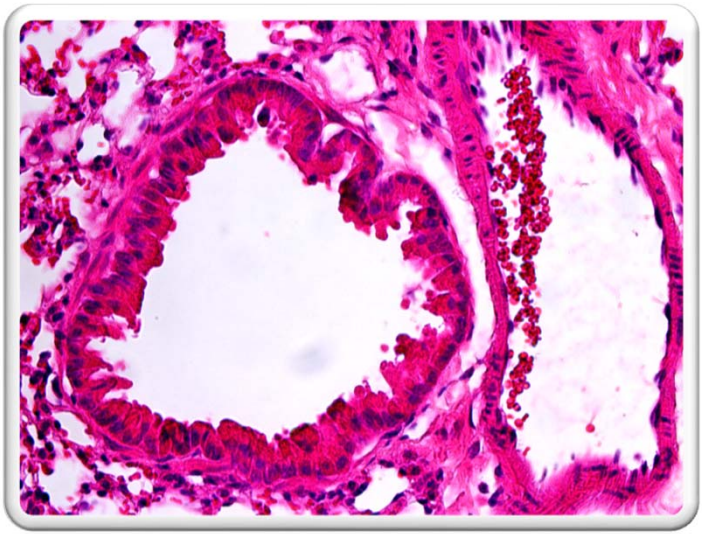


Institute of Molecular Biology

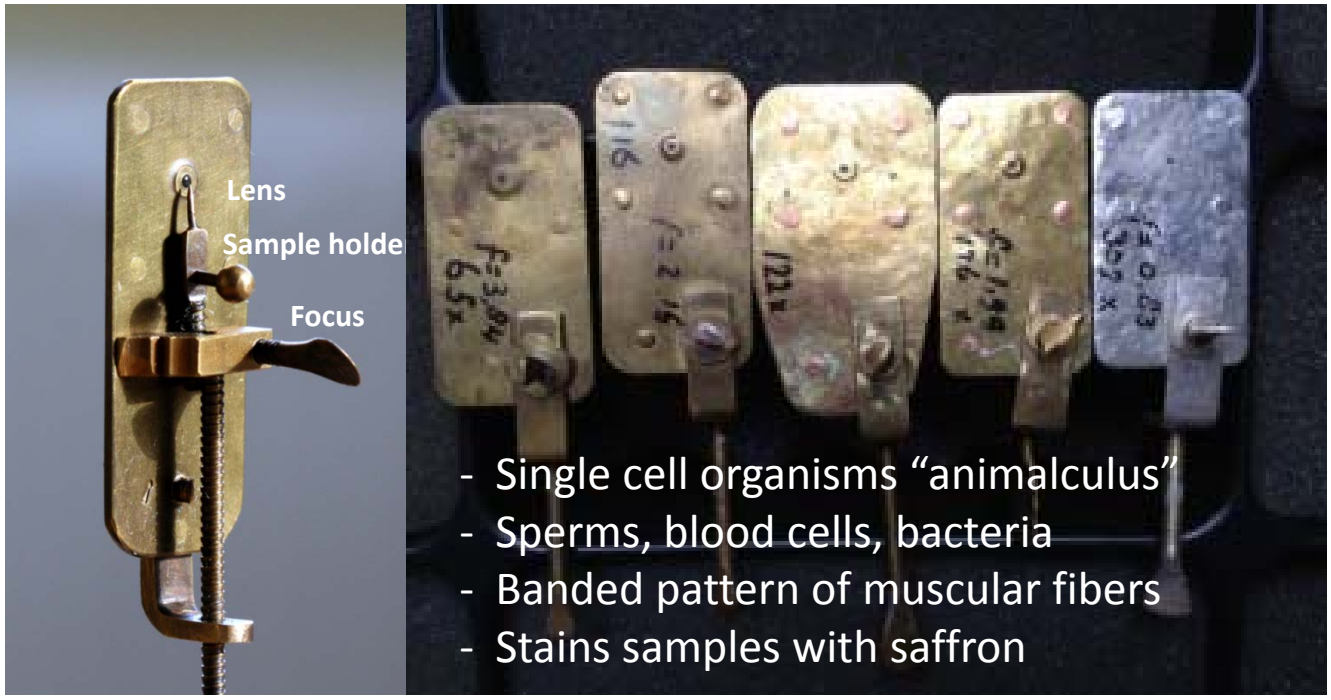
HISTOLOGY AND TISSUE STAINING

Modern Techniques in Life Sciences

09.05.2017, Sandra Ritz

Microscopy & Histology & Staining

- Greek: ἱστός *histos* „tissue“ und -logy, gr. λόγος *logos* „study of“
- Marcello Malpighi (1628-1694): Malpighian tubules, Malpighian corpuscles…)
- Antonie van Leeuwenhoek (1632-1723): magnifying lens, dyes



Jeroen Rouwkema - http://www.flickr.com/photos/rouwkema/2262158965/

Microscopy & Histology & Staining

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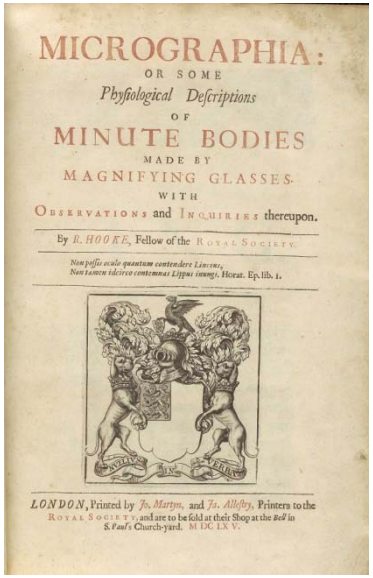
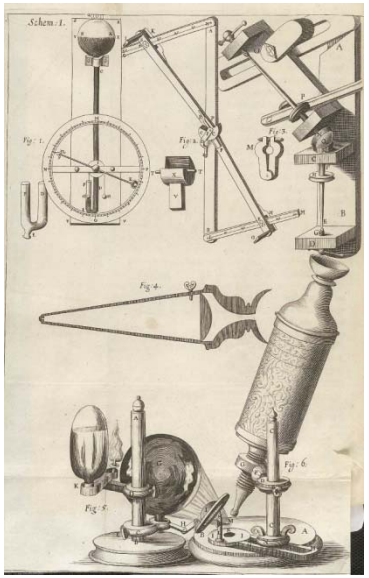
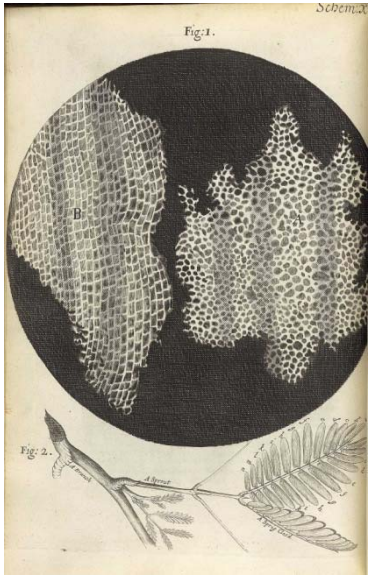
- **Robert Hooke (1635-1703): *Micrographia* (1665)**
- **1832: Matthias Schleiden and Theodor Schwann: Cell theory „All plant and animal tissues are composed of cells.“**


MICROGRAPHIA:
OR SOME
Physiological Descriptions
OF
MINUTE BODIES
MADE BY
MAGNIFYING GLASSES.
WITH
OBSERVATIONS and INQUIRIES thereupon.
By R. HOOKE, Fellow of the ROYAL SOCIETY.
Non possis oculo quantum contendere Lynceus,
Non tamen idcirco contemnas Lippus inungi. Horat. Ep. lib. 1.
LONDON, Printed by Jo. Martyn, and Ja. Allestry, Printers to the ROYAL SOCIETY, and are to be sold at their Shop at the Bell in S. Paul's Church-yard. MDCLXV.





Microscopy & Histology & Staining

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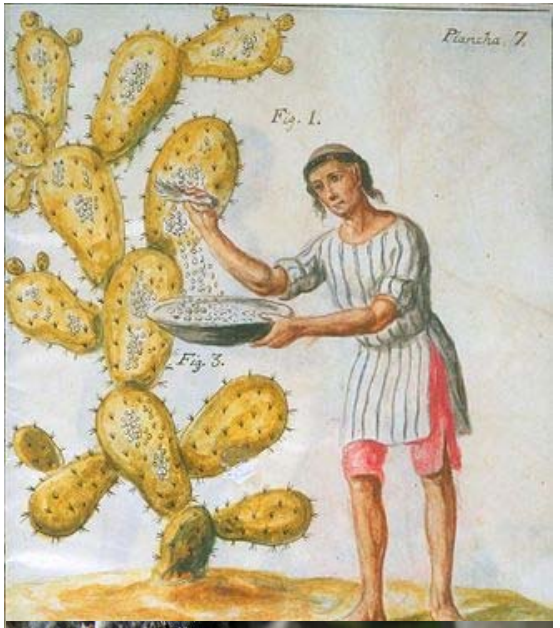
- **Joseph von Gerlach (1820-1896): carmine/gelatin mixture for staining**
- **natural stainings: saffron, carmine/cochineal (E120), hematoxylin**




Cochineal *(Dactylopius coccus)*

Microscopy & Histology & Staining

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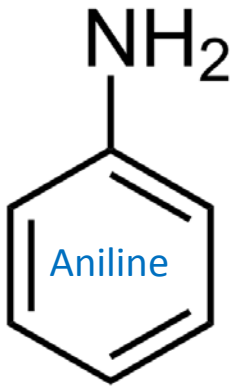
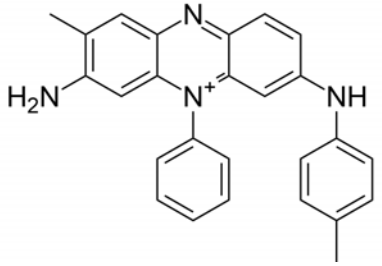
- natural stainings: saffron, carmine/cochineal
- **19th century: WH Perkin synthesized the first synthetic aniline dye „aniline purple or Mauveine“ (Malve) extracted from coal tar (Steinkohlenteer).**



Aniline



„Mauveine“
or
aniline purple

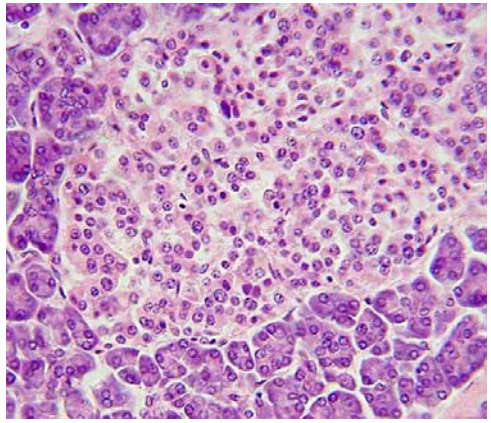
https://de.wikipedia.org/wiki/Cochenilleschildlaus#/media/File:Indian_collecting_cochineal.jpg

Histology/Histopathology today

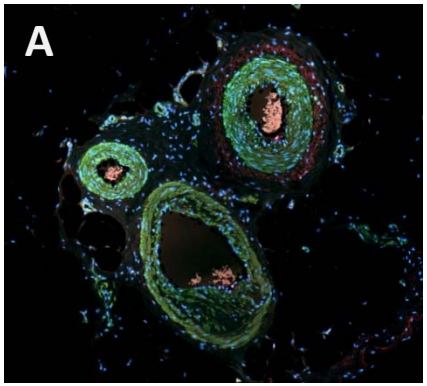
Depends entirely on the experiment. E.g.:

- Which molecules are you interested in?
- What kind of tissue are you using?
- Do you want to detect active enzymes?
- Does your AB work in fixed tissue?

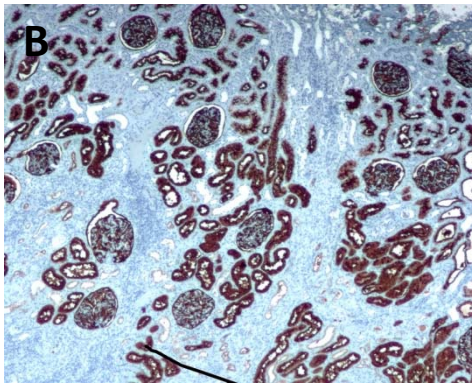
HE



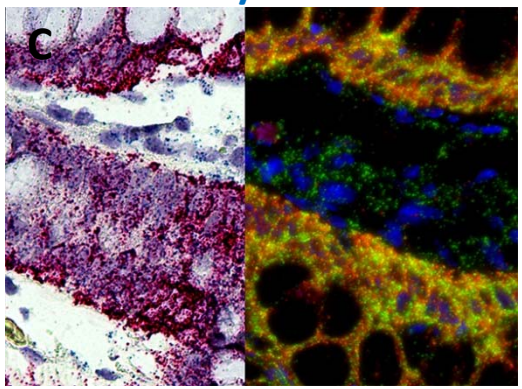
IF



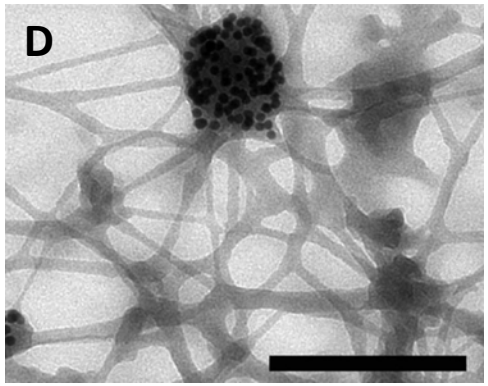
IHC



ISH/FISH



EM



HE: Pankreas by user:Polarlys - Eigenes Werk, CC BY 2.5, https://commons.wikimedia.org/w/index.php?curid=1032935
A: Porcine blood vessels by Alizee Pathology LLC. - http://alizeepathology.com/samples/1, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=33313176
B: Kidney IHC agianst CD10, by Nephron - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=5961392
C: ISH and FISH, by Ryan Jeffs - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=21438920
D: Immunogold for mtDNA, by Francisco J Iborra1 , Hiroshi Kimura2 and Peter R Cook - The functional organization of mitochondrial genomes in human cells, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=9085139

Histology

Tissue (from organ preparation/biopsy)

↓

Fixation/snap freezing/fixing and freezing

↓

Sectioning

↓

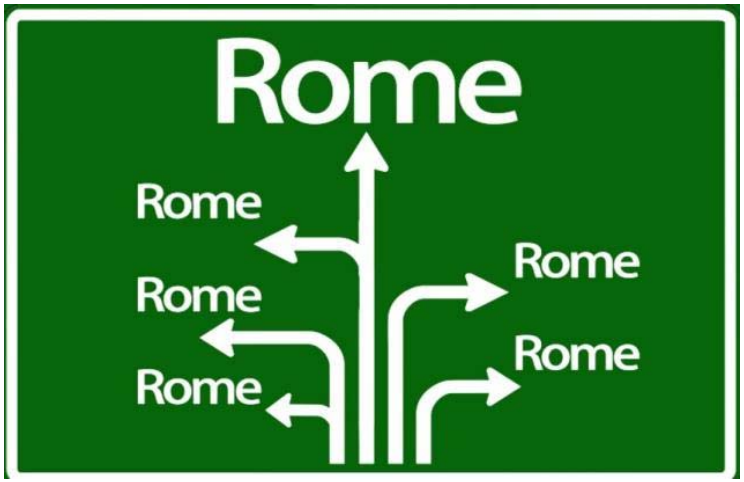
Staining

↓

Mounting

↓

Microscopy



https://www.sciencedump.com/sites/default/files/styles/article_width/public/field/image/Schermafbeelding%202015-12-16%20om%2021.28.07.jpg?itok=GCsDueTC

Which molecules/structures do I want to preserve?

- **proteins:** precipitation and crosslinking
- **nucleic acids:** precipitation and crosslinking
- **polysaccharides:** indirect fixation through crosslinking of proteins
- **lipids:** often extracted
- **small molecules:** go live!
- **3D structure:** chemical fixation, freezing and chemical fixation

….there are four general fixation methods:

- drying
- heating (55°C)
- freezing
- chemical fixation

Chemical fixation (i): coagulating fixatives

- alcohol (methanol, ethanol, 99%)
- acetone
- salt (ammonium sulfate)

Hydration state of molecules are changed

+

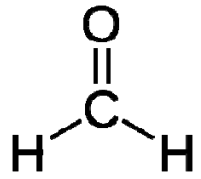
- fast penetration
- the antigen recognition sites for immunolabeling are preserved very well
- works nice for microtubules

-

- the specimen shrinks almost 50%
- morphology is poorly preserved
- extraction of **lipids/membrane proteins**
- incompatible with **phalloidin staining for actin fibers**

Chemical fixation (ii): crosslinking fixatives

- Aldehydes
  - Formaldehyde



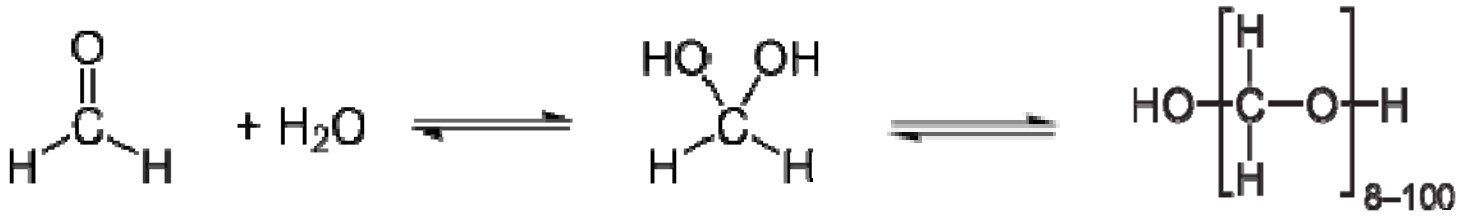
  - Glutaraldehyde

- Ethylene Glycol bis-Succinimidyl Succinate (EGS)

  (reversible; the crosslinks are cleaved at a pH 8.5. It is useful for membrane-bound proteins but it is not good soluble in water)

Covalent crosslinks that are determined by the active groups in each compound.

Formaldehyde/Formalin/Paraformaldehyde



formaldehyde
(gas)
Mw 30 g/mol

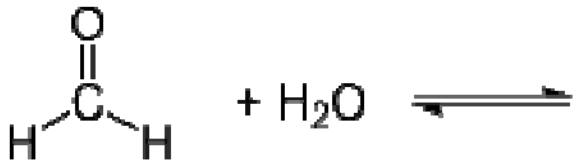
methylene hydrate
(solved in water)
(formalin)

paraformaldehyde
(solid)

Most widely used fixative in histology

- good tissue penetration (~1mm/h)
- penetration-fixation paradox

Formaldehyde/Formalin/Paraformaldehyde



formaldehyde
(gas)
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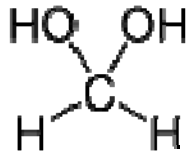
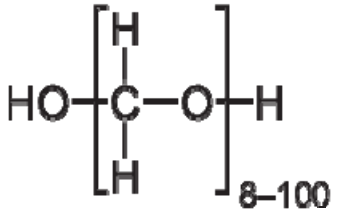


methylene hydrate
(solved in water)
(formalin)



paraformaldehyde
(solid)

	Formalin (100%)	Paraformaldehyde
form	40% (v/v) or 37% (w/v) formaldehyde in water contains stabiliz… meth…	insol… le white powder
degree of polymerization		n = up to 100
depolymerization	directly when diluted at pH 7.4	pH 7.2-7.6 and heating (65°C)

10% formalin = 4% formaldehyde (1.3 molar)

Formaldehyde fixation

Formaldehyde solution needs to be fresh!

Prepare from Paraformaldehyde:

Paraformaldehyd (4%)

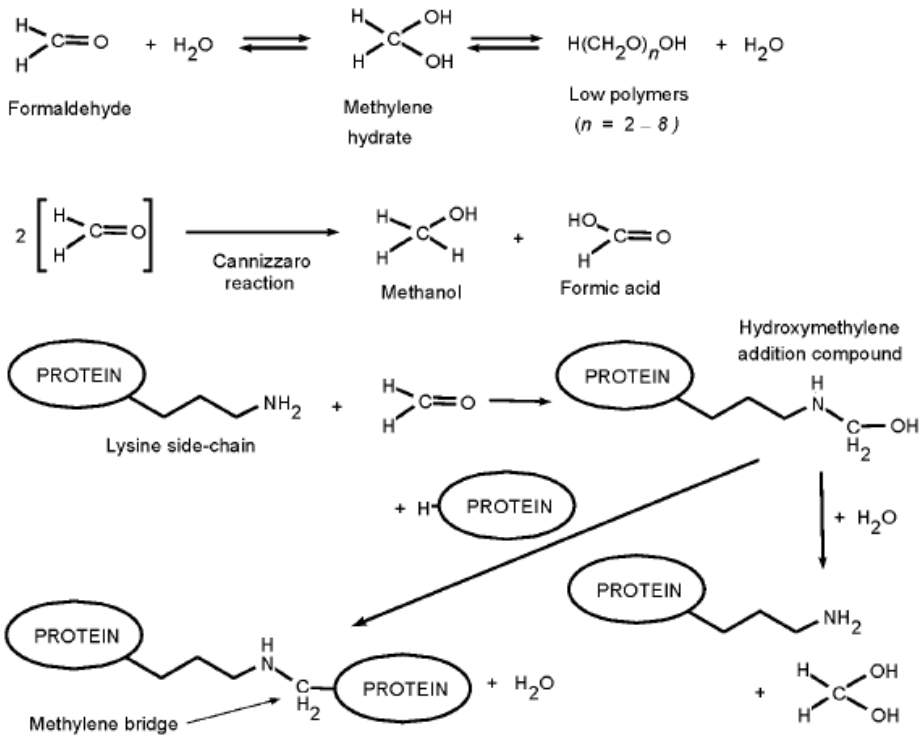
- Mix 40 g PFA (Sigma) with 500 mL ultrapure water
- Add 10 mL 1 M NaOH and heat to 65°C (magnetic stirrer under fume hood)
- Add 100 mL 10xPBS and cool down to room temperature
- Adjust pH 7.4 (1M HCL ; 1mL);
- Fill to 1000 mL with ultrapure water
- Sterile filtration (0.45 µm Filter)
- Store aliquots at -20°C (ca. 6 month)

Quenching solution

50 mM Tris-Cl (pH 8.0)

100 mM NaCl

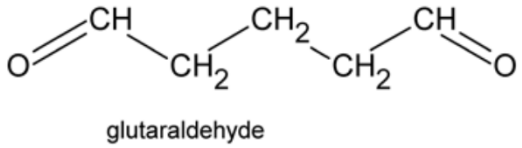
Formaldehyde fixation reaction



- Formaldehyde crosslinks proteins by forming methylene bridges (-CH_2-) between reactive groups (**protein-protein** or **protein-nucleic acid** (C, A, G, no T)
- Does not crosslink lipids.
- Labile structures such as microtubules might not be well preserved.
- Fixation is partially reversible: heat induced antigen retrieval (HIER).

Kiernan JA. Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do. Microscopy Today. 2000;1:8–12.

Glutaraldehyde

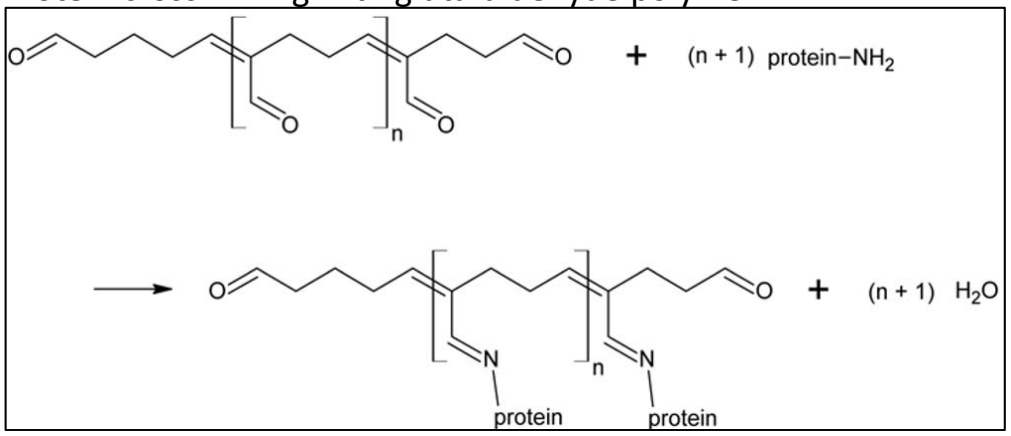


glutaraldehyde

Mw 100.12 g/mol

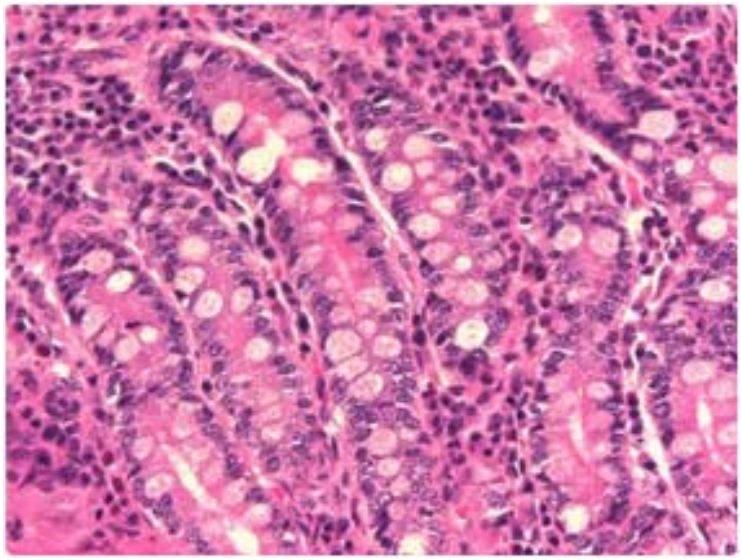
Protein cross-linking with glutaraldehyde monomer

Protein cross-linking with glutaraldehyde polymer

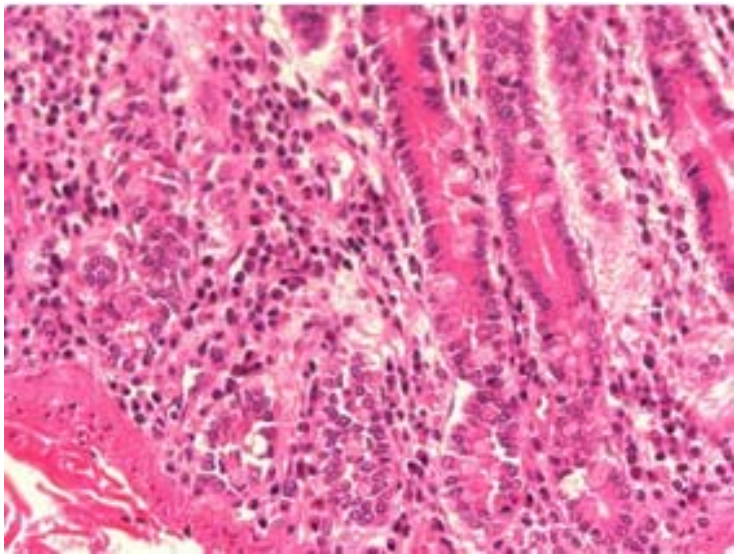


- Introduced 1962 as EM staining (Sabatini et al.)
- Bifunctional crosslinker
  small enough to penetrate tissue/ slower than formaldehyde)
- Present in aqueous solutions as monomers and polymers of variable size

Fixation causes artifacts



Neutral buffered formalin fixation



95% ethanol fixation

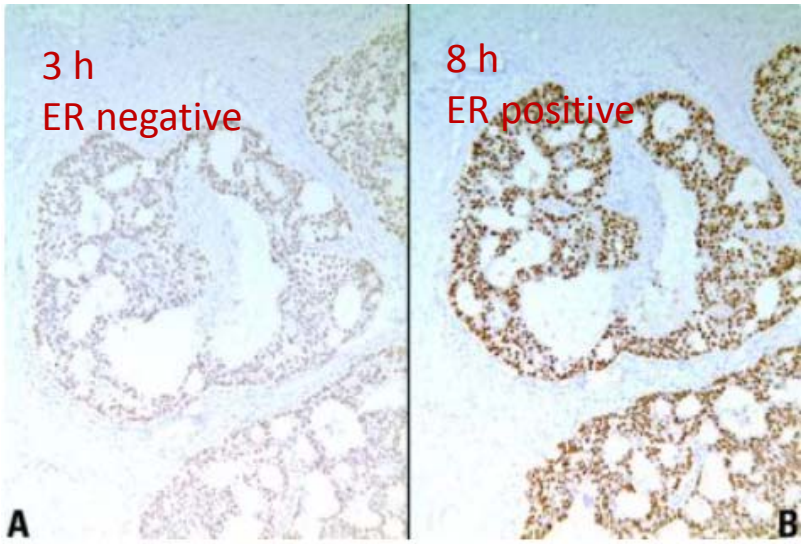
A paraffin section from the mucosa of small intestine imaged at same magnification.

- Shrinkage (sometimes also swelling)
- Diffusion of unfixed material (streaming)
- Improper fixation
- Solubilization of hydrophobic cell components
- Loss of protein tertiary/secondary structure
- Antigen masking
- DNA/RNA: low read length, bad probe binding

Some artifacts can be avoided, some reverted, some can be compensated…

Some important points…

Fixation in neutral buffered formalin(NBF, 10%)



Estrogen receptor (ER) staining of breast carcinoma.

http://www.leicabiosystems.com/pathologyleaders/effects-of-fixation-and-tissue-processing-on-immunocytochemistry/
Effects of Fixation and Tissue Processing on Immunocytochemistry, Peter Jackson

Goldstein NS, Ferkowicz MT, Odish E, Mani A, Hastah F. Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. Am J Clin Pathol 120:86-93 2003.

No standards exist!
Recommentations from the College of American Pathologists for the fixation of breast tissue in NBF

- sample 3-4 mm
- minimum fixation time of 6-8 h
- maximum fixation time 48h (24h better to prevent overfixation)
- fixation volume 15-20 times higher than the bulk of the tissue
- NBF solution less than 1 month old
- storage of fixed samples in 70% ethanol until processing

Different kinds and combinations of fixatives

Fixative	Method of fixation	Contents
B5	Denaturing	5.4% Mercuric Chloride (w/v), 1.1% Sodium Acetate (w/v), 4% Formaldehyde (v/v), Water
Bouin's	Denaturing, cross-linking	25% of 37% formaldehyde solution, 70% picric acid, 5% acetic acid
Carnoy's	Denaturing	60% ethanol, 30% chloroform, 10% Glacial acetic acid
Glutaraldehyde	Cross-linking	Generally, 2% v/v of glutaraldehyde to water/PBS
Methacarn	Denaturing	60% methanol, 30% chloroform, 10% Glacial acetic acid
Neutral buffered formalin (NBF)	Cross-linking	10% of 37% formaldehyde solution, in a neutral pH
Paraformaldehyde (PFA)	Cross-linking	Generally, 4% w/v of paraformaldehyde to Water/PBS
Zenker's	Denaturing	5% Mercuric Chloride (w/v), 2.5% Potassium Dichromate (w/v), 5% Glacial acetic acid (v/v), Water

RNA read lengths following tissue fixation for RTPCR.

Fixative	Read lengths DNA	Read lengths RNA
HOPE	600 bp [54]	300 bp [54]
Methacarn	1900 bp [35]	850 bp [35], 463 bp [57]
PAXgene	571 bp [41]	712 bp [41]
RCL2	600 bp [58], 850 bp [57]	377 bp [58], 463 bp [57]
UMFIX/RTP	1.4 kb [20], 450 bp [19]	816 bp [20], 450 bp [19], 700 bp [59]
Z7	2400 bp[21]	361 bp [21]

Formaldehyde: ~200bp read length

Fixatives: Alternatives to NBF

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Fixative	Publication	Better in alternative fixative	As good as NBF	Better in NBF	Antibody type[a]	Optimised for alternative?	Scoring[b]
AFA	Nietner et al. [24]	1/3		2/3	C	Y	SQ
Bouin's	Mitchell et al. [45]	3/4	1/4		C	N	SQ
Carnoy's	Mitchell et al. [45]	3/4	1/4		C	N	SQ
Ethanol	Gillespie et al.[59]		1/1		C	N	V
FineFix	Kothmaier et al. [51]	2/5	3/5		C/R	N	SQ
	Nykanen et al. [52]		2/2		C	N	Q
	Paavilainen et al. [25]			72/72	C/R	N	SQ
Glyo-fix	Paavilainen et al. [25]			72/72	C/R	N	SQ
Histochoice	Vince et al. [53]	4/21	15/21	2/21	C	N	Q
HOPE	Kothmaier et al. [51]	3/5	2/5		C/R	N	SQ
	Braun et al. [54]		3/3		C/R		V
	Goldmann et al. [55]		4/4		C	Y	V
Methacarn	Mitchell et al. [45]	3/4	1/4		C	N	SQ
	Delfour et al. [57]		10/10		C	N	SQ
Neo-Fix	Paavilainen et al. [25]			72/72	C/R	N	SQ
PFA (4%)	Burns et al. [43]		8/10	2/10	R(ph)	N	SQ
PAXgene	Nietner et al. [24]	1/3		2/3	C	Y	SQ
	Belloni et al. [41]		5/10	3/10	C/R	Y	Q
	Staff et al. [56]		7/7		C	N	V
	Kap et al. [26]		33/33		C	Y	V
RCL2	Van Essen et al. [42]	10/85	51/85	24/85	C	N	SQ
	Kothmaier et al. [51]	1/5	3/5		C/R	N	SQ
	Staff et al. [56]		7/7		C	N	V
	Delfour et al. [57]		10/10		C	N	SQ
	Preusser et al. [58]		12/12		C	Y	V
Streck's	Burns et al. [43]	6/10	4/10		R(ph)	N	SQ
UMFIX/RTP	Nadji et al. [44]	23/70	42/70	5/70	C	Y	V
	Nassiri et al. [40]		2/2		C	Y	SQ
	Vincek et al. [19]		29/29	1/29	C	N	V
Z7	Lykidis et al. [21]	3/3			C	Y	SQ
	Staff et al. [56]		7/7		C	N	V
ZF	Paavilainen et al. [25]			72/72	C/R	N	SQ
ZBF	Paavilainen et al. [25]	10/72	62/72		C/R	N	SQ
	Wester et al. [23]	5/9	2/9	2/9	C	Y	SQ

ZBF – Zinc based fixative.

[a] C – clinical, R – research, (ph) – phospho.

[b] Q – quantitative, SQ – semi-quantitative, V – visual.

If AB performs poorly, maybe try a different fixative

Guidelines for choosing a fixative

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Table 3. Guidelines for choosing a fixative

Antigen	Fixative
Most proteins, peptides and enzymes of low molecular weight	Cells / cytological preparations: 4% formaldehyde Tissue sections: 10% Neutral-buffered formalin (NBF)
Delicate tissue	Bouin's fixative
Small molecules such as amino acids	4% formaldehyde
Blood-forming organs (liver, spleen, bone marrow)	Zenker's solution
Connective tissue	Helly's solution
Nucleic acids	Carnoy's solution
Large protein antigens (e.g., immunoglobulin)	Ice-cold acetone or methanol (100%)
Nuclear morphology	Zinc formalin
For electron microscopy	4% formaldehyde - 1% glutaraldehyde

http://docs.abcam.com/pdf/kits/immunohistochemistry-ihc-application-guide.pdf

Freezing: SNAP freezing

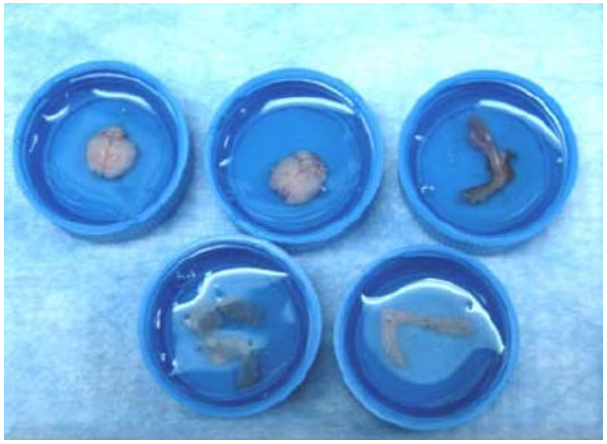
- tissue blocks (max. 1cm) or organs could be snap frozen in
  - Liquid N_2 (-196°C), use vapor phase to prevent freeze damage
  - Isopentane on dry ice bath (-80°C)
  - Isopentane on liquid nitrogen (ca. -150 to -160°C)

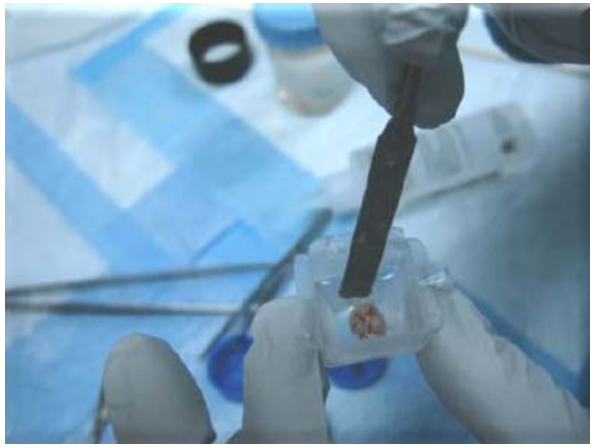
- remove residual isopentane with a paper
- place the sample in an ice-cold cryo-vial
- storage -80°C or liquid nitrogen

Images: Donna J. Emge, HT-ASCP, MHPL Manager

Fresh frozen tissue in OCT

- acclimate tissue to OCT (Optimum Cutting Temperature, TissueTek)
- OCT (10.24% polyvinyl alcohol, 4.26% polyethylene glycol, 85.5% non-reactive ingredients)
- cover freshly dissected tissue for a few minutes in OCT in a labeled small petri dish/ small weight boat
- transfer and orientate in fresh OCT (labeled cryomold, prevent bubles)
- sectioning surface is the bottom of the cryomold





Images: Donna J. Emge, HT-ASCP, MHPL Manager

Fresh frozen tissue in OCT

- start freezing
  - Liquid N_2 (-196°C), use vapor phase to prevent freeze damage
  - Isopentane on dry ice bath (-80°C)
  - Isopentane on liquid nitrogen (ca. -150 to -160°C)

- store on dry ice during processing
- wrap the cryomold in foil and cool down before storage at -80°C or liquid nitrogen



Images: Donna J. Emge, HT-ASCP, MHPL Manager

PFA fixed frozen sucrose protected tissue

- Perfusion with 4% PFA (~7.4 pH, 310mOsm) in PBS
- PFA fixation of fresh tissue for 6-8 h
- 3 x 10 min PBS wash
- 15% sucrose/PBS at 4°C until is sinks (ca. 30 min)
- 30% sucrose/PBS at 4°C until samples sinks (ca. 15 min)
- Partially fill a container with dry ice (small crushed dry ice if available)
- Label cryomolds and fill with OCT (TissueTek)
- Remove excess sucrose from tissue by blotting on Kimwipes and place tissue
- Orient tissue into the bottom of the well and transfer them to the dry ice
- Ready to be sliced after they are frozen completely or wraped in alu foil
- Storage at -80°C

Histology

Tissue (from organ preparation/biopsy)

↓

Fixation/snap freezing/fixing and freezing

↓

Sectioning

↓

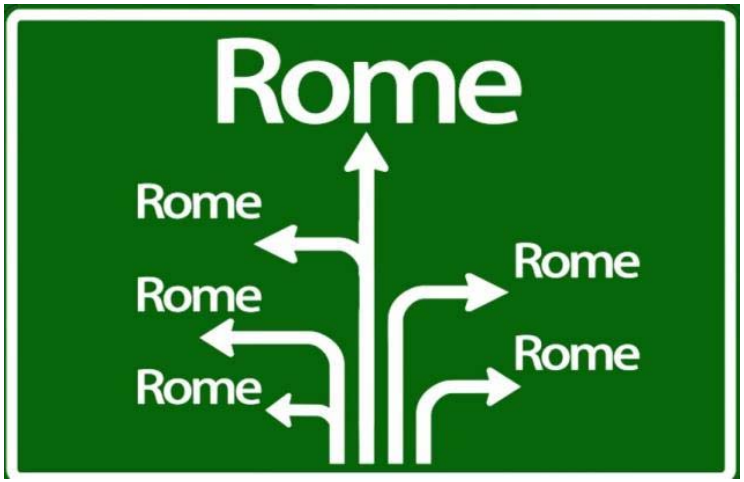
Staining

↓

Mounting

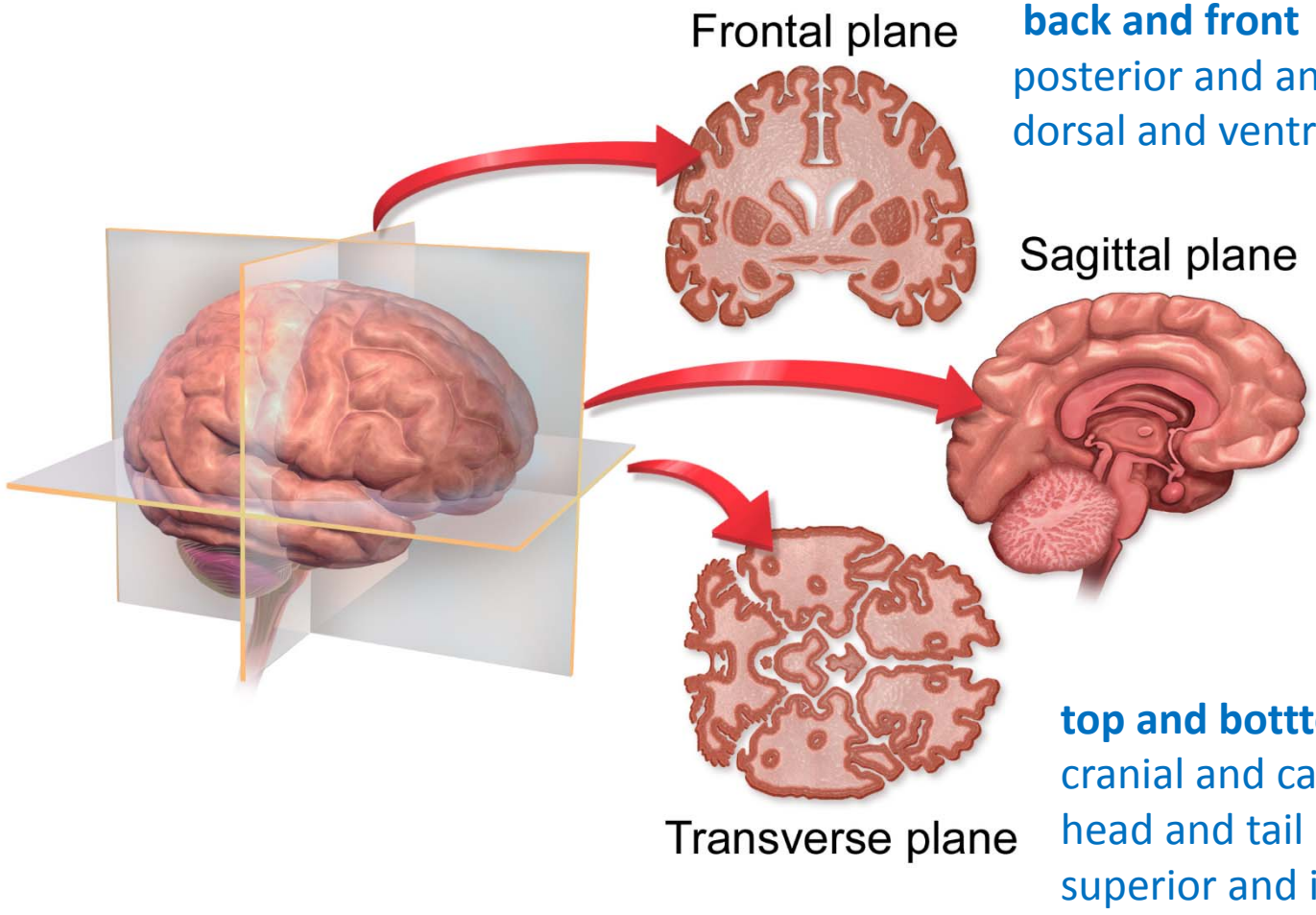
↓

Microscopy



https://www.sciencedump.com/sites/default/files/styles/article_width/public/field/image/Schermafbeelding%202015-12-16%20om%2021.28.07.jpg?itok=GCsDueTC

Sectional Planes or Anatomical Planes



= Coronal plane
back and front
posterior and anterior
dorsal and ventral

(lt. sagitta (arrow))
left and right

top and botttom
cranial and caudal
head and tail
superior and inferior

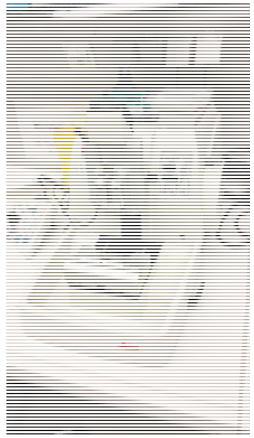
Blausen.com staff. "Blausen gallery 2014". *Wikiversity Journal of Medicine*. DOI:10.15347/wjm/2014.010. ISSN 20018762. -

Machines for sectioning

	Typical section thickness
➢ Microtome (LM)	3 - 10 µm
➢ Cryotome (LM)	3 - 10 µm
➢ Vibratome (vibrating microtome) (LM)	10 - 30 µm (fixed)
used for soft tissue	100 - 300 µm (living)
➢ Ultramicrotome (EM), glass or diamond blades	50 - 100 nm

Workflow: Paraffin Sections

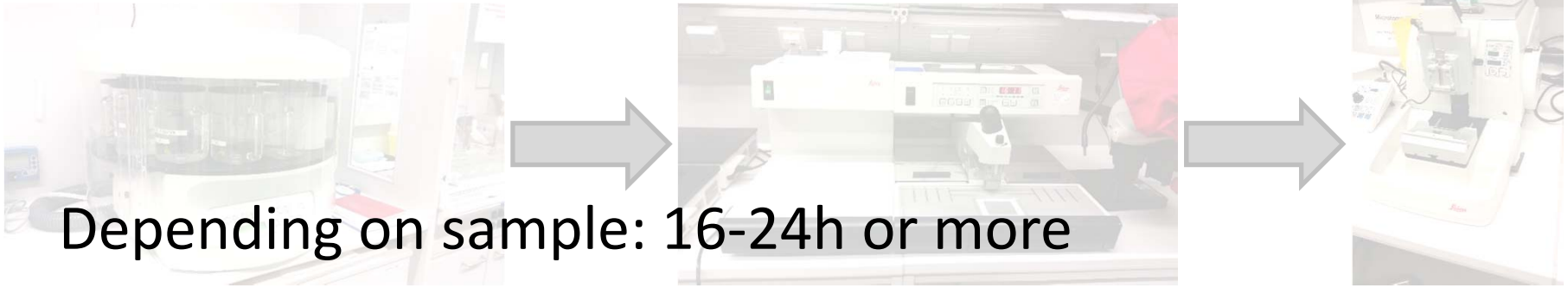
- Tissue blocks or organs are fixed (chemically)
- Dehydration and clearing: Ethanol series to Xylene
- Paraffin Embedding
- Sectioning
- De-waxing and rehydration for staining: Xylene, Ethanol series, staining
- Usually dehydration again. Mounting in Xylene based medium



Workflow: Paraffin Sections

- Tissue blocks or organs are fixed (chemically)
- Dehydration and clearing: Ethanol series to Xylene
- Paraffin Embedding
- Sectioning
- Rehydration for staining: Xylene, Ethanol series, staining
- Usually dehydration again. Mounting in Xylene based medium

➢ 3-10 µm sections



Depending on sample: 16-24h or more

Paraffin sections (fixed tissue)

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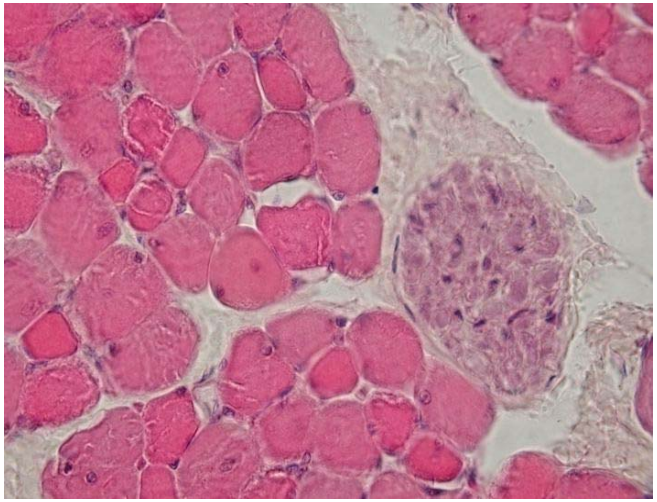
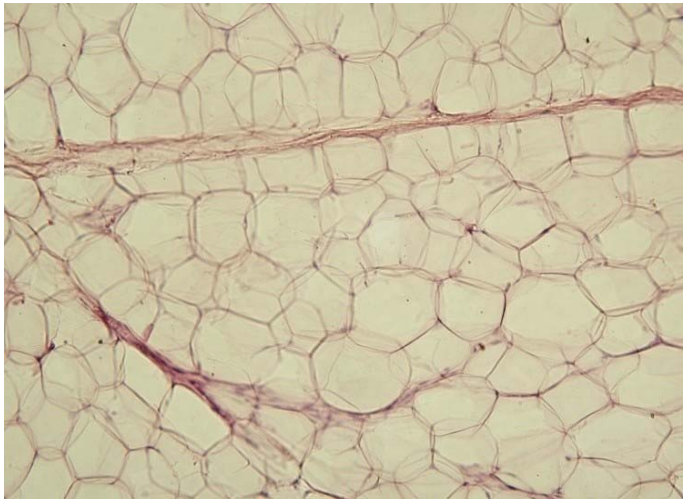
- Preservation (autolysis, putrefaction)
- Tissue can be stored for a long time
- Inactivation of pathogens
- Structure stabilization, good tissue morphology

-

- Can lead to antigen masking
- Antigen retrieval might be necessary
- Fixation artifacts
- Hydrophobic components like lipids will be lost





Skeletal muscle with nerv bundle: CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=657552
White adipose tissue: CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=600755

Workflow Frozen Sections

- put samples on wet ice, saline-dampened gauze, freeze within 5 -10 min for DNA/RNA isolation
- remove excess liquid on the outside (otherwise ice crystals!)
- tissue blocks (max. 1cm) or organs could be snap frozen in
  - Liquid N_2 (vapor phase)
  - Isopentane on dry ice

  place the sample in an ice-cold cryo-vial

  storage -80°C or liquid nitrogen
- mounting in cryo embedding medium (OCT, optimal cutting temperature), wrap the cryomold in foil and cool down before storage
- E.g. PFA fixation, 3 x 10 min PBS wash, 15% sucrose/PBS at 4°C until is sinks, 30% sucrose/PBS at 4°C until samples sinks
- Sectioning
- Staining or fixation + staining
- Mounting in H_2O based medium or dehydrate and proceed like for paraffin sections

10 minutes to 1 h

- 3-10 µm sections

View inside the cryotome working space

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Use a brush

Using the Anti-Roll-Plate

Frozen sections (fresh or frozen)

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- Fastest of all methods
- In general best for IHC
- Preservation of hydrophobic substances and antigens
- Presence of endogenous enzymes (enzyme assays)

-

- Poor morphology (compared to paraffin)
- Freezing artifacts
- Presence of endogenous enzymes
- Fresh tissue: prone to degradation

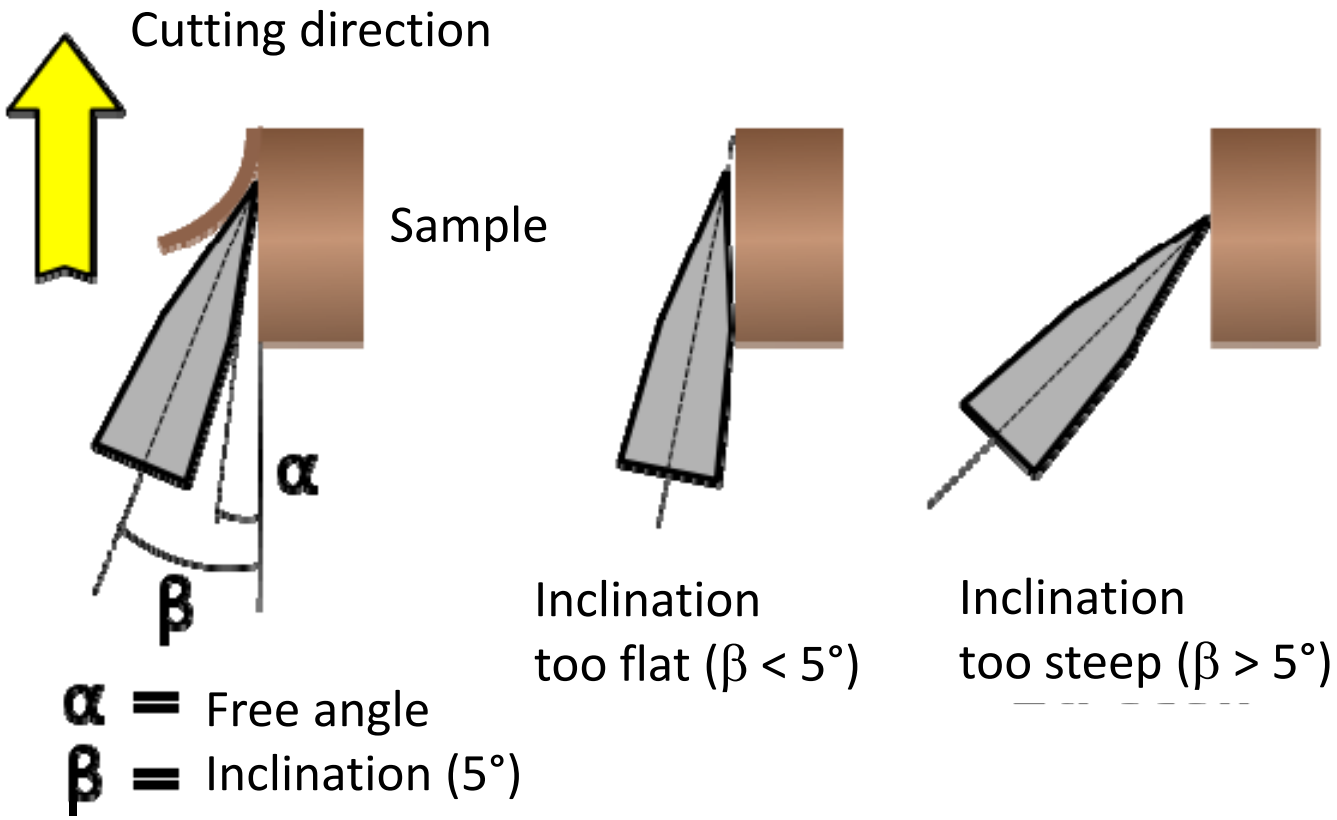
Vibratome

- 10 - 30 µm (fixed) used for soft tissue
- 100 - 300 µm (living)

- No dehydration
- No Paraffin embedding
- No high temperatures, no harsh chemicals
- Use of ordinary razor blades
- No paraffin or freezing artifacts
- Short time between tissue preparation and observation

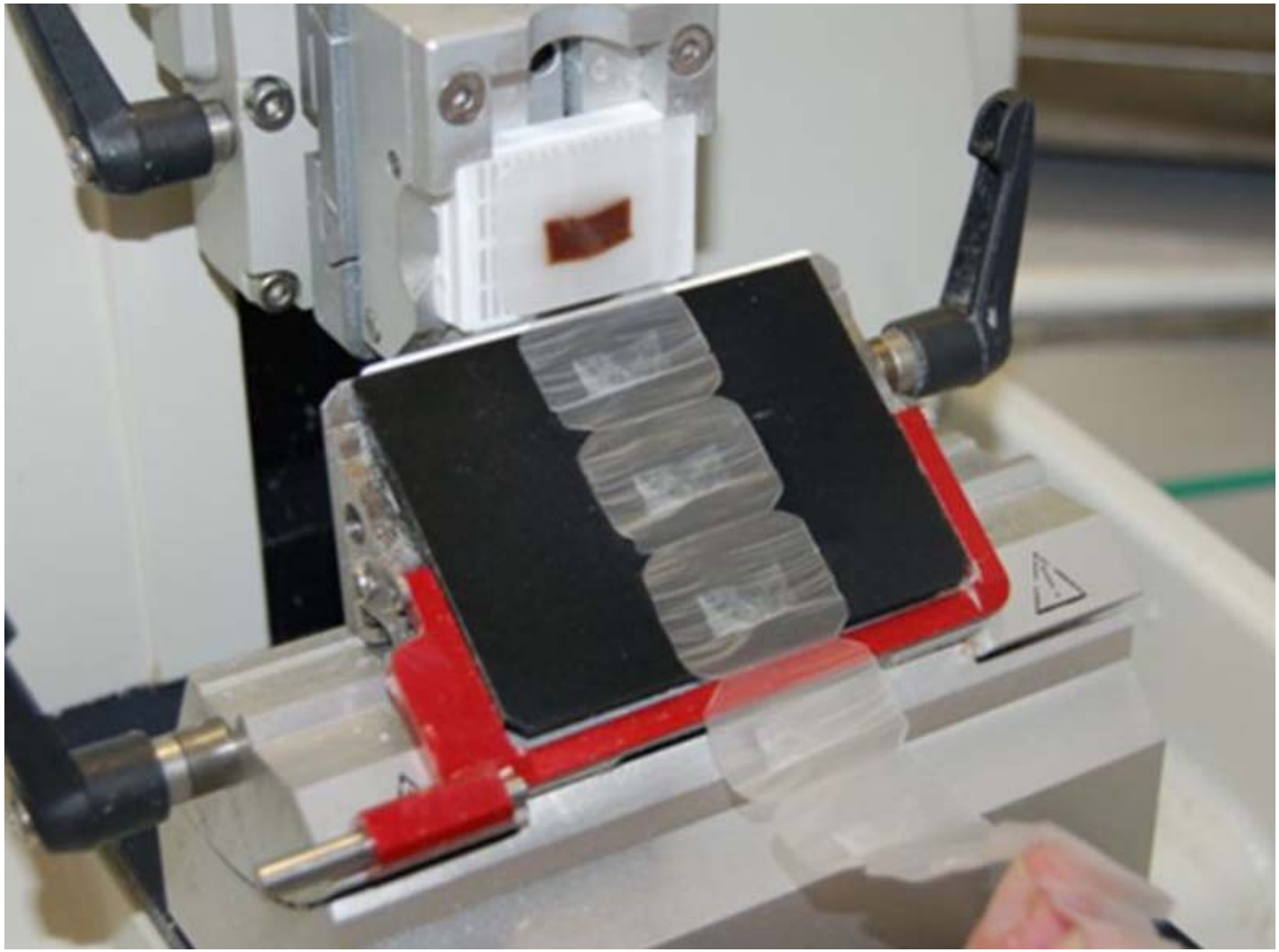
No ribbons, thicker sections: maybe longer AB incubation time

Sectioning (Inclination)



Inclination: angle of the knife in relation to the block surface.

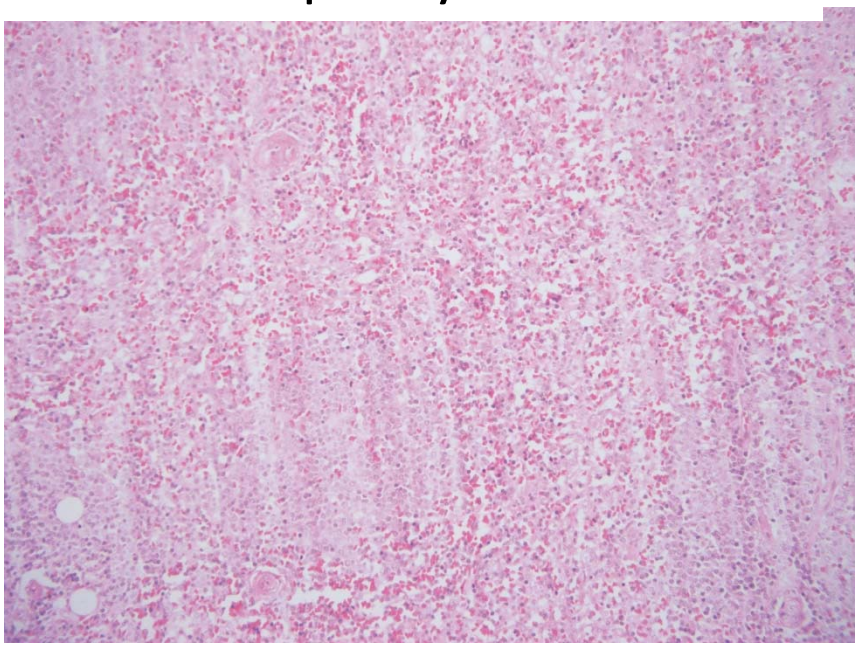
Beautiful „ribbon“ cutted with the microtome



The relevance of knife angle and quality

Inclination too flat

Poor knife quality



101 steps to better histology. Geoffrey Rolls et al.. Copyright © 2016 by Leica Biosystems Melbourne Pty Ltd, Melbourne, Australia

Histology

Tissue (from organ preparation/biopsy)

↓

Fixation/snap freezing/fixing and freezing

↓

Sectioning

↓

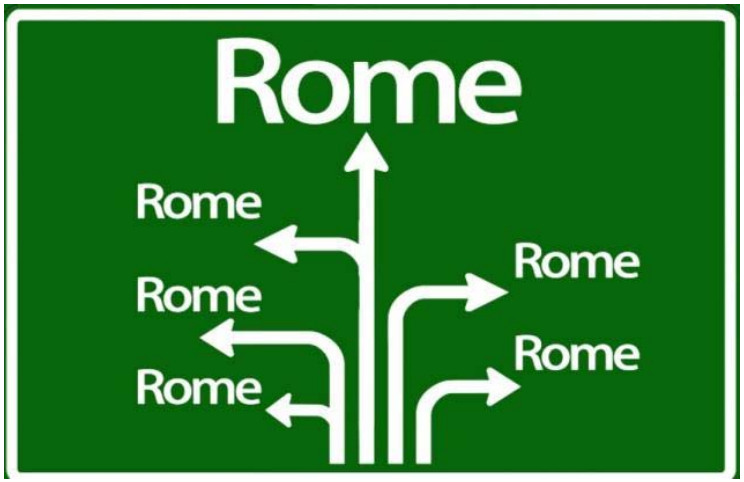
Staining

↓

Mounting

↓

Microscopy



https://www.sciencedump.com/sites/default/files/styles/article_width/public/field/image/Schermafbeelding%202015-12-16%20om%2021.28.07.jpg?itok=GCsDueTC

Three common types of stainings…

1. Histological staining

2. Immunhistochemistry (IHC-P, IHC-F)

3. Immunofluorescence (IF)

Pretreatment paraffin slices

- classical staining, dry for 20 min at 60°C
- enzyme or antigen detection: dry for several hours or over night at max. 40°C
- deparaffinization: xylene or replacement, xylene 1:1 100% ethanol, 5-10 min each
- rehydrate 100%, 100%, 96%, 90%, 80%, 70%, 50% ethanol; 2-5 min each
- Stop at correct alcohol concentration (like staining solution)
- antigen retreaval (HIER, heat in citrate buffer pH6 for 20 min, or enzymatic)

Pretreatment of cryoslices

- dry at 60°C or 40°C, depending on the staining method
- alternatively, 4% PFA for 10 minutes
- if staining in alcoholic solution, dehydreation with increasing alcohol solution

1. Histological staining

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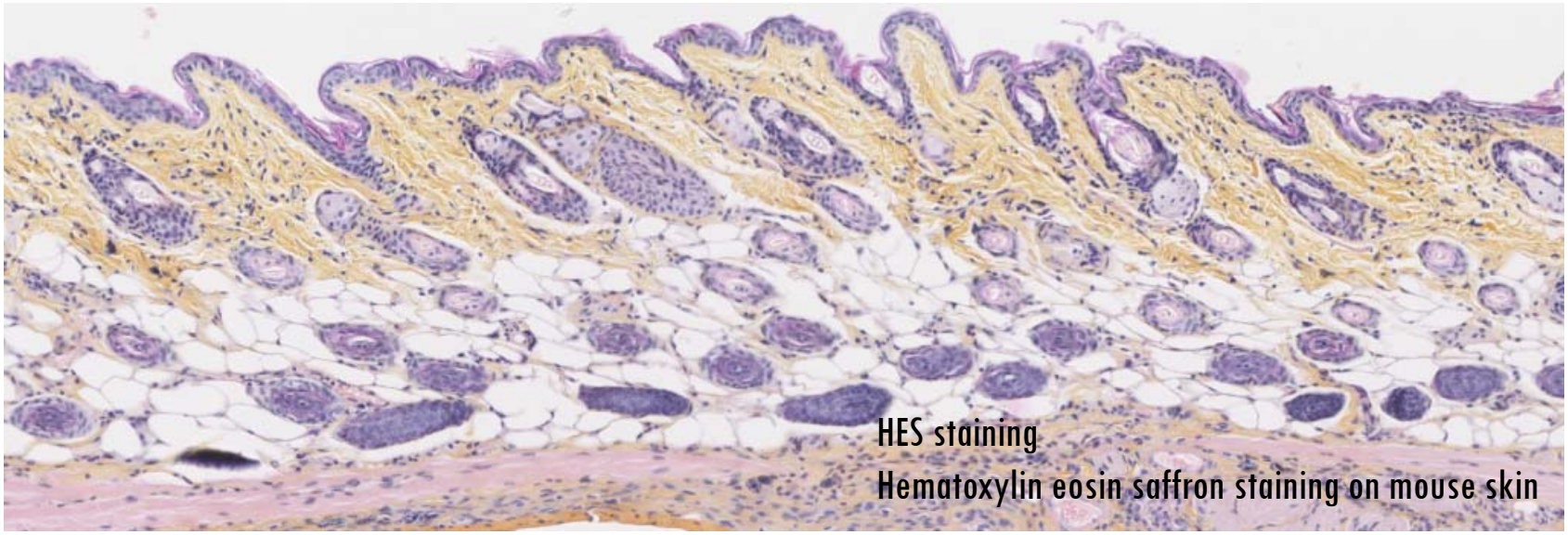
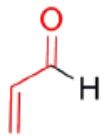
1. Histological staining → dye (chromophore) interact with cells/tissue/organells → Light-Microscope (transmitted light) color camera

Uniform therory of histological dye staining does not excist.

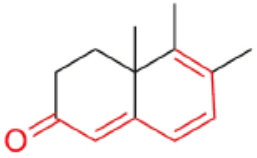


Dye (chromophore + auxochrome)

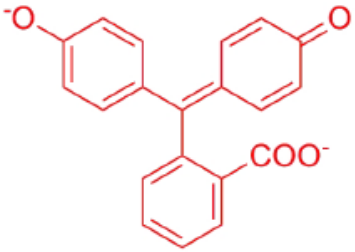
chromophores: chemical groups responsible for the color of the dye



λ_{max} 207 nm
no color



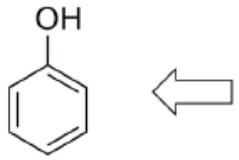
λ_{max} 388 nm

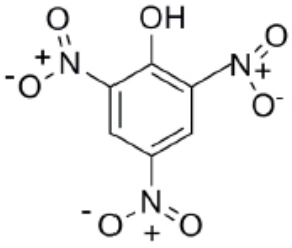


λ_{max} 552 nm
red

C=C ethylene,
C=O carbonyl,
C=N carbimin
N=N azo,
N=O nitroso,
NO_2 nitro

auxochrome: (Greek αὐξάνειν *auxánein*: "to increase" and χρῶμα *chrōma*: "colour“)
Ionizable groups which increase the color and the binding to tissue





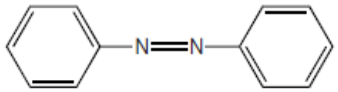
phenol (white)

picric acid (yellow)

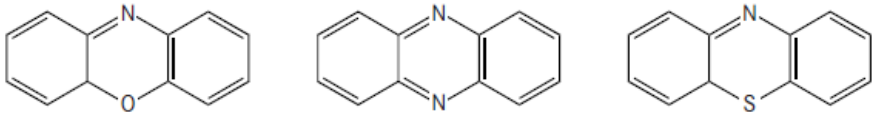
acidic groups		basic groups
COOH	carboxy	NH2 prim. amino
OH	hydroxy	NHR sec. amino
SO3H	sulfo	NR2 tert. amino
		NH imido

Chemical classification of important dyes

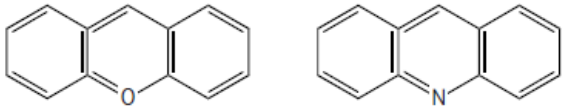
Azo dye
e.g Congo red, sudan IV



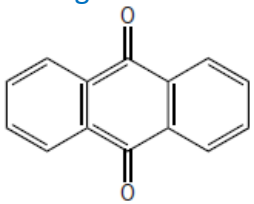
Quinone-imine dyes e.g Methylene Blue



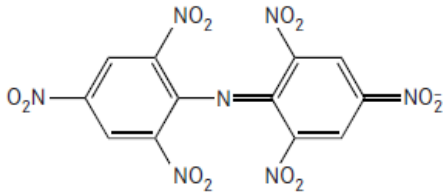
Xanthene dyes e.g rhodamins,
phenolphtalein, fluorescein



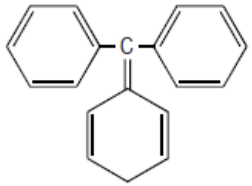
Anthrachinone
e.g Alizarin



Nitro dye e.g.
picric acid



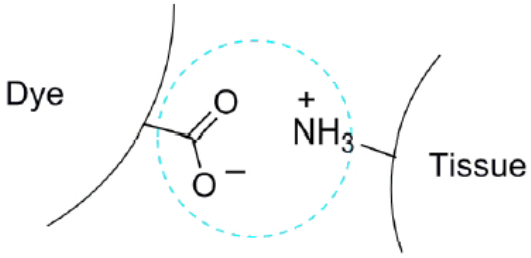
Phenyl-Methane dyes
e.g Malachite Green, Basic Fuchsin



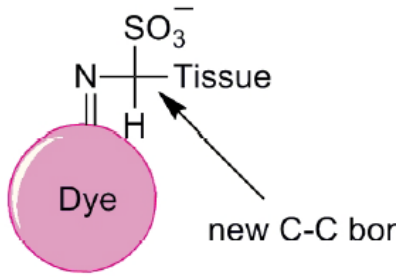
- Color index international (reference database, 13000 CI)
- Stainsfile (www.stainsfile.info/StainsFile/bdl.htm)
- Chroma (www.chroma.de)

General binding mechanisms

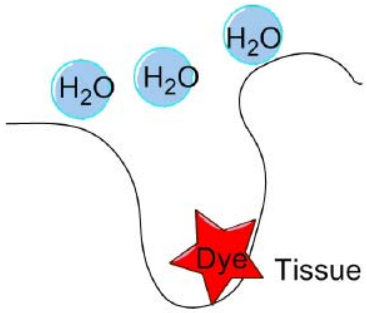
1. Direct binding by chemical interaction (substantive)



electrostatic attraction/ionic bound (40-110 kcal/mol)



covalent bond (35-212 kcal/mol)



hydrophobic attraction (4-8 kcal/mol)

Classification based on electric charge

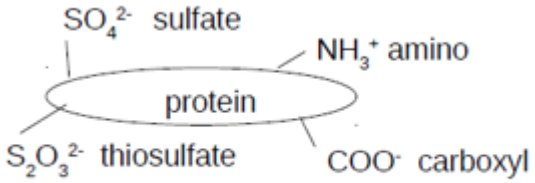
acidic dyes/negative charge/anionic dye

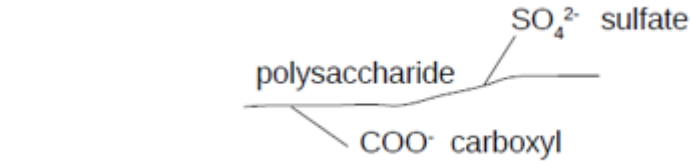
- eosin
- binds to acidophilic structures: Cytoplasma, secretory granula
- better soluble in water than in ethanol

basic dyes/positive charge/cationic dye

- Haematoxylin, fuchsin, crystal violet
- binds to basophilic structures: Chromatin, granula, nissle bodies, mucus
- most better soluble in ethanol than in water

- Molecule with many negative and positive charged groups: net charge determines behavior





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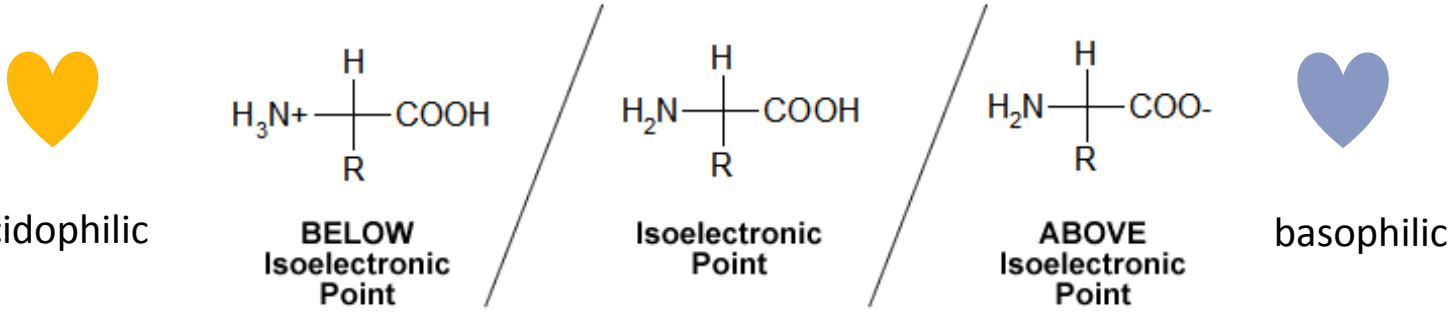
basic dyes/positive charge/cationic dye

- Haematoxylin, basic fuchsin, crystal violet
- binds to basophilic structures: Chromatin, granula, nissle bodies, mucus
- most better soluble in ethanol than in water

- Many negative and positive charged groups: net charge determines behavior
- Isoelectric Point (PI): pH at which a molecule carries no electrical net charge
- Interactions depends on the pH of the stainig solution, charge of dye and charge of target molecule



Classification based on electric charge

acidic dyes (anionic)
basic dyes (cationic)

amphotheric dyes: equal amounts of positive and negative charge in one molecule, net charge depends on pH

neutral dyes: mixture of acid and basic dye (anionic and cationic structures can be colored), Romanowsky stain (hematology, methylene blue +, eosin Y -)

Non-ionic/indifferent dye/sudanophilic: no charge; =0, $-OCH_3$, $-OC_2H_5$ compounds, staining of fats (sudan dyes, scarlet R)

II. Lake (chromophore + mordant)

2. Indirect binding by the formation of chelate complexes

- mordant (Beize, *mordre* = bite in French): polyvalent metal ion solution
- common mordants: alum, chrome, iron, tin, copper – salts
- alum (double sulfate salts, Aluminium Potassium Sulfate, $KAl(SO_4)_2 \times 12\ H20$
- chrome (Potassium Dichromate, $K_2Cr_2O_7$)
- lake (Lack): chromophore + mordant = coordination complex or metal ion chelating complex between mordant and chromophore forms a colloid
- can be added before, with or after the dye (pre/meta/postmordating)
- toxic but very stable (substansive)

Histochemical staining intensity depends on…

Bonding parameters

- dye concentration and staining time
- dye nature (size, lipophilicity, hydrophobic index)
- pH – most profound effect, basic dyes better in alcalic solution and vice versa
- ion/salt concentration: decrease stainability of proteins, supresses dissociation, dye particle can aggregate
- temperature (increase dye diffusion, less aggregates)
- tissue density and permeability
- fixation (keep in mind, ethanol removes lipids)
- simultaneous or successive addition of multiple dyes

Progressive: dye interact with tissue until a propper staining is reached
Regressive/differentiation: tissue is overstained and excessive stain is removed

Famous stainings for tissue components..

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Carbohydrates:
Alcian Blue
Periodic Acid-Schiff (PAS)

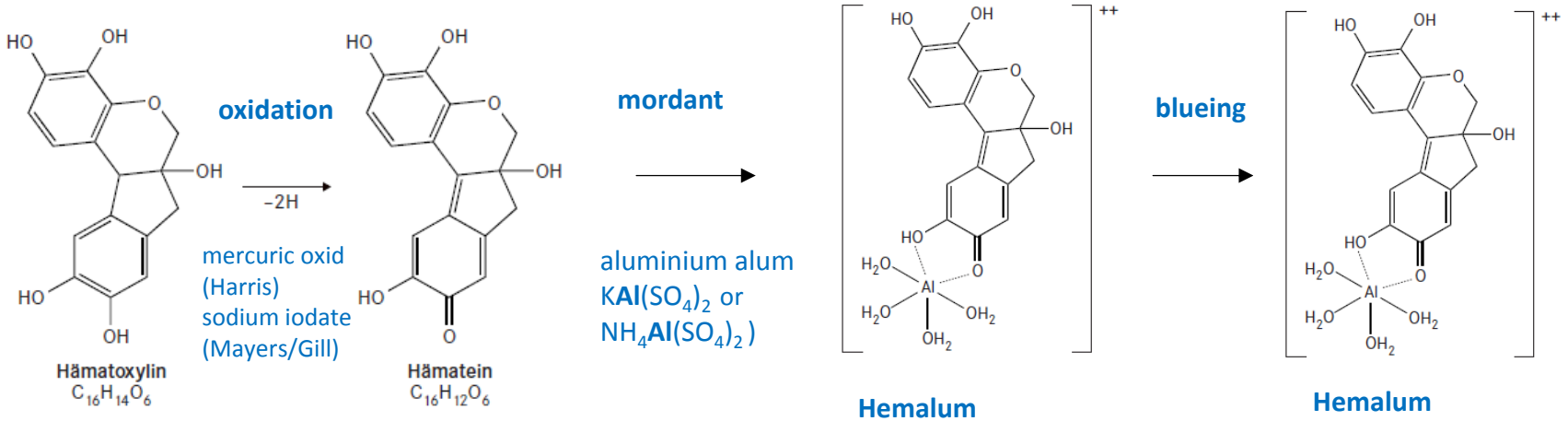
Connective tissue
Collagen fibers (Masson´s trichrome, Mallory´s trichrome, Gomori´s trichrome)
Elastic fibers (Verhoeff-Van Gieson, Weigert´s Resorcin-Fuchsin, Orcein)
Reticular fibers (silver impregnation: Wilder, Gordon and Sweet)

Lipids (Sudan III, IV, black, Oil red O)
Minerals (Ca: von Kossa, Alizarin red S, Cu: p-Dimethylaminobenzidine, rhodamin (DMABR)

Nucleic acids (Haematoxylin, Feulgen stain, Ethyl green-Pyronin-Y, nuclear fast red, cystal violet, cresyl violet acetate)

Pigments
Iron (Prussian Blue)
Melanin (Masson-Fontana silver, Schmorl´s ferricyanide)

Hematoxylin staining



Nuclear staining

- tissue structure and pathological changes
- dead and damaged cells, tumor cells, every cell type in the body can be recognized from the nuclear structure
- Stains also mucins, bacteria, acidic tissue, some secretory granules

Hematoxylin (staining developed around 1876)

- isolated from wood of the central americal logwood tree (*Haematoxylon campechianum*)
- has to mature to **Hematein** by oxydation
- reaction with metal salt (Al = Hemalaun, Fe = iron hematoxylin, Cr = chromium hematoxylin)
- Addition of acid increase staining specificity
- Rinsing with tap water or basic buffer leads to a stable blue staining

Hematoxylin fomulations

Autor	Hämatoxylin, Hämatein	Oxidations-mittel	Al+++ als	Stabilisatoren	Säurezusatz
Mayer (Kap. 3.6.1.3.1)	0,1 %	NaJO3	Kalialaun	0,5 % Chloral-Hydrat	0,1 % Zitronensäure
Harris (Kap. 3.6.1.3.2)	0,5 %	HgO	Kalialaun oder Ammoniumalaun	5 % Ethanol *	0,4 % Essigsäure
Delafields Stammlösung (Kap. 3.6.1.3.3)	0,65 %	Luft	Ammoniumalaun	5 % Ethanol 16 % Methanol 16 % Glycerin	–
Ehrlich (Kap. 3.6.1.3.4)	0,6 %	NaJO3	Kalialaun	30 % Ethanol 30 % Glycerin	3 % Essigsäure

* Nach Rerabek (1960): Zusätze bis 25 % Glycerin

Mayer's acid haemalum - very selective, strong blue staining
- does not overstain, short staining is sufficient for strong color
- there should not be too much oxidizing agent as it will overoxidize the haematoxylin

Harris – contains mercury

Delafields – no oxidative agent – has to be oxidized by light and air for several days before use. Staining time 4-24 h, as there is little haematein.
- can be stored for a long time as it will not overoxidise

Ehrlich – 2 weeks maturation time, can be stored for a long time. Very strong dark blue staining

Procedure

- stain **progressively** – solution contains enough ions and is acidic → no danger of overstaining

Harris or Mayer – 5 min a good staining, 8 min not too much, 12 min looks too strong
- time varies with concentration and age of the solution
- check staining progress and continue if necessary. blueing in warm tap water

differentation, if necessary, in acid medium, use weak acids

blueing – fixes the dye to the tissue
- in warm tap water or weak base: 0.1% ammonium or sodium bicarbonate
- haemalum changes colour and solubility – it is not well soluble in neutral and alcalic solutions
- haemalum binds first through weak electrostatic interactions, later through covalent bonds – cannot be removed by ethanol rinsing
- rinse in distilled water before blueing
- for very specific nuclear staining: briefly put in 0.1-1% aqueous potassium alum. same to correct overstaining

procedure
- 8 min in haemalum
- rinse with distilled water or 0.1% HCl until there is no more dye elution
- 5 min blueing in warm tap water

Cytosol staining

eosin and erythrosine

- 0.1% aqueous solution 5-15 min, rinse in distilled water, differentiate in 80% ethanol
- for a stronger staining: add a drop of acetic acid
- cytoplasm, collagen, erythrocytes

- eosin Y: soluble in water and ethanol
- eosin B: blueish
- ethyl- and methyleosin: only ethanol soluble

- erythrosine B

structure (Romeis p. 206)

- eosin tetrabromofluorescein
- erythrosine: iodine instead of bromine

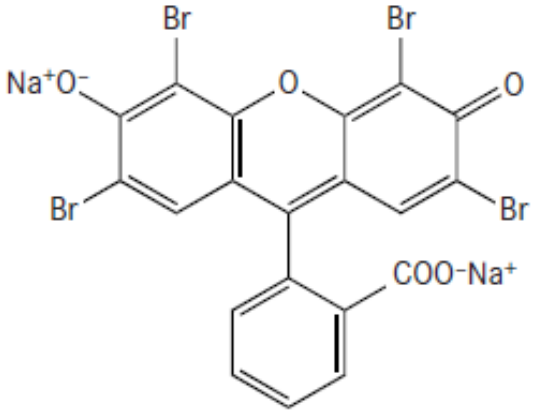


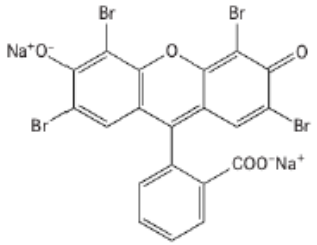
Abb. 3.13: Formel Eosin Y

azophloxine, orange G, fuchsin S, lightgreen SF yellowish (acid green, lissamine green)

Eosin staining

eosin

- tetrabrom-fluorescein-natrium
- weakly acidic solution
- most cytoplasmic proteins have a low isoelectric point – they are negatively charged. H+ ions of the acidic eosin solution give the proteins a positive charge – the negatively charged dye can bind.
- stains positively charged cytoplasmic protein and collagen
- acid treatment of tissue, e.g. during decalcification, produces a stronger staining
  - H+ bind to protein amino groups.
- overstaining+differentiation in 70% ethanol



procedure

- 1-5 min in 1% aqueous eosin solution (slight overstaining)
- rinse in distilled wate
- differentiate: 70% ethanol - rinse shortly
- dehydrate in ethanol series: 96% 1 min, 96% 1 min, 100% ethanol 2-5 min, 100% ethanol 2-5 min. xylene 2-5 min, xylene 2-5 min.
- mounting in xylene soluble medium.

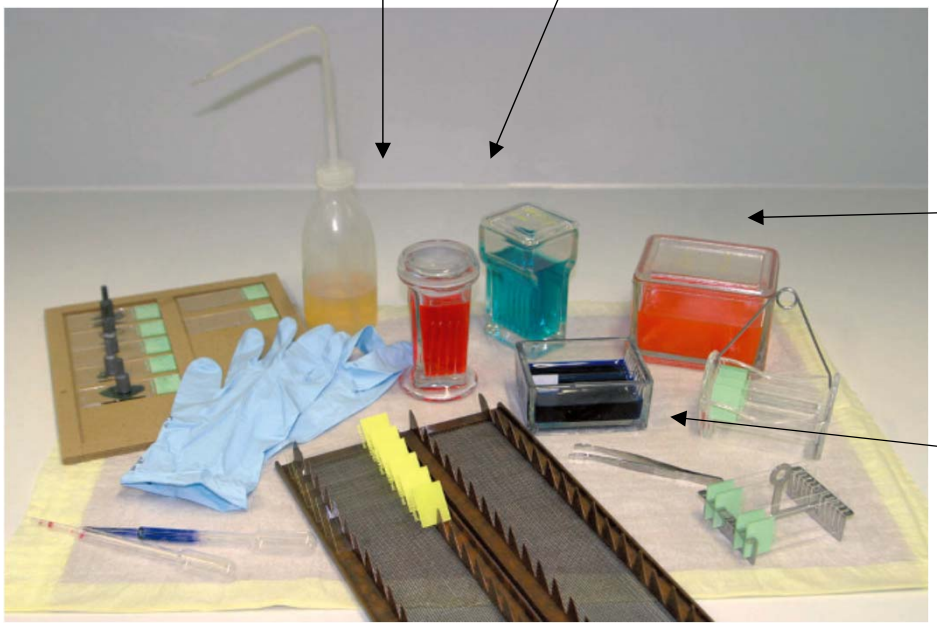
solutions

- aqueous: 1% eosin Y, 1 drop acetic acid to 100 ml (not too much acid because the heamatoxylin staining will suffer)
- ethanolic: 1% eosin in 50% ethanol, 1 drop of acetic acid per 100 ml

Staining cuvettes

Coplin jars
10 slides
60 mL

Hellendahl
8-16 slides
80 mL



Glass box with
glass/metal insert
10-20 slides
200 mL

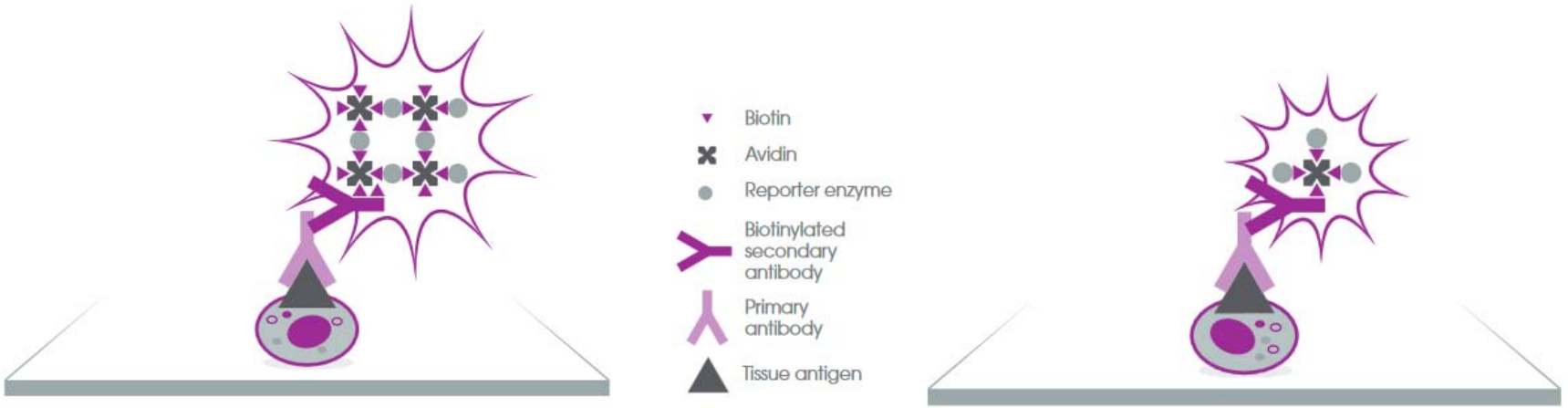
Schiefferdecker
10-20 slides
100 mL

- Staining in microwave: use plastic cuvettes
- Store dye solution in glass bottles with a cap or a plug (prevents loss of solvent)

Three common types of stainings…

2. Immunhistochemistry (ICH-P, IHC-F)

Biotinylated primary/secondary Ab
- Streptavidin-peroxidase
- avidin/biotin-enzyme **ABC/LSAB**



Avidin-Biotin Complex (ABC)

Labelled Streptavidin-Biotin (LSAB)

http://docs.abcam.com/pdf/kits/immunohistochemistry-ihc-application-guide.pdf

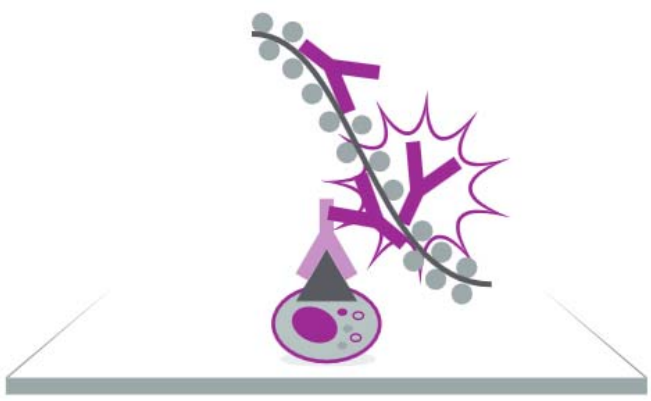
Three common types of stainings…

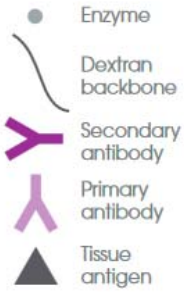
Institute of Molecular Biology

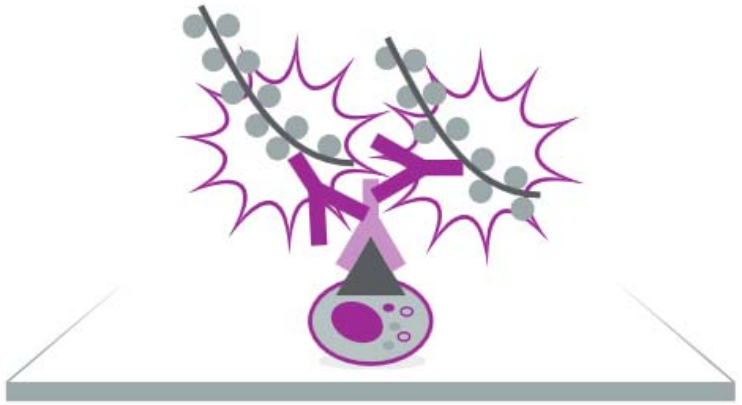
2. Immunhistochemistry (ICH-P, IHC-F)

primary/secondary Ab labeled with enzyme

- horseradish peroxidase **HRP**
- alkaline phosphatase **AP**







Polymer method

Micro-polymer method

http://docs.abcam.com/pdf/kits/immunohistochemistry-ihc-application-guide.pdf

Three common types of stainings…

Institute of Molecular Biology

2. Immunhistochemistry (ICH)

primary/secondary Ab labeled with enzyme
- horseradish peroxidase **HRP**
- alkaline phosphatase **AP**

Biotinylated primary/secondary Ab
+
Streptavidin-peroxidase

Chromogenic substrate is transformed by enzyme in colorized product
HRP + DAB (Diaminobenzidine) or AEC*
AP + BCIP/NBT ** or Fast Red (fluorescent)

* AEC (3-Amino-9-Ethylcarbazol), soluble in ethanol
** 5-bromo-4-chloro-indolyl-phosphate + n-nitroblue tetrazolium chloride

Three common types of stainings…

Institute of Molecular Biology

2. Immunhistochemistry (ICH)

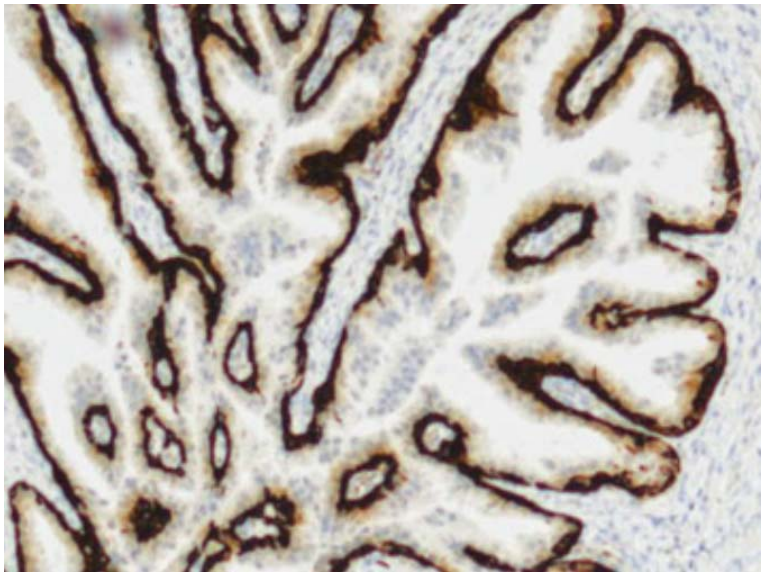
primary/secondary Ab labeled with enzyme
- horseradish peroxidase **HRP**
- alkaline phosphatase **AP**

Biotinylated primary/secondary Ab
+
Streptavidin-peroxidase

Chromogenic substrate is transformed by enzyme in colorized product

Counterstaining with dye

Light-Microscope (transmitted light) color camera



How to prevent crossreaction with endogeneous enzymes?

If the enzymatic activity is also endogenous to the tissue being studied, the endogenous enzymes must be blocked before the detection step.

1 . Horseradish peroxidase (HRP)-conjugated antibody:
- endogeneous peroxidase activity e.g. in kidney, liver, vascular tissue/red blood cells
- Test: Incubate tissue with DAB prior first antibody indubation.
- tissue becomes brown?
- Blocking with hydrogen peroxide (0.3% H_2O_2, 10-15 min) after secondary Ab

2. Alkaline Phosphatase (AP)-conjugated antibody:
- Endogeneous AP activity e.g. in kidney, intestine, osteoblast, lymphoid tissue, placenta
- More prevalent in frozen tissue
- Test: incubate with BCIP/NPT prior first antibody incubation
- Tissue becomes blue?
- Blocking with levisamole (chromogenic substrates + levisamole commercial available)
- Intestinale AP is unaffected by levisamole, treat tissue with weak acid.

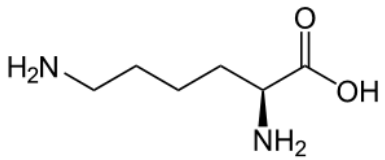
Formaldehyde fixation and paraffin embedding can destroy epitopes and impede access for Ab

- AR - method was developed by Shi et al. (1991)
- mechanism not fully clear
- proposed mechanism: linear epitopes available after AR (secondary, tertiary structure lost)

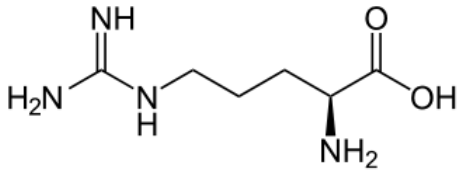
Three types of epitopes and their status after formaldehyde fixation:

1. Irreversible destroyed (epitope contains lysine)
2. Reversibley destroyed (epitopes or adjacent sequences contain arginine or tyrosine)
3. Insensitive (no lysin and arginine in epitope)

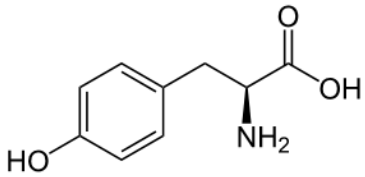
Shi SR, Key ME, Kalra KL. 1991. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 39:741–748 [PubMed]



lysine



arginine



tyrosin

Antigen retrieval can unmask some epitopes in formalin-fixed, paraffin embedded (FFPE) tissue

	HIER (heat induced epitope retrieval)	EIER (enzyme induced epitope retrieval)
advantage	more defined epitope retrieval	for difficult epitopes
temperature	95-100°C	30 – 37°C
time	20 - 40 min	10-15 min (range 10-30)
buffer	Citrate-HCl pH 6, Tris-EDTA pH 9	buffer for enzyme (pepsine, proteinase K, trypsin)

HIER can be performed in a
- Microwave
- pressure cooker
- waterbath
- autoclave

Example heat induced epitope retrieval (HIER)

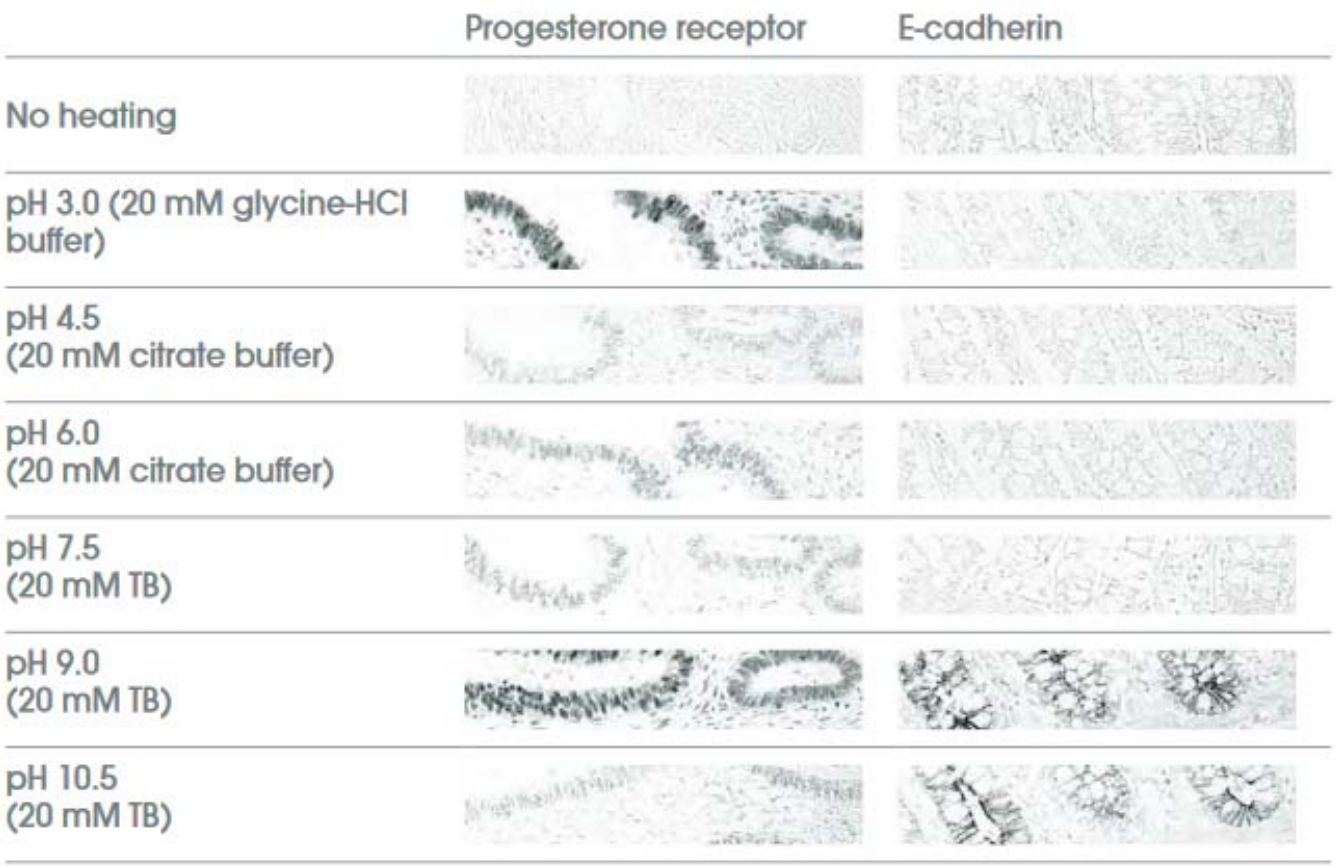


Figure 2. Effects of pH on heat-induced antigen retrieval in human tissues

Adapted: Mechanisms of Heat-induced Antigen Retrieval: Does pH or Ionic Strength of the Solution Play a Role for Refolding Antigens? Katsura Emoto, Shuji Yamashita, Yasunori Okada, Volume: 53 issue: 11, page(s): 1311-1321, Cytochemistry

Three common types of stainings…

3. Immunofluorescence (IF)

primary/secondary Ab labeled fluorescent dye

Counterstaining with fluorescent dye (nucleus, organell specific)

Fluorescent microscopy (PMT, camera b&w)

Immuno Fluorescence Protocol

Institute of Molecular Biology

Fixation with freshly made Formaldehyde (10 – 20 min, RT)
Fixation solution: 3.7 – 4% Formaldehyde in either Medium or PBS buffered with 10 mM Hepes 7.0,

Quenching (optional)
Quenching solution: 50 mM TRIS/100 mM NaCl (pH 8.0)

Permeabilisation with (1) Triton X-100 or (2) Saponin (5 – 20 min, RT)
Permeabilisation solution (1): 0.1% - 0.25% Triton X-100 in PBS plus 0.5% BSA or 10% Goat Serum
Permeabilisation solution (2): 0.05% Saponin in PBS plus 0.5% BSA or 10% Goat Serum

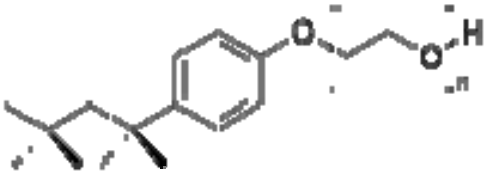
Blocking (30 min – 1h at RT)
Blocking solution: 3% BSA in PBS or 20% Goat Serum in PBS or 10% Goat Serum + 5% FCS in PBS,
when Saponin is used as permeabilisation one has to add also here: 0.05% Saponin;

Staining
Staining solution: 1st Ab, (30 min-1h), washing, 2nd Ab (30 min), in PBS or blocking solution, when Saponin is used as permeabilisation one has to add also here: 0.05% Saponin;

And thoroughly **washing** between all the steps (5 x 5 min at horizontal shaker)
Washing solution: PBS or PBS + 0.05% Saponin, when Saponin was used as permeabilisation

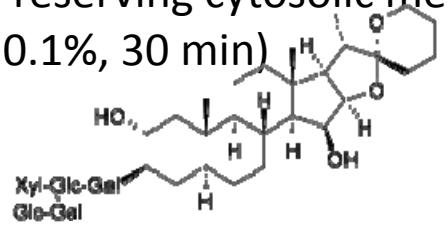
Permeabilization

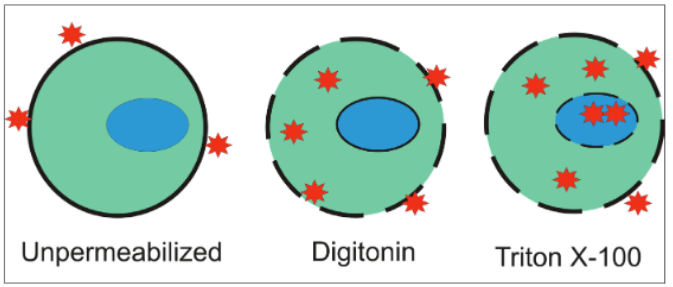
Triton X-100 or **NP40 are** detergents and makes irreversible big holes in the plasma membrane.
Stainig proteins in mitochondria and nucleii.
(0.1 – 0.5%)



Permeabilization create holes in the plasmamembrane (access for antibodies and fluorophores)

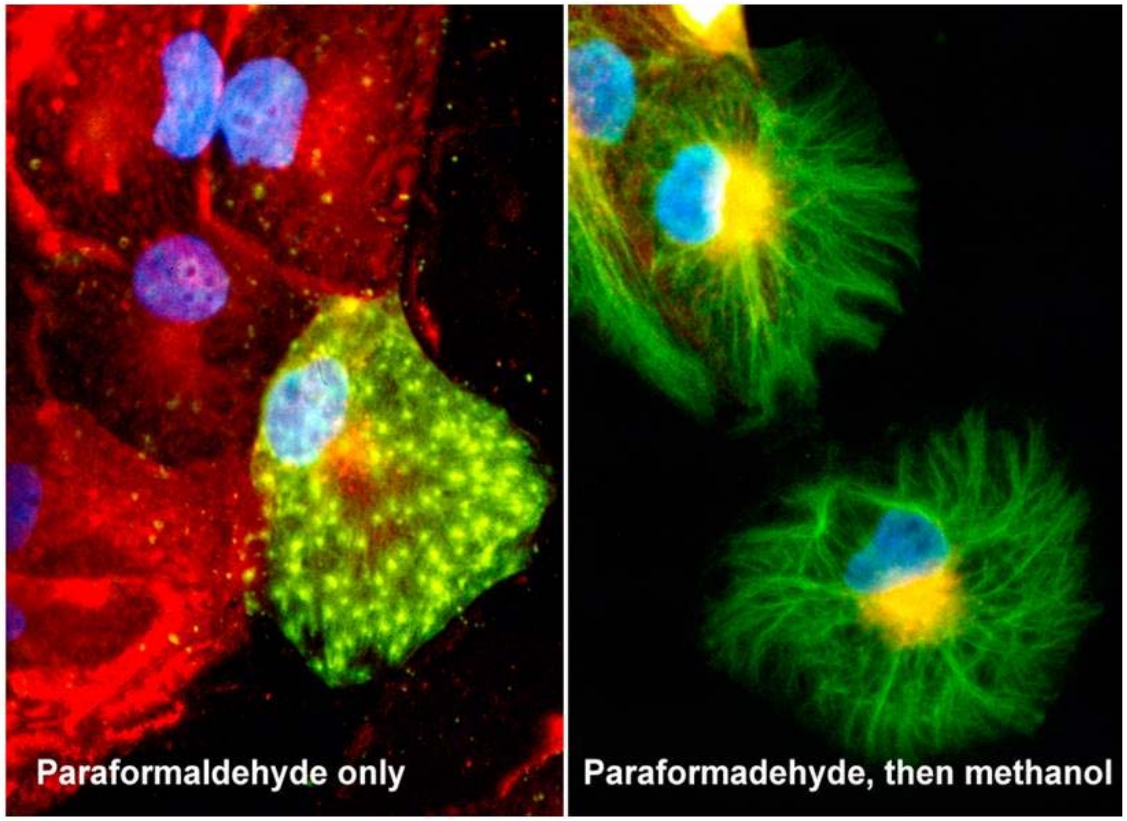
Saponin *quillaja bark* (plant) or **Digitonin** *digitalis purpurea* are detergents, but produces smaller holes and their effect is reversible by washing.
Preserving cytosolic membranes.
(0.1%, 30 min)





..of course, fixing with organic solvents needs no permeabilization.

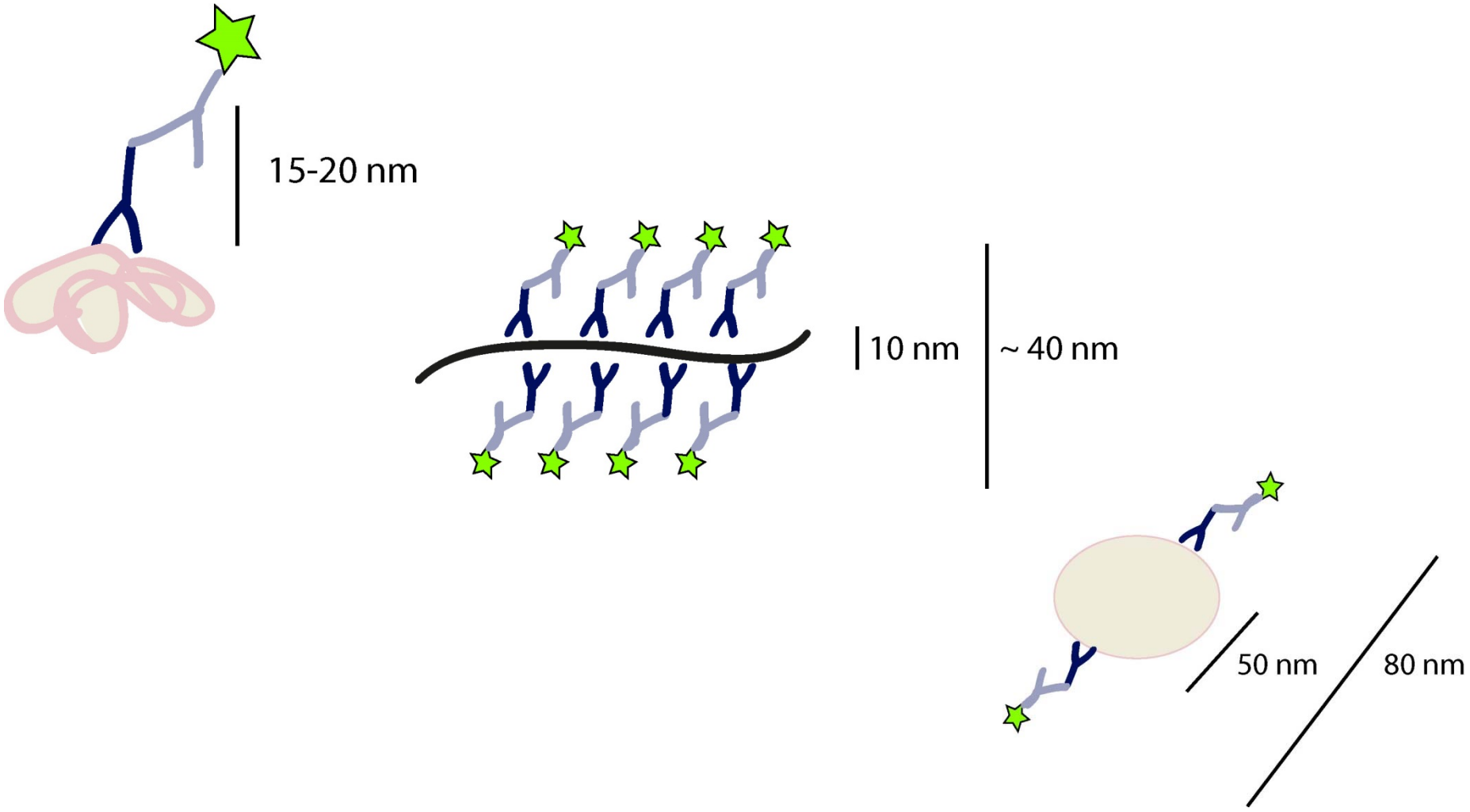
Think actively about your target protein!



Cells fixed with paraformaldehyde (PFA) only (L), or paraformaldehyde followed by methanol (R), and stained for a carbohydrate antigen (red) and a a cytoskeletal protein (myosin, green). The myosin staining shown in the right image is more 'true', however the carbohydrate surface staining present in the left image has been removed by permeabilization with methanol (R). The green staining in the cell (L) indicates a leaky cell, in which a number of small holes have been formed by PFA fixation through which the antibody could gain partial access to the interior (the bright spots). More rigorous permeabilization on the same cell type (R) allows the antibody into the cell to detect the entire cytoskeletal network, largely inaccessible in most of the non-permeabilized cells (L). Whether the cell (L) was leaking before the formaldehyde treatment, or leaking was caused by the fixation process, is unknown. What is clear, however, is that without the image on the right, it could be erroneously concluded that only a few cells contain the antigen and it stains in a punctate pattern.

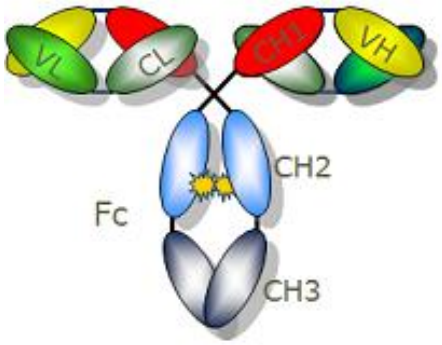
http://blog.ptglab.com/index.php/immunostaining-cultured-cells/

Label size



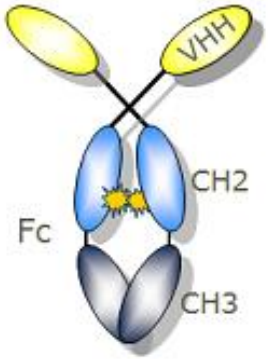
Labeling strategies for super-resolution microscopy

Conventional antibody

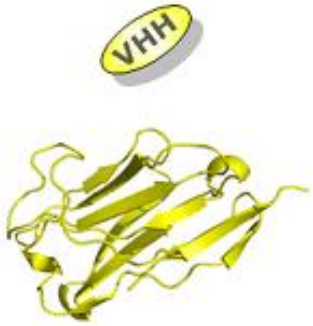


150–160 kDa

Camel Heavy-Chain antibody



VHH or Nanobody

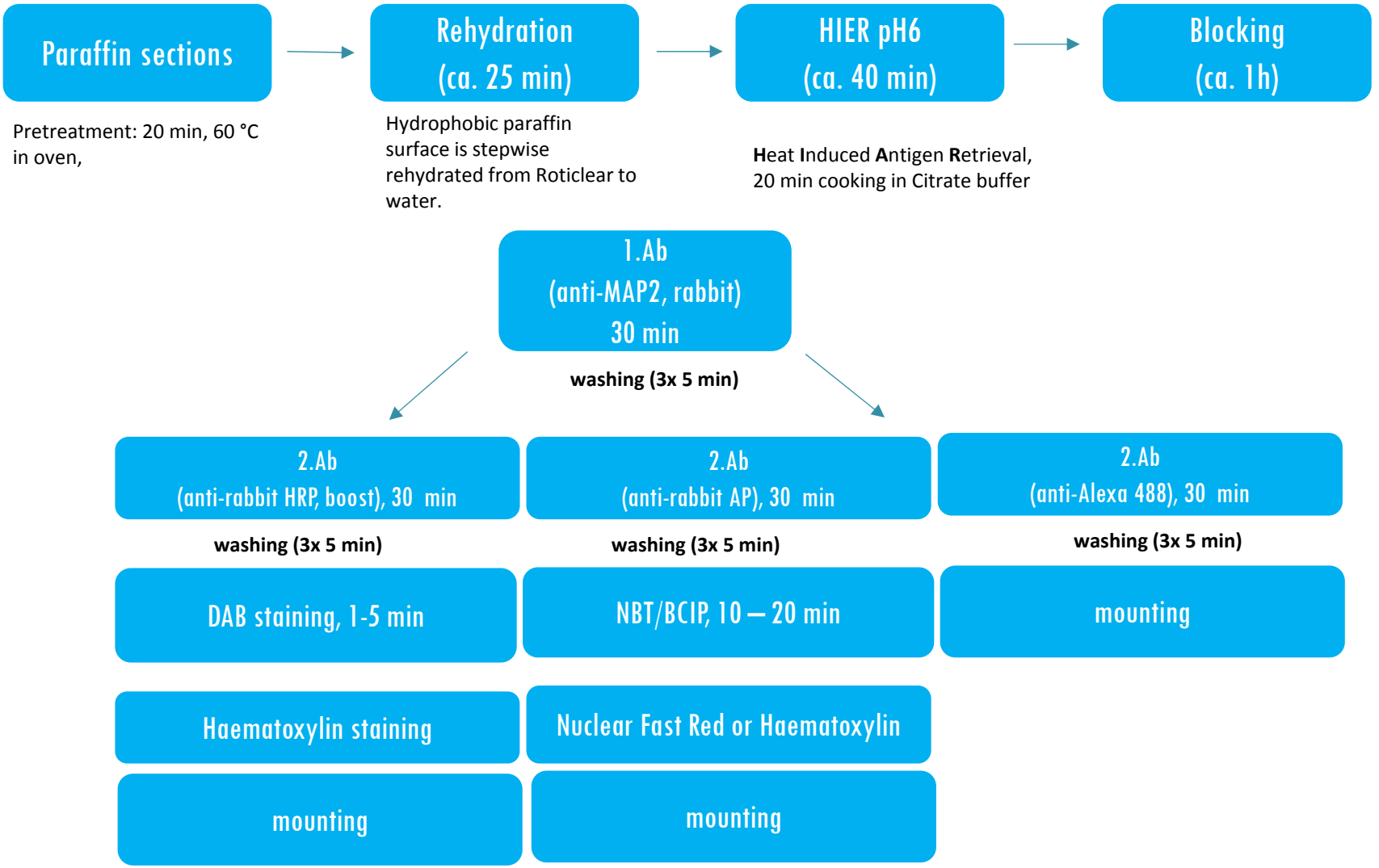


12-15 kDa

If possible:

- Dye labeld primary antibody (150 – 160 kDa)
- Fab fragments (~ 50 kDa)
- Nanobodies (12-15 kDa)
- Aptamers
- Small protein tags (SNAP, HaLo)

Overview IHC-P with counterstaining or IF



Coverslip Thickness

Use Coverslips with a thickness of 0.17 mm. This is # 1.5!!!

class	ideal thickness	range
#0	100 µm	80 - 130 µm
#1	150 µm	130 - 170 µm
#1.5	170 µm	160 - 190 µm
#2.0	220 µm	190 - 250 µm

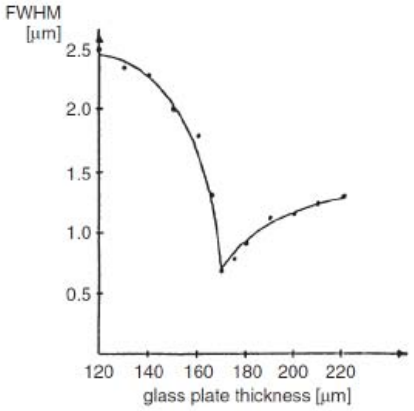


FIGURE 7.9. Changes in the half-width of the intensity distribution with changing coverslip thickness. Plan-Neofluar 63×, NA 1.2 water.

Source: James B. Pawley, Handbook of Biological Confocal Microscopy, 3rd edition

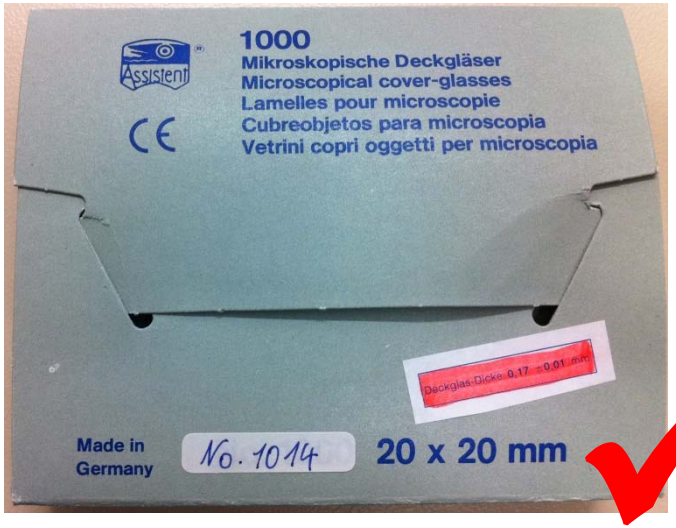
10 µm thickness deviation deteriorates approximately 50 % of resolution & intensity for objectives with high NA

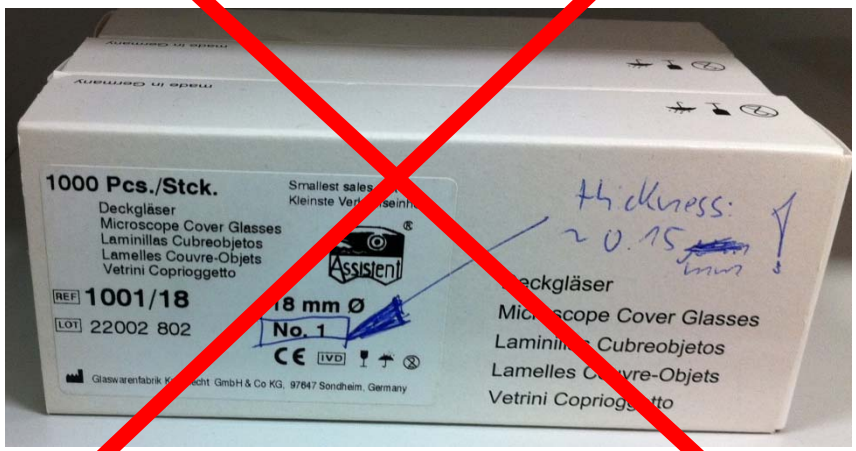
high precision coverslips:

"Assistent" Cover glasses, selected thickness

1014 **thickness** 0,17 ± 0,01 mm
1015 **thickness** 0,17 ± 0,02 mm

www.hecht-assistent.com


1000
Mikroskopische Deckgläser
Microscopical cover-glasses
Lamelles pour microscopie
Cubreobjetos para microscopia
Vetrini copri oggetti per microscopia
Made in Germany
No. 1014
20 x 20 mm

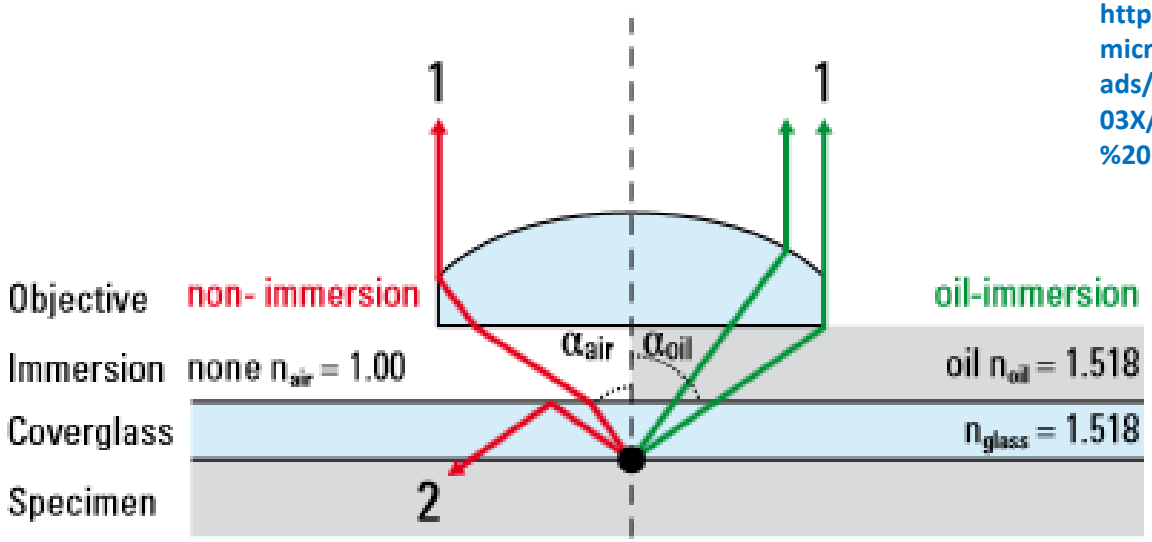

1000 Pcs./Stck.
Deckgläser
Microscope Cover Glasses
Laminillas Cubreobjetos
Lamelles Couvre-Objets
Vetrini Coprioggetto
REF 1001/18
LOT 22002 802
No. 1
Thickness: ~ 0.15 mm !


ibidi
ibiTreat
054 u-Slide 8-well ibiTreat
80826
1 item


The invisible man
Herbert George Wells
1897

Refractive index match!

https://www.leica-microsystems.com/fileadmin/downloads/Leica%20TCS%20SP8%20STED%203X/Brochures/Leica%20TCS%20SP8%20Objective-Brochure_EN.pdf



1 Limiting ray
2 Total internal reflection

Mountant	Manufacturer	RI
Fluoromount-G™	Southern Biotech Assoc. Inc.	1.40
ProLong®/ProLong® Gold	Molecular Probes	1.46 after curing
VECTASHIELD®	Vector Laboratories	1.44
VECTASHIELD® Hard+Set™	Vector Laboratories	1.46 after hardening
Mowiol®	Kuraray Europe GmbH	1.41–1.49
TDE/Water	–	1.33–1.52

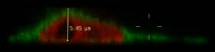
Immersion Medium	RI
Air	1.000
Water	1.333
Immersion Type G at 23°C (Glycerol/Water)	1.450
Glycerol 100%	1.474
Immersion Type F (Oil)	1.518

Tools


High Precision
Deckgläser
Microscope Cover Glasses
Laminillas Cubreobjetos
Lamelles Couvre-Objets
Vetrini Coprioggetto
200 St./pcs.
22 x 22 mm
170±5µm
No. 1.5H

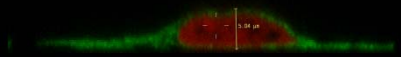

IMMU-MOUNT

Fluoromount G



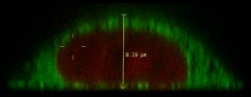
5,2 ± 0,7 µm

Fluoromount



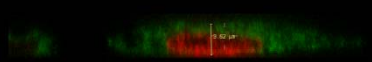
4,8± 0,3 µm

Ibidi Mounting Medium



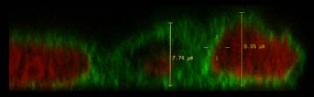
7,6± 0,7 µm

FluorSave



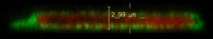
3,7± 0,5 µm

Prolong Gold Antifade



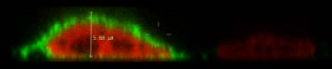
6,1± 0,9 µm

Vectashield Hard Set



2,5 ± 0,3 µm

Shannon Immu-Mount



4,1 ± 1,0 µm

References and sources

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- Special Stains and H&E, second edition, Dako
- Dyes and stains: From molecular structure to histological application, Frontiers in Bioscience, Jan 2014, article
- Knife angle in Microtomy, Resolution No.5, Leica
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Thank you for your attention!

Questions?