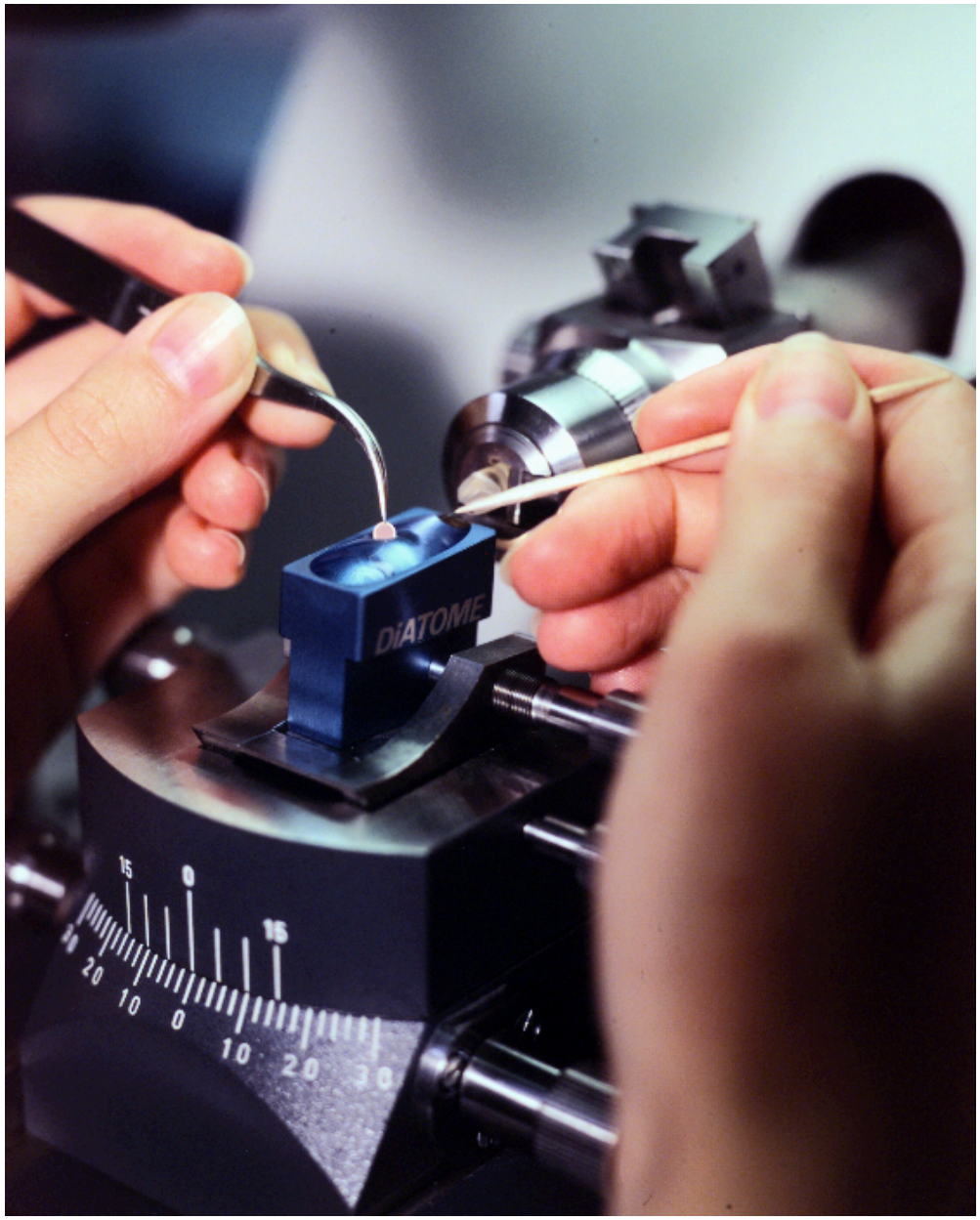
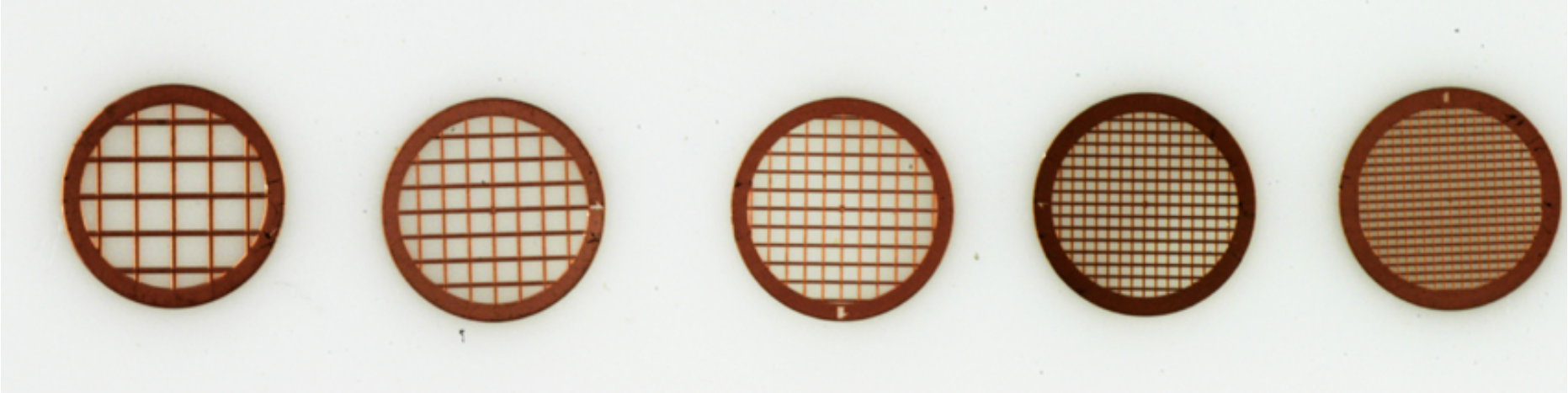
# Methods in Transmission Electron Microscopy and their application

**Day 1**

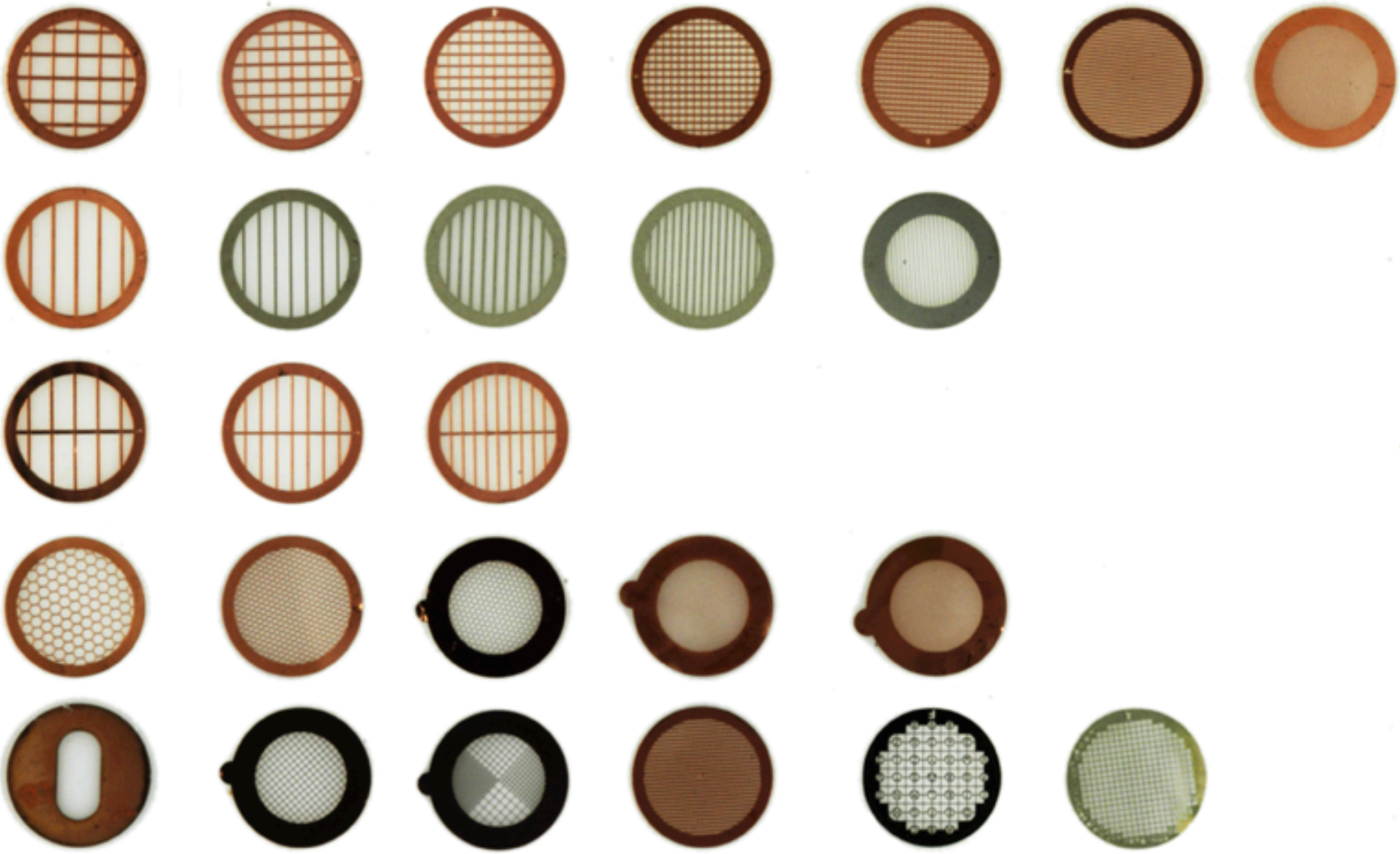


Standard EM-grids:

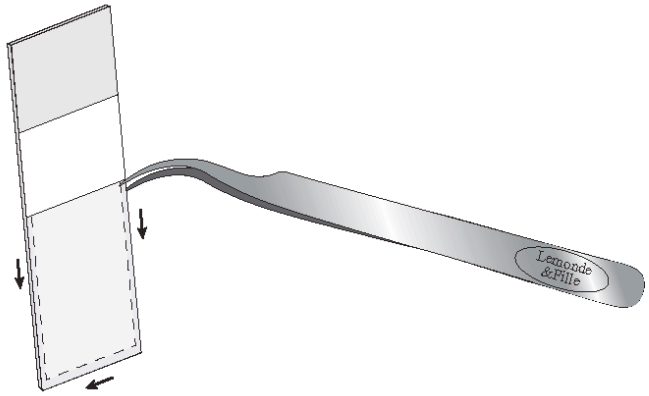
‚mesh' = number of bars per inch (2.54 cm).

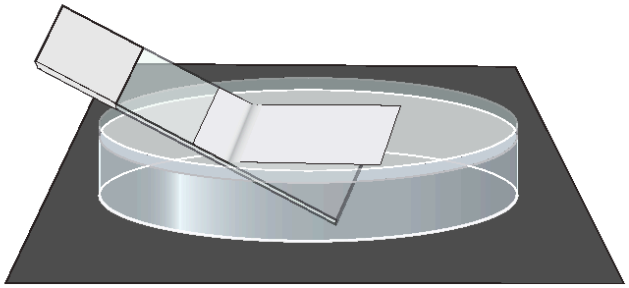


Different types of EM-grids. For our applications, grids with enhanced bar-free areas are very important (Slot-Grids).



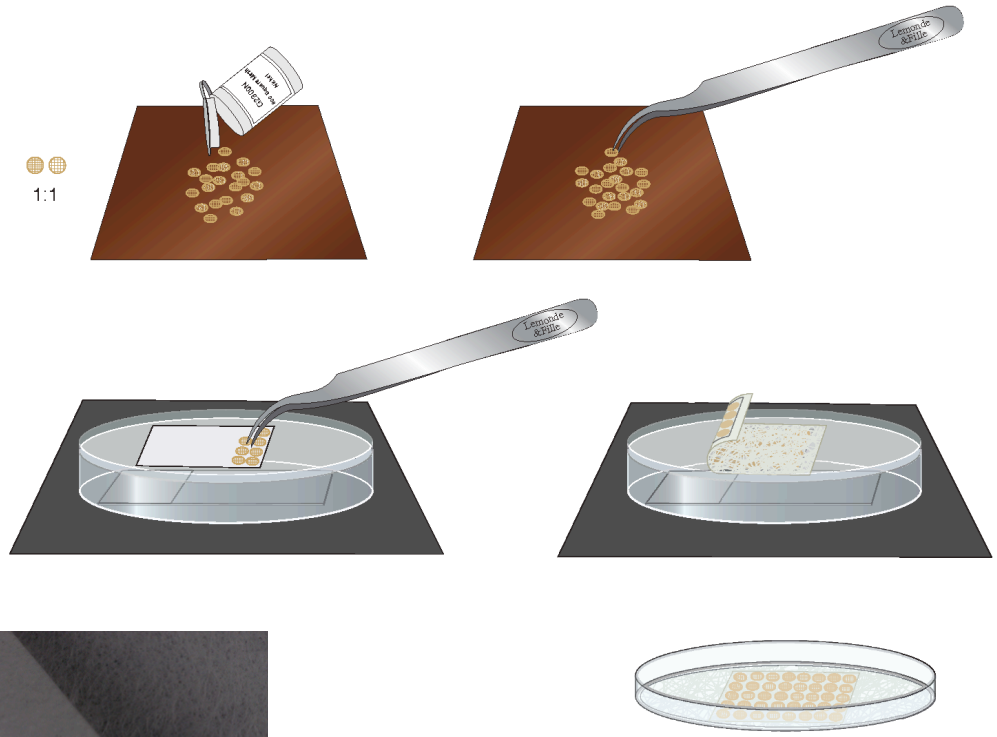
The plastic support film consists of e.g. Collodium, Formvar der Pioloform.

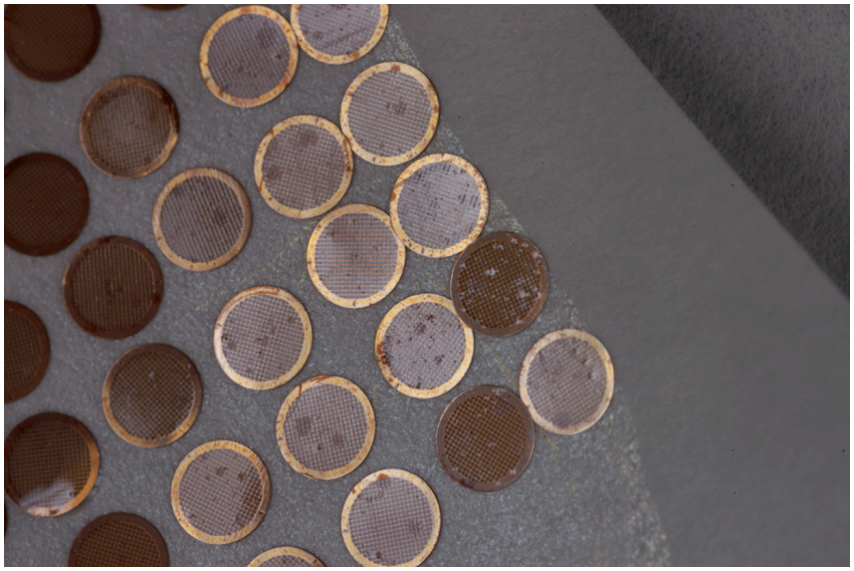


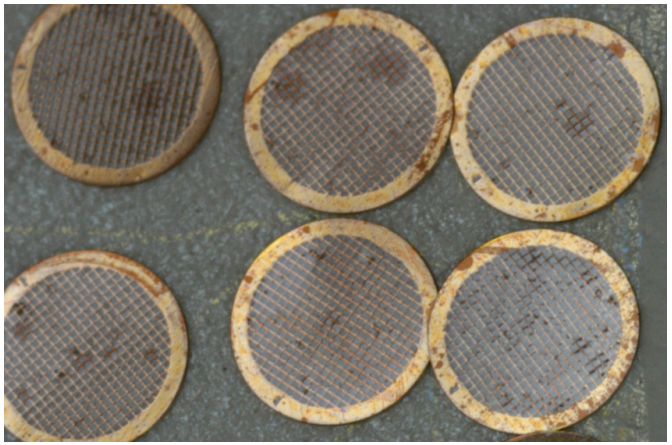



Floating of the support film on water after scratching it at the edges.

EM-grids with Collodium-plastic foil





# Sampling

- Incubation in bacterial growth medium
- Centrifugation at 1.000 g or 14.000 g for 3 min?
- Resuspension in growth medium

# Fixation-temperature

In contrast to animal samples, this is of minor importance for plant material.

**Room temperature**: much faster for standard questions; good results in 99% of experiments.

**0-4°** : Immuno localization, histochemistry (z.B. detection of catalase), but: all incubation times have to be 4 times longer!

# Cacodylate buffer

Sodium salt of dimethyl arsenic (cacodylic) acid

$(CH_3)_2As(=O)ONa$

MG: 214.05 g/mol

**Toxic!!**

Blocks glycolysis and thus fermentation of cells → „Agent Blue“

„storable“

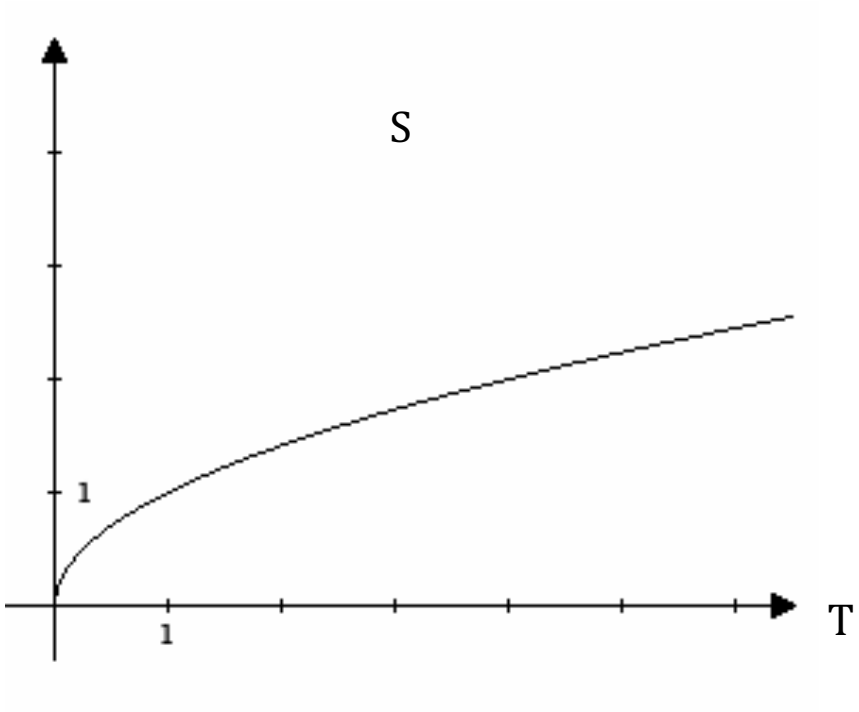
Concentration: 50-75 mM; osmolarity can be adjusted with e.g. NaCl .

Dissociates 1:2 also at high concentration(50 mMol = 100 mOsmol)

pH: 7.0 (or adjusted to the pH of medium respectively)

# Fixation time

Diffusion: square function (double distance = 4x time!).




**Diffusionsgeschwindigkeit von Fluorescein in Wasser**

| durchwanderte Strecke | Zeit |
|---|---|
| 1 nm | 130 psec |
| 10 nm | 13 nsec |
| 100 nm | 1,3 µsec |
| 1 µm | 130 µsec |
| 10 µm | 13 msec |
| 100 µm | 1,3 sec |
| 1 mm | 2 min |
| 1 cm | 3,5 Std |
| 10 cm | 15 Tage |
| 50 cm | 1 Jahr |
| 1 m | 4,2 Jahre |

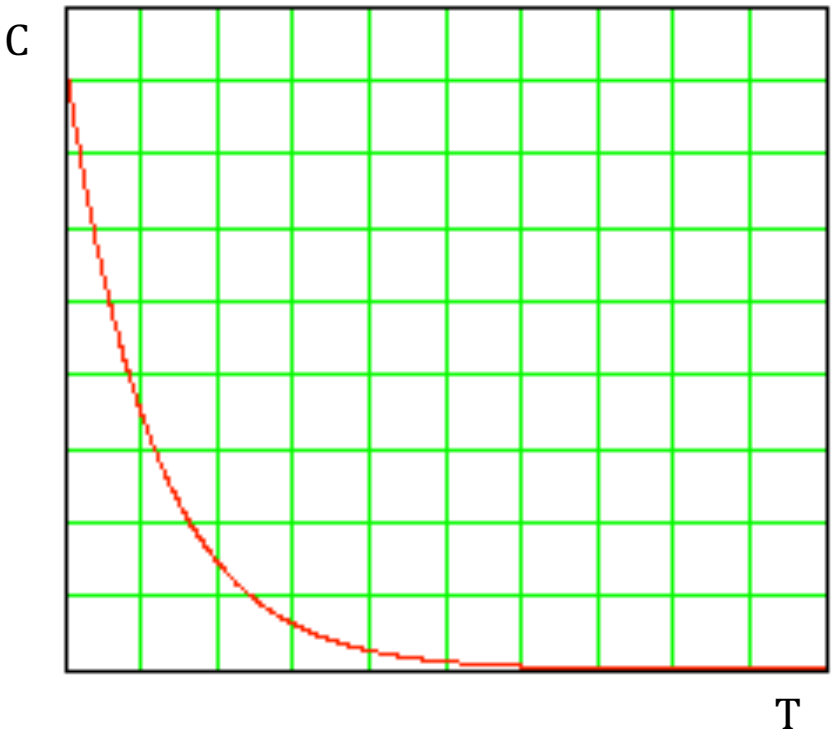
Diffusion is temperature dependent ($Q_{10}$ = ca. 2).

Glutardialdehyde takes 20 min for complete fixation of a molecule with a diameter of 100 nm (at RT).

Diffusion in a tissue sample with 1 mm edge length:
at least 30 min – i.e. complete fixation takes at least 1 h.

# Washing times

Elution process: $e^{-x}$ function (exponential decrease!)
Rule of thumb Washing time = 2 x (fixation time)




Wrong: 30 min – 30 min – 30 min – 30 min – 30 min – 30 min
Correct 5 min – 15 min – 30 min – 60 min – 120 min

# Classical/chemical fixation

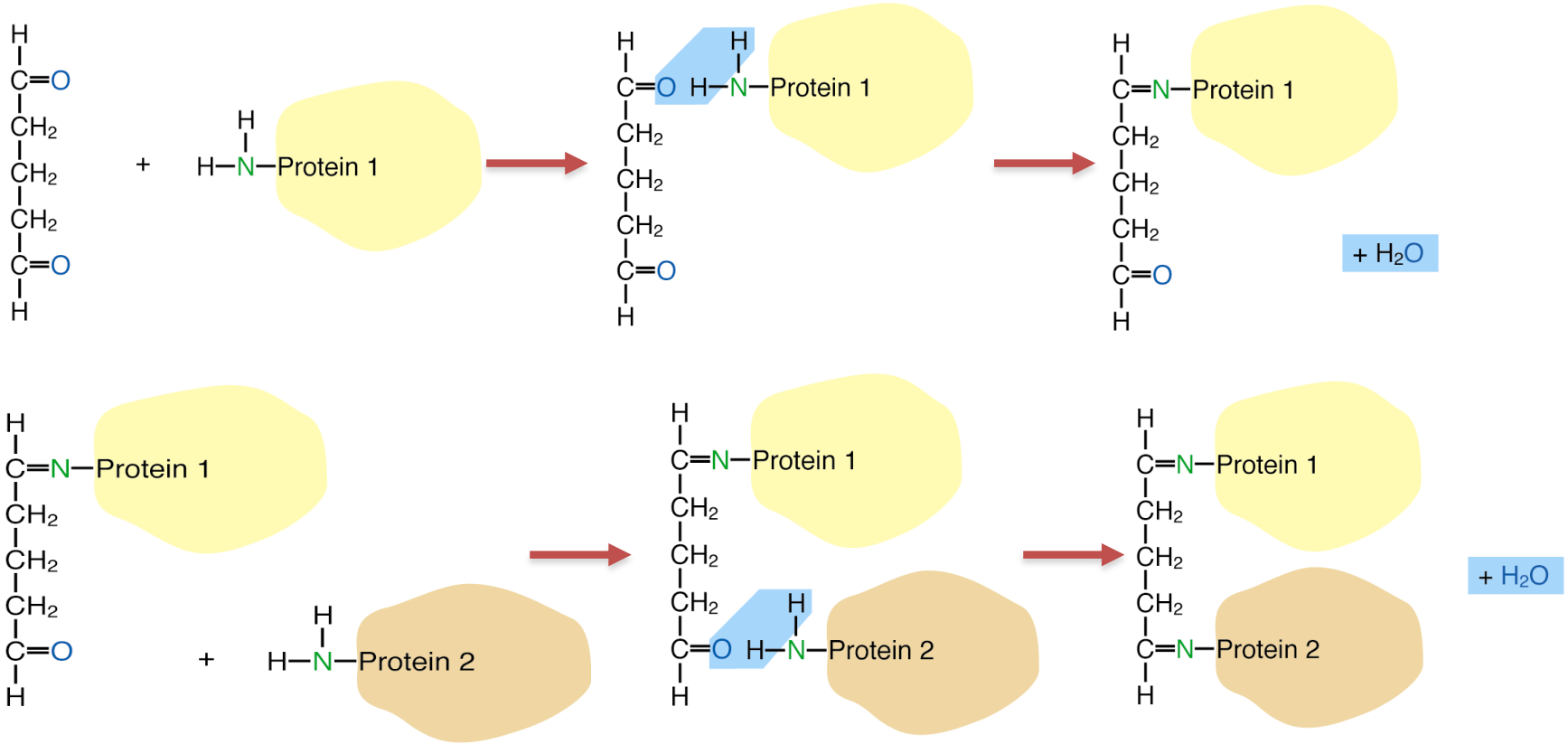
1. Fixation buffer: cacodylate buffer
2. Fixative: 2.5 % glutaraldehyde (in some cases + 1-4% formaldehyde)
3. Post-fixation: osmium tetroxide ($OsO_4$)
4. (en bloc staining with uranyl acetate)
5. Dehydration in a graded acetone series
6. Resin embedding
7. Post/positive staining

# Glutar(di)aldehyde

Concentration: 2.5%

pH: 7.0

buffer: cacodylate
phosphate



# Chemical fixation by aldehydes

- Crosslinking of proteins by aldehydes: fixation
- Length of aldehyde molecules is important
- Do not prevent lipid loss from bilayer
- Exception: phospholipids containing amino groups: phosphatidylserine, phosphatidylethanolamine

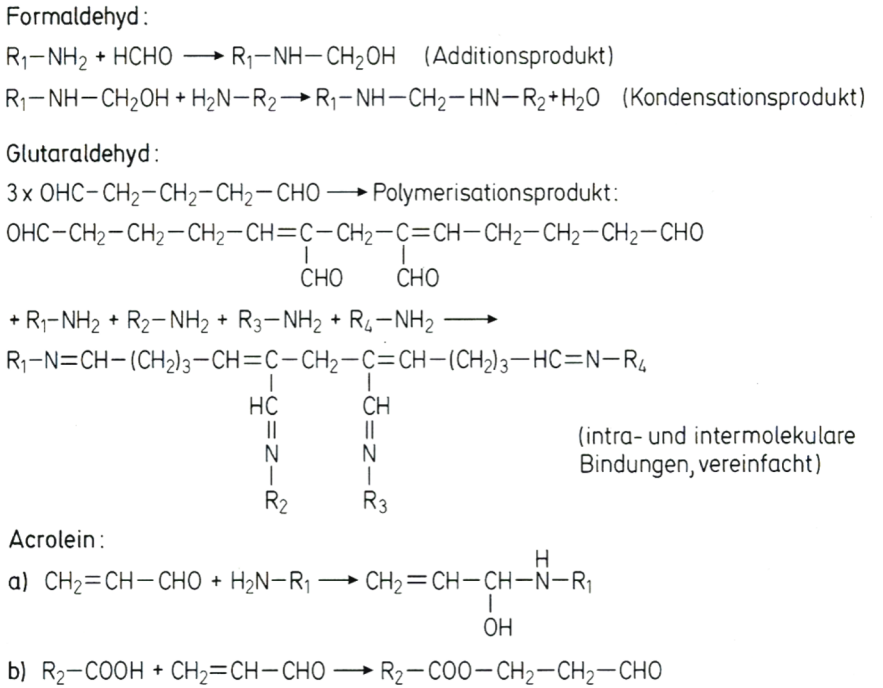



Abb. 18: Reaktionsschemata der Quervernetzung von Proteinen durch Formaldehyd (Fraenkel-Conrat und Olcott, 1948), Glutaraldehyd (Richards und Knowles, 1968) und Acrolein [(a) Jones, 1972; (b) Hall und Stern, 1955]. R-$NH_2$ bezieht sich auf Aminogruppen im Protein.

# Osmium tetroxide

Yellowish crystals in pharmaceutical phials under $N_2$ atmosphere

**Toxic!!**

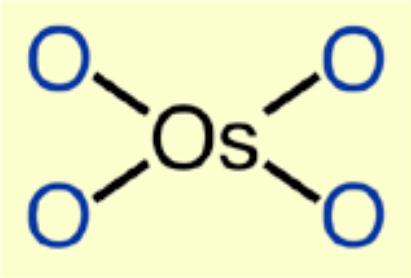
Diffusion significantly slower than glutaraldehyde

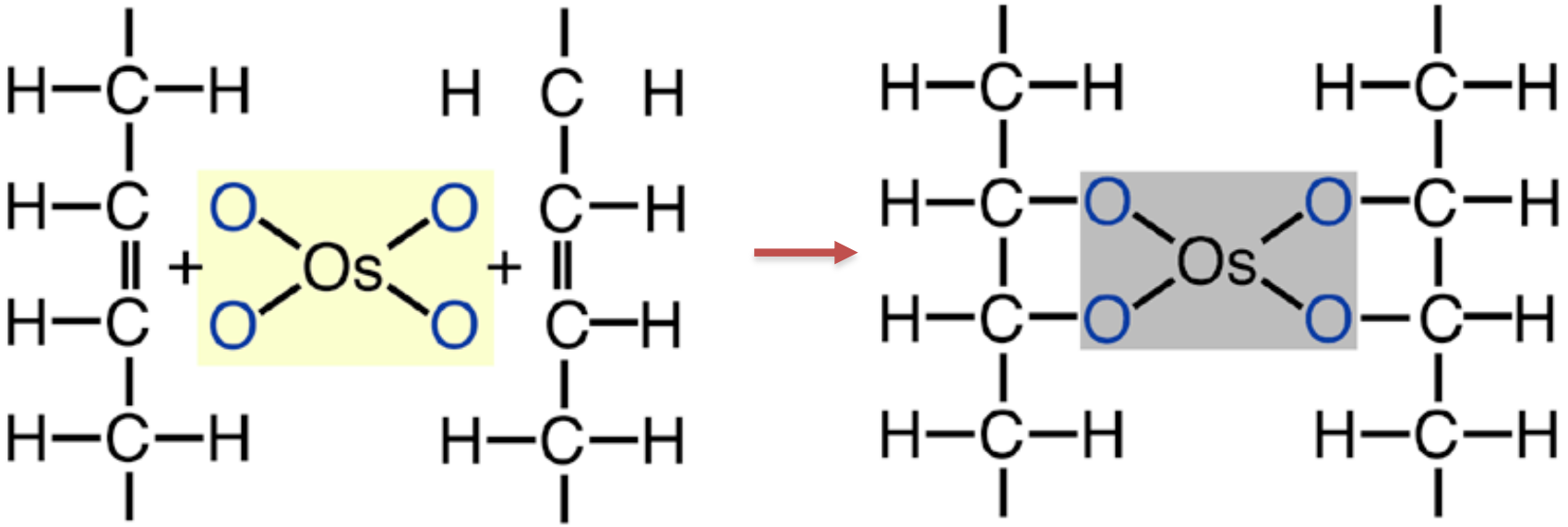
Cross reaction with glutaraldehyde (black precipitates)

Concentration: 1 – 2%

pH: 7.0

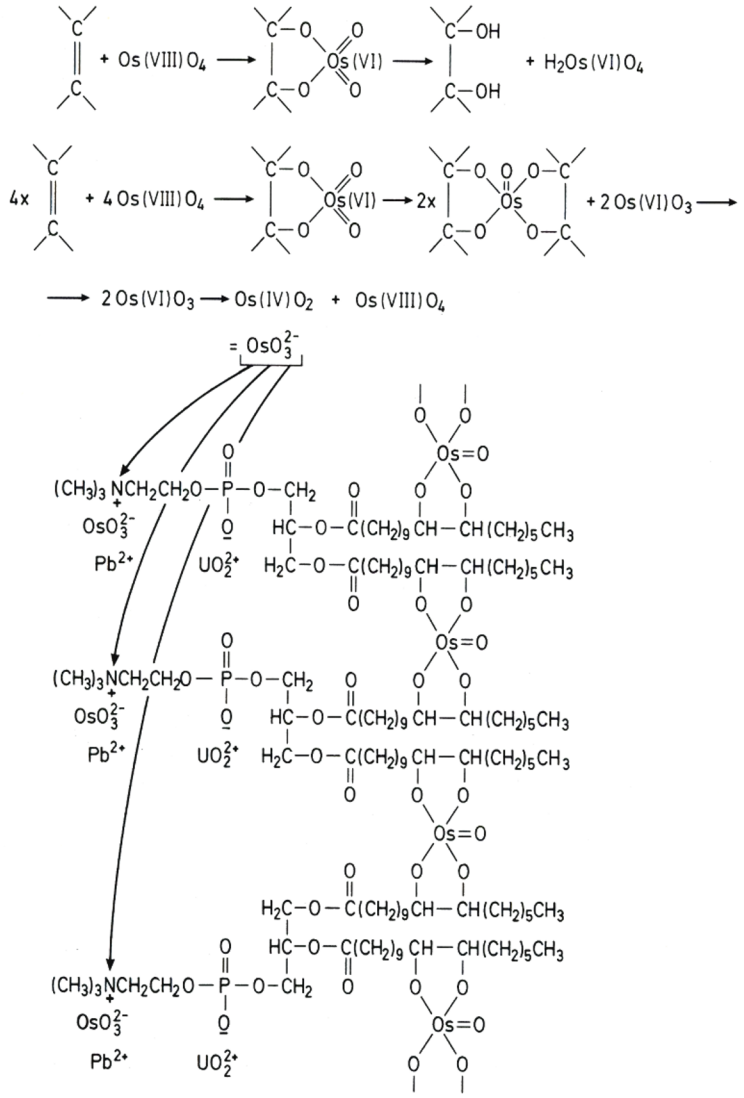
buffer: cacodylate
phosphate
Tris
Pipes





# Fixation/staining with $OsO_4$

- Crosslinking and preservation of lipids/unsaturated fatty acids (oleins and oleic acids)
- Point of attack: C=C-double bond
- Proteins: reaction with double bonds of tryptophan
- Additional side effect: contrast enhancement
- Disadvantage: low rate of penetration -> fine structure may change before completion of fixation
- Osmates and osmium dioxide most likely migrate to cations of phospholipid head groups
- Trilaminarar appearance of membranes treated with $OsO_4$: preferential deposition of $OsO_2$ at the two hydrophilic faces, some residual osmium at original olefinic site



# Chemical fixation: major problems

- Lipid extraction in dehydration process and resin infiltration/embedding
- Glutaraldehyde (GA) may cause artifacts like blebs or mesosomes
- GA is unable to prevent movement of phospholipids and intramembrane particles
- GA is not very effective at temperatures below -20° C, Acrolein remains active even at -80° C
- Formaldehyde (FA) fixation alone leads to lipid-depleted membranes that consist largely of protein. The same is true for Acrolein, although to a smaller extent
- Difficult handling of Acrolein: polymerization on exposure to air, light and several chemicals
- Fixation is often too slow (e.g. slow penetration of $OsO_4$)
- Loss of lipids after $OsO_4$ fixation often higher than expected (often without significant structural alterations)

# Cacodylate buffer (1000 ml); pH 7.0 with 3% NaCl

- **Cacodylate:** Sodium salt of dimethyl arsenic (cacodylic) acid

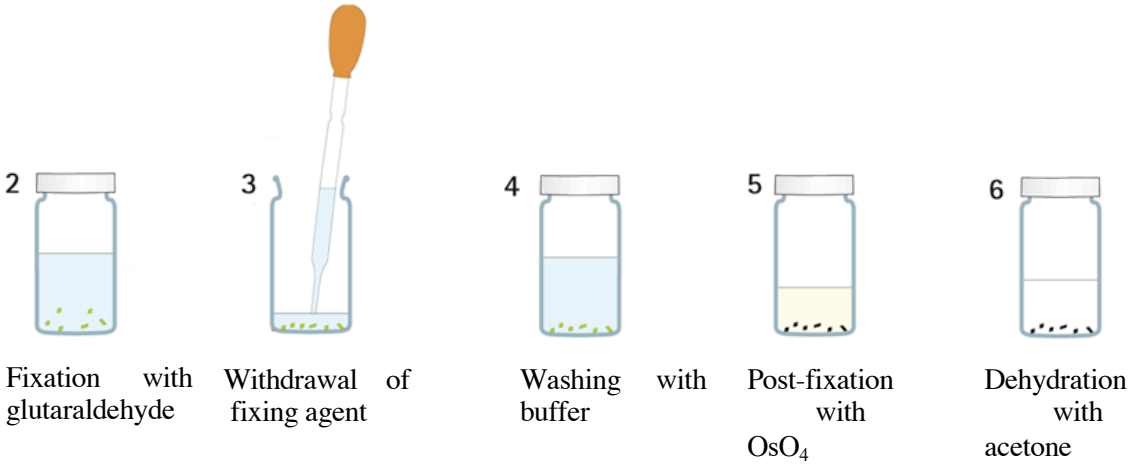
  $(CH_3)_2As(=O)ONa$

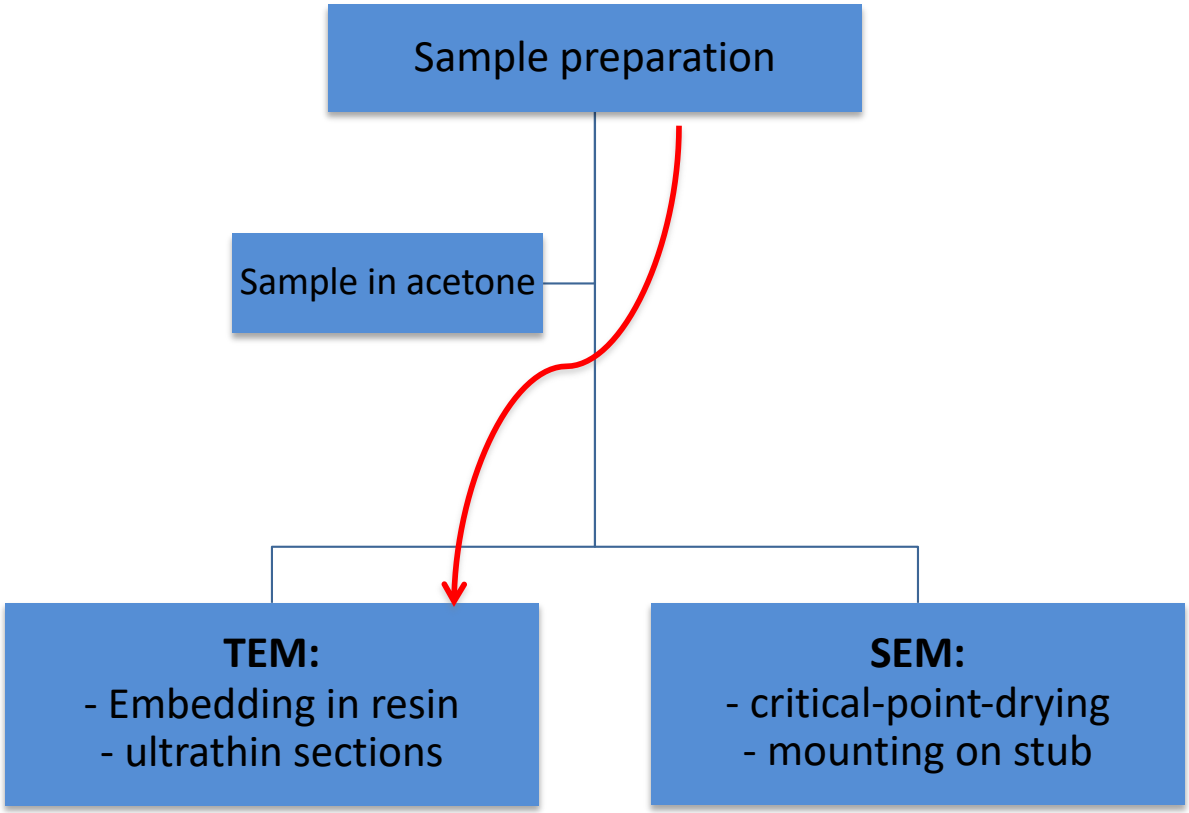
  MW: 214.05 g/mol

- **$MgCl_2$ x 6 $H_2O$:** MW: 203,30 g/mol

How much do we need of each substance to make a 50 mM Cacodylate buffer with 2 mM MgCl2 and 3% NaCl?

# Sample preparation



# Embedding material

**epoxy resin** **methacrylate** **polyester**

The embedding material depends on the research purpose:

• good/excellent ultrastructural preservation (bad for immunological detection):

Embedding in epon (812) oder Spurr`s resin

• good preservation of epitopes/antigens (limited ultrastructural preservation):

Embedding in Lowicryl or LR-White (methacrylates)

# Embedding in epon resin

(Dehydration: dehydration with graded ethanol or acetone series)

**Embedding in Epon812:**

1. Epon812: Epoxy monomer, glycidether
2. MNA: methyladenicanhydride – hardener
3. DDSA: Dodecylsuccinicanhydride – hardener
4. DMP: Dimethylaminomethylphenol – catalyst, accelerator

- Mixing ratio determines final mechanical hardness of plastic
- Polymerization of epoxy monomers over night (minimal time 12h) up to 72 h at 60° C

# Silicone embedding molds