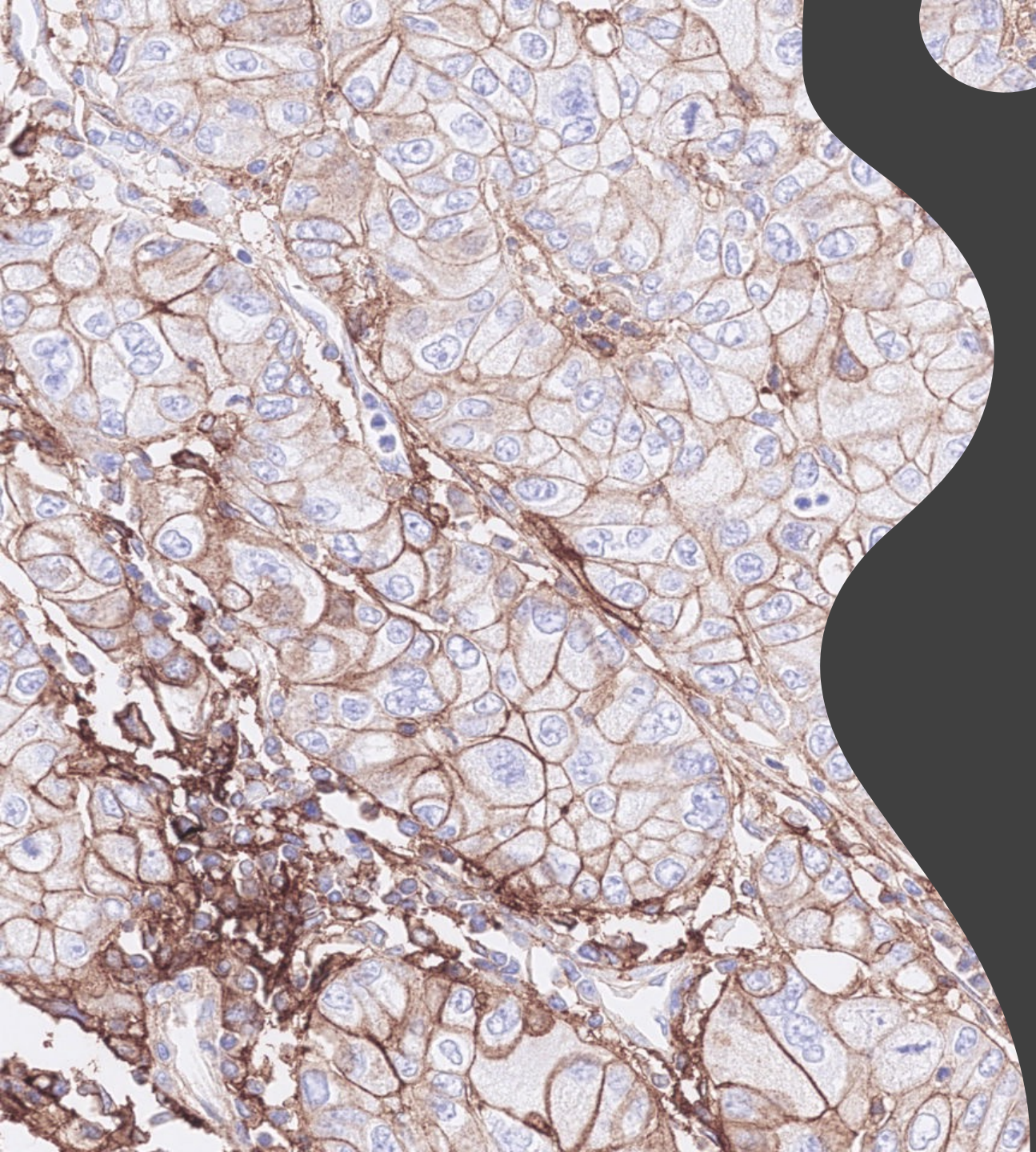


The technical approach Pre-analytical phase NordiQC workshop 2024

By Tanya Julio

Histotechnologist

Dept. of Pathology

Aarhus University Hospital, DK

The total test paradigm

"Immunohistochemistry is technically complex, and no aspect of this complexity can be ignored, from the moment of collecting the specimen to issuance of the final report"
Taylor CR. Arch Pathol Lab Med 2000; 124:945

Preanalytical

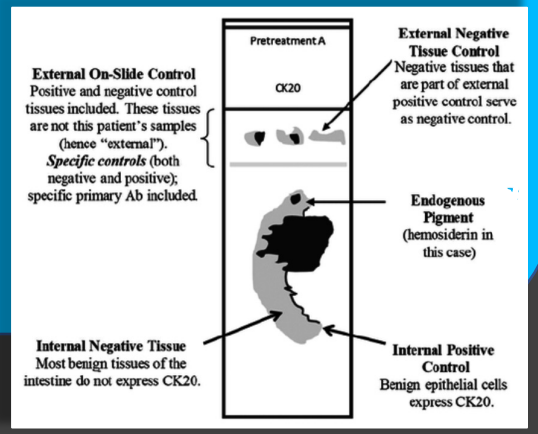
Operating method
Ischemia
Fixative and fixation
Tissue processing
Paraffin embedding
Paraffin sectioning
Slide choice
Storage

Analytical

Choice of platform
Epitope retrieval
Blocking
Primary Antibody
Diluent
Detection system
Chromogen
Counter stain
Mounting

Interpretation

Design of controls
Positive controls
Negative controls
Interpretation
Critical stain indicator



60-70% Errors in pathology estimated to be related to preanalytical…

Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

Kelly B. Engel, PhD; Helen M. Moore, PhD

Arch Pathol Lab Med—Vol 135, May 2011

Table 1. Potential Sources of Preanalytic Variation During Specimen Fixation and Processing

Prefixation
- Duration and delay of temperature
- Specimen size
- Specimen manipulation (pathology ink)

Fixative
- Formula
- Concentration
- pH
- Age of reagent
- Preparation source

Fixation
- Tissue to fixative volume ratio
- Method (immersion, injection, and sonication or microwave acceleration)
- Conditions of primary and secondary fixation
  - Movement
  - Light exposure
  - Primary container
  - No. and position of cofixed specimens

Postfixation
- Washing conditions and duration
- Storage reagent and duration

Processing
- Type of processor, frequency of servicing and reagent replacement
- Tissue to reagent volume ratio
- No. and position of coprocessed specimens

Dehydration and clearing
- Reagent
- Temperature
- No. of changes
- Duration (total and change-specific)

Paraffin impregnation
- Type and melting point of wax
- No. of changes
- Duration (total and change-specific)
- Method (immersion and sonication or microwave acceleration)

Paraffin sectioning
- Type of blade and frequency of replacement
- Frequency of servicing and wax replacement
- Temperature of block during sectioning
- Slide pretreatment
- Water bath conditions, if used
- Chemical adhesives, if used
- Temperature and duration of slide drying

Storage
- Temperature and duration of paraffin block storage
- Temperature, duration, and manipulation of slide-mounted tissue sections

Decalcification:
Type, Time, Temperature

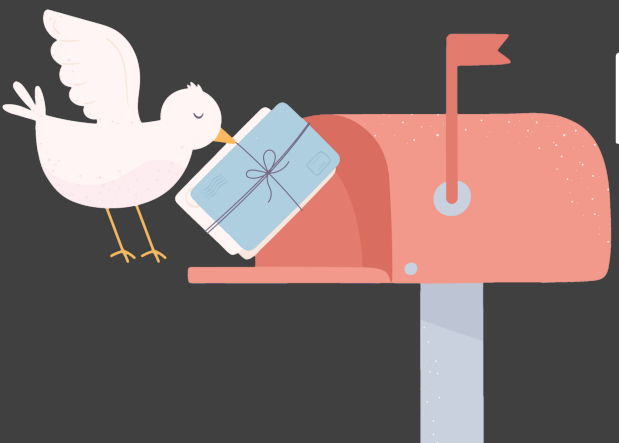
By courtesy Ole Nielsen

Preanalytics and Precision Pathology

Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine

Carolyn C. Compton, MD, PhD; James A. Robb, MD; Matthew W. Anderson, MD, PhD; Anna B. Berry, MD; George G. Birdsong, MD; Kenneth J. Bloom, MD; Philip A. Branton, MD; Jessica W. Crothers, MD; Allison M. Cushman-Vokoun, MD, PhD; David G. Hicks, MD; Joseph D. Khoury, MD; Jordan Laser, MD; Carrie B. Marshall, MD; Michael J. Misialek, MD; Kristen E. Natale, DO; Jan Anthony Nowak, MD, PhD; Damon Olson, MD; John D. Pfeifer, MD, PhD; Andrew Schade, MD; Gail H. Vance, MD; Eric E. Walk, MD; Sophia Louise Yohe, MD

NordiQC

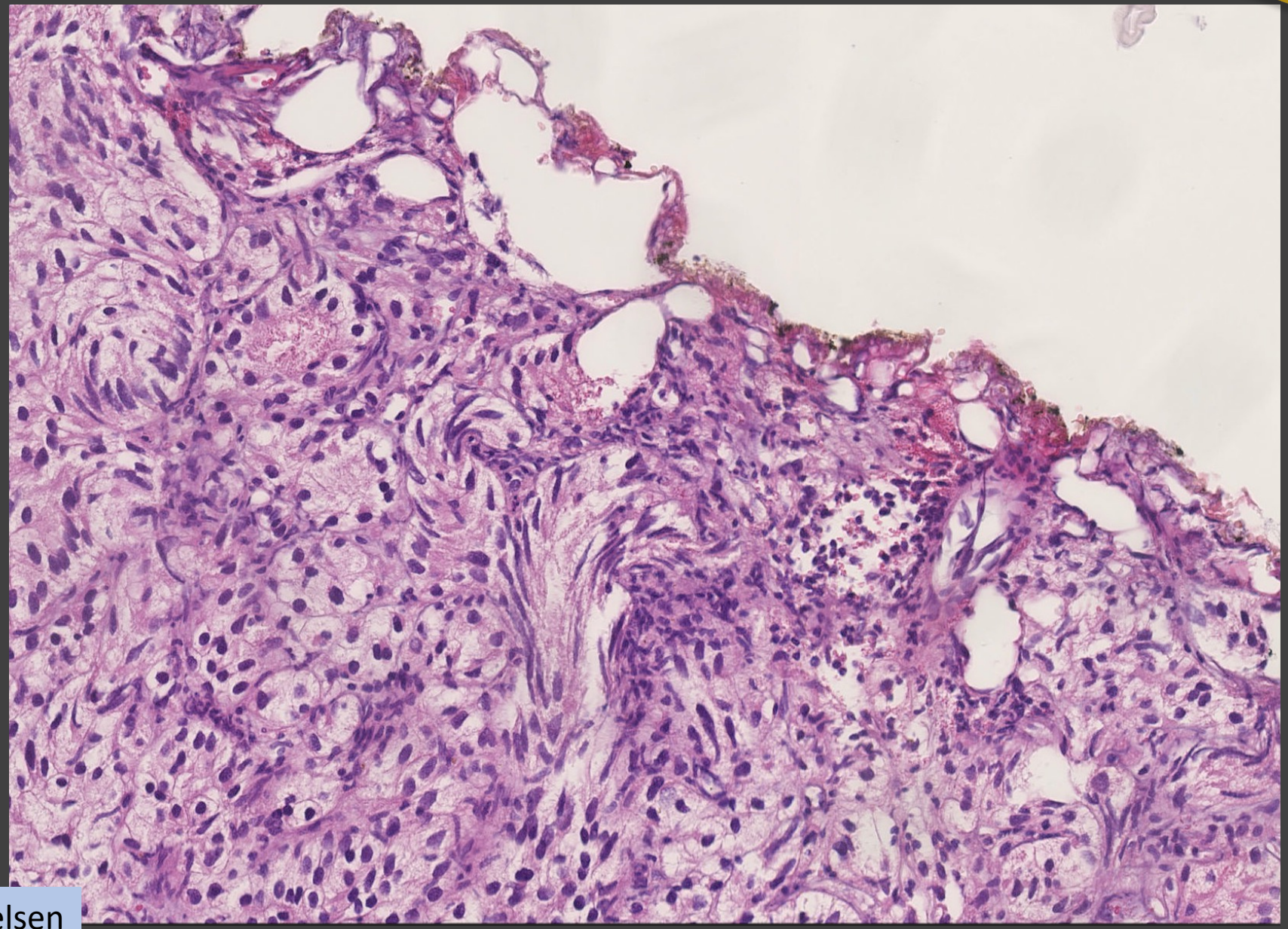


Prefixation

- Surgical procedure
- Fixation delay (cold ischemia)
- Specimen size
- Specimen manipulation (pathology ink)

Electrosurgery – Heat impact

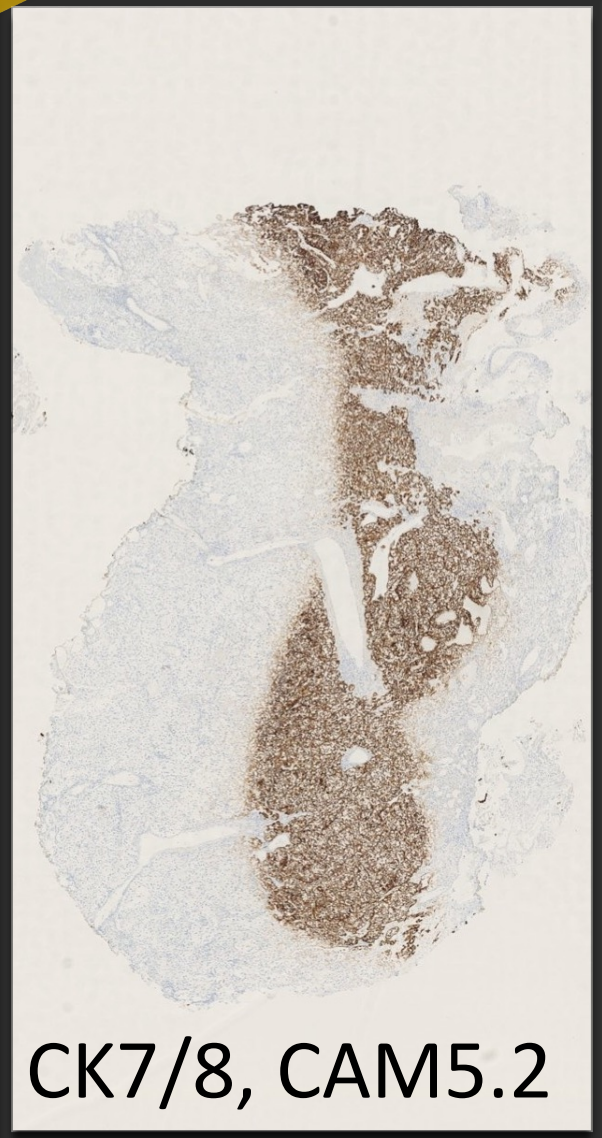
RCC



Photos by Ole Nielsen

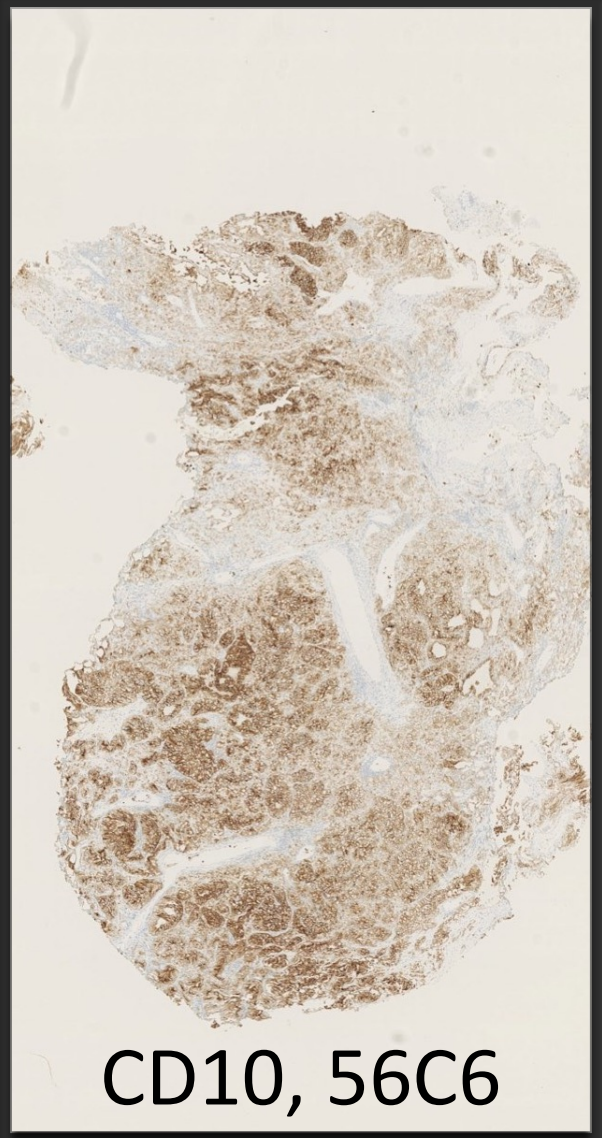
Surgical procedures - Impact on IHC

RCC



CK7/8, CAM5.2

CK8, EP17



CD10, 56C6

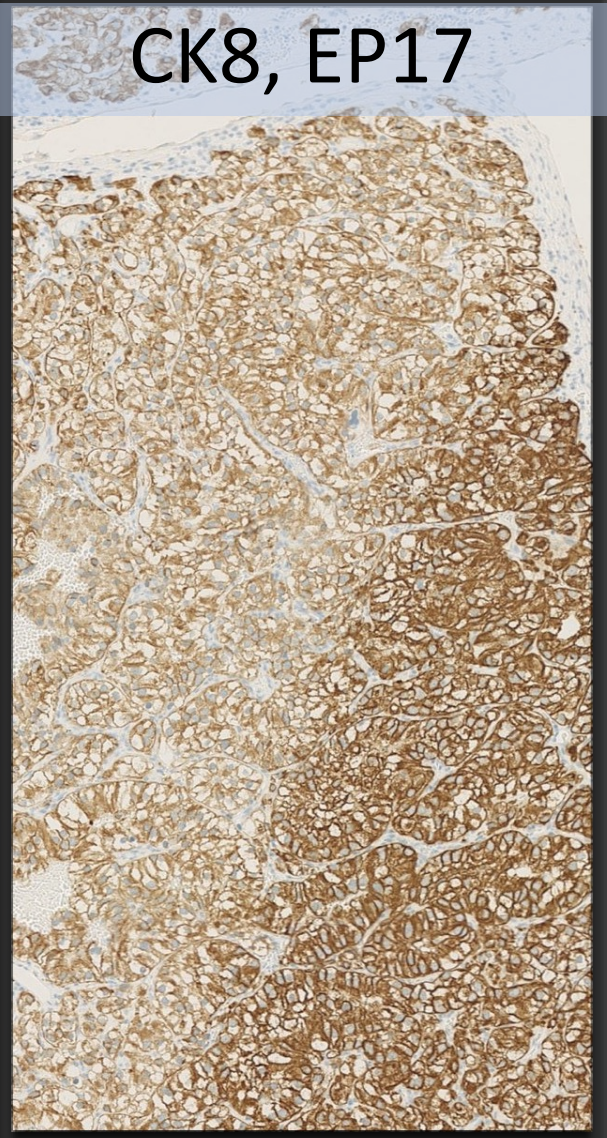
Photos by Ole Nielsen

Surgical procedures - Impact on IHC

RCC

CK7/8, CAM5.2

CK8, EP17

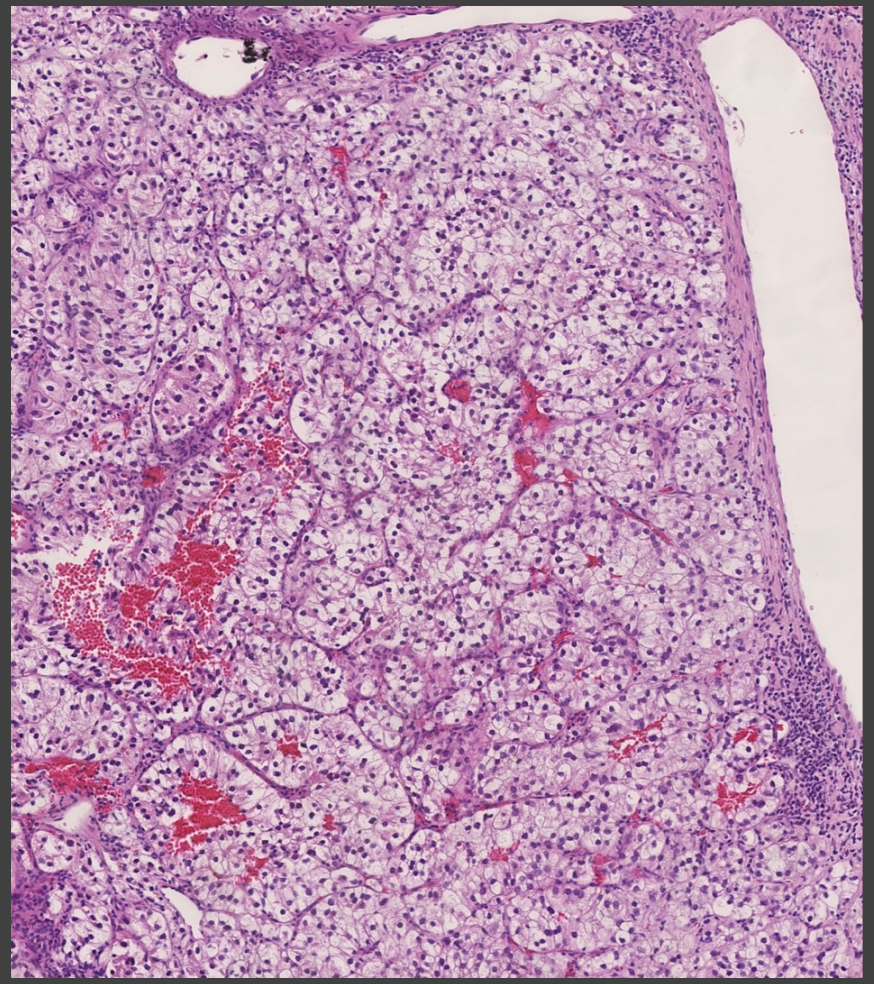


CD10, 56C6

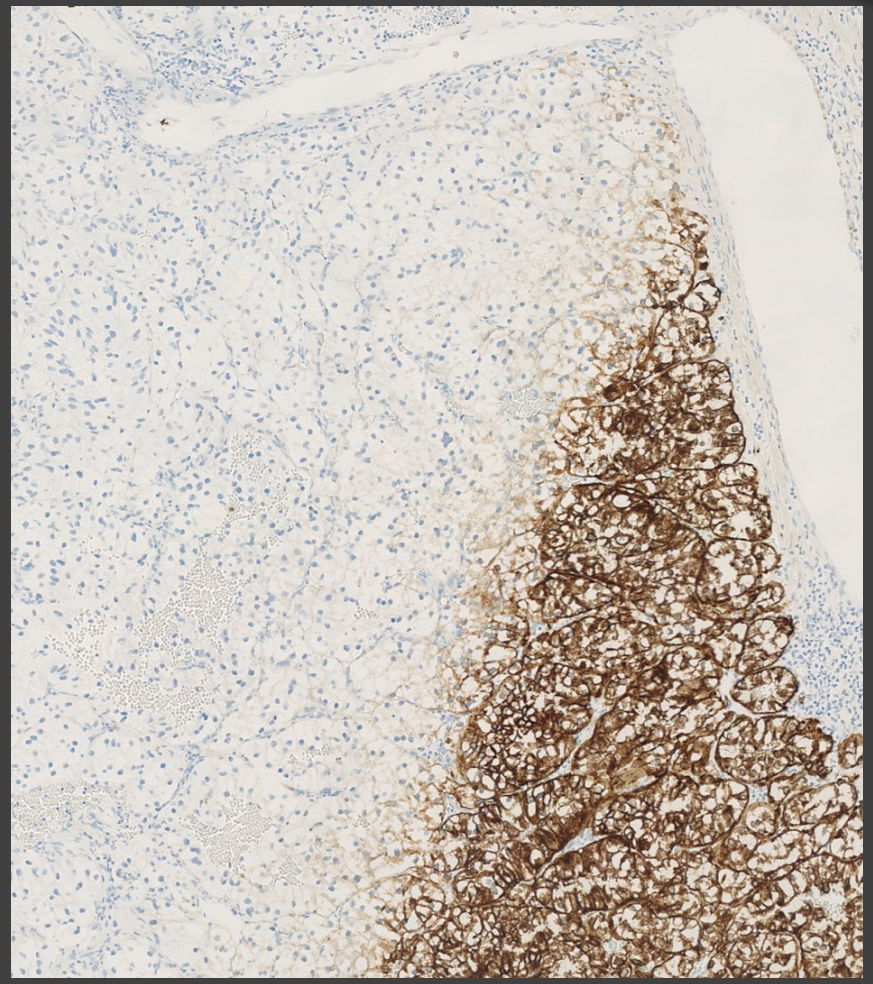
Photos by Ole Nielsen

CK, CAM5.2 simple marker of electrosurgery

RCC



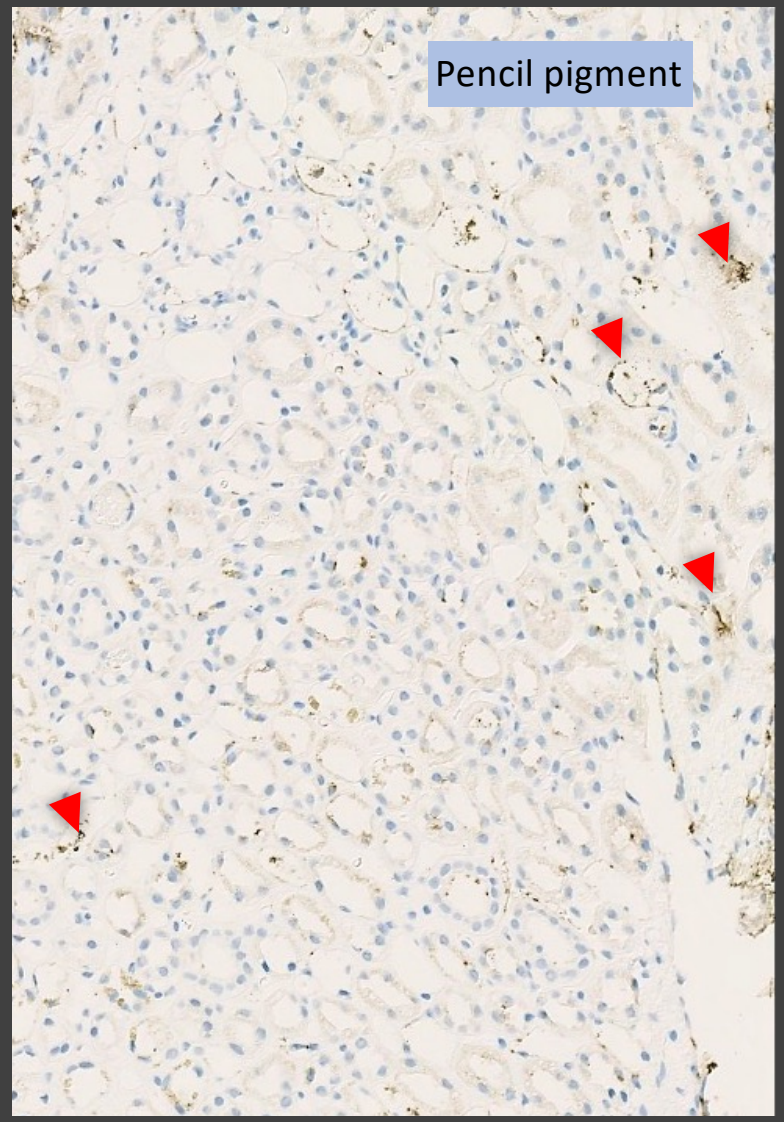
HE



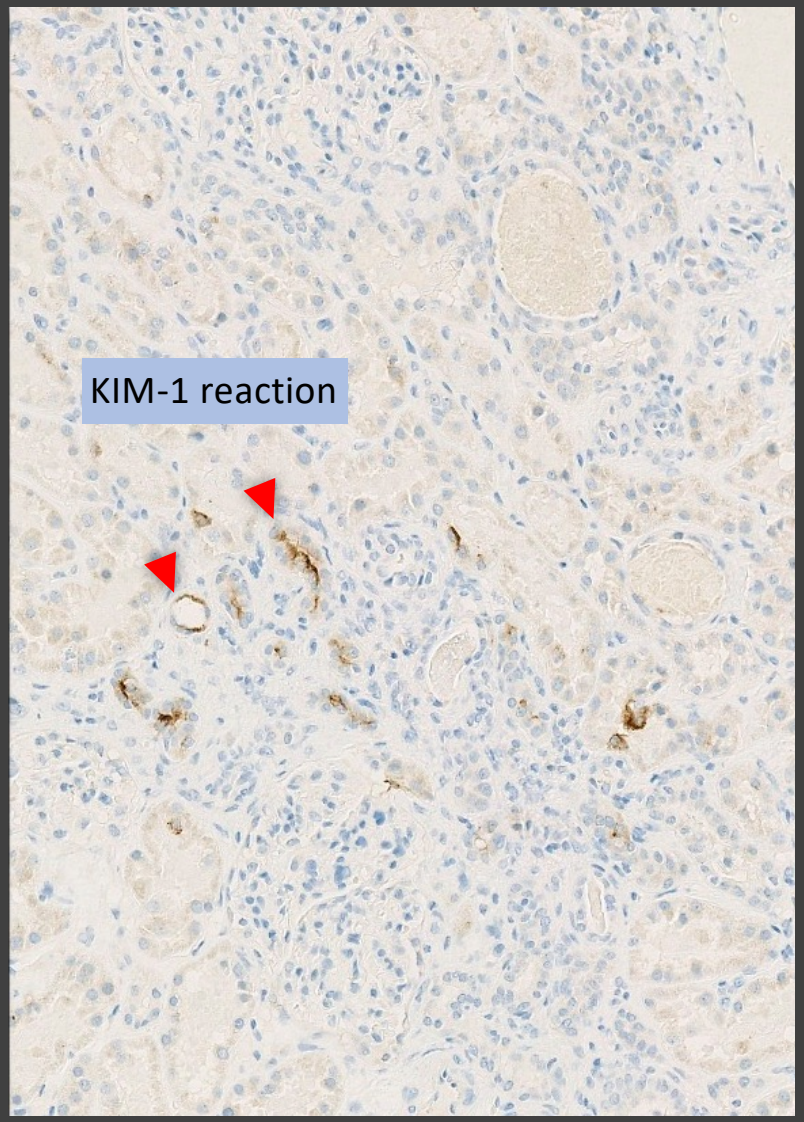
CK, CAM 5.2

Photos by Ole Nielsen

Pencil marking of small biopsies



KIM-1 (Kidney with marking)



KIM-1 (Kidney without marking)

Photos by Ole Nielsen

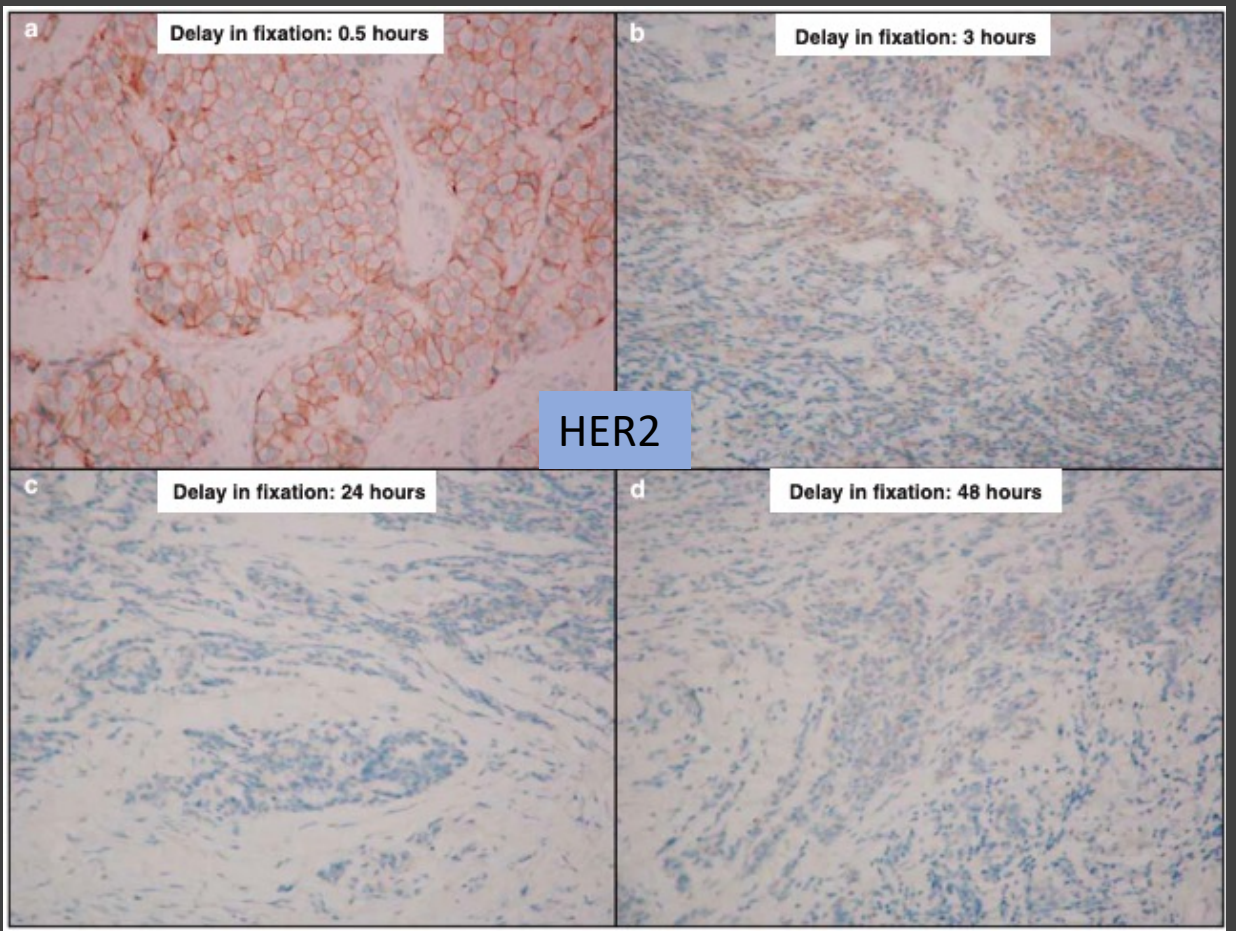
Cold ischemia – time from removal to fixation

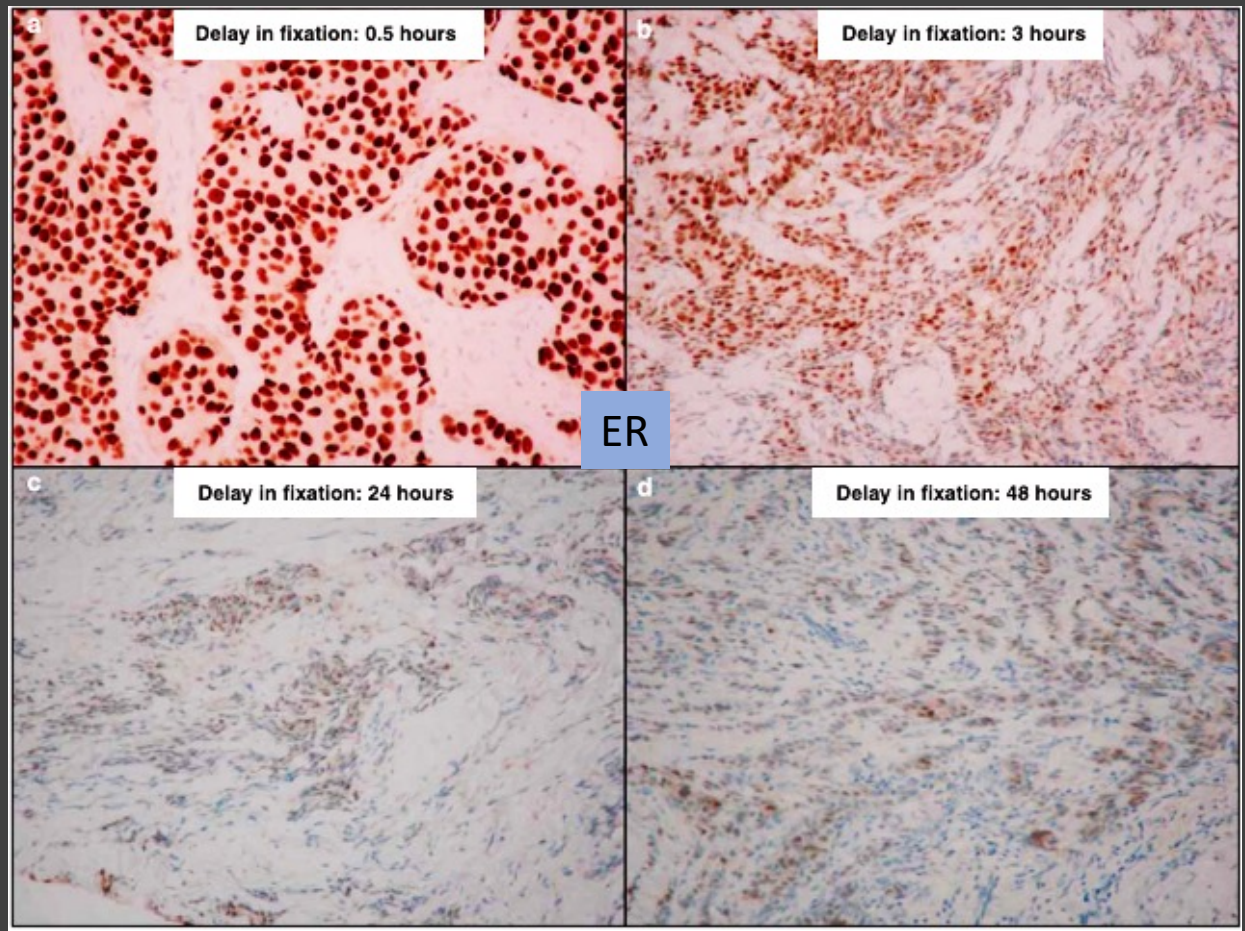
The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma

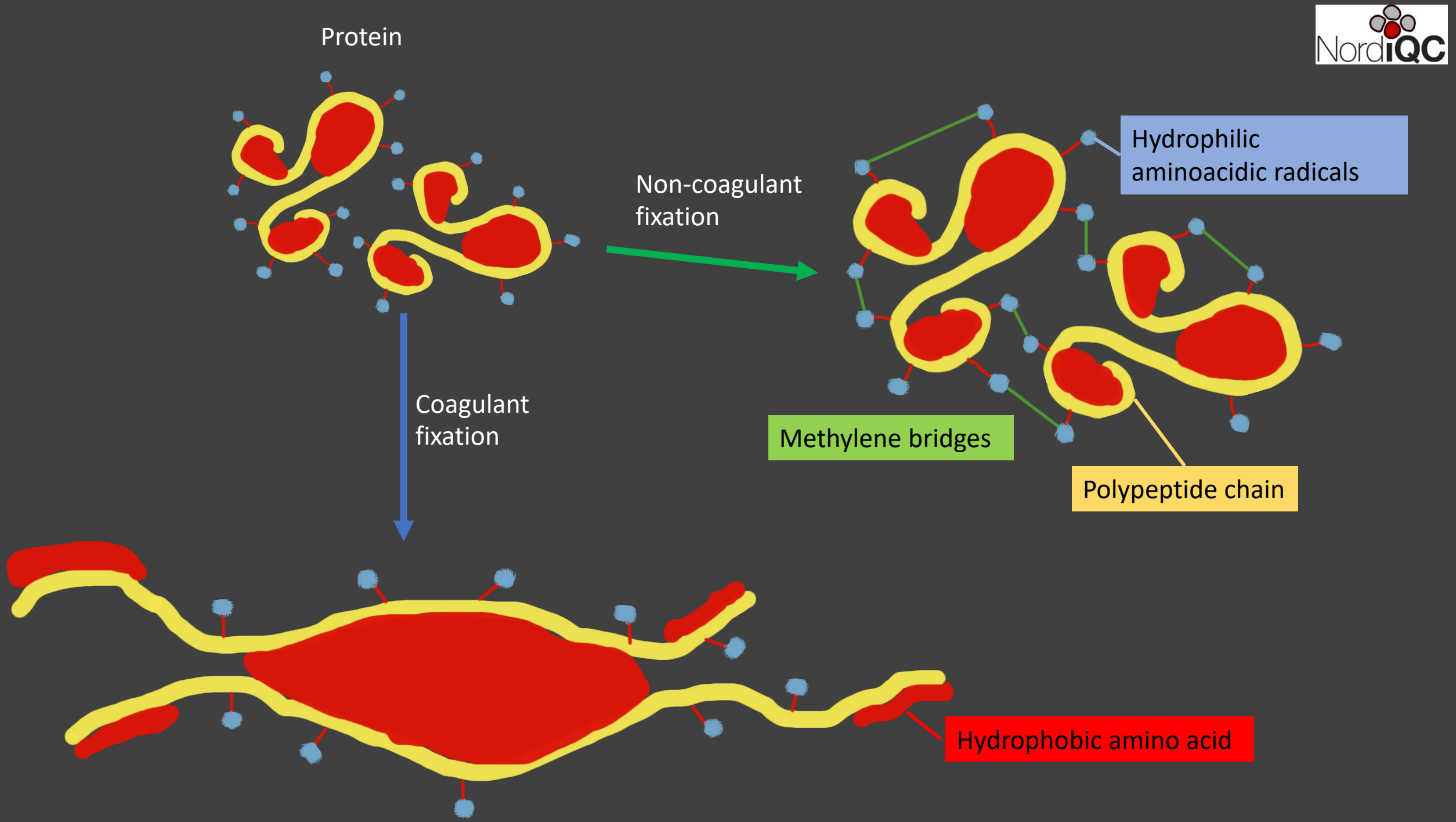
Isil Z Yildiz-Aktas, David J Dabbs and Rohit Bhargava

Department of Pathology, Magee-Womens Hospital, University of Pittsburgh Medical Center, Pittsburgh, PA, USA

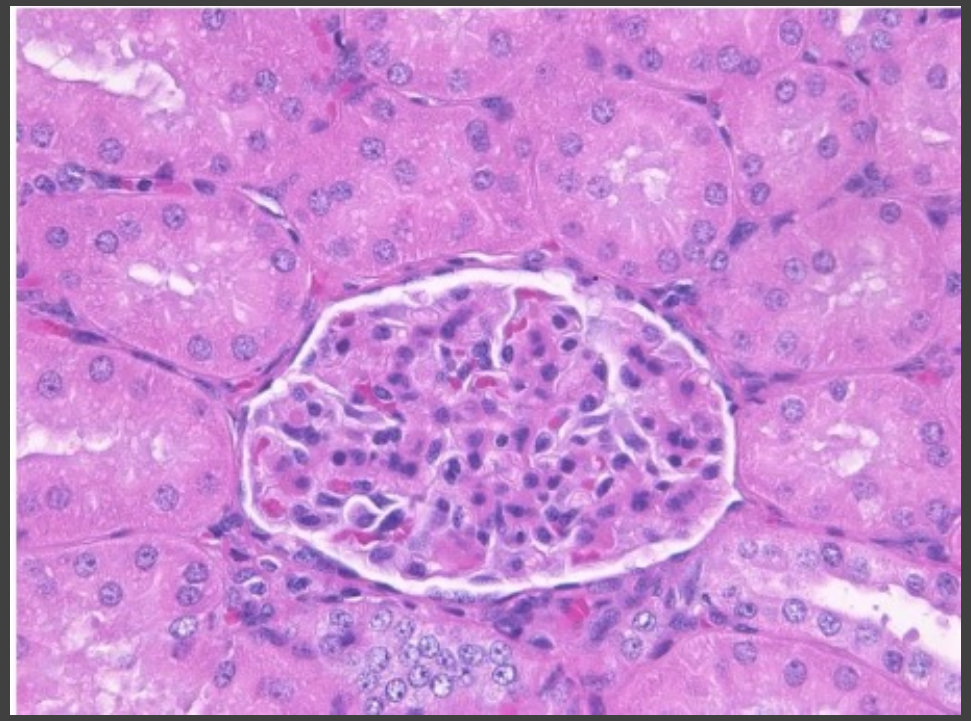
ASCO/CAP: time before fixation < 1h room temperature



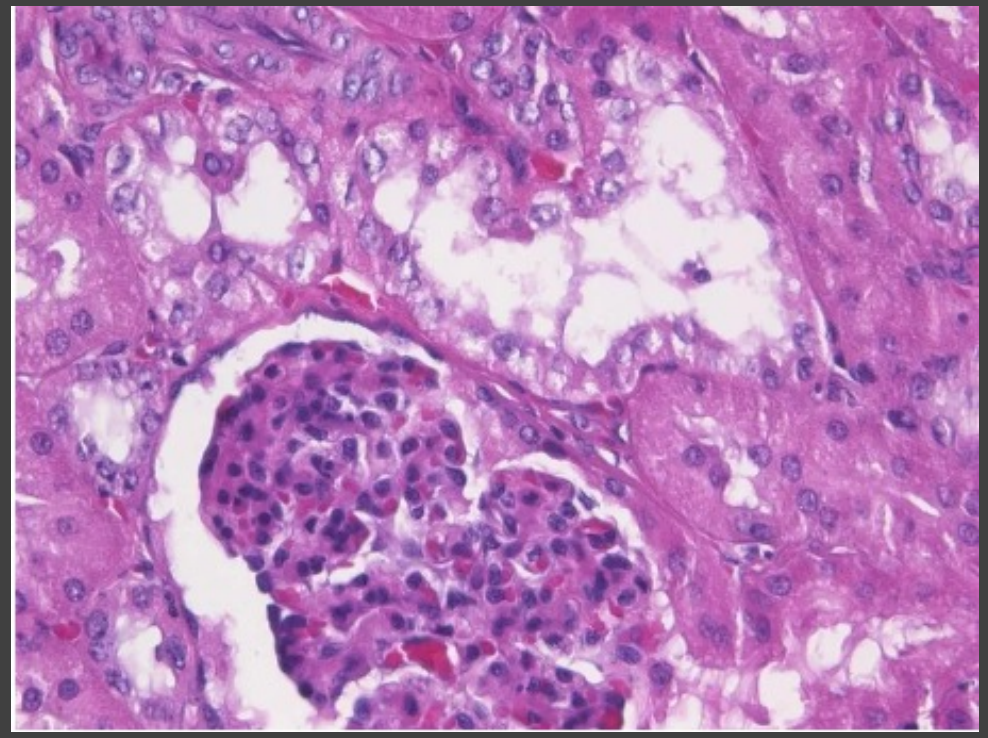



Protein
Non-coagulant fixation
Coagulant fixation
Hydrophilic aminoacidic radicals
Methylene bridges
Polypeptide chain
Hydrophobic amino acid

Noncoagulating vs coagulating fixative



Kidney fixated in neutral buffered formaldehyde. The structures are well preserved.



Kidney fixated in neutral buffered formaldehyde but too short time. The tissue display excessive shrinkage and poorly defined cell structures.

Fixation

Detection of Changes in Immunohistochemical Stains Caused by Postmortem Delay and Fixation Time

Lundström, Yasmin Med Stud*; Lundström, Patrik Med Stud*; Popova, Svetlana N. MD, PhD*,†; Lindh

1098 MODERN PATHOLOGY (2012) 25, 1098–1105

The effect of cold ischemic time on the immunohistochemical evaluation of estrogen receptor, progesterone receptor, and HER2 expression in invasive breast carcinoma

Isil Z Yildiz-Aktas, David J Dabbs and Rohit Bhargava

Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen

Maartje van Seijen[1,2] · Luka Brcic[3] · Atilio Navarro Gonzales[4] · Irene Sansano[5] · Matyas Bendek[6,7] · Iva Brci
Birgit Lissenberg-Witte[8] · H. Ibrahim Korkmaz[1] · Thomas Geiger[9] · Rosita Kammler[9] · Rolf Stahel[9,10] ·
Erik Thunnissen[1] · On behalf of ETOP[9]

MODERN PATHOLOGY (2009) 22, 1457–1467

Delay to formalin fixation effect on breast biomarkers

Thaer Khoury[1], Sheila Sait[2], Helena Hwang[1], Rameela Chandrasekhar[3], Gregory Wilding[3], Dongfeng Tan[4] and Swati Kulkarni[5]

The Influence of Tissue Ischemia on Biomarker Expression in Colorectal Cancer

Havelund, Birgitte M. MD*,†; Olsen, Dorte A. MSc‡; Andersen, Rikke F. PhD‡; Spindler, Karen-Lise G. MD, PhD*,†; Brandslund, Ivan MD, DMSc†,‡; Jakobsen, Anders MD, DMSc*,†; Soerensen, Flemming B. MD, DMSc†,§

Author Information

Applied Immunohistochemistry & Molecular Morphology: July 2013 - Volume 21 - Issue 4 - p 298-307
doi: 10.1097/PAI.0b013e31826f4475

[Delay in formalin fixation and HER2 testing in gastric cancer]

[Article in Chinese]
Lixia Zeng [1], Junqi Huang, Yun Ma, Yixiao Liu, Yuying Wei, Qian Zheng, Hongtao Ye [2]

Effect of Delayed Formalin Fixation on Estrogen and Progesterone Receptors in Breast Cancer

A Study of Three Different Clones

Jingxin Qiu, MD, PhD,[1] Swati Kulkarni, MD,[2] Rameela Chandrasekhar,[3] Mark Rees, PhD,[4,6] Kathryn Hyde,[5] Gregory Wilding, PhD,[3] Dongfeng Tan, MD,[6] and Thaer Khoury, MD[1]

Key Words: Breast cancer; Biomarkers; Delay to formalin fixation

Delay to formalin fixation 'cold ischemia time': effect on ERBB2 detection by *in-situ* hybridization and immunohistochemistry

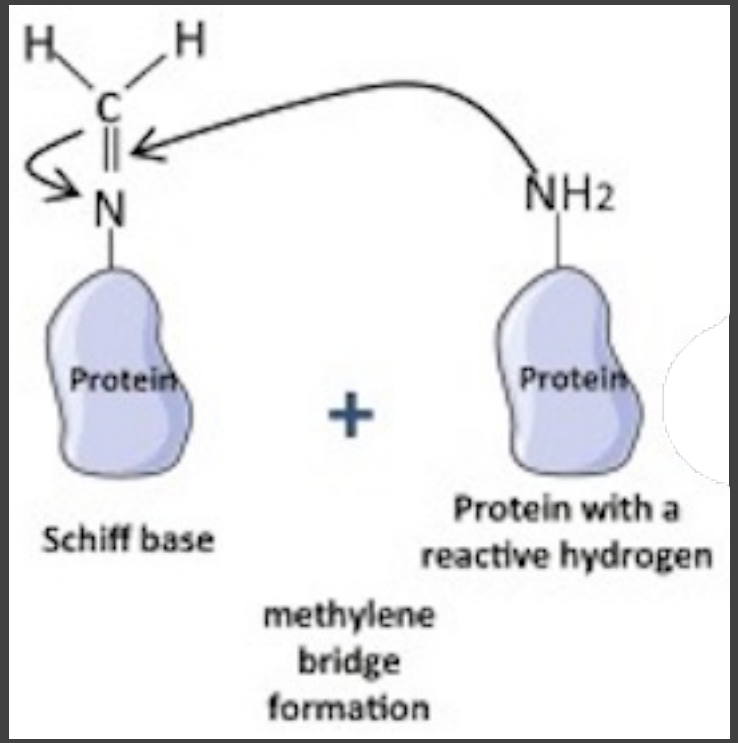
Bryce P Portier, Zhen Wang, Erinn Downs-Kelly, Jordi J Rowe, Deepa Patil, Chis Lanigan, G Thomas Budd, David G Hicks, David L Rimm & Raymond R Tubbs

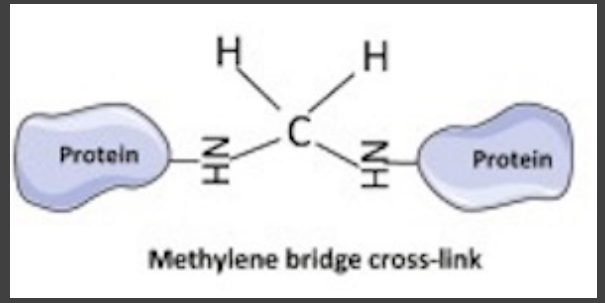
Modern Pathology **26**, 1–9 (2013) | Cite this article

Fixation

- 10% Neutral buffered formaldehyde
- For more than 70 years
- You should insure enough liquid for your sample size (10-20:1)
- Formaldehyde is one of the fastest solutions regrading tissue penetration but one of the slowest regarding fixation

Phase I	**Penetration**	**Fast**
Phase II	**Binding**	**Moderate**
Phase III	**Cross-linking**	**Slow**





Fixation

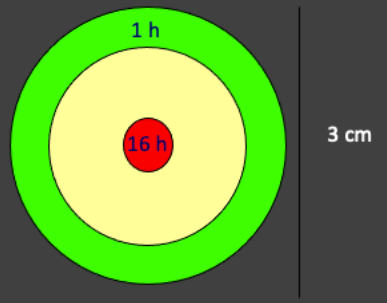
Penetration time at K = 3.6 (Baker's coeficient)

d = penetration in mm

K= 3,6 (Bakers coeficient)

t = time in hours

$$d = K \times \sqrt{(t)}$$



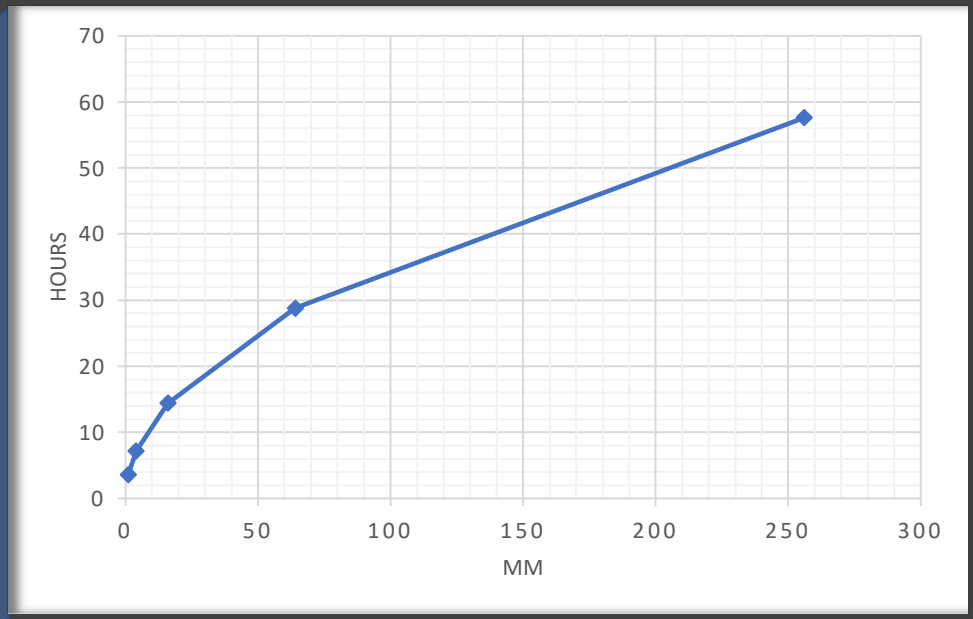
1 hour = 3.6 mm

4 hours = 7.2 mm (1.8 mm/hr)

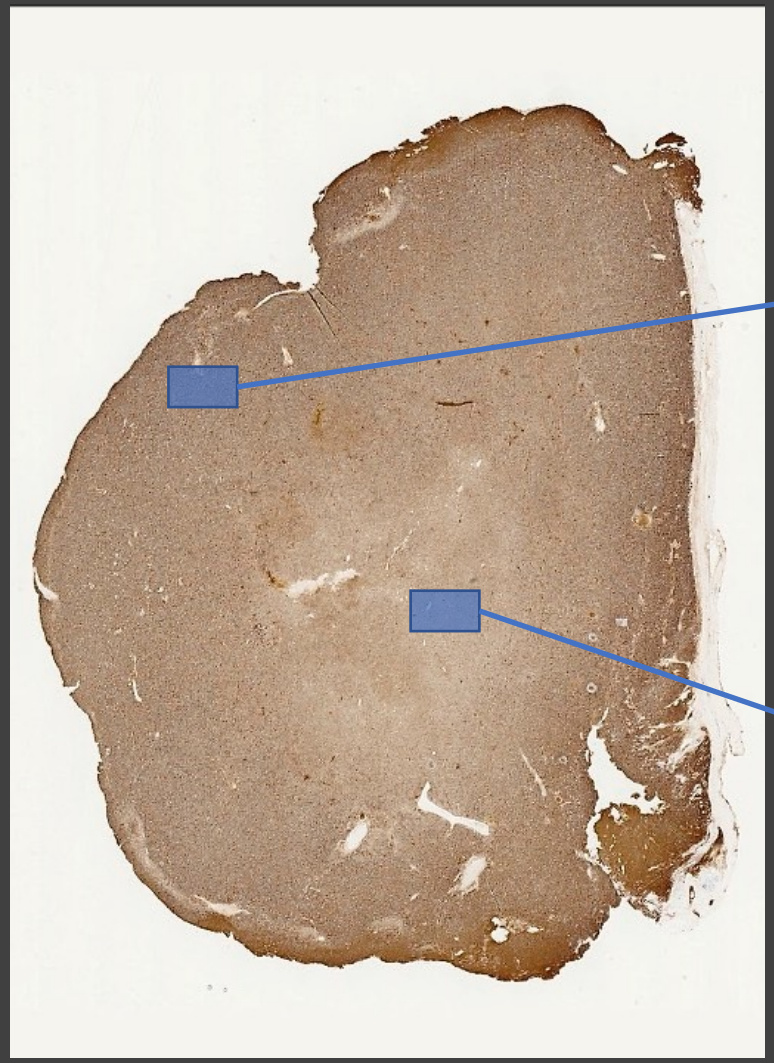
<u>16 hours = 14.4 mm (0.9 mm/hr)</u>

64 hours = 28.8 mm (0.45 mm/hr)

256 hours = 57.6 mm (0.225 mm/hr)

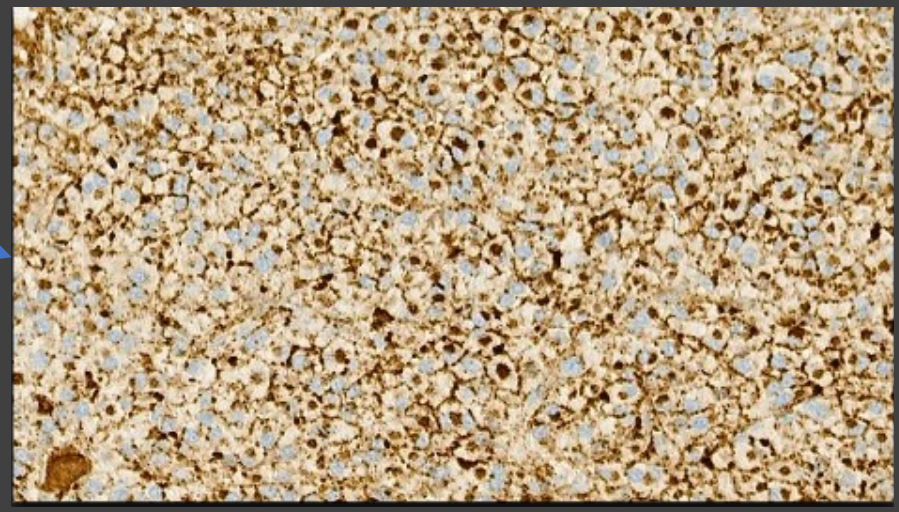


Plasmacytoma



CD138

Edge

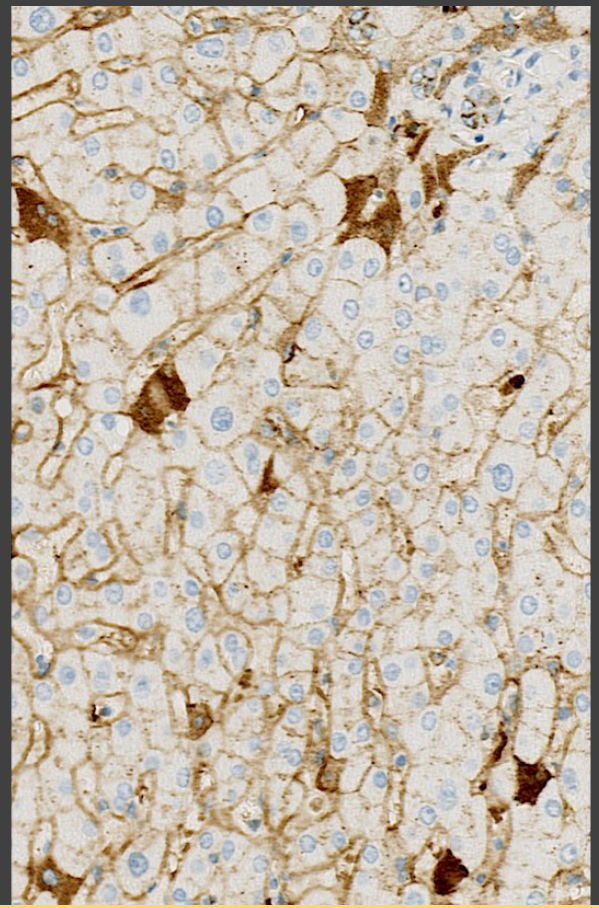


Center

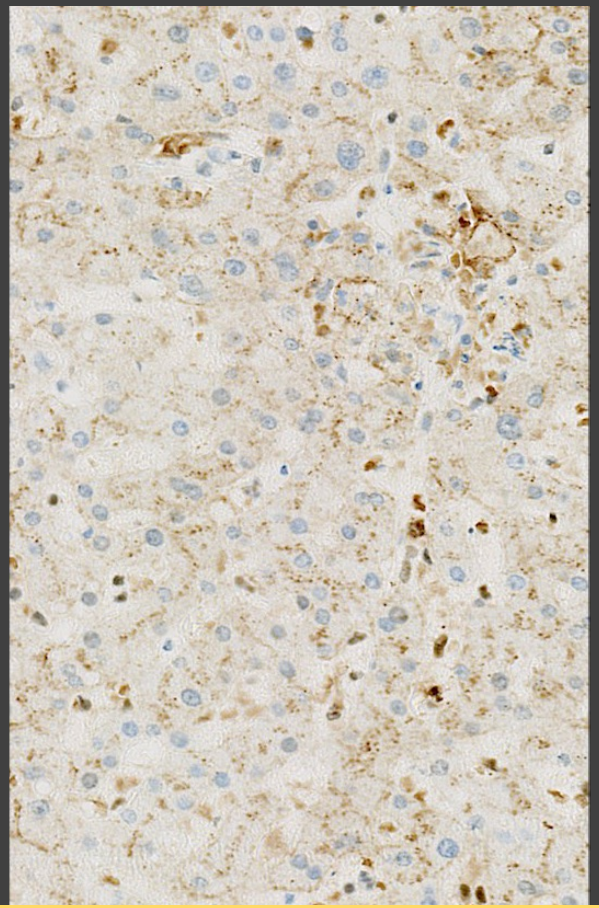
Photos by Ole Nielsen

CD138: Simple marker of fixation delay

Liver: No Fix delay



Liver 16 hrs delay

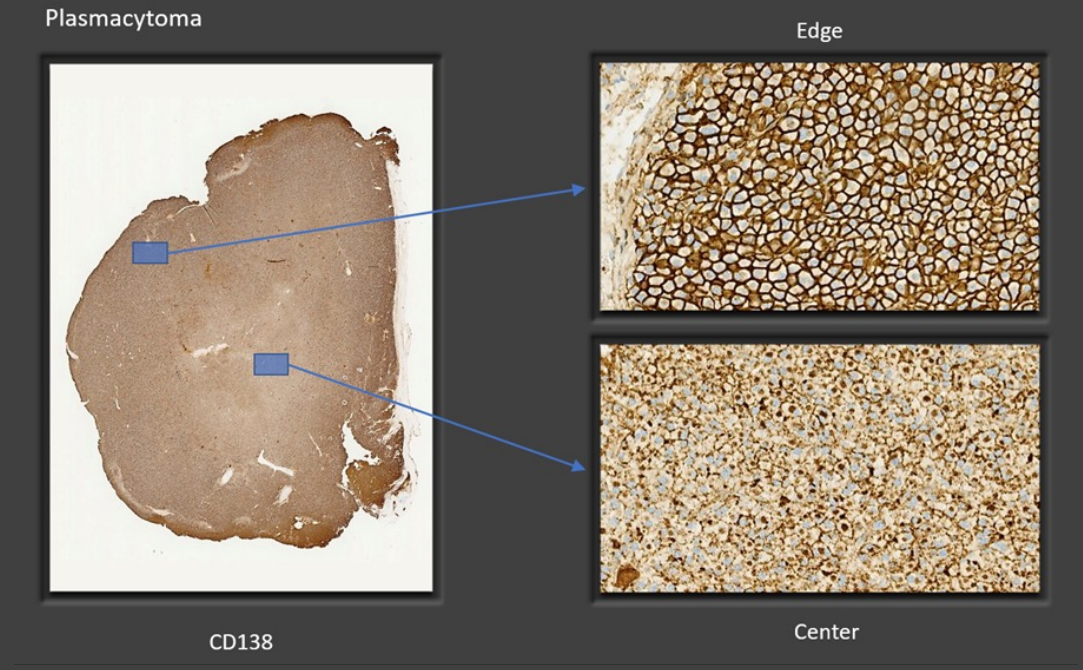


Liver 48 hrs delay

Photos by Ole Nielsen

Targets sensitive to short formalin fixation / delayed time to formalin;

Target
ALK
Bcl6
BRAF
CD138
Cyclin D1
MLH1
p53
PD-L1
PMS2
PAX8
PRAME
TTF1



Clinical sample

Control design

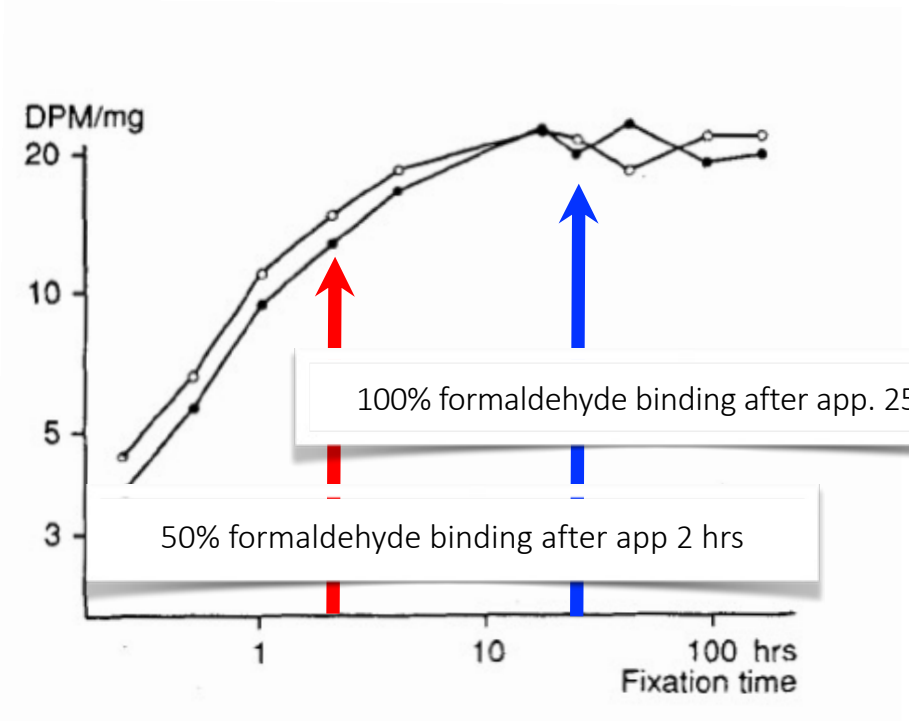
By courtesy Ole Nielsen

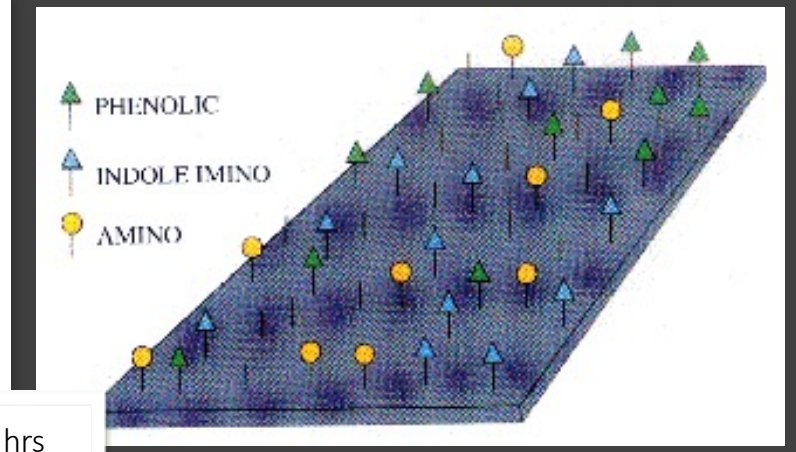
Kinetic Studies of Formaldehyde Binding in Tissue

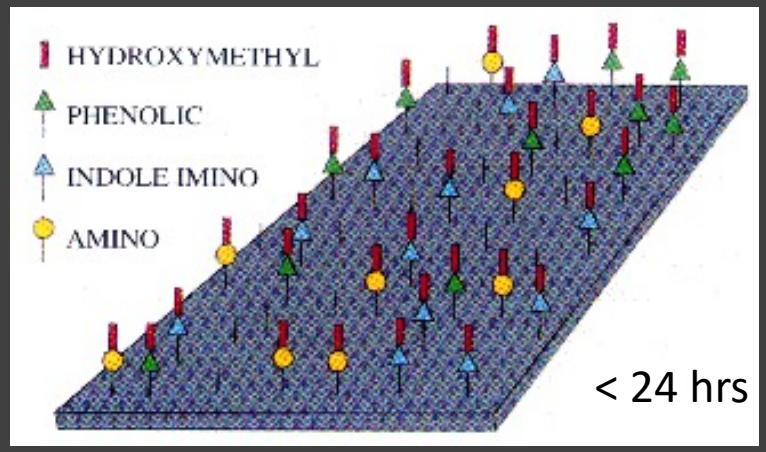
Kerstin G. Helander
Laboratory of Membrane Biology, Center for Ulcer Research and Education, University of California, Los Angeles, California 90073

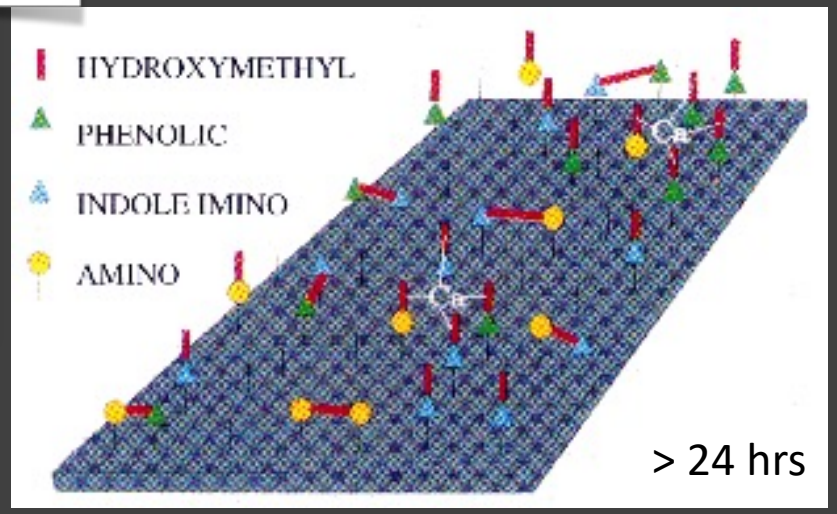
"There is a misconception that smaller biopsy samples will fix more quickly than larger resection specimens and therefore require less time in formalin."

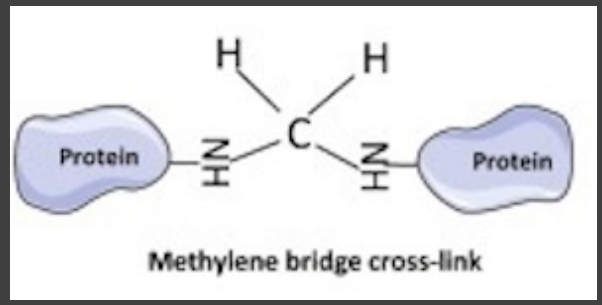
David G. Hicks



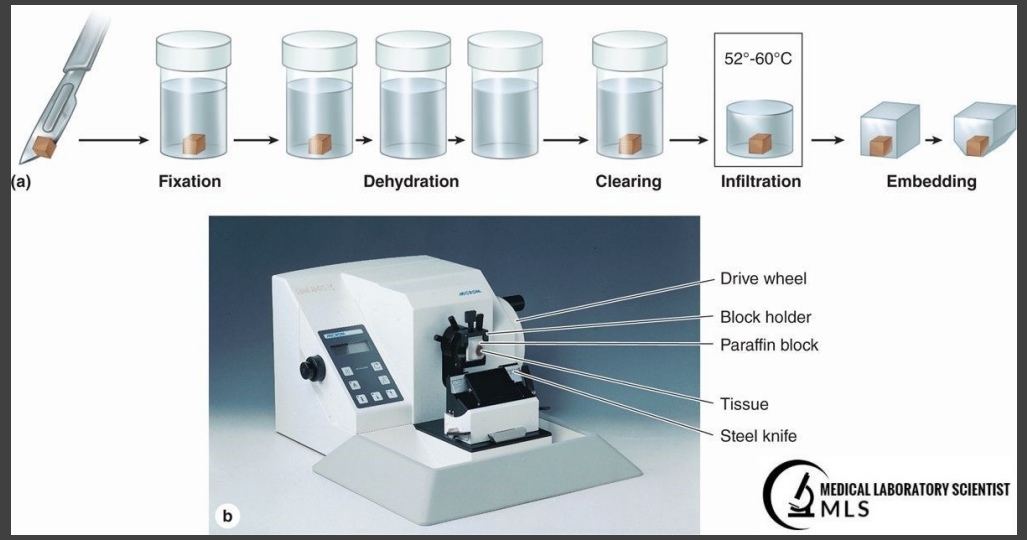


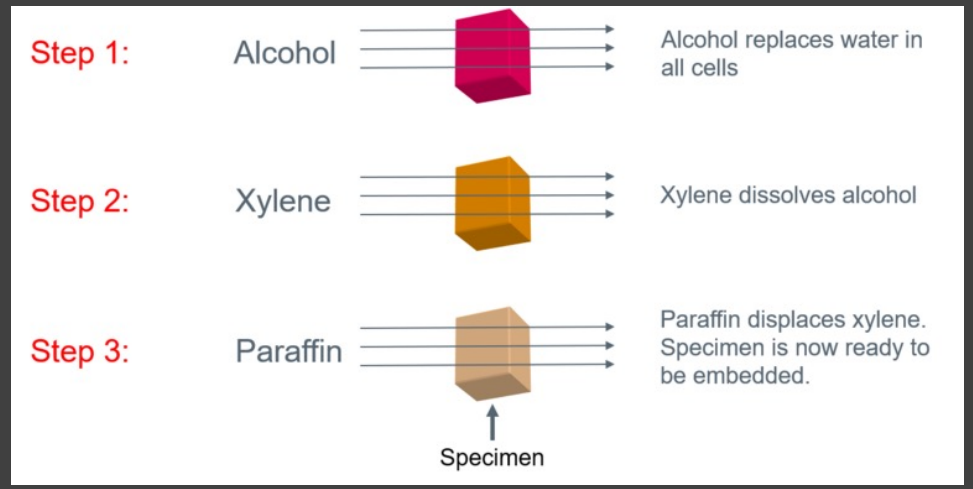






Tissue processing

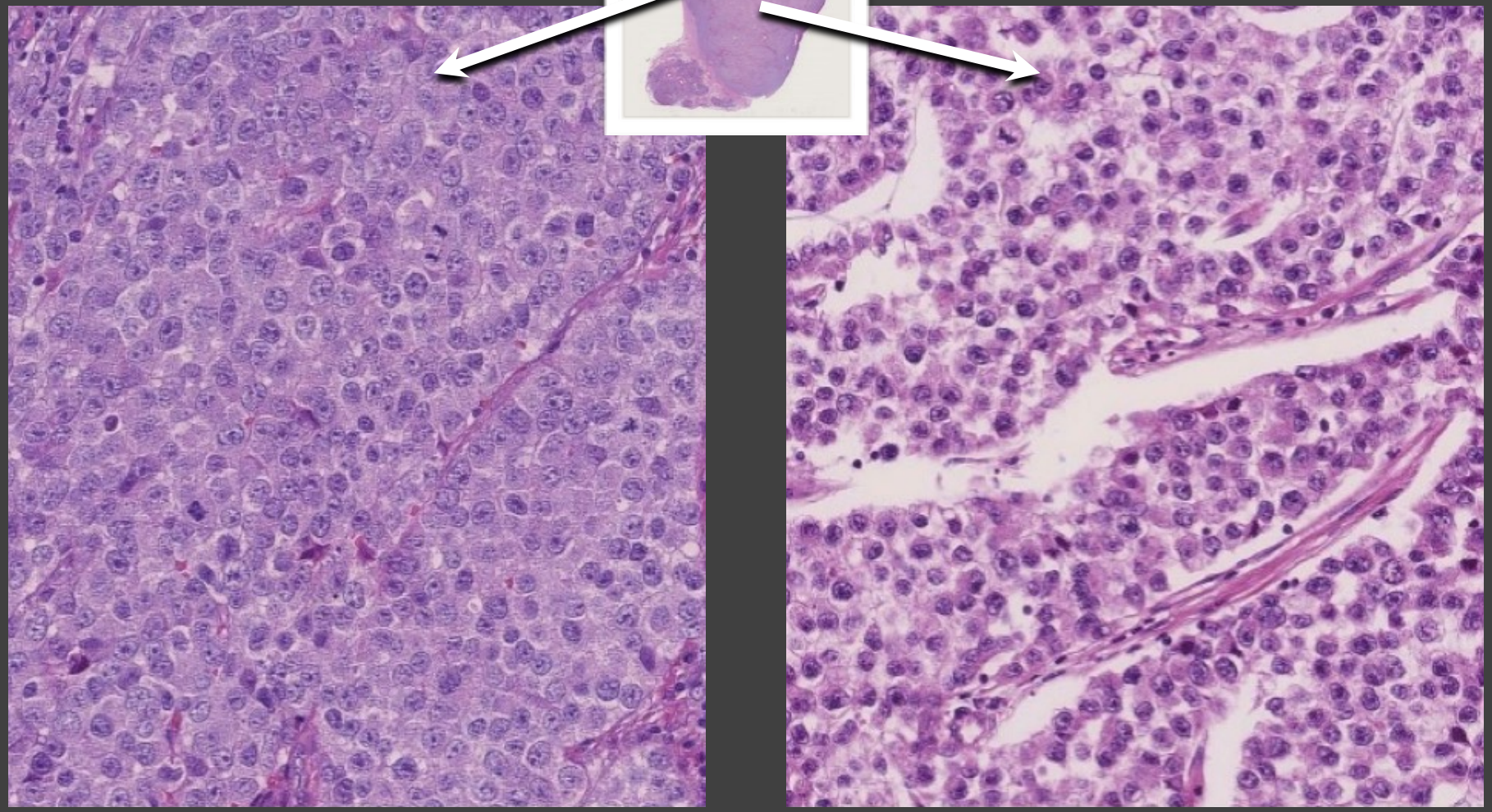




Sta	Solution	Time	Temp (°C)	Mix
1	Formalin	0:10	37	Slow
2	Alcohol (70%)	1:00	37	Slow
3	Alcohol (96%)	0:45	37	Slow
4	Alcohol (96%)	1:00	37	Slow
5	Alcohol (99%)	1:00	37	Slow
6	Alcohol (99%)	1:15	37	Slow
7	½ Alcohol (99%) ½ Histoclear	1:00	37	Slow
8	1/3 Alcohol (99%) 2/3 Histoclear	1:00	37	Slow
9	Histoclear	1:30	37	Slow
10	Histoclear	2:00	40	Slow
11	Paraffin	0:45	65	Slow
12	Paraffin	1:00	65	Slow
13	Paraffin	1:00	65	Slow
14	Paraffin	1:15	65	Slow

Too short time in formalin induces a hybrid fixation with alcohol affecting some antigens / targets

Seminoma

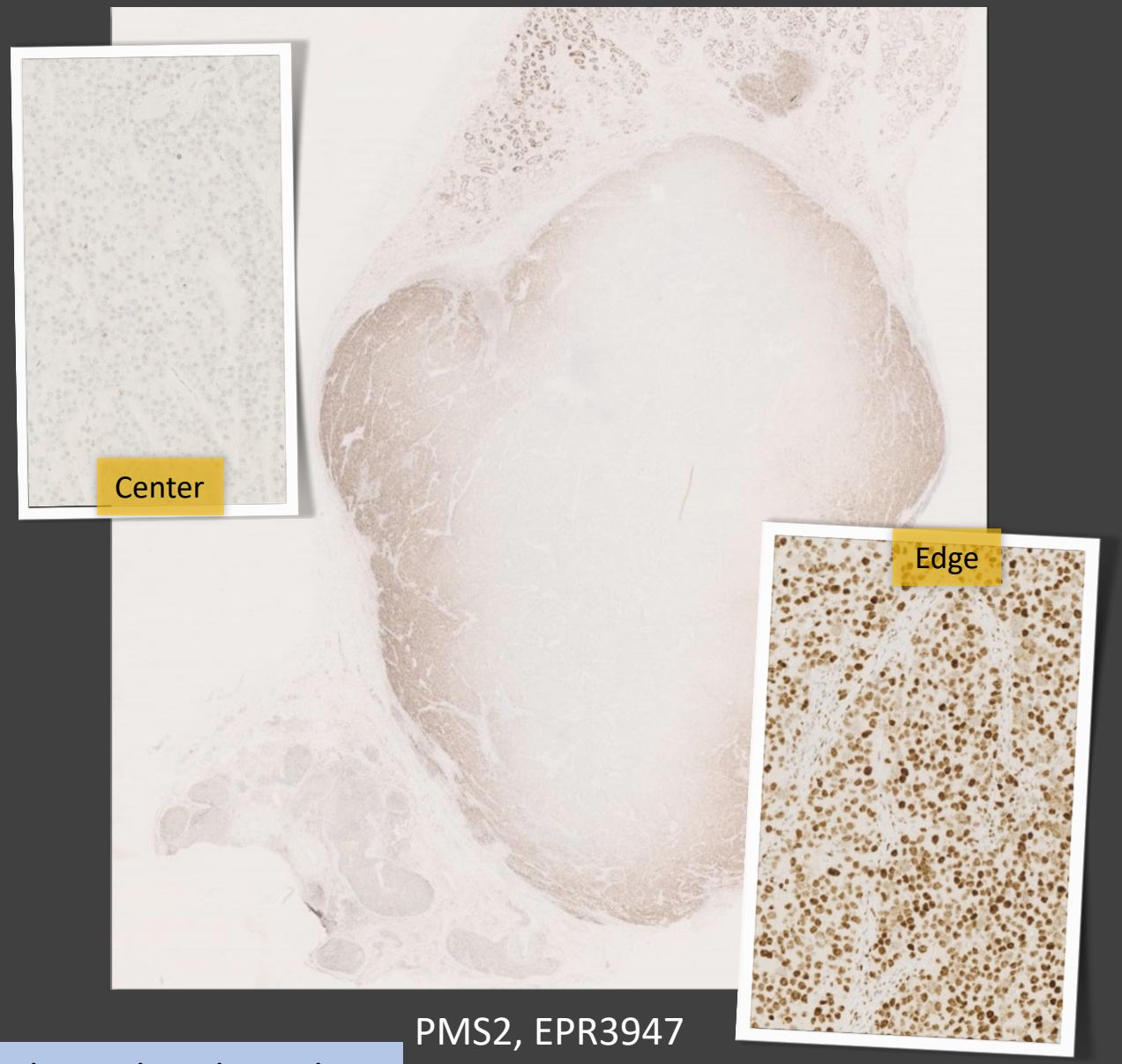


Edge

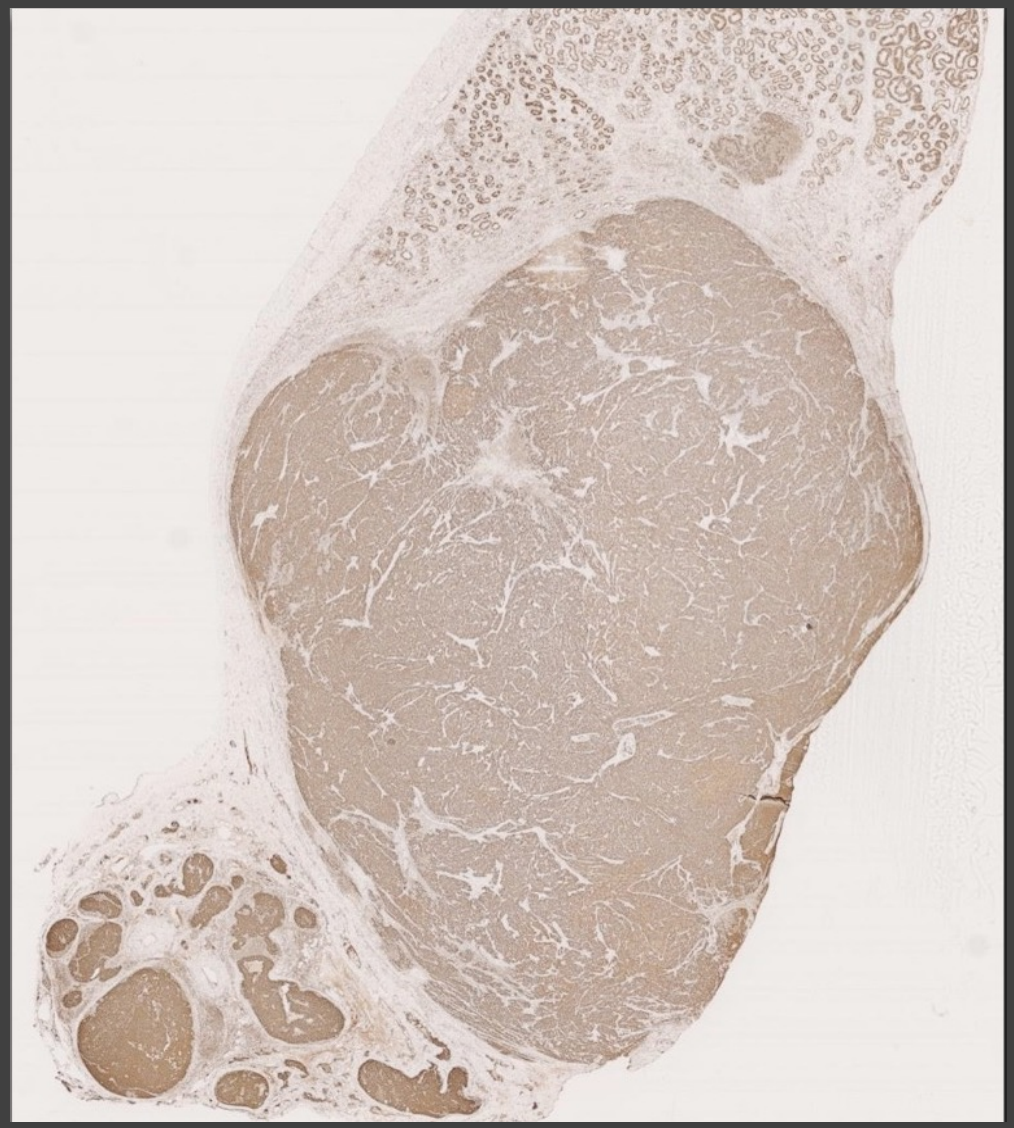
Center

Photos by Ole Nielsen

Seminoma



PMS2, EPR3947



MSH6, EP49

Photos by Ole Nielsen

Cold ischemia – time from removal to fixation

HE

ER

Delay to Formalin Fixation (Cold Ischemia Time) Effect on Breast Cancer Molecules

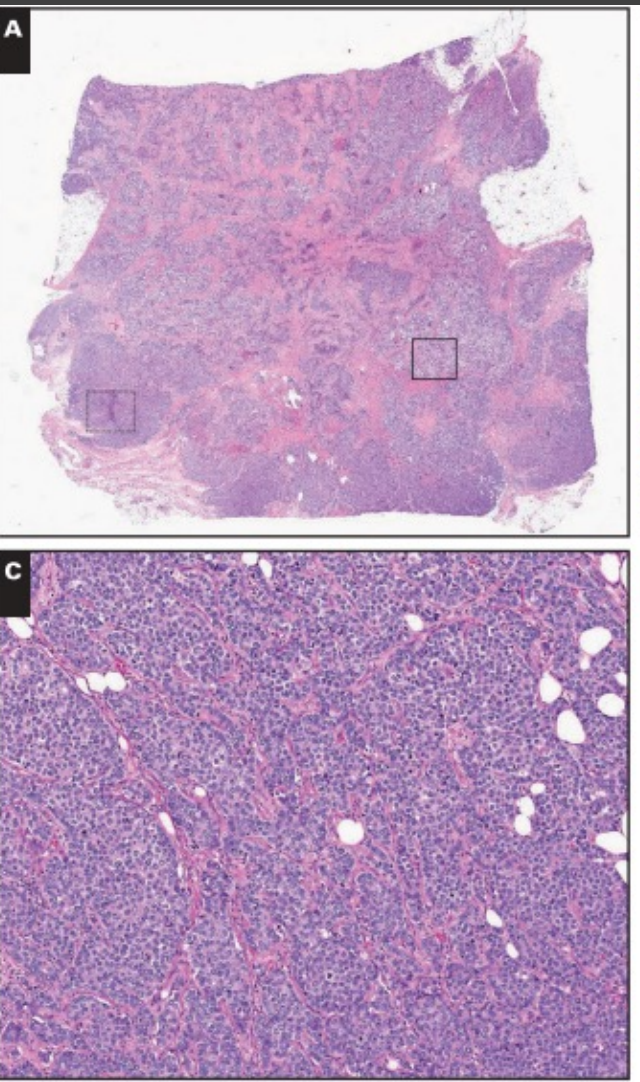
Thaer Khoury, MD

From the Department of Pathology, Roswell Park Cancer Institute, Buffalo, NY.

Key Words: Breast cancer; Delay to formalin fixation; Breast biomarkers; Review

Am J Clin Pathol April 2018;149:275-292

DOI: 10.1093/AJCP/AQX164



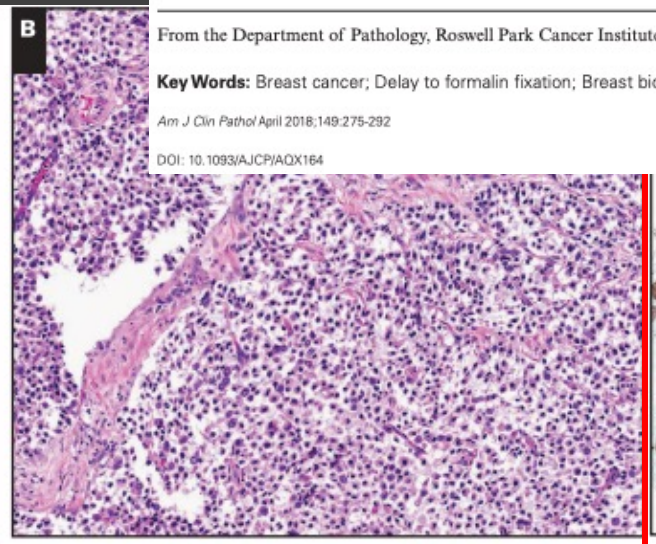
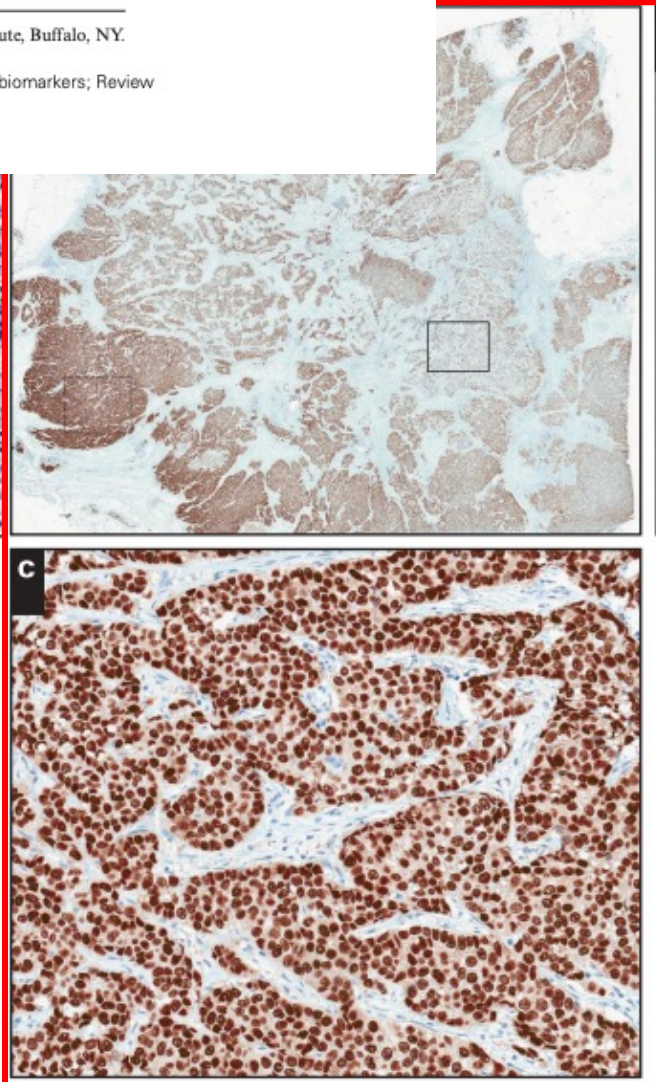


Image 1 Tissue immersed in formalin without sectioning (H&E): (**A**) scanning magnification, (**B**) center of the section (solid outline) with poor fixation (×10), and (**C**) periphery of the section (dotted outline) showing proper fixation (×10).



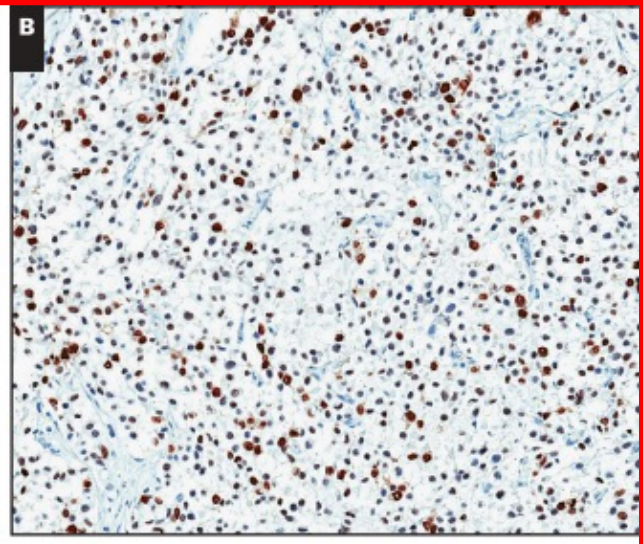


Image 2 Estrogen receptor (ER) staining of the section in Image 1: (**A**) scanning magnification (note the strong diffuse staining at the periphery compared with the weak sparse staining in the center), (**B**) center of the section (solid outline) with decreased ER staining (×10x), and (**C**) periphery of the section (dotted outline) with strong diffuse staining (×10).

Cytology cell blocks are suitable for immunohistochemical testing for PD-L1 in lung cancer

H. Wang[1,2,3*], J. Agulnik[3,4], G. Kasymjanova[3,4], A. Wang[4], P. Jiménez[5], V. Cohen[3,4], D. Small[3], C. Pepe[3], L. Sakr[3], P. O. Fiset[1,2], M. Auger[1,2], S. Camilleri-Broet[1,2], M. Alam El Din[1,2], G. Chong[1,2], L. van Kempen[1,2,3] & A. Spatz[1,2,3,4]

Annals of Oncology 29: 1417–1422, 2018
doi:10.1093/annonc/mdy126
Published online 12 April 2018

Cytology/EBUS isn't clinical validated and NOT included in the labeling for PD-L1 IHC 22C3 pharmDx

EBUS

- In their 86 paired samples of NSCLC with both histological blocks and cytology material, they reported an overall agreement of 94% with the 22C3 clone. It is not clear why the cytology cell blocks were more often associated with high PD-L1 expression than surgical resections in our study.

- In this study, they assessed the effect on PD-L1 IHC of 10% buffered formalin only, methanol/alcohol only versus prefixation with Cytolyt/alcohol followed by 10% formalin. They did not find difference of PD-L1 expression, nor in morphology, with or without Cytolyt/alcohol pre-fixation.

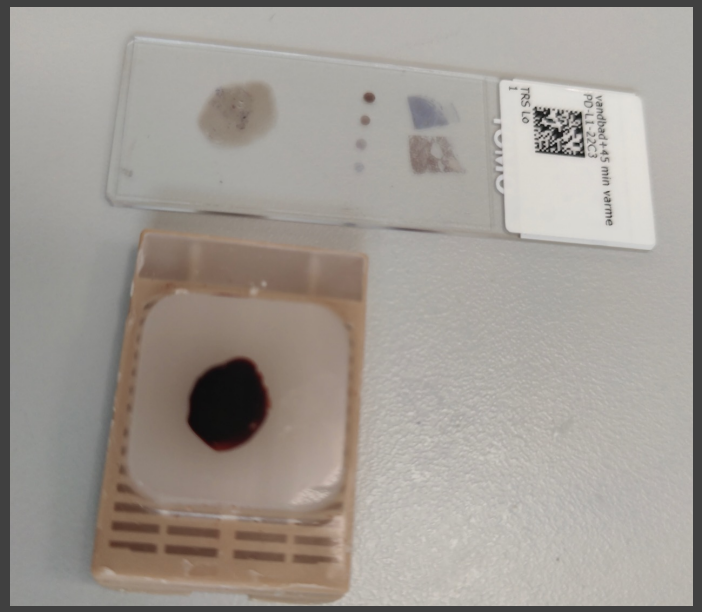
Cell block procedure – pros and cons

Received: August 20, 2020; **Revised:** September 28, 2020; **Accepted:** September 30, 2020

Formalin Fixation for Optimal Concordance of Programmed Death-Ligand 1 Immunostaining Between Cytologic and Histologic Specimens From Patients With Non–small Cell Lung Cancer

Bregje M. Koomen, MD [1]; Jose van der Starre-Gaal, MD, PhD[2]; Judith M. Vonk, PhD[3]; Jan H. von der Thüsen, MD, PhD[4]; Jacqueline J. C. van der Meij, MD[5]; Kim Monkhorst, MD, PhD[6]; Stefan M. Willems, MD, PhD[1,7]; Wim Timens, MD, PhD[7]; and Nils A. 't Hart, MD, PhD[2,7]

Reduced PD-L1 TPS expression in Cellient processed and alcohol fixed cell blocks compared to Agar / Plasma trombin and formalin processed cell blocks



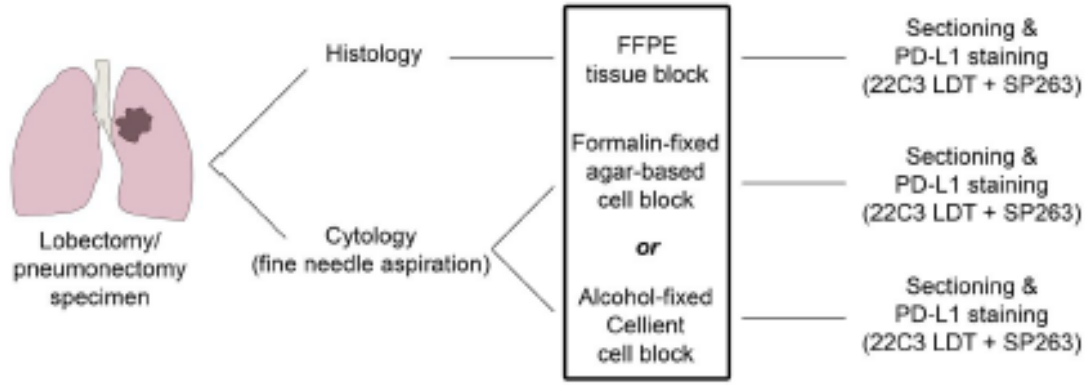
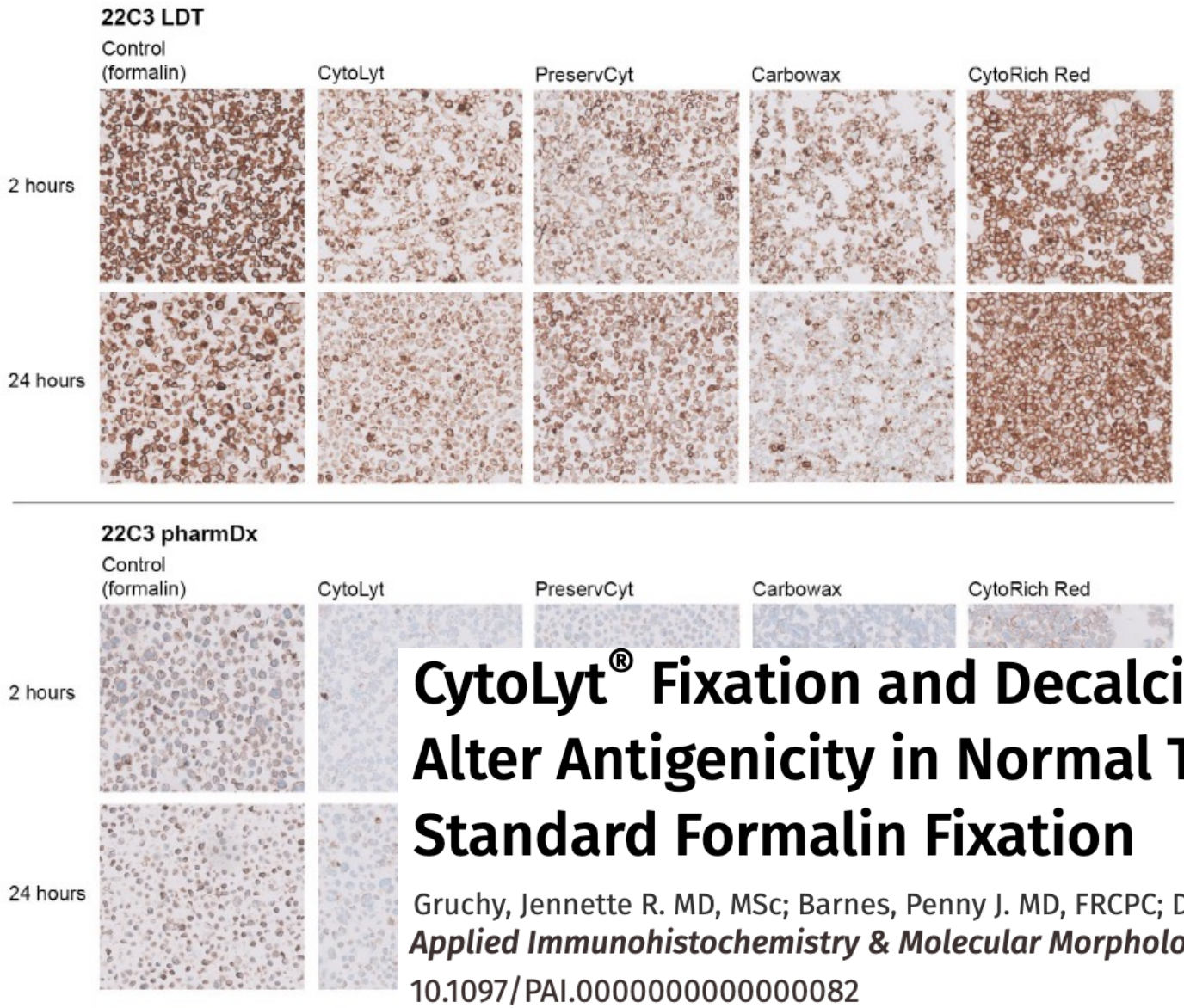


Figure 1. This is a schematic representation of the study design. FFPE indicates formalin-fixed, paraffin-embedded; LDT, laboratory-developed test (using the 22C3 antibody); PD-L1, programmed death-ligand 1; SP263, antibody used in the standardized assay.

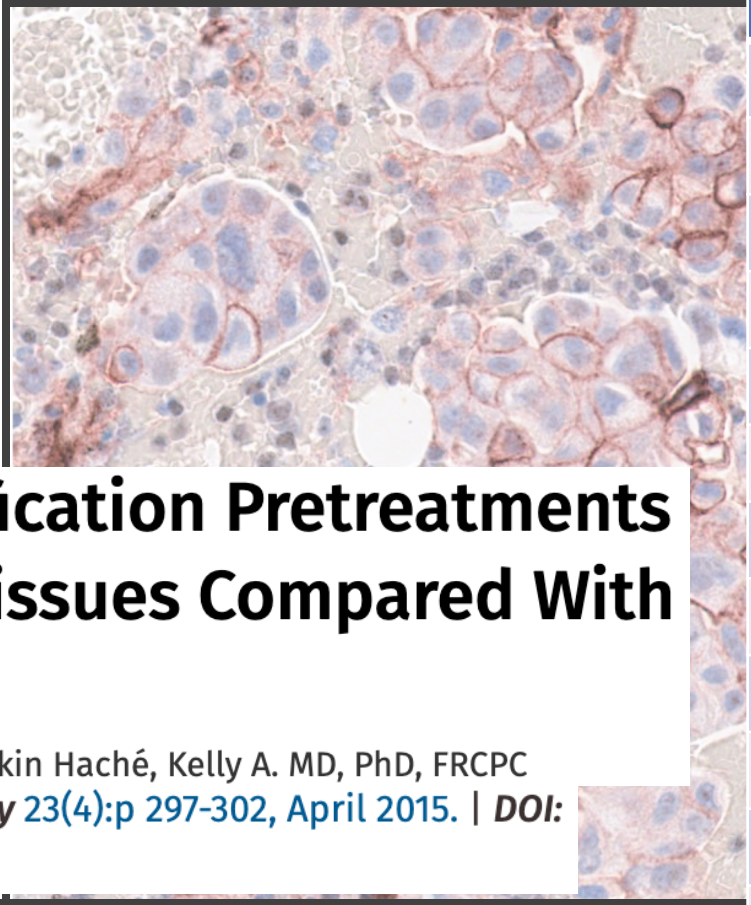
Cell block procedure – pros and cons



Received: August 20, 2020; **Revised:** September 28, 2020; **Accepted:** September 30, 2020

Formalin Fixation for Optimal Concordance of Programmed Death-Ligand 1 Immunostaining Between Cytologic and Histologic Specimens From Patients With Non–small Cell Lung Cancer

Bregje M. Koomen, MD [1]; Jose van der Starre-Gaal, MD, PhD[2]; Judith M. Vonk, PhD[3]; Jan H. von der Thüsen, MD, PhD[4]; Jacqueline J. C. van der Meij, MD[5]; Kim Monkhorst, MD, PhD[6]; Stefan M. Willems, MD, PhD[1,7]; Wim Timens, MD, PhD[7]; and Nils A. 't Hart, MD, PhD[2,7]



Target
ALK
Bcl6
BRAF
CD138
Cyclin D1
MLH1
p53
PD-L1
PMS2
PAX8
PRAME
TTF1

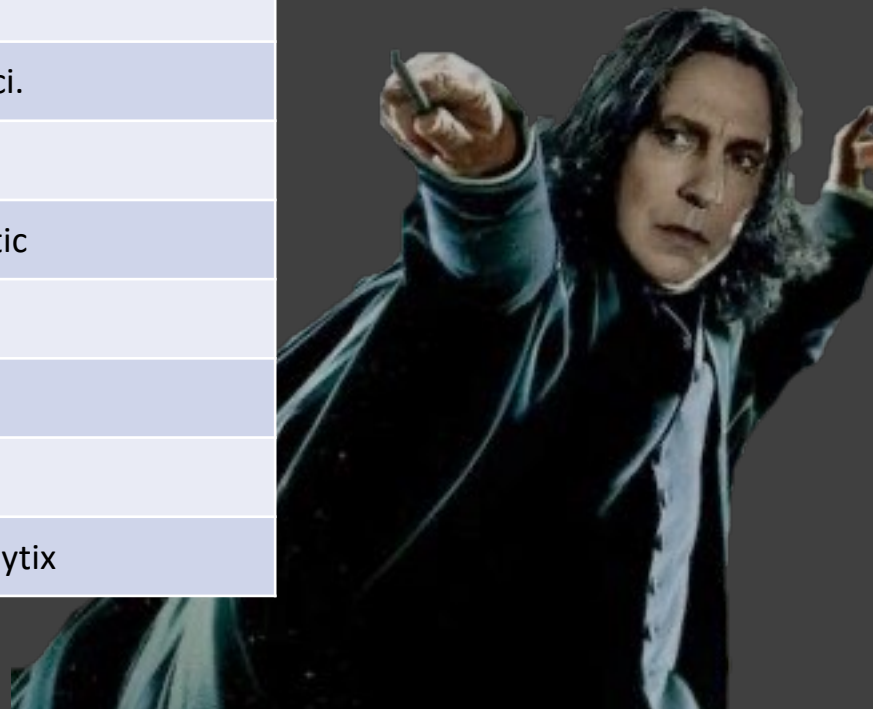
CytoLyt® Fixation and Decalcification Pretreatments Alter Antigenicity in Normal Tissues Compared With Standard Formalin Fixation

Gruchy, Jennette R. MD, MSc; Barnes, Penny J. MD, FRCPC; Dakin Haché, Kelly A. MD, PhD, FRCPC

Applied Immunohistochemistry & Molecular Morphology 23(4):p 297-302, April 2015. | ***DOI:*** 10.1097/PAI.0000000000000082

Name	Contains...	Company
F-solv	Denat. EtOH / Aldehyde derivate / Stabiliser	Yvsolab
GreenFix	Ethandial / Ethanol	Diapath
CyMol	Ethanol / Methanol / 2-Propanol	Copan
RCL-2	Ethanol / Acetic acid / Complex carbohydrates	Alphelys
FineFix	Ethanol / Glycerol / PVA / Simple carbohydrates	Milestone
Formaldehyde-EtOH	Formaldehyde / Ethanol / Buffer	BBC Biochemical
Zn-Formalin	Formaldehyde / Methanol / Zn-sulfate	Richard-Allen
Prefer	Glyoxal / Ethanol	Anatech
Davidson's AFA	Formaldehyde / Ethanol / Acetic acid	Electron Micr. Sci.
Molecular Fixativ	Methanol / Polyethylenglycol	Sakura
All-Fix	Glyoxal / Ethanol	Cancer Diagnostic
Histochoice	Glyoxal / Zn-sulfate / Butandial	Ameresco-Inc.
O-Fix	Formaldehyd / Ethanol / Acetic acid	SurgiPath
GTF	Glyoxal / Ethanol	StatLab Medical
PAXgene Tissue-fix	Alcohols / Acid / A soluble organic compound	Qiagen- PreAnalytix

Alternatives to NBF??



J Int Oral Health. 2013 Feb; 5(1): 31–38.
Published online 2013 Feb 26.
PMCID: PMC3768083
PMID: 24155575

Revelation in the Field of Tissue Preservation – A Preliminary Study on Natural Formalin Substitutes

Shankargouda Patil, Senior lecturer, BR Premalatha, Senior lecturer, Roopa S Rao, Professor and Head, and BS Ganavi, Postgraduate student

Natl J Maxillofac Surg. 2018 Jan-Jun; 9(1): 14–21.
doi: 10.4103/njms.NJMS_57_17
PMCID: PMC5996645
PMID: 29937654

Probing natural substitute for formalin: Comparing honey, sugar, and jaggery syrup as fixatives

Amritaksha Bhattacharyya, Bhavana Gupta,[1] Anil Singh,[2] Kunal Sah,[2] and Vivek Gupta[3]

Table 5: Problems encountered with different fixatives and their remedies

PROBLEM	FIXATIVES	REMEDY
Breach in continuity of sections	• Honey • Sugar syrup • Jaggery syrup	• Re-impregnate the tissue for another hour • Use new blades • Handle the sections carefully
Intense staining with eosin	• Honey • Sugar syrup	• Minimize the staining time with eosin
Folding of the tissue sections	• Sugar syrup	• Difficult to avoid • Careful microtomy and floatation techniques

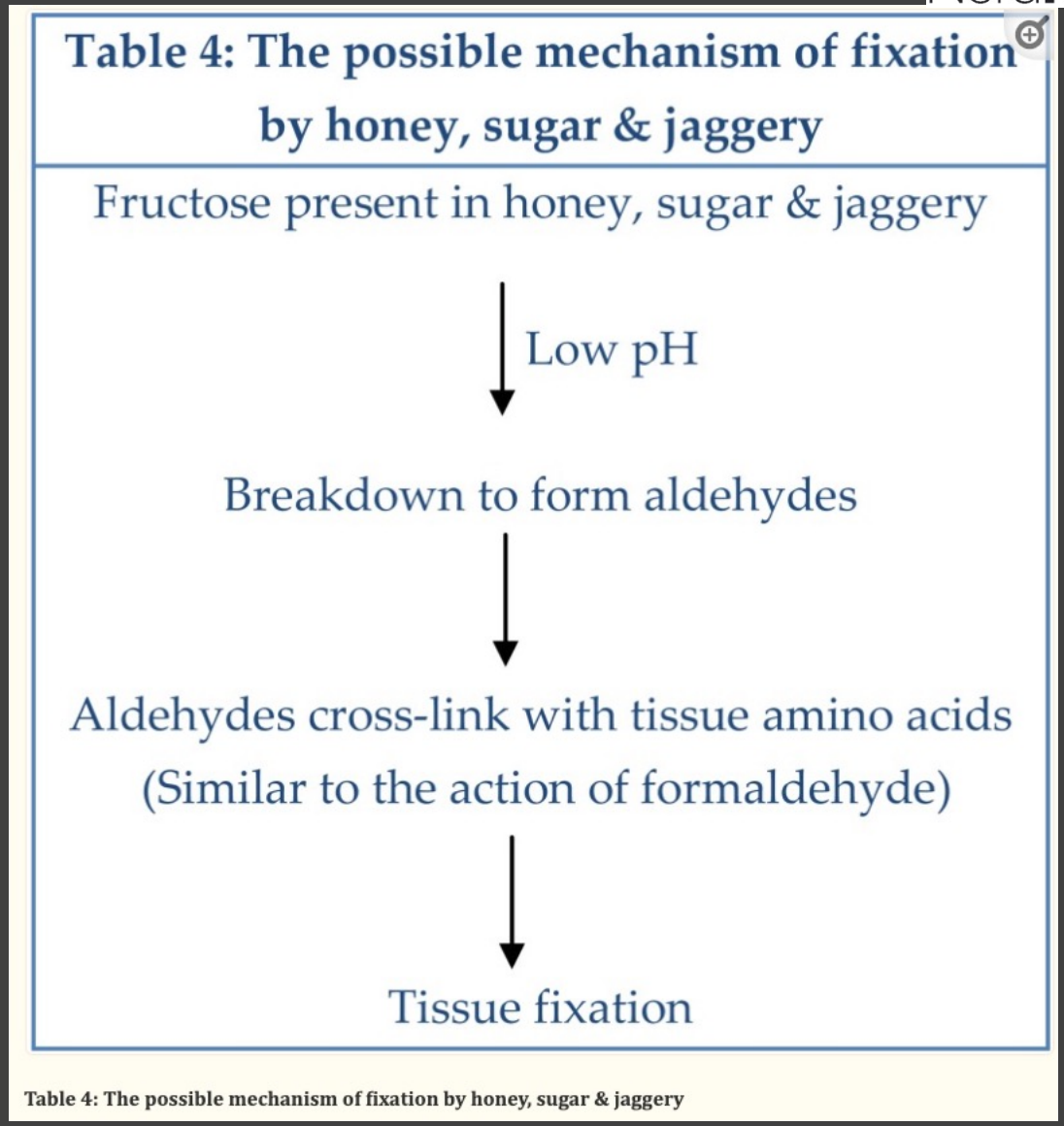
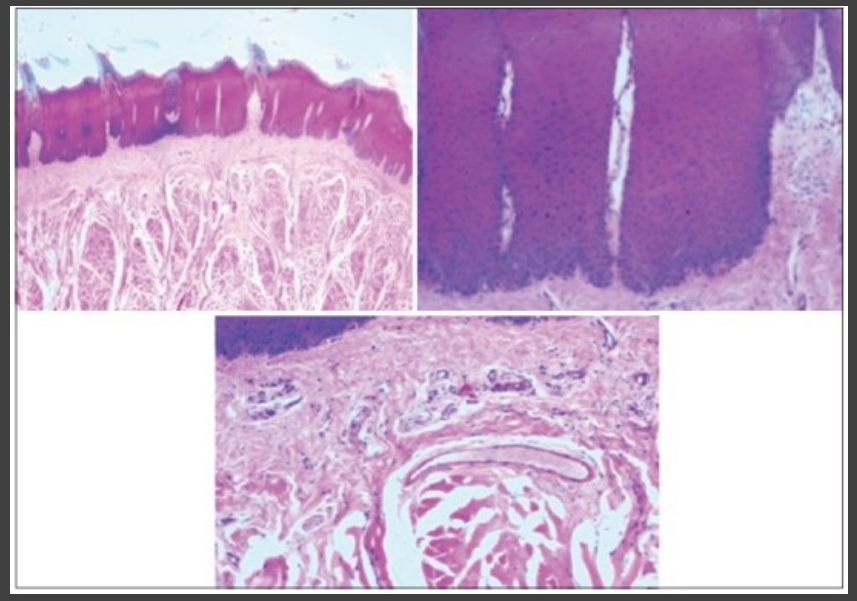
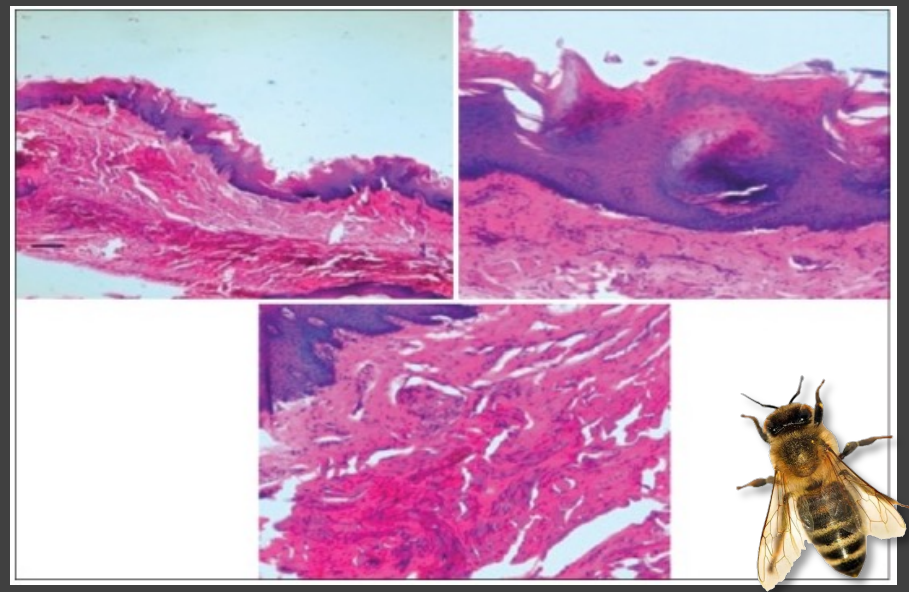


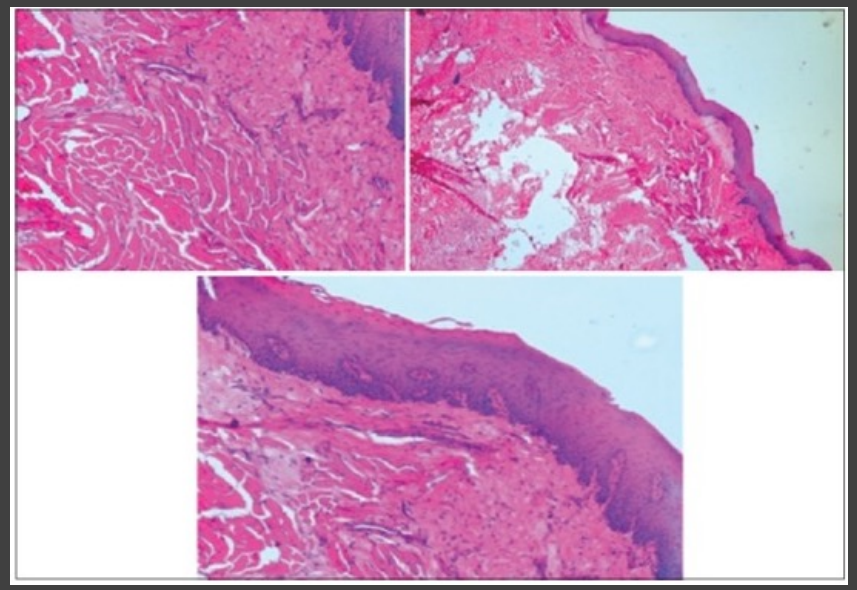
Table 4: The possible mechanism of fixation by honey, sugar & jaggery



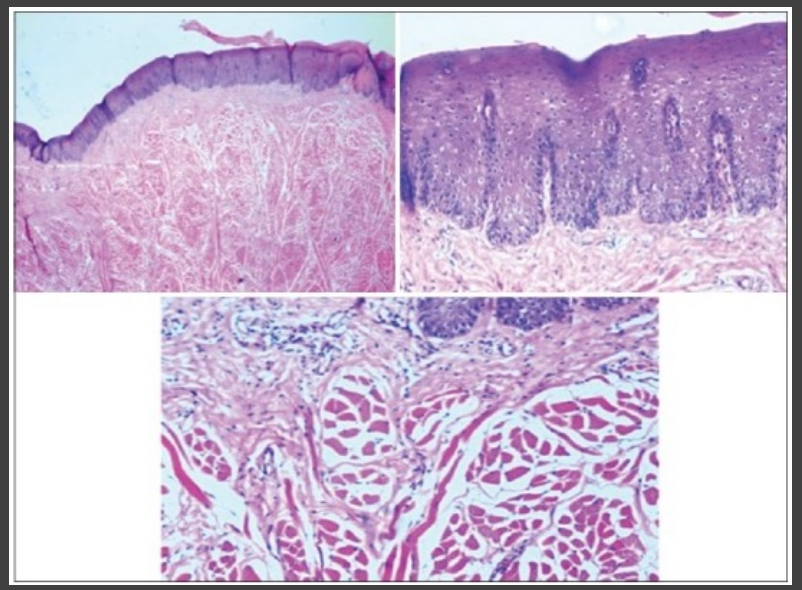
Formalin



Honey



Jaggery



Water

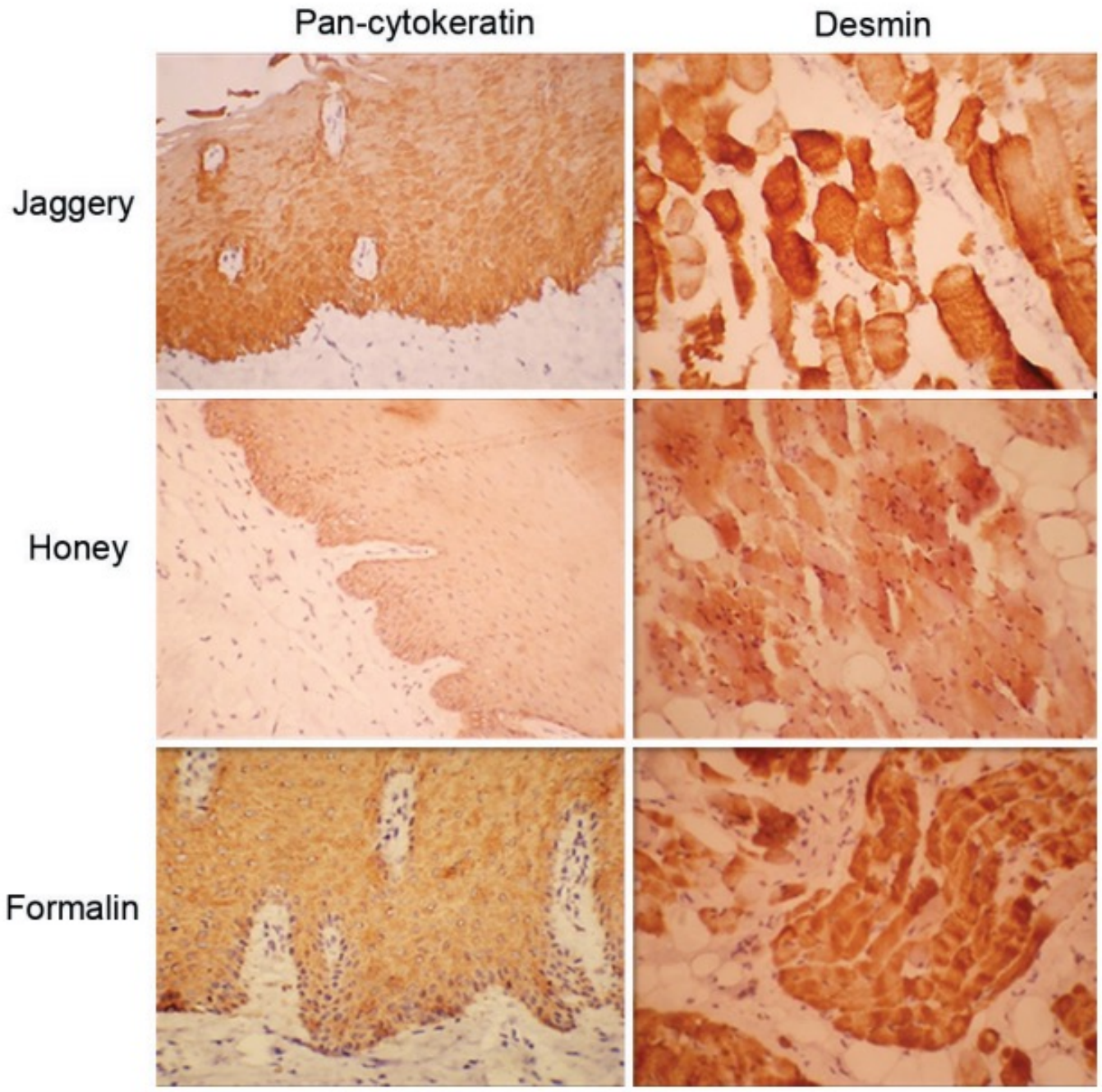
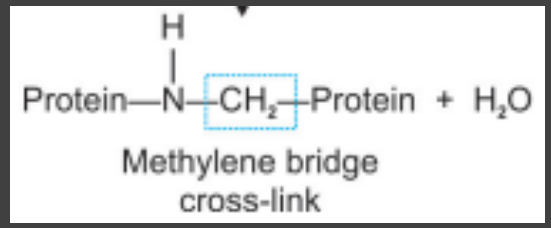


Fig. 1: Intensity of the immunohistochemical stains

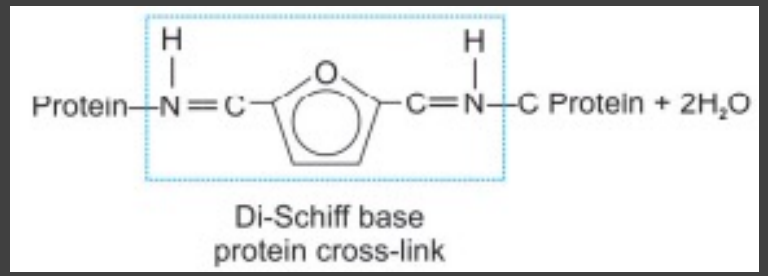
10.5005/jp-journals-10015-1371
ORIGINAL RESEARCH

Tissue Preservation with Natural Fixatives: An Immunohistochemical Evaluation

[1]Barnali Majumdar, [2]Roopa S Rao, [3]Shankargouda Patil



Formalin



Natural fixative

Fixation – take home message

- Formaldehyde is at the moment the golden standard
- Fixation needs to happen <1h from collecting the specimen
- Fixation needs minimum 24-48 hours dependent on the sample size
- Overfilling your cassette will cause problems during processing
- If changing to other fixations types all immunohistochemistry needs reevaluation – be careful with alcohol fixatives

Virchows Arch. 2019; 475(2): 191–199. PMCID: PMC6647403
Published online 2019 Jul 1. doi: 10.1007/s00428-019-02595-9 PMID: 31264038

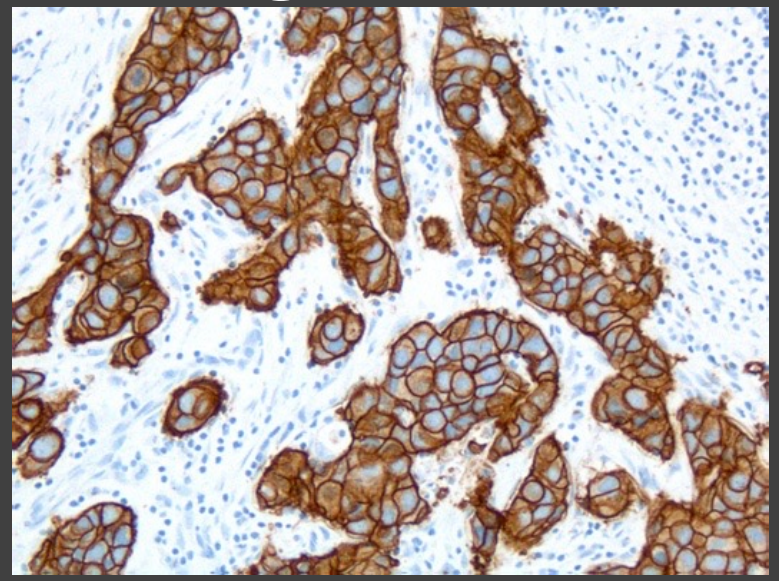
Impact of delayed and prolonged fixation on the evaluation of immunohistochemical staining on lung carcinoma resection specimen

Maartje van Seijen,[1,2] Luka Brcic,[3] Atilio Navarro Gonzales,[4] Irene Sansano,[5] Matyas Bendek,[6,7] Iva Brcic,[3] Birgit Lissenberg-Witte,[8] H. Ibrahim Korkmaz,[1] Thomas Geiger,[9] Rosita Kammler,[9] Rolf Stahel,[9,10] Erik Thunnissen,[1] and On behalf of ETOP[9]

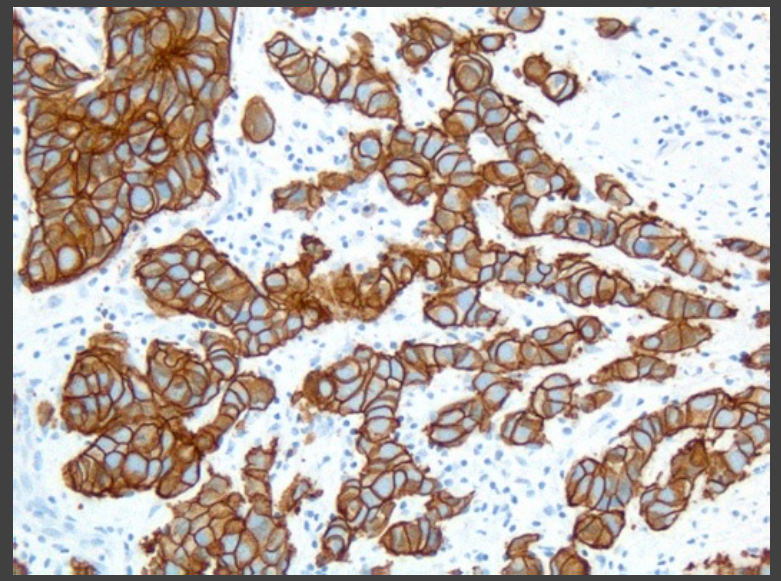
”Prolonged fixation had no influence on the performance of immunohistochemical stains. Delay of fixation negatively affects the expression of different immunohistochemical markers, influencing diagnostic (cytokeratins) and predictive (PD-L1) testing.”

Prolong fixation - HER-2 PATHWAY, rmAb 4B5

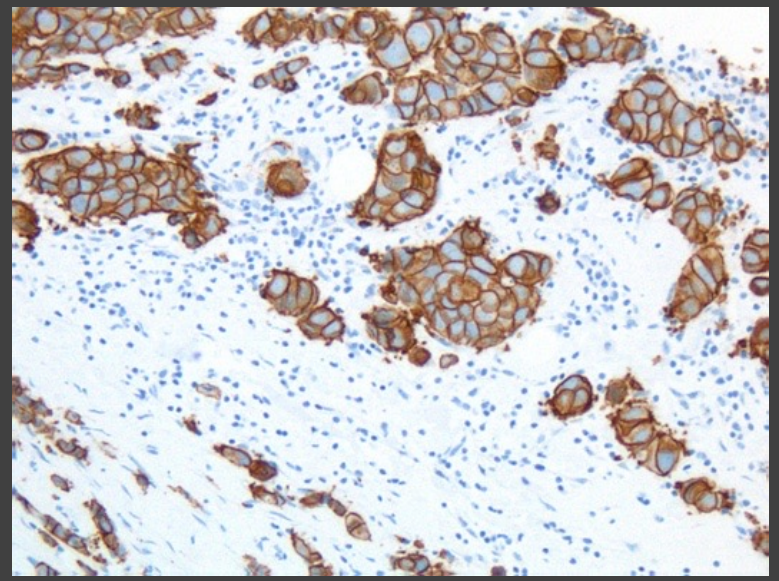
4 h



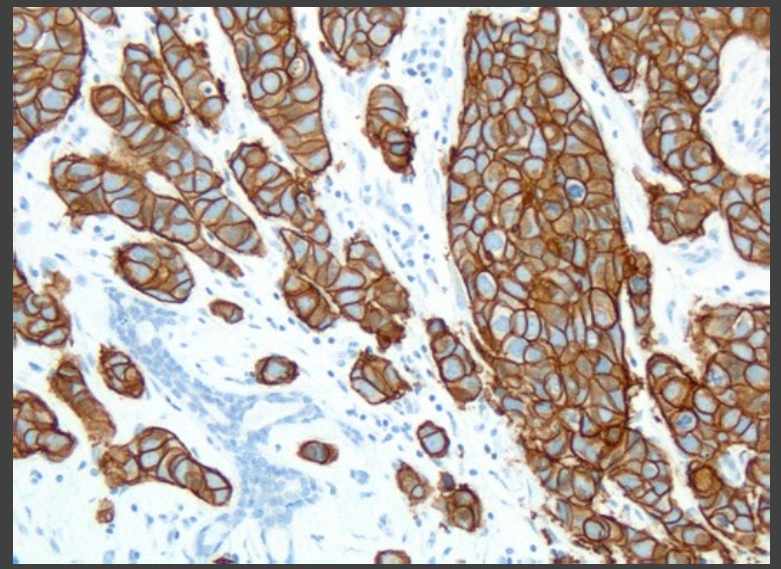
24 h



48 h



168 h



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

Photos by Søren Nielsen

Prolonged fixation

HER2 rmAb 4B5

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	1+	1+	1+	1+
Tumour 2 3+	3+	3+	3+	3+
Tumour 3	0	0	0	0
Tumour 4	1+	1+	1+	1+
Tumour 5	0	0	0	0
Tumour 6 3+	3+	3+	3+	3+
Tumour 7	0	0	0	0
Tumour 8	0	0	0	0
Tumour 9	0	0	0	0

ER rmAb SP1

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	-	-	-	-
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6	+	+	+	+
Tumour 7	-	-	-	-
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

PR rmAb 1E2

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	-	-	-	-
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6+	+	+	+	+
Tumour 7	-	-	-	-
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

ECAD mAb NCH-36

Internal IHC validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	+	+	+	+
Tumour 2	+	+	+	+
Tumour 3	+	+	+	+
Tumour 4	+	+	+	+
Tumour 5	+	+	+	+
Tumour 6	+	+	+	+
Tumour 7	+	+	+	+
Tumour 8	+	+	+	+
Tumour 9	+	+	+	+

Conclusion: IHC biomarkers not affected by NBF fixation time and patient material and control material can be fixed from 4 - 168h in 10% NBF …. **but**

Courtesy of Søren Nielsen

Prolonged fixation

Internal SISH validation	4 h. NBF	24 h. NBF	48 h. NBF	168 h. NBF
Tumour 1	-	-	-	FN
Tumour 2 Amp	+	+	+	+
Tumour 3	(?)	-	FN	FN
Tumour 4	-	-	FN	FN
Tumour 5	-	-	-	-
Tumour 6 Amp	+	+	+	+
Tumour 7	-	-	-	FN
Tumour 8 poly.	-	-	-	FN
Tumour 9 poly.	-	-	-	FN

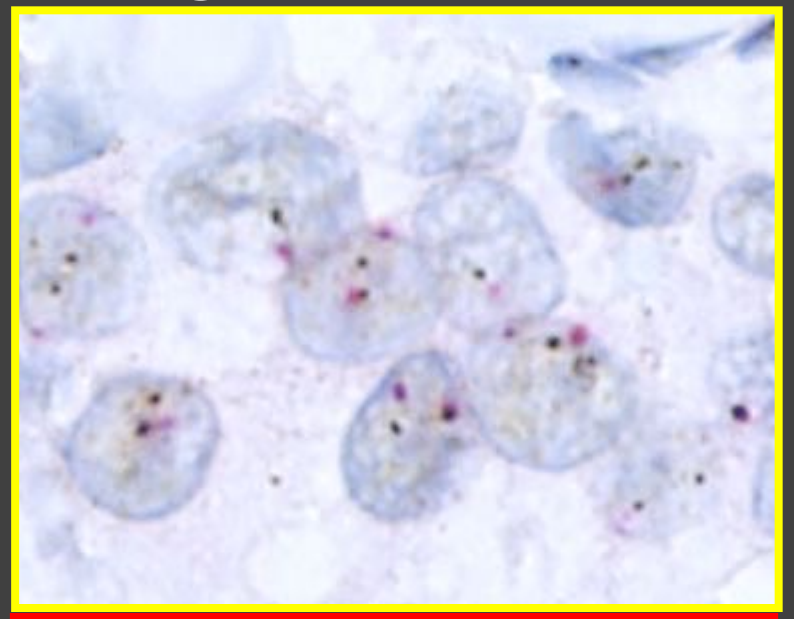
HER-2 ISH: *8/36 cores could not be assessed..!*

Breast carcinomas, Dual SISH

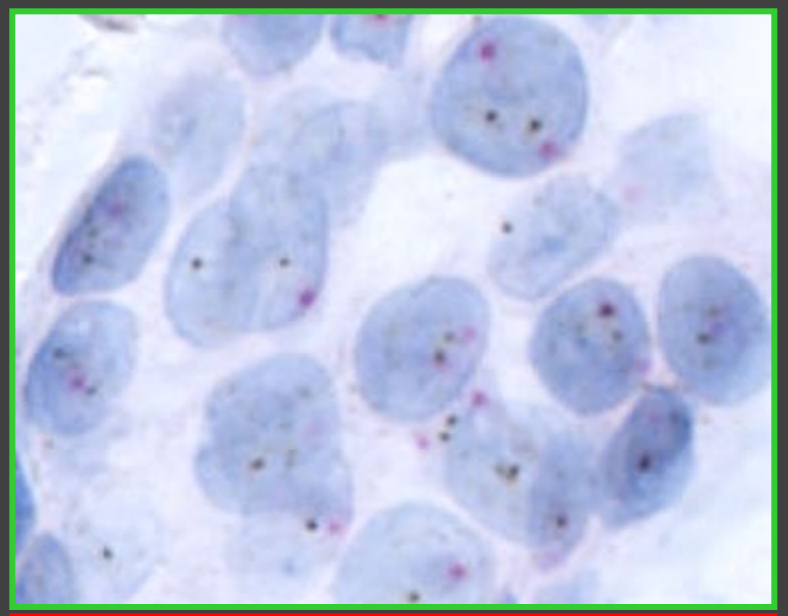
Courtesy of Søren Nielsen

Prolonged fixation

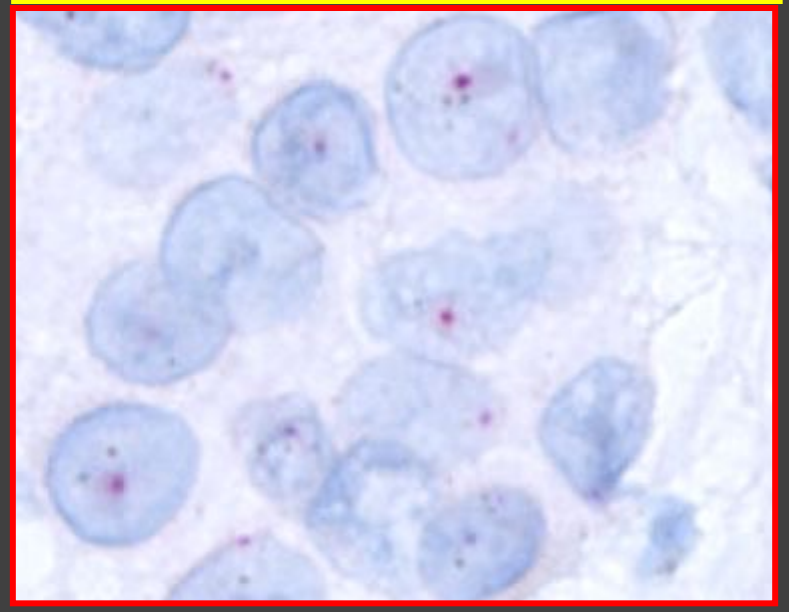
4 h



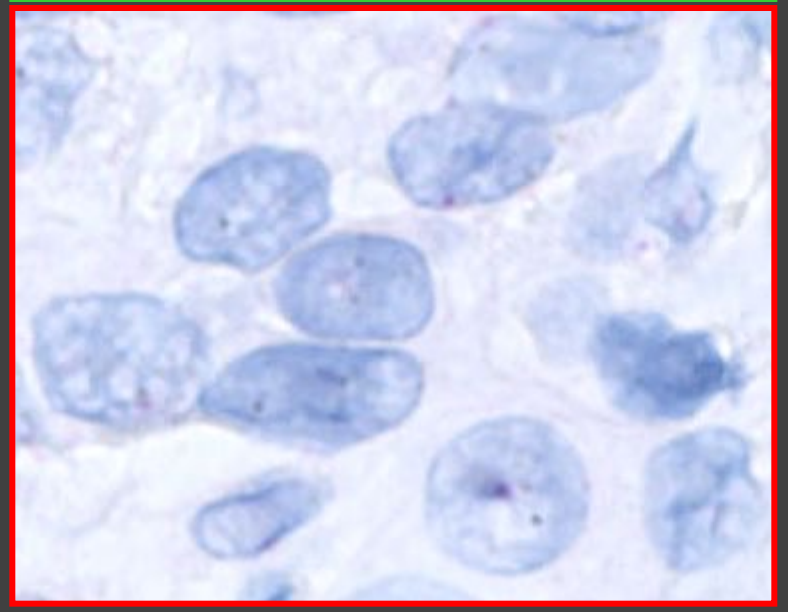
24 h



48 h



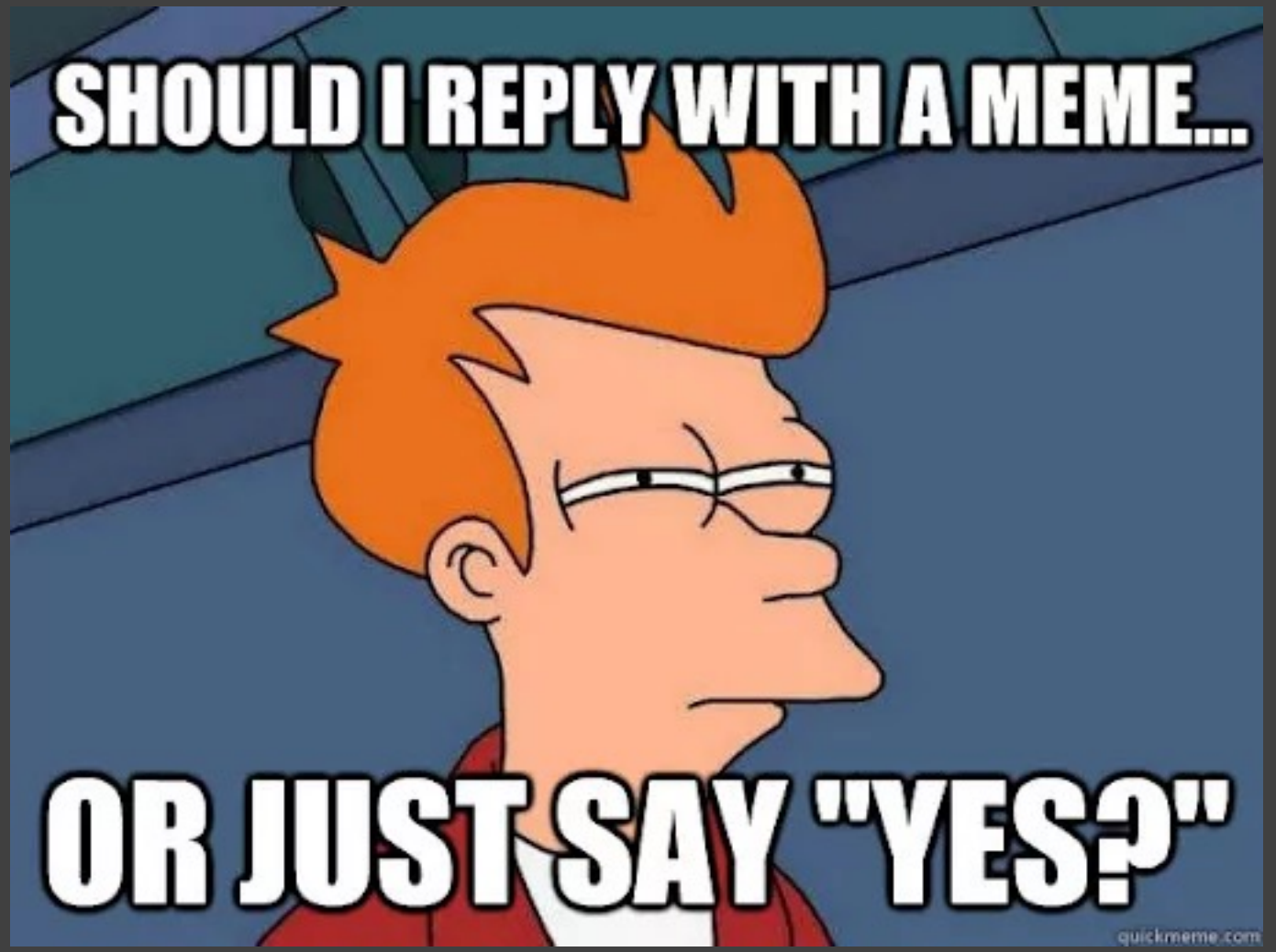
168 h



Courtesy of Søren Nielsen

Breast carcinoma, 1+ Dual SISH

Did you realize we are more than halfway through and still talking about fixation…. Important?



Decalcification

- Type
  - Strong acid (e.g. HCl)
  - Weak organic acid (e.g. formic acid) 2-2,5 timer longer
  - Chelating agents (e.g. EDTA) 8-16 times longer
  - Time, Temperature
- Time in fixative before decalcification

Modern Pathology (2020) 33:1505–1517
https://doi.org/10.1038/s41379-020-0503-6

USCAP

ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

Elodie Miquelestorena-Standley[1,2] · Marie-Lise Jourdan[3] · Christine Collin[3] · Corinne Bouvier[4] · Frédérique Larousserie[5] · Sébastien Aubert[6] · Anne Gomez-Brouchet[7] · Jean-Marc Guinebretière[8] · Matthias Tallegas[1,2] · Bénédicte Brulin[9] · Louis-Romée Le Nail[2,9,10] · Anne Tallet[3] · François Le Loarer[11] · Jessica Massiere[11] · Christine Galant[12] · Gonzague de Pinieux[1,2,9]

Modern Pathology (2020) 33:1505–1517
https://doi.org/10.1038/s41379-020-0503-6

USCAP

ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

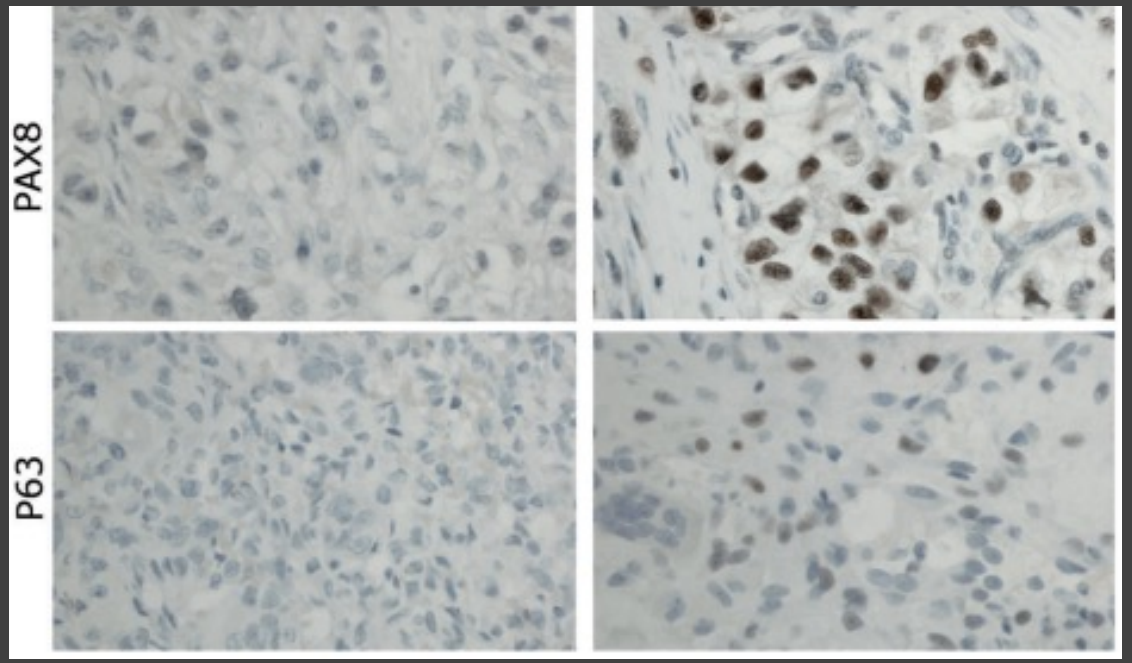
Elodie Miquelestorena-Standley[1,2] · Marie-Lise Jourdan[3] · Christine Collin[3] · Corinne Bouvier[4] · Frédérique Larousserie[5] · Sébastien Aubert[6] · Anne Gomez-Brouchet[7] · Jean-Marc Guinebretière[8] · Matthias Tallegas[1,2] · Bénédicte Brulin[9] · Louis-Romée Le Nail[2,9,10] · Anne Tallet[3] · François Le Loarer[11] · Jessica Massiere[11] · Christine Galant[12] · Gonzague de Pinieux[1,2,9]

Table 1 Content and pH provided by manufacturers of commercial decalcifying agents.

	Decalc	DC2	DC3	DC1	TBD2	EDTA
Manufacturer	Histolab, Gothenburg, Sweden	VWR, Radnor, PA, USA	VWR, Radnor, PA, USA	VWR,Radnor, PA, USA	Thermo Fisher Scientific, Waltham, MA, USA	Promega, Madison, WI, USA
Content	Hydrochloric acid 10–20%	Hydrochloric acid 10–25%	Hydrochloric acid 5–10% Alcohols, C12–14, ethoxylated, propoxylated <1% EDTA disodium salt <0.1%	Formic acid 5–15% Formaldehyde 5–10%	Water 77–80% Formic acid 21–23% Fluorad >1% Sodium citrate >1% Polyvinyl pyrrolidone >1%	EDTA 0.5 M
pH	<1	<1	<1	1.3–2.7	2.3–2.4	8

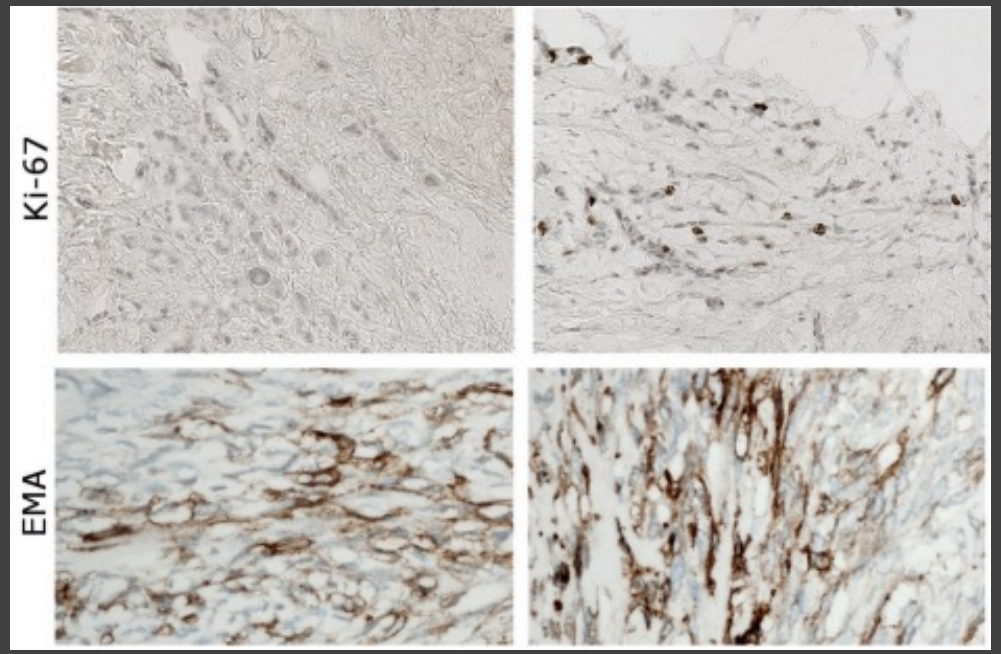
Hydrochloric acid

Formic acid

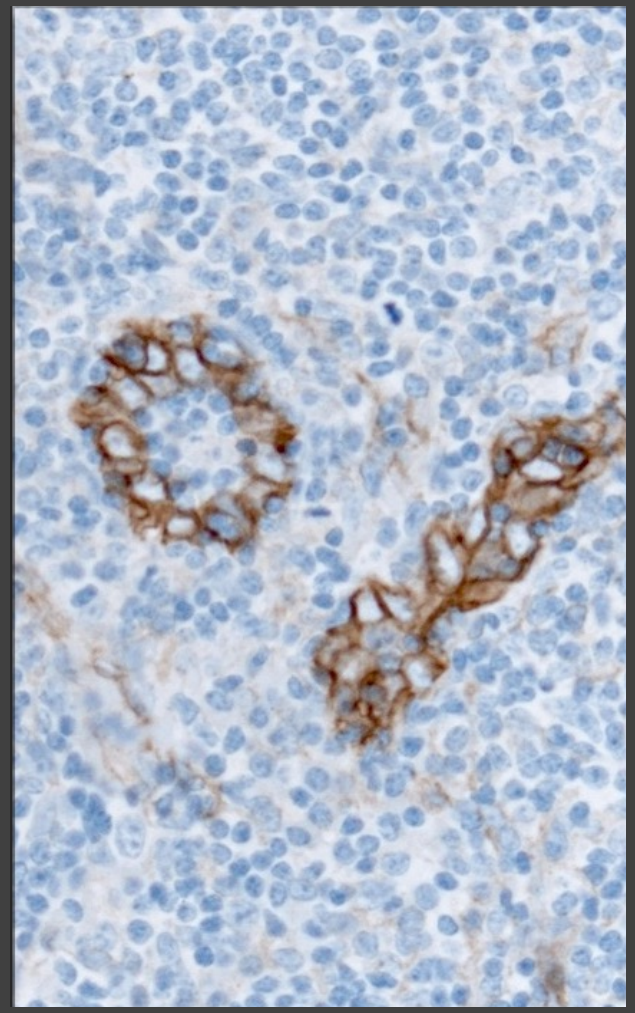


Hydrochloric acid

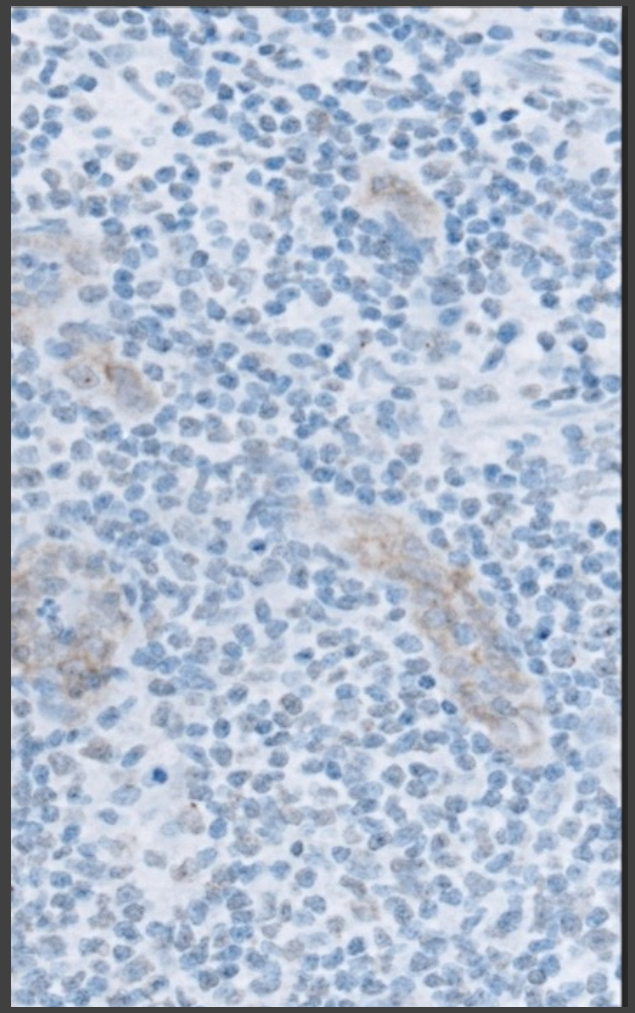
Formic acid



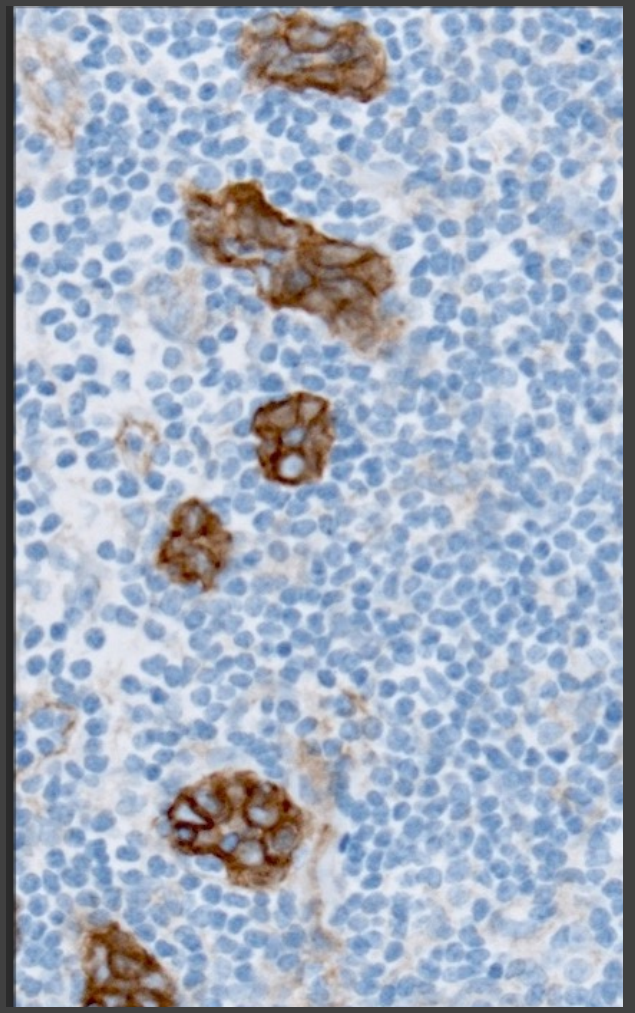
Decalcification and CD105, SN6h



No decalcification



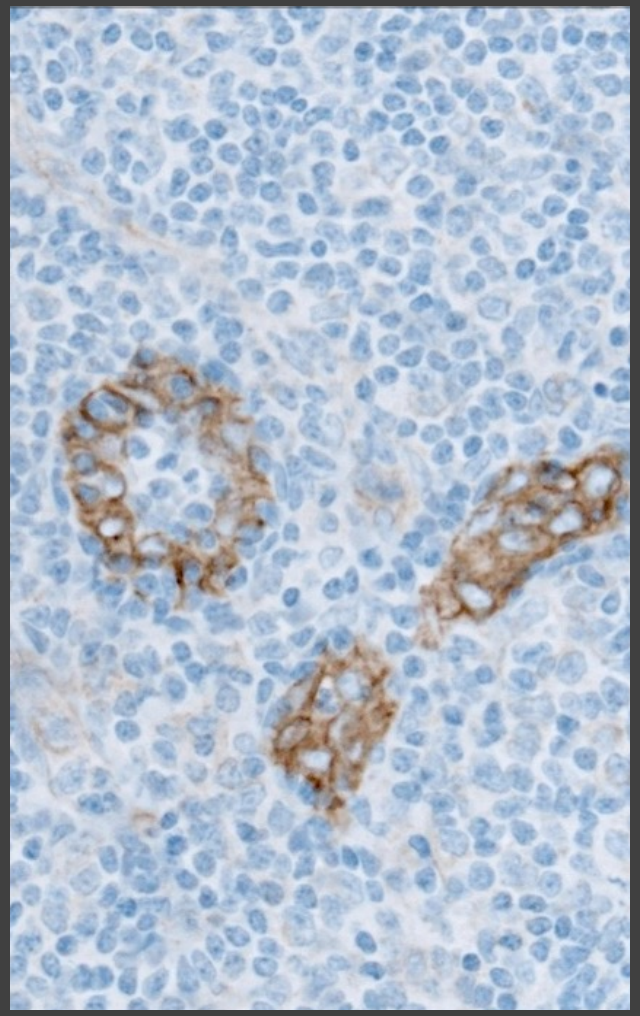
Formic acid 16hrs



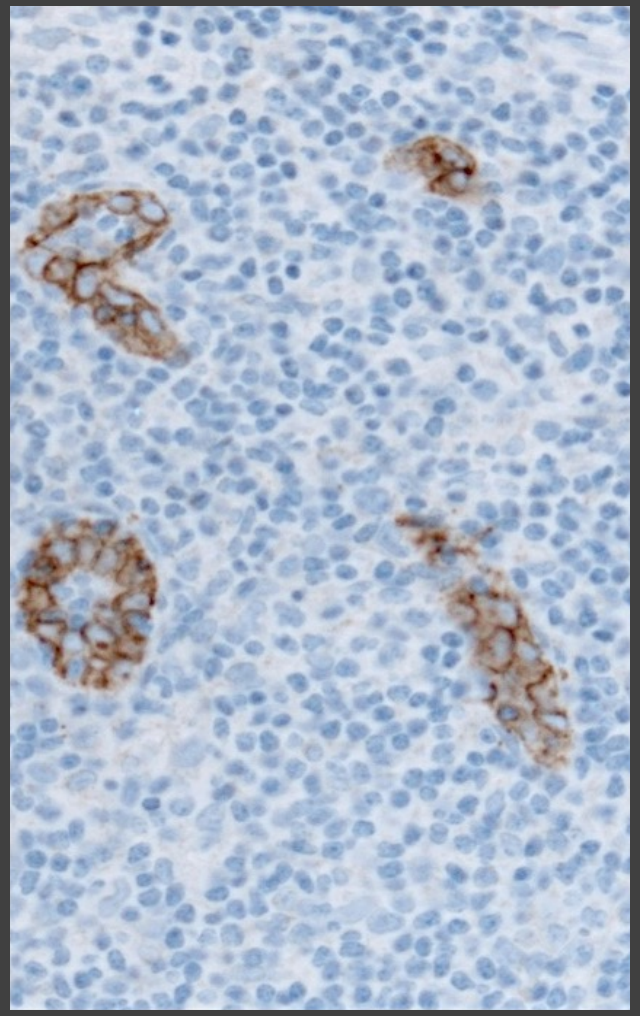
EDTA 96hrs

Photos by Ole Nielsen

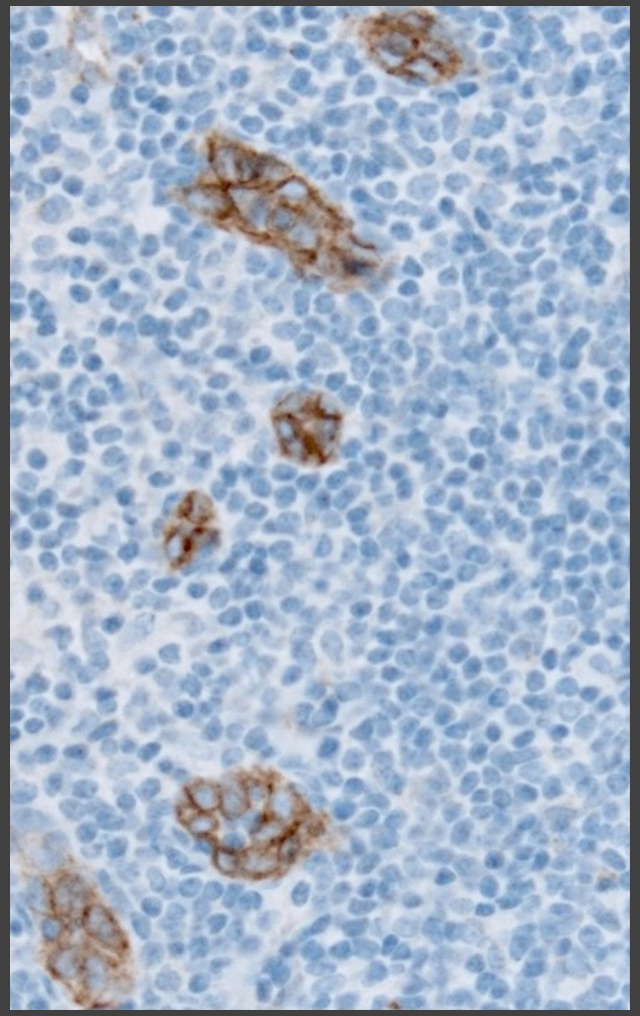
Decalcification and CD105, 4G11



No decalcification



Formic acid 16hrs



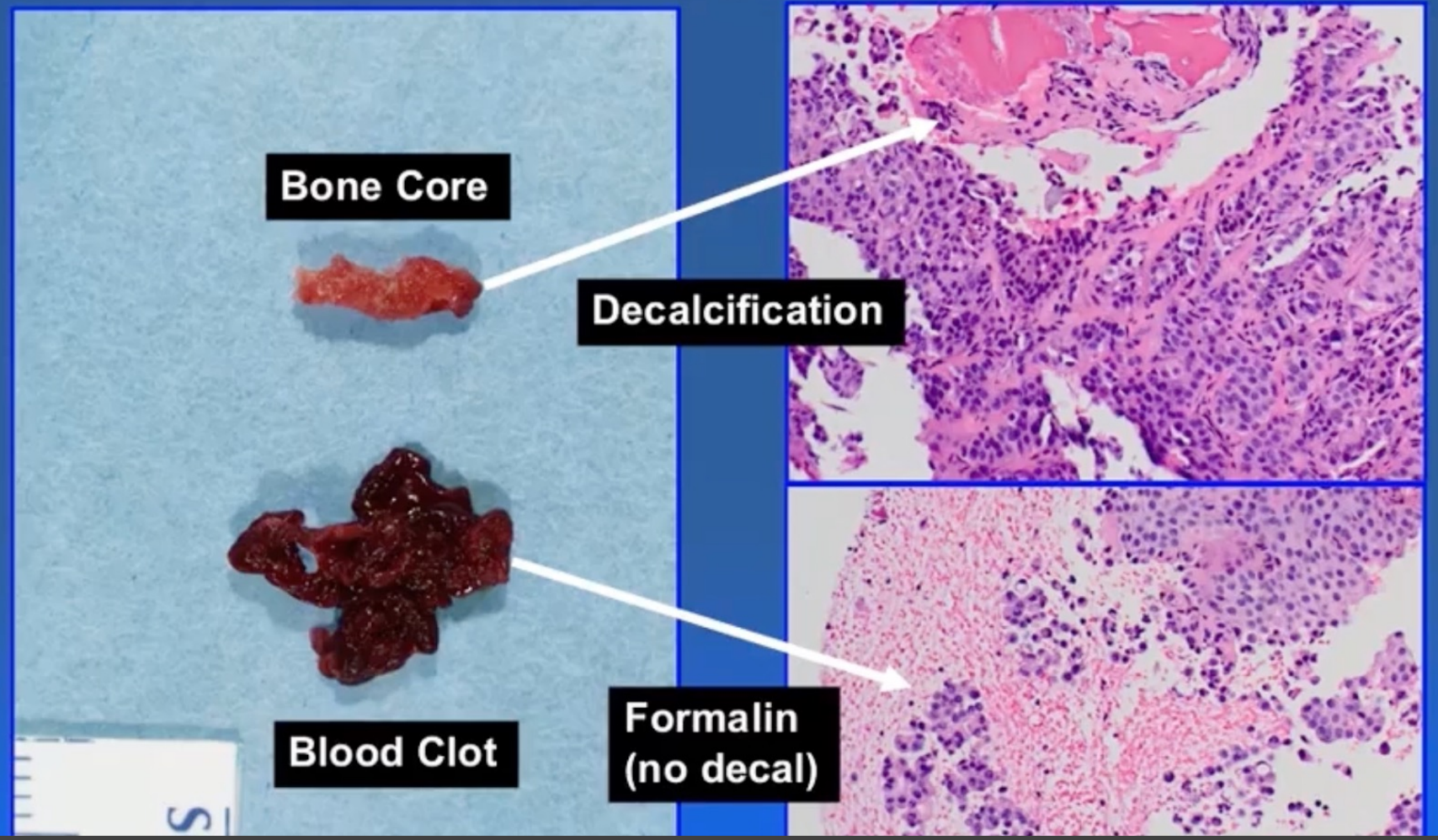
EDTA 96hrs

Photos by Ole Nielsen

The Impact of Decalcification on Staining

Leica Biosystems

Core Biopsy of Bone for Suspected Breast Cancer



Courtesy of Loralee McMahon

EDTA - slow and steady win the race

Modern Pathology (2016) 29, 1460–1470

Influence of decalcification procedures on immunohistochemistry and molecular pathology in breast cancer

Willemijne AME Schrijver[1], Petra van der Groep[1,2,3], Laurien DC Hoefnagel[1,3], Natalie D ter Hoeve[1], Ton Peeters[1], Cathy B Moelans[1] and Paul J van Diest[1]

Human Pathology (2021) 117, 108–114

ELSEVIER

Human PATHOLOGY

www.elsevier.com/locate/humpath

Original contribution

Effect of EDTA decalcification on estrogen receptor and progesterone receptor immunohistochemistry and HER2/neu fluorescence in situ hybridization in breast carcinoma☆

Erik Washburn MD*, Xiaoyu Tang MD, PhD [1], Carla Caruso MD [2], Michelle Walls, Bing Han MD, PhD

Modern Pathology (2020) 33:1505–1517
https://doi.org/10.1038/s41379-020-0503-6

USCAP

ARTICLE

Effect of decalcification protocols on immunohistochemistry and molecular analyses of bone samples

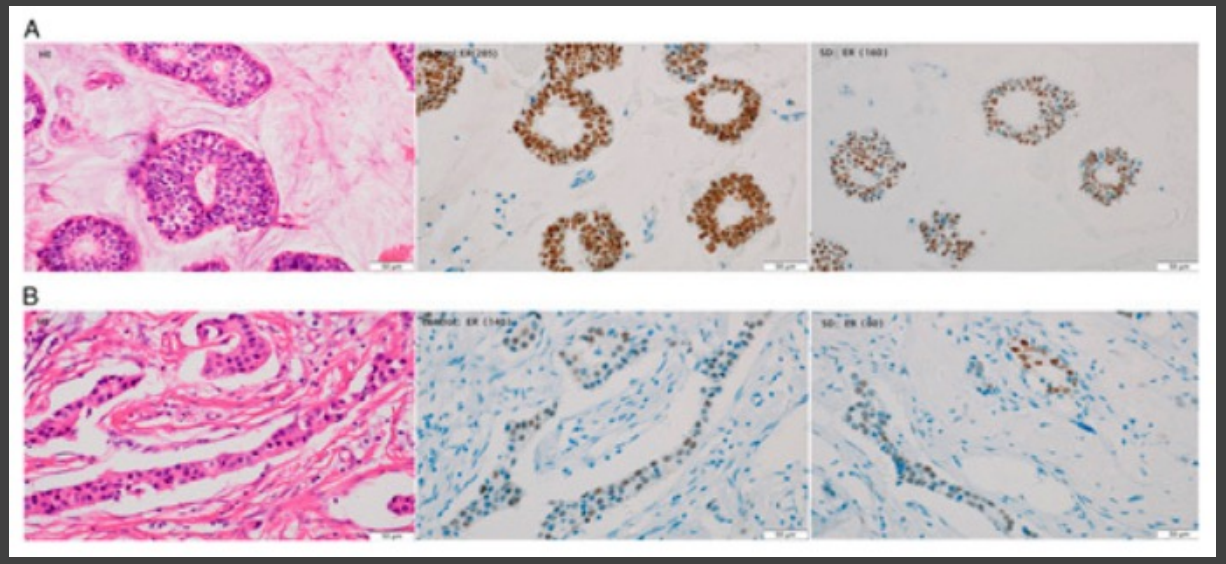
Elodie Miquelestorena-Standley[1,2] · Marie-Lise Jourdan[3] · Christine Collin[3] · Corinne Bouvier[4] · Frédérique Larousserie[5] · Sébastien Aubert[6] · Anne Gomez-Brouchet[7] · Jean-Marc Guinebretière[8] · Matthias Tallegas[1,2] · Bénédicte Brulin[9] · Louis-Romée Le Nail[2,9,10] · Anne Tallet[3] · François Le Loarer[11] · Jessica Massiere[11] · Christine Galant[12] · Gonzague de Pinieux[1,2,9]

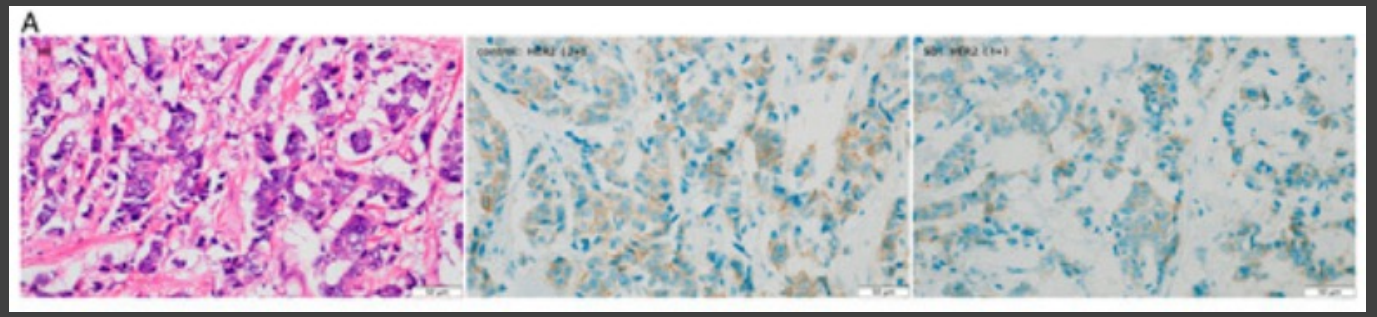
Longer incubation, but minimal negative effects on immunohistochemistry, molecular analysis (DNA/RNA) and CISH.

Appl Immunohistochem Mol Morphol. 2023 Apr; 31(4): 232–238. PMCID: PMC10072208
Published online 2023 Mar 8. doi: 10.1097/PAI.0000000000001111 PMID: 36883948

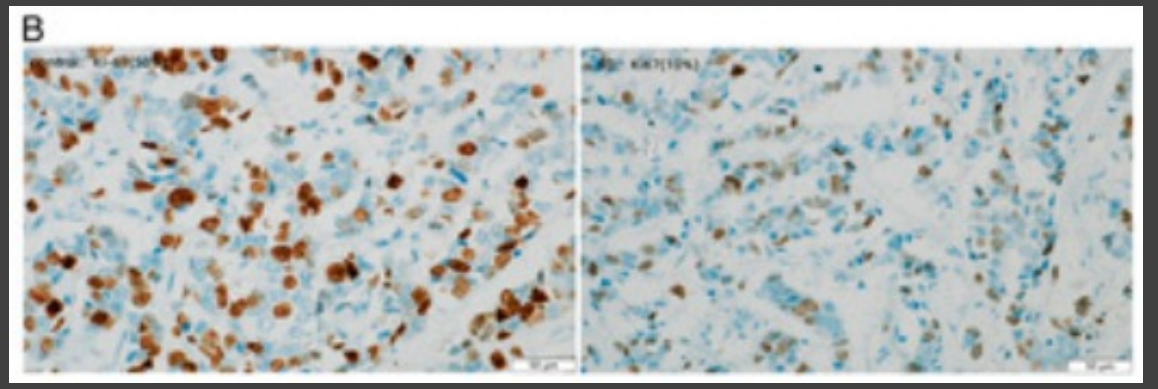
Effect of Surface Decalcification With Hydrochloric Acid on the Determination of Estrogen Receptor, Progesterone Receptor, Ki67, and Human Epidermal Growth Factor Receptor 2 Expressions in Invasive Breast Carcinoma Based on Immunohistochemistry and Fluorescence In Situ Hybridization

Wu Ping, MD,*† Rao Xin, MD,‡§ Zhang Li, MD,*† Chen Yupeng, MM,*† Song Fangling, MM,*† Ren Caihong, MD,*† Hu Shun, MD,*† and Zhang Sheng, MD✉*†





44 Breast metastasis to bone included



Ki67 decreased from 22% to 13% after HCL

ER – 9/31 cases decreased expression, but all still positive

Her2	0-1+	2+	3+
EDTA	23	12	8
HCL	27	8	8

4 cases no FISH

Effects of Preanalytical Variables on the Detection of Proteins by Immunohistochemistry in Formalin-Fixed, Paraffin-Embedded Tissue

Kelly B. Engel, PhD; Helen M. Moore, PhD

Arch Pathol Lab Med—Vol 135, May 2011

Table 1. Potential Sources of Preanalytic Variation During Specimen Fixation and Processing

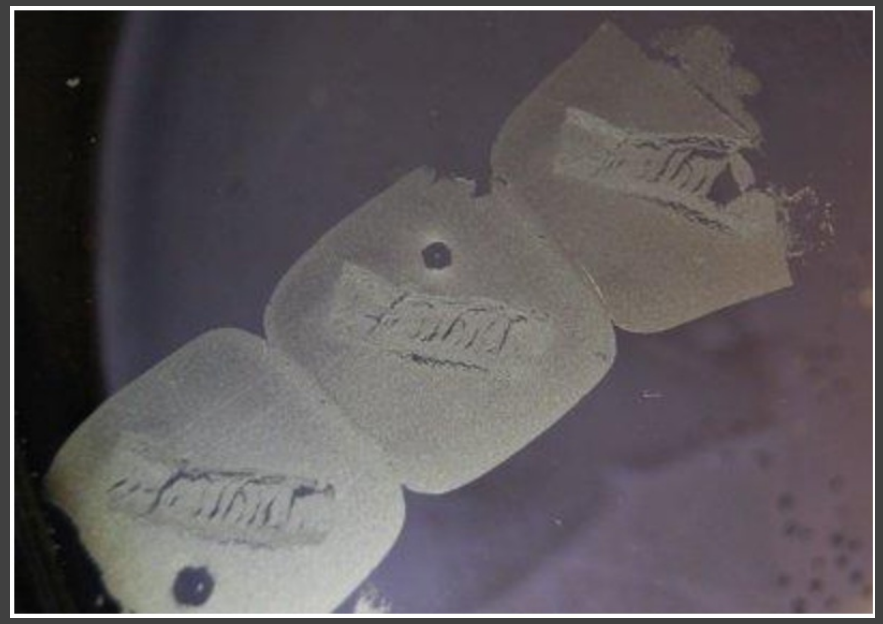
- Prefixation
  - Duration and delay of temperature
  - Specimen size
  - Specimen manipulation (pathology ink)
- Fixative
  - Formula
  - Concentration
  - pH
  - Age of reagent
  - Preparation source
- Fixation
  - Tissue to fixative volume ratio
  - Method (immersion, injection, and sonication or microwave acceleration)
  - Conditions of primary and secondary fixation
    - Movement
    - Light exposure
    - Primary container
    - No. and position of cofixed specimens
- Postfixation
  - Washing conditions and duration
  - Storage reagent and duration
- Processing
  - Type of processor, frequency of servicing and reagent replacement
  - Tissue to reagent volume ratio
  - No. and position of coprocessed specimens
- Dehydration and clearing
  - Reagent
  - Temperature
  - No. of changes
  - Duration (total and change-specific)
- Paraffin impregnation
  - Type and melting point of wax
  - No. of changes
  - Duration (total and change-specific)
  - Method (immersion and sonication or microwave acceleration)
- Paraffin sectioning
  - Type of blade and frequency of replacement
  - Frequency of servicing and wax replacement
  - Temperature of block during sectioning
  - Slide pretreatment
  - Water bath conditions, if used
  - Chemical adhesives, if used
  - Temperature and duration of slide drying
- Storage
  - Temperature and duration of paraffin block storage
  - Temperature, duration, and manipulation of slide-mounted tissue sections

Decalcification:
Type, Time, Temperature

Paraffin sectioning

- Type of blade and frequency of replacement
- Frequency of servicing and wax replacement
- Temperature of block during sectioning
- Section thickness 3-5 μm
- Speed of cutting
- Tissue orientation
- Water bath conditions, if used
- Temperature and duration of slide drying

Water bath

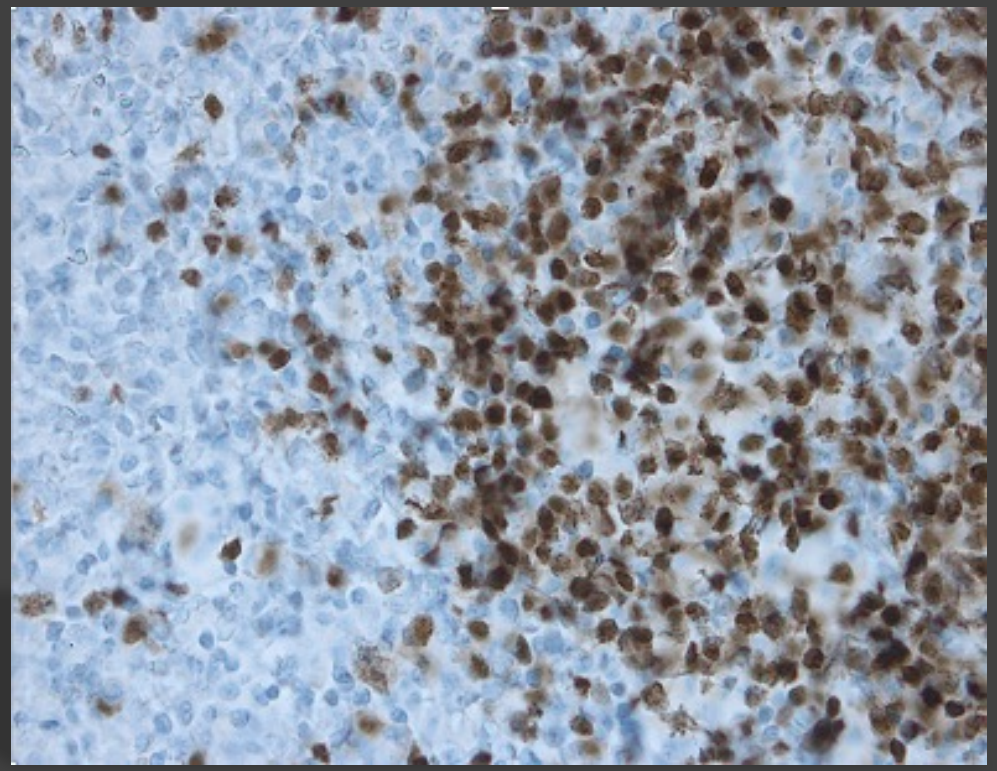


Flotation bath temperature is carefully checked. A temperature 4–9°C below the melting point of the wax is optimal. Sections should readily flatten but the wax should not melt.

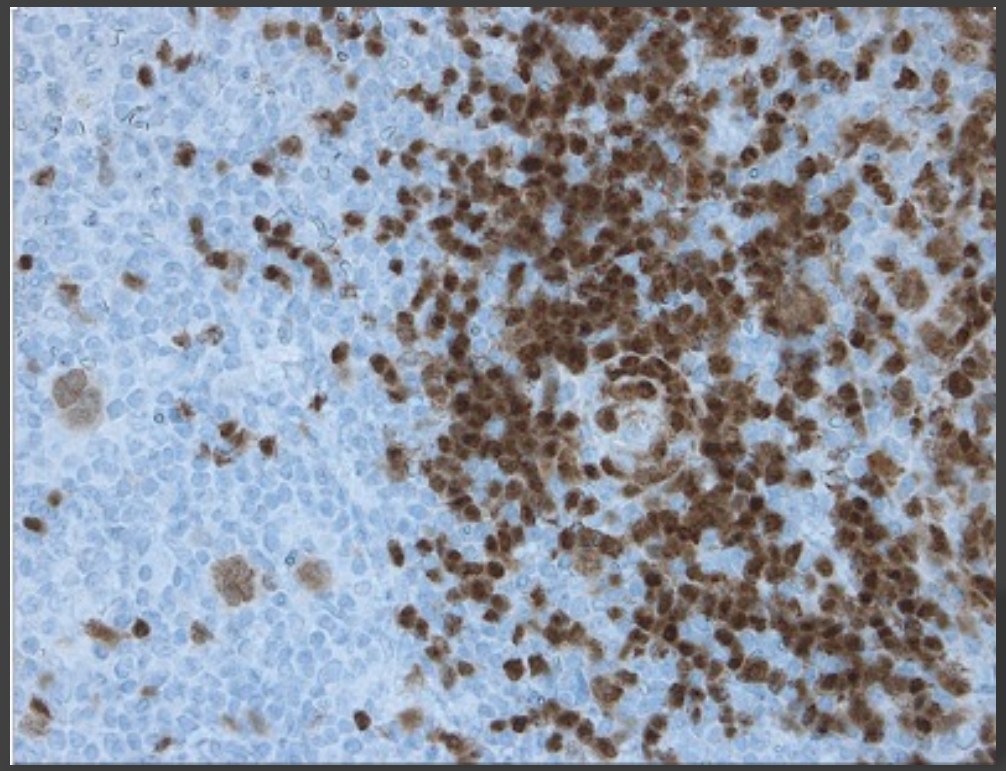
Fatty tissues may need a lower temperature.

If sections are left on the flotation bath for more than 15 seconds, the wax melts. Although this may seem to make the process faster, it can rapidly cause over-expansion and tissue and cell damage.

Pax5 in HD



42 °C/5 sec



52 °C/10 sec

From 2015, in house test from Odense, DK

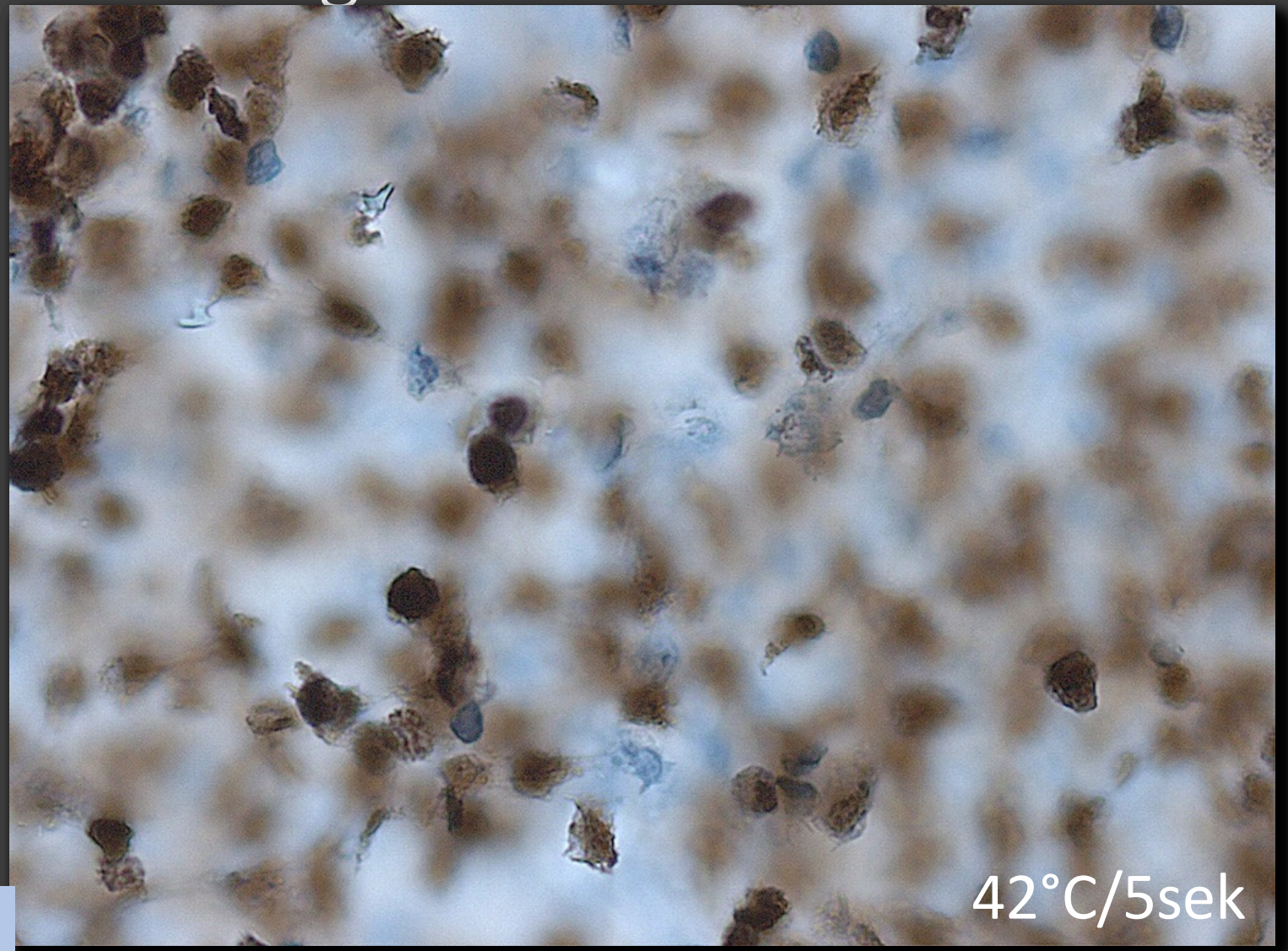
Sectioning – remember to stretch

Sections

Slides

Courtesy of Ole Nielsen

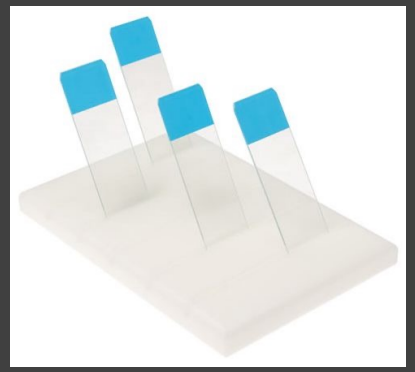
Pax5 in HD – is not ready for digital pathology without streching



Courtesy of Ole Nielsen

Oven after cutting

- Because we have Omnis in our lab we never leave the slide on the rim of the water bath to stretch.
- We place it vertical immediately and insure as little as possible water is trapped under the tissue.
- Set to dry in oven with circulating heat at 40 °C/15 min
  - Different type of slides may need different conditions
- Baked in oven with circulating heat at 60 °C/45 min



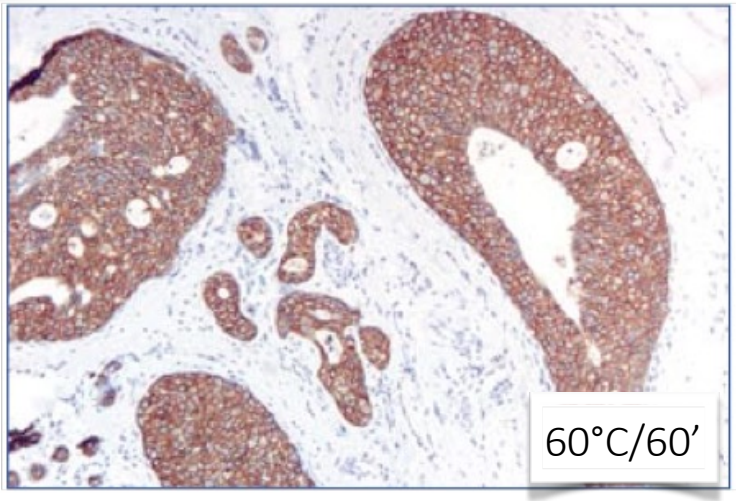
Immunocytochemistry 2008; Volume 6 Issue 3

TECHNICAL ARTICLE

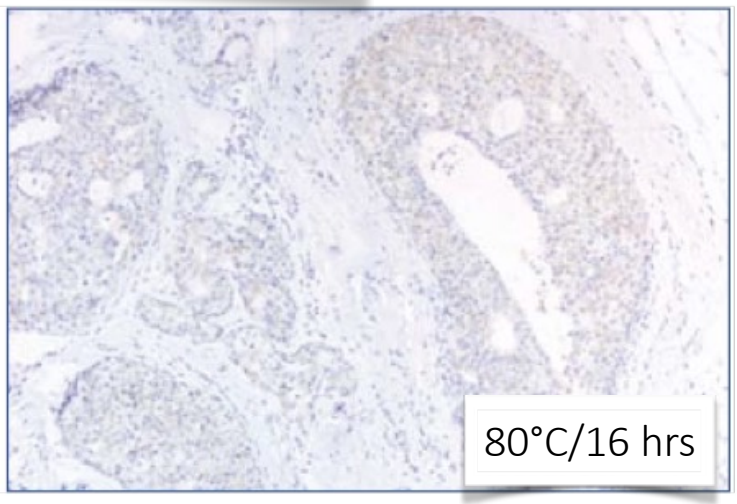
EXCESSIVE SECTION DRYING OF BREAST CANCER TISSUE PRIOR TO DEPARAFFINISATION AND ANTIGEN RETRIEVAL CAUSES A LOSS IN HER2-IMMUNO-REACTIVITY

Bent Lundgaard Hansen, Henrik Winther and Kristian Moller

Dako A/S, DK-2600, Glostrup, Denmark

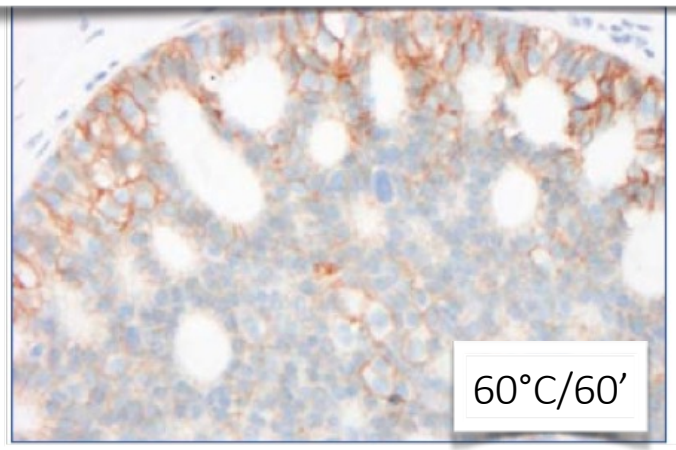


60°C/60’

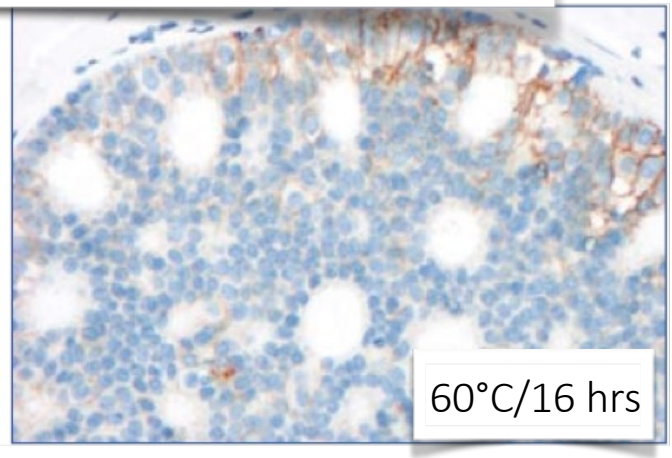


80°C/16 hrs

“Procedure for drying of tissue prior to deparaffinization: The drying temperature should be 60°C for a maximum of one hour, 37 °C for a maximum of 24 hours, or ambient temperature for 24 hours or longer”.

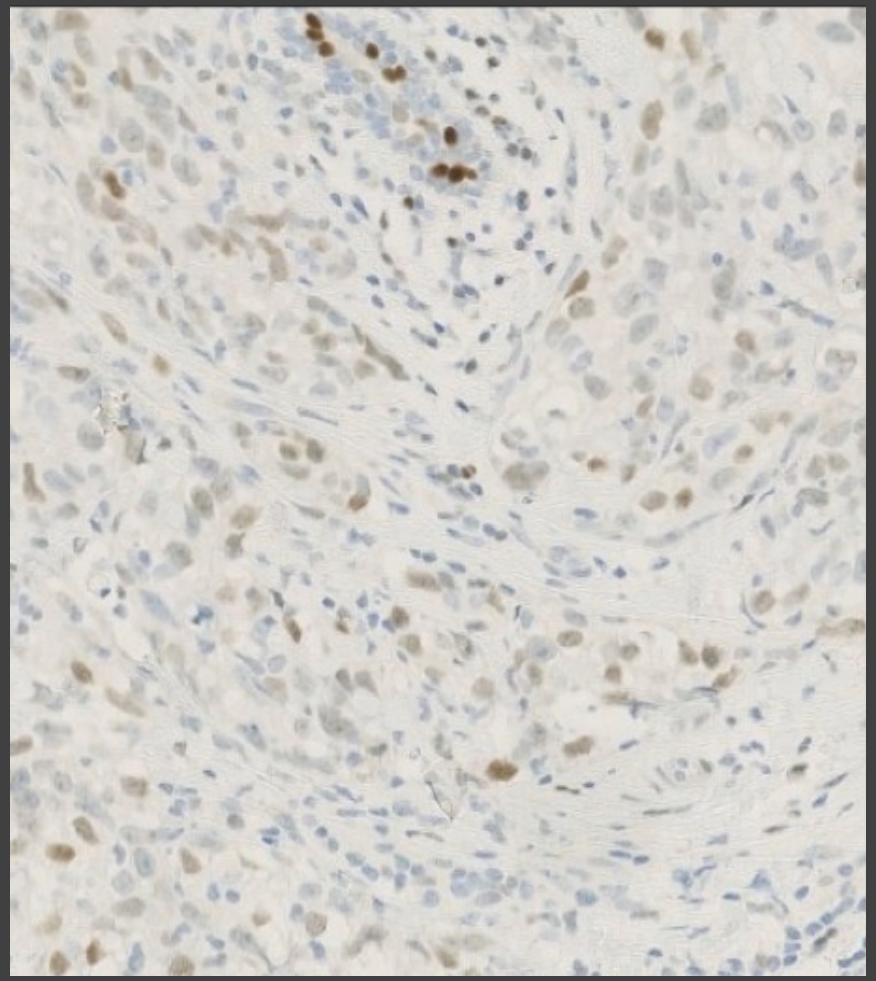


60°C/60’

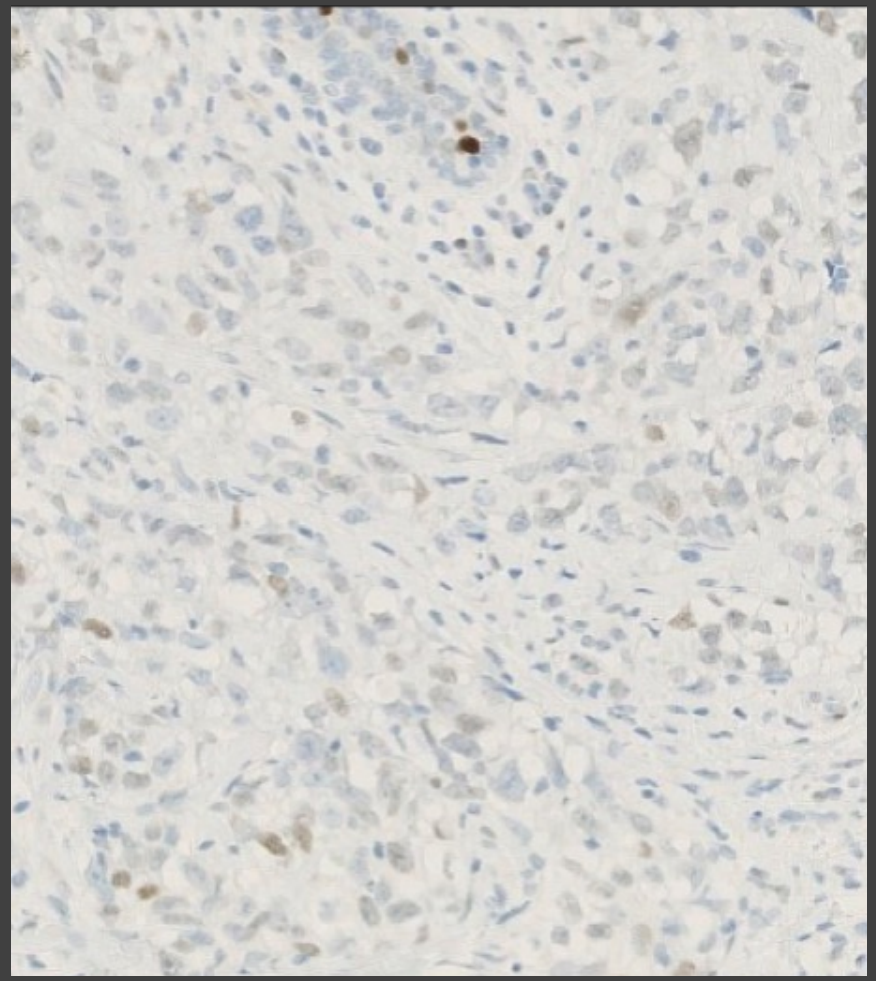


60°C/16 hrs

Drying of sections - ER, SP1

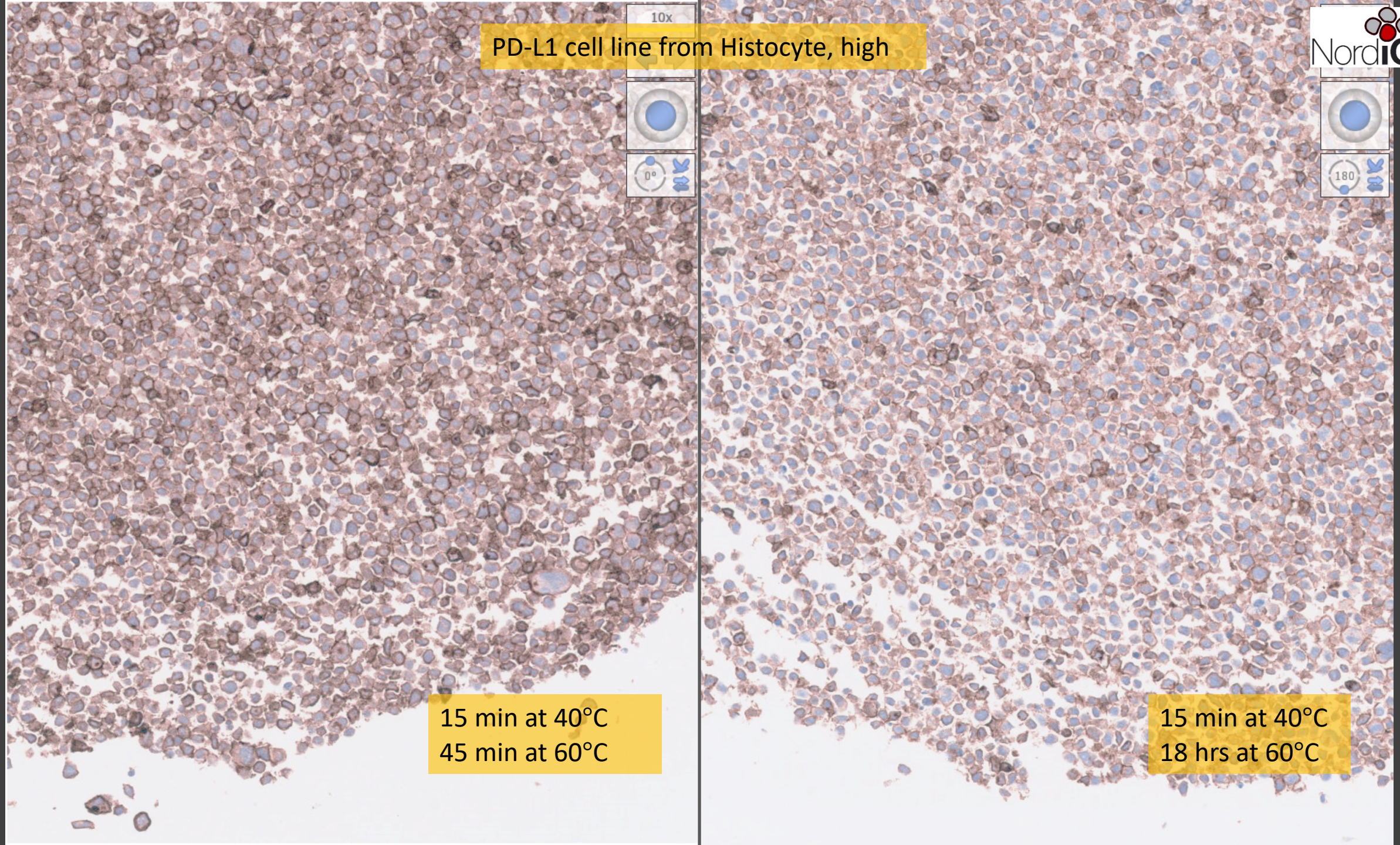


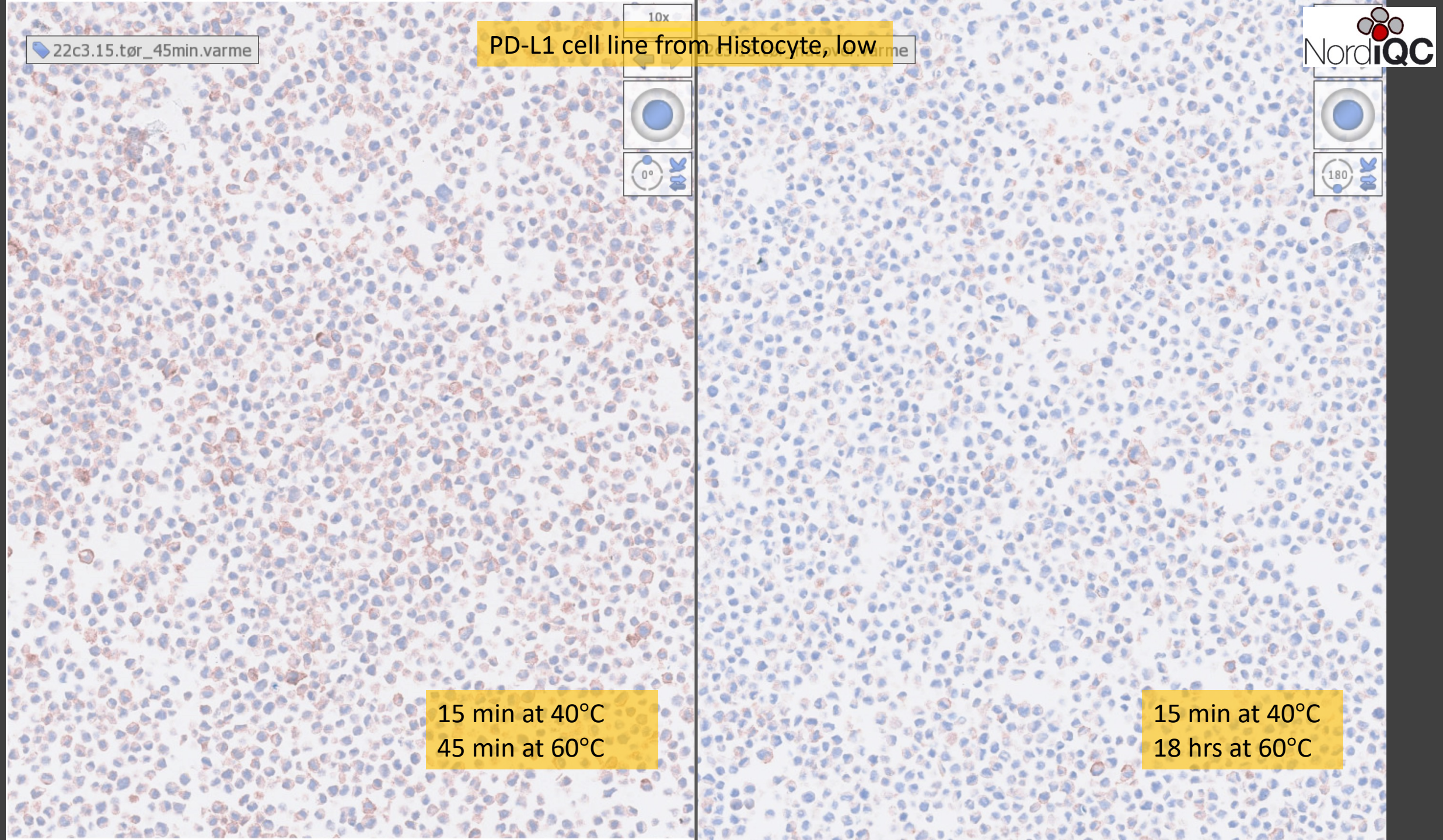
60 min at 60°C

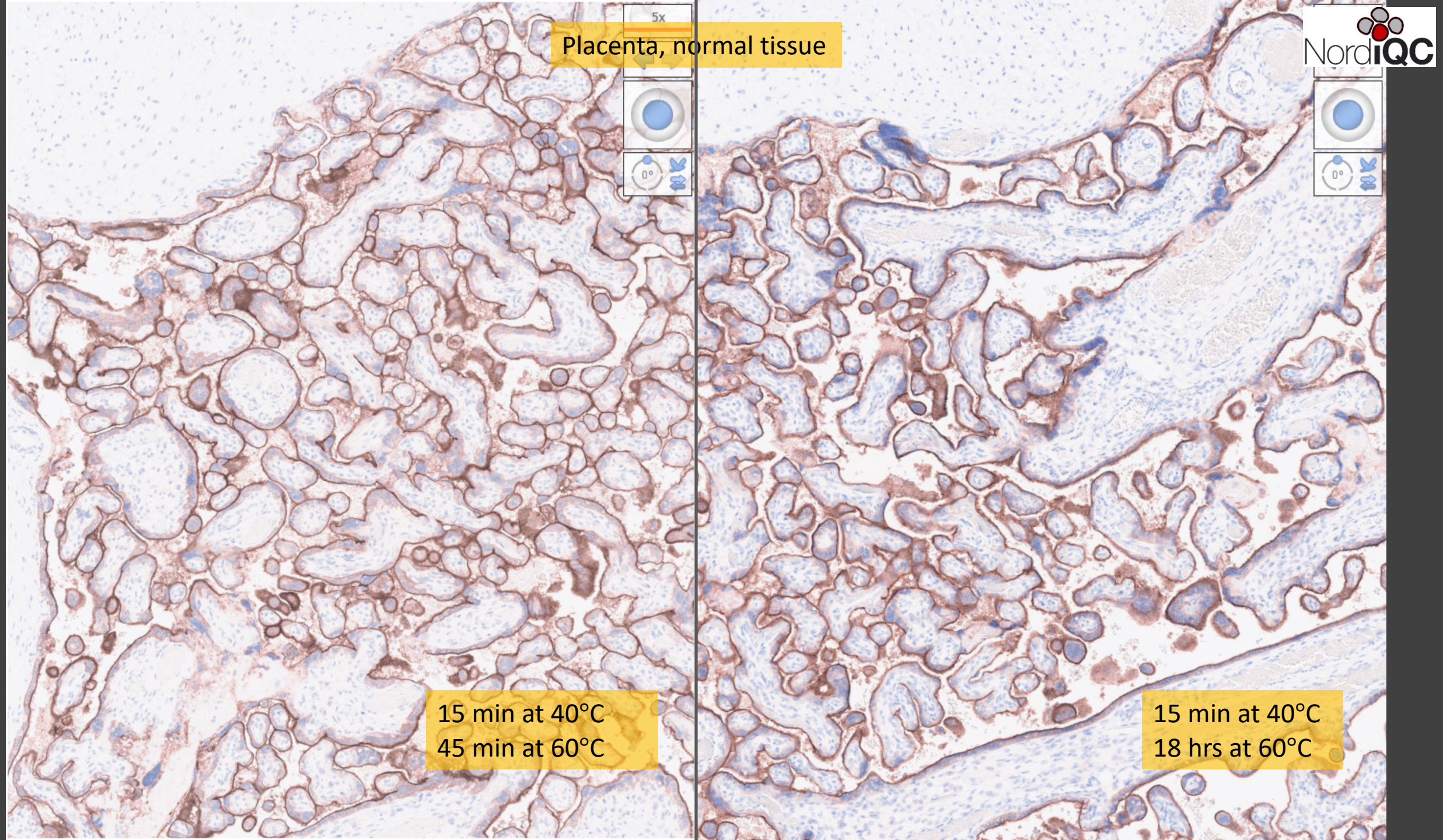


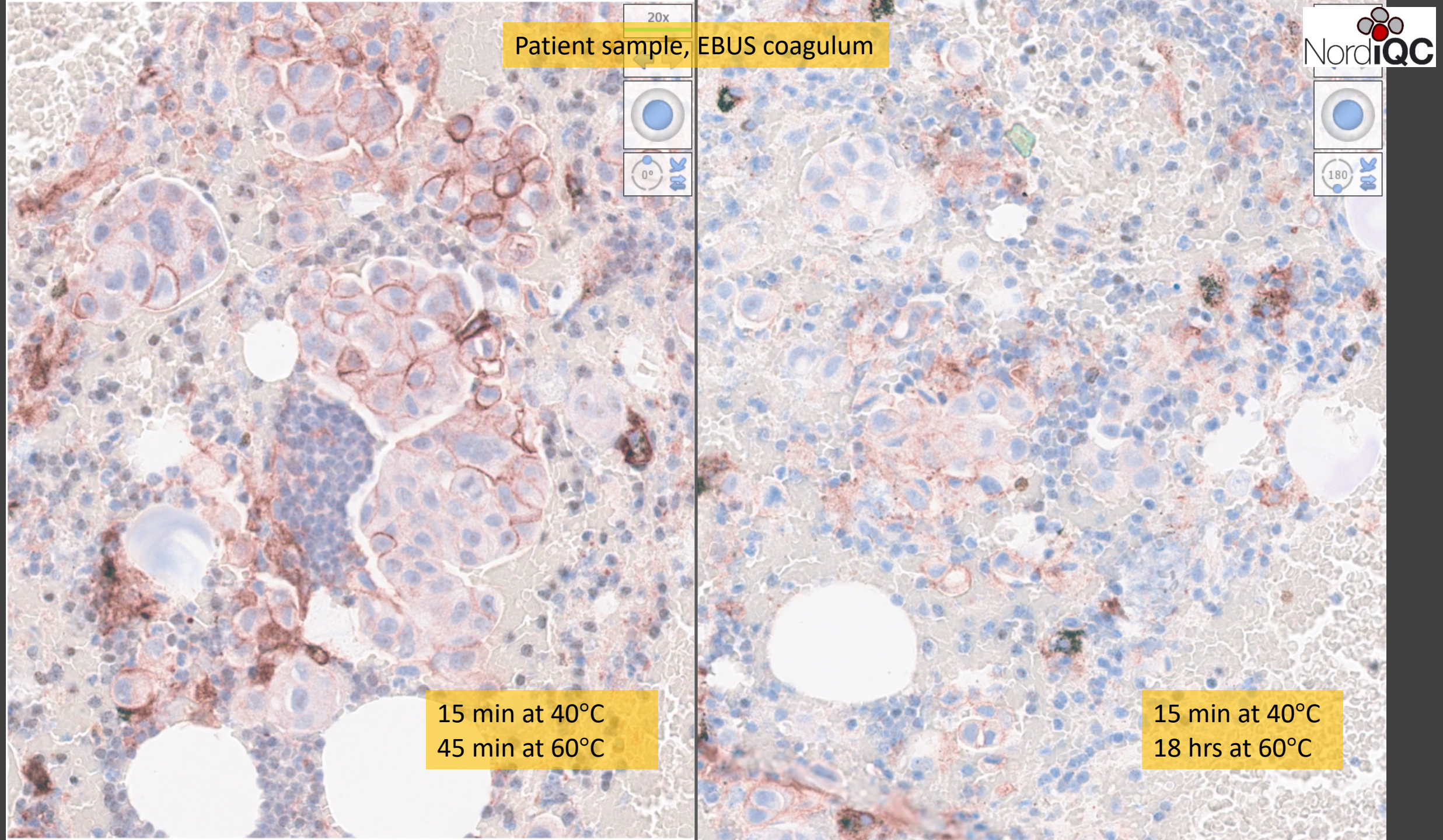
16 hrs at 80°C

Photos by Ole Nielsen


PD-L1 cell line from Histocyte, high
15 min at 40°C
45 min at 60°C
15 min at 40°C
18 hrs at 60°C

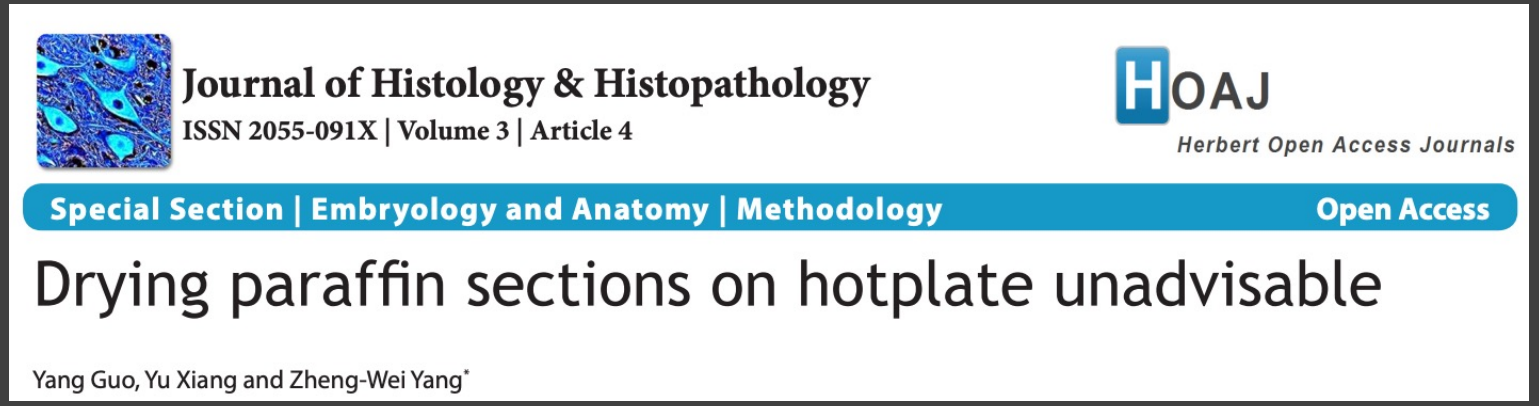

22c3.15.tør_45min.varme
PD-L1 cell line from Histocyte, low
15 min at 40°C
45 min at 60°C
15 min at 40°C
18 hrs at 60°C


Placenta, normal tissue
15 min at 40°C
45 min at 60°C
15 min at 40°C
18 hrs at 60°C


Patient sample, EBUS coagulum
15 min at 40°C
45 min at 60°C
15 min at 40°C
18 hrs at 60°C

Drying of sections

Preanalytical variable	Published Guidelines and Recommendations	Literature-Based Recommendations	Aarhus University Hospital
Drying of sections	ASCO/CAP | CLSI 24 hrs at RT or 1 hr at 50°C - 60°C	Engel KB, Moore HM. Arch Pathol Lab Med. 2011;135:537–543 24 hrs at RT or overnight at 37°C	15 min at 40 °C in circulating oven, then 45 min at 60°C



Journal of Histology & Histopathology
ISSN 2055-091X | Volume 3 | Article 4

HOAJ
Herbert Open Access Journals

Special Section | Embryology and Anatomy | Methodology — Open Access

Drying paraffin sections on hotplate unadvisable

Yang Guo, Yu Xiang and Zheng-Wei Yang*

"Taken together, we consider it unadvisable to dry paraffin sections (freshly floated onto slides from water bath) on hotplate or at a horizontal position. When the drying temperature is high, the section will be destructed and compressed; when the temperature is low, there will be no drying effect or that the section maybe deformed."

Modern Pathology (2004) 17, 1414–1420

www.modernpathology.org

Influence of slide aging on results of translational research studies using immunohistochemistry

Martina Mirlacher, Marlis Kasper, Martina Storz, Yvonne Knecht, Ursula Dürmüller, Ronald Simon, Michael J Mihatsch and Guido Sauter

Fresh sections (F) vs. sections stored at 4°C for 6 months (O)

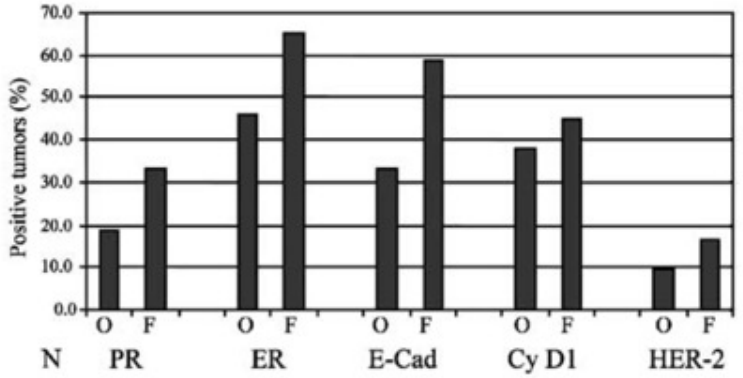


Figure 2 Influence of slide aging on the fraction of positive cases. For each antibody, the frequency of positive cases is shown as separate bars for old (O) and fresh (F) sections.

8.2 Cut section storage recommendation

To preserve antigenicity, tissue sections, once mounted on slides, should be held in the dark at 2-8 °C (preferred), or at room temperature up to 25 °C. Slide storage and handling conditions should not exceed 25 °C at any point post-mounting to ensure tissue integrity and antigenicity.

8.2.1 NSCLC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 ºC (preferred), or at 25 ºC.

8.2.2 Urothelial carcinoma cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 °C (preferred), or at 25 °C.

8.2.3 Esophageal cancer cut section storage recommendation

Cut sections must be stained within 4.5 months when stored at 2-8 °C (preferred), or within 1 month when stored at 25 °C.

8.2.4 HNSCC cut section storage recommendation

Cut sections must be stained within 6 months when stored at 2-8 ºC (preferred), or within 4 months when stored at 25 ºC.

8.2.5 TNBC cut section storage recommendation

Cut sections must be stained within 7.5 months when stored at 2-8 °C (preferred), or within 4 months when stored at 25 °C.

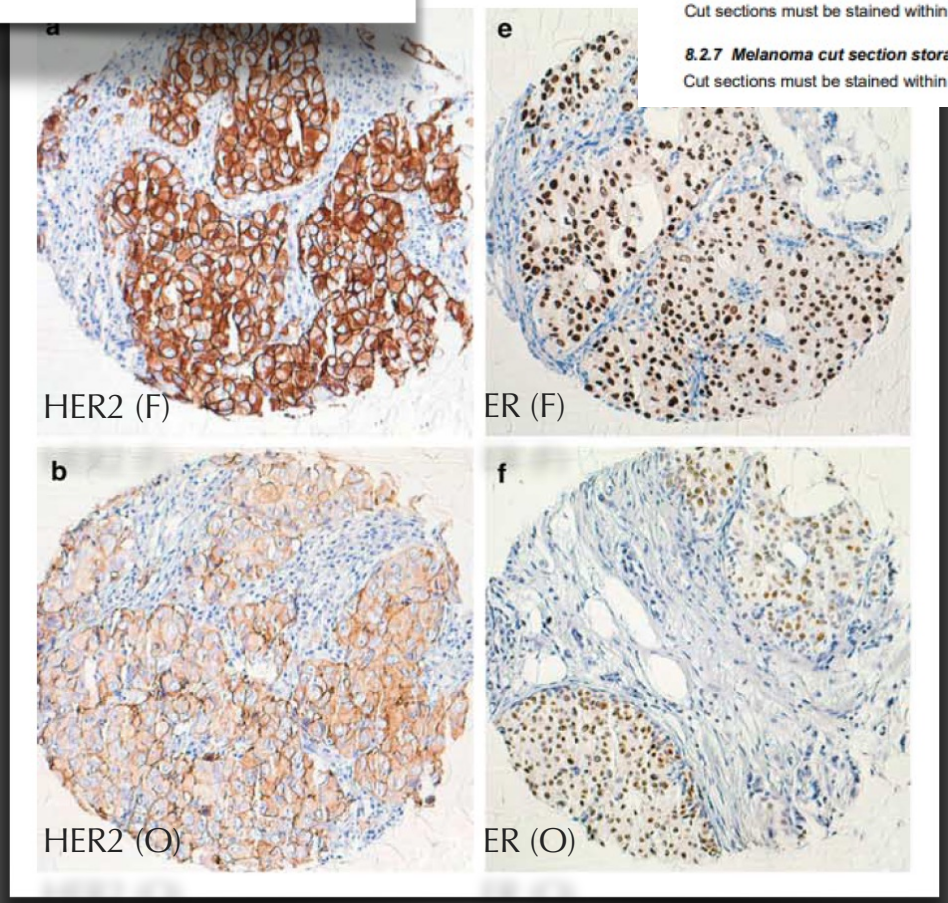
8.2.6 Cervical cancer cut section storage recommendation

Cut sections must be stained within 2 months when stored at 2-8 ºC (preferred), or within 1 month when stored at 25 ºC.

8.2.7 Melanoma cut section storage recommendation

Cut sections must be stained within 4 months when stored at 2-8 ºC (preferred), or within 2 months when stored at 25 ºC.

Agilent



NordiQC

Controls in storage

Negative factors influencing antigen preservation in cut sections

- Time
- Temperature
- Water amount in slide
- Moist / humidity in room
- Light

All with negative effects

Storage time	Storage temp.
Days	Room temp.
Weeks	4°C
Months	-20°C
Years	-80°C
Cut sections, mount on charged slides and dry overnight or up to 48 hours and store in closed boxes without baking. Immediately before IHC bake 30-60 min at 60°C	

Paraffin coating of single slides or
Paraplast sealing of boxes have not proven to be efficient

Is there an expiration date on the tissue blocks?

Loss of antigenicity with tissue age in breast cancer

Susan E Combs[1], Gang Han[1], Nikita Mani[1], Susan Beruti[2], Michael Nerenberg[3] and David L Rimm[1]

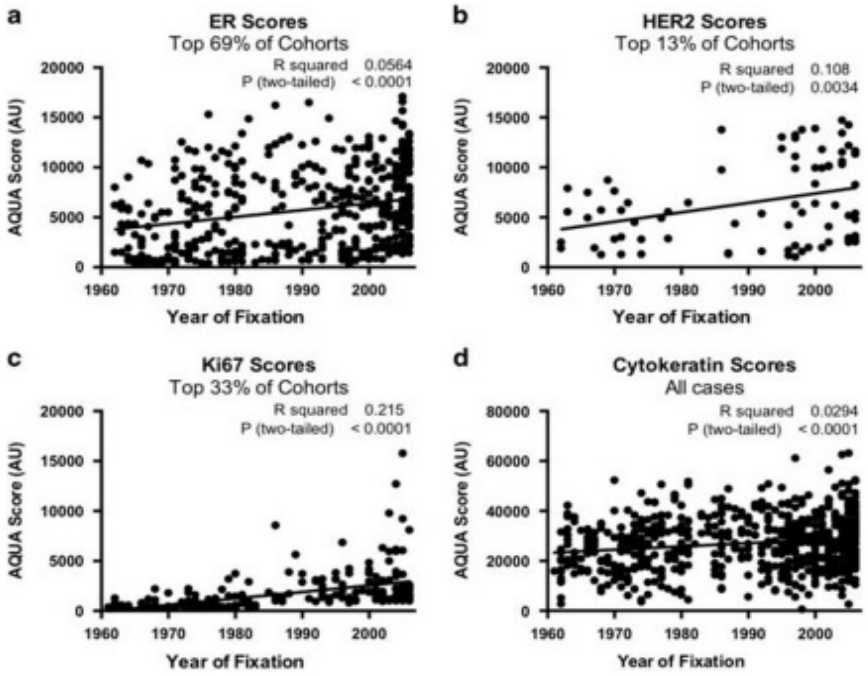


Figure 2 The distribution of scores for each biomarker as a function of tissue age after omitting the fraction of expected negative cases. (**a**) ER, (**b**) HER2, (**c**) Ki67 and (**d**) cytokeratin. The fraction of positive cases is shown by percentage beneath the biomarker in the title. The regression value and *P*-value are presented in the insets. Au, arbitrary unit.

The average signal decreased with preservation time for all biomarkers measured. For ER and **HER2**, there was an average of 10% signal loss after 9.9 years and **8.5** years, respectively, compared with the most recent tissue. Detection of **Ki67** expression was lost more rapidly, with **10% signal loss in just 4.5 years**. Overall, these results demonstrate the need for adjustment of tissue age when studying FFPE biospecimens. **The rate of antigenicity loss is biomarker specific and should be considered as an important variablefor studies using archived tissues.**

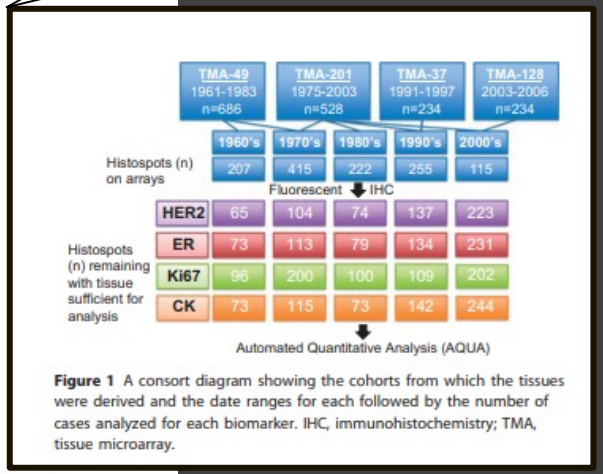


Figure 1 A consort diagram showing the cohorts from which the tissues were derived and the date ranges for each followed by the number of cases analyzed for each biomarker. IHC, immunohistochemistry; TMA, tissue microarray.

Correlation between PD-L1 expression and clinicopathological characteristics of non-small cell lung cancer: A real-world study of a large Chinese cohort

J Thorac Dis 2019;11(11):4591-4601

Yan Jin[1,2], Xuxia Shen[1,2], Yunjian Pan[2,3], Qiang Zheng[1,2], Haiquan Chen[2,3], Hong Hu[2,3#], Yuan Li[1,2#]

The surgical resection group consisted of 827 recently resected and 329 archived (>5 years old) NSCLC samples

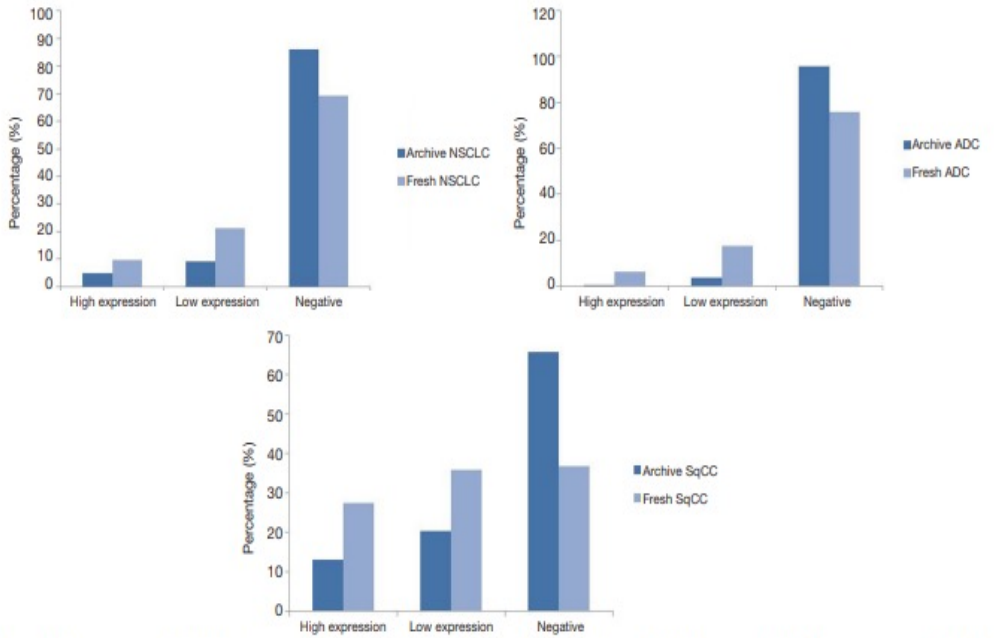
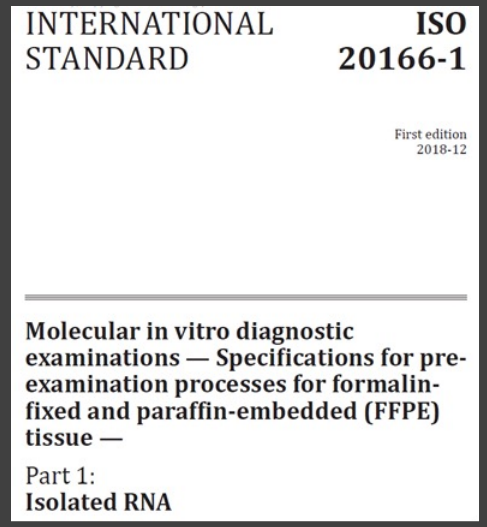
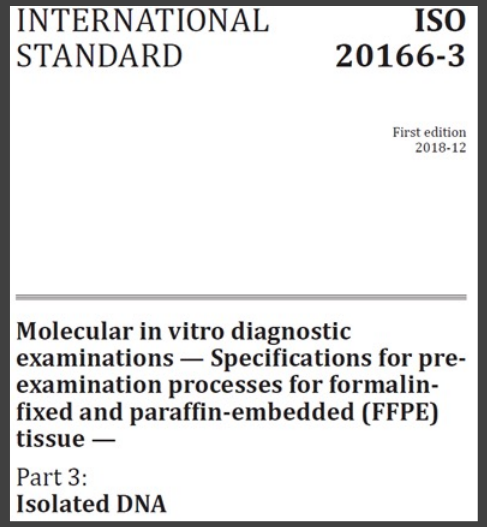


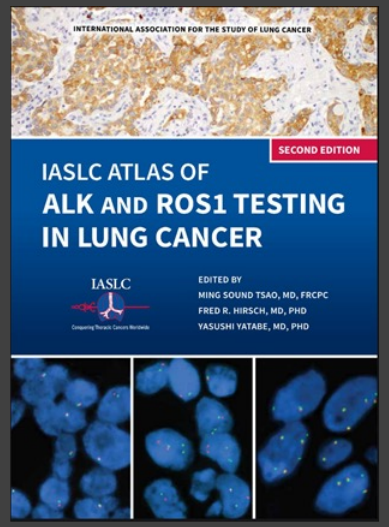
Figure 2 Comparison of PD-L1 expression in recently acquired samples and archived NSCLC samples. PD-L1, programmed death ligand-1; NSCLC, non-small cell lung cancer; ADC, adenocarcinoma; SqCC, squamous cell carcinoma.

PD-L1 high expression was observed in 9.7% of 827 NSCLC patients, including 6.5% with adenocarcinoma (ADC, n=690), and 27.4% with squamous cell carcinoma (SqCC, n=117). These results showed higher expression rates than those in archived samples (>5 years old, n=329).

Need to secure IHC testing quality – Guidelines pre-analytics







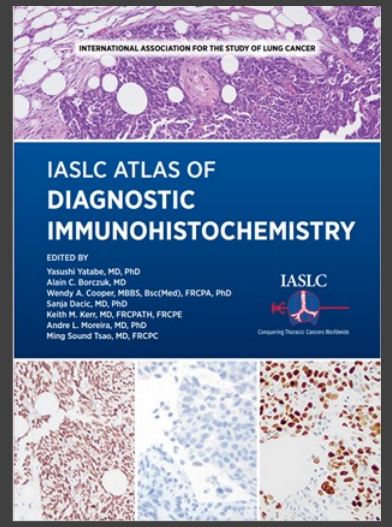


Table 2. Guidelines for core pre-analytical procedures for tissue from international and national authorities

Pre-analytical step	ASCO/CAP*	IASLC**	ISO/TC 212***
Biomolecule/method	ER-, PR-, HER2-IHC	PD-L1-IHC	Isolated DNA, RNA
Ischemic time	60 min. or less.	30 min. or less	Avoid or as short as possible
Type of fixative	10% NBF	10% NBF	10% NBF
Time in fixative	6-72 hours	6-48 hours	12-24 hours
Tissue thickness/fixative ratio	5 mm/-	-/10:1	5 mm/4-10:1
Storage time/temp. for slides	6 weeks at RT#	8 weeks at RT#	Avoid/short at 2-8°C
Storage time/temp. for blocks	-	3 years/2-8°C or RT#	/2-8°C or RT#

* American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), ** International Association for the Study of Lung Cancer (IASLC), *** European Committee for Standardization, ISO 20166, # Room temperature

Artificial Intelligence Applications in Human Pathology
Huss et al; S Nielsen Stain Quality Management & Biomarker Analysis https://doi.org/10.1142/9781800611399_0004

That was Preanalytical – important?

Special thanks to the wizards of Immunohistochemistry

- Ole Nielsen, DK
- Michael Bzorek, DK
- Søren Nielsen, DK