TISSUE ENGINEERING
Volume 5, Number 1, 1999
Mary Ann Liebert, Inc.

Method for Automated Cartilage Histomorphometry

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ABSTRACT

We have developed and tested a color-based method for automated computerized histomorphometric analysis of cartilage. Histological sections stained with safranin O from 29 rabbit periosteal agarose-cultured explants were selected with various amounts of cartilage (0–100%). Color photomicrographs of these sections were visually assessed by five expert observers who estimated the percent area occupied by cartilage and outlined (in pen) the areas they considered to be cartilage. Manual histomorphometry was performed by cutting out and weighing the outlined areas. The average area for each of the five observers ranged from 31% to 43% (intraclass correlation coefficient = 0.70). The average of these values was used as a "gold standard" against which to compare the computer measurements. When point counting histomorphometry was performed on the 29 sections, the data agreed with the measurements made by the other five cartilage experts ($r^2 = 0.96; p < 0.0001$). The analysis of cartilage is based on safranin O stain, using a custom-designed Vidas 2.1 Image Analysis Program (Zeiss). The computer-based results correlated very closely with those obtained by manual ($p = 0.0001$; $r^2 = 0.92$) and point counting ($r^2 = 0.92$; $p < 0.0001$) histomorphometry. The mean percentage of the sections occupied by cartilage measured in the automated mode was only 6% higher than that using the gold standard. Histological complexity had only a minor effect on the computerized values. The automated computerized image analysis system has the advantages of objectivity, accuracy, repeatability, and ease of use.

INTRODUCTION

THERE IS A RAPIDLY GROWING INTEREST in the experimental repair and regeneration of articular cartilage, particularly tissue engineering that involves growing cartilage *in vitro*. Accurate, reliable, scientific outcome analyses are necessary to communicate and compare the findings of one experiment with another. Although molecular and biochemical markers of chondrogenesis are well known, they do not replace the need for detailed histological and histochemical analysis of the tissue following neochondrogenesis (new cartilage growth). Traditionally, histological/histochemical scoring systems have been used to assess the quality or quantity of cartilage.[1–8] However, subjective scoring systems can only be semiquantitative, and the possibility for substantial inter- and/or intraobserver variability is a concern.

Histomorphometry, with its greater degree of objectivity, accuracy, and reproducibility, is more appro-

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priate for statistical analyses.[9] There are several histomorphometry methods available ranging from time-intensive manual techniques to computer-based programs that offer the possibility of automation. Though manual histomorphometry can be performed by outlining photomicrographs then cutting and weighing the relative areas, current techniques are usually based on the principle of point-counting, on intersection counting, or by linear integration. Employing any of these methods for analyzing cartilage (as in new chondrogenesis for repair) still requires an observer to look at the histological section and make a decision whether to call the tissue in that region cartilage or not. Thus, there is still an element of observer dependence. For this reason, interobserver variability can be high, as has been found in bone histomorphometry.[10]

A number of investigators have demonstrated that computerized histomorphometry can reduce intra- and interobserver variation compared to manual methods for bone histomorphometry.[11,12] Such automated histological measuring has found wide acceptance and application in other areas, such as engineering. It can be semi or fully automatic. In orthopedics, it is perhaps used most commonly for bone histomorphometry, but would be highly useful for cartilage histomorphometry. Hacker et al.[13] reported the usefulness of a semiautomatic cartilage histomorphometry system to evaluate cartilage repair and to compute surface irregularities in repair tissue.

We have spent several years developing and testing a method for automated computerized histomorphometric analysis of cartilage. The purpose of this study was to scientifically evaluate its accuracy and reliability for analyzing cartilage grown in tissue culture.

MATERIALS AND METHODS

Technical Description

We employed a Zeiss Axioskop microscope, though any microscope can be used. Color temperature was maintained by always setting the light intensity on the microscope at the maximum brightness, which is standardized for color temperature. Kohler illumination is maintained through the use of two polarizing filters in the microscope. By adjusting the orientation of the polarizing filters with respect to each other, it becomes possible to control the brightness of the viewing field without changing the light distribution therein. Brightness adjustments made by adjusting the aperture size of the substage light condenser or by changing the light bulb output would have resulted in the loss of Kohler illumination and color fidelity.

The microscope was fitted with a substage light condenser specifically designed to be able to provide Kohler illumination with the low-power objectives (×1.25 and ×2.5) that were used in this particular automated analysis technique. Kohler illumination is crucial to the proper analysis of cartilaginous tissues with this system. Without the proper Kohler illumination, the white balance will not be uniform across the entire viewing field and the computer will not be able to accurately discriminate the boundaries of the tissue(s) being analyzed. To ensure that there were no white balance problems that could interefere with the histomorphometric analysis, the program checked the white balance in two different places in the viewing field to make sure that they were indeed white and not different from each other. If the computer detected that the white balance of the viewing field was "out," the computer stopped the analysis and asked the user to correct the problem before continuing.

A Hitachi FP-C1H high-resolution three-chip RGB video camera and a VA-C1 RGB adapter was attached to the microscope, and the output was sent to a Kontron 486 DX_2 microcomputer, where it was digitized at 640 × 480 pixels using a "frame grabber" board, stored and analyzed. All hardware and software was purchased from Carl Zeiss Canada (Don Mills, Ontario, Canada).

The Vidas 2.1 Image Analysis software, which is commercially available from Zeiss, was customized for cartilage analysis. This software has been designed for real color separation and the camera is high resolution to permit analysis using low-power objectives (×1.25 to ×2.5), which is necessary for analyzing large fragments of cartilage.

The definition of cartilage is based on color. A software algorithm discriminates each region based on its color. Though safranin O stain may appear to be red, it is actually a mixture of primary colors (red, green, and blue) when viewed from a video source. Therefore, to describe it to the computer, it must be defined as all colors from an RGB video source are defined, that is, in terms of its red, green, and blue

components. When these primary colors are being used in a video signal, they are referred to as channels. By assigning the appropriate value (between 0 and 255) to each of these channels, any one of 1.67×10^6 colors can be depicted by an RGB video source. The first step is the conversion of the RGB video image into its hue, luminance, and saturation equivalents. The Vidas image analysis software package has a built-in command ("dis2lev") to perform the multichannel color discrimination necessary for separating a colored region from a contrasting background, as is necessary in the case of cartilage sections stained with safranin O and counterstained with fast green. This discrimination is performed using the color channel opposite to the color being discriminated (blue in the case of safranin O stain). Upper and lower numerical constants (thresholds) between 0 and 255 are assigned to control the sensitivity of this discrimination. The "dis2lev" settings that we used, and continue to use, for our analyses were 40 and 170. Red areas of each section (as discriminated through the blue channel) that fell below 40 were ignored for not being red enough (i.e., too light), and red areas that were above 170 were ignored as being too red (i.e., too dark).

True color separation is an important component of this system. Cartilage is discriminated using a different channel (blue) from that used to discriminate the whole tissue section (red). This independent functioning prevents constraints created by one portion of the analysis being imposed on the other. This permits optimal discrimination.

Holes or empty spaces within the tissue (lacunae or artifacts) are discriminated mathematically using the built-in Vidas command "disdyn," which works on the basis of size and shape (sphericity) using size (1–255) and threshold (5–50) settings. This function is particularly useful for the discrimination of small objects and narrow linear structures on varying backgrounds. The "disdyn" size and threshold settings we used, and continue to use, for our analyses are 15 and 30, respectively. The areas of these discriminated holes are calculated separately; the lacunae are included in the cartilage measurement, while the artifacts are not. In this manner, lacunar spaces are included in the calculation of percent cartilage despite the fact that they do not stain with safranin O. Visual outlines are made on the video image for confirmation of the derived data (Figure 1). The lacunae–artifact discrimination is presented visually for observer confirmation.

These color and geometrical parameters are set initially at the time of software installation and adjusted in the first few trial applications. From that point, it is intended that no further adjustments be made. In other words, the program is designed and recommended for use in the automated mode. However, it is not meant to be simply a "black box," and indeed it does permit observer interaction by presenting an optional interactive mode. The observer is permitted the option of making adjustments to any of the parameter settings.

The central processing unit (CPU) of the computer does not have to be tied up during the day, and operator time can be minimized to approximately 40 sec per sample by storing the images without analyzing them and then analyzing all images overnight in an automated process. This has made work much easier. The final numerical result is automatically saved to disk in a separate file, which can be imported into a statistical package for analysis.

Histological Test Sections

Twenty-nine histological sections from rabbit periosteal explants cultured *in vitro* were used. The model used was the agarose/TGF-β model of O'Driscoll et al.,[6–8] which has been shown to promote chondrogenesis in periosteal explants cultured for 6 weeks. The techniques used for this study were identical to those previously published. The sections were selected so that the percentage of the total area that was cartilage ranged from 0% to 100%, in increments of 5–10%. Additionally, we wanted to know whether or not the computer could evaluate complex sections. Therefore, nine of the sections chosen were selected based upon the presence of artifacts, mottling, or blending of cartilaginous and fibrous tissues, multiple islands of cartilage surrounded by noncartilaginous tissue, and/or variable staining with safranin O. We anticipated explants such as these to be potentially problematic for the computer to analyze accurately. The sections were all 3-μm thick, and had been stained with safranin O and counterstained with fast green.

The tissues were prepared and stained using the same techniques used over the past decade in our previously published studies.[2,6–8,14–20] Prior to paraffin embedding, the tissues were fixed in 10% buffered formalin for at least 4 h (proteoglycan leachout can occur with prolonged fixation times). The staining proto-

col, a modification of that published by Rosenberg in 1971,[21] is as follows: The sections are rinsed in distilled water and then stained with 5% iron haemotoxylin for 5 min. Next, the sections are washed with running water for 5 min and then dipped twice in 0.5% HCl in ethyl alcohol. They are then washed under running water for 5 min and rinsed in distilled water. Next, they are stained with 0.4% aqueous fast green (color index: 42053) for 4 min. After staining with fast green, the sections are rinsed for 3 min in aqueous acetic acid (1%), then rinsed again for 2 min in a fresh solution of aqueous acetic acid (1%). They are then stained for 5 min with 0.125% aqueous safranin O (C.I. 50240, Fisher Scientific, Wood Dale, IL). After staining, the sections are dehydrated by dipping them five times in 95% ethanol (total time 5 sec) and then by dipping them in three changes of absolute ethanol for 3 sec each. Finally, the sections are cleared in three changes (1 min each) of straight xylol and then mounted in mounting media.

Color photomicrographs of these sections, taken in random order with respect to cartilage content, were printed at final magnifications of $\times 100$ or $\times 200$ so that the whole explant was included in each photograph. The photomicrographs were given to five expert observers from three different centers who had significant experience in cartilage research and histology and were blinded to any information regarding the explants. The instructions were simple; they were asked to make a visual estimate of the percent area occupied by cartilage and to outline with a marking pen the area(s) on each photomicrograph that they would consider to be cartilage. They were told that they were free to decide what criteria they would use to define "cartilage," and no reference to color or appearance of the matrix, nor of cellular morphology was made to them.

Manual histomorphometry was performed on the areas described above that were thought to be cartilage and were outlined with a marking pen on the photomicrographs. The images of the whole explants were cut out and weighed on a Mettler AT261 analytical balance (Mettler Instrument Corp., Hightstown, NJ). Then the areas outlined as cartilage were also cut out and weighed. The percent area of cartilage was defined as the ratio of these two weights. This method of cutting and weighing, though time consuming, is known to be accurate for the measurement of areas.[9,22]

All measurements were made blind, i.e., without knowledge of the results of the visual estimate. Reproducibility was assessed by the same individual repeating the measurements after a 6-month interval.

Point-counting histomorphometry was also performed as an additional control measure of accuracy. A 100-μm grid was used to count an average of 144 points per section. First, using a microscope, the number of points of the grid that fell within the boundaries of the section was counted. Second, the number of points that fell within the boundaries of the safranin O–stained areas of the section was counted. Points that fell on the left edge of the area being quantified were counted, and points that fell on the right edge were not counted. The percent cartilage was defined as the ratio of the number of points in the safranin O–stained region to the number of points in the total section. The point counting was repeated to ensure reproducibility, and the values were averaged.

Statistics

The data were analyzed as follows. Interobserver variability was assessed for both manual histomorphometry and the visual estimates using the intraclass correlation coefficient (ICC).[23] The mean results using each of the four methods (manual, point counting, automated and interactive computer) were compared by a two-way analysis of variance (ANOVA) for repeated measures and post hoc testing with Duncan's multiple range test. The automated computer histomorphometry results were compared to those obtained by manual histomorphometry using regression analysis. The automated and interactive computer histomorphometry results were compared similarly. Complex and noncomplex sections were analyzed separately and together. For manual histomorphometry, the complex and noncomplex sections were compared by two-way analysis of covariance (ANCOVA).

RESULTS

Manual Histomorphometry ("Gold Standard")

The average percent of the section area that was considered to be cartilage by each observer ranged from 31% to 43% (Figure 2). The ICC was 0.70, indicating good concordance among the observers' ratings tak-

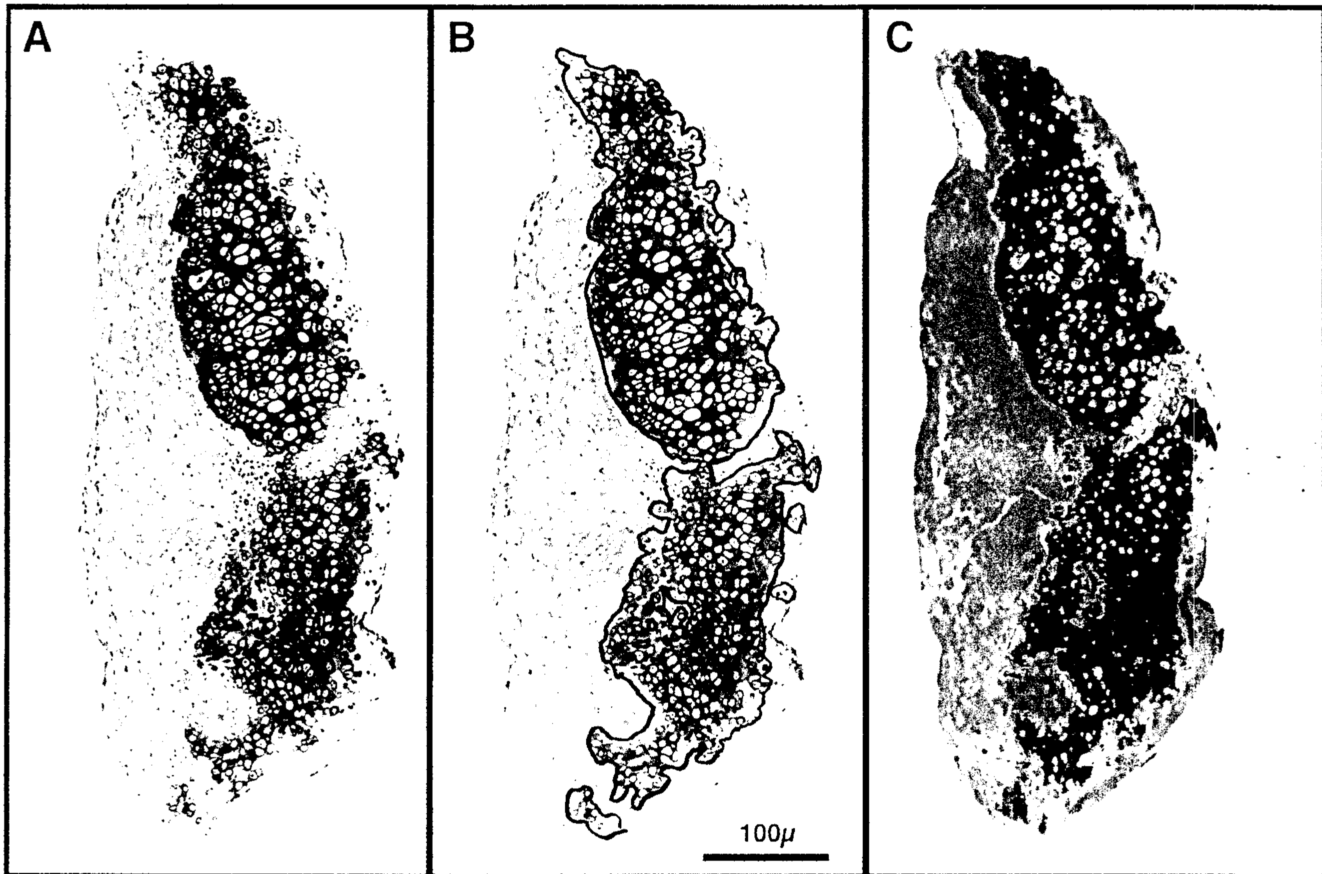

FIG. 1. (A) Histological section of a periosteal explant cultured in agarose for 6 weeks and supplemented with 10 ng/ml TGF-β1 for the first 2 weeks. The cartilage is clearly seen containing chondrocytes in lacunae surrounded by cartilaginous matrix that takes up the red safranin O stain (safranin O/fast green stain, ×175 magnification). (B) Pen tracing performed by one of the cartilage experts defining what tissue he would consider to be cartilage for the purpose of manual histomorphometry. In this case, the areas outlined by the five observers ranged from 47% to 55%. (C) Visual confirmation of the area(s) determined to be cartilage (outlined in green) by image analysis using the automated histomorphometry program. In this case, the cartilage area measured 54%. This figure includes the holes representing lacunae, but not artifacts, which are distinguished by the software based on size and shape.

ing into consideration all of the histological sections. The concordance is considered good if the ICC is 0.4–0.75 and excellent if the ICC is >0.75.[23] Thus, there is relatively close agreement among expert observers as to what one would refer to as cartilage on histological/histochemical sections. We did note that the correlation was the closest among the first three observers, who were from our lab, although they performed their work completely independently of one another.

There was a slight, but statistically significant effect of section complexity that was dependent on the observer. Two-way ANCOVA revealed that not only were observer and section complexity significant, but so was the interaction between these two factors ($p < 0.001$).

The means from the manual histomorphometry can be used as a reliable "gold standard" against which to compare the computer measurements. This method of cutting and weighing the areas outlined on paper (as it is used for integrating areas under curves, etc.) is highly accurate and is limited only by how accurately the observer uses his pen and the technician cuts out the marked areas.[22] Assuming that these aspects were done with care and precision, the values represent true measurements of what these experts call cartilage on histological sections.

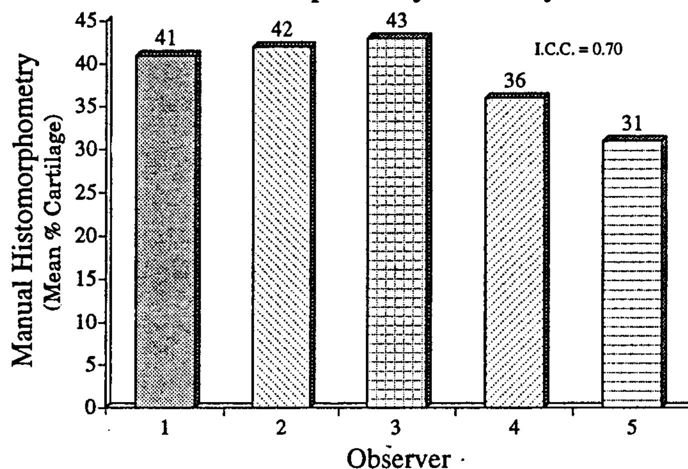

FIG. 2. Five expert observers outlined the areas they considered to be cartilage on 29 photomicrographs with a marking pen. This graph represents the means of those values for each observer. There was strong concordance (ICC = 0.70) among them, with a total range of only 12% from the highest to lowest mean values. This establishes that such a method can be considered a reliable "good standard" against which to compare other methods.

Computerized Histomorphometry

Regression analysis revealed that there was a highly significantly fit to a linear model ($p = 0.0001$) between the values measured by the computer in the automated mode and the means of those obtained by manual histomorphometry ($p = 0.0001$; $r^2 = 0.92$) (Figure 3). This was true not only for the means, but also for each of the observers individually. Regression analyses comparing the measurements by manual and computerized histomorphometry for each individual revealed significant correlations in each case ($p =$

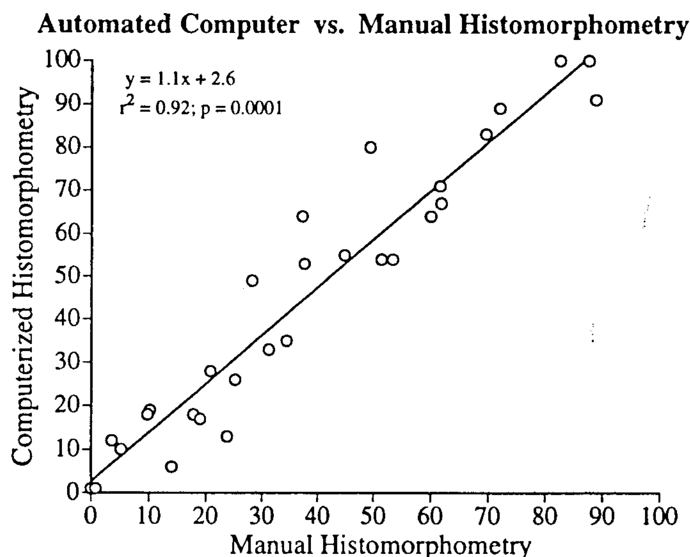

FIG. 3. The values obtained by the computer in the automated mode were compared to those from the "gold standard," manual histomorphometry. In this case, the means of the manual histomorphometry values were used (x-axis). There is very good correlation in a linear fashion with $r^2 = 0.9$. The computer tends to slightly overestimate the percentage of the section areas that are cartilage compared to the values obtained by manual histomorphometry, but by less than 10%.

0.0001), with $r > 0.86$ for all observers and $r > 0.97$ for three of the five observers (Pearson product moment correlation coefficients).

The mean percentage of the sections occupied by cartilage was measured by the computerized image analysis program in the automated mode to be 45%. This was only 6% higher than the mean obtained by manual histomorphometry ($p < 0.05$).

To determine what the difference might be between analyzing the sections entirely automatically (i.e., in the automated mode) versus manually adjusting the settings for color (i.e., in the interactive mode), we also measured each of the sections using the optional interactive mode function and again found an average of 45% cartilage. Although the difference of the means was 0, the mean difference was 3 ± 6%. Two-way ANOVA confirmed that there was no statistically significant or scientifically important difference in outcome between the interactive and automated modes. Regression analysis on the histomorphometry data revealed a very close correlation between the results obtained by the automatic and interactive mode ($r^2 = 0.96$; Figure 4). The effect of assisting the computer is thus negligible, and therefore the simpler and less subjective automatic mode would generally be appropriate to use.

Complexity of the histological sections (mottled cartilage, cartilage islands, poor staining, and artifacts) had only a minor effect on the computerized values. For noncomplex sections, the auto-computer values were on average within 5% of the manual histomorphometry values, but with the complex sections they differed by 9%. This was not statistically significant. The mean difference between the automated and interactive methods was 3 ± 5% for the noncomplex sections and 5 ± 7% for the complex sections.

Finally, comparison of the mean percentages of the areas considered to be cartilage by all of the three methods used revealed a significant difference, but this difference was very small (6%) and possibly within the range of experimental measurement error.

Repeat measurements made by the computer on the automated mode 6 months after the first measurements correlated with the originals very closely, with a correlation coefficient of $r^2 = 0.98$. The means (±1 standard deviation) for the test and re-test data were 45 ± 31 and 45 ± 32, respectively ($p > 0.99$).

Point-Counting Histomorphometry

As a further measure of validation, we compared the results obtained using the automated computerized histomorphometry to those obtained using a current point-counting technique with a 100-μm grid. The point

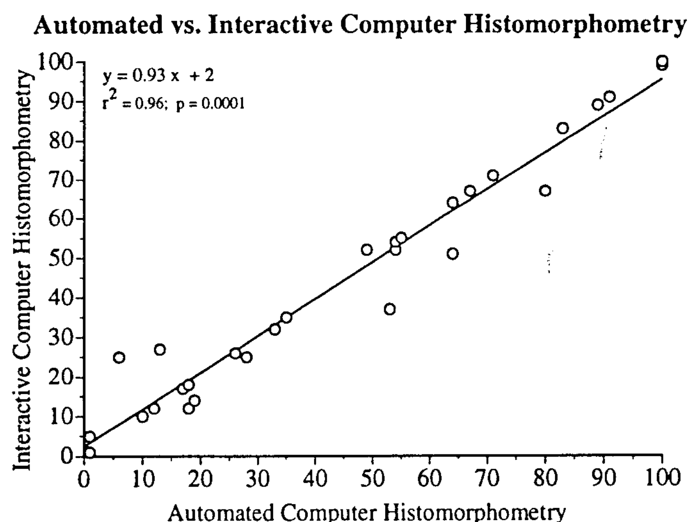

FIG. 4. The computer can be used in an interactive mode, with the user adjusting the threshold and other parameters. There was no significant difference in the results between the automated and interactive modes of use, and the two correlated strongly. This offers a real advantage because it means that it is not necessary for the user to make adjustments to the program for each section. This greatly reduces the time required and the potential for subjective error.

counting was confirmed to be reliable by performing it twice on the entire set of 29 sections. The correlation between the two sets of observations was linear ($y = x - 0.71$; $r^2 = 0.98$, $p < 0.0001$). Also, the point counting data obtained by a technician agreed with the measurements made by the other five cartilage experts ($r^2 = 0.96$; $p < 0.0001$). The correlation between point counting and the values obtained from the computer in the automated mode was very good ($r^2 = 0.92$; $p < 0.0001$).

Not surprisingly, the fastest histomorphometry method among those used here was the computerized histomorphometry method. It took about 1.5 min to analyze each section using the computer, 2.5 min per section using the point-counting method, and at least 10 min per section using the cutting and weighing technique. The more complex samples took longer to analyze using the point-counting, and cutting and weighing techniques. The complexity of the section did not affect the analysis speed of the computer.

DISCUSSION

In this study, we have defined what we would call a "gold standard" as that which a group of experts would agree upon histological examination to be cartilage. This first step was necessary, as a gold standard against which to validate an automated measure of cartilage in histological sections did not previously exist. We did this by confirming that five independent observers with experience in cartilage research could agree closely on what they would refer to as cartilage on histological sections without having been given any guidelines or criteria. The average manual histomorphometry values from the five observers were within 12% from lowest to highest. This level of agreement among expert observers validated the definition of our gold standard. We also confirmed a high degree of correlation between our gold standard measure obtained by traditional cutting and weighing method and the more commonly used method of point counting ($r^2 = 0.96$). Finally, we showed strong correlations between our automated computerized histomorphometric method and both the gold standard ($r^2 = 0.92$) and manual point-counting ($r^2 = 0.92$). In absolute terms, the measurements obtained using the automated histomorphometry differed from those of the gold standard (average of the values determined by manual histomorphometry by the five expert observers) by an average of only 6%.

No attempt was made in this study to determine the importance of the experience of the observers on manual histomorphometry. This was not one of the questions being addressed—the purpose of the study was to evaluate the feasibility of the computerized image analysis system, and to do this we required the opinions of experts in the field. All observers were experienced in the histology of cartilage. In evaluating the data from each observer, some observers called tissue "cartilage" if it stained moderately or intensely with safranin O and/or if the lacunae were well formed, whereas others required both criteria. As they were only provided low-power photomicrographs, there were sometimes regions that stained well, but in which the lacunae were not distinct, that were not marked as cartilage.

Having already confirmed the feasibility of applying this technology to research in cartilage regeneration, we have demonstrated that such an automated computerized image analysis system can be reliably and accurately applied to cartilage histomorphometry. It is designed and intended to be used in the automatic mode without interaction on the part of the investigator, virtually eliminating subjectivity responsible for inter- and intraobserver variability. Indeed, we have used this program in a fully automated fashion to analyze several thousand explants without the need to make adjustments. We have randomly re-analyzed sections 6 months apart and obtained identical measurements.

This system is not intended to function only as a "black box." There may be reasons for an investigator to make adjustments to the parameters being analyzed, and this is possible. The optional "interactive mode" permits the investigator to change the threshold settings for the percentages of red, green and blue in the total color composition, the hue, intensity, etc. This might be desirable if one were analyzing specimens that were stained with a stain other than safranin O (i.e., toluidine blue, von Kossa, etc.) or if there was cartilage containing little proteoglycans such as degenerative cartilage. As a built quality control measure, the program shows visual images of the section, outlining what it has called cartilage and asks if the observer wishes to use the automatic or interactive mode. Thus, a confirmatory check is required for each specimen analyzed.

To evaluate the interactive mode, in which the user is allowed to set or adjust the threshold settings, we measured our samples using both the automatic and the optional interactive functions available. The average difference between the results obtained by these two methods was less than 5%, indicating that the easier and less subjective automatic mode is advantageous. The measurement variability is increased in complex sections, such as those with intermixed areas of cartilage and noncartilage tissues, but only by 2% versus the noncomplex sections. We believe this is acceptable and still use it in the automatic mode for such sections.

Additional flexibility exists in the criteria set for excluding artifacts, and inclusion of lacunae. These are mathematical measurements of size, shape, perimeter to area ratios, etc., which are incorporated into the software customized for cartilage analyses.

Variability in safranin O staining has not been a significant problem for three reasons. First, staining irregularities can be detected by previewing the quality of the staining prior to analysis. Second, measures can be taken to ensure the reproducibility of our safranin O/fast green staining. Working with fresh solutions is important. The fast green counter-stain has a shelf life of up to 1 month, and the safranin O stain is prepared fresh every time. Meticulous clearing of the counter stain before safranin O staining is required to prevent a muddy looking section. We have been able to use this system to analyze sections prepared and stained in two different laboratories over nine years. In each case, the initial staining consistency was inadequate, but with appropriate quality control measures quickly became reliable. We always stain control samples of cartilage. In our experience overall, fewer than 5% of the sections require re-staining. Third, some flexibility in detecting the safranin O stain is permitted by independent discrimination of cartilage versus the whole tissue using separate video channels. This independent discrimination accommodates a wide range of colors in the red range—i.e., red, magenta, pink, orange, etc. This feature was specifically built in to the software for the very reason that safranin O staining is variable. This versatility has been validated by the successful use of this method in thousands of explants over 9 years in two different centers.

True color separation is an important component of this system. This is relevant to color variability with safranin O staining. Cartilage is discriminated using a different channel (blue) from that used to discriminate the whole tissue section (red). This independent functioning prevents constraints created by one portion of the analysis being imposed on the other. In other words, the novel feature of this approach is that it does not simply identify the specific colors associated with the safranin O and fast green staining, but rather the two colors are maximally distinguished by using separate processes for each. This optimizes color discrimination in a manner that is not possible with gray-scale separation. This latter point has been confirmed by employing the gray-scale function of this and other systems that did not permit such distinction.

We have in the past cultured explants for up to one year, and in that experiment noted gradual loss of safranin O staining after 3 months. As such, the values for cartilage histomorphometry were artificially low. However, the visual confirmation screen images made that readily apparent. In such cases, point-counting or computer tracing/outlining is more accurate.

This program is easy to use and has the advantages of objectivity and repeatability. Automation of such measurements should improve the communication and interpretation of results among investigations relating to cartilage research. During the past 9 years, we have used this program in the automated mode to analyze several thousand histological sections, and it has proven reliable, without requiring adjustments for the past 4 years.

There is a potential limitation of this or any other computerized system; the threshold values can be set or changed by the investigator. Thus, the possibility of subjective error still exists. This would affect the comparison of results from different investigators, but not those among different study groups in any one experiment if the investigator used the system in the automatic mode. We have used it in the automatic mode for several years and have therefore benefited tremendously in being able to compare our own results across studies. Of course, as with any computerized system, knowledge and experience are necessary for troubleshooting.

Experience with a system such as this indicates that automated computerized cartilage histomorphometry is a useful tool for cartilage research and has as its main benefit the objective and reproducible quantification of cartilage on histological sections. Histology is still highly useful in cartilage research despite newer techniques such as molecular biology. This method adds a dimension of credibility to traditional his-

tology that has been believed by some to have been lacking. The key contribution is that a system such as this one can be used in a completely automated fashion, without intervention on the part of the investigator. Thus, once the initial parameters have been set there is no subjectivity involved.

In conclusion, the concept of automated computerized histomorphometry can and should be applied in the study of tissue engineering for cartilage regeneration. It is not important which program is used, but rather that the methodology be adopted.

ACKNOWLEDGMENTS

We are grateful to Yasushi Miura, M.D. and Stephen Gallay, M.D. of our laboratory, Robin Poole, Ph.D., D.Sc. and Nelson Mitchell, M.D. of the Shriner's Hospital, McGill University, Montreal, Canada, and Robert Salter, O.C., M.D., F.R.S.C., F.R.C.S.(C), of the Hospital For Sick Children, University of Toronto for their participation and input in this study. This study was supported in parts by research grants to S.W.O. from the Medical Research Council of Canada (MA 10879) and Mayo Foundation. We are grateful to Thomas J. Wronski, Ph.D.; Professor, Department of Physiological Sciences; University of Florida, College of Veterinary Science, Gainesville, FL for carefully reviewing this manuscript and for his helpful suggestions. None of the authors nor the institutions with which they are or have been affiliated have any commercial or financial interest in the products or processes related to the histomorphometry, nor the computer software or hardware described in this paper. Furthermore, none have received, nor will they receive, any benefits, personal or professional, from anything related to this image analysis system. This paper was presented as a poster at the 27th Annual meeting of the Canadian Orthopaedic Research Society, May 30 to June 3, 1993, Montreal, Quebec, Canada.

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