IMAGE ANALYSIS OF TRACHEID DIMENSIONS FOR DENDROCHRONOLOGICAL USE

M. A. R. MUNRO, P. M. BROWN,[1] *M. K. HUGHES* and *E. M. R. GARCIA*

Laboratory of Tree-Ring Research, The University of Arizona, Tucson, Arizona 85721 USA

ABSTRACT. An image analysis system measures cell widths and double cell-wall thicknesses with a resolution and precision of ca. 0.3 μm. We avoid the distortion and preparation problems associated with thin sections by using epi-illumination fluorescence microscopy on cut wood surfaces. A CCD camera and frame grabber capture microscope images, which we combine into a mosaic spanning a ring using the NIH-Image program on a Macintosh computer. We select lines along radial cell files, which the program measures by taking the areas above a manually specified brightness threshold as cell walls and those below it as lumina. Measurement errors arc small relative to the variability of files within a ring.

INTRODUCTION

Traditional dendrochronology studies the attributes of complete tree rings. Even when techniques such as X-ray densitometry have produced multiple measurements from each ring these have been used to form time series with an annual resolution. This approach neglects the wealth of information that can be obtained from within each ring by measurements at the cellular level, which provide a direct record of changes in the physiology of the tree occurring over periods of weeks or days. However, there is increasing interest in using cell measurements to reconstruct short-term fluctuations in the environment of the tree during the growing season, inspired in part by mechanistic models that predict how the cells within a ring form as the processes of cambial division and cell enlargement are influenced by daily variations in variables such as temperature and precipitation (*e.g.*, Fritts *et al.* 1991, 1992, 1994).

Some measurements of cellular features within tree rings provide only a single set of values for each ring, such as the ratio of measurements of vessel area in ring-porous species (*e.g.*, Woodcock 1989; Sass and Eckstein 1992) or the ratio of cell wall area to lumen area in the latewood tracheids of conifers (*e.g.*, Jagels and Telewski 1990; Park and Telewski 1992; Park 1993). These contain only a little additional temporal information from the separation of measurements from earlywood and latewood (identical to the division commonly made in X-ray densitometry). In contrast to such measurements, the tracheidograms described by Vaganov (1990) provide a summary of the individual cell sizes or wall thicknesses throughout the ring, providing much higher temporal resolution. Sass and Eckstein (1992) suggest that an analogous approach can be applied to the vessels of ring-porous species, but here we will concentrate on conifer tracheid measurements.

In principle, each cell could provide values for many different variables representing its size, shape and orientation; the choice of variables determines the details of the measurement process, since the details of cell wall structure require relatively higher precision than gross cell sizes. Vaganov *et* al. (1983) describe a system which has been extensively used for cell file measurements at the Institute of Forestry, Russian Academy of Sciences in Krasnoyarsk, Siberia. **A** skilled operator views a thin section through a transmitted light microscope which superimposes a crosshair on the field of view; a mechanical stage moves the section relative to the crosshair and the operator records when it intersects the boundary of a feature of interest, while an automatic data logger converts these events to a

[1]Present address: Rocky Mountain Station Tree-Ring Laboratory, 240 W. Prospect Road, Ft. Collins, Colorado 80526 USA

Tree Rings, Environment and Humanity, edited by J. S. Deon, D. M. Meko and T. W. Swetnam. RADIOCARBON 1996, pp. 843–851

permanent record of distance measurements with micrometer resolution. We have attempted to produce a similar system using image analysis technology.

METHODS

Sample Preparation

The usual practice in tracheid measurement has been to obtain a series of measurements from a number of radial cell files within each ring. Each cell file records the activity of a patch of cambium only one cell wide, so any relative changes within the series reflect physiological changes rather than variations between different parts of the cambium. Some files may appear distorted because the plane of the transverse section being measured passes through the tapering ends of the tracheids, and the cambium will not alwaysdivide along a radially directed file, but in everything apart from juvenile wood it will be possible to find files unaffected by these problems.

We initially attempted to measure microtomed thin sections, following the example of Park (1990) and Vaganov *et al.* (1983), but encountered two problems: if the sections were thicker than ca. 20 μm, vertical distortions flattened the earlywood cell walls so that they were no longer normal to the plane of the cut, making it difficult to see the true wall thickness; if the sections were thinner than this, it was impossible to prepare large areas without lateral distortions. We now prepare a surface on the original increment core and measure this directly (Smith 1965). Increment cores are first glued into slotted wooden core mounts using a waterproof resorcinol-based marine glue. Proper alignment of cells perpendicular to the field of view is critical at this stage, since there can be no realignment of the core once set in the core mount. Excess glue should be avoided lest it penetrate deeply into the core. We cut long cores into sections with oblique band saw cuts so that they will fit on the microscope stage, then place them in a beaker containing a mixture of five parts water to two parts glycerol. A microwave oven heats this mixture until it starts to boil, then we cover it tightly with plastic or aluminum foil and let it stand until it has cooled to 20°C before shaving the previously sanded core surface with a microtome. Three cuts are generally necessary before the surface is even enough to measure, the final cut removing 25 μm of the surface. We apply a very dilute (ca. 1 mg l^{-1}) solution of alkaline-buffered solution of the stain Aurarnine O to the cut surface, avoiding soaking the entire thickness of the core in the stain.

Microscope Technique

If reliable cell wall measurements are required, the microscope must provide a relatively high magnification. We use a ×40 objective of moderate numerical aperture (Nikon Plan 40, NA 0.65). This constrains the illumination that can be used, since the short working distance makes it difficult to illuminate the field of view evenly with an independent light source, and we use an epi-illumination system through the image-forming objective (on a Nikon Optiphot microscope). A 100-W mercury vapor lamp and interchangeable sets of mirrors and filters allow us to view the surface either by reflected white light or by yellow fluorescence from illumination with blue light (470–490 nm excitation filter, 510 nm dichromatic mirror and 520 nm barrier filter). Surprisingly, the fluorescence images are much better for the semiautomatic measurement of cell dimensions than those using reflected light. The cell walls show consistently bright fluorescence across the cut surface, but the reflected light shows many irregularities, possibly because reflections from surfaces at different orientations to the illumination are more directional than fluorescence.

Wood surfaces exhibit autofluorescence over a wide range of visible wavelengths, so in principle stains are unnecessary, but they can be used to adjust the brightness of the image to convenient levels, and to improve the contrast between the walls and lumina if applied only to the surface of the

sample. Because we are not concerned with measuring different components of the cell wall, the stain should be uniformly absorbed, ideally producing uniform fluorescence across the wall. We initially used a fluorescent brightener that was not well matched to the combination of excitation and barrier filters (Calcofluor White), then obtained better results with Auramine O, which also has the advantages of efficiently staining the wood, being less toxic than some other stains with similar properties, and being easily obtainable. Other combinations of filters and stains should also give good results. Heavy staining should be avoided, since the fluorescence may then be too bright for the camera to record it.

Fluorescence microscopy does suffer from an important disadvantage. The fluorescence becomes increasingly dim the longer the sample is illuminated, a process known as photobleaching (Rost 1992). Since we do not attempt to extract any information from the intensity of the fluorescence, this would not appear to be a problem, but in practice it causes effects similar to misaligned illumination. Because we follow single radial files of tracheids across the ring at a magnification that makes it impossible to fit a complete ring into a single field of view, we must examine a succession of overlapping fields. When we move to a new field of view one edge has already been illuminated (by the light used when viewing the previous field), but the other edge has remained in darkness. The fluorescence from the previously illuminated edge will be relatively dim, and there is a gradient from dark to light across the entire width of the field. Some fluorescent substances are less affected by photobleaching than others, but even the autofluorescence of the unstained cell walls will dim in this way. If the sample is illuminated for short intervals only (by blocking the illumination with a shutter), the effect is reduced, but cannot be removed.

Video Technique

Computers are now commonly used to manipulate images that have been converted to arrays of distinct picture elements (pixels), each encoding the brightness (and possibly color) of that part of the image at one point. Russ (1992) provides a review of these techniques and Inoué (1986) and Russ (1990) describe in detail their application to microscopy. Computer image analysis enables measurements that would be extremely tedious in conventional microscopy (such as the areas of particular structures), but is far from being a panacea. The process of turning a microscope image into a pixel array within a computer loses information in several ways. The spatial detail in the microscope image, originally limited only by factors such as the objective's numerical aperture, is limited by the number of pixels in the array; details smaller than a single pixel cannot be resolved. The rectangular pixel array will represent only part of the circular optical image formed by the microscope. All but the faintest images will have an effectively continuous range of brightnesses, but the pixel array must represent this as a series of discrete codes, possibly using a single code to represent gray values that are visually distinguishable in the optical image. The spectral information present in the optical image will at best be represented by encoding a few discrete channels (such as red, green and blue), or ignored altogether by having the pixel array record only the average gray value of the image over a broad range of wavelengths. The use of fashionable technology is no justification for this information loss; image analysis must provide some compensatory advantages if it is to be used.

The video equipment is very similar to that used by Sheppard and Graumlich (1996), Thetford *et al.* (1991) or Sass and Eckstein (1992), with a charged coupled device (CCD) camera occupying the position of the camera body in a conventional photomicrography configuration. There is a lens between the camera and the barrier filter of the microscope, but this does not contribute to the magnification of the image, which is determined only by the objective lens. CCD cameras contain arrays of discrete light-sensing elements, and so immediately constrain the size and resolution of the image that can be represented as a pixel array within the computer. In the camera we use (Dage-MTI

CCD72) the CCD array measures 8.8 x 6.6 mm with 768 x 493 elements, which with a ×40 objective yields a spatial resolution of ca. 0.3 μm. The camera is restricted to a single broad spectral channel (limited to visible wavelengths by an infrared filter) but the 520 nm barrier filter used in the fluorescence optical system makes color discrimination unnecessary. Output from the CCD is turned into a conventional RS-170A video signal by analog electronics that allow manual or automatic control of the brightness and contrast of the image, but conceal the spatial granularity imposed by the discrete light sensitive elements. A device called a frame grabber performs the final step of converting the video signal into a pixel array within a computer. To avoid discarding additional information we use a frame grabber (Scion LG-3) that approximates the spatial resolution of the camera, yielding a 640 x 480 pixel array; its analog-to-digital converter circuitry can resolve 256 different gray levels. The computer (a Macintosh Centris 650) can display both live and previously captured images directly in windows on a monitor screen. We find it convenient to use a 1152 × 870 pixel display so that a single image does not fill the entire screen.

Processing and Measurement

We have slightly modified the NIH-Image program (Wayne Rasband, National Institutes of Health; freely available from ftp://zippy.nimh.nih.gov/pub/nih-image/) to produce the tracheid measurement software. Operators can display a live video image on the monitor screen, manually adjust the focus and position of the image, then average a series of between 16 and 32 video frames to reduce the noise from the camera electronics (which manifests itself as brief fluctuations in brightness randomly distributed across the image). Corrections for the uneven fluorescence can also be applied at this stage. The newly acquired image is then transferred onto a composite image of the part of the ring that has already been recorded, and features such as cell walls visually aligned so that the new image becomes the next part of a long mosaic, before the microscope stage is advanced and the entire process repeated. In this way an image of the entire ring is formed before the measurements begin, although the individual video frames display a much smaller area of the surface. This approach allows operators to measure several parallel cell files on the same image, reducing the photobleaching that would be inflicted on the surface were they to measure them individually, and removing any ambiguities about the assignment of cells to particular radial files.

Quantitative measurements require that distances measured in arbitrary pixel units on the images can be translated into absolute units, but fortunately once a calibration is established once for a particular optical configuration it is then valid for all subsequent measurements, and can be easily determined by measuring graticules of known absolute size. Operators choose the position at which the measurements will be made by marking the image with a temporary line, which should be normal to the orientation of one or more cell walls. The program immediately presents them with a profile of the gray values along the line, combined with a display of the threshold that will determine what is considered cell wall as opposed to lumen. They can move this threshold up or down, and allow it to vary along the measurement line to compensate for uneven illumination. Once they choose the threshold, the program automatically measures the double cell wall thicknesses and lumen widths, marking the positions of the measurements and the sequence number of the cell so that obvious errors can be seen and corrected (*cf.* Fig. 1). The program logs the measurements, cell file identification and sample description information to a permanent file.

This approach differs from the usual image analysis approach to cell measurement, which would be to first segment the image into areas corresponding to the structures of interest, in this case walls and lumina, and only then to measure them. It is difficult to select a single threshold that will allow accurate measurements of cell wall thicknesses in all parts of the image, so by allowing operators to vary

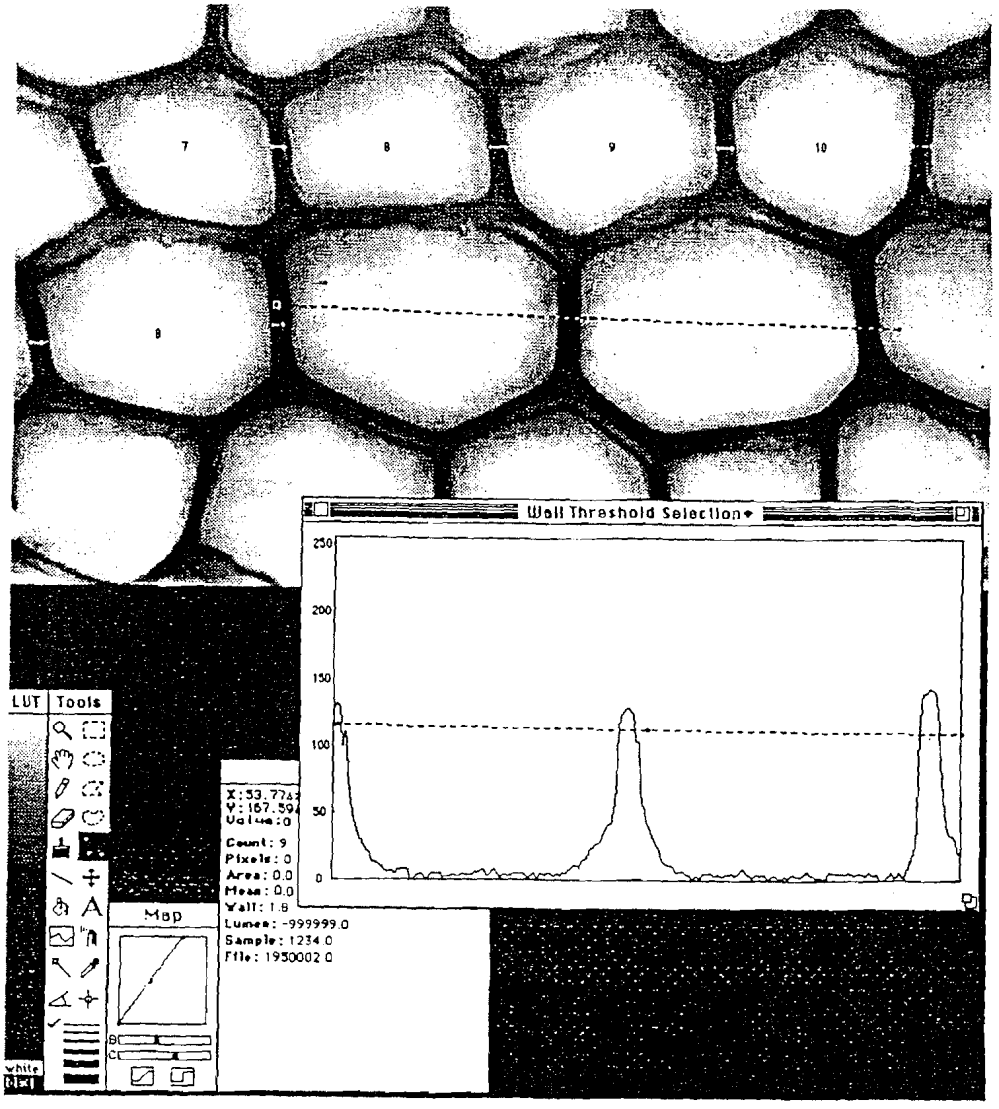

Fig. 1. The monitor screen during measurement. The operator has assembled the individual video images into a mosaic spanning the entire ring, and has already measured one radial tracheid file (cells 7, 8 and 9 of this file are towards the top of the picture). The measurement line is currently on cells 9 and 10 of the second file and the program is displaying a profile of the gray levels along this line. The operator can move the second dotted line (superimposed on this profile) to set the threshold defining the cell wall boundaries. The program has marked the position of previous wall measurements with short white ticks, and not only records the measurements. bur also maintains a count of the current cell number, which it places on the image at the position of the lumen measurements.

the threshold in a flexible way we can obtain more accurate measurements at the expense of greater subjectivity.

The measurement program produces files that can be read by commonly used spreadsheet programs and by additional software to convert the double cell wall thicknesses and lumen diameters to cell sizes. In some cases the middle lamella separating the walls of contiguous cells is visible, but we always ascribe to a cell a wall thickness based on the mean of the radial double cell wall thickness (apart from the first and last cells in the file, where this would be unrealistic). The software produces

standard tracheidograms from the measurements by a method similar to that described by Vaganov (1990). We keep a permanent record of the original lumen and double cell wall measurements, but delete the images within a week of measuring them. If remeasurement is required later we can return to the original core as the ultimate archive of the reality behind the results, but we can re-examine the images (marked with the measurement positions) during the period before they are deleted.

RESULTS

We have concentrated on measuring cores from living *Sequoiadendron giganteum* from the Giant Forest in Sequoia National Park, California, USA, with the ultimate goal of producing cell chronologies that can be used for climatic reconstruction, but initially attempting to demonstrate that our system produces useful measurements. It has two potential defects that could prevent a single operator from reproducibly measuring an image. Operators might choose different subjective thresholds for discriminating between cell walls and lumina each time they measured an image, and if the cell wall thicknesses were small relative to the size of the individual pixels of the image there might be a quantization error, with even tiny changes in the threshold causing large jumps in the wall sizes, corresponding to increases or decreases by the width of a pixel.

An experimental test where a single operator remeasured an image ten times (Fig. 2) demonstrates that these errors are small relative to the meaningful differences between cells (the mean variance of

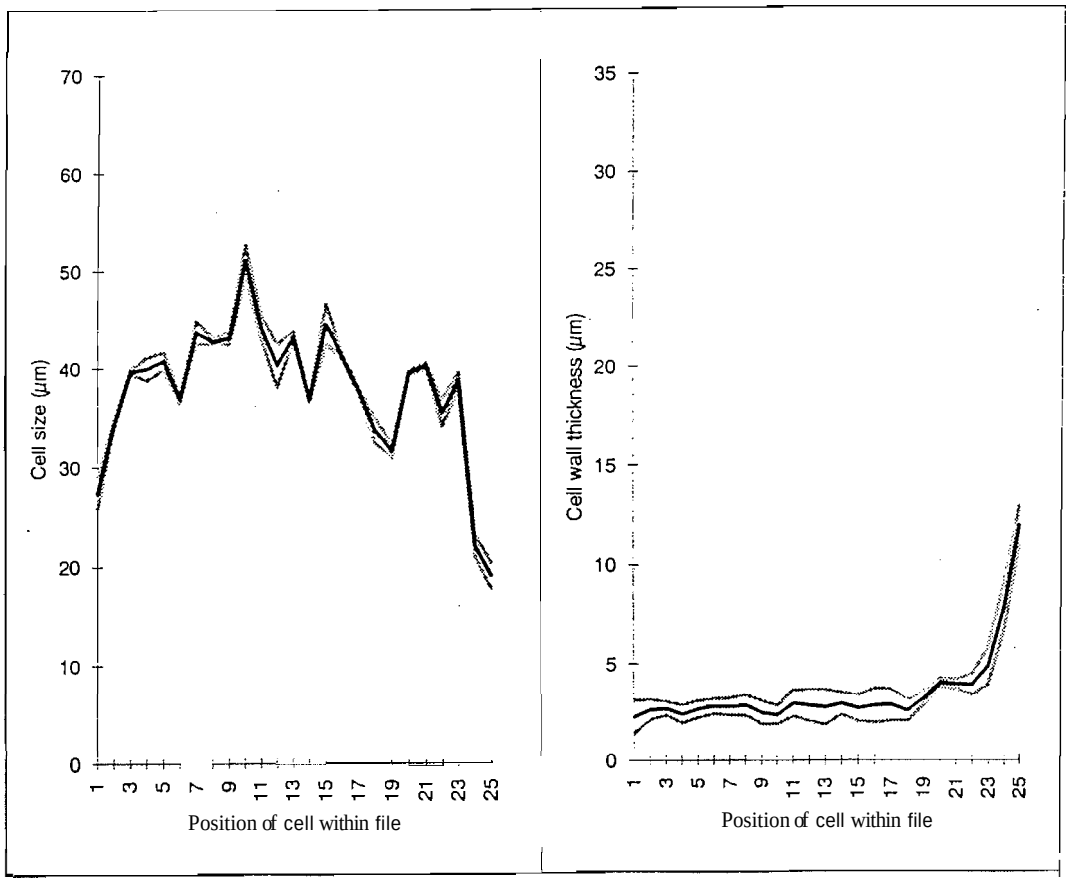

Fig. 2. Replicate measurements of a single image by the same operator. The black lines are the mean of ten replicate measurements; the gray lines represent the ±2 standard error bounds.

the size and wall thickness measurements within the groups of replicate cell wall measurements were 2.7 and 1.1 μm^2, respectively). Other sources of error have a much greater influence on the production of standard tracheidograms. Figure 3 shows three of these, based on the means of five cell files from three rings. The variance within the groups of size measurements for particular cell numbers within a ring is much higher than would be expected from the replicate measurements of a single file (the mean within-group variances for sizes and wall thicknesses in this case are 31.5 and 0.6 μm^2, respectively). This has a biological cause, rather than arising from the measurement process: parallel cell files within a ring are not identical, and those in *Sequoiadendron giganteum* are more variable than in several other conifer species (I. V. Sviderskaya, personal communication 1993).

Differences in the subjective choices made by different operators are another potential source of error. We investigated these by coinparing the mean tracheidograms produced by two different operators measuring the same samples, each based on five cell files. The measurements were made

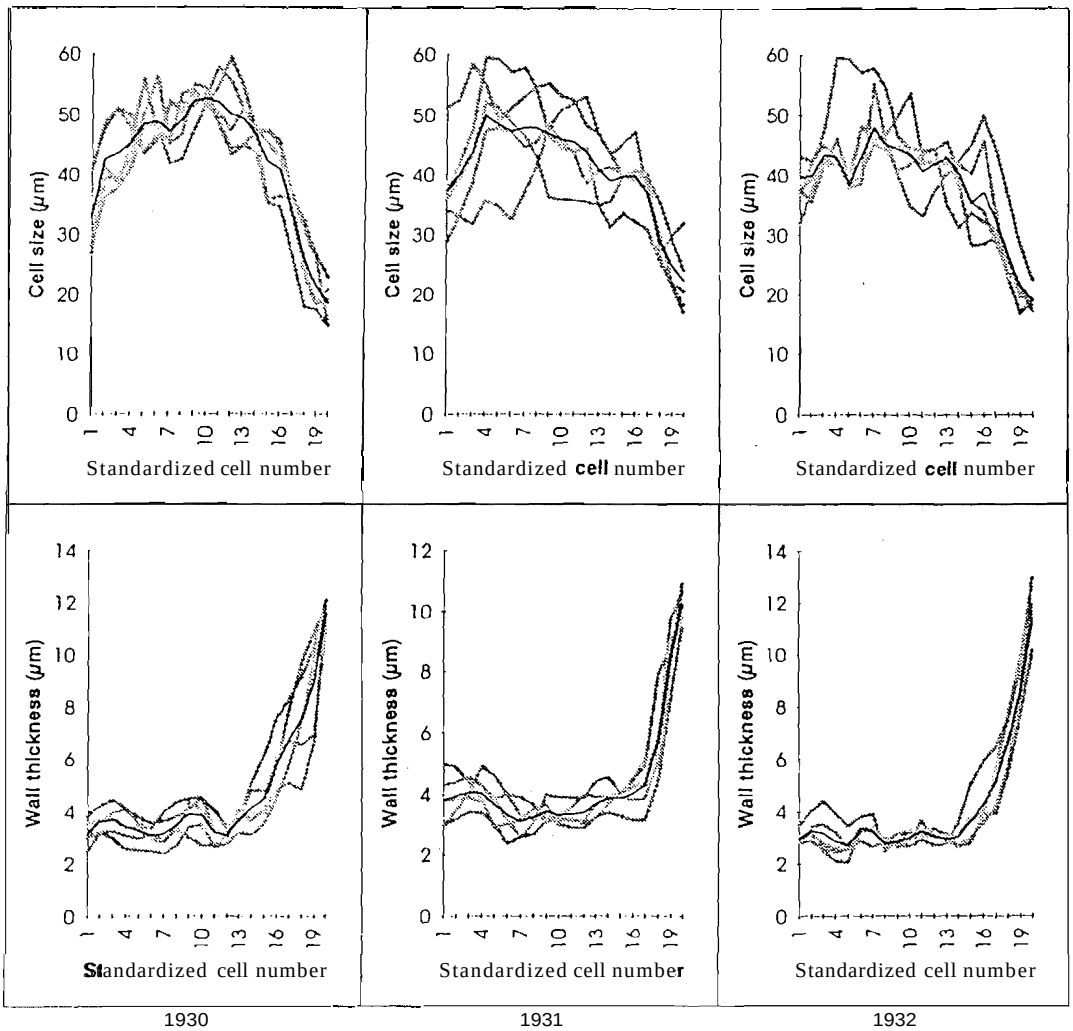

Fig. 3. Examples ofstandard tracheidograms measured (*Sequoiadendron giganteum* from the Giant Forest in Sequoia National Park, AD 1930–1932). The black lines are the cell size and wall thickness means, the gray lines are the measurements of the five radial files used to produce the mean. The trachcidograms are standardized to a length of 20 cells.

before we had developed our sample preparation techniques, making it difficult to see clear details in the latewood of some rings. Some large cell wall thicknesses were measured as a result, but we removed these from the comparison by imposing an arbitrary limit of 20 μm on wall thicknesses. Figure 4 shows that there is a good agreement between the operators (r^2 = 0.98 and 0.91 for sizes and walls, respectively; N = 1468), and although there is a significant bias between the observers (0.26 μm and 0.12 μm for sizes and walls, respectively), is too small to be of practical importance. The most discordant values seem to arise from cases where the operators made different decisions about which cell files on the image they were measuring, again reflecting the biological variability of the files rather than a measurement problem. Some of the values measured from latewood cells would undoubtedly be more in agreement had we used our present sample preparation methods.

CONCLUSION

We can justify using image analysis techniques for tracheid measurements, since they help the operator make long series of measurements. The measurement errors are small relative to the other sources of error affecting the description of cambial activity by mean tracheidograms, and although it would be possible for observers using a conventional eyepiece micrometer to duplicate the results of the image analysis system they would need considerable skill and effort to do this within the same time.

ACKNOWLEDGMENTS

We would like to thank Valerie Best, Jim Burns, Ikuo Furukawa, Leonard Gomez, Patty Jansma, Ron Lynch, Andreas Mergner, Alex Shashkin, Frank Telewski and Emma Williams for their help during

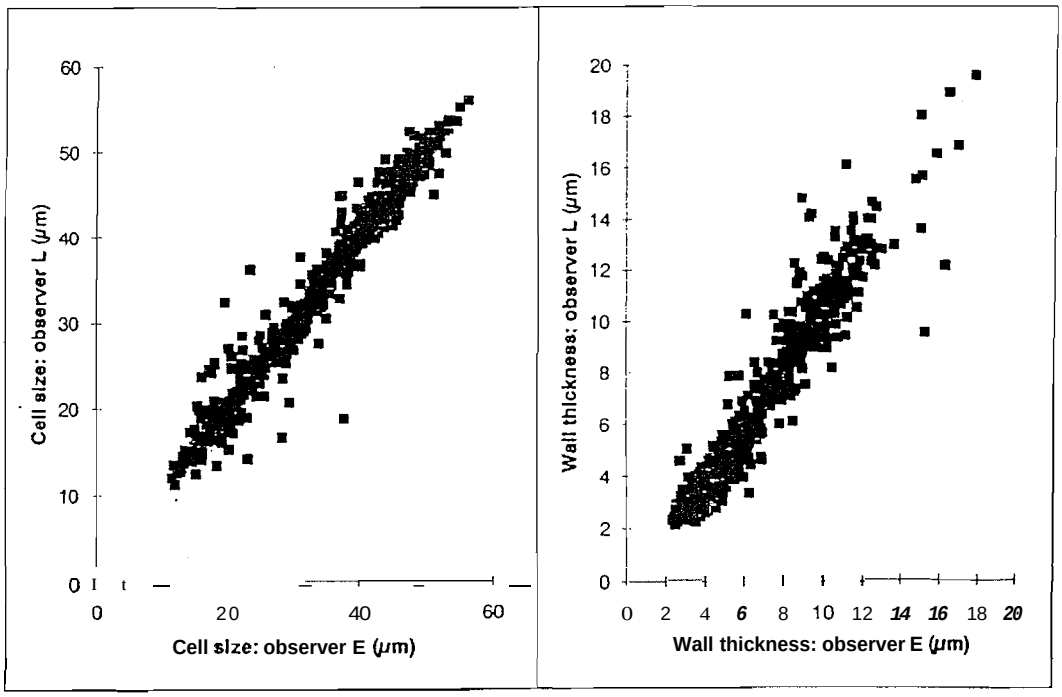

Fig. 4. Comparison of measurements made by two operators on the same *Sequoiadendron* samples. The points represent corresponding pairs of values from the standard tracheidograms produced by the two operators for a particular ring, but the operators may have made different choices of which cell files to measure.

the development of the system described here. The work was supported under Cooperative Agreement CA 8000-1-0002 between the U.S. National Park Service and the University of Arizona.

REFERENCES

Fritts, H. C., Vaganov, E. A., Sviderskaya, I. V. and Shashkin, A. V. 1991 Climatic variation and tree-ring structure in conifers: Empirical and mechanistic models of tree-ring width, number of cells, cell size, cell-wall thickness and wood density. *Climate Research* 1(2): 97–116.

Fritts, H. C., Vaganov, E. A., Sviderskaya, I. V. and Shashkin, A. V. 1992 Modeling tree-ring climatic relationships. In *Tree Rings and Environment: Proceedings of rhe International Dendrochronologica! Symposium.* Lundqua Report 34: 104–108.

Fritts, H. C. and Shashkin, A. V. 1995 Modeling tree-ring structure as related to temperature, precipitation and day length. *In* Lewis, T. *E.*, ed., *Dendrochronology and Dendrochemistry in Forest Health Monitoring.* Boca Raton, Florida, CRC Press: 17–57.

Inoué, S. 1986 *Video Microscopy.* New York, Plenum Press: 584 p.

Jagels, R. and Telewski, F. W. 1990 Computer-aided analysis of tree rings. In *Methods of Dendrochronology: Applications in the Environmental Sciences*. Boston, International Institute for Applied Systems Analysis, Kluwer Academic Publishers: 76–93.

Park, W.-K. (ms.) 1990 Development of anatomical tree-ring chronologies from southern Arizona conifers using image analysis. Ph.D. dissertation, University of Arizona: 234 p.

——1993 Measuring maximum latewood density by image analysis at the cellular level. *Wood and Fiber Science* 25(4): 326–332.

Park, W.-K and Telewski, F. W. 1992 Anatomical parameters for dendrochronology: An image analysis of ponderosa pine from southeastern Arizona, USA. In *Proceedings, 2nd Pacific Regional Wood Anatomy Conference, 15–21 Ocrober 1989.* Forest Products Research and Development Institute, Laguna, Philip pines: 301–320.

Rost, F. W. D. 1992 *Fluorescence microscopy.* Vol. *1.* Cambridge, Cambridge University Press: 253 p.

Russ, J. C. 1990 *Cornpuler-Assisted Microscopy: The Measurement and Analysis of Images.* New York, Plenum Press: 453 p.

——1992 *The imageprocersing handbook* Boca Raton, Florida, CRC Press: 445 p.

Sass. U. and Eckstein, D. 1992 The annual vessel area of beech as an ecological indicator. In *Tree Rings and Environmenr: Proceedings of the International Dendrochronological Symposium,* Lundqua Report 34: 281–285.

Sheppard, P. R. and Graumlich, L. J. 1996 A reflected-light video imaging system for tree-ring analysis of conifers. This volume.

Smith, D. M. 1965 Rapid measurement of tracheid cross-sectional dimensions of conifers. *Forest Products Journal* 15(8): 325–334.

Thetford, R. D., D'Arrigo, R. D. and Jacoby, G. C. 1991 An image analysis system for determining densitometric and ring-width timeseries. *Canadian Journal of Forest Research* 21: 1544–1549.

Vaganov, E. A. 1990 The tracheidograrn method in tree-ring analysis and its application. In *Methods of Dendrochronology: Applications in the Environmental Sciences.* Boston, International Institute for Applied Systems Analysis, Kluwer Academic Publishers: 63–76.

Vaganov, E. A., Krasovskii, K. F., Svidcrskaya, I. V. and Shashkin, A. V. 1983 An automatic device for measuring and treating data on annual ring structure. *Lesovedenie* 2: 73–81.

Woodcock. D.W. 1989 Climate sensitivity of wood-anatomical features in a ring-porous oak (*Quercus macrocarpa*). *Canadian Journal of Foresr Research 19:* 639–644.