tiggelman

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Waar gaan we het deze ochtend over hebben?

Pre-analyse

- Variabelen bij prefixatie
- Warme ischemie en koude ischemie

- Belang van fixeren
- Niet-coagulerende en coagulerende fixatieven
- Variabelen bij fixeren
- Fixatie artefacten

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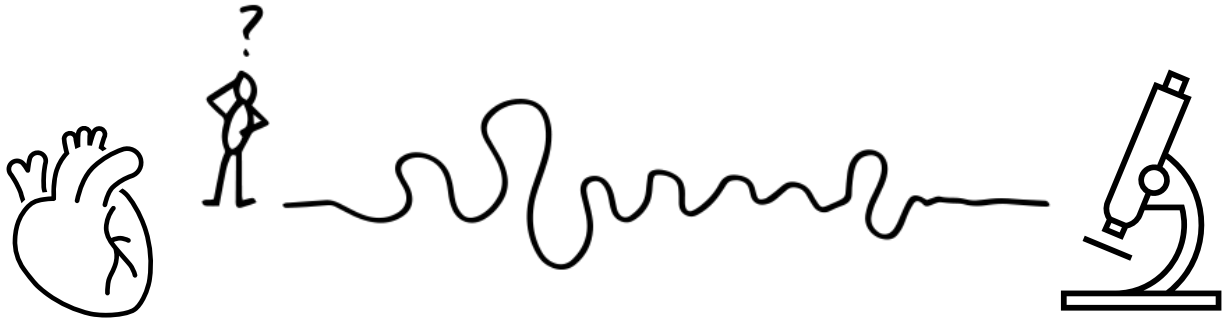
Pre-analyse

Welke stappen horen bij pre-analyse?



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Pre-analyse

- Prefixatie
- Fixatie
- Postfixatie / ontkalken
- Processing (doorvoeren, dehydreren, ophelderen, paraffine)
- Snijden, plakken
- Drogen, bewaren

Analyse

Post-analyse

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Pre-analyse

- Prefixatie
- Fixatie
- Postfixatie / ontkalken
- Processing (doorvoeren, dehydreren, ophelderen, paraffine)
- Snijden, plakken
- Drogen, bewaren

Analyse

Post-analyse

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Variabelen bij prefixatie

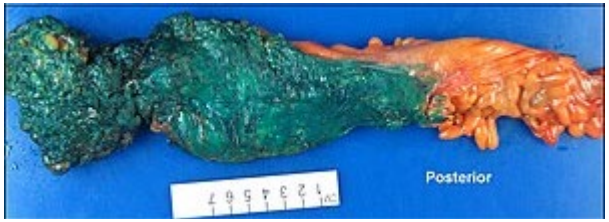
Afname weefsel

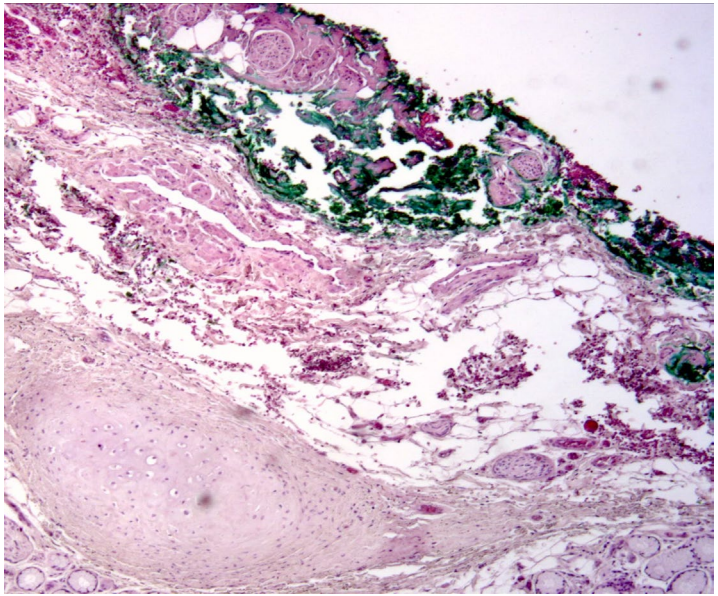
Vertraging voordat weefsel in fixatief gaat

- Tijd, temperatuur
- Warme – koude ischemie

Grootte / type weefsel
(biopt, resectie, histologie, cytologie)

Inkt





https://librepathology.org/wiki/Marking_ink

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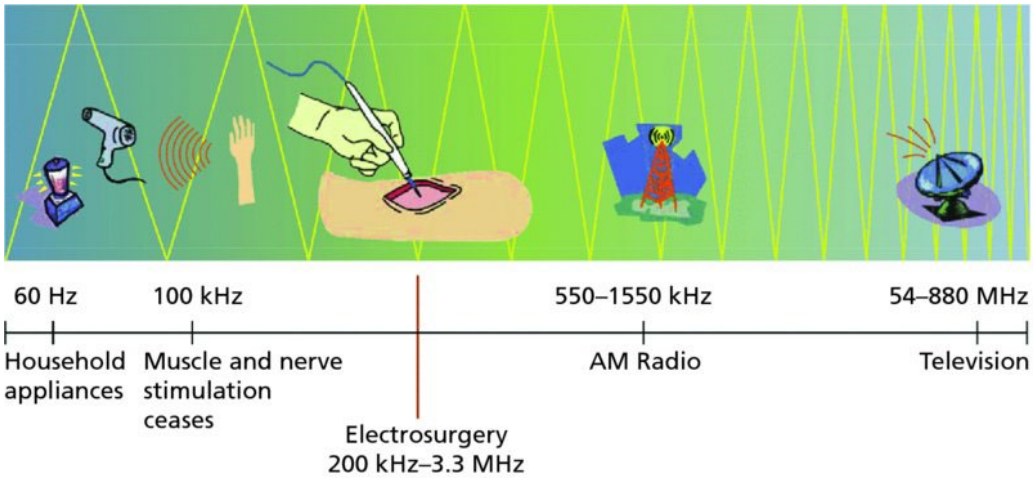
Effect afname weefsel bij OK

Electro-chirurgie - Hitte denaturatie

Wat gebeurt er met eiwitten?



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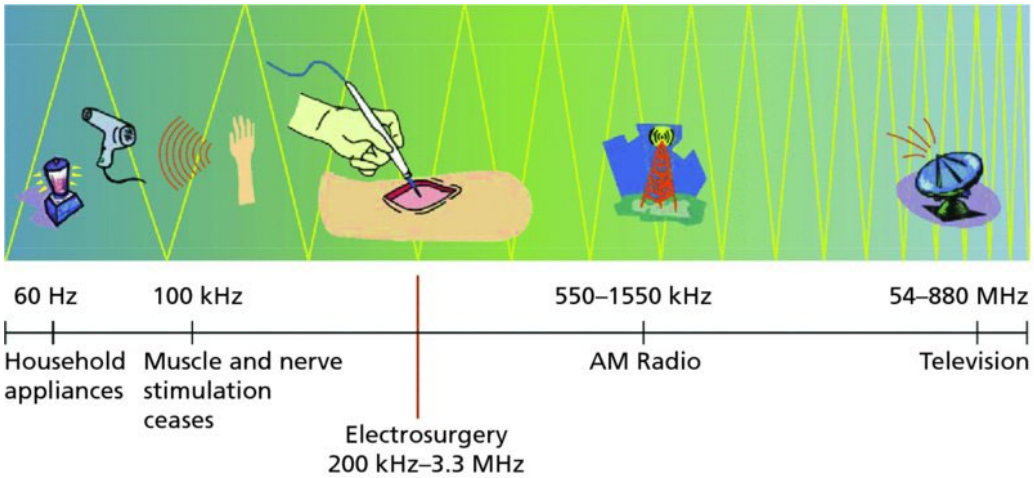
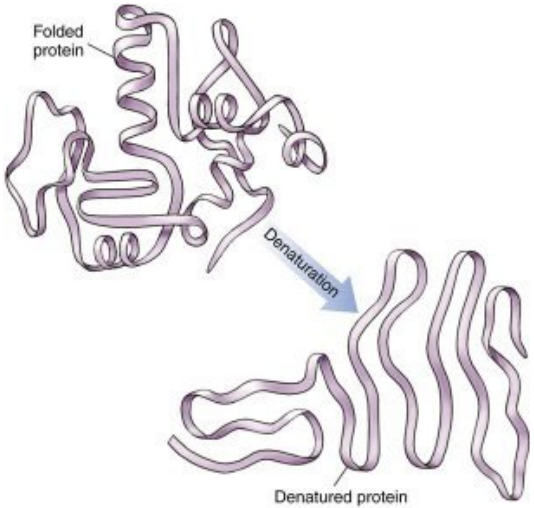
Effect afname weefsel bij OK

Electro-chirurgie - Hitte denaturatie

Wat gebeurt er met eiwitten?





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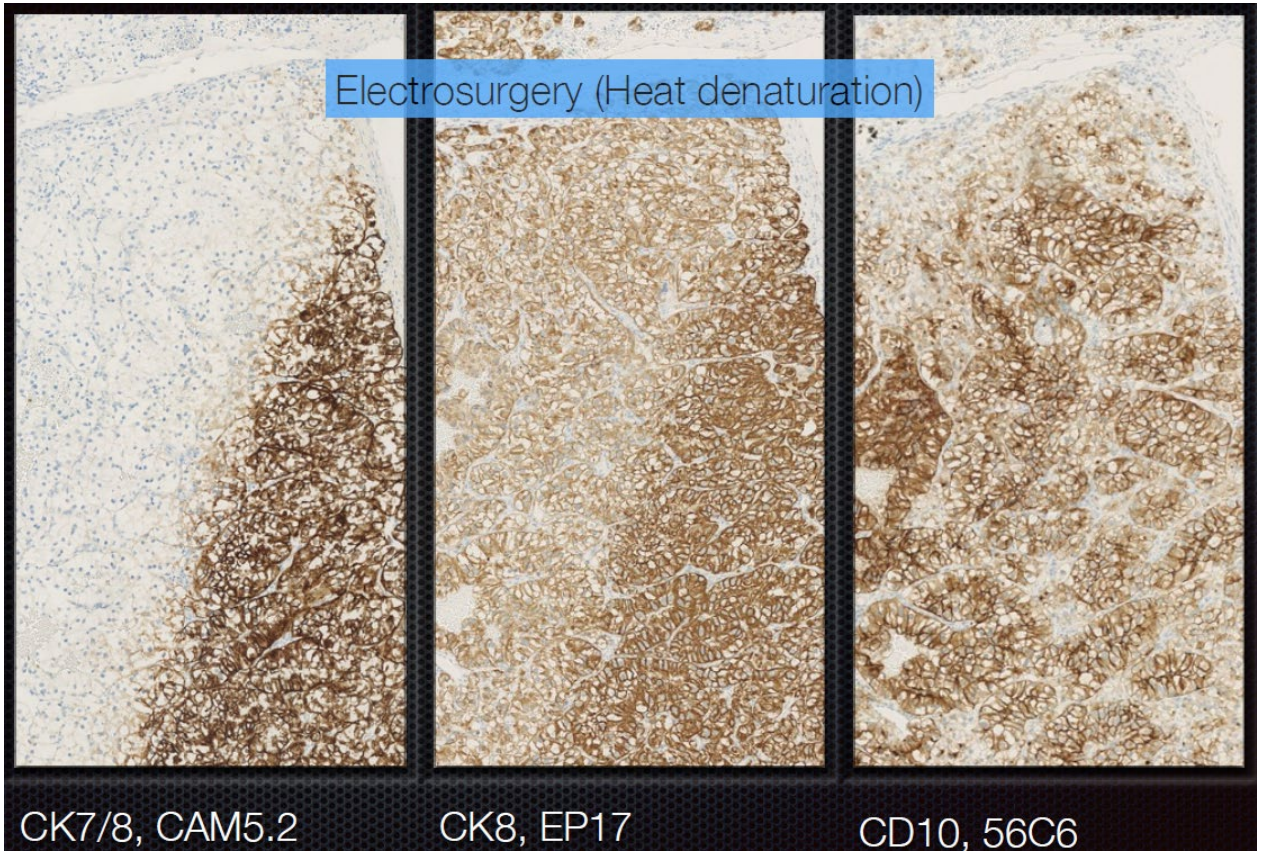

https://www.nordiqc.org/downloads/documents/8.pdf

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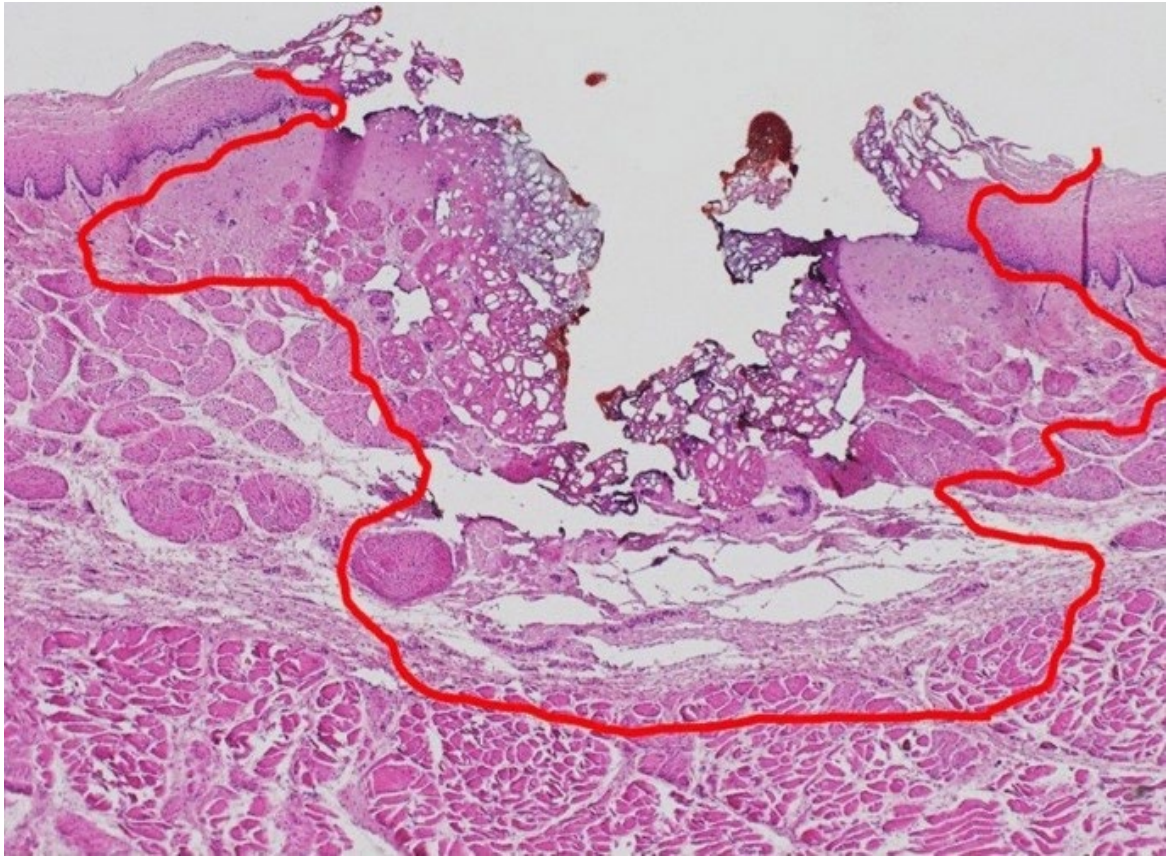

https://www.nature.com/articles/s41598-022-06533-9

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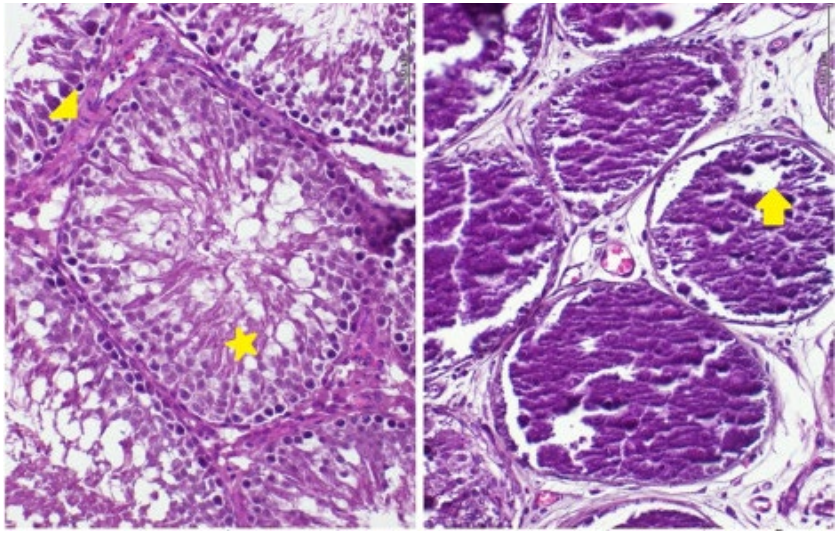
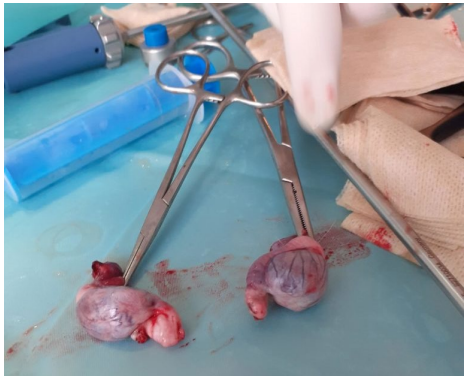
Effect afname weefsel bij OK

Afbinden orgaan – Warme ischemie





https://www.sciencedirect.com/science/article/pii/S1477513124004455

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Variabelen bij prefixatie

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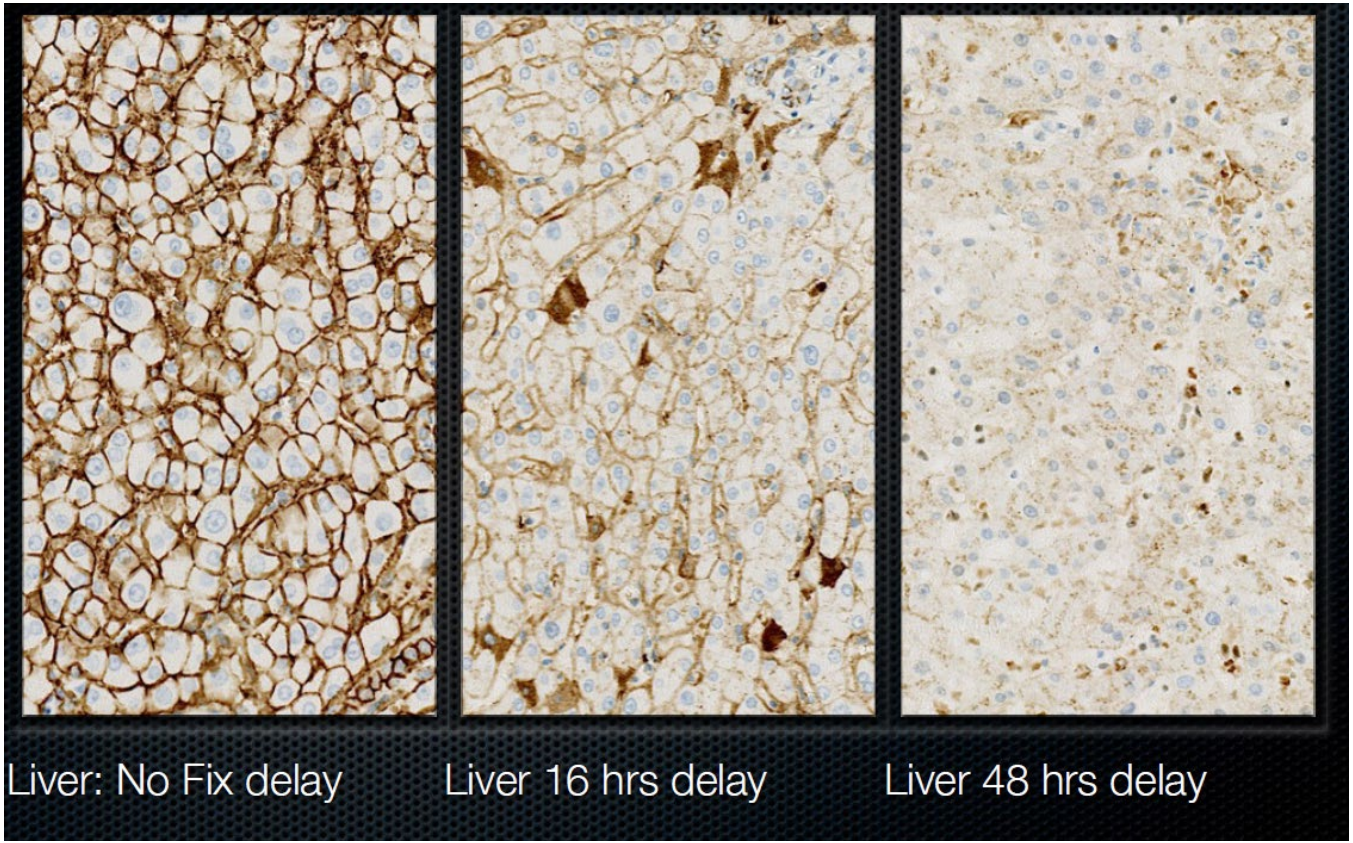

https://www.nordiqc.org/downloads/documents/8.pdf- CD138

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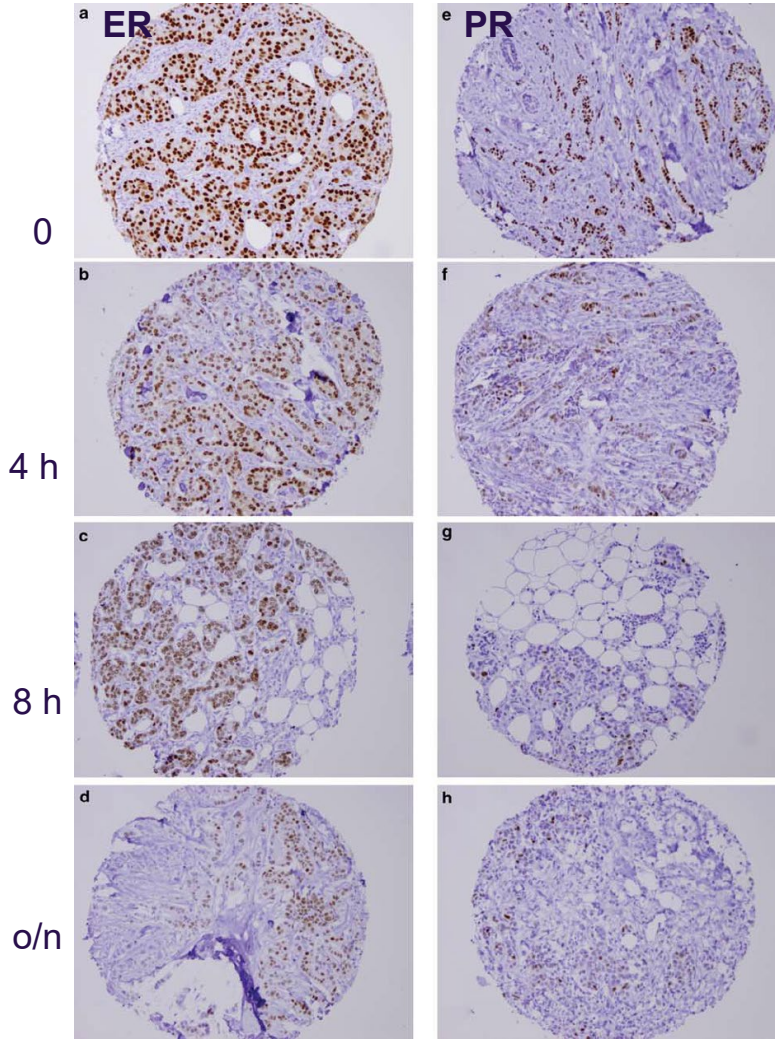
Koude ischemie

Tijd tussen afname weefsel en fixatie



https://www.researchgate.net/figure/26793880_fig1_Figure-1

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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 URMC 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+Breast+Cancer

Meest recente update: https://ascopubs.org/doi/10.1200/JCO.19.02309#T2

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Koude ischemie

Fixeren!

Gepubliceerde richtlijnen en aanbevelingen (ASCO en CLSI)
Fixeren binnen één uur

Literatuur-gebaseerde aanbevelingen
Fixeren binnen 12 uur

Waarom is op tijd fixeren zo belangrijk?

https://www.nordiqc.org/downloads/documents/8.pdf

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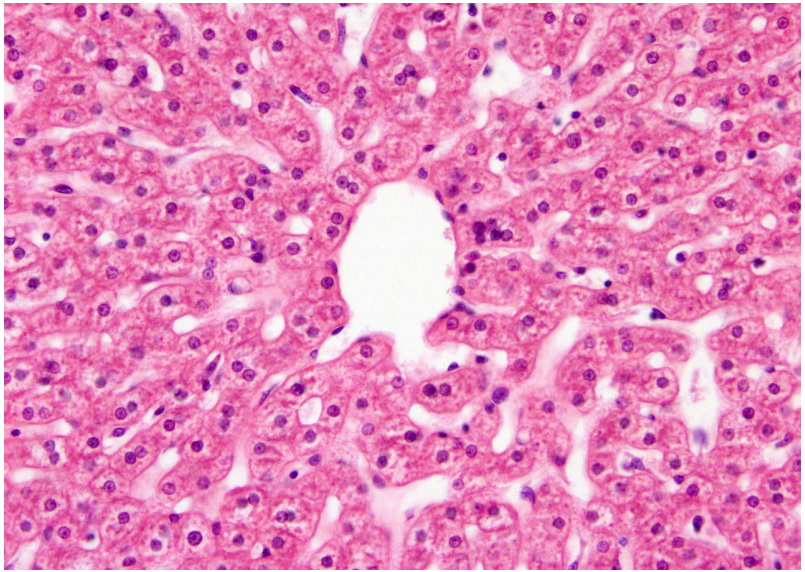
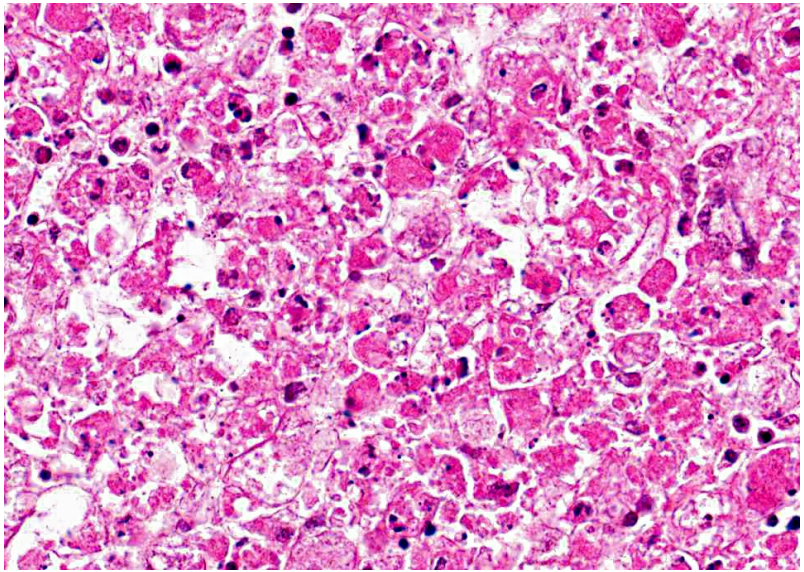
Koude ischemie – Autolyse





Zoek de verschillen…

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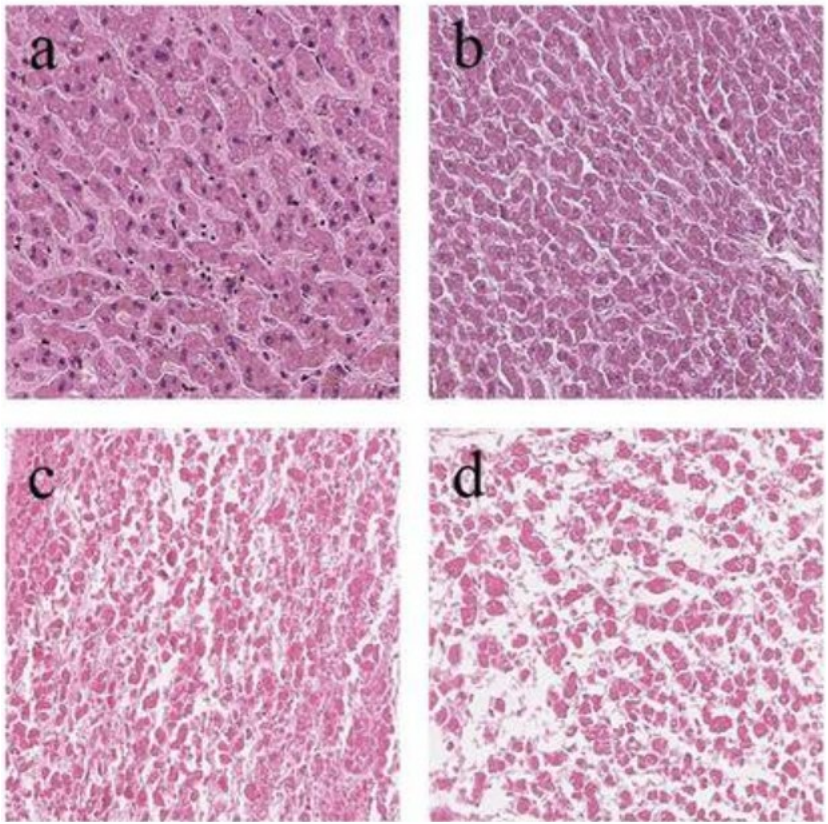

Kenmerken autolyse:

- Zwelling, lysis ery's
- Zwelling, krimp cellen
- Cytoplasma: eosinofieler, bleker, verdwijnt
- Nucleolus: eosinofiel, verdwijnt
- Kern: bleker, verdwijnt

https://www.researchgate.net/figure/Histological-changes-at-a-0-b-48-c-96-and-d-144-h-postmortem-for-liver_fig1_318352054

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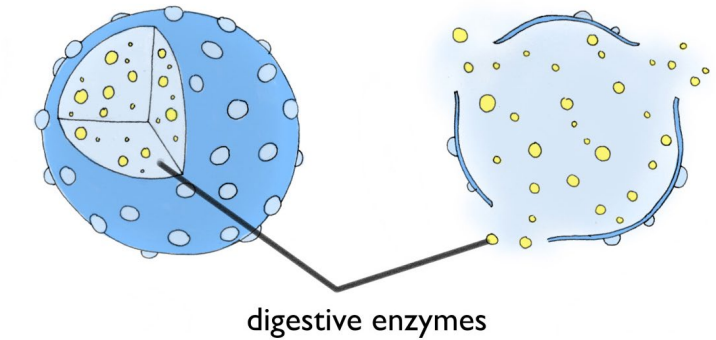
Koude ischemie – Autolyse

‘zelf vertering’

- afbraak processen
- afvalstoffen
- membraanpermeabiliteit
- lekken lysosomen



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Hoe kun je autolyse voorkomen of beperken?

Zonder fixatief

Met fixatief

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Hoe kun je autolyse voorkomen of beperken?

Zonder fixatief

Koelen

Met fixatief

Zo snel mogelijk!!!

Fysisch = invriezen

Chemisch = fixeren

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Het belang van fixeren

Met welke doelen wordt weefsel gefixeerd?

Welke manieren van chemisch fixeren zijn er?

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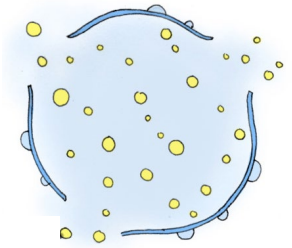
Doelen fixeren

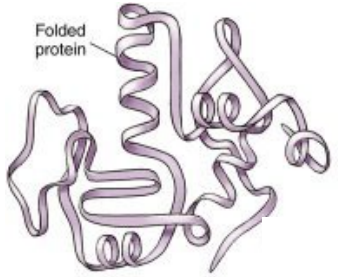
Afbraak (katabole processen) remmen / voorkomen

- autolyse
- heterolyse



3D structuur vastleggen

- behoud van eiwit structuur
- antigeniciteit



Bestand maken tegen histotechnische bewerkingen

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Chemisch fixeren

Niet-coagulerend (cross-link)

- Formaline
- Formaldehyde

Coagulerend

- Aceton
- Ethanol, methanol
- Azijnzuur

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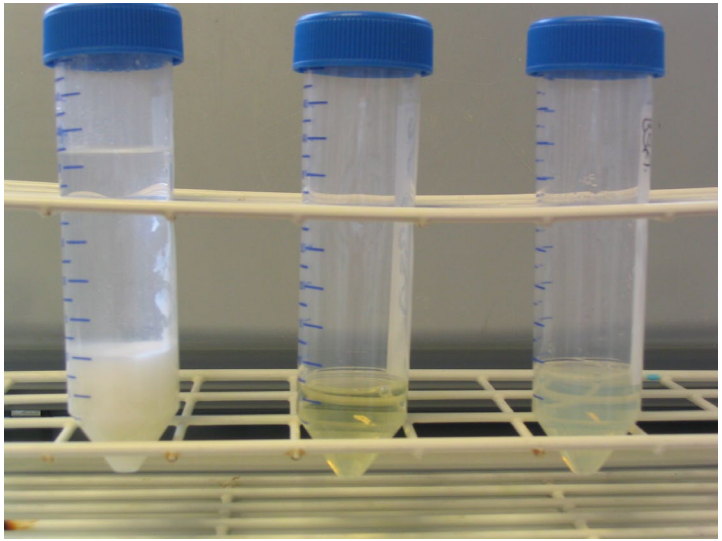
Wat zit waar in?

In alle drie de buizen zit een BSA oplossing

Aan één buis is formaline toegevoegd

Aan één buis is ethanol toegevoegd

Wat gebeurt er met
de eiwitten in deze buizen?



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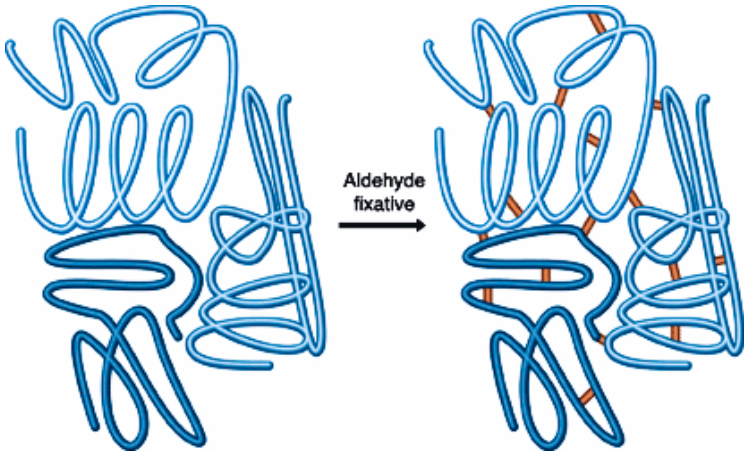
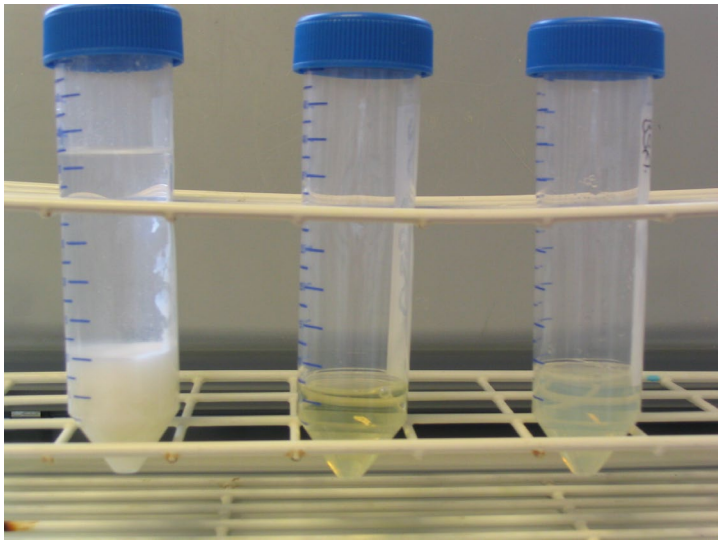
Wat zit waar in?

Aan één buis is **formaline** toegevoegd

Niet-coagulerend (cross-link)





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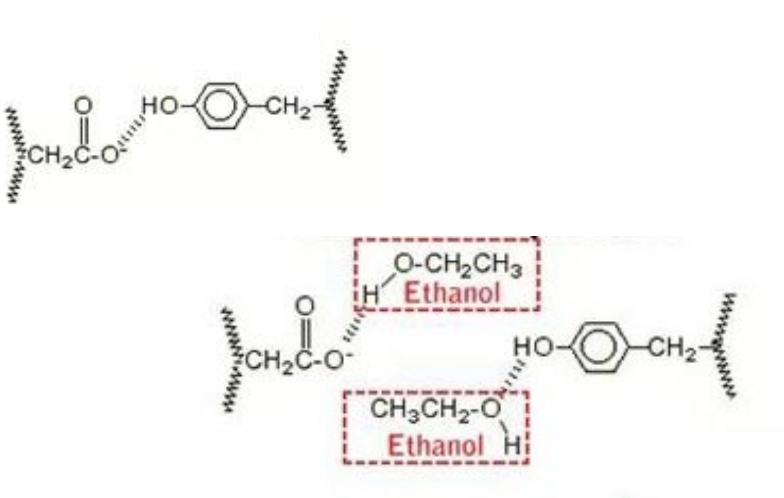
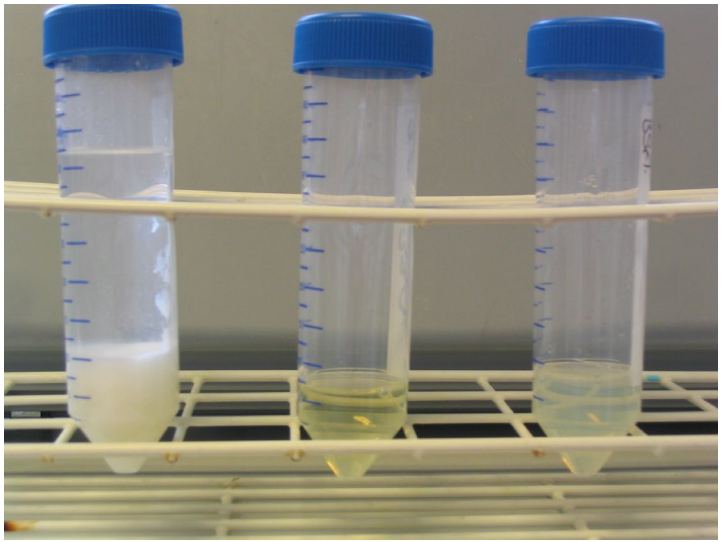
Wat zit waar in?

Aan één buis is **ethanol** toegevoegd

Coagulerend (klonteren)





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Niet-coagulerend (cross-link)

Aldehyde fixatieven

Formaline (NBF, FFPE)
(Para)formaldehyde

4% gebufferde formaldehyde

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Formaline – Formaldehyde

Formaldehyde (g)

→ oplosbaar tot 37% → (100%) formaline

→ 10x verdunnen → 10% formaline

Paraformaldehyde (s), polymeer van formaldehyde

→ 4g / 100 ml → 4% formaldehyde

4% formaldehyde ≈ 10% formaline

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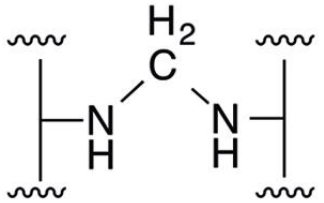
Formaldehyde reactie

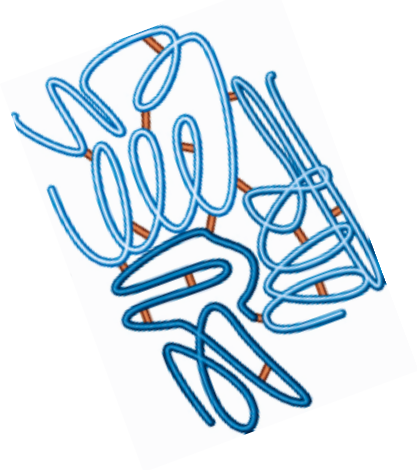
1: permeatie

2: additie

3: condensatie (cross-linking)



methylene bridge



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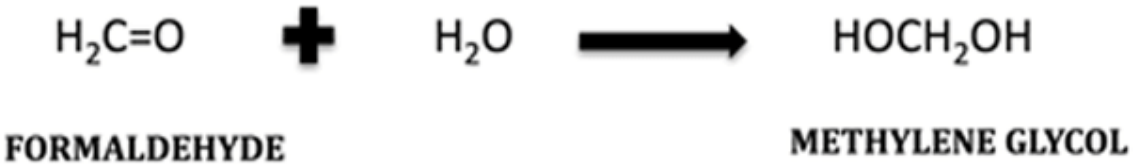
Formaldehyde reactie

1: permeatie

Hydratatie van formaldehyde tot formaline (methyleenglycol)



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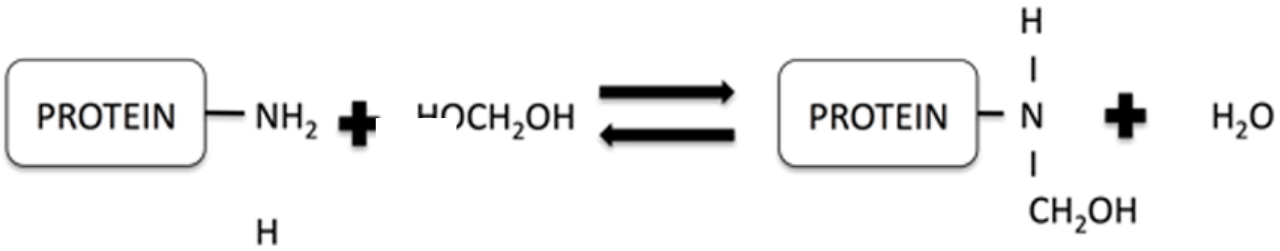
Formaldehyde reactie

2: additie (snel; minuten)

Covalente additiereactie waarbij formaline bindt met een NH_2 groep (instabiel)



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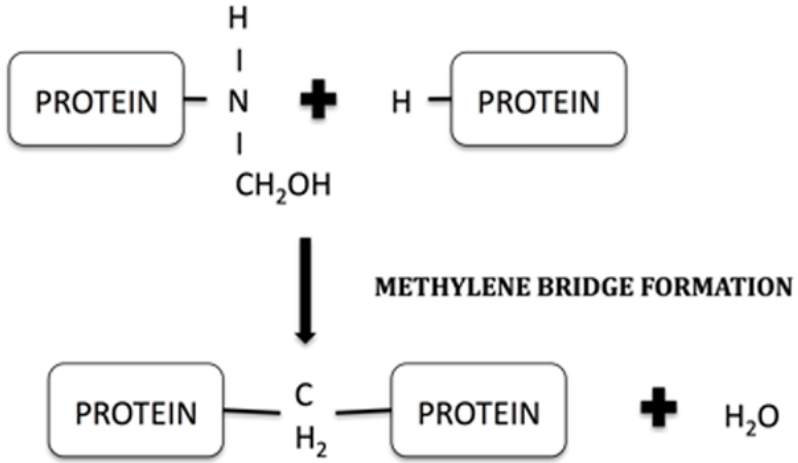
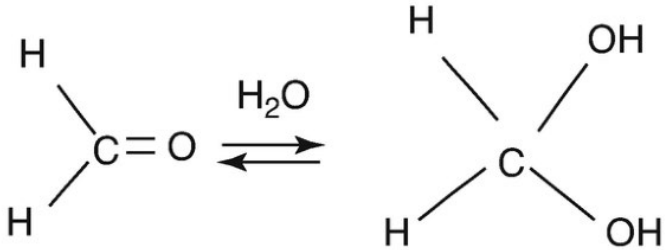
Formaldehyde reactie

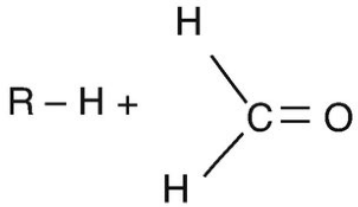
3: condensatie (traag; dagen)

Vorming cross-links (methyleenbruggen)





Formaldehyde Methylene glycol

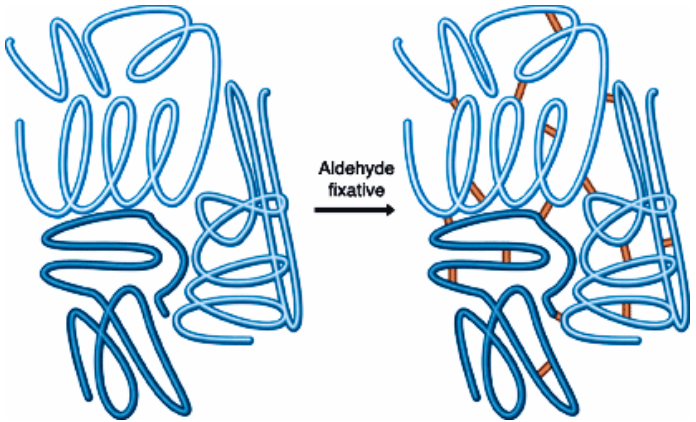


$= \quad R - CH_2OH$

Bound hydroxymethy group

$$R - CH_2OH + R - H = R - CH_2 - R + H_2O$$

Metyhylene bridge



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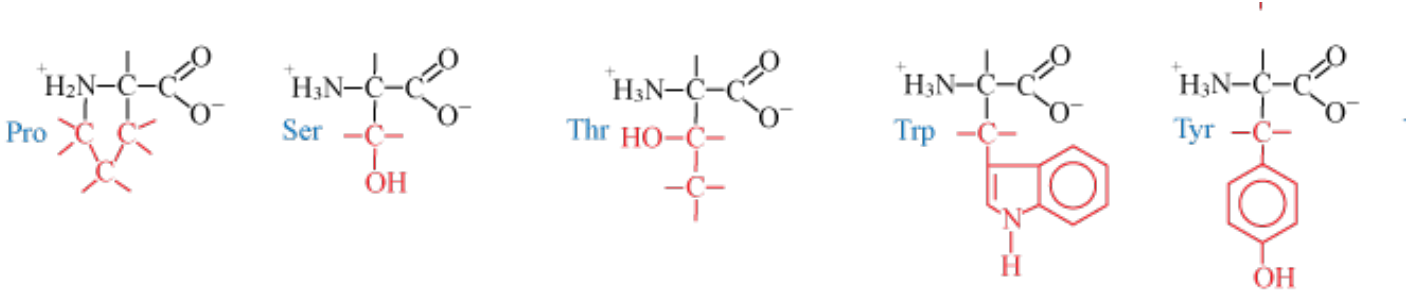
Formaldehyde reactie

Reactie met macromoleculen

Aminozuren -NH_2, -COOH, -OH, -SH

Nucleïnezuren -NH_2, -OH, -PO_4

Lipiden -C=C-



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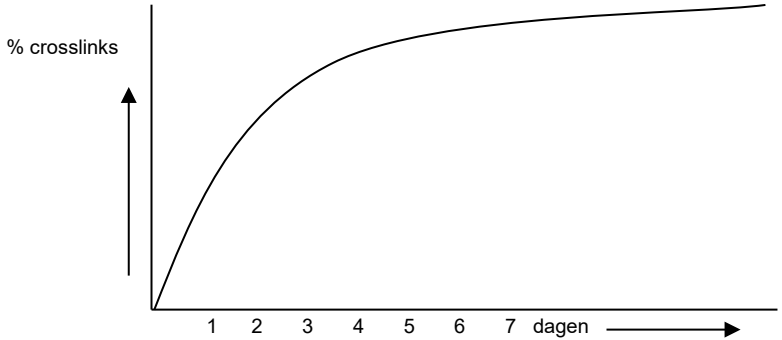
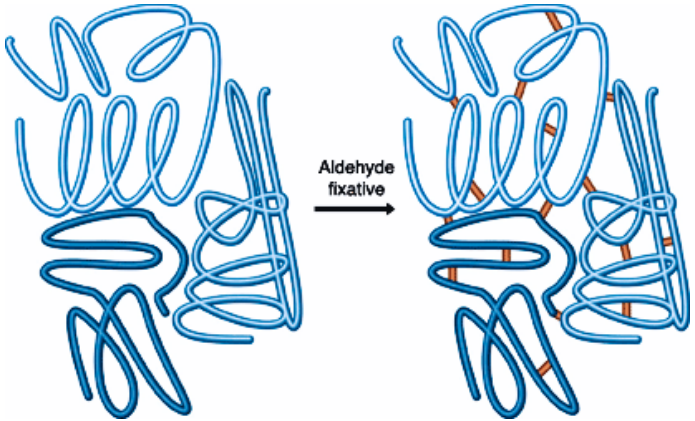
Fixatiesnelheid

Fixatie reactie van formaline met weefsel

Pathologie, diagnostiek: 24 uur formaline fixatie





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Variabelen bij fixatie

Fixatie tijd

- Onderfixatie, optimale fixatie, overfixatie

Buffer (type, concentratie)

Concentratie fixatief

Fixatie temperatuur (kT versus 4 ºC)

Aard van weefsel

- Cytologisch versus histologisch preparaat

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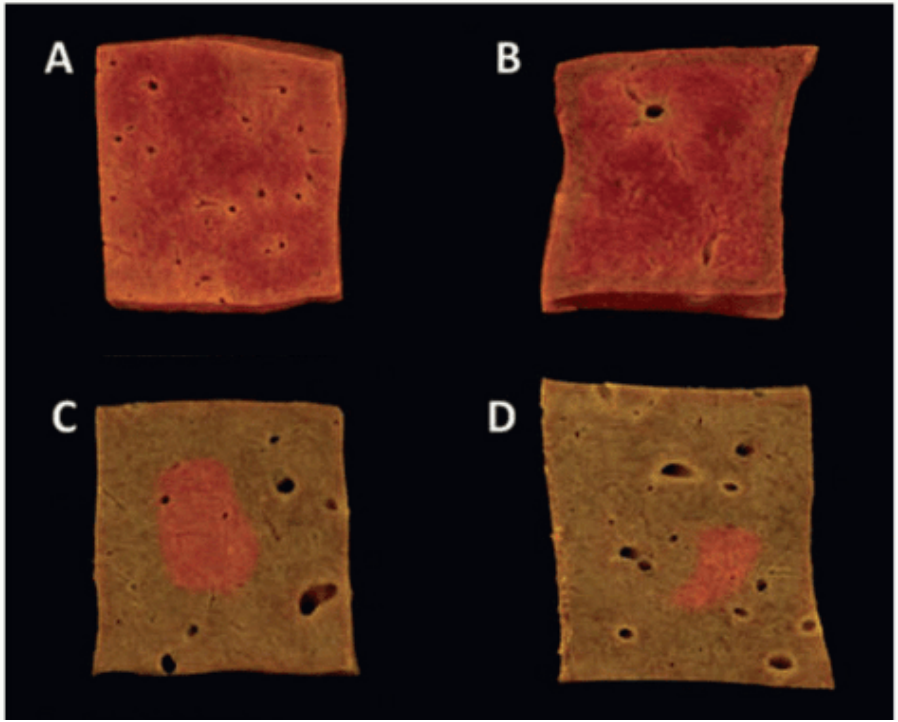
Belang fixatietijd - macroscopie



https://basicmedicalkey.com/gross-processing-of-liver-specimens/

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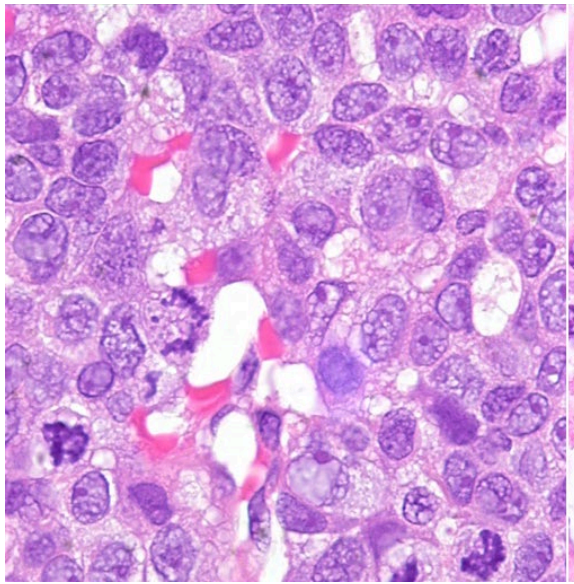
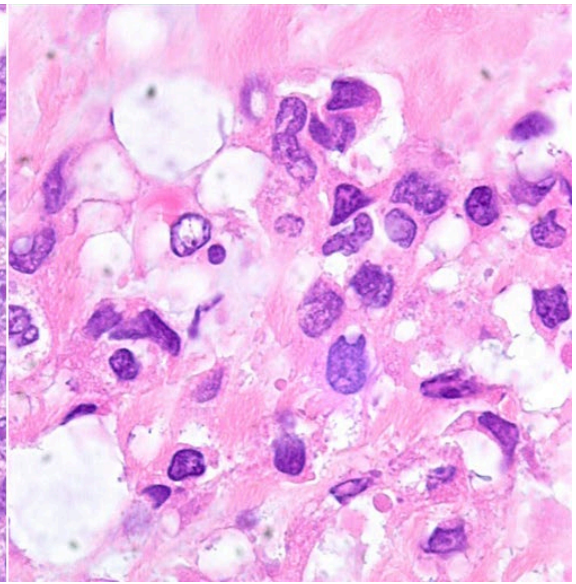
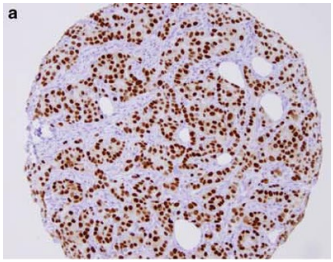
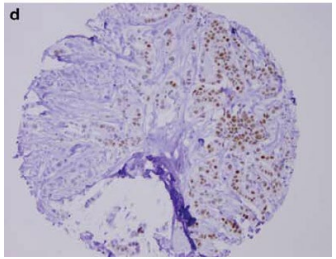
Belang fixatietijd - microscopie









https://oraclebio.com/pathologists-fixation-histology-histopathology/

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Belang fixatietijd – microscopie - IHC

NordiQC CD4 – SP35

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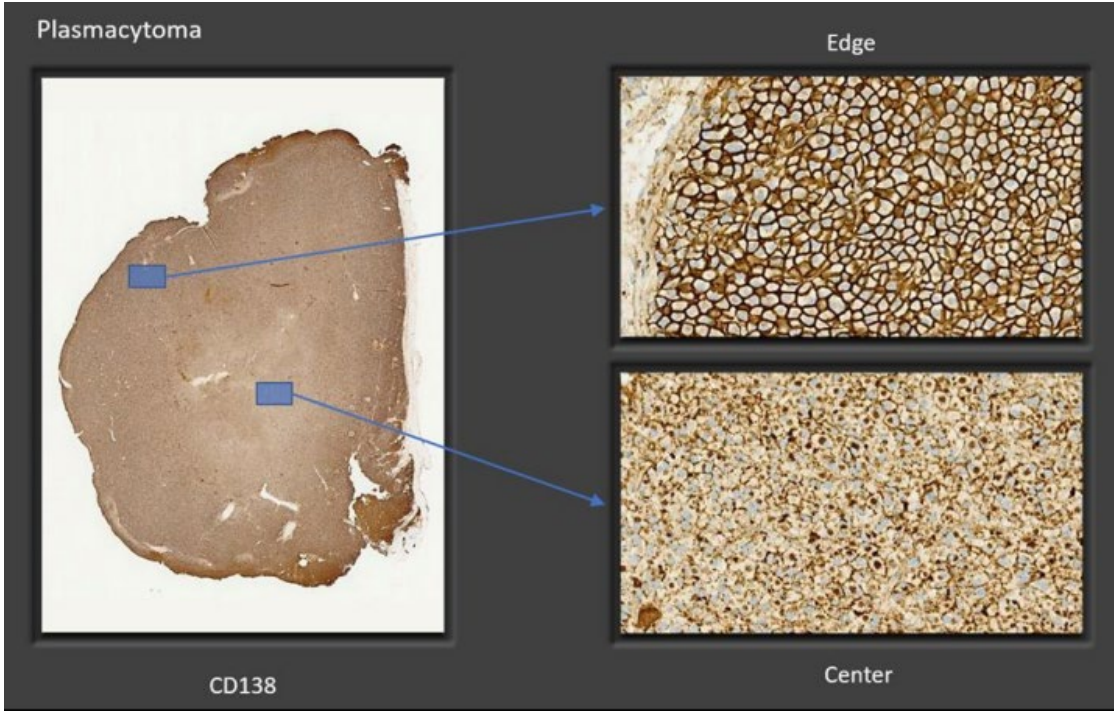
Belang fixatietijd – microscopie - IHC



https://www.nordiqc.org/downloads/documents/154.pdf

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Diffundeerbaarheid fixatief

Coëfficiënt van diffundeerbaarheid
(Medawar 1941)

$$D = k * \sqrt{t}$$

D	halve diameter van het weefsel = afstand van rand tot midden weefsel (mm)
k	diffusiecoëfficiënt
t	uur

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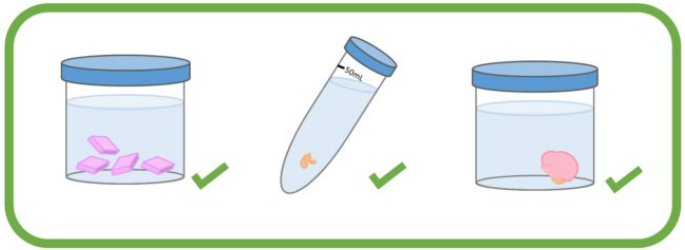
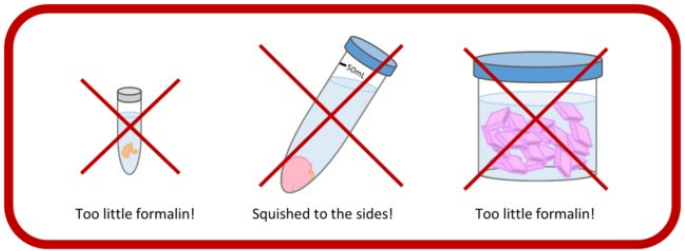
Diffundeerbaarheid fixatief



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



Minimale ratio

weefsel : fixatief = 1 : 10

https://uwhistologyandimaging.org/histopathology-work-order/general-histology-guidelines/

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Minimale fixatietijd voor ER

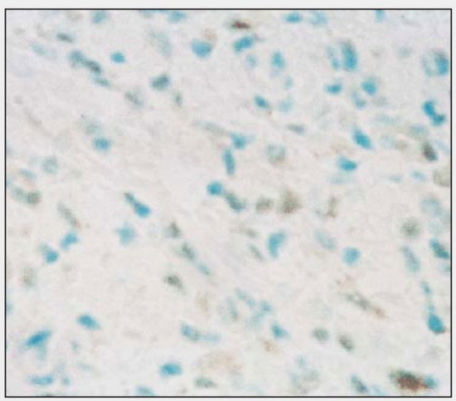

Image 1 Fixation, 3 h; antigen retrieval, 40 min.

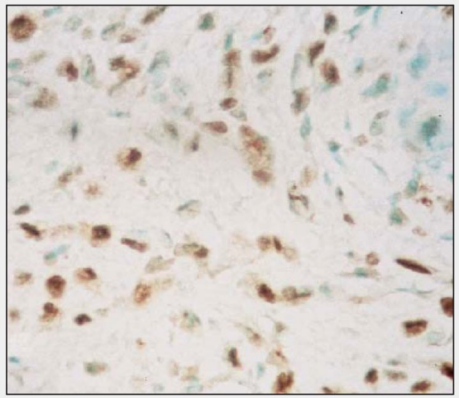

Image 2 Fixation, 6 h; antigen retrieval, 40 min.

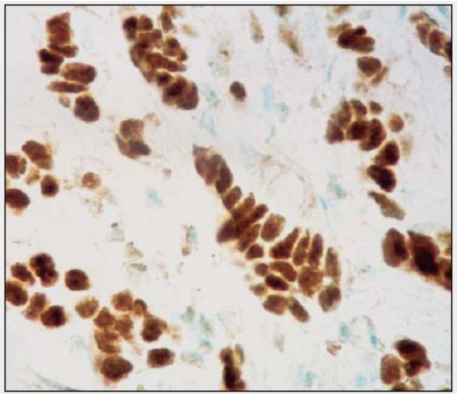

Image 3 Fixation, 8 h; antigen retrieval, 40 min.

NordiQC

mAb clone 1D5

" The minimum formalin fixation time for reliable immunohistochemical ER results is 6 to 8 hours in our laboratory, regardless of the type or size of specimen"

Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F. Am J Clin Pathol. 2003 Jul;120(1):86-92

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ASCO/CAP: 6-72 uur, 10% NBF

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 ≤ 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.

2019: http://www.cap.org/ShowProperty?nodePath=/UCMCon/Contribution%20Folders/WebContent/pdf/er-pgr-summary.pdf

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Is er een maximum fixatietijd voor ER?

Waarom zou er technisch een maximum fixatietijd zijn?

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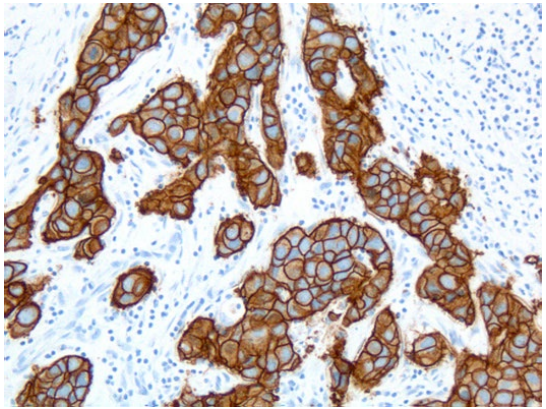
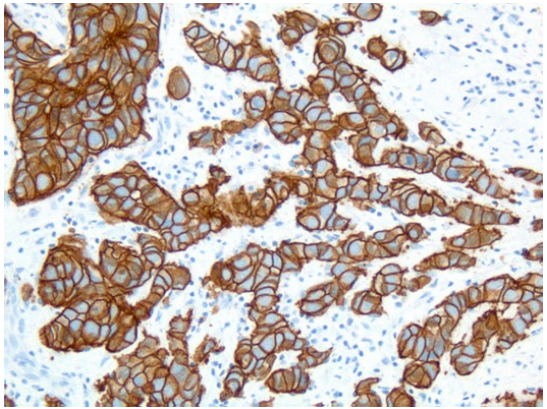
Breast carcinoma 3+, HER-2 PATHWAY, rmAb 4B5

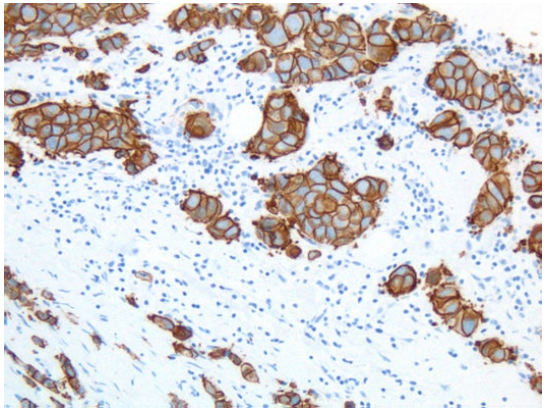
4 h



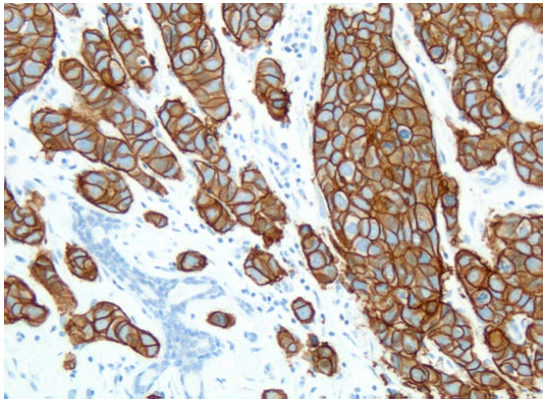
24 h



48 h



168 h



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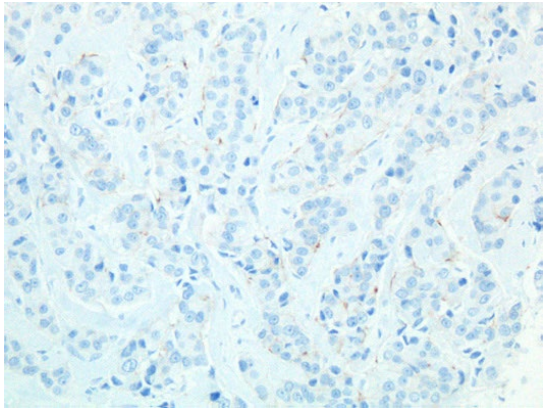
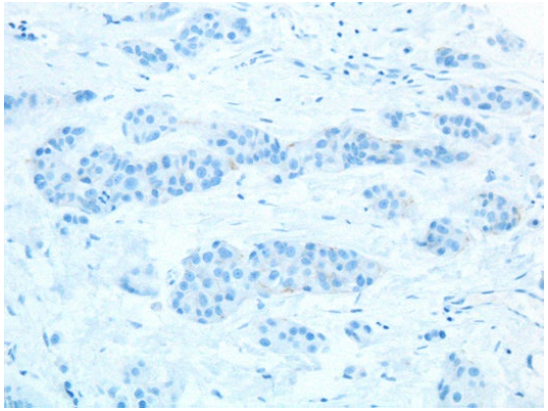
Breast carcinoma 1+, HER-2 PATHWAY, rmAb 4B5

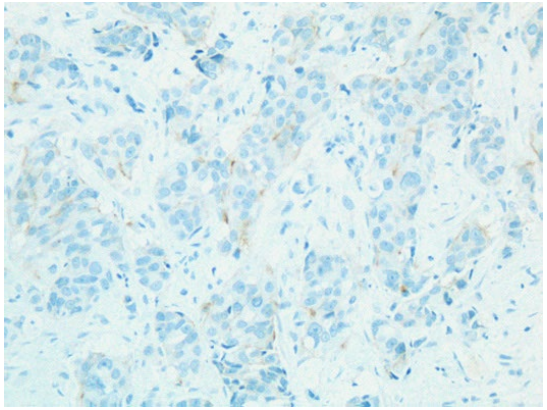
4 h



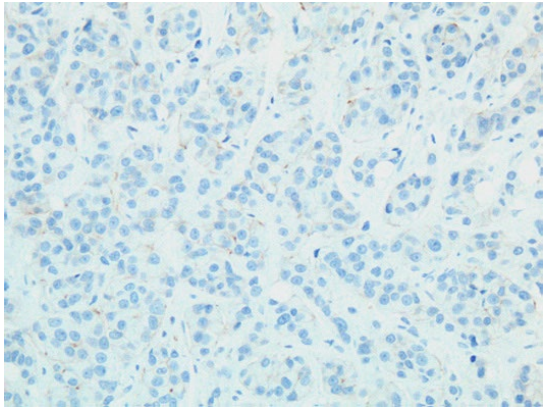
24 h



48 h



168 h



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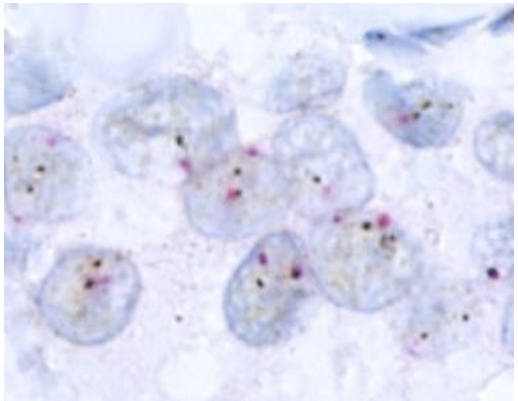
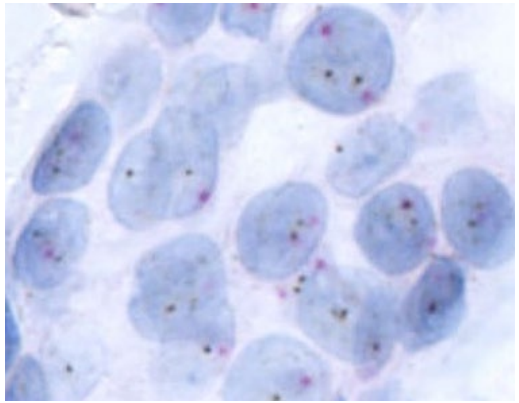
Breast carcinoma, 1+ Dual SISH CCrb ext, P3. 8 m

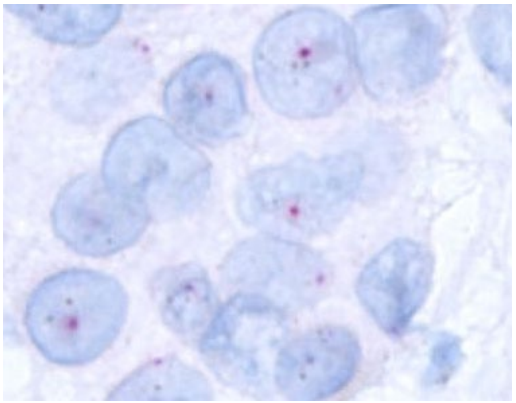
4 h



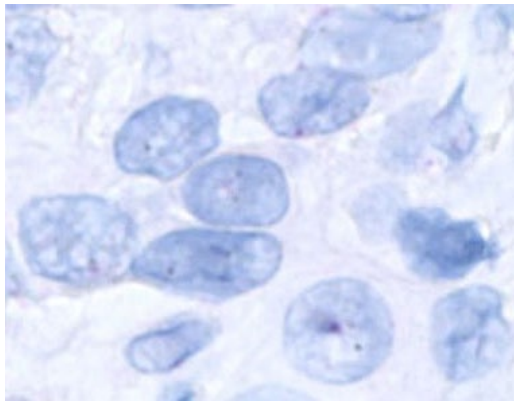
24 h



48 h



168 h



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ASCO CAP richtlijnen HER-2 test

	2007	2013	Laatste update 2023
Optimal tissue handling requirements	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. Samples should be sliced at 5-mm to10-mm intervals after appropriate gross inspection and margins designation and placed in sufficient volume of neutral buffered formalin	Duration of fixation has been changed **from 6–48 hours to 6–72 hours.** Any exceptions to this process must be included in report.	

http://www.cap.org/ShowProperty?nodePath=/UCMCon/Contribution%20Folders/WebContent/pdf/her2-summary.pdf

Meest recente update: https://meridian.allenpress.com/aplm/article/147/9/993/493531/Human-Epidermal-Growth-Factor-Receptor-2-Testing

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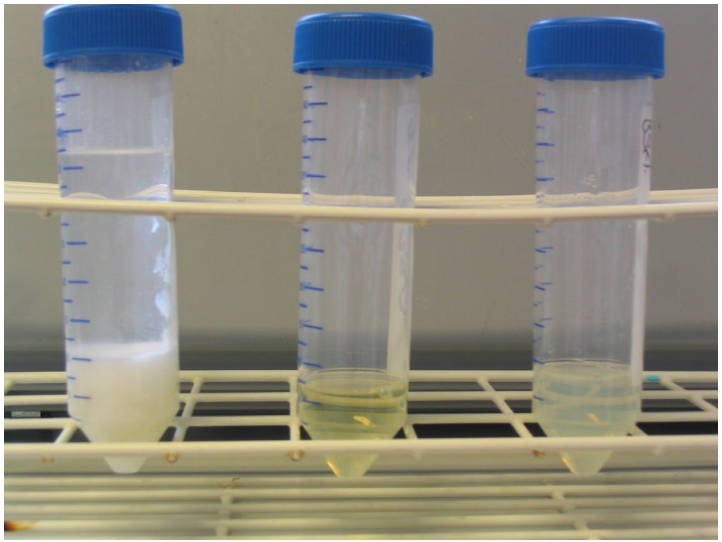
Chemisch fixeren

Niet-coagulerend (cross-link)

- Formaline
- Formaldehyde

Coagulerend

- Aceton
- Ethanol, methanol
- Azijnzuur



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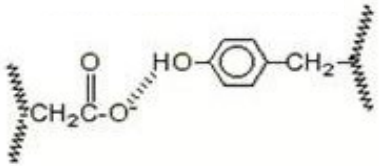
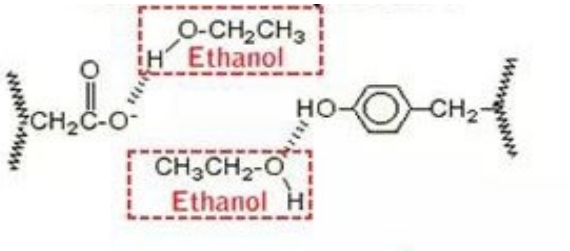
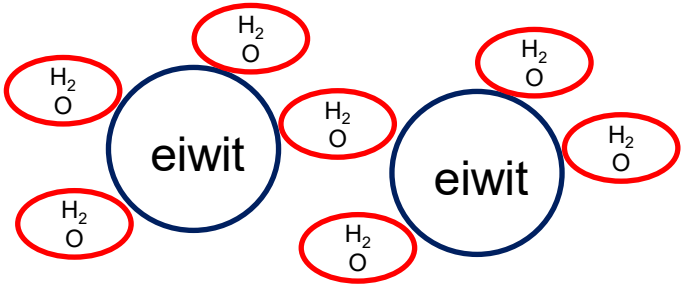
Coagulerende fixatieven

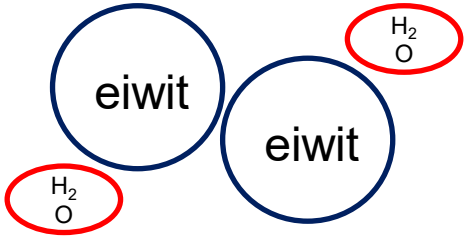
Onttrekken watermantel (waterstofbruggen)

Eiwitten klonteren (coaguleren) en slaan neer









http://www.exobiologie.fr/wp-content/uploads/2008/12/image1-8.jpg

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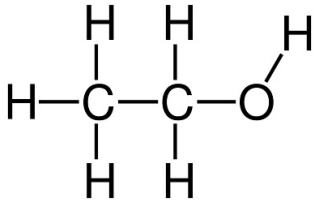
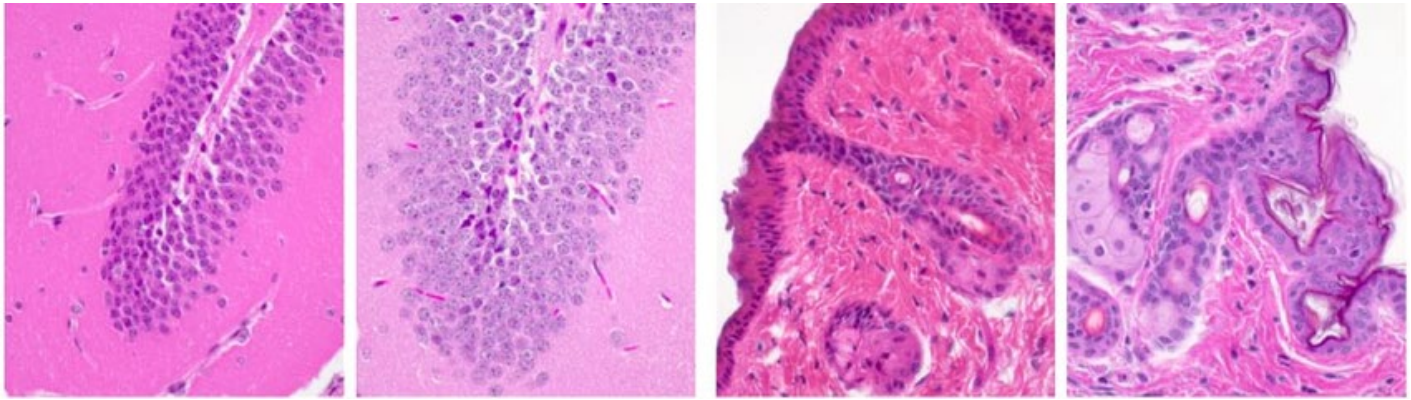
Coagulerende fixatieven

Aceton

Ethanol, methanol





https://www.researchgate.net/figure/Comparison-between-buffered-ethanol-70-BE70-and-neutral-buffered-formalin-NBF_fig2_303511316

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Richtlijnen fixeren

Tijd van afname, dan wel tijd van aankomst op het lab is bekend

Gebruikte fixatief is bekend

Fixatietijd is bekend

2019: http://www.vapweb.nl/files/page/wihc-kwaliteitsrichtlijnen-1-weefselbewerking-20121009.pdf

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Fixatie artefacten

Hoe te herkennen?

LM “normaal”

erytrocyten (hemolyse)

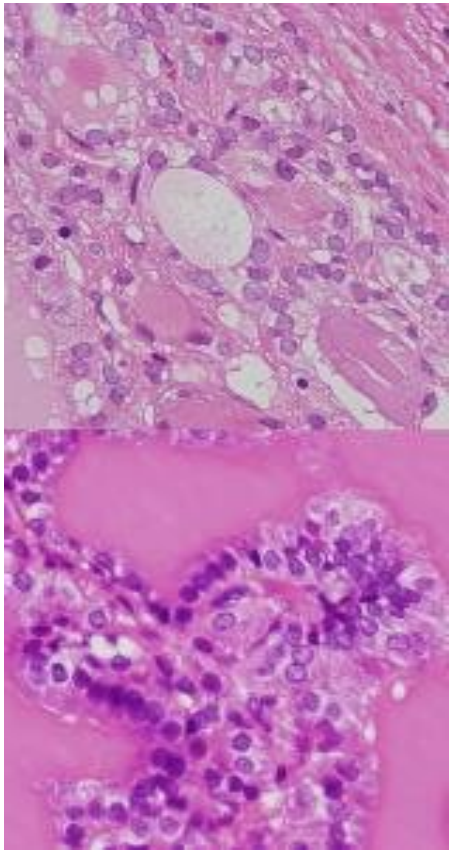
geen scheiding weefselstructuren

chromatine patroon (‘poederig’)

nucleolus

cytoplasmagrenzen

“normaal” contrast



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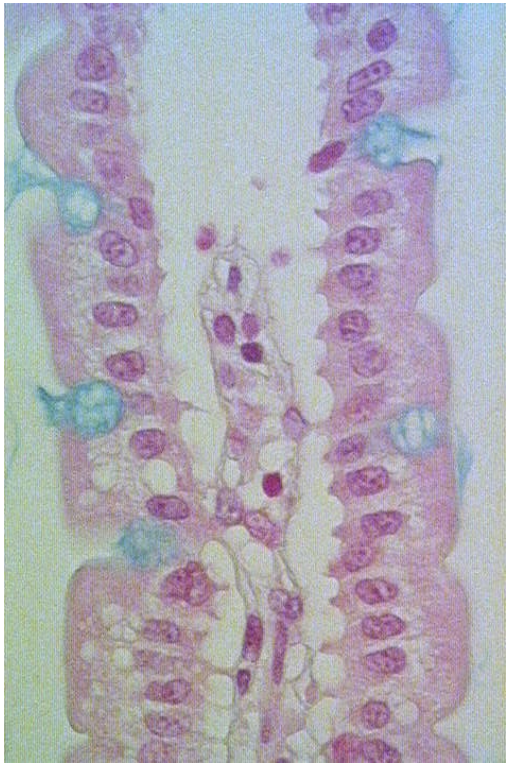
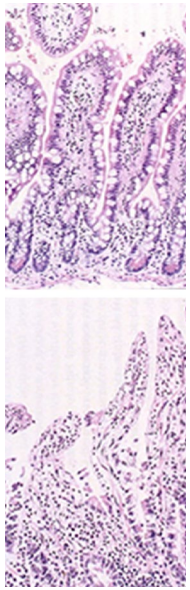
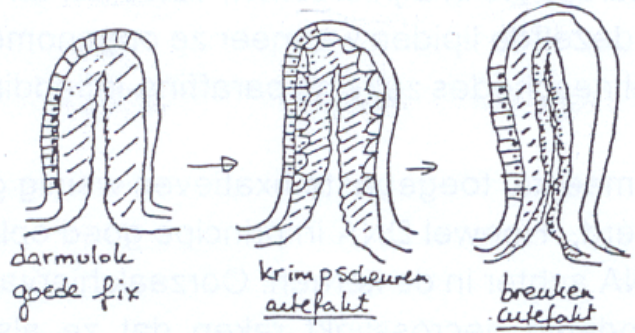
Fixatie artefacten - Krimp







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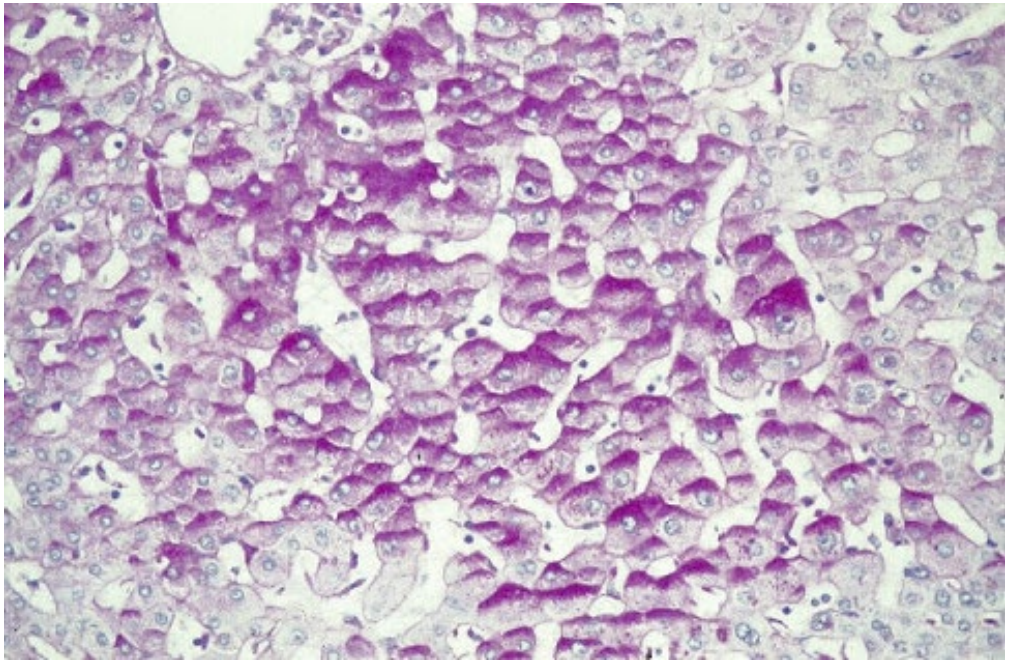
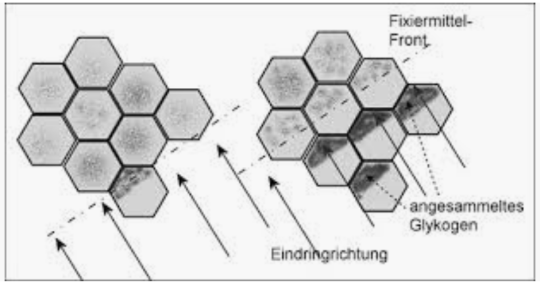
Fixatie artefacten - Krimp





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Waar hebben we het deze ochtend over gehad?

Pre-analyse

- Variabelen bij prefixatie
- Warme ischemie en koude ischemie

- Belang van fixeren
- Niet-coagulerende en coagulerende fixatieven
- Variabelen bij fixeren
- Fixatie artefacten

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Wat vinden jullie?

Waar

Niet waar

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Wat vinden jullie?

Fixeren is een zeer belangrijk onderdeel van de pre-analyse fase

Waar – Ga staan

Niet waar – Blijf zitten

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Wat vinden jullie?

De concentratie van 37% formaline wordt standaard toegepast voor fixatie

Waar – Ga staan

Niet waar – Blijf zitten

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Wat vinden jullie?

De verhouding tussen fixatief en weefselvolume moet minstens 1:10 zijn

Waar – Ga staan

Niet waar – Blijf zitten

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Wat vinden jullie?

Cross-linkende fixatieven verwijderen de watermantel rond eiwitten

Waar – Ga staan

Niet waar – Blijf zitten

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Wat vinden jullie?

De aanbevolen maximale tijdsvertraging voor weefselfixatie is 1 uur

Waar – Ga staan

Niet waar – Blijf zitten

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Fixation matters!

Nynke Hahn
Hahn.n@hsleiden.nl
Met dank aan Anke Tiggelman