Histomorphometry of the Ligaments Using a Generic-Purpose Image Processing Software, a New Strategy for Semi-Automatized Measurements

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Abstract Gold chloride technique can be combined with Adobe Photoshop® software to yield a quantitative assessment of the different areas in heterogeneous structures as are ligament. A semi-automatized method based on the sum of two- and three-dimensional morphological criteria upon colorimetric criteria allows the identification and measurement of the area occupied by a structure of interest. It also allows the quantification of color intensity to differentiate structures with similar staining avidity, like vessels and nerves. This computer-assisted, semiquantitative procedure for computerized morphometry is relatively simple to perform. The accuracy, efficiency, and reproducibility of this method based on a commercially available imaging program were considered adequate when tested on the anterior

cruciate ligament of the cat. Image normalization by trained observers using a commercially available software package designed for photography, applied to a sample randomly chosen, has provided the means of making reproducible measurements of heterogeneous structures.

Keywords Adobe Photoshop® · Proprioception · Gold chloride · Anterior cruciate ligament · Colorimetry · Computer-assisted morphometry

Introduction

Neural element distribution in articular tissues remains a matter of controversy when interpreting the role of these structures in the healthy and the diseased articular function. An element of particular difficulty is the anterior cruciate ligament (ACL), a complex three-dimensional structure with regional variations in its collagen frame, vascularization, and innervation, which precludes an automatic computerized histomorphometry. Insertional and central (middle) thirds in the ligament body present tight collagen fascicles, with also loose interfascicular and subsynovial spaces where vessels and nerves can be found.

Histological techniques can produce multiple artifacts that must be taken into consideration when interpreting the results of any quantification (tissue shrinking, wrinkles and folds during processing, microtome indentations...). Partial volume effect, the lighter staining observed when only part of a structure is included, is another frequent error. Monitoring structures along the slide or the use of serial sections may be necessary to avoid these potential misinterpretations (Fig. 1). Morphological criteria help to enhance the tissue staining discrimination, particularly in heterogeneous structures, such as ligaments, where three types of criteria have

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Fig. 1 Partial volume effect. A structure (**a**) when included partially in a histological section (*lower line*) absorbs only part of the staining. When the same structure (**b**) is completely included in the histological preparation (*upper line*), it is stained more intensely. To avoid misinterpretation of both the partial volume effect and of the artifacts, it is necessary either monitoring the structures along the preparation or using serial sections

been used: colorimetric, two-dimensional morphological criteria, and three-dimensional morphological criteria. Colorimetry in stained material helps to differentiate vascular and nerve structures based on their morphology and dye appearance. Two-dimensional morphological criteria help the histological identification of vascular and nerve structures in the connective tissue, but continuity in the serial sections is the most important morphological criteria to differentiate a vessel or a nerve. Therefore, a third dimension is necessary, minimizing the error due to partial volume effect in the volumetric study. According to Koch et al. [1], many of the classical papers on articular neurosensory elements interpreted the nature of the structures based only upon nonquantitative colorimetric criteria and two-dimensional morphological criteria, without serialization. Colorimetric measurements in different histological structures are relative to the collagen background. As collagen bundles are heterogeneous, more or less dense collagen background used as a reference varies depending on the area of interest (AOI). This problem is solved, in part, when the same morphological criteria (appearance, uniformity, absence of artifacts, etc.) are used to choose the reference collagen area. In this case, colorimetric assessment of the structures under study may be given as a percentage of the reference collagen. Digital processing of images obtained from the samples may be based in different software packages. Most common packages offer the possibility of selecting image areas based on several criteria. Initially, one must select structures whose appetite for staining is similar and are stained with the same intensity. The area showing any specific color range can be specified using tools such as the magic wand, associating manual pixel selection in the AOI, in case of large intensity differences

(Fig. 2). These tools allow the observer to make a more accurate selection of pixels. These software packages may allow a colorimetric quantification of the tinctorial appetite of the structure under study and of the area occupied by the selected pixels.

Our hypothesis is that a semi-automatized method based on the sum of two- and three-dimensional morphological criteria upon colorimetric criteria allows the identification and measurement of the area occupied by a structure of interest. It also allows the quantification of color intensity to differentiate structures with similar staining avidity. The purpose of this study was to evaluate a new method of histomorphometry of heterogeneous structures, specifically neural structures in the articular ligaments, using a commercial, non-purpose designed software, with regard to its reliability and validity. We also tried to determine the criteria to discriminate between neural and vascular structures within the ligament. The aim was to determine the intraobserver variability of the images using Adobe Photoshop® (AP) and the interobserver variability when the same images were analyzed by different observers.

Material and Methods

Sample Processing

Twenty normal ACL of cat were stained “in toto” with the standardized gold chloride technique of Gómez-Barrena. Serial sections of 80 µm thick were obtained in a cryostat, coverslipped, and examined under light microscopy with a Zeiss Axioscop. Approximately 40 histological sections of 80 µm thick were obtained per ligament, providing ten images of representative areas per section, with a ×200 augmentation each. No areas were considered representative if they had artifacts (folds, staining lumps, broken tissue, defects in the slide or the cover...), and only homogeneous areas that visually (small, ×100; large, ×200 magnification) seemed to represent the structure of the tissue under study were selected. Thus, a large number of images were digitized from 20 ACLs, representative of each of the study areas (body of the ligament and synovial/subsynovial region in the middle third and in the insertional area), and randomly (1 in 50 images), a sample of each area was chosen for measurement: 40 images in the ligament insertion area, 40 in the central ligament area, 40 in the proximal subsynovial, and 40 in the central subsynovial area. A total random sample of 160 images was chosen and used for measurements (Table 1, Fig. 3).

Images were uploaded into a computer (Power Macintosh G5, Apple Computer Inc., Cupertino CA, USA) by means of a digital camera (Kodak Professional DCS 200-ci. Kodak, NY, USA) through the microscope. The following

Fig. 2 Selection of area of interest using the various tools of Adobe Photoshop. *Left*: encapsulated mechanoreceptor within an interfascicular space of the middle third of the ACL—*up*. *Center*: histograms with the luminosity and surface of the selected area in the histological preparation. *Right*: selection tools applied to a figure with curved boundaries and homogeneous intensity. **a** Manual selection, which is used by the examiner to select the desired area drawing its contour by

hand with the mouse. **b** Magic wand tool, which is used by the examiner to select contiguous areas of color within a narrow range of a selected point. **c** Magnetic lasso tool, which allows the examiner to select an area following approximately its defined contour. **d** Polygonal lasso tool, which allows the examiner to select an area drawing polygonal lines over its contour. Although the histogram is very similar, the different tools used can improve the selection

photographic variables were standardized: microscope light intensity, microscope magnification, aperture, shutter speed,

movie mode, etc. The images were defined by pixels, and the power of discrimination of each pixel was 2^{24} . Each

Table 1 Sampling system and distribution of the sample

Sampling system				
Anterior cruciate ligament (ACL)	20			
Histological slides/ACL	40		800 histological slides	
Images/histological slide	10		8,000 images	
Random selection of the sample	1/50 images		160 images	
Distribution of the sample				
	Insertional area		Central area	
	Images of representative areas	Analyzed sample	Images of representative areas	Analyzed sample
Ligament body	2,000	40	2,000	40
Synovial/subsynovial region	2,000	40	2,000	40

Fig. 3 Selection of “representative areas” of the tissue, defined as areas considered by a trained observer as representative of the study area. Middle third of the body of the cat’s ACL stained with gold chloride

image was saved in raw TIFF format, with a standardized resolution of 1.5 million pixels per image. The color of each pixel was expressed in the red, green, and blue (RGB) channels, whose value ranged from 0 (black) to 255 (white). The brightness intensity of every picture element, composed of an image from each band (red, green, and blue), was measured.

Morphometric and Color Criteria and Image Protocol

The defining characteristics of nerve or blood vessel in a structure found in the selected images are shown in Table 2 and Fig. 4. The color quantification has been carried out with AP software version 7.0 (Adobe Systems Inc., CA, USA). In the image menu, gray levels or luminosity (color) of all pixels within the selected area (including mean, median, and standard deviation...) are depicted. Statistical analysis was performed using a two-tailed paired *t* test between values of staining from vascular and neural structures, observations of the same samples by different observers, and observations of the same samples by the same observer along time in each of the RGB channels. Level of significance was established in $p=0.05$.

Intraobserver and Interobserver Variability

To calculate the intraobserver variability of the method, the same observer blinded to the sample selection performed two series of 200 measurements of the surface of the structures stained with gold chloride and the intensity of staining. Between the first and second measurement, the time interval was 1 month. In each measurement, a stained area was selected, adding by magic wand tool in all areas with the same intensity of staining. In this way, and for a given colorimetric intensity, we calculated the relative area of

stained structures. The selected structures responded only to colorimetric criteria (regardless of morphological criteria) and included vascular structures and nerve structures. To calculate the interobserver variability of the colorimetric method, two observers blinded to the sample selection performed a series of 200 surface structure measurements with staining appetite by gold chloride in connective tissue, responding only to colorimetric criteria, regardless of morphological criteria. To solve the problem of collagen background, the color of the structures under study was standardized as a percentage of collagen staining and according to the formula:

$$\text{Structure channel color} = \frac{\text{structure channel color}}{\text{collagen channel color}} \times 100$$

Results

Colorimetric Data

Table 3 shows the results of colorimetry in different areas, after the colorimetric quantification has been corrected as a percentage of the background staining. These data correspond to the colorimetry of all structures impregnated with gold chloride, including artifacts. After applying morphological criteria (two- and three-dimensional) to the stained structures, we obtained the color data of each type of structure, vascular and nervous, corrected for the collagen background. When comparing the quantitative differences between the staining of vascular structures and neural structures (Fig. 5), we obtained statistically significant differences in all channels under study in the interfascicular spaces of the insertional area of the ACL ($p=0.0005$, $p=0.0008$, $p=0.0008$, $p=0.0006$), with higher staining values for nerves. In the interfascicular spaces of the central area of the ligament and in the subsynovial/synovial insertional area, nerves also show a trend towards higher tinctional intensity than vascular structures, although those differences did not reach statistically significant values. In the central area of the subsynovial/synovial region, paradoxically, vascular structures display a tendency towards higher colorimetric intensity than nerves, although not all of these differences were significant (p values 0.04/0.02/0.08/0.04). This result is paradoxical because nerves are expected to stain more densely than vessels due to their structural differences. Although there are areas where the colorimetry has a high resolution power (interfascicular spaces of the insertional area of the ACL), the discrimination of vascular and nerve structures solely based on colorimetric criteria is low, due to the proximity of values and to the fact that the color intensity is reversed in some areas. In summary, the colorimetry of structures stained with gold chloride has little

Table 2 Two-dimensional morphological criteria

Vascular structures	Sections parallel to the axis of the vessel	Identifiable light, clear, or with blood cells (forming lumps)	
		Tubular appearance: collapse of the vascular structure lumen when it undergoes torsion (increased density)	
		Characteristic and relatively constant wall diameter/lumen diameter ratio	
		Vascular ramifications in V shape, with continuous lumen and/or continuity of the wall	
	Sections perpendicular to the axis of the vessel	Formation of large vascular loops	
		Presence of lumen with or without lumps inside, surrounded by framing walls	
	Other features	Arteries	Striations parallel to the axis of the vessel due to the longitudinal orientation of the intimal elements
			Wall formed by palisade cells, which appears as a discontinuous dot line and corresponds to the nuclei of smooth muscle cell walls
		Veins	Geographic pattern of staining, by impregnation of endothelial intercellular cement
		Capillaries	In addition to their small diameter and many ramifications, there are indentations in the lumen due to the staining of endothelial cells
Neural structures	Sections parallel to the axis of the nerve	Solid appearance, without lumen	
		Nerve trunk formed by intensely stained fiber bundles	
		Barcode appearance	
		Typical ripple (meanders)	
		Peripheral nerve trunk consists of loose fibers (bundles decrease their size as they emit isolated fibers)	
		Great length compared to its diameter	
		Constant diameter	
		Small number of branches	
	Sections perpendicular to the axis of the nerve	Dotted intensely stained area surrounded by a continuous (epineurium)	
		Myelinated fibers	Halo of stained myelin that surrounds the fiber or indentations that correspond to the nodes of Ranvier
	Other features	Unmyelinated fibers	Smaller size and lack of indentations
		Nerve endings	Receptors as described by Freeman and Wyke

discriminatory power when used in isolation, which prevents automation of the analysis.

Intraobserver and Interobserver Data

The intraobserver analysis with only colorimetric criteria shows no significant differences between the two sets of observations. Therefore, the results are comparable, with the same measured surface in both observations. When the staining intensity (RGB levels) is considered, differences between the first and the second value reach statistical significance. That is, colorimetry varies from one observation to another and cannot be used exclusively for the differentiation of structures. To calculate the interobserver variability, two observers of our working group conducted, in a blind way, an estimate of the stained surface on the same digitized images of different regions of the studied ACLs. Using pure colorimetric criteria to measure the

Fig. 4 Two-dimensional morphological criteria. **a** Vascular structure: identifiable lumen, empty, or with blood cells forming lumps, tubular appearance, wall diameter/lumen diameter ratio characteristic, and relatively constant, geographical pattern of staining. **b** Neural structure: solid appearance, without lumen, with intensely stained fiber bundles, and meandering waves of constant diameter

Table 3 Colorimetric data

Colorimetry of dyed material (including artifacts) corrected for the collagen background												
Anterior cruciate ligament body												
Insertional area			Central area			Subsynovial/synovial			Central area			
Bright	R	G	Bright	R	G	Bright	R	G	Bright	R	G	
Mean	64.8	70.6	60.6	65.1	68.7	72.2	77.2	68.7	73.0	50.1	56.5	B
SD	12.4	13.8	11.6	11.9	8.0	8.3	8.1	7.8	7.5	11.4	12.2	B
Vascular structures' colorimetry applying two- and three-dimensional morphological criteria corrected for collagen, the background												
Anterior cruciate ligament body												
Insertional area			Central area			Subsynovial/synovial			Central area			
Bright	R	G	Bright	R	G	Bright	R	G	Bright	R	G	
Mean	48.1	55.5	42.7	48.9	33.0	40.8	27.2	33.2	49.7	60.5	67.7	B
SD	11.4	12.9	10.4	11.2	15.1	13.7	17.1	15.5	7.7	13.9	15.2	B
Nerve structures' colorimetry applying two- and three-dimensional morphological criteria corrected for collagen, the background												
Anterior cruciate ligament body												
Insertional area			Central area			Subsynovial/synovial			Central area			
Bright	R	G	Bright	R	G	Bright	R	G	Bright	R	G	
Mean	73.1	78.3	69	73.3	42.7	49.6	37.7	43.8	53.6	49.3	55.2	B
SD	13.7	11.8	16	13.5	14.5	16.0	14.5	15.2	17.4	16.1	15.7	B
Quantitative differences between vascular and nerve staining (<i>p</i> values for Student's <i>t</i> test)												
Anterior cruciate ligament body												
Insertional area			Central area			Subsynovial/synovial			Central area			
Bright	R	G	Bright	R	G	Bright	R	G	Bright	R	G	
<i>p</i>	<0.01	<0.01	<0.01	<0.01	0.23	0.28	0.22	0.20	0.48	0.04	0.02	B

Fig. 5 a Nervous structure colorimetry selected by magnetic lasso tool, with their corresponding histograms showing brightness, RGB channels, and selected surface area. **b** Vascular structure colorimetry selected using magic wand tool with the corresponding histograms showing brightness, RGB channels, and selected surface area

stained surface, we found no statistically significant differences between measurements by two observers (Table 4).

Discussion

Quantitative image analysis turns microscopic data that might otherwise be merely descriptive into reliable mechanistic information [2]. The computerized morphometry is useful in several medical fields for objective color analysis and characterization of biological tissues. It is used in very different fields, for example, for the quantitative measurement of liver fibrosis [3], quantification of erythema and pigmentation [4, 5], and calibration of human wounds [6]. Our group has been conducting histomorphometric studies on ligaments [7] using a nonspecific commercial image analysis. Initially, we attempted the histometric study with two common analytical applications, NIH image and AP. We chose AP due to its versatility and ease of application since it is an intuitive software, and we performed the first studies on irregular structures. Adobe Photoshop® has been used to enhance digital images in different areas, and its power has been confirmed in diagnostic imaging [8–10] including construction of three-dimensional images [11], in the study of friction surfaces in arthroplasties [12] or adjustment implant fixation to bone anatomy [13], in different histologic [14–18] and clinical studies [19–24] and even in forensic studies [25]. Our group has applied this software to quantify proprioceptive structures of ACL reconstructions [26–29] and the quantification of fracture callus in rats under experimental conditions [30]. In all these studies, error rates were very low and correlation coefficients

demonstrated inter-rater and test–retest reliability, making this technique fast, applicable, and highly reproducible.

Most software programs for image analysis are custom-made, expensive, and with limited applicability to selected parameters and study end points [19]. The precision was better and the errors of the data obtained using AP were significantly smaller than those of data obtained by other imaging techniques currently in clinical use [31]. Brunner et al. [32] presented evidence of the technical accuracy of the software tools and high degree of interobserver reliability. Dahab et al. [15] designed a fully computerized technique with no manual interference at any step, reliably for objectively quantifying any pattern of fibrosis in liver biopsy. This is true for homogeneous structures histomorphometry but is not applicable to heterogeneous structures such as ligaments. Romer et al. [33] used a modified standard microscope to generate virtual slides with the help of a robotic stage and specific software with specific scanning algorithms to generate images that enable morphometric analysis. This technique, while promising, is too complex to be applied to heterogeneous structures. Other authors have used this technology for the quantification of tissue stained with gold impregnation techniques. Thus, Bisland et al. [34] described the use of this software to quantify the accumulation and distribution of gold-labeled constructs within two-dimensional cell sections in electron microscope images. Likewise, Lahm et al. [35] performed a histology study of cartilage tissue samples and determined the content and synthesis of collagen types and proteoglycans in knee joint cartilage. The content of these structures was colorimetrically measured using AP. These authors, as their samples are relatively homogeneous in contrast to our study, used specifically colorimetric data, regardless of morphological features.

Table 4 Intraobserver and interobserver variability

Intraobserver variability of the colorimetric measurement												
<i>p</i>	Anterior cruciate ligament body				Subsinoval/sinoval				Central area			
	Insertional area				Insertional area				Central area			
Stained surface	0.99				0.6				0.92			
Colorimetry	Bright	<i>R</i>	<i>G</i>	<i>B</i>	Bright	<i>R</i>	<i>G</i>	<i>B</i>	Bright	<i>R</i>	<i>G</i>	<i>B</i>
	0.09	0.14	0.05	0.08	0.04	0.04	0.05	0.04	0.06	0.04	0.09	0.08
Interobserver variability of the colorimetric measurement												
<i>p</i>	Anterior cruciate ligament body				Subsinoval/sinoval				Central area			
	Insertional area				Insertional area				Central area			
Stained surface	0.91				0.23				0.77			
<i>P</i> values for Student's <i>t</i> test												

In our experiment, an experienced observer obtained multiple representative areas (small and large magnification), choosing optimal staining histological areas without artifacts. We digitized a large number of representative images of each of the study areas and a random sample of these images was chosen blindly. This method allows more accurate measures than automatic methods. The camera sensor enables the independent capturing of the color information for any spatial point within its field-of-view [36]. Then, a series of computer operations were performed on a number of acquired fields of these randomly selected areas.

In this study, first we strictly defined morphological criteria (two- and three-dimensional) on which observers must be based for histomorphometry of the ligament. The intraobserver variability observed when only colorimetric criteria were applied supports the conclusion that the differences between both sets of observations are not statistically significant. When the intensity of staining (RGB levels) is evaluated, intraobserver variability is statistically significant. The interpretation of these results is complex. We measured the color brightness of the stained substance (without using morphological criteria) relative to the collagen background, obtaining a percentage. This percentage has been subjected to statistical tests. According to Oberholzer et al. [37], the noise is mainly present in the image background. For an optimal discrimination between different objects or features in an image, these defects must be minimized by shading correction, usually dividing the original image values by those of the background. Small differences in the dye uptake may be increased depending on the intensity of staining of the collagen background, which would explain the results of statistical tests and a lower accuracy. Therefore, the proposed method (comparison of percentages of staining relative to the collagen background) is not valid to compare images of different histological preparations. Colorimetry alone represents only one feature of the structure under study and it is useful to differentiate tissue structures within the same image when used in combination with morphological criteria. However, colorimetry is not a valid method to compare images of different histological preparations because of the large intraobserver variability. Stained area assessment was performed by two examiners. Using pure colorimetric criteria, we found no statistically significant differences in the various regions of the ACL. Therefore, the differences between the two observers are not significant and the method is valid as long as there is a previous training of observers to increase its accuracy. The variability of these measurements for the stained surface is therefore limited. One must keep in mind that validation of the method is based on heterogeneous tissues (synovium and ligament) and only stained material was measured (colorimetric criteria exclusively). Despite

this fact, the method seems reproducible and reliable. The sensitivity and specificity must increase if more homogeneous tissues are studied and if, in addition to the colorimetric criteria, two- and three-dimensional morphological criteria are used. Therefore, the colorimetric quantification does not allow absolute identification of a given structure, but represents a feature that, combined with two- and three-dimensional morphological characteristics, allows identification and quantification of structures in a much more accurate way.

This is a histomorphometric method that falls into the category of semiautomatic [38], faster, more capable of discrimination and with fewer errors than manual methods (rectangular grid, Merz's grid, etc.). When compared to other semiautomatic histomorphometric methods, it has the advantage of using non-specialized software, with very high resolution, easy handling, and a cheaper, nonspecific hardware. Semiautomatic methods, in general, and especially in heterogeneous solid tissues, are superior to the automatic methods because, in the words of one of the fathers of histomorphometry, we must not forget that a number of misunderstood and possibly irrelevant measurements will never replace an observer with clear ideas and common sense [39].

It is concluded that colorimetric features help to discriminate structures within a given image, but do not allow comparison between different images. Colorimetry with gold chloride has little discriminatory power when used in isolation. When it is combined with two- and three-dimensional morphological criteria, discriminatory power increases. The developed histomorphometric method is considered valid, applicable to any variety of histological staining and tissue studies (solid tissue) and histological techniques for calculating area. The greater the staining differences in the tissues under study (especially stained preparations to achieve a high level of contrast between the different structures being measured) the higher the resolution of the method.

Conclusions

In conclusion, gold chloride technique can be combined with AP software to yield a quantitative assessment of the different areas in a heterogeneous structure as is the ACL. We developed a new technique in computerized image analysis to apply to heterogeneous collagenous structures based on AP software. The histomorphometry of the ligaments using AP is available for any computer and it is useful for improving the measurement accuracy. This computer-assisted, semiquantitative procedure for computerized morphometry is relatively simple to perform. The accuracy, efficiency, and reproducibility of this method based on a

commercially available imaging program were considered adequate. Image normalization by trained observers using a commercially available software package designed for photography, applied to a sample randomly chosen, has provided the means of making reproducible measurements of heterogeneous structures.

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