Automated Measurement of Myofiber Disarray in Transgenic Mice With Ventricular Expression of ras

WILLIAM J. KARLON, JAMES W. COVELL, ANDREW D. MCCULLOCH, JOHN J. HUNTER, AND JEFFREY H. OMENS*

1Department of Bioengineering, University of California, San Diego, La Jolla, California 92093
2School of Medicine, University of California, San Diego, La Jolla, California 92093

ABSTRACT

Quantitative assessment of myofiber disarray associated with diseases such as familial hypertrophic cardiomyopathy (FHC) can be performed by estimating local angular deviation of fiber orientation in histologic sections. The large number of measurements required to estimate angular deviation prohibits manual measurement. We describe methods for automated measurement of local orientation and angular deviation in tissue sections from transgenic mice with ventricular expression of ras, proposed as a model of FHC.

Images of histologic tissue sections from normal and transgenic mice were analyzed using image processing techniques to estimate local orientation of myofibers. Results from the automated methods were compared with manual measurements.

Automated methods estimated differing mean orientation in 7–20% of normal sections and 17–29% of transgenic tissue sections with differing dispersions in 23–30% of normal sections and 25% of transgenic tissue sections. Automated methods estimate 24.47 ± 13.03% of total ventricular mass affected by disarray that is comparable to a previous estimate of 21.7% in the same mouse model.

Automated methods are a rapid and accurate alternative to manual measurement for estimation of mean orientation and angular deviation in myocardial tissue sections. Differences between manual and automated methods may be attributed to the substantially larger number of measurements made by automated methods. Automated methods are particularly appropriate for use in determining local variation in orientation such as focal myofiber disarray associated with FHC. The generality of these methods suggests they may have use in other biological fields such as quantifying cellular alignment. Anat Rec. 252:612–625, 1998.

Key words: myofiber disarray; hypertrophic cardiomyopathy; fiber orientation; angular deviation

Ventricular myocardium has a hierarchical organization of muscle fibers with a fiber orientation that varies with transmural depth. This continuous organization of fibers was first described quantitatively by Streeter (1969). It has been postulated that this ordered arrangement of myocytes helps to distribute wall stress more uniformly, generate torsion, and is implicated in the development of substantial cross-fiber shortening (Arts, 1979, 1984; Rade-makers, 1994). An accurate quantitative description of

Grant sponsor: NIH; Grant number: HL0744-17; Grant sponsor: American Heart Association Grant-In-Aid; Grant number: AHACA92-291.

*Correspondence to: Jeffrey H. Omens, University of California, San Diego, School of Medicine, 9500 Gilman Drive, Mail Code 0613-J, La Jolla, CA 92093. E-mail: omens@be-research.ucsd.edu

Received 24 April 1998; Accepted 7 July 1998
myocardial fiber organization is also required for predicting regional cardiac mechanical function (Costa, 1996).

Disruption of fiber organization is a hallmark of the disease familial hypertrophic cardiomyopathy (FHC), which is caused most commonly by genetic mutation of the sarcomeric proteins myosin, tropomyosin, or troponin (Watkins, 1995). Focal myofiber disarray is often preferentially located in the septal wall (Maron, 1987). Recently, transgenic mouse models of this disease have been developed that exhibit ventricular hypertrophy and focal myofiber disarray similar to that found in the human disease (Hunter, 1995; Vikstrom, 1995; Geisterfer-Lowrance, 1996).

Previously, estimation of the percentage of ventricular mass exhibiting disarray has been performed by manual assessment of histologic tissue sections using stereologic techniques (Maron and Roberts, 1979; Gottshall, 1997). Characterization of fiber orientation is also classically performed by manual measurement on histologic sections. Estimation of variation in fiber orientation found in areas of myofiber disarray has been proposed by Masuda (1996) as a quantitative measure of disarray. However, a large number of manual measurements would be required to estimate local variation in orientation. Additionally, psychophysical studies indicate that humans may over- or underestimate angles by up to 10° even when reproducing angles that are in view (Jastrow, 1892; Fisher, 1969). Since human estimation of angles is poor, it is likely that manual measurement of fiber orientation may be error-prone and subject-dependent. Ideally, an automated method for estimating local fiber orientation would provide a standard method for estimating orientation and would be capable of making large numbers of measurements in any given tissue section to estimate local variation.

Automated methods for assessing orientation have been developed for image processing of textural patterns, such as a herringbone weave in cloth and for analysis of materials containing short carbon fibers embedded in a resin matrix (Kass and Witkin, 1987; Rao, 1990; Chaudhuri, 1993; Denslow, 1993; Gadala-Maria and Parsi, 1993; Gonzalez, 1994). These algorithms make use of image processing methods, such as the Fourier transform, edge-detection methods, and gradients of image intensity to highlight areas of interest and assess orientation. We have developed automated methods for measuring orientation in microscopic images based on these image processing algorithms. The methods have use in assessing fiber angle distribution in normal and disarrayed myocardial tissue and for quantifying myofiber disarray found in mice with ventricular expression of the ras oncogene. We estimate 24.47 ± 13.03% of ventricular mass is affected by disarray in this transgenic mouse model, which compares favorably...
Fig. 2. (a) Performance of automated methods on test images: variation in mean orientation. * = Hough method significantly different from Hand ($P < 0.05$), † = Gradient method significantly different from Hand ($P < 0.05$). (b) Variation in SD, symbols as above.
with measurements made manually. The generality of these methods also may be useful in other biological applications such as quantifying in vitro cellular alignment.

**MATERIALS AND METHODS**

**Tissue Preparation**

Hearts from two NIH Swiss White mice were removed under anesthesia and the right ventricle dissected away to expose the septum. Hearts were fixed in Telly's fixative (formaldehyde, alcohol, glacial acetic acid) for 24 hours. The apex and base regions of the heart were removed and the remaining portion of the left ventricle was infiltrated for 48 hours in JB-4 plastic resin (Polysciences, Warrington, PA). The tissue was embedded in fresh resin and mounted for sectioning on an automatic microtome (LKB, Bromma, Sweden). The blocks were mounted for cutting sections in a plane tangent to the central region of the epicardial (RV side) septum between the insertion points of the right ventricle on the anterior and posterior walls of the heart. Measurements were made only in the central region of the septum. This orientation was parallel to the arrangement of fibers in the normal heart. The blocks were sectioned at 10 μm from the epicardium to the endocardium, mounted on glass slides, and stained using Gomori trichrome to enhance contrast of the myocardium.

Hearts were also removed from transgenic mice with cardiac-specific expression of the human oncogene ras proposed as a model of familial hypertrophic cardiomyopathy (FHC) by Hunter (1995). Transgenic mice exhibited a 58% increase in left ventricular mass and focal myocyte disarray similar to that found in human FHC. Two hearts from transgenic mice were processed and sectioned as described above.

**Image Acquisition**

Greyscale images of the myocardial sections were obtained using a Nikon Optiphot 2 upright microscope with a 10× objective (final magnification 100×). Images were acquired with Sony CCD camera Model DXC-151 using

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**TABLE 1. Performance of Computer Algorithms**

<table>
<thead>
<tr>
<th>Test images</th>
<th>Actual</th>
<th>Hough method</th>
<th>Gradient method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average measurements per section</td>
<td>336</td>
<td>400 ± 23</td>
<td>640 ± 25</td>
</tr>
<tr>
<td>Sections with different dispersion (P &lt; 0.05) —</td>
<td>2/9 (22%)</td>
<td>6/9 (67%)</td>
<td></td>
</tr>
<tr>
<td>Sections with different mean (P &lt; 0.05) —</td>
<td>1/8 (12.5%)</td>
<td>0/8 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD. Differences in dispersion and mean are with respect to actual distribution of orientation of line segments in images.
NIH Image software on a Macintosh Quadra 900 computer with a video capture board (Data Translation, Marlboro, MA). Acquired images measured 640 × 480 pixels with 8-bit greyscale resolution. From 12–18 sections were chosen with approximately uniform transmural spacing from each of the four hearts. Sections were oriented on a rotating microscope stage so that the circumferential axis of the heart corresponded with the horizontal axis of the acquired image. The user was not blinded to the source of the tissue, but random areas in the central region of septum were imaged in each section. The same images were processed manually and using two automated techniques for estimation of local orientation and angular deviation of myofibers.

**Manual Measurements**

Manual measurements of fiber angle with respect to the image horizontal axis (heart circumferential axis) were made using NIH Image software. A uniform 5 × 4 grid was overlaid on the image and 20 measurements were made at the grid line intersections. The time required to make these measurements was ~2 minutes, which was similar to the time required to process the images automatically using either of the methods described below.

**Method 1: Hough Transform-Based Technique**

Local orientation of fibers in the images was determined by using the Hough transform as suggested for analysis of cloth fibers. Full details of the algorithm are given by Gowayed (1996). Briefly, the following procedure was used:

1. Edges in the image were highlighted by applying a Difference of Gaussians (DOG) operator, proposed by Marr (1983) as a model of visual processing used by the visual cortex. Two Gaussian filters were applied to the image. A larger filter convolved with the image tends to smooth out images features, whereas a smaller filter highlights sharp transitions in intensity (defining edges). Subtraction of the two operations highlights edges and removes background intensity, resulting in the DOG image.
2. Four directional gradients were calculated using 5 × 5 arrays, so-called Sobel (gradient) filters (see Gonzalez and Woods, 1992). Gradient arrays oriented at 0°, 90°, +45°, and −45° were each convolved with the DOG image resulting in four directional images.
3. An automatic threshold value for each of the four directional images was chosen by selecting a fixed percentage (12.5%) of each image.
4. These four images were converted into binary images where pixel values equal to or greater than the threshold were set equal to 255 and those below the threshold were set equal to zero.
5. Continuous regions with pixel values equal to 255 were defined as relevant features in the image. Continuous
regions with area above a maximum (7,000 pixels square) or below a minimum (50) threshold value were eliminated as noise. A roundness factor for each of the remaining connected regions was computed:

\[ R = 4 \frac{\text{Area}}{\pi [(y_{\text{max}} - y_{\text{min}})^2 + (x_{\text{max}} - x_{\text{min}})^2]} \]

The value of \( R \) approaches 1.0 as the region becomes circular and approaches zero as the region become more linear. Image features with roundness values above a threshold (0.14) were eliminated.

6. The Hough transform (described in Gonzalez and Woods, 1992) was used to determine the primary orientation of each of the remaining regions in each of the four directional images. The Hough transform is used to fit a line through the group of pixels defining each region, and the slope of the line was used to determine the orientation. The orientation of the region was assigned a location at the centroid of that region.

The algorithm was implemented using the C programming language on a Silicon Graphics O2 workstation. Approximately 2 minutes were required to process each image. Output consisted of a list of image feature centroid locations, feature areas, and angles. Between 100 and 500 measurements were made per image with the number of measurements made dependent upon image quality and features present in the image. Dust or other unwanted features caused by tissue sectioning or processing present in the image may cause erroneous measurements; these can be removed interactively by the user.

Method 2: Intensity Gradient Technique

Local orientation of the images was determined by examining local intensity gradients in small subregions of each image. Full details of the algorithm used are given by Chaudhuri (1993). Briefly, the algorithm used was:

1. Intensity gradients in the horizontal and vertical directions were calculated by convolution of the image with 9 × 9 gradient arrays similar to the Sobel filters used above. This procedure calculates a horizontal and vertical gradient value for each pixel in the image.
2. For each image pixel, the gradient magnitude \( G \) was computed as the sum of the squares of the horizontal and vertical gradients. The direction of the gradient, \( \phi \), was calculated with respect to the horizontal image axis as the inverse tangent of the ratio of the vertical to horizontal components.
3. The dominant local orientation was determined in 20-pixel-square subregions using an accumulator scheme. A 180-element array $A_u^W$ represented the possible angles $0^\circ$–$179^\circ$ in subimage $W$, quantized in $1^\circ$ intervals. Each accumulator bin value was determined by summing a weighted contribution from each pixel in the subimage (modified from Chaudhuri’s original algorithm):

$$A_u^W = \sum_{(i,j)\in W} G(i,j) \frac{\exp(2 \cos[2(\theta - \phi_{ij})])}{\exp(2)}$$

where $0^\circ \leq \theta < 180^\circ$

This scheme assumes that each pixel has an orientation with an associated probability density given by $\exp(2 \cos[2\theta - \phi_{ij}])$, describing a von Mises distribution, analogous to a normal distribution, but for circular data as described by Fisher (1993). The distribution has a mean value of $\phi_{ij}$ and angular deviation of $\sim 25^\circ$. The probabilities are weighted according to the strength of the gradient and summed. Dominant local orientation in the subimage was determined by the largest accumulator bin value and converted to range from $-89^\circ$ to $+90^\circ$.

The algorithm was implemented using the C programming language on a Silicon Graphics O2 workstation. Less than 2 minutes were required to process each image and output consisted of a uniform distribution of measurements made for every nonoverlapping $20 \times 20$ subregion region in the image ($\sim 700$ measurements total). Subregion region size could be varied or overlapped to increase or decrease number of measurements made. Measurements that did not represent fiber orientation (due to noise in the image or unwanted tissue features) were removed after processing.

**Test Images**

A series of images was created to test the performance of the computer measurement techniques. Images consisted of short line segments generated on a uniform background. The length, width, mean orientation, and standard deviation of orientation were specified and a random normal distribution of segments with the specified characteristics were placed at regular intervals in the image. A mean and standard deviation of the created line segments was then computed, weighted by the length of the segments. Both computer algorithms were used on the test images to determine the ability of each to estimate the known mean and standard deviation.

**Analysis of Results**

Calculation of the mean orientation and circular standard deviation were calculated using circular statistics as
described by Fisher (1993). The mean orientation was computed by treating each measurement as a unit vector and averaging the vector components. Since the fiber angles were only measured within the range \(-90 \leq \theta \leq +90\), all angular values were doubled before computation, and results halved:

\[
C = \sum \cos 2\theta_i \\
S = \sum \sin 2\theta_i
\]

where \(\theta_i\) are individual fiber angle measurements

\[
R^2 = C^2 + S^2 \\
\cos 2\theta_{\text{mean}} = C / R, \; \sin 2\theta_{\text{mean}} = S / R
\]

where \(\theta_{\text{mean}}\) is the mean angle. The circular standard deviation is given by:

\[
s = \frac{1}{2} \left( -2 \log(R_{\text{mean}}) \right)^{1/2}
\]

where

\[
R_{\text{mean}} = R / n
\]

and \(n\) is the total number of measurements.

Testing for differences in mean orientation of two sets of angular measurements was performed using the Watson-Williams test proposed by Zar (1996). This test is equivalent to the Student's t-test for linear statistics. Differences in standard deviation of two samples was determined using a test proposed by Fisher (1993), which assumes the samples have a von Mises distribution (equivalent to a normal distribution in linear statistics). Nonparametric ranking tests appropriate for data that was not von Mises-distributed yielded the same results in all cases.

**RESULTS**

A sample test image with overlayed fiber angles estimated by the Gradient method is shown in Figure 1. Two sets of test images were analyzed using each of the computer algorithms:

1. Images containing segments with zero mean orientation and standard deviations ranging from 0° to 40°.
2. Images containing segments with mean values ranging from \(-80°\) to \(+80°\) and standard deviation of 15°.

The two methods estimated the mean orientation in each image to within \(\pm 3°\), shown in Figure 2a. Each of the methods was capable of accurately predicting the standard deviation over the given range, shown in Figure 2b. However, the Gradient method consistently underestimated the standard deviation by \(\sim 3°\), whereas the Hough transform method underestimated the standard deviation in \(\sim 22\%\) of the images. Table 1 summarizes the performance of each method in comparison with the actual distribution of oriented segments in the test images.

Digitized images of normal and disarrayed tissue were processed with each of the automated methods. Angles measured with the Hough transform method superimposed on sample images of normal and disarrayed tissue are shown in Figure 3a,b. Angles obtained with the Gradient method superimposed on the same images are shown in Figure 3 are shown in Figure 4a,b. Figure 5a,b shows the distribution of angles in the normal and disarrayed tissue sections shown in Figures 3 and 4 determined using the Gradient method. Figures 6 and 7 show the mean orientation and standard deviation in a transmural set of sections measured manually and with the two computer techniques in a normal and a ras heart. Each of the methods produced similar mean orientations, but there was significant difference in dispersion as determined using Fisher's von Mises test. Table 2 summarizes the results comparing the two automated methods with measurements made manually on the same tissue sections.

**DISCUSSION**

We present two methods for automated measurement of fiber orientation in myocardial tissue sections using image processing techniques. These methods make large numbers of measurements faster and more objectively than manual techniques. Local angular variation can be determined from these local measurements, which offers a quantitative approach to examining the myofiber disarray found in mice with ventricular expression of ras.

The Hough method, based on the Hough transform, used edges defined by directional intensity gradients as input to a Hough transform. This method produced similar results to manual measurements, with \(\sim 70\)–\(80\%\) of sections analyzed having the same mean (and all computer estimated means within 7° of manual measurements) as determined using circular statistical analysis. Since measurements were taken from the same tissue sections, the underlying variation in fiber orientation is identical, which makes statistical comparisons valid. Statistical tests comparing the Hough transform method to manual measurements indicated that in 25–30% of the sections, the methods produced similar results. Differences in mean and standard deviations were likely due to the smaller number of manual measurements.

The Gradient method used the inverse tangent of two orthogonal directional gradient masks to determine local orientation. This method produced a regular array of measurements at evenly spaced intervals in each image. The Gradient method also produced similar results to manual measurements (\(\sim 80\%\) of images with same mean and \(\sim 75\%\) with same angular dispersion). As with the Hough transform method, differences were attributable to the smaller number of samples made manually in each image. The results from each of the computer methods suggest that making a small number of measurements on images of tissue sections manually may produce significant errors in both mean fiber orientation and angular dispersion contained in these sections.

Both methods were able to make accurate estimates of mean orientation and standard deviation on a series of test images that were generated to span the range of expected values. The Hough transform method produced significant differences in the estimated standard deviation in \(\sim 15\%\) of the images. The Hough transform method relies on a gradient in image intensity to recognize features in the
Fig. 5. Histograms of angle distribution from sample (A) normal and (B) disarrayed tissue sections.
Fig. 6. (A) Mean fiber orientation vs. % depth for normal heart. * = Hough method significantly different from Hand ($P < 0.05$), † = Gradient method significantly different from Hand ($P < 0.05$). (B) Estimated fiber angle standard deviation vs. % depth, symbols as above.
Fig. 7. (A) Mean fiber orientation vs. % depth for transgenic heart. * = Hough method significantly different from Hand ($P < 0.05$), † = Gradient method significantly different from Hand ($P < 0.05$). (B) Estimated fiber angle standard deviation vs. % depth, symbols as above.
image. Automated selection of a threshold in the gradient does not account for large numbers of features in strongly oriented images and thus recognizes fewer features with a given orientation than are actually present in the image, which may influence the measured standard deviation. Additionally, the Hough transform method may reject image features based on size or shape that may correspond with oriented structures in the image, also influencing measured variation. The Gradient method averages orientation in small subregions of the image, which has a smoothing effect and results in a relative decrease in the measured standard deviation of orientation. Altering the size of the subregion analyzed alters the reported standard deviation (smaller area results in greater deviation). Each of the methods is consistent in the way the standard deviation is estimated; thus results from different images processed with the same technique can still be analyzed using circular statistical methods such as the Watson-Williams test.

Each of the computer methods was tested on transmural sets of tissue sections (shown in Figures 6 and 7), which include a wide range of tissue orientations. The similarity between manual and computer methods suggests that any of the three methods can be used to estimate mean orientation accurately. The significant differences in estimated standard deviation between the manual and computer methods suggest that a large number of measurements must be made in a tissue section to obtain an accurate estimate. The computer methods each produce hundreds of measurements in each image, which would be prohibitively time-consuming if performed manually.

Some areas of normal myocardium are known to contain a large variation of fiber orientation as well as the right ventricular wall inserts into the left ventricle. Our methods make no assumptions about the origin of the tissue and therefore would find large angular deviation in these areas similar to the large deviation found in disarray associated with disease. We tested our method on an image from a normal heart near the insertion of the RV into the LV and found an angular standard deviation of 38°, which was comparable to the standard deviation found in tissue sections from disarrayed myocardium. This suggests that presence of disarray alone is insufficient to determine if tissue is from a diseased or transgenic heart or simply from a region of normal heart with a large variation of fiber orientation. The angular standard deviation can be used as a sensitive indicator of the presence of disarray, but only if sufficient measurements are made in the tissue section to estimate accurately standard deviation. Our methods can be used to differentiate between tissue sections that contain disarray from those that do not, but since disarray was not found at all wall depths in the ras transgenic mice, we expect that the absence of disarray alone would not be sufficient to determine whether tissue samples originated from control or transgenic mice.

Masuda (1996) proposed that the standard deviation of fiber orientation could be used as a method for quantifying disarray associated with FHC. By making a large number of measurements of fiber orientation, local estimates of the standard deviation in fiber orientation can be made. The average standard deviation in fiber orientation from the control tissue sections was significantly smaller than for transgenic tissue (control = 11 ± 3, ras = 19 ± 6, P < 0.0001, compared using a t-test assuming unequal variances). Since local standard deviation in fiber orientation rarely exceeded 15° in normal tissue sections and approximately half of the tissue sections from ras hearts had standard deviations >20°, we evaluated local regions with >20° in standard deviation to be disarrayed. By taking the ratio of disarrayed regions to total sampled regions, we estimated the total disarray present in ras mice to be 24.47 ± 13.03% (n = 7) compared with 0.024 ± 0.0011% (n = 4) in normals. Our estimate of disarray compares favorably with the estimate of 21.7% made by Gottshall (1997) in the same transgenic mouse model. Although the transgenic mouse model we examined has been proposed as a model of FHC, the expression of ras using the alpha myosin promoter throughout the myocardi-um may result in more substantial disarray than that found in mice with similar genotype to the human disease. We believe the Gradient method in particular would be capable of quantifying less substantive disarray found in other transgenic models or in human FHC. By altering the area of the subregion examined, a greater density of measurements can be made in an image, allowing for detection of disarray occurring on a smaller length scale than that found in the ras transgenic mouse.

Since these methods rely on intensity gradients in grayscale images to quantify orientation, the methods also should be appropriate for use in measuring orientation in other microscopic images, such as aligned cells in culture. A sample image of myocytes oriented by adherence to an aligned matrix is shown in Figure 8 with overlaid orientation measurements as determined by the Gradient method. A Fourier transform method for quantifying cellular orientation described by Palmer and Bizios (1997) indicated that Fourier methods can accurately determine mean cellular orientation, but not angular dispersion. In comparison with manual measurement of cellular orientation, our new methods may be able to estimate accurately both mean orientation and dispersion in images of cells in various states of alignment.

Our results indicate that computer-based methods for automated fiber angle measurement are fast, accurate, objective alternatives to manual measurement techniques. These methods are particularly appropriate when variation in orientation is to be examined, as in the myofiber

### Table 2. Comparison of Three Fiber Angle Measurement Techniques

<table>
<thead>
<tr>
<th></th>
<th>Manual</th>
<th>Hough method</th>
<th>Gradient method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average measurements per section</td>
<td>20</td>
<td>151 ± 43</td>
<td>640 ± 65</td>
</tr>
<tr>
<td>Sections with different dispersion (P &lt; 0.05)</td>
<td>—</td>
<td>9/30 (30%)</td>
<td>7/30 (23%)</td>
</tr>
<tr>
<td>Sections with different mean (P &lt; 0.05)</td>
<td>—</td>
<td>6/30 (20%)</td>
<td>2/30 (7%)</td>
</tr>
<tr>
<td>ras tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average measurements per section</td>
<td>20</td>
<td>171 ± 67</td>
<td>653 ± 64</td>
</tr>
<tr>
<td>Sections with different dispersion (P &lt; 0.05)</td>
<td>—</td>
<td>6/24 (25%)</td>
<td>6/24 (25%)</td>
</tr>
<tr>
<td>Sections with different mean (P &lt; 0.05)</td>
<td>—</td>
<td>7/24 (29%)</td>
<td>4/24 (17%)</td>
</tr>
</tbody>
</table>

aValues expressed as mean ± SD. Differences in dispersion and mean are with respect to manual measurements.
disarray found in mice with ventricular expression of ras. The methods are a more objective means of assessing fiber alignment than manual measurement, which can be influenced by human perception of orientation. The automated methods also make substantially larger numbers of measurements than could be made in a similar amount of time manually. The generality of the methods used suggest that these methods have other biological applications such as quantifying alignment of cultured cells.

**LITERATURE CITED**


