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# TREPHOR: A NEW TOOL FOR SAMPLING MICROCORES FROM TREE STEMS

**Sergio Rossi, Tommaso Anfodillo** and **Roberto Menardi**

Treeline Ecology Research Unit, Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova, viale dell'Università 16, 35020 Legnaro (PD), Italy
[E-mail: sergio.rossi@unipd.it]

SUMMARY

Detailed analyses of cambium activity and wood formation during growth need repeated sampling of newly formed xylem. In order to be minimally invasive, wood samples are extracted as microcores. Despite the research done on xylem cell development and the increasing interest in intra-annual studies of xylogenesis, few tools are available for microcore sampling. Methods originally designed for other purposes have often been used, but no details are available on their efficiency and usefulness. Information is also lacking on laboratory preparation techniques for cell analysis of tree-ring formation, leading to difficulties in carrying out these experiments. The advantages and limits of the tools used up to now are described. A new tool, named Trephor (patent pending n° PD2004A000324), specifically designed for long-lasting use is presented. Trephor is chisel-shaped for a fast recovery of 2 mm diameter microcores. The cutting tube is inserted into the wood using a hammer and no other accessory is required. Simple technical characteristics allow high quality samples to be collected from both softwood and hardwood species with minimum damage to the sampled trees. Trephor was tested during the 2004 growing season, demonstrating good resistance to wear and tear and mechanical stress. Embedding the microcores in paraffin for fast section preparation is described.

***Key words:*** Trephor, xylogenesis, cambium, xylem, phloem, paraffin, rotary microtome.

## INTRODUCTION

Cell development in wood follows cambium reactivation according to several physical and biochemical processes, such as water movement into vacuoles or lignin and cellulose accumulation, in order to enlarge cell lumens and constitute cell walls (Plomion *et al*. 2001; Abe *et al*. 2003). During the growing season, different phases of xylem and phloem cell development are found within a few microns, going from the living bark to the mature xylem. The anatomy of the developing tree rings is therefore highly heterogeneous and characterized by enlarging and wall thickening cells close to both mature and cambial cells.

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The timing and mechanisms of wood formation on short-time scales are essential information for understanding growth dynamics in trees and the main factors affecting xylogenesis (Chaffey 2002). In the last decade or so, several studies have been published on intra-annual analysis of radial growth. These studies are principally based on repeated observations of the developing tree rings on wood samples during the growing season (Bäucker *et al*. 1998; Camarero *et al*. 1998; Horacek *et al*. 1999; Deslauriers *et al*. 2003; Rossi *et al*. 2003; Jones *et al*. 2004). Wood samples extracted in spring or early summer are likely to be damaged because the cambium and enlarging cells have thin primary walls. There is therefore a need for specific extraction tools and suitable sample preparation methods.

In order to be as little invasive as possible, wood samples are repeatedly extracted as small cores around the stem (microcores, Fig. 1A) during the growing season (Deslauriers *et al*. 2003). Generally, sampling is done by means of a small cutting tube hammered into the stem (after bark removal) and then extracted. The portion of wood removed with the cutting tube must then be extracted from the tool for xylem tissue analyses. Although apparently easy to do, this sampling procedure involves difficulties connected to the core size and the above-described features of the collected tissues. Cross sections are cut from the microcores in order to make anatomical observations on developing cells. The quality of these sections is directly linked to the sharpness of the cutting tube. Inadequate tools with unsharpened blades inevitably produce samples with deep cracks or flaking on the outer surface of the microcore and consequently poor sections. Moreover, when the microcore is removed from the cutting tube, compression of the unlignified tissues can occur thus increasing cell deformation in the meristems, collapse of enlarging tracheids or cracks in the thickening walls.

Although there is growing interest in intra-annual studies of xylogenesis (Downes *et al*. 2002; Wimmer 2002; Eckstein 2004), tools for microcore sampling have only seldom been described (Forster *et al*. 2000). In many cases, products originally designed for other purposes are used (Bäucker *et al*. 1998; Deslauriers *et al*. 2003; Mäkinen *et al*. 2003) but no information is available on their efficiency and usefulness in specific situations such as large numbers of samples, sampling on hardwood species or trees with thick bark or low branches along the stem. Furthermore, few details have been given of laboratory preparation techniques related to cell analysis in tree-ring formation (Antonova *et al*. 1995; Camarero *et al*. 1998; Horacek *et al*. 1999; Schmitt *et al*. 2003), leading to difficulties in the accomplishment of such experiments. This paper describes: 1) the tools used up to now for extracting microcores, 2) a new tool specifically designed to overcome the limitations of these tools, and 3) a method for preparing sections of developing wood tissues. The tools have been grouped into two separate categories, non-specific and specific tools, depending on the main purpose for which they had been designed.

## NON-SPECIFIC TOOLS

The surgical bone needle (Fig. 1B) is used in the medical field for bone marrow aspiration and consists of a stainless steel cutting tube with a sharp fluted beak and plastic

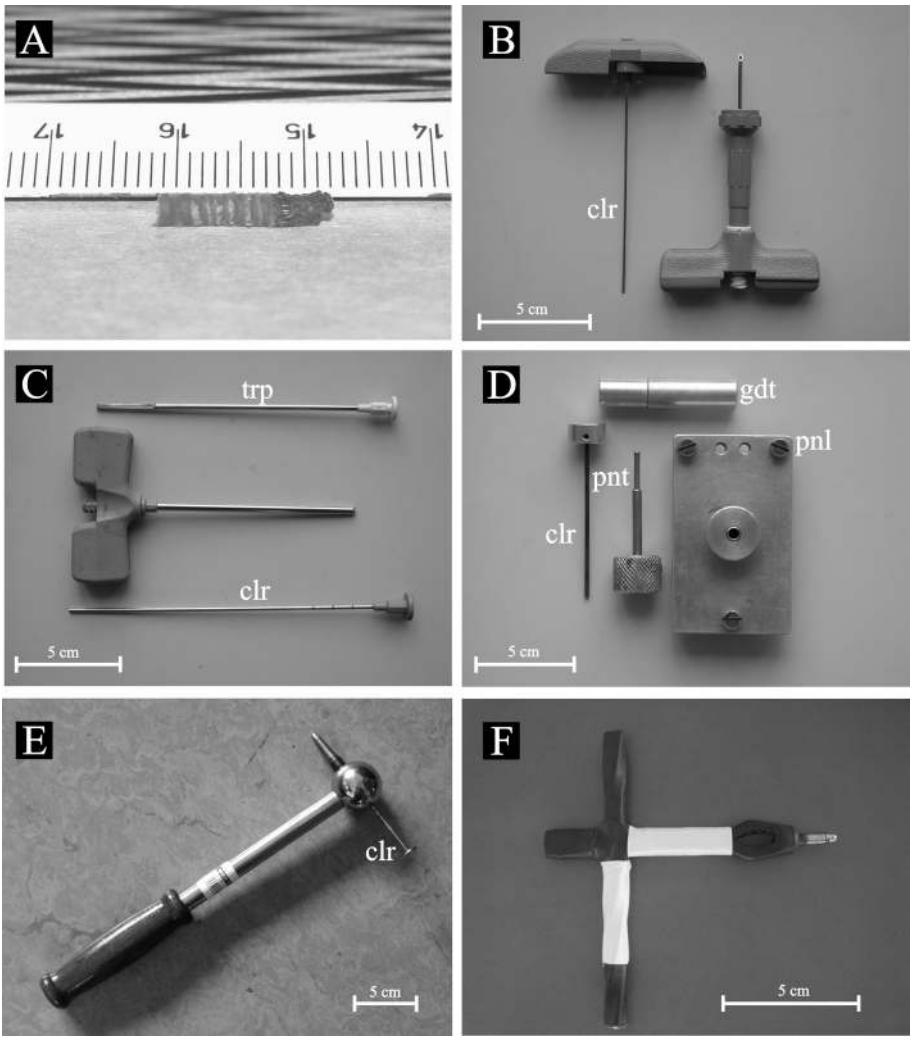


Fig. 1. A: Microcore. – B: Surgical bone needle, clearing rod (clr). – C: Trapsystem needle, clearing rod (clr), Trapsystem® rod (trp). – D: Increment puncher, clearing rod (clr), punchertube (pnt), guide tool (gdt), panel (pnl). – E: Increment hammer, clearing rod (clr). – F: Trephor.

handle. Encircling the tree trunk with one arm, the surgical bone needle is pushed in with the other hand. The necessary pressure can be applied only if the sampling is at about breast height. Inserting the needle into the stem otherwise becomes difficult and, for sampling above shoulder height, almost impossible. The cutting tube has a weakly ellipsoid inner section, so the microcores can be easily oriented. As the microcores are more than 20 mm in length, they contain many tree rings and allow current and previous years' radial growth to be compared. However, the inner size of the cutting tube is only about 1.2 mm in diameter, so very few cell rows can be measured. Samples

are extracted from the cutting tube using the clearing rod (clr, Fig. 1B), but this is done pushing against the side of the bark and phloem thus compressing the meristematic and elongating cells and deforming the developing tree ring.

The trapsystem® needle (Fig. 1C) is a plastic-handled cutting tube with concave-sharpened tip. It is inserted into the stem in the same way as the surgical bone needle. Before extraction of the cutting tube, the sample is trapped by the Trapsystem rod (trp) through the dentate semicircular-section tip. In the case of very long samples, a clearing rod (clr) can be used to remove the microcores from the Trapsystem rod.

Surgical bone and Trapsystem needles are readily-available tools originally produced for surgical biopsies. The cutting tube therefore rapidly becomes blunt, or chips or bends, leading to cracked or flaked microcores after a few samplings. The insertion technique also requires a close approach to the stem, which is difficult when trees retain low branches.

## SPECIFICALLY DESIGNED TOOLS FOR MICROCORE EXTRACTION

The increment puncher (Fig. 1D, Forster *et al*. 2000) partially resolves the problems described for surgical needles. The cutting tube (punchertube, pnt), gliding along a guide, is hammered into the stem by means of pressure from the palm of the hand. The guide consists of a panel (pnl) fixed to the stem with a strap or belt. The inclination of the panel, and thus sample direction, can be regulated by means of 3 screws. The clearing rod is introduced in the punchertube by the cutting side, thus avoiding damage to immature cells. In order to avoid damage to the blade, the clearing rod and punchertube are connected through a guiding tool (gdt).

The increment puncher is specifically designed to extract microcores 15 mm in length and 2 mm in diameter from living plants. However, its disadvantages include the long time required to fix the panel on the stem and the difficulty of sampling on hardwood species or trees with thick bark. Moreover, spare blades often go missing or are difficult to find. In the field, the smaller components can easily get lost on the ground and cause delays. The procedure of inserting the punchertube by means of a blow applied with the palm of the hand becomes uncomfortable when repeated on several trees, even for sampling of softwood species.

The increment hammer (Fig. 1E) is formed by a spherical head with a cutting tube at right angles to the metal handle. The microcore is sampled by thrusting the cutting tube hard into the stem. The clearing rod on the opposite side of the head is used to push out the microcore, but pressure occurs from the phloem side and so compresses the weak cells. Moreover, the sampling point on the stem is not precisely assessed.

*Trephor* (patent pending in Italy, n° PD2004A000324) was designed and produced at the Centro Studi per l'Ambiente Alpino in San Vito di Cadore (Italy) for extracting microcores from living trees. A Trephor for microcores of 15 mm in length and 2 mm in diameter is shown in Figure 1F. The tool is named after a mountain in the Cinque Torri group (Cortina, Italy), the location of some of the study sites and where the tool was tested throughout the 2004 growing season. Trephor is designed specifically for long-term microcore sampling in forests. The simple and innovative technical characteristics offer: 1) high-quality samples on both softwood and hardwood species,

2) minimum damage to sampled trees, 3) ergonomic shape, 4) simple use, 5) robustness, 6) operative safety. It is a chisel-like tool with fast sample recovery. The cutting tube is inserted into the wood using a hammer and no other accessories are required.

Trephor is composed of a single, compact and easily-transported tool. A main element (mnl) is fixed to two coplanar asymmetric arms at right angles, a back axis (tail, tl) and a piercing head (prh) in tempered stainless steel (Fig. 2A). The cross shape allows a hammer to be used to ease penetration in the stem of any tree species. The two asymmetric arms allow a comfortable grip for easy and fast extraction of the Trephor from the stem. The piercing head (Fig. 2B) consists of a cutting tube (ctt) cylinder-shaped outside and cone-shaped inside connected by two supports (fork, frk) with a U-shaped main element. The fork opens the back of the head for collecting the extracted microcores. The inner cone shape limits metal friction and abrasive effects on the microcore and damage to xylem tissues. After extracting the piercing head from

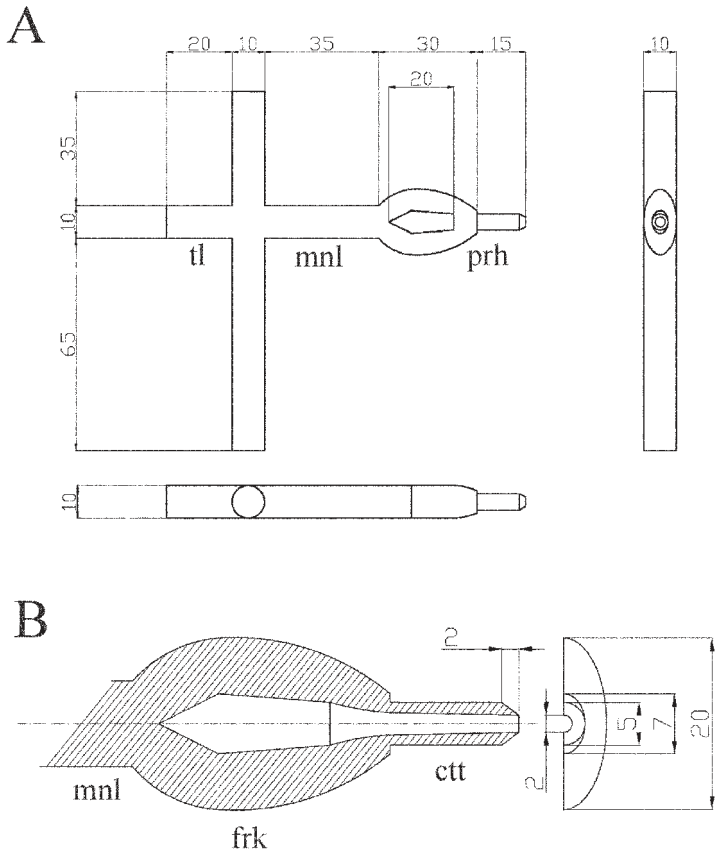


Fig. 2. A: Trephor (version for sampling microcores 2 mm in diameter and 15 mm in length) in the three orthogonal projections, main element (mnl), back axis (tail, tl), piercing head (prh). – B: side and front views of Trephor piercing head, main element (mnl), fork (frk), cutting tube (ctt) (all measures in millimetres).

the stem, the wood sample enclosed in the cutting tube can be removed by a slight push from the sharp side of the tube without any cell deformation. The tool, weighing about 120 g, can be protected by thermo-insulating paint or covered with thermo-restricting plastic. The shape and constructional choices of the cutting tube are a compromise between elasticity and hardness. The stainless steel is tempered at 300 °C for 12 hours to confer specific characteristics on each part of the cutting tube in order to withstand wear and tear and mechanical stress. The harder sharp part can be hammered into the hardwood without any damage to the blade and the more elastic basal part resists when struck by the hammer during sampling. Innovative shape, technical characteristics and the materials used allow high-quality microcores to be obtained even after hundreds of samplings, unlike the other blades that at best must be sharpened after a few dozen samples. When moving in the forest, the cutting tube is protected by a hood and the tool stored in a pocket or small bag for user safety and to prevent blade damage.

## HOW TO USE TREPHOR

As the cutting tube is only 1.5 cm long, thick barks should be removed in order to sample the living tissues. For young trees or species with thin bark, no preliminary operation is generally needed, thus minimizing damage to the stem. The Trephor piercing head is held against the sampling position with three fingers, checking that the asymmetric arms do not hit the hand while the cutting tube is being hammered into the wood. The microcore is separated from the xylem by holding the asymmetric arms and rotating the tool like a corkscrew, thus extracting the cutting tube with movements coaxial to the main element. When Trephor is held in the palm of the hand with the blade facing the fingers and the longer asymmetric arm towards the outside, delicate pressure is sufficient to separate the sample from the cutting tube. The microcore slides along the inside of the cutting tube and falls directly onto the hand from the back of the piercing head. The microcores are then placed in Eppendorf microtubes in an ethanol solution (0.5 in water) and stored at 5 °C. The overall time spent for the sampling is generally half a minute.

## MICROCORE EMBEDDING AND CUTTING

After having extracted the microcores, the wood sections for light microscope can be prepared by means of sledge microtomes or hand cuts. Both methods are useful when samples can be easily handled or fixed on a support. The microcores consist of two parts with different consistency and density: a compact side composed of the mature xylem and a soft side with phloem and differentiating cells, thus precluding a stable fixing of the whole sample. Microcores also often break in half close to the cambial zone, leading to irreversible damage to the tissues and loss of wood material for observations. However, use of a rotary paraffin microtome solves the problems related to the small size and fragility of the samples.

Sample preparation involves the embedding of the microcores in paraffin by means of dehydration with ethanol and D-limonene and the successive immersion in liquid

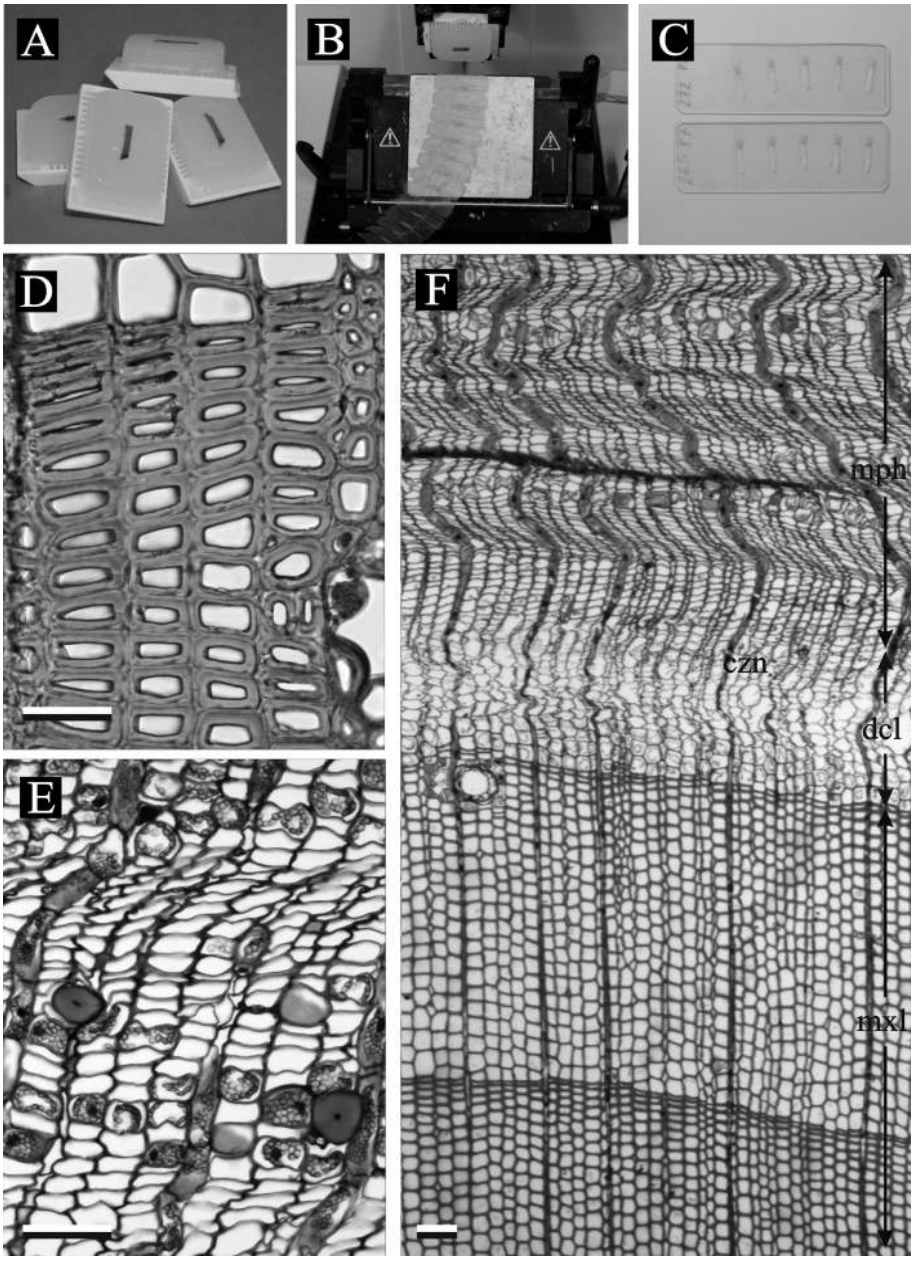


Fig. 3. A: Paraffin blocks containing microcores. – B: Paraffin strip with microcore sections obtained by rotary microtome. – C: Microcore sections placed on microscope slides. – D: mature tracheids of *Picea abies* in polarized light (×500). Scale bar = 20 μm. – E: Phloem of *Larix decidua* (×500). Scale bar = 20 μm. – F: Microcore section of *Pinus cembra* (×100) containing mature xylem (mxl), mature phloem (mph) and developing cells (dcl), including the cambial zone (czn). Scale bar = 50 μm.

paraffin at 65 °C, as reported in Table 1 (Anderson & Bancroft 2002). The microcores are then fixed on supports (biocassettes) by means of paraffin blocks (Fig. 3A). In this way, the paraffin penetrates into the wood and fills the cell lumens in both xylem and phloem tissues.

Before making the final cut, the outer excess layer of paraffin is removed. The paraffin blocks are then immersed in water at room temperature for 20–120 minutes, depending on the sample size and wood hardness, in order to lubricate the walls and facilitate the cut. Microtome Feather R35 blades are generally used for cutting 20–40 samples of both hardwood and softwood species, producing sections without apparent damage to the cell wall (Fujii 2003). The samples, blocked on the microtome clamp, oscillate vertically and meet the knife. Each run, the clamp advances towards the knife and paraffin films containing sections of pre-set thickness (8–12 μm) are cut. After some runs, the continuous paraffin strip produced (Fig. 3B) is plunged in water at 45–55 °C in order to stretch the sections and to place the films on the microscope slides. For greater adhesion of the sections, the slides should previously be treated with albumin or polylysine. Sections are dried at 50 °C for 1 hour and cleaned of residual paraffin by immersing in D-limonene and ethanol. After complete elimination of the paraffin, the sections are ready for staining and observation under the microscope (Fig. 3C).

Table 1. Steps for paraffin embedding. D-limonene is a natural agent with low toxicity specifically formulated to replace xylene during the clearing steps.

| 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 | 90 |
| D-limonene | 90 |
| D-limonene | 90 |
| Paraffin | 120 |
| Paraffin | 120 |

Microcores extracted during the growing season are composed both of cells already completely provided with secondary walls (mature xylem cells, Fig. 3D) and cells with thinner secondary walls (phloem, Fig. 3E) or lacking secondary wall (cambium). The paraffin embedding technique and the use of a rotary microtome for the analyses of developing wood tissues allow suitable sections of these heterogeneous tissues to be obtained even when both are present in the same sample (Fig. 3F). Moreover, paraffin blocks can be stored and preserved for further cuts and observations.

## CONCLUSIONS

Study of forest tree xylogenesis requires specific methods of wood sampling and section preparation. Although few details are available on these techniques, several tools exist for extracting and cutting microcores, but not all of them have been specifically designed for small wood samples and heterogeneous tissues. Trephor and the rotary paraffin microtome have been shown to be suitable tools for microcore sampling and the fast preparation of wood sections for the analysis of both mature cells and developing xylem.

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