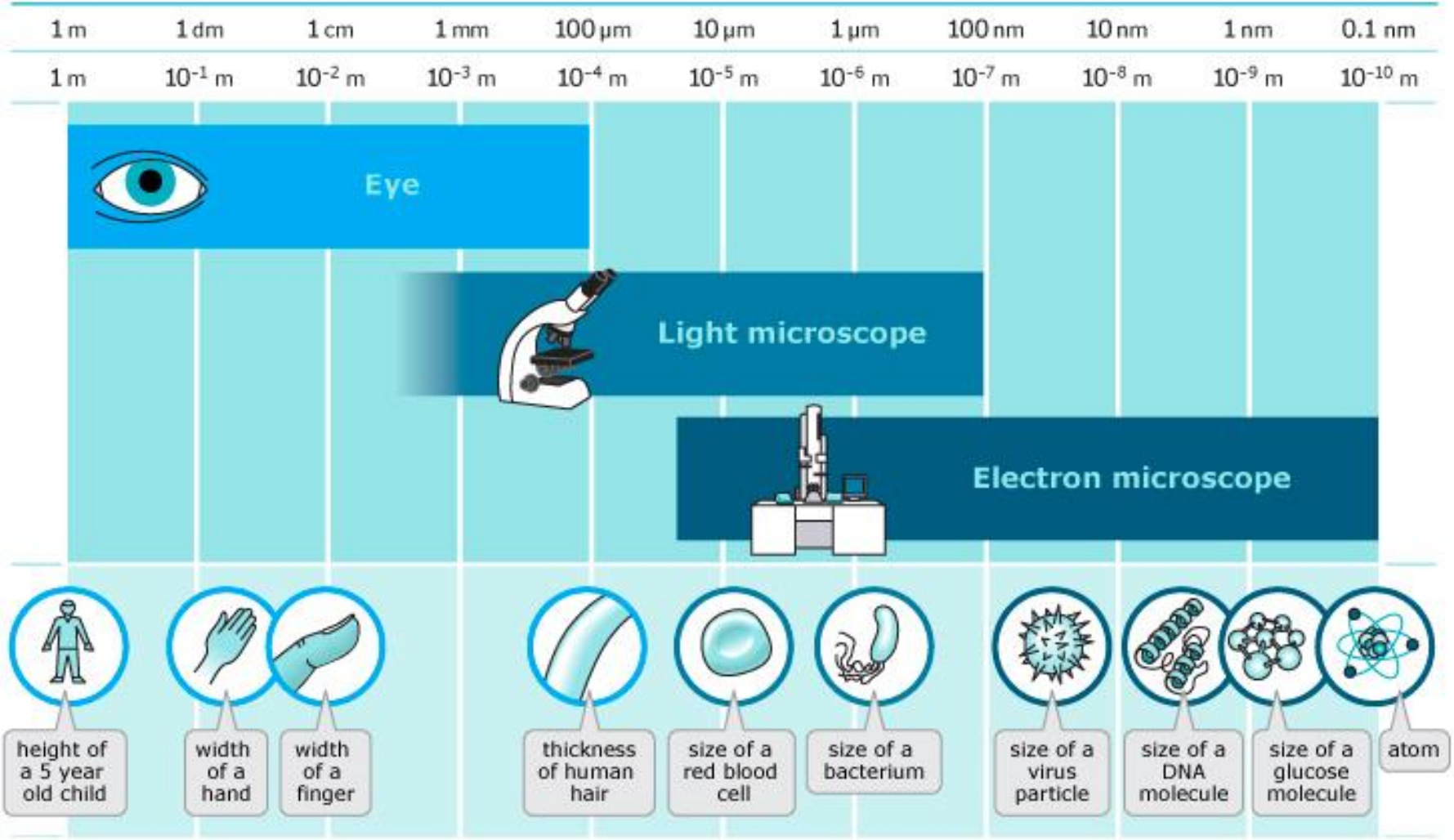
Scanning electron microscopy & microtomic techniques

2017.1.30

Lab meeting

Yu-Ling Huang

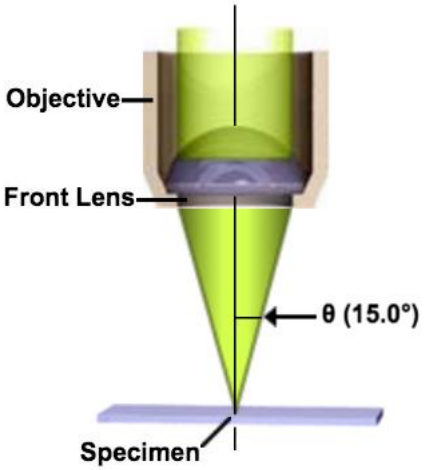
Resolving power of microscopes



	Magnification	Resolution	Depth of field
Light microscope	1-1000x	200nm	2µm
SEM	2-300,000x	3.5-6nm	Up to 1 cm
TEM	2,00-900,000x	0.21-0.5nm	0.11µm

$$r = \frac{1.22\lambda}{2n\sin\theta} = \frac{0.61\lambda}{\mathrm{NA}}$$

where



r is the minimum distance between resolvable points, in the same units as λ is specified

λ is the wavelength of light, emission wavelength, in the case of fluorescence,

n is the index of refraction of the media surrounding the radiating points,

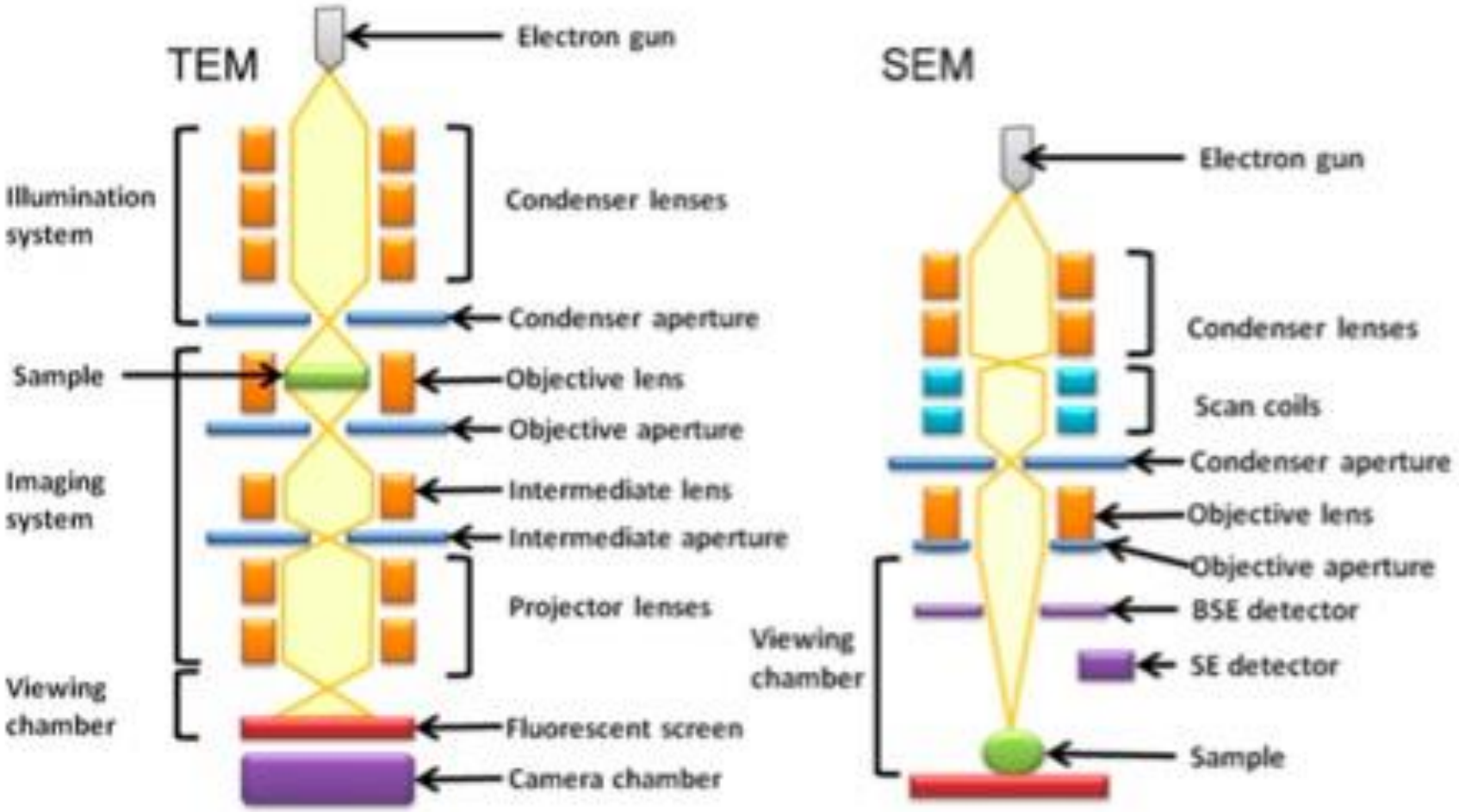
θ is the half angle of the pencil of light that enters the objective, and

NA is the numerical aperture

Visible light wavelength= 400-700nm

Electron wavelength= 0.004nm

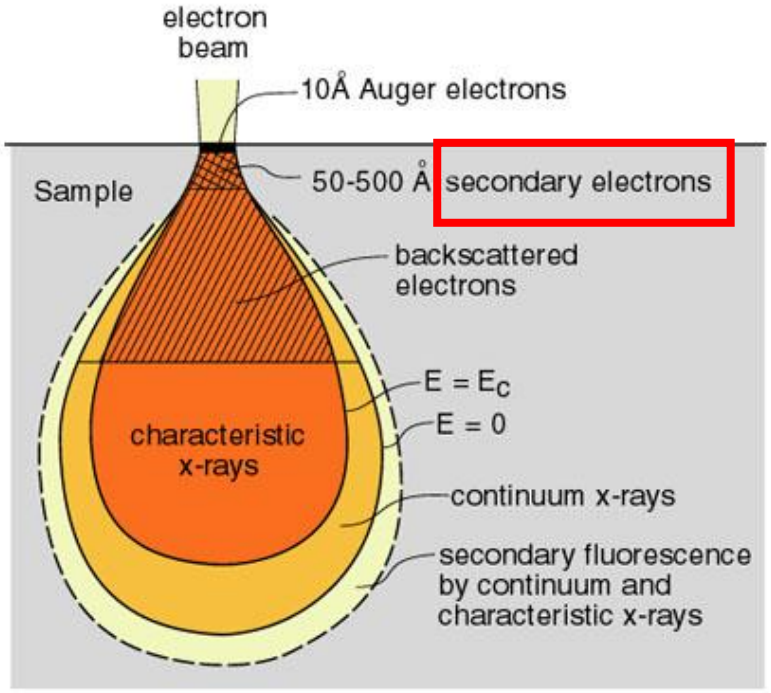
Electron microscope



http://bitesizebio.s3.amazonaws.com/wp-content/uploads/2016/07/08150412/Fig1.jpg

SEM

- Useful for observing surface details
- Electron beam is focused on surface of specimen with electromagnetic lenses in a vacuum chamber
- Electron beam scans back and forth across specimen
- Electron beam emit different electrons from the sample surface



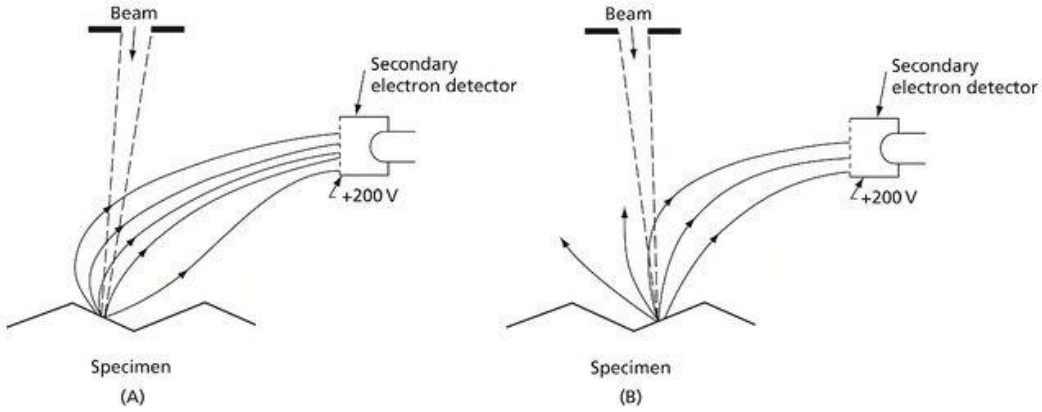
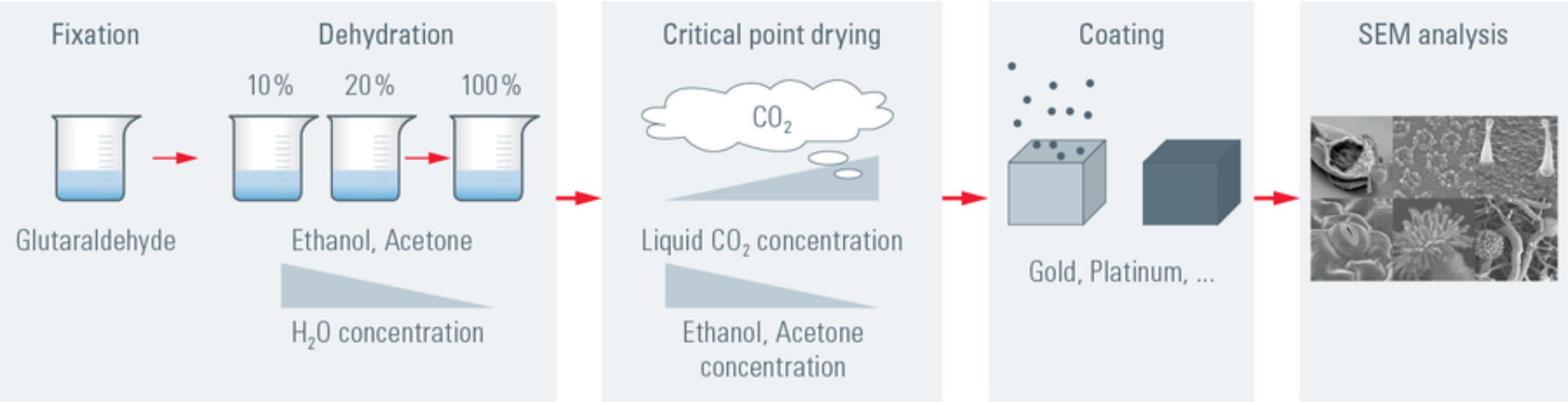


FIG. 2.24 An illustration of how the scanning electron microscope can reveal surface relief when used with a secondary electron detector

SEM sample preparation

- Fixation
- Dehydration
- Critical point drying
- Coating



http://www.leica-microsystems.com/fileadmin/_processed_/csm_ACPD---Workflow-SEM-analysis_8a314f6462.png

Fixation

- Problems of biological specimens:
  - Contrast lost
  - Redistribution or degradation of organelles
  - Wound induced changes
- Fixation preserves sample morphology, anatomy, cell and subcellular structure to keep the sample close to living status

- **Chemical fixation**: slow, general method.
- **Cryofixation**: fast, but ice crystal may break the tissue. Shrinkage is another problem.
- **Microwave fixation**: fast, but heat may affect subcellular structure.

Factors affect chemical fixation

- sample size and penetration rate
- pH and osmolarity
- Freshness and concentration of fixatives
- Fixation duration
- Temperature
- Amount of fixative
- Air space in tissue

Fixatives

- Coagulating Fixatives:
  - fix rapidly, change hydration state, proteins are coagulated. Not for high resolution
  - Example: acetone, methanol, ethanol

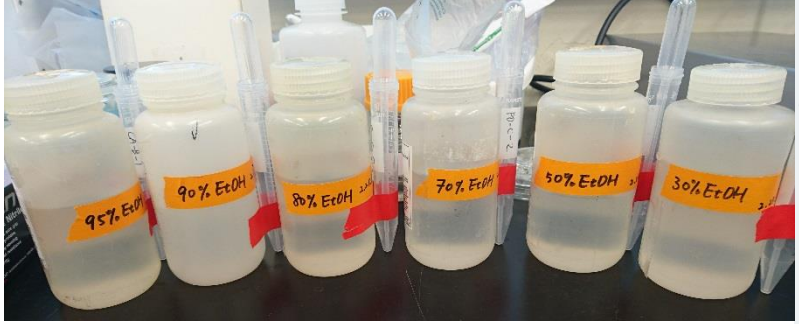
- Non-coagulating fixative
  - Slow penetration, form crosslink to proteins. Good for high resolution
  - Example: formaldehyde, glutaraldehyde, OsO_4

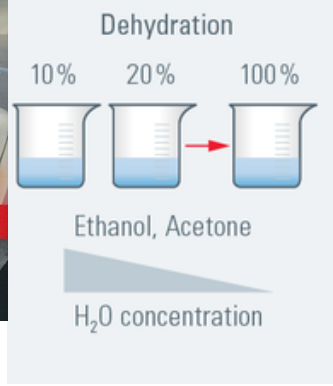
General fixatives for SEM

- 2.5-4% Glutaraldehyde (GA) in phosphate buffer (pH 6.8-7.4)
  - GA is good for preserve ultra-structure
  - Slow penetration

- Karnovsky's fixative (half-strength)
  - 2% Paraformaldehyde (fast)
  - 2.5% Glutaraldehyde (slow)
  - 0.1M phosphate Buffer (pH 6.8-7.4)

Dehydration





- SEM samples are in vacuum chamber, so the samples must be 100% dry
- Dehydration in ethanol series to remove water from tissue through substitution without causing extreme shrinkage
- Dehydration time depends on sample size, but general is 10-20min.
- Do not store samples in ethanol for extended time which may cause shrinkage (except in 70% ethanol)
- For samples with high water content, starts from 30% ethanol, otherwise 50% ethanol is fine.
- Keep samples ‘wet’ all the time

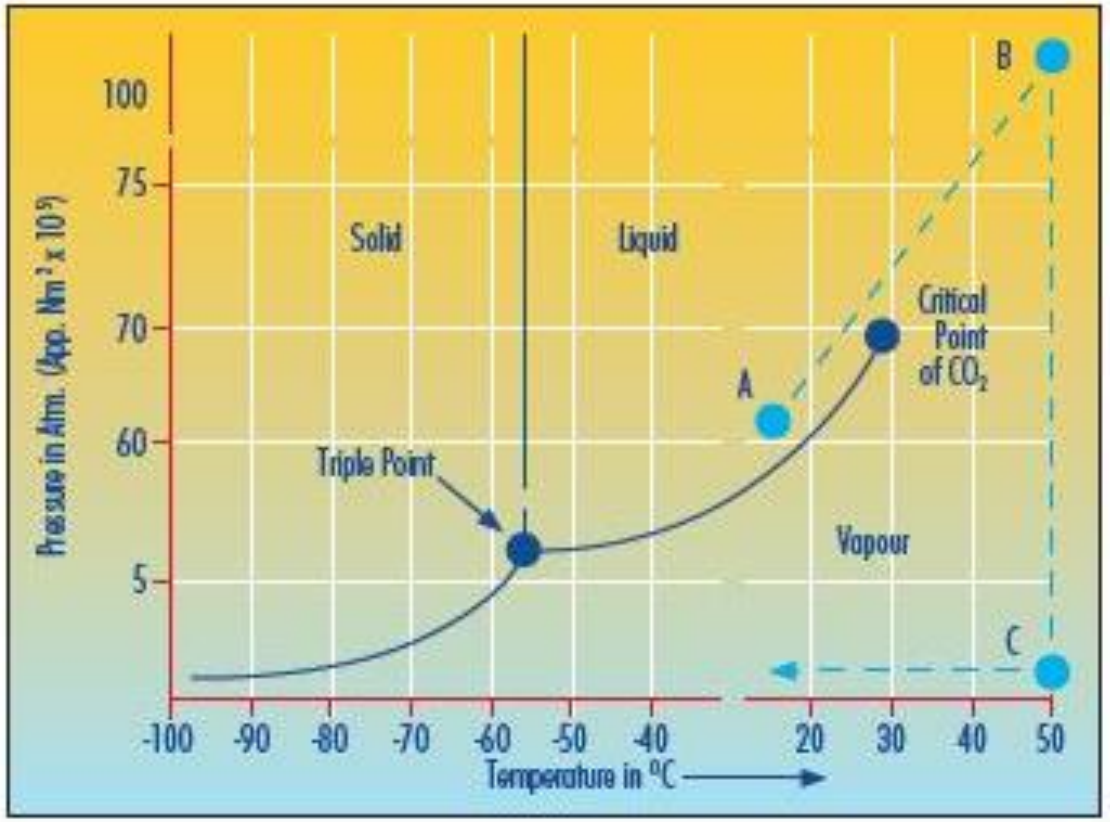
(30%→)50%→70%→80%→90%→95%→100%

Critical point drying (CPD)

- Water leaves tissue could cause breakage due to high surface tension
- No surface tension, no liquid/gas boundary in supercritical fluid

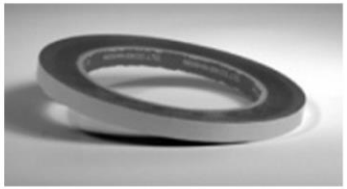
- CO2 is safe and has lower temperature and pressure than water to reach critical point

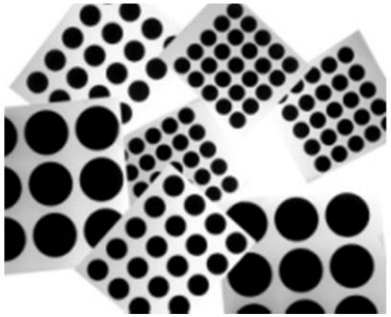
- Ethanol or acetone is compatible with CO2



Critical point dryer

Sample mounting





Carbon adhesive tapes/ tabs

SEM sample stub

Sputter coating

- Biological samples is non-conductive
- Protect samples from heat damage
- Reduce sample charging
- Gold or platinum
- Thickness
- Direction

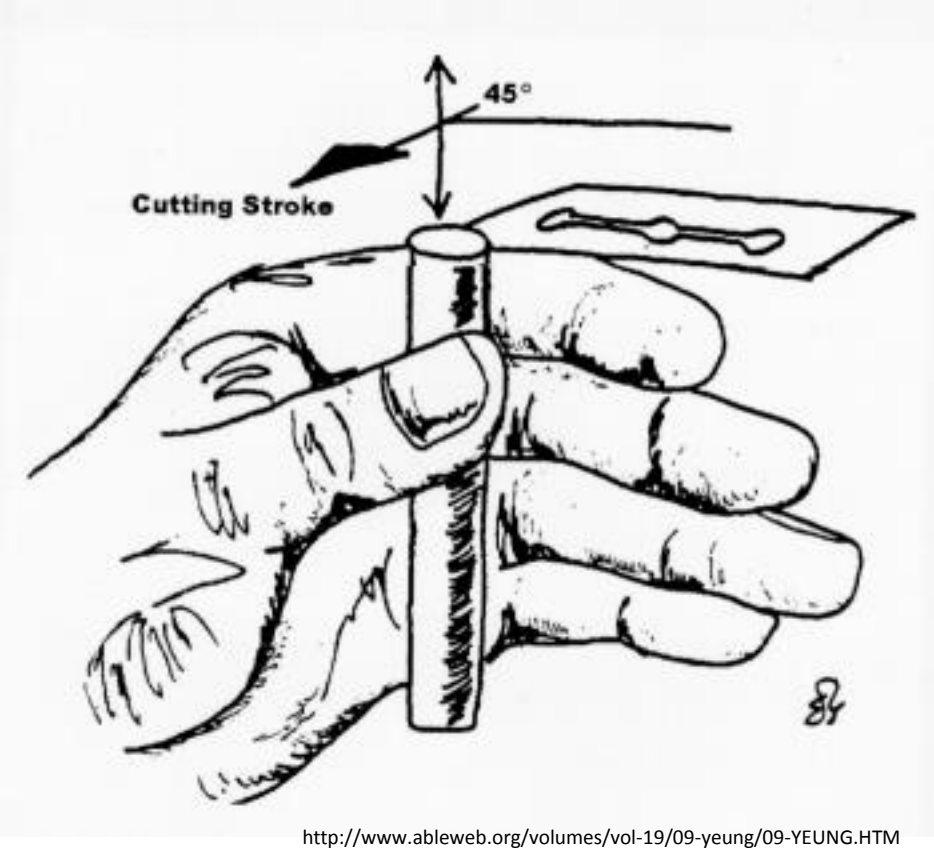
Hitachi S-4800 Type II Ultra-High Resolution Field Emission Scanning Electron Microscope

Microtomy

- Microtome: a tool for cutting samples into thin slices
- Normal thickness of the section: 1-10µm
- Ultra-thin section: < 1 µm (TEM)
- Types of microtome:
  - Free hand sections
  - Sliding/sledge microtome
  - Rotary microtome
  - Cryomicrotome
  - Ultamicrotome

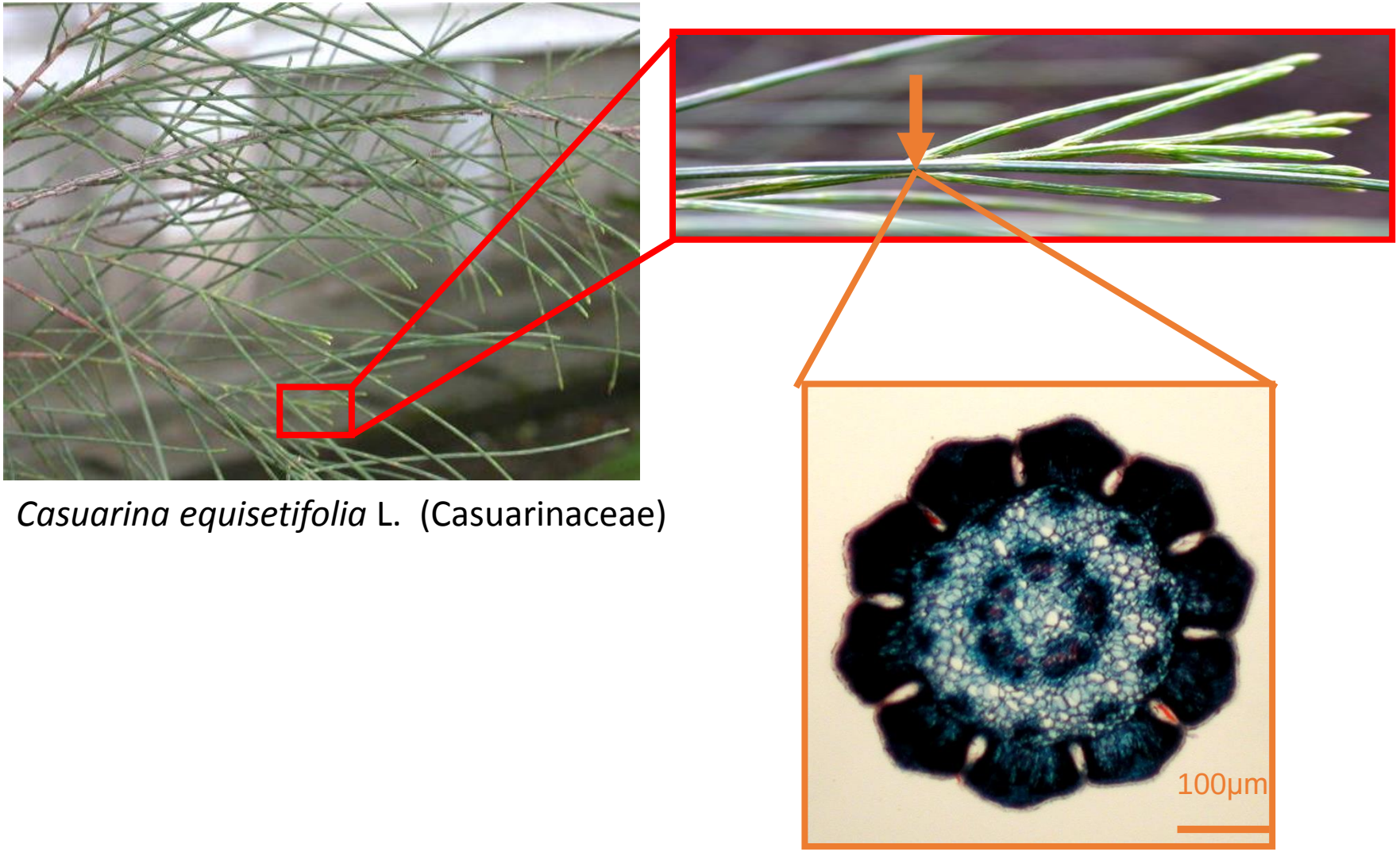
Free hand section

- Fast
- Thick sections
- Temporary preparations

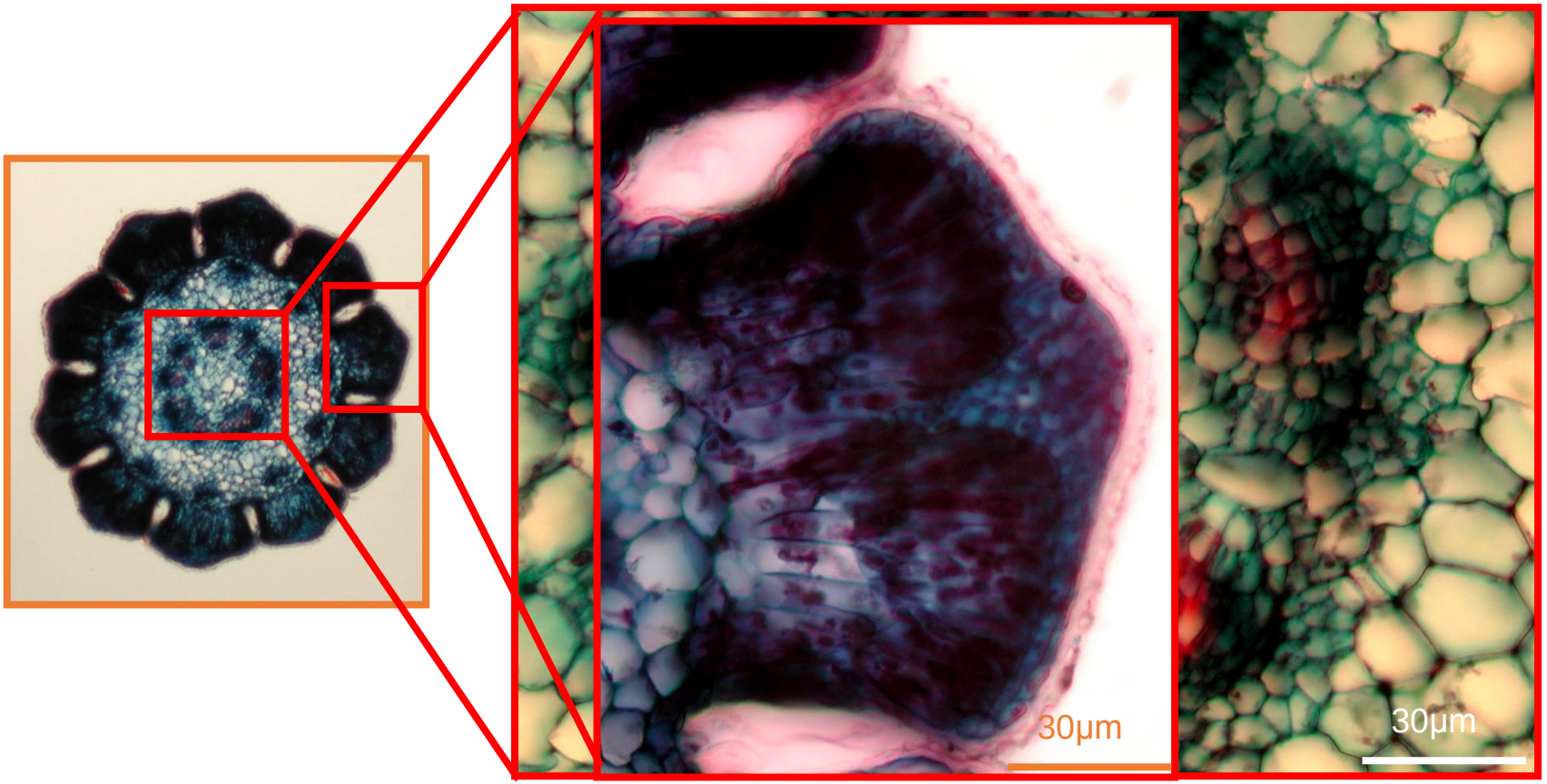


http://www.ableweb.org/volumes/vol-19/09-yeung/09-YEUNG.HTM

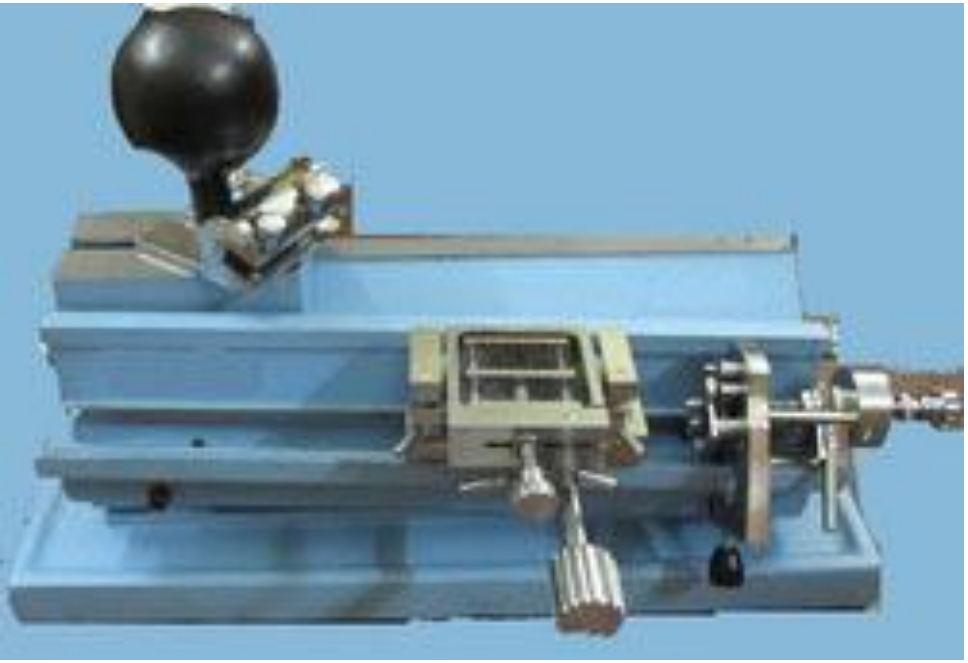
Free hand section

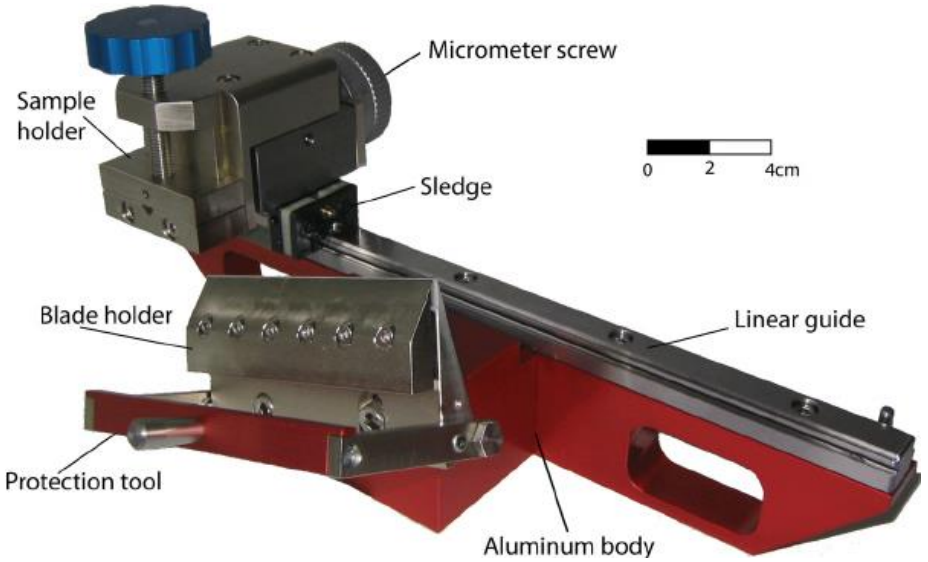


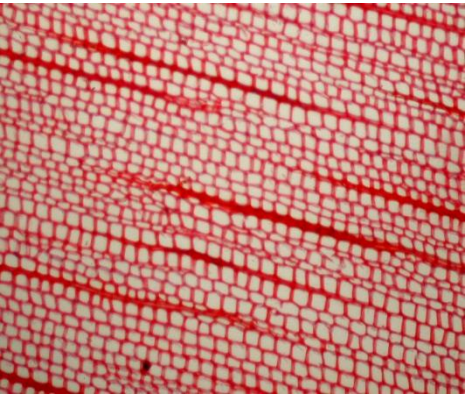
Casuarina equisetifolia L. (Casuarinaceae)

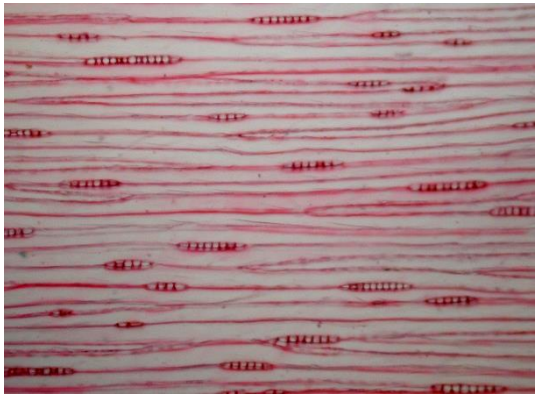

30µm
30µm

Sliding/sledge microtome



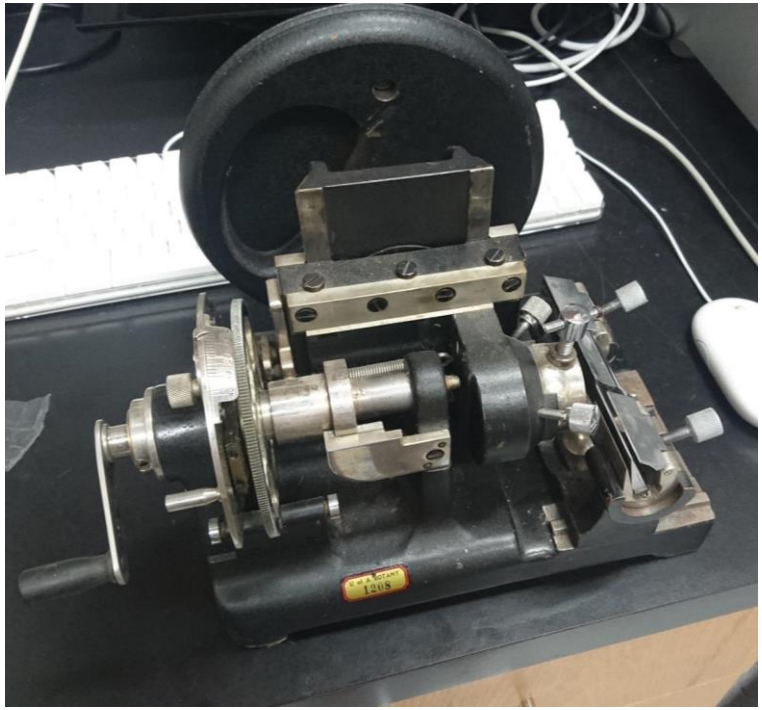






https://youtu.be/VcOmtP566b4

Rotary microtome



https://youtu.be/qGyE4p9XanA

Cryomicrotome

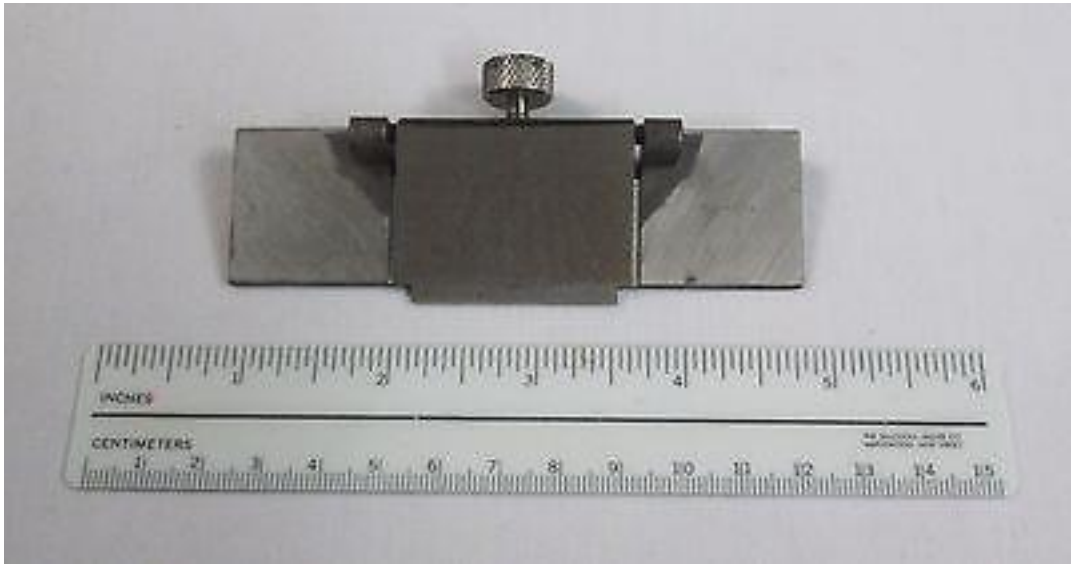
Ultramicrotome

Microtome knife

- Steel knife

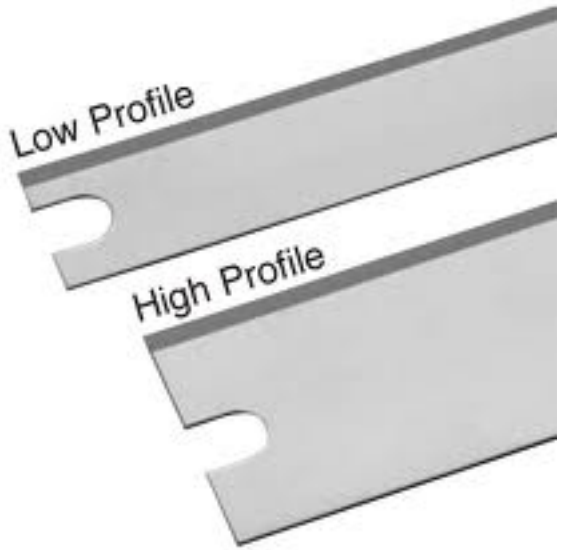
Microtome knife

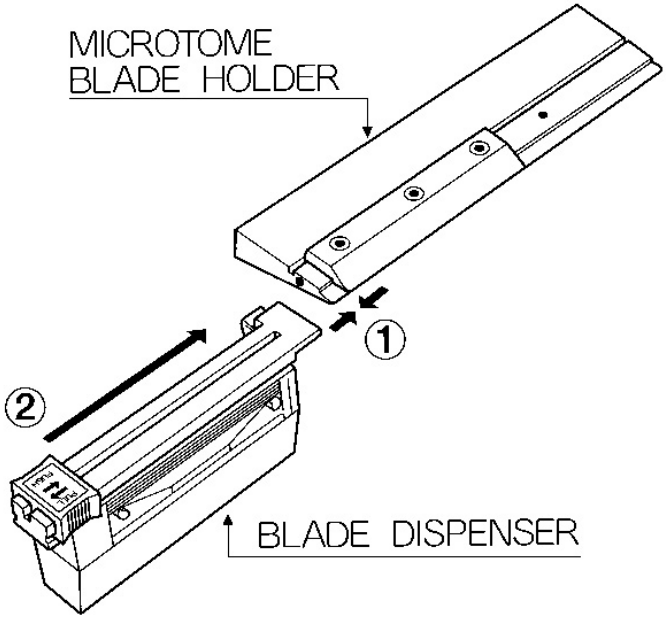
- Steel knife
- Razor blade



Microtome knife

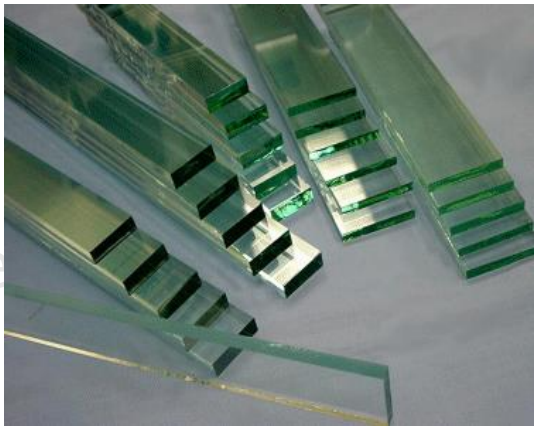
- Steel knife
- Razor blade
- Disposable steel blade

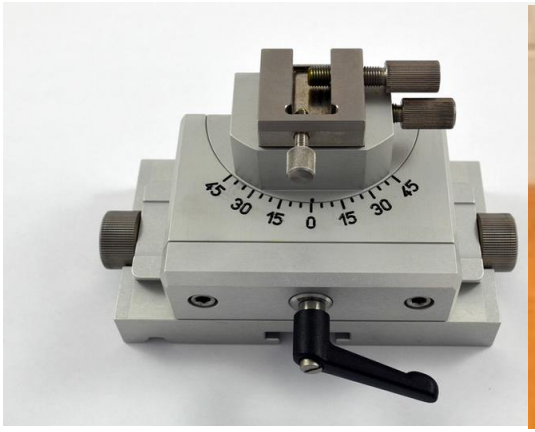


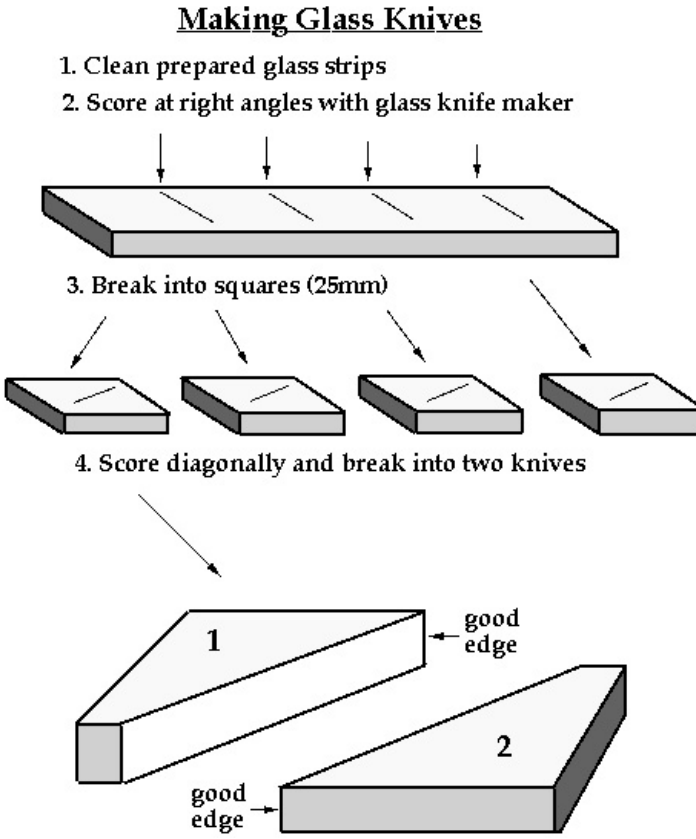


Microtome knife

- Steel knife
- Razor blade
- Disposable steel
- Glass knife







Microtome knife

- Steel knife
- Razor blade
- Disposable steel blade
- Glass knife
- Diamond knife

Embedding medium

Paraffin

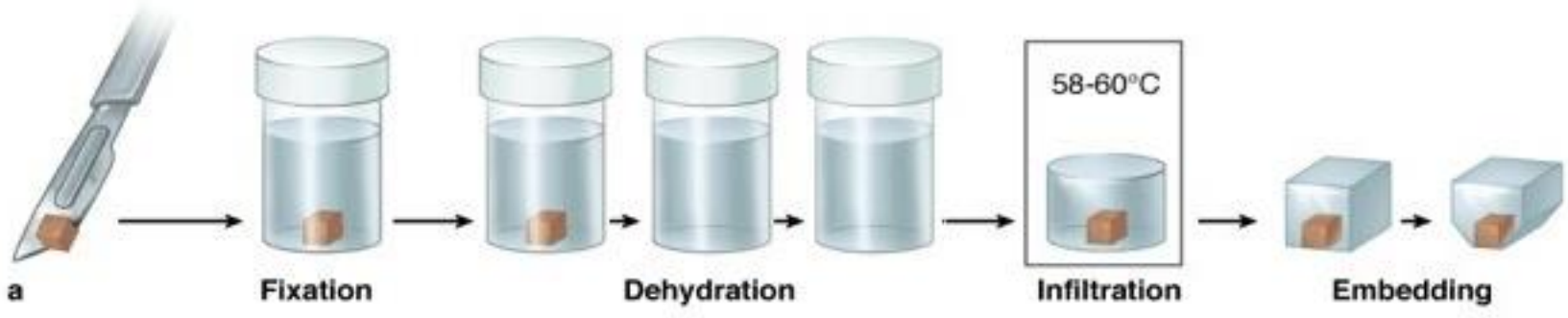
- Low cost
- Fast
- Soft and easy to cut
- Need de-paraffin before staining
- Section 5-10 µm

Technovit 7100
(Glycol Methacrylate)

- High cost
- Slow
- Hard to cut
- Easy to stain
- Section 1-5 µm

Sample preparation for rotary microtome

- Fixation
- Dehydration
- Infiltration
- Embedding



Fixation

- Similar to the SEM process

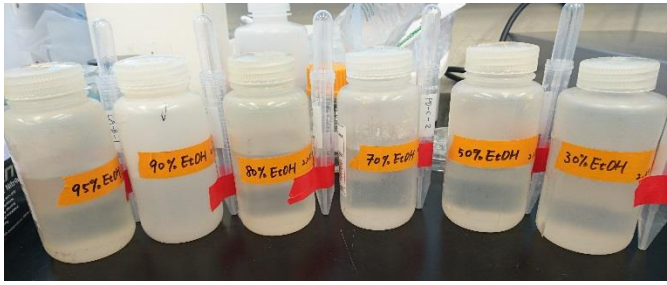
- Common fixative in botanical studies:
  - Formalin- Acetic Acid –Alcohol (FAA)

50% (or 70%) Ethanol	90 ml
Glacial Acetic Acid	5 ml
Formaldehyde (38%)	5 ml

Dehydration

- Select the dehydration solution which is compatible with the embedding medium
- Series of t-butanol (TBA) in 95% ethanol and water for paraffin
- Series of Ethanol for Technovitt 7100

  50%→70%→80%→90%→95%→100%

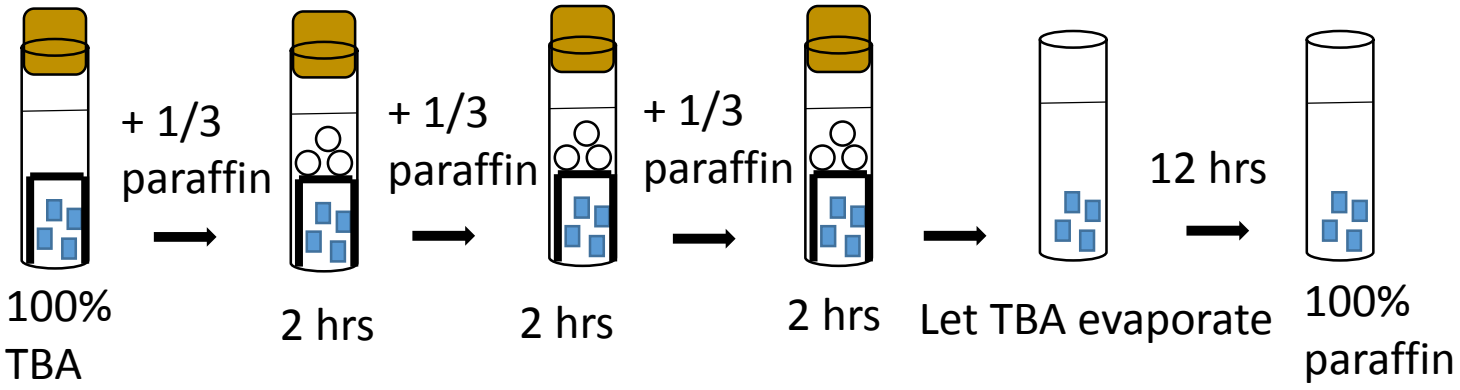


TBA dehydration series

steps	t-butanol	95% ethanol	dH_2O
1	10	40	50
2	20	50	30
3	35	50	15
4	55	45	0
5	75	25	0
6	100	0	0

Paraffin infiltration

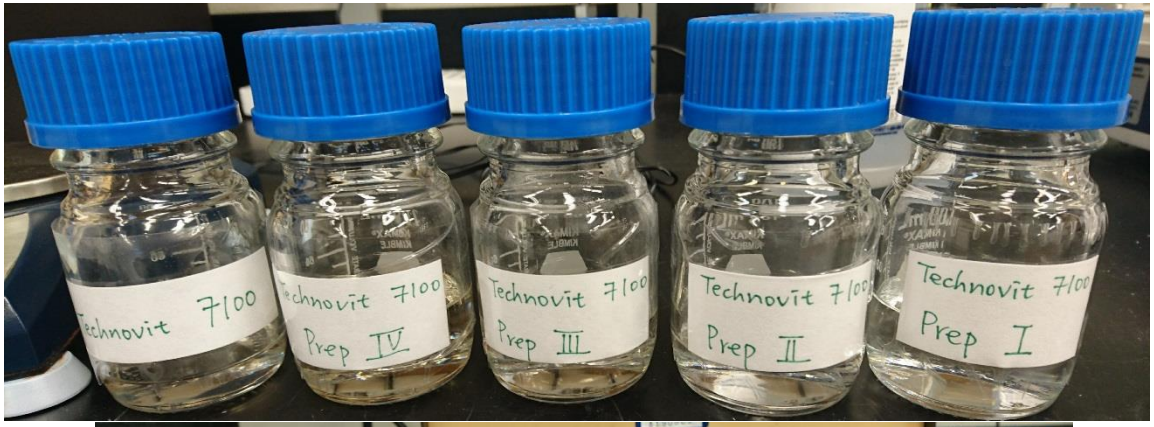
- Let embedding medium infiltrate into tissue
- Paraffin infiltration in oven around 60 °C
- Lower temperature is better but must higher than the melting point of paraffin



Technovit 7100 infiltration

Prep	100% Ethanol	“Technovit 7100”
I	5	1
II	3	1
III	2	3
IV	1	5

“Technovit 7100”: 100mL Technovit 7100 + 1 g hardener I (1 pack)



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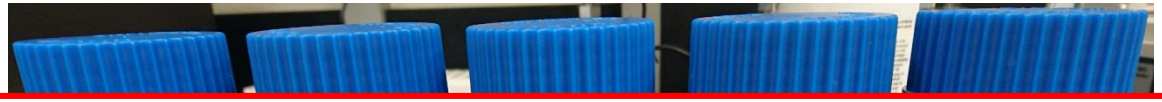


Infiltration steps:

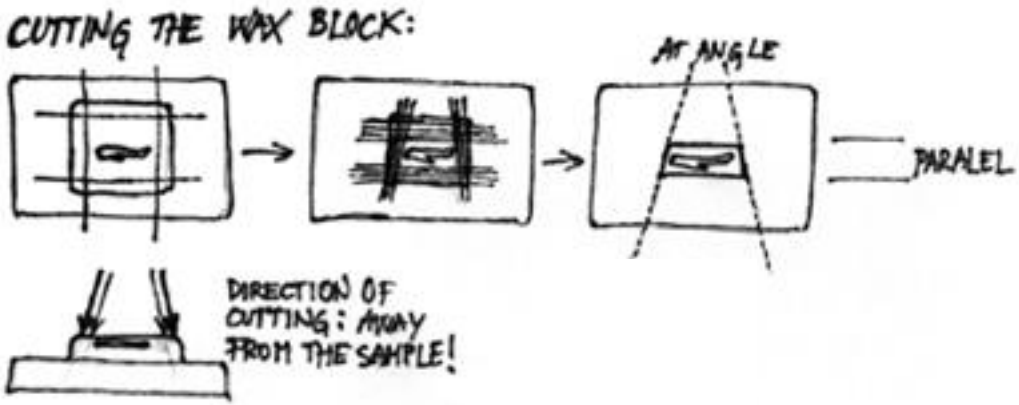
Technovit 7100 Prep I	12 hours
Technovit 7100 Prep II	12 hours
Technovit 7100 Prep III	12 hours
Technovit 7100 Prep IV	12 hours
"Technovit 7100"	12 hours
"Technovit 7100"	24 hours**

**If longer than 24 hours, put in 4 ˚C to prevent polymerization.

Paraffin embedding

Paraffin sample block

- Trimming
- Attach to a wood block



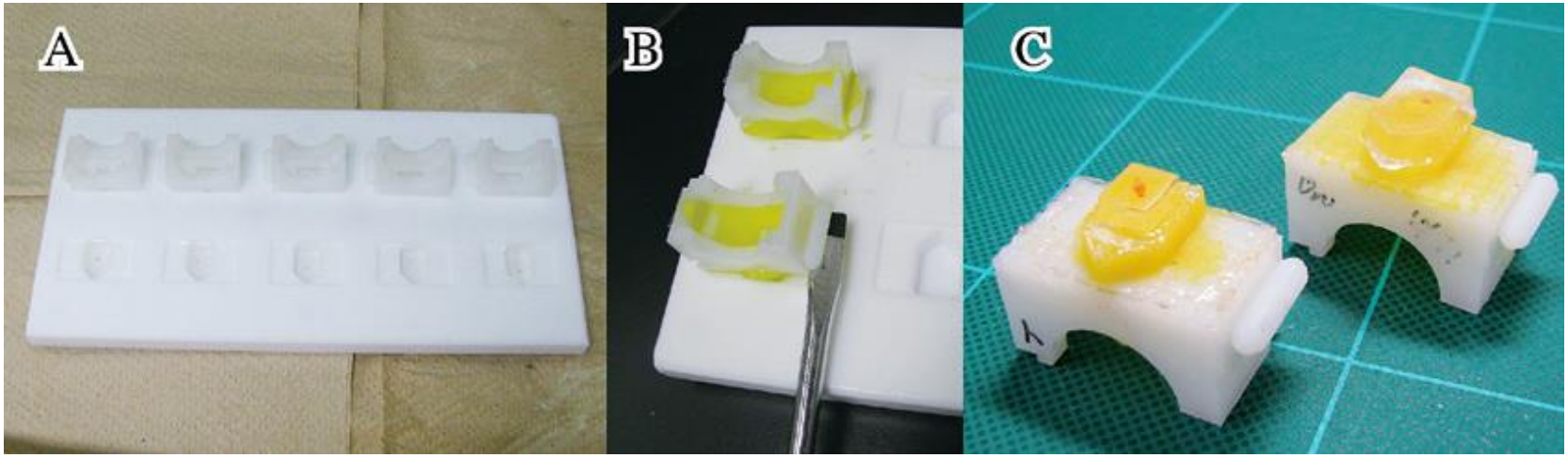
Technovit 7100 embedding

- Prepare embedding medium:

  15 mL “Technovit 7100”
  1 mL Technovit 7100 harderner II
  0.6 mL PEG 400

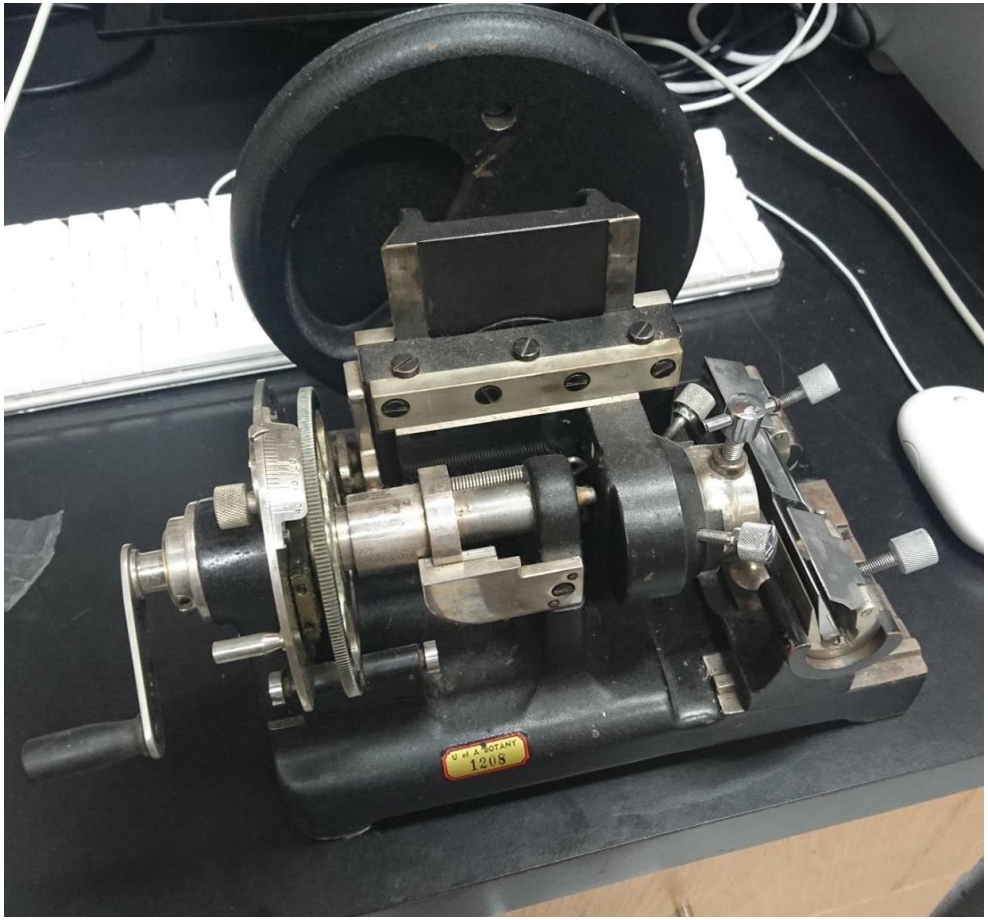
- Fill each block of HistoForm S with the embedding medium using micropipette (P1000)
- Transfer each sample to each block with tweezers, toothpicks or pipette tips
- Wait samples sink to bottom, and adjust the orientation of the samples
- Put the HistoForm S horizontally in 4 °C, overnight (~ 1 day) for polymerization

Technovit 7100 sample block



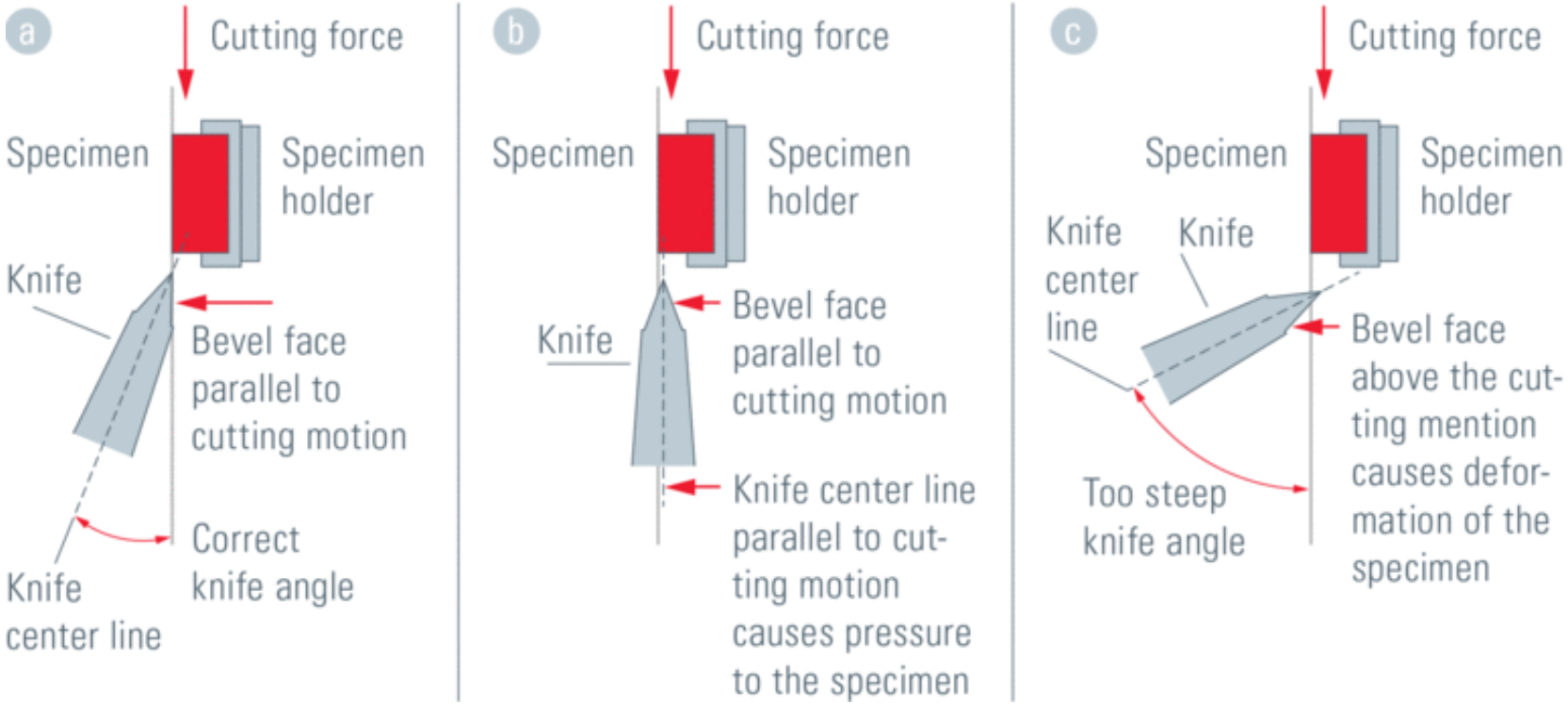
*Put sample block on 80°C heat plate to soften the block before trimming
** re-embedding if the sample orientation is not right

Microtome operation



AO Spencer 815 Rotary Microtome

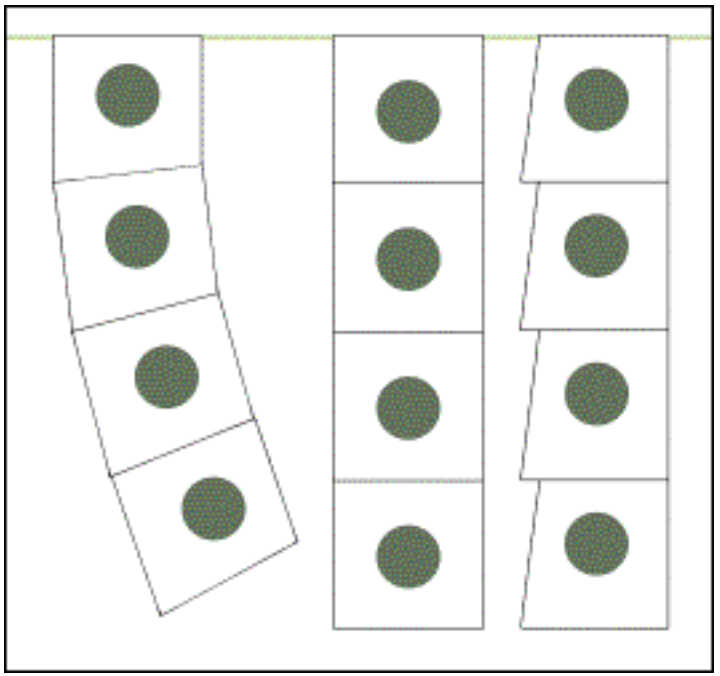
Knife angle (clearance angle)



paraffin section: 4-6 degree
Technovit : 10 degree

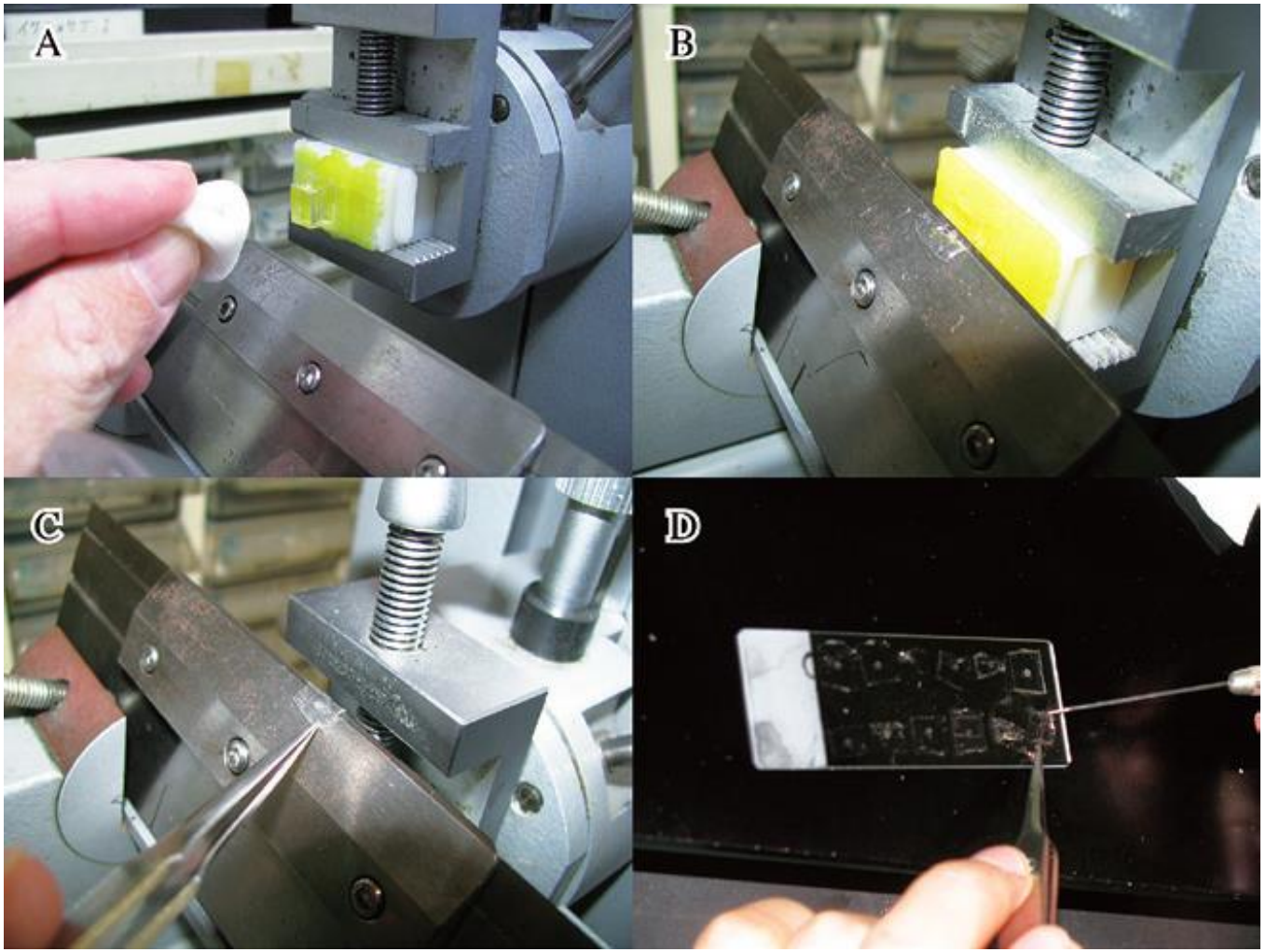
Paraffin ribbon

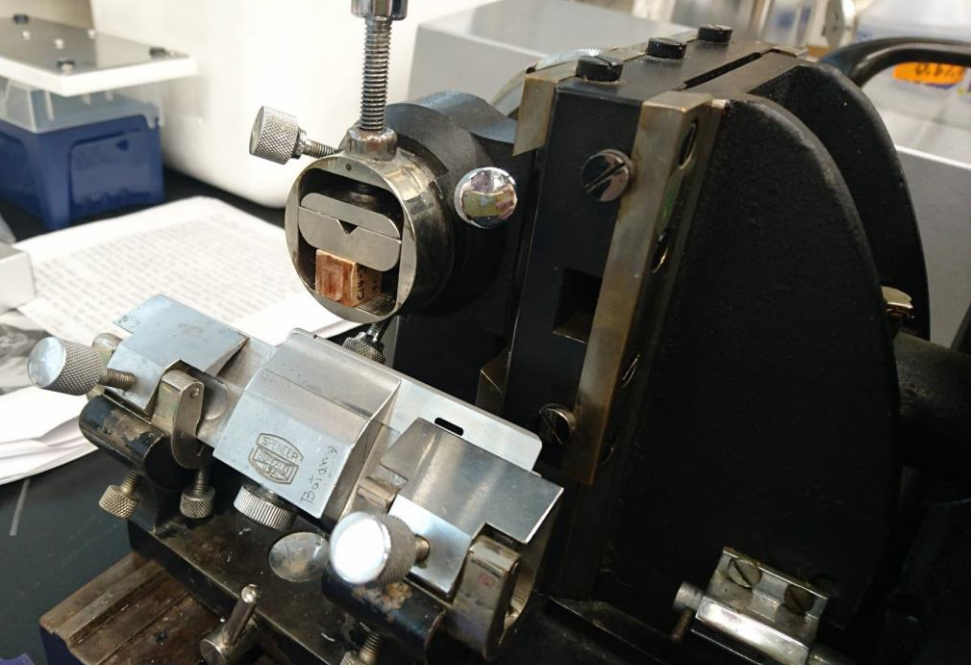
Paraffin sections could form a straight and long ribbon if everything is good

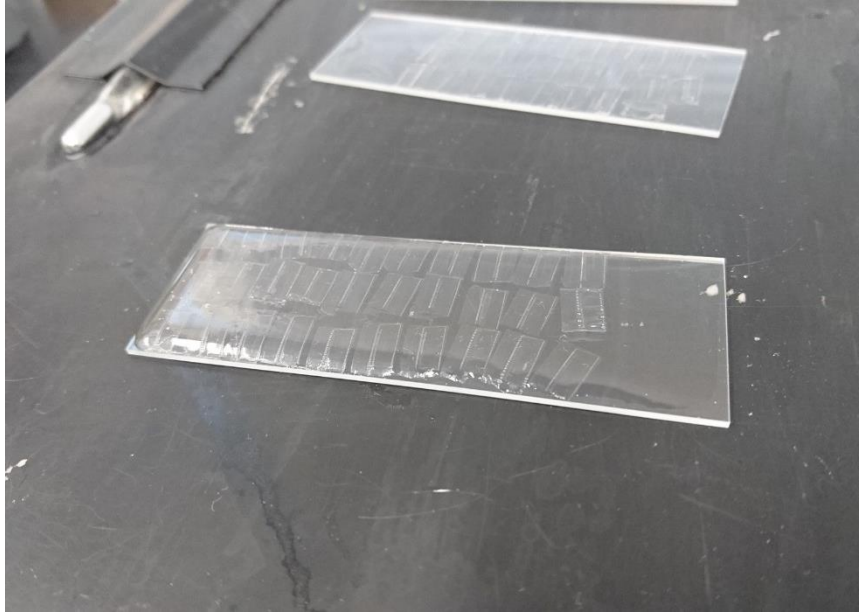


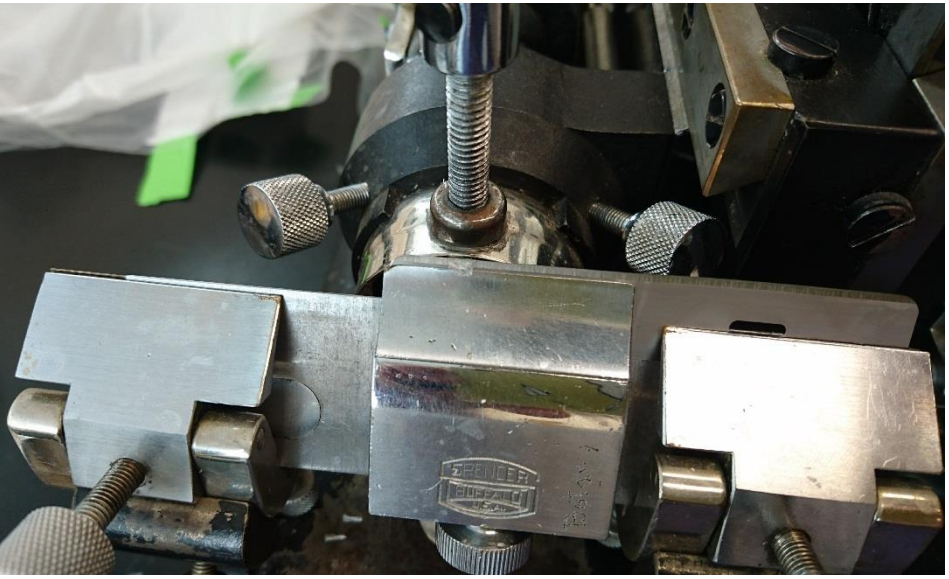
Inadequate trimming

Technovit 7100 section









Staining

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New staining technique for fungal-infected plant tissues

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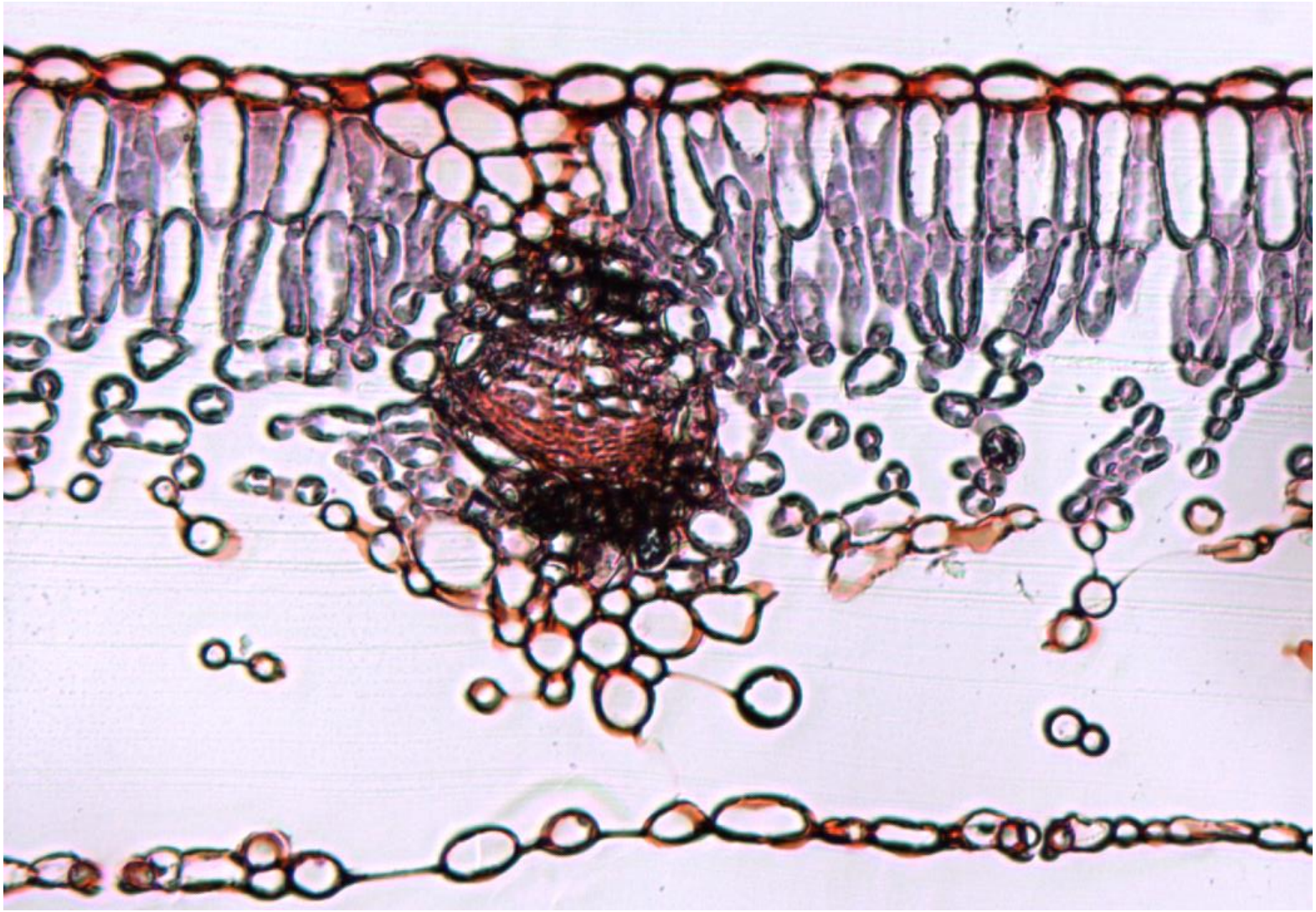
2.4. Staining procedure

1. Staining in 5% blue cotton in lactophenol for 20 min (Macedo, 1997);
2. Three 1-min washes in distilled water;
3. Staining in 1% aqueous safranin O for 10 s;
4. Three 1-minute washes in distilled water;
5. After drying, the slide can be mounted with an Entellan® synthetic resin.



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