# Wood Sample Preparation for Microscopic Analysis

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# Protocol for preparation of high quality slides
## (A slightly more time consuming method)

In wood/phloem formation studies at cellular and ultrastructural levels, tissues containing phloem, cambium and outer xylem are collected from living trees. The time intervals of samplings are relatively short, i.e., one to two weeks, and depend on the goal of the study. For observations of the seasonal dynamics of different phases of wood or phloem formation, small micro-cores are usually collected with tools causing minor damage on tree stems.

A Trephor tool has recently become widely used for sampling (Rossi et al. 2006) but an increment puncher (Forster et al. 2000) and injection needles (Jyske et al. 2011) are also used. Due to the small size of wounds caused by these tools, repeated sampling on the same tree can be performed in more than one growing season, without affecting the vitality of the tree. However, micro-cores (e.g., diameter c. 2 mm and length c. 10 mm) are difficult to handle and they can be easily damaged or the tissue can be affected.

The following protocol describes, how to produce high quality samples for observation under light microscope.

### *A. Sample collection*

**Equipment needed:**

- Chisel
- Hammer
- Trephor
- Histosetts
- FAA (500 ml of FAA contains: 25 ml of 37% formalin, 450 ml of 50% ethanol and 25 ml of 100% acetic acid)

The samples are usually collected at tree's breast height (1.3 m). In case of wood formation studies samples are collected during the vegetation period. To avoid wounding effects the samples should be collected a spiral up the stem and sampling locations should be separated by at least 10 cm.

Immediately after removal from the trees, the samples were fixed in ethanol-formalin acetic acid solution (FAA) (Gričar, 2007).

The samples can be placed directly into Eppendorf tubes filed with FAA or directly into Histosetts (which are then transferred into a bottle filled with FAA). Usually we collect two micro-cores from each of the tree; one sample is archived and the other is prepared for further processing.

The collected samples should be stored in the fixation solution for maximum one week.

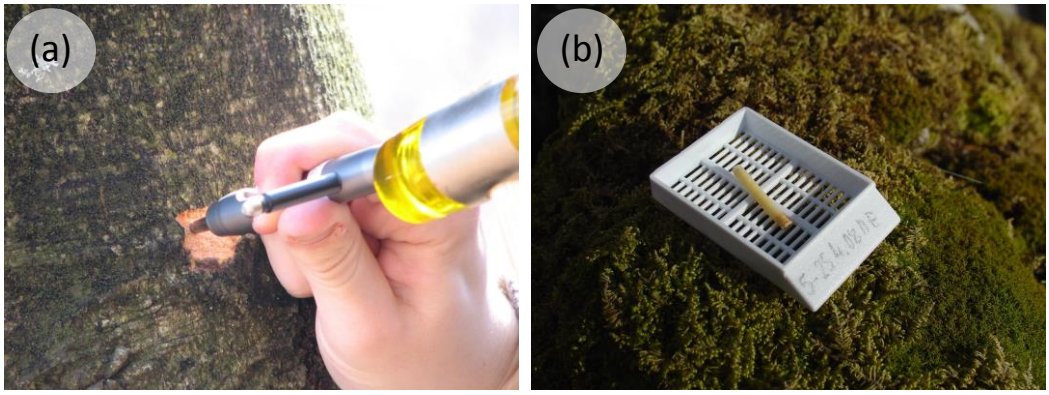


**Figure 1**: Micro-cores are collected with the Trephor tool (a) and moved into properly marked Histosettes.

## B. *Micro core preparation*

**Equipment needed:**

- razor blade
- pencil
- tissue processor

Before further processing the samples should be reduced in size and contain only non-collapsed and collapsed phloem, vascular cambium and at least two of the last formed xylem growth rings.

1. First, the excess bark and xylem growth rings are removed with a razor blade.
2. Then the transverse plane of the microcore is identified and marked with the pencil. Usually, radial plane can be easily distinguished from the transverse one because the transverse plane appears darker and the radial one brighter.
3. Samples prepared for further processing are placed into a basket of the tissue processor containing 70% ethanol. When the basket is filled with the samples it can be moved to the tissue processor.

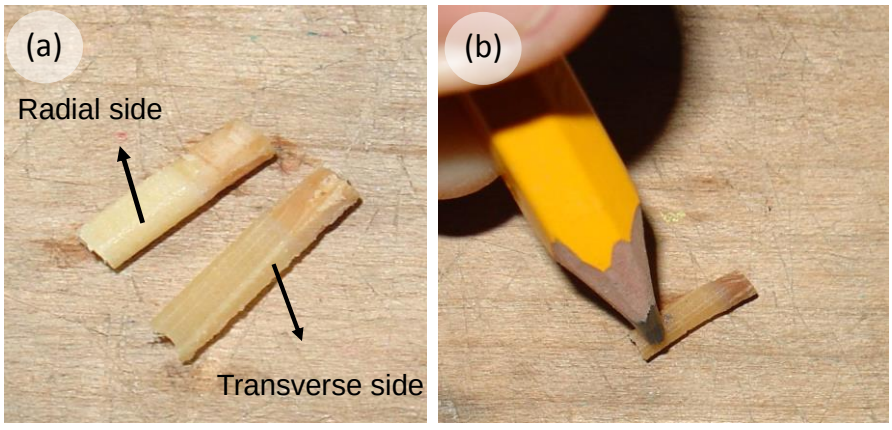


**Figure 2**: The transverse plane of the microcore is identified (a) and marked with the pencil (b)

## C. *Dehydration and infiltration*

**Equipment needed:**

- tissue processor
- ethanol
- clearing solvent (e.g. D-limonene)
- paraffin

1. The tissue processor enables automatic dehydration and infiltration of the samples with paraffin.
2. The first seven baskets contain different concentrations of ethanol, the next three baskets are filled with the clearing solvent (instead xylene other less toxic clearing solvent should be used; for example D-limonene), however other odour free solutions can also be used.
3. The last two baskets are heated up to 60° C, and contain paraffin.
4. The whole dehydration and infiltration procedure lasts 20 hours. After this, the samples can be embedded in paraffin.

**Table 1**: Steps for paraffin embedding in the tissue processor according to Rossi et al 2006.

| Reagent | Time (min) |
|---|---|
| Ethanol 70% | 120 |
| Ethanol 70% | 120 |
| Ethanol 90% | 90 |
| Ethanol 90% | 90 |
| Ethanol 95% | 90 |
| Ethanol 100% | 90 |
| Ethanol 100% | 90 |
| D-limonene 9 | 90 |
| D-limonene 9 | 90 |
| D-limonene 9 | 90 |
| Paraffin | 120 |
| Paraffin | 120 |

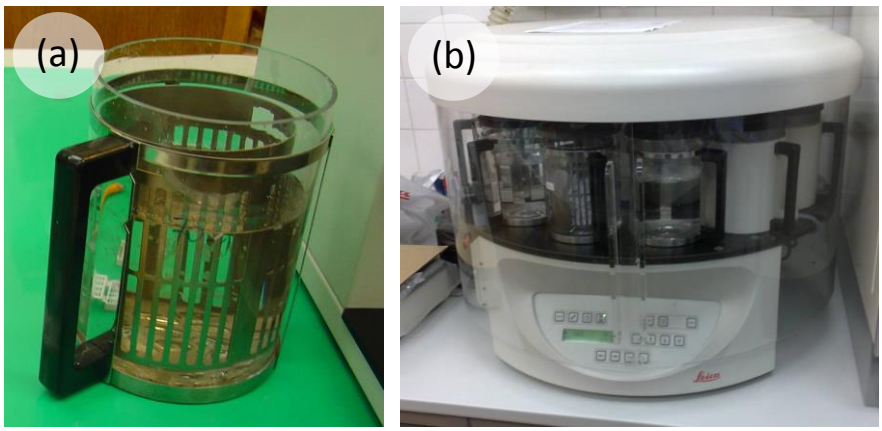


**Figure 3**: The prepared samples are placed into a basket of the tissue processor (a). The tissue processor contains twelve vessels/containers filled with different concentrations of Ethanol, clearing solvent and paraffin (b).

## D. *Preparation of paraffin blocks*

**Equipment needed:**

- Paraffin dispenser with integrated hot plate
- Electrically heatable forceps
- Metal moulds
- Petri dish filled with cold water
- Gloves

1. For preparation of paraffin blocks we use a paraffin dispenser with a heated plate. Both, the container with the paraffin and the plate, should be heated to around 60 degrees, depending on the type of paraffin.In addition, hateable forceps, metal moulds and a Petri dish filled with cold water are needed.
2. During the embedding procedure histosettes with samples should be kept in baskets containing paraffin or placed on the heated plate of the paraffin dispenser. Also, the metal moulds should be preheated to the same temperature.
3. Before placing a microcore into the mould, a small amount of paraffin should cover the bottom. Then the sample is placed into the mould in a diagonal direction, and the bottom of the mould should be immersed into the cold water so the paraffin can harden. This will stop any movement of the microcore.
4. When the mould is full, the top part of the histosette is placed on top of the metal mould and filled with additional paraffin.
5. After that the samples should be cool down to room temperature (for approximately half an hour). For easier separation of the paraffin block from the metal mould, they can be placed in to the refrigerator or freezer for approximately 15 to 20 minutes.

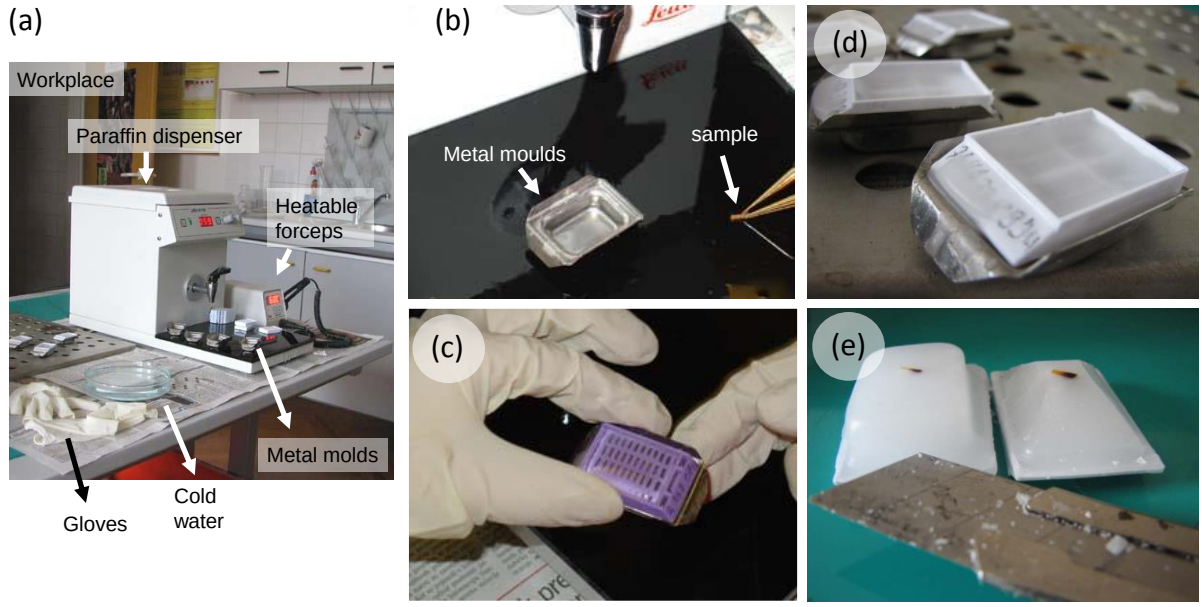


**Figure 4**: For preparation of paraffin blocks paraffin dispenser, hateable forceps, moulds and a dish of cold water is needed (a). Samples are placed into a metal mould containing paraffin (b). The top part of the histosette is placed on top of the metal mould and filled with additional paraffin (c). The samples are the cooled down to room temperature (d). The surface of the paraffin blocks should be reduced with a knife, to ensure the smallest possible surface for preparation of slices with rotary microtome (e).

## *E. Reduction of paraffin blocks*

1. The surface of the paraffin blocks should be reduced with a knife, to ensure the smallest possible surface for preparation of slices with rotary microtome.
2. The cutting surface should have a trapezoid shape, with the size of 1 cm2 for optimal formation of the ribbon at the rotary microtome.

## *F. Trimming of the paraffin blocks and cutting of samples*

**Equipment needed:**

- Half-automatic rotation microtome
- Knives for mounting into rotation microtome
- Slides
- Glycerin Albumin
- Wooden sticks
- Glass of water
- Slide holder
- Water bath
- Pencil

1. Before sectioning, the paraffin blocks should be trimmed with a rotary microtome. This step removes excess paraffin and levels the surface.
2. The trimmed blocks should now be placed into cold water for at least two hours (or overnight) to soften the woody tissue. This will produce slices of higher quality.
3. When the tissue is moistened enough, it is prepared for cutting.
4. Before cutting the object glasses should be coated with a drop of Glycerin Albumin for better adhesion of the slices to the glass. It is important that the amount of the adhesive is appropriate; too much Glycerin can result in opaque and dirty samples. Too little Glycerin can cause that the sections will be washed away in the subsequent steps.
5. Furthermore, it is important not to forget to label the sample on the object glass.
6. For cutting, paraffin block are mounted to the clamping device of the rotary microtome. The thickness of the slices is usually 8 to 10 um.
7. The formed ribbons should be transferred with two sticks, first into the cold water, and then into water heated to 40° C, to stretch them.
8. It is important that transfer of sections to the object glass is done as quickly as possible; otherwise Glycerin Albumin is washed away.
9. Then the object glass with the sections can be moved to a glass basket or other object glass holder.
10. When the basket is full the samples should be heated in a dryer up to 70° C in order to ensure the best adhesion between the section and object glass.
11. When this process is finished, let the samples cool off, before proceeding with further steps.

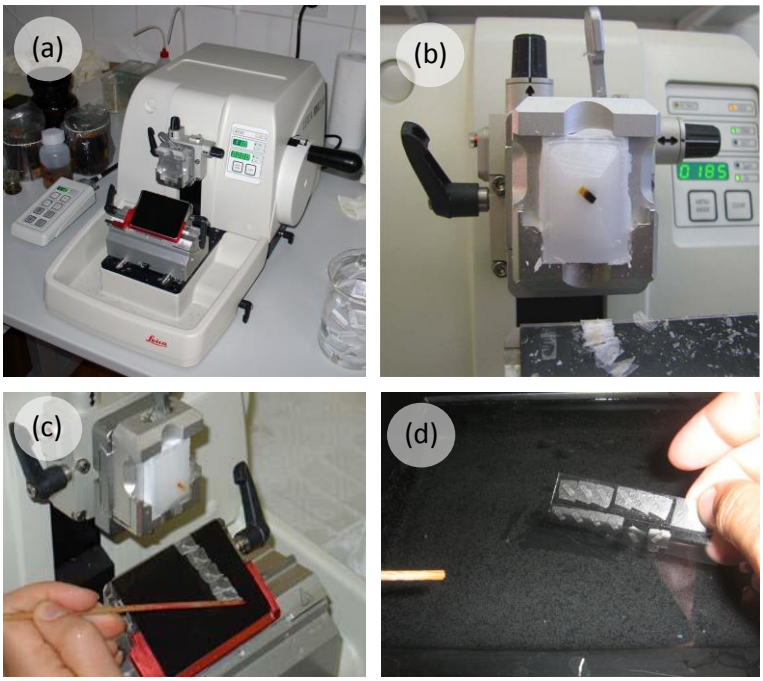


**Figure 5**: Samples are cut with a rotary microtome (a). Before sectioning, the paraffin blocks are trimmed to removes excess paraffin and level the surface (b). Sectioning is performed (c) after the trimmed samples were placed for at least two hours into water. The formed ribbons should be transferred into warm water (40° C warm) to stretch them. Sections are then transferred to the object glasses (d).

## G. *Staining*

**Equipment needed:**

- Gloves
- Two baskets filled with clearing solvent
- Four baskets filled with 100% ethanol
- One basket filled with water solution of safranin and astra blue
- One basket filled with distilled water

1. Before staining the sections, the paraffin should be removed from the slices.
2. Therefore the samples are placed into two baskets with clearing solvent (e.g. D-limonene or Ultraclear), for 15 minutes in each basket, and after that in two baskets filled with 100 % ethanol, also for 15 min in each.
3. Then the sections can be coloured in a water solution of safranin and astra blue (van der Werf et al., 2007) for at least 15 min.
4. After that the sections are washed clean. First with distilled water and followed by 70 and 100 % ethanol (for a few seconds).

## H. *Preparation of permanent slides*

- Gloves
- Cover glasses
- Euparal mounting medium
- Preparation needle

- Tweezers
- Thin paintbrush
- Weights

1. Before mounting in Euparal, clean the object glass and remove twisted or damaged sections.
2. Put a drop or two of the mounting medium Euparal onto the sections and cover them with the cover glass.
3. Finally remove the air bubbles and the excess of the mounting medium.
4. Weight the sections with 200 g metal weights and leave them for at least 24 hours, so that the resin hardens.

The samples are now ready for observation under the light microscope.

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