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Gold chloride technique to study articular innervation. A protocol validated through computer-assisted colorimetry

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Summary. We studied variations in gold chloride techniques to elicit neural elements within articular samples, after "in toto" staining. These techniques attempt the differentiation of neural and vascular structures. Major changes in differential staining were observed when the gold chloride concentration was empirically modified. After the rest of the technique was standardized, we selected three gold chloride solutions to perform quantitative color experiments: 1%, 0.75%, and 0.5%. Significant sections of the same thickness were acquired with a digital camera to perform computer-assisted colorimetry. Color was measured through RGB (red-green-blue) channels in vessels, nerves, and background connective tissue as an internal control. By means of multivariate regression analysis, we compared differences in color measurements after 1%, 0.75% and 0.5% gold chloride preparation. Statistically significant coefficients confirmed that red color signals in vessels after the 0.75% and the 0.5% solution were both less intense than after the 1% preparation. Green and blue signals in vessels were also significantly less intense after the 0.5% protocol than after using the 1% solution. Red color signals in nerves between the 1% and the 0.75% preparation protocols were more intense and not significantly different, while the 0.5% preparation produced significantly less intense red signals in nerves. Non-significant differences were observed in green or blue signals from nerves after any protocol. We concluded that the 0.75% gold chloride solution protocol produced more intense red signals in nerves and less intense red signals in vessels. This was the most discriminant protocol in our series, based on color signals.

Key words: Gold chloride, Articular innervation, Histological technique, Computer-assisted colorimetry

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Introduction

Metallic impregnation histological techniques, mainly based on silver and gold salts, have been used and quoted by classical and modern authors throughout this century to demonstrate neural elements within a variety of tissues (Cohnheim, 1866 -quoted by Ehrlich et al., 1910; Ranvier, 1889; Ruffini, 1897; Ehrlich et al., 1910; Ramón y Cajal, 1910; Gairns, 1930; Carey, 1941; Gabi, 1968; Kiernan, 1981). To obtain satisfactory results, these empirical techniques heavily rely on the experience of the researcher and on the tissue and reactant quality. Due to stain variability, the remarkable but capricious discrimination offered by these techniques has been widely challenged. When the technique is completed however, the morphological evidence can be brilliant.

Metallic impregnation techniques differentiate neural structures based on their increased affinity for metallic ions. Gold staining is based on gold chloride or golden sodium and potassium salts. Gold chloride can usually be obtained as "pure" (about 65% Cl_3Au), di- or trihydrated (about 50% + $2(3)H_2O$), or with clorhydric acid (about 50% + HCl). Gold chloride techniques are believed to rely on gold ion fixation to neural proteins, whether they form myelinic fibres, amyelinic fibres or neural endings.

Renewed interest has been paid to gold chloride techniques to elicit neural elements in joints (Skoglund, 1956; Freeman and Wyke, 1967; O'Connor and McConnaughey, 1978; O'Connor and Gonzales, 1979; De Avila et al., 1989; O'Connor, 1984; Schultz et al., 1984; Zimny et al., 1985, 1986, 1987; Schutte et al., 1987; Yahia et al., 1988; Zimny, 1988; Sjölander et al., 1989; Wink et al., 1989, 1992; Yahia and Newman, 1991; Gómez-Barrena, 1992; Gómez-Barrena et al., 1991, 1992a-c, 1993; Amir et al., 1995). These studies require the penetration of metallic gold chloride into the tissue. Different authors originally considered some tissue preparation in gold chloride techniques (formic acid -Loewit, quoted by Ramón y Cajal, 1910-, lemon juice -Ranvier, 1889, and subsequent modifications-...)

to allow that penetration. However, small amounts of tissue have been processed without any preparation (like the cornea, by Cohnheim, 1867 -quoted by Ranvier, 1889-). The final demonstration of the gold-protein complex requires metal reduction, which is performed by most authors in an acidic medium (formic acid -from Loewit, quoted by Ramón y Cajal, 1910; to Zimny et al., 1985-, oxalic acid -Boccardi, quoted by Ehrlich et al., 1910).

Significant criticism has been raised on the use of gold chloride techniques to study articular innervation. Difficulties in reproducing the technique, confusing images doubtfully considered as neural endings, controversial quantification and overestimation due to vascular artifacts, are some of the issues provoked by these techniques, to the point of raising considerable skepticism about their results.

In view of the widely different techniques, the unpredictable results, and the misleading artifacts, we undertook the present study. Our preliminary aim was to obtain a standardized, reproducible gold chloride protocol. We focused on empirical techniques to determine a controlled composition of the reactants, with particular interest in the gold chloride solution quality. When the technique was precisely isolated, we set the aims of this study: to compare the effect of different gold chloride concentrations in the color discrimination of vascular and neural structures in the joints; and to propose the most discriminant gold chloride protocol to histologically differentiate neural structures in the articular tissues. To complete these aims, we focused on the development of a computer-assisted method to assess quantitative colorimetry in the stained sections.

Materials and methods

Four hundred articular specimens underwent gold chloride techniques. Two hundred samples were processed as described by Gairns (1930), and modified by Zimny and coauthors (1985), to obtain empirical data about the current technique. One hundred and fifty samples underwent trial experiments by modifying timing, composition and concentration of reactants, to obtain data about the different steps of the technique. These samples included knee structures (anterior and posterior cruciate ligaments, collateral lateral and medial ligaments, lateral and medial menisci, patellar ligament, fat pad and perimeniscal soft tissues) from cats (*Felis catus*), non-human primates (*Macaca nemestrina*), and human knees (adults, 17 to 84 years of age). The samples were processed 'in toto', keeping their bone insertions to avoid tissue distortion caused by harvesting. Their diameter varied from 2 to 5 mm, except for human ligaments, which ranged from 10 to 20 mm. These big ligaments showed higher variability due to different penetration of the reactants. The samples were fresh, processed immediately, or otherwise stored in saline, frozen at -20 °C and thawed just before processing. "In toto"-stained samples underwent cryosectioning between 60 and 150 μm. Sections were routinely mounted on coated slides, dehydrated in crescent alcohols followed by Xilol, and then coverslipped.

Empirical staining technique

Following Gairns (1930) and Zimny and coworkers (1985), three stages of the technique were performed: 'in toto' specimens were immersed in a lemon juice (3/4 in volume) and 88% formic acid (1/4 in volume) solution. After rinsing the tissue, it was bathed in a 1% gold chloride solution. Then, the reduction was performed in 25% formic acid.

As lemon juice was a non homogenous reactant, we considered the composition of 100gr of natural lemon juice (water content, 7.7gr of carbohydrates, 6gr of citric acid, 0.4gr of proteins, 0.3gr of malic acid, 0.13gr of K^+, 0.05gr of ascorbic acid, 0.014gr of Ca^{++}, under 0.01gr of other ions and vitamins). The ionic, acidic, and carbohydrate composition largely vary, due to different fruit ripeness. Of the different components in the natural lemon juice, we empirically kept citric acid and ascorbic acid as the basis of the reactant to perform tissue acidic preparation, the first step in the technique. We prepared 100gr of "laboratory lemon juice" with 100ml deionized water through a high purification MiliQ system (Millipore, France), adding 6gr of citric acid, 0.1gr of malic acid, 0.04gr of anhydrous $CaCl_2$, 0.03gr of $MgCl_2$, and also adding 0.1gr of L-ascorbic acid immediately before use. As 88% formic acid seemed to be the purest formic acid commercially available at the time of the description of the technique (Gairns, 1930), we used 98% formic acid, the purest formic acid today available, that allowed us an easier preparation of the reactants in our routine. When these two formic acid concentrations were experimentally compared, no observable difference was detected in the examined sections.

Our routine was performed as an empirical technique with a first step of tissue preparation (3/4 volume of "laboratory lemon juice", plus 1/4 volume of 98% formic acid). The control of this phase timing was tissue transparentness in the sample. When this point was obtained, the tissue was withdrawn and rinsed. The second step (gold impregnation) was empirically performed in a 1% gold chloride solution (Gold Chloride trihydrate, Tetrachloroauric [III] acid HCl_4Au+3H_2O (50.0% Au), Sigma Chemical Co., USA). The control relied on the gold yellow color of the tissue, after a minimum time similar to that of the first step (empirical penetration time). The sample was rinsed again. A third step (reduction) was performed in a 25% formic acid solution, where the tissue was left overnight (over 12 hours).

All the reactions were performed in the dark, at room temperature, and no metallic instruments were used to manipulate the samples within the gold solution. No differences were observed due to light exposure in the first step, but an acceleration of the reduction occurred during light exposure. A significant

deceleration of tissue reduction was observed with decreasing temperature. At 4 °C, the reaction was stopped. Metallic instruments used to section or manipulate the "in toto" sample during tissue preparation did not significantly affect the final result. However, metallic instruments used within the gold solution were not allowed to avoid metallic ion competition, although no clear effect could be identified in the sections.

When the technique was standardized in the different steps, we empirically observed that major variations occurred after changes in gold chloride concentration when using 1% freshly prepared, 1% "old" (recycled solution with more than 2-3 uses) and 0.5% fresh solution. These have been the three main solutions previously used in articular tissue gold chloride preparation. We could observe the best empirical results with some samples stained with the confusing 1% "old" gold chloride solution, and we then focused on its reproducibility.

Gold chloride quality analysis

We prepared 1%, 0.75% and 0.5% gold chloride solutions using deionized water through a high purification MiliQ system (Millipore, France), and Gold Chloride trihydrate (Sigma Chemical Co., USA). We also obtained 1% "old" gold chloride solution, that had been repeatedly used (3 samples of the solution after 2 uses, and 3 samples of the solution after 5 to 7 uses). We prepared 5ml samples and performed absorption spectrophotometry in a Shimadzu UV-3000 double wave length spectrophotometer (Shimadzu Co., Japan). Fig. 1A shows the absorption spectrum for a 1% "old" gold chloride solution after 2 uses and after 6 uses, compared with the curve for 1% "new" (freshly prepared) gold chloride solution, before any use. Fig. 1B shows intervals where the curves fall for the samples of 1%, 0.75% and 0.5% fresh gold chloride solutions that underwent the spectrophotometry. As a part of the method, we obtained the preliminary conclusion that some 1% "old" gold chloride solutions could be substituted by the predictable 0.75% freshly prepared solution, to be used in our experiments.

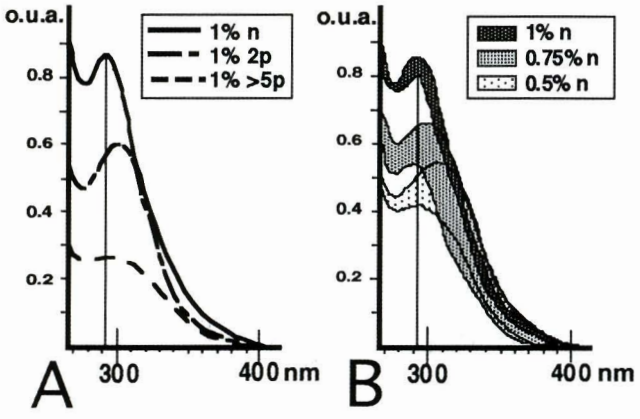


Fig. 1. A. Absortion spectrum curve of 1% gold chloride solutions ("new" freshly prepared n, "old" after 2 uses 2p, "old" after more than 5 uses, >5p). In the X axis, wave length in nanometers (nm). In the Y axis, optical units of absorbance (o.u.a.). **B.** Curve intervals where the obtained absorption curves fall from gold chloride solutions (1% freshly prepared, 1%n; 0.75% fresh, 0.75% n; 0.5% fresh, 0.5% n). Same axis as Fig. 1A. Note closeness of 1% 2p curve (Fig. 1A) and 0.75% n interval (Fig. 1B).

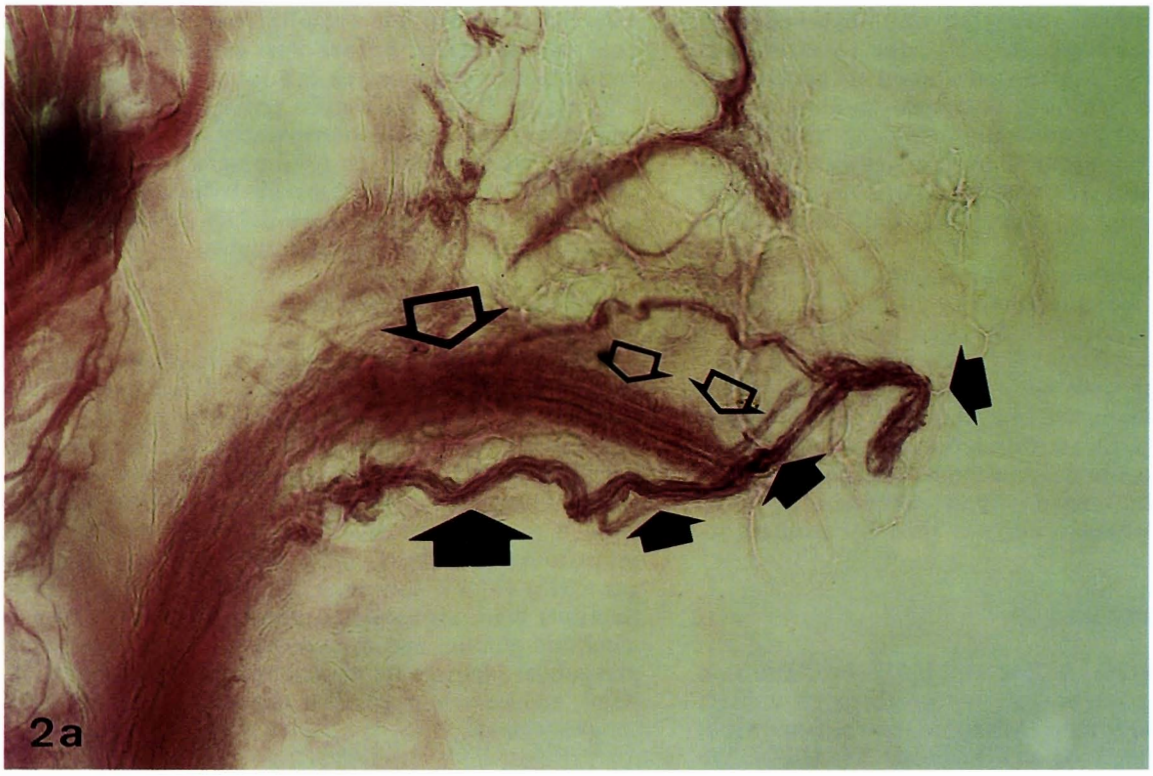
Experimental staining technique

We performed the previously standardized gold chloride technique to "in toto" stain 10 articular samples with the 1% freshly prepared gold chloride solution, 30 samples with the 0.75% solution, and 10 samples with the 0.5% solution. All the samples followed the same protocol, except for the gold chloride solution that was used. Cryosections of 80 μm were obtained with a microtome (Reichert, Austria) connected to a cold generator (MGW-Lauda, Leitz Kryomat, Germany). Sections were collected in phosphate-buffer, then in alcoholic gelatin, and then mounted on gelatin-coated glass slides, dehydrated in crescent alcohols followed by Xilol, and coverslipped. The same technique was used for every series.

Color analysis

We selected 11 fields in the 10 samples processed with the 0.5% gold chloride solution, where histological discrimination of vessels and nerves was definite (Fig. 2a). Also, 77 fields were selected in the 30 samples processed with the 0.75% solution (Fig. 2b), and 24 fields were selected in the 10 samples after the 1% preparation (Fig. 2c). All these fields were studied with the same magnification (x 100, with a x 10 lens) and light intensity, without any filter, in an Axioscop optical microscope (Zeiss, Germany).

All the fields were imaged with a Kodak DCS200ci digital camera (Eastman Kodak Co., USA), then acquired in TIFF format by Adobe Photoshop 3.0 software (Adobe Systems Inc., USA) in a Power Macintosh 7100/80 computer (Apple Computer Inc., USA). The images were defined by pixels, and the power of discrimination of each pixel was 2^{24}. The color of each pixel was expressed in the RGB channels (red-green-blue), whose value ranged from 0 to 255 (256 units). When a particular structure was selected (first automatically, by selecting the pixels with close RGB values, and then completed manually), the program made histograms, corresponding to the selected structure, for each color (red-gree-blue) in the range 0 to 255 in the X axis, being 0 the most intense and 255 the lighter the color (Figs. 3a,b). In the Y axis, the number of selected pixels with a particular value were expressed. The program also produce the mean value and the standard deviation for each channel. The gray channel expressed the pixel brightness, which was also included



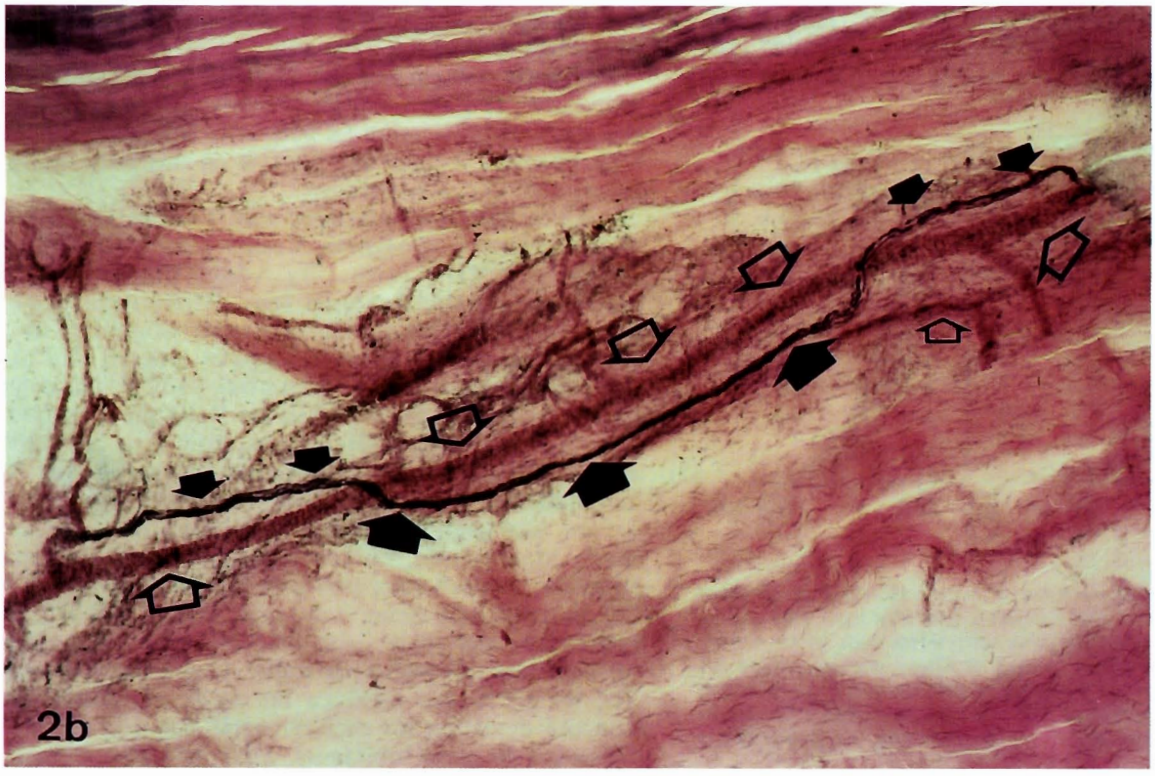


Fig. 2. a. Micrograph from the cat perimeniscal tissue after processing with 0.5% freshly prepared gold chloride solution. Note scarce discrimination between vessels and nerves. Vessels are signaled by empty arrows, and nerves by solid ones. x 100. **b.** Micrograph from an interfascicular space in the cat meniscofemoral ligament after processing with 0.75% freshly prepared gold chloride solution. Note distinct differentiation between vessels and nerves. Vessels are signaled by empty arrows, and nerves by solid ones. x 100. **c.** Micrograph from an interfascicular space in a human anterior cruciate ligament after processing with 1% freshly prepared gold chloride solution. Note darkness and scarce discrimination between vessels and nerves. Vessels are signaled by empty arrows, and nerves by solid ones. x 100

in the RGB channels. With this technique, RGB values were determined in each field for the neural (Fig. 3a) and vascular (Fig. 3b) structures, and also for the connective tissue that appeared in the background and served as an internal control of the color input.

Statistical analysis

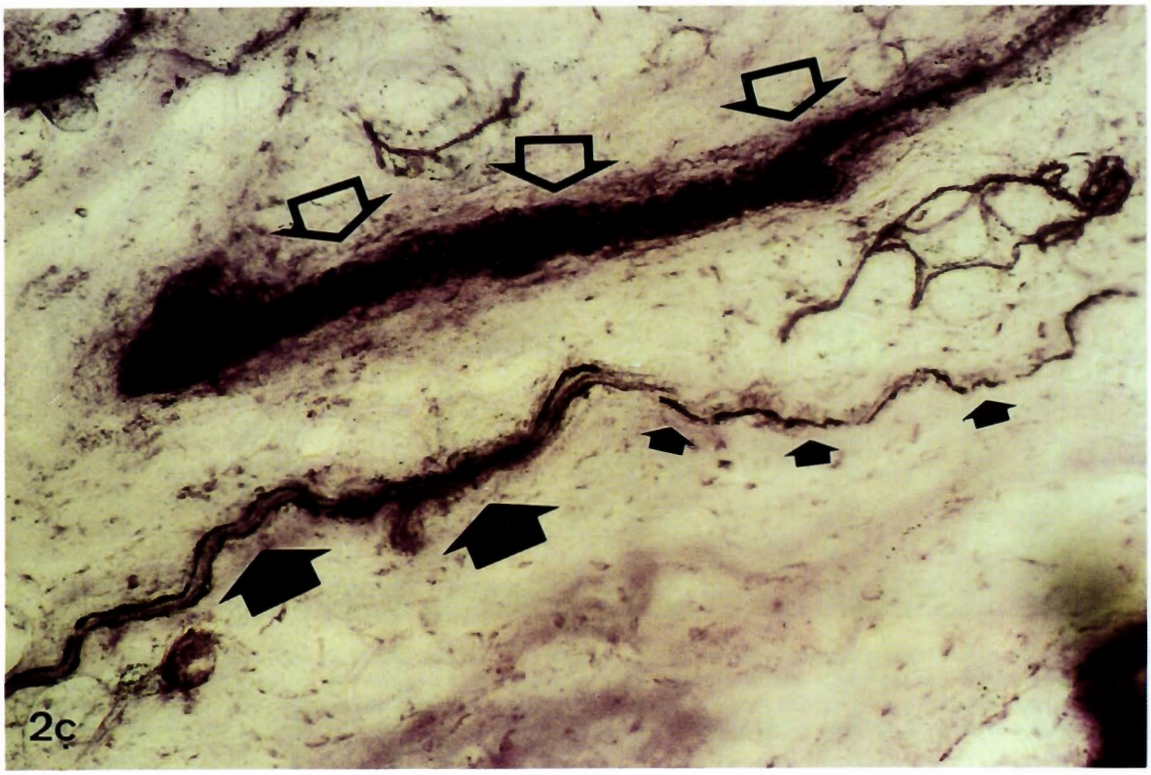
Univariate statistics were calculated to describe the stained samples, protocols, and output (color measurements of vascular and neural structures, as well as connective tissue as a reference). Continuous variables were summarized by the mean and standard deviation. Color measurements ranging from 0 to 255 were treated as continuous variables. To evaluate the impact of gold concentration on the color measurements from the stained samples, a variable was generated for each gold chloride solution to compare the samples stained under each protocol with the rest of the series. To assess the association of each protocol with every single color measurement in connective tissue, and vascular and neural tissues, crude analyses were performed using simple linear regression and Student's t test, for dichotomous, polytomous and continuous dependent variables. All P-values were two-sided. Statistical inference was also summarized using 95% confidence intervals.

To further assess the association of color measurements and gold concentration, due to interdependence among color variables, we performed multivariate regression analysis to jointly regress several dependent variables (our color measurements) on the same independent variables (gold concentration variables). To avoid any external factor, due to the sample, we included control color measurements obtained in every sample (connective tissue color measurements) as independent variables. A multivariate regression model was calculated with the above-mentioned variables. Single equations showing p(F) under 0.05 were considered as significant. Significant coefficients expressed significant differences in color measurements in a particular location between the protocols in the model and the one used as a reference. The coefficient amount and sign determined the amount and sign of the difference. Coefficients with p(t) under 0.05 were considered as significant when found in a significant equation.

Statistical analyses were performed using the Statistical package STATA, version 4.0 (Stata Co., USA).

Results

The standardized protocol, originated after empirical modifications of the previous techniques as outlined in the methods section, is proposed as the first, preliminary result of our study. A first step consisted of tissue preparation (3/4 volume of "laboratory lemon juice", plus 1/4 volume of 98% formic acid) until the tissue was transparent (about 15 to 20' in the 3mm diameter samples, such as small cat ligaments). Thick human



ligaments were observed not to become fully transparent but translucent, even after 60' preparation. The tissue was then withdrawn and rinsed. Secondly, gold impregnation was performed in a gold chloride trihydrate (Sigma Chemical Co., USA) solution, whose concentration varied from 0.5% to 1% (Figs. 2a-c). The control relied on the gold yellow color of the tissue, after a minimum time similar to that of the first step. After the sample was rinsed again, the reduction of metallic deposits was performed overnight in a 25% formic acid solution. Our main contribution relies on the substitution of the unpredictable lemon juice by a solution of 6gr of citric acid, 0.1gr of malic acid, 0.04gr of anhydrous $CaCl_2$, 0.03gr of $MgCl_2$, in 100ml deionized water, also adding 0.1gr of L-ascorbic acid immediately before use.

The required impregnation time in minutes for the 10 samples processed with the 1% gold chloride solution was 19.8±1.0 (range 15' to 20'). All the 30 samples processed with the 0.75% solution required 20 minutes of impregnation. The 10 samples processed with the 0.5% solution required a mean 26.4±10.0 minutes (range 15' to 40'). We obtained measurements in the 24 fields analyzed in the group of 1% stained samples, expressed as mean ± standard deviation (range) (Table 1, 1% column); measurements in the 77 fields selected from the samples prepared with the 0.75% solution (Table 1,

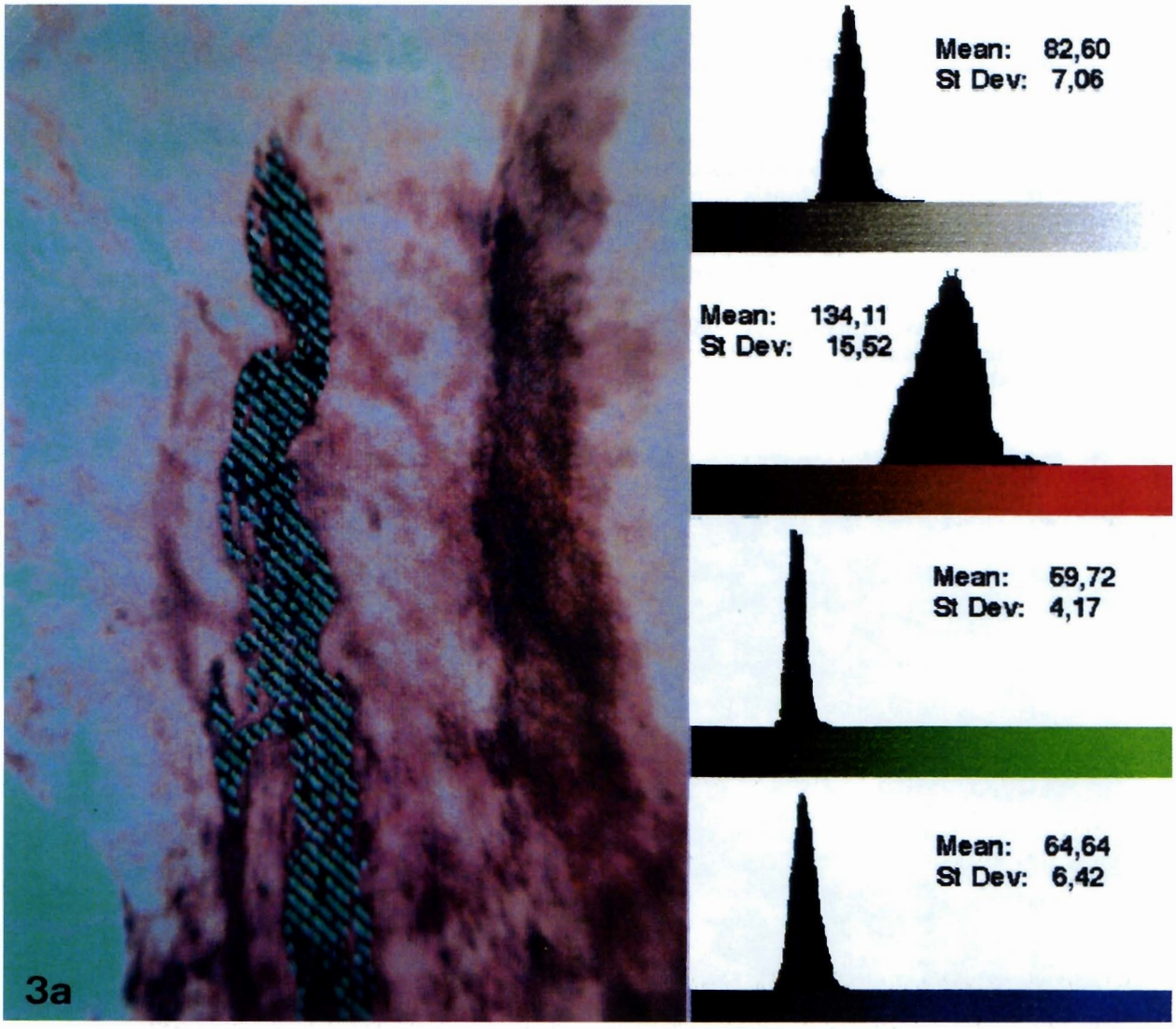


Fig. 3. a. Composed micrograph from an interfascicular space in the cat anterior cruciate ligament after processing with 0.75% freshly prepared gold chloride solution. After selecting the neural area (first automatically and then corrected manually), the software produces histograms, and mean and standard deviation for gray, red, green, and blue channels (RGB mode). x 100. **b.** Composition of the same micrograph in Fig. 3a. The vascular area is selected and new histograms, mean and standard deviation values are given for gray, red, green, and blue channels, to allow for color comparison with Fig. 3a. x 100

Table 1. Color measurements through the RGB (red-green-blue) mode in analyzed fields from the samples stained under the 1%, 0.75%, and 0.5% gold chloride solution protocols.

	1%	0.75%	0.5%
	Mean±Std dev (range)	Mean±Std dev (range)	Mean±Std dev (range)
RED (connective)	205.9±42.0 (127-254)	250±6.7 (240-255)	232.2±27.7 (191-255)
GREEN (connective)	156.9±35.6 (83-201)	127±23.7 (86-153)	149.7±24.4 (116-182)
BLUE (connective)	192.5±42.8 (99-251)	140.7±32.5 (109-202)	144.1±23.4 (104-166)
RED (vascular)	74.5±29.9 (30-133)	117.6±43.7 (39-231)	185±37.6 (126-247)
GREEN (vascular)	40.9±14.7 (17-63)	62.3±26.6 (7-147)	70.7±17.1 (43-93)
BLUE (vascular)	59.2±24.0 (19-99)	79.8±31.8 (17-179)	70.1±16.5 (43-90)
RED (neural)	87.9±43.6 (37-171)	117.5±39.8 (32-213)	178.6±45.5 (114-241)
GREEN (neural)	49.7±23.5 (21-95)	64.8±26.5 (16-138)	72.7±28.7 (37-123)
BLUE (neural)	64.4±30.3 (4-127)	79.0±32.2 (19-162)	83±33.3 (39-138)

Mean ± standard deviation (range) for each color, in a scale of 256 units (0-255).

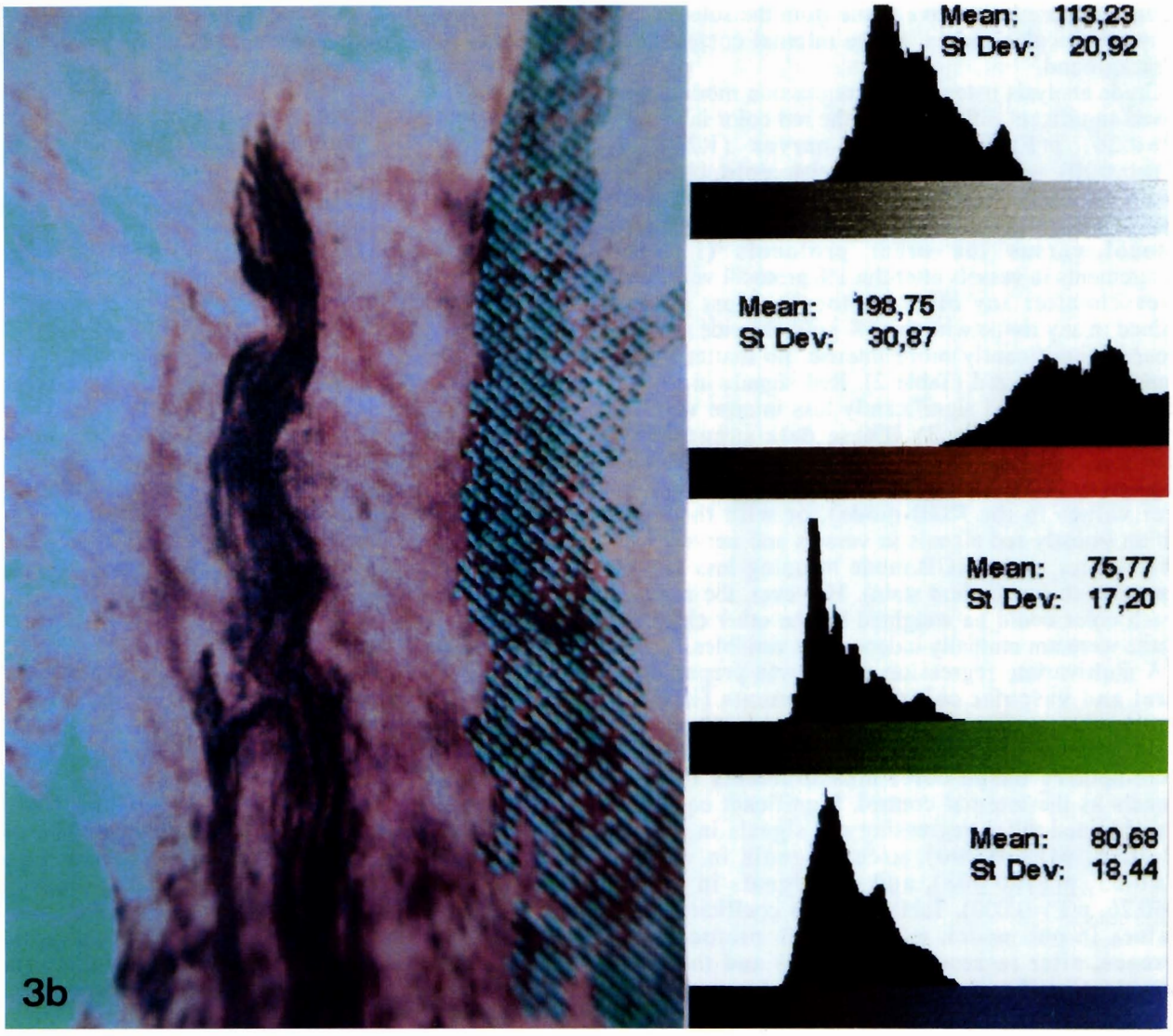


Table 2. Comparison of mean color measurement for each protocol with the rest.

	1%	Other	p	0.75%	Other	p	0.5%	Other	p
RED (connective)	205.9	239.8	0.001	250	213.5	0.034	232.2	216.1	0.311
GREEN (connective)	156.9	140	0.139	127	154.9	0.056	149.7	150.0	0.983
BLUE (connective)	192.5	142.6	0.000	140.7	178.7	0.054	144.1	180.6	0.039
RED (vascular)	74.5	126.7	0.000	117.6	119.5	0.866	185	109.6	0.000
GREEN (vascular)	40.9	63.4	0.001	62.3	53.1	0.111	70.7	58.3	0.129
BLUE (vascular)	59.2	78.5	0.018	79.8	63.6	0.017	70.1	75.9	0.545
RED (neural)	87.9	127.5	0.001	117.5	118.2	0.952	178.6	109.2	0.000
GREEN (neural)	49.7	66.1	0.017	64.8	57.4	0.231	72.7	60.55	0.182
BLUE (neural)	64.4	79.7	0.065	79.0	70.6	0.257	83	74.9	0.461

Means and p-values (p) after a Student's t-test comparing the mean color measurement obtained with each gold chloride protocol (1%, 0.75%, and 0.5%) versus the mean measurement obtained with the other protocols (Other, in each column).

0.75% column); and measurements in the 11 fields from the samples processed with the 0.5% gold chloride solution (Table 1, 0.5% column). Table 1 shows color measurements through RGB channels in nerves (obtained as in Fig. 3a), in vessels (obtained as in Fig. 3b), and in loose connective tissue from the subsynovial and interfascicular spaces, as the internal control from the background.

Crude analysis using simple regression models only showed significant differences in the red color in vessels (R2=0.26, p(F)=0.000) and nerves (R2=0.21, p(F)=0.000) when regressed with gold chloride concentration. Student's t-test was revealed as more sensitive when comparing color measurements after each protocol versus the other protocols (i.e. red measurements in vessels after the 1% protocol versus red in vessels after any other protocol). Most signals obtained in any tissue with the 1% gold chloride solution appeared significantly more intense, no matter which channel was analyzed (Table 2). Red signals in vessels and nerves appeared significantly less intense with the 0.5% solution (Table 2). These data suggest less discriminant power with the 1% solution (each color being too dark due to higher intensity, represented by lower values in the RGB mode), or with the 0.5% solution (mostly red signals in vessels and nerves, with higher values in the RGB mode meaning less intense colors, due to insufficient stain). However, the analysis of each color could be weighted by the other channels, as these were not mutually independent variables.

A multivariate regression model was prepared for neural and vascular color measurements (in RGB channels) that were jointly regressed on the independent variables generated for each gold chloride solution, and on connective tissue color measurements in RGB channels as the internal control. Significant equations were obtained when regressing red signals in vessels (R2=0.76, p(F)=0.000), green signals in vessels (R2=0.52, p(F)=0.008), and red signals in nerves (R2=0.76, p(F)=0.000). Table 3 shows coefficients and p-values in our model, using the 1% protocol as a reference, after regressing the 0.75% and the 0.5% protocols. Significant differences in red signals from vessels were found between samples processed with the 0.75% protocol and the reference (1% protocol), but not in red signals from nerves. The coefficient sign indicated a positive difference, meaning a higher number of color units, and thus a less intense color, after the 0.75% protocol. Note significant differences, not only in vessels but in nerves, between the 0.5% and the reference protocol. Every positive coefficient after 0.5% staining indicated less intense colors.

Table 3. Multiple regression obtained when regressing RGB color measurements for vessels and nerves with 0.75%, and 0.5% gold chloride solution protocols.

	0.75%		0.5%	
	Coef. ± std.dev.	p	Coef. ± std.dev.	p
RED (vascular)	88.1±26.7	0.004	104.0±21.1	0.000
GREEN (vascular)	15.8±12.2	0.210	32.7±9.6	0.003
BLUE (vascular)	12.0±15.9	0.458	27.9±12.6	0.039
RED (neural)	51.2±27.4	0.076	97.6±21.6	0.000
GREEN (neural)	0.9±21.3	0.976	24.5±16.9	0.162
BLUE (neural)	-8.4±25.0	0.740	29.2±19.8	0.154

Coefficients ± standard deviation (coef.±std.dev.) and p-values (p). Coefficients express the difference with the 1% protocol, used as a reference. Note significant differences in red signals from vessels between the 0.75% and the reference (1% protocol), but not from nerves. Note significant differences not only in vessels but in nerves between the 0.5% and the reference protocol.

Discussion

Gold chloride techniques are considered "old fashioned" by some histologists. However, today's interest in old gold chloride techniques is due to the renewed interest in articular innervation. Knee propioception studies in the last decade pursue answers to unsolved clinical problems on knee instability and its treatment. The increased incidence of sports injuries, frequently impairing knee stability, has prompted the development of surgical and rehabilitation techniques. A deeper knowledge on knee propioception will hopefully permit advances in the management of the unstable knee. Morphological studies about the density and

distribution of neural endings are needed, in the knee and other joints at risk. However, unless morphological data are solid enough, these will be of no help or may even be misleading.

Morphological studies on articular innervation require techniques to elicit neural fibres and endings within articular tissues. In the search for specificity, classical histological techniques are based on metallic impregnation. Silver and gold techniques focus on metallic ion selective fixation on neural proteins. Gold chloride techniques have been claimed to provide better quality stain to observe neural endings in thick articular tissues, if compared with silver stains (Skoglund, 1956). When the difficult gold chloride techniques are successfully performed, they permit a detailed study of well-defined neural endings (Ranvier, 1889; Ramón y Cajal, 1910; Skoglund, 1956). However, the main advantage is sensitivity, as "in toto" gold chloride staining may avoid section artifacts and neural material loss prior to processing. Also, "in toto" staining allows for thick sectioning, as sections need no further reactant penetration. Precise topographical studies require "in toto" histological techniques providing thick serial sections, in order to collect the whole specimen in the slides without significant material loss. Thick serial sections may provide data on nerve distribution within the section, and also allow for complete reconstruction of the processed samples. These reconstructions are determinant in clearly outlining the total neural ending and fibre distribution within a particular ligament.

Modern techniques to enhance neural specificity rely on electron microscopy and immunohistological protocols. Ultrastructural studies on neural endings within articular tissues provided detailed information about their structure, location and relationship with the host ligament (Halata et al., 1985; Halata and Haus, 1989; Haus et al., 1992). However, ultrastructural studies need the definite support of histology to assess neural element distribution. On the other hand, different authors developed immunohistological techniques in joints using protein-gene-product 9.5 (PGP 9.5), substance-P (SP), calcitonin gene-related peptide (CGRP), and other techniques (Grönblad et al., 1985, 1991; Konttinen et al., 1990; Mapp et al., 1990; Hanesch et al., 1991; Hukkanen et al., 1991; Marinozzi et al., 1991; Marshall et al., 1994; Aune et al., 1996). Although these techniques are reproducible, valuable, and widely recognized as "promising", no study has shown definite superior specificity when compared with classical gold chloride techniques. Besides, their sensitivity is also doubtful, as topographical information about big neural endings (length up to 200-600 μm for paciniform endings, as signaled in the dog by O'Connor, 1984; or to 500 μm for Golgi endings, as signaled in the cat by Skoglund, 1956, or Freeman and Wyke, 1967) is certainly scarce in non-serial thin sections (10-15 μm) obtained before immunohistological processing. As immunohistological techniques require thin sections (to allow for antiserum and antibody penetration), previous sectioning is mandatory. To totally collect a small ligament (such as a cat's knee ligament, 2-3mm in diameter), about 200-300 serial 10 μm-sections would be required, but for a human ligament (10-20mm in diameter), this would represent the unfeasible task of collecting 1000 to 2000 sections. Quantitative innervation estimates based on a few sections, in studies that discard most of them, disregard the variable topographical distribution of neural elements, and thus may be incorrect.

Criticism against gold chloride techniques as a way to study articular innervation mainly focus on difficulties in reproducing the technique, confusing images doubtfully considered as neural endings, and controversial quantification due to vascular artifacts (De Avila et al., 1989; Halata and Haus, 1989; Haus and Halata, 1990; Koch et al., 1995).

Difficulties in reproducing the technique rely on inconstant reactants (such as lemon juice or repeatedly-used gold chloride solutions). This has been addressed by other authors through overstaining (Zimny et al., 1985, 1986; Zimny, 1988), using the 1% gold chloride solution, and through commercially available reactants (O'Connor and McConnaughey, 1978; O'Connor and Gonzalez, 1979; O'Connor, 1984; De Avila et al. 1989), using Borden ReaLemon™. We chose to obtain constant reactants to perform a reproducible technique, and concluded that freshly prepared, controlled reactants produced more controlled results. However, tissue quality is still a variable difficult to control. Tissue storage may influence this quality, and fresh tissue is thus recommended, although we did not find differences with -20 °C frozen tissue, thawed immediately before staining.

Stained material not clearly defined as "neural" is a source of confusing results. These artifacts greatly increase with higher gold chloride concentrations, thus 1% solutions produce sections very difficult to interpret. Dark stains such as obtained with the 1% gold chloride solution, promote artifacts and uncertain quantification. Serial sections after "in toto" staining are often determinant. This is not the case when sections are discarded or stained separately, instead of "in toto". We also focused on the higher discrimination based on color, followed by careful study of not overstained structures. We could observe that lighter staining provided the best criteria to discriminate vessels and nerves, and then we focused on its reproducibility. We were able to develop a computer-assisted technique to obtain quantified color data to statistically define color differences. This method may support other uses, and proved to be efficient in color discrimination of vascular and neural structures. We then concluded that 0.75% gold chloride solutions provided statistically significant, observable color differences to support our protocol in small samples of articular tissues. Bigger samples associate problems related to differential penetration and thus impregnation. In these big samples, better, more homogenous staining was obtained with longer time and lower concentration, but these were uncontrolled results, if compared to those

obtained in small samples.

Clinical relevance of morphological studies on joint innervation relies on the correlation between the density and location of neural endings and the mechanical function of articular structures (ligaments, menisci). Histological techniques with high specificity are probably today our best tool to assess density and topography. To obtain the highest specificity, gold chloride techniques must be standardized, artifacts detected and validity of morphological data ascertained. Our work focused on the gold chloride technique standardization to increase its reproducibility, and on the assessment of its discrimination power, in the belief that histological techniques will remain the gold standard to further study the most important issues in the morphology of articular innervation: its density and topographical distribution.

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