Comparison of techniques to measure the fibril angle

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SUMMARY

The different techniques available for the measurement of fibril angle are reviewed. A number of comparative sets of data in the literature are evaluated for information on the overall accuracy of fibril angle measurements. Recently, several new techniques for the measurement of fibril angle, which take advantage of the optical sectioning power of a confocal microscope, have been published. These techniques are reviewed and the relative advantages and disadvantages of each technique are discussed.

The cellulose polymer molecules in wood fibres are aligned in long, highly crystalline microfibrils wound helically around the lumen. The S2 layer of the fibre wall contains as much as 90% of the fibre mass, and therefore largely determines the fibre properties. The pitch of the helix in the S2 layer defines the microfibril angle. As the fibrils in the S2 layer are tightly packed and the S2 layer is itself surrounded by the S1 and S3 layers and is not exposed, it is not possible to simply place a fibre under a microscope and measure the fibril angle from visual observation, except in a limited number of special cases (eg. softwood compression wood). There is a wide variation in the properties of fibres from different parts of a tree and between different species of trees.

Theoretical and experimental work (*1-3*) have shown that while the elastic modulus and strength of fibres are approximately constant at very low fibril angles, they fall rapidly for fibril angles beyond 5 or 10°. At fibril angles of 40° and above the tensile strength is reduced to about a third (*1*) and the stiffness is reduced to about a fifth (*2*) compared to fibres with fibril angles between 0 and 10°. The fibril angle also strongly affects the mechanical and shrinkage (*4*) properties of wood.

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Despite the importance of the fibril angle as a variable, influencing wood, fibre and paper properties, it is a quantity that is relatively rarely measured.

LITERATURE REVIEW

Over the years, a number of different techniques have been used to measure the fibril angle. The work of Page (*5*) and Mark (*6*) includes comprehensive reviews of the earlier literature in this area.

In this review techniques used to measure the fibril angle have been divided into four categories. The first of these is direct observation methods, where the fibres are treated to allow the fibrils to be directly observed from a microscope image taken of the fibre. In the second, x-ray diffraction techniques for the of fibril angle will be examined. Finally, there are two categories of polarised microscopy techniques labelled here as 'birefringence polarised microscopy' and 'excitation polarised microscopy'.

Direct observation

There have been a number of attempts to develop treatment techniques which will make the fibril angle directly visible by separating the fibrils from each other. The most recent publication in this area is the work of Huang (*7*) who used ultrasonic treatment in the presence of congo-red dye to generate checks in the cell wall, which were assumed to represent the fibril angle. It was found that three hours at an ultrasonic frequency of 47kHz was required for optimal production of check marks and that the technique worked best with thick-walled fibres with high fibril angles.

The technique of iodine staining involves a series of treatments designed to deposit iodine crystals between the fibrils. The fibril angle can then be directly observed in a microscope. Meylan (*8*) used a five-stage treatment to fix the iodine crystals in place and reported that for fibres of low fibril angle, this treatment often failed initially and the whole process had to be repeated several times.

Crosby and Mark (*9*) have developed a technique in which the fibril angle is determined from phase contrast microscopy under near ultraviolet illumination. Under these conditions, the structural orientation of the fibrils in the S2 layer becomes visible. The technique seems to have been relatively slow and to have required considerable experimental skill to obtain accurate results.

In general, the problem with the direct techniques so far presented in the literature is that they have all required extensive sample preparation or sophisticated experimental technique, which has limited their applicability in routine measurements.

One additional semi-direct technique is the use of pit apertures to measure the fibril angle. It has been observed that pits in fibres often present an elliptical shape and it is assumed that the direction of the long axis of the ellipse is the fibril angle (*10,11*). This cannot be considered as a completely direct technique because the orientation of the fibrils is not directly observed.

X-Ray diffraction techniques

X-ray diffractometry has been used to estimate microfibril angle in cellulosic fibres for over 65 years. Both the (002) and (040) cellulose I reflections have been used, each with its advantages. Although the (040) reflection can be used to estimate the microfibril orientation distribution directly the (002) reflection has much greater intensity and is more suited to rapid analysis (*12,13*). The technique gives the average fibril angle of hundreds of fibres at a time and cannot be used to measure fibril angles of single fibres, since the diffracted intensity is too weak.

The general relationship (*14*) between the measured variance of the (002) peak and the fibril angle is $S^2 = \mu^2/2 + \sigma^2$ where S^2 is the variance of the (002) peak, μ is the average fibril angle and σ^2 represents the variance due to sample geometry, instrumental effects,

as well as fibril angle variance from all scales of organisation within the wood. More recently (*15,16*) a method has been developed to allow the rapid scanning of large numbers of increment core samples for forest assessment and tree improvement programs.

Birefringence polarised microscopy techniques

These techniques take advantage of the natural birefringence of cellulose. That is, the orientation of the fibrils with respect to the direction of polarisation of the incident light determines the intensity and polarisation of the light transmitted through a cell wall. In order to measure the fibril angle it is necessary to isolate a single cell wall since, if light passes through opposite cell walls, the effect on the light polarisation from the first cell wall is cancelled out as the light passes through the second. This isolation of a single cell wall has been achieved either mechanically, by slicing the fibre at an angle (*8*) or along its length (*17*), by using pit apertures to view light transmitted through a single cell wall (*18*) or by impregnating the fibre lumen with mercury (Page's technique) (*5*).

In each case, the sample is illuminated with polarised light and the reflected (mercury impregnation) or transmitted (single fibre wall) light is passed through an analyser crossed with the polarisation direction of the incident light. The fibre is rotated until maximum extinction occurs. The angle between the fibre axis at maximum extinction and the polarisation axis of the polariser is taken as the fibril angle.

Recently a technique was published that uses the depth-resolution capabilities of a confocal microscope to optically isolate one cell wall from the other, thus allowing a crossed polarisation experiment to be conducted without extensive sample preparation (*19*). The technique is rapid, requires no sample preparation and can be used on single fibres and solid wood samples.

El-Hosseiny and Page (*20*) have modelled the effect of layer thicknesses on the extinction position measured using Page's technique. For the case where the S1 layer was set at a fixed value of 0.2 μm, it was found that only for an S2 wall thickness between 1.3 and 3.4 μm was the measured extinction position within 5° of the true fibril angle, set for the purposes of the study at 30°. For the other cases studied, where the S1 and S3 layer thicknesses combined were up to 15% of the S2 layer thickness, the measured extinction position was always within 5° of the true fibril angle for a S2 layer thickness less than 3.4 μm. The effect of frequency on the difference between the true fibril angle and measured extinction position was investigated and found to be negligible for an S2 layer thickness of 2.27 μm, but to be a significant source of error as the cell wall thickness increased.

A variation on the birefringence polarised microscopy techniques that does not require optical isolation of a single fibre wall has been published (*21,22*). The fibre is placed with its axis at 45° to the polarisation of the incident light. The intensity of light transmitted through an analyser is then measured as the analyser is rotated, as a function of the wavelength of the incident light. From a fit to the data using the optical theory of the system it is then possible to estimate the fibril angle. The biggest weakness of the technique seems to be that no account is taken of the effect of the S1 and S3 layers on the measured result.

Excitation polarised microscopy techniques

These techniques rely on the absorption and re-emission of light within the cell wall, with the intensity of the re-emitted light being dependent on the orientation of the fibrils.

Two techniques have been published, one using confocal microscopy and fluorescent dyes (*23,24*) and one using micro-Raman spectroscopy (*25*). The techniques have been categorised under the new label 'excitation polarised microscopy' because both techniques use light scattering within the cell wall, rather than light reflected at an interface or allowed to pass completely through the fibre wall, as is the case with the birefringence polarised microscopy techniques. Both of these new techniques also rely on the optical sectioning capability of the confocal microscope in order to isolate light scattered from a single cell wall.

The fluorescence technique relies on dyes such as congo red or acridine orange which, after absorption, tend to be oriented parallel to the fibrils in the cell wall (*24*). These dyes are inherently difluorescent, ie, the alignment of the dye relative to the direction of polarisation determines the fluorescent intensity. Maximum fluorescent intensity occurs when the fibrils are aligned with the polarisation direction of the laser light.

The micro–Raman technique (*25*) depends on inelastic scattering within the cell wall and produces a spectrum with characteristic peaks determined by the frequencies of the modes of vibration in the cellulose chains. The alignment of cellulose chains relative to the direction of polarisation of the incoming light, which is exciting the vibrations within the cellulose, has been shown to determine the intensity of many of the Raman peaks (*26*).

It was found that the most reliable micro–Raman determinations of fibril angle were made when the ratio of the 1095 cm^{-1} peak (varies strongly with orientation of fibrils to laser polarisation direction) and the 1120 cm^{-1} peak (almost independent of orientation of fibrils to laser polarisation direction) was used. Taking the ratio of peak intensities compensates for changes in the overall intensity of the Raman spectra. The fibril angle is then determined from the angle at which the maximum in the ratio of the 1095 cm^{-1} to 1120 cm^{-1} peaks occurs (*25*). The two main disadvantages of the technique is that fluorescence from any lignin present will drown the Raman signal, thus the measurements can only be made on fibres that have been completely delignified. Also, fibril angle measurements with Raman spectroscopy take much longer than the other polarised microscopy methods because Raman scattering gives only a weak signal compared to that given fluorescence and polarised light microscopy.

LITERATURE COMPARISONS

Meylan (*8*) has compared fibril angles determined by x-ray diffraction, optical replicas, compression wood striations and major extinction position (crossed polarisation analysis) with fibril angles measured by iodine staining. In each case, the iodine method was used as the reference technique. The data was collected from *Pinus radiata* early wood, late wood and compression wood. Apart from the x-ray measurements, each point was the average of 25-30 fibres. For this study, the iodine method

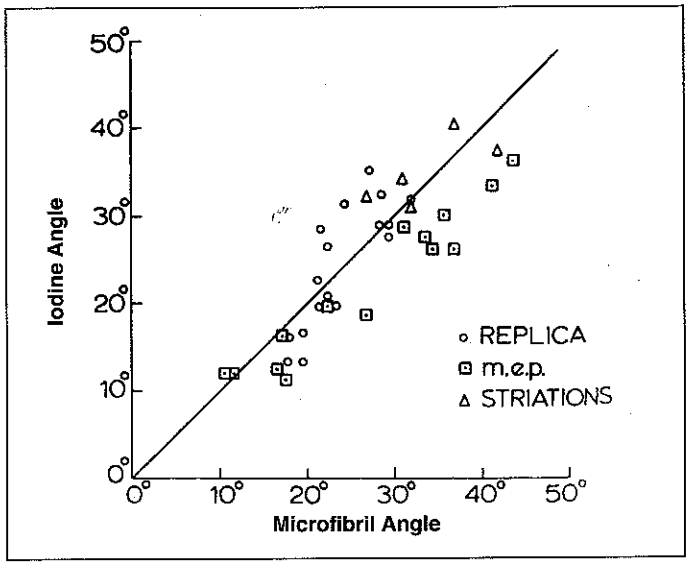

Fig. 1 Data from Meylan comparing fibril angles determined by iodine deposition with fibril angles determined by optical replicas, polarisation microscopy and compression wood striations.

was used as a reference value against which all other techniques were compared. This is because in the iodine method, the fibrils in only the S2 layer become clearly visible under a microscope and can then be directly measured. Meylan's results comparing the iodine, replica, compression wood striations and major extinction position are shown in Figure 1.

The solid line drawn in the figure indicates a one-to-one correspondence in fibril angles measured by the different techniques. It can be seen that there is good agreement for both the optical replicas and the compression wood striations with the iodine measurements. The points measured in the two techniques are scattered around the line of one-to-one correspondence. The scatter in the results is indicative of the error in the techniques and differences of up to 7-8° can be observed. Thus, even when fibril angle measurements from two direct techniques are compared there is still significant error.

When the crossed polarisation results are compared with the iodine results, it can be seen that the microfibril angles measured by polarisation are generally larger than those measured with the iodine technique. On average the microfibril angles measured by polarisation were about 8° more than those measured by iodine staining. This discrepancy has been given by Mark (*6*) as a reason for doubting the reliability of fibril angle measurements using polarisation.

In retrospect there were probably several factors that were impacting on the accuracy of the results reported by Meylan. The typical errors, as a result of the presence of the S1 and S2 layers, calculated by El-Hosseiny and Page (*20*) might explain up to half of this discrepancy. Also, at this point the technique of mercury injection had not been developed and so single cell walls were isolated for measurement mechanically. The mechanical action of the microtome on the fibres may have had some effect on the measured fibril angle. Finally, the measurements appear to have been made only under white light and not at a single frequency. This is not stated directly in the text, but the instrument used to perform the polarisation studies is only stated as being a 'polarizing microscope'. El-Hosseiny and Page have shown that for a given true fibril angle, frequency affects the measured major extinction position, although under normal measurement conditions the effect is relatively small (*20*). Dispersion with light frequency of the extinction position would imply that complete extinction is unattainable.

Jang (*24*) has compared the fibril angle measured with the fluorescence technique with those measured using pit apertures and crossed polaroids using mercury impregnation. The comparison between the pit aperture and fluorescence techniques was presented as measurements on approximately 30 individual fibres, softwoods and hardwoods, fibres produced by TMP and by the kraft process. Fibril angles ranging from 0° to 70° were measured. The results showed a one-to-one correlation between the fibril angles measured by the two techniques. However, the data showed that there was considerable experimental scatter in the measurements, with apparent deviations between fibril angles measured by the two techniques of up to 10°.

When Jang compared the fluorescence and the mercury impregnation results, it was not as measurements on individual fibres, but rather as comparisons of the mean fibril angles of four Bauer-McNett fractions of a bleached softwood kraft pulp. Each point was described as being the average of at least 100 measurements. It was found that for the R (14+28) and the R48 fractions the average fibril angle measured by the two techniques was almost identical, but that for the R100 and the R200 fractions that the averages determined by Page's mercury technique was approximately 5° higher than those determined by the confocal-fluorescence method. The difference in the values determined by the two techniques was ascribed to the influence of the S1 and S3 layers on the fibril angles determined by Page's technique (*24*).

Figure 2 shows the comparison between the micro–Raman technique and the cell wall striation method for seven pine compression wood fibres (circles) and between micro–Raman and measurements on pit apertures for twenty-nine eucalypt fibres. The solid line shows one-to-one correspondence. The data is combined from two sets of data from Pleasants et al (*25*).

It can be seen that micro–Raman results are somewhat higher than those determined by the cell wall striation method with the average fibril angle determined by the micro–Raman method being about 3° higher than that determined by the compression wood striations. This systematic error was suggested as being most likely due to inaccurate measurement of the laser polarisation axis (*25*).

When the pit aperture data is compared with the micro–Raman data it can be seen that there is a very large degree of scatter, with an apparent lack of correlation between the two sets of data. It is interesting to note that despite the lack of correlation between the two sets of data, the average fibril angles determined by the two techniques agreed to within 2°, even though the average discrepancy between fibril angles measured by the two techniques was 6°.

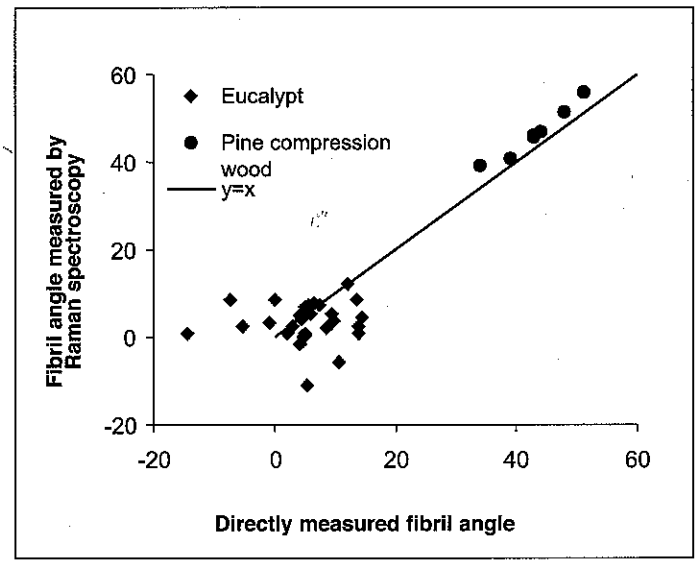

Fig. 2 Comparison of fibril angles measured by Raman spectroscopy with fibril angles measured by two direct methods, cell wall striations (pine compression wood) and pit apertures (eucalupts). Data taken from Pleasants et al (25).

The scatter in the data reflects some of the intrinsic difficulties in performing the measurements on the eucalypt fibres used here. The relative fragility of the eucalypt fibres means that distortion of the cell wall structures during the sample preparation stages can occur, leading to variation in fibril angle along the fibre. The fibril angle measured by micro–Raman spectroscopy must be measured at some distance from the pit aperture, with which it is correlated, so as to avoid the distortion of the pit aperture structure. These problems are in addition to the natural measurement uncertainties of each technique, which are at least $\pm 3°$.

Jurbergs (*10*) compared the fibril angle determined by x-ray diffraction with that determined by the pit aperture method for ten different trees. Jurbergs found a generally close correspondence between fibril angles measured by the two techniques. Although the individual fibril angles calculated by the two techniques differed by as much as 10°, the averages of the two sets of fibril angle data differed by only 1.4°. Prud'homme and Noah (*27*) have used a distribution of fibril angles measured by the mercury reflection method to calculate the azimuthal distribution of the 002 peak in the x-ray diffraction pattern of wood. Very good agreement was found between the theoretical distribution and the measured distribution.

Long et al (*28*) have compared fibril angles measured by x-ray diffraction with those determined by the new birefringence confocal technique. Good agreement between the two techniques was found when a factor of $\sigma = 9.4°$ was used.

DISCUSSION

Comparison of new confocal microscopy techniques

It is worthwhile to compare the two new confocal microscopy techniques since it is likely that these two techniques will be

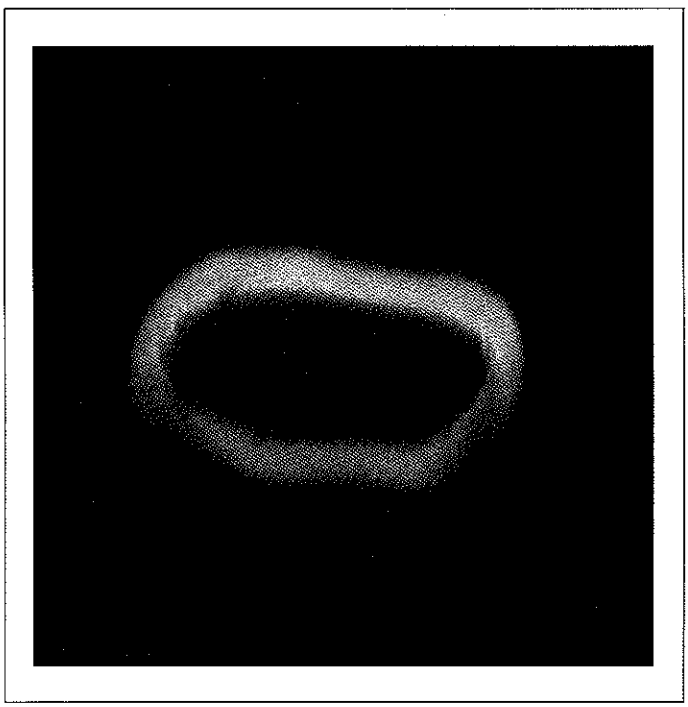

Fig. 3a Fluorescence confocal cross-section of a Pine TMP fibre stained with acridine orange. The fluorescence signal is detected from all parts of the fibre. (The image taken on a Leica TCS NT confocal microscope with a 63x, 1.2NA water immersion objective lens. Image size 80 x 80μm.)

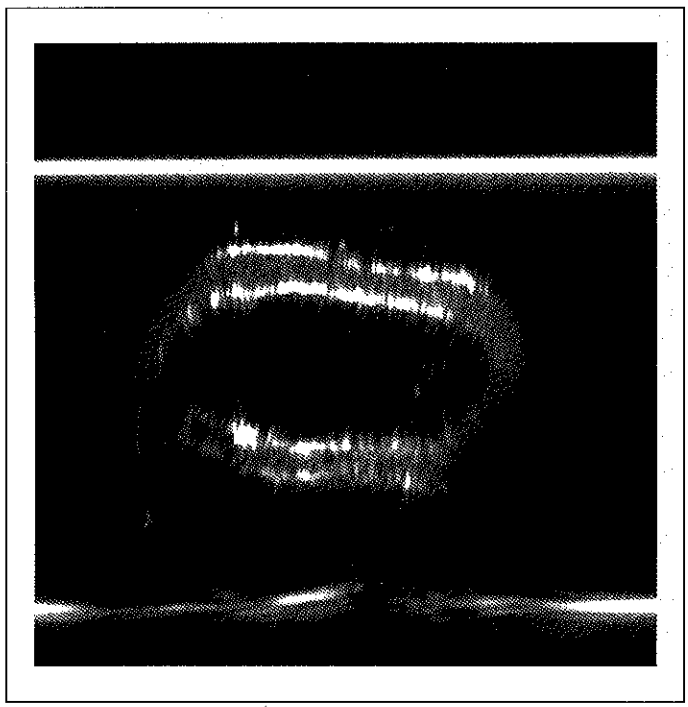

Fig. 3b Reflectance confocal cross-section for comparison with fluorescence image 3a. The reflectance signal is isolated to the top and bottom fibre and lumen surfaces where significant changes in refractive index occur.

most widely used for single-fibre measurements.

Both our birefringence confocal microscopy technique and the fluorescence confocal microscopy technique of Jang take advantage of the optical sectioning ability of polarised confocal microscopy. However, the two techniques 'scatter' light by different means and from different parts of the fibre. Figure 3a shows a fluorescence (excitation technique) image of a pine TMP fibre cross-section. The same fibre cross-section is shown in reflectance (birefringence technique) in figure 3b.

In the fluorescence image a significant signal is detected from the whole-fibre cross-section, including the sides, with absorption and other factors reducing the contrast on the lower section of the image. By comparison the reflectance signal is isolated to the fibre-water interfaces oriented at approximately 90° to the direction of the incident light. Light reflected from the sides of the fibre is at too great an angle to re-enter the objective and hence the sides of the fibre are not visible in reflectance.

As there is no change of polarisation of the light reflected from the top fibre surface, this top surface is not detected under crossed polarisation conditions. Also, in the birefringence technique the fibre is usually mounted on a glass slide. The close matching of the refractive index of the fibre wall with that of the glass slide greatly reduces any reflection from below the second cell wall. This significantly limits the possibility of interference from the second wall in the measurement of the fibril angle of the first cell wall. Thus, under crossed polarisation conditions, most of the reflected intensity detected will come at the two lumen-fibre interfaces. As the confocallity of the microscope will limit most of the detected signal to the upper lumen surface (unless the fibre is near collapse) making the technique optically equivalent to Page's.

The advantages of the birefringent technique include minimal sample preparation; no reflection from the curved fibre edges to distort the fibril angle measurement and limited interference from the second cell wall through the confocal optical sectioning and the refractive index matching of the second cell wall with the glass slide mounting. The disadvantages include the inability to measure the fibril angle of collapsed fibres and the presence of the S1 and S3 layers can produce errors in the measurement as per Page's technique (*20*).

The fluorescence technique described by Jang also has problems with collapsed fibres and care must be taken to sample the top cell wall (not the lower or side walls). However the fluorescence method is less susceptible to errors from the presence of the S1 and S3 layers and has improved resolution through the use of oil immersion lenses.

Accuracy of techniques

One of the striking features when data available in the literature is examined is that the average fibril angles, determined by different techniques for a set of wood or fibre specimens, usually seem to be within a few degrees of each other. This is in strong contrast to the much poorer correlation of fibril angles measured on the elements that make up the set. An excellent example of this can be seen in the comparison of the pit aperture data and the micro–Raman fibril angle data shown in Figure 2. For these measurements, the largest difference in fibril angle measured by the two techniques was 16°, yet the averages of fibril angles for the two sets of data were still within 2° of each other.

It is likely that the large scatter observed when the results of different measurement techniques on the same sample are compared is due to a combination of the inherent errors in the measurement as well as point-to-point variation within the fibre. For example, to measure a single fibril angle in the birefringent polarised microscopy technique, requires the determination of the axis of polarisation of the laser light on the sample, the orientation of the sample (not necessarily easy, given that samples are rarely fully straight) and the angular position of the minimum in the reflected intensity.

The best conclusion that can be drawn seems to be that, on average, the different techniques give similar results but that individual measurements are subject to considerable error.

CONCLUSIONS

Average fibril angles for sets of fibres, measured with different techniques, generally agree closely with each other, but measurements on individual fibres using different techniques can show large discrepancies.

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