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A method for quantitative histomorphometric evaluation of soft tissue reactions to implants

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The spatial distribution and concentration of distinct cellular elements, and the width of the reaction zone are of particular interest in the evaluation of the compatibility of implant materials. There is a dilemma in the use of interactive histomorphometric evaluation system, At low magnifications cell differentiation is difficult and at high magnification (×400) correct cell differentiation is possible but the evaluated area adjacent to the implant border is small and therefore not necessarily representative. The solution could be the evaluation of random samples at precise localization under high magnification. A light microscope with a software-controlled motorized stage is connected to a personal computer. The software allows definition of one or more polygonal areas at low magnification. A random generator determines the coordinates of the microscopic fields to be analysed and the motorized stage moves automatically to these coordinates. The number of microscopic fields which it is necessary to evaluate in each sample is calculated by the statistical methods described by Stein which take the heterogeneity of the histological structures into account. A software package ranks the various cells at selectable class intervals off the material–tissue interface (distance histogram). Data are stored in ASCII format, which allows importation into any evaluation software. The use of statistical methods seems to be justified for quantitative biocompatibility testing when the tissue encapsulating the implant is heterogeneous and larger than the suitable optical field of the microscope.

1. Introduction

To assess the potential usefulness of an implant material, the biological reaction to the material, the spatial distribution and concentration of distinct cellular elements, and the width of the reaction zone are of particular interest. Automated cell differentiation and counting is not feasable when discrimination has to be based exclusively upon morphological criteria. Since specific staining techniques, e.g. a monoclonal antibody staining method [1, 2] in combination with a fully automatic image analysis system, are only available for a limited spectrum of specimens, interactive assessment, controlled by the human eye, is still necessary.

Histomorphometric evaluation systems using a light microscope with drawing tube attachment and a digitizer tablet have made computer-assisted data acquisition possible [3]. Electrochemical implant dissolution guarantees optimal preservation of the implant–tissue interface [4], whereas sectioning with metallic implants in place may damage the implant–tissue interface. A combination of these two aspects was further developed by Christel [5].

These techniques are very useful as long as the samples allow the evaluation of tissue very close to the implant–tissue interface. This may be the case for small rodents, whereas in sheep and rabbits the thickness of a reaction zone may exceed the suitable field of view, and in human implant retrieval analysis a capsule thickness of 1–2 mm is found around a titanium implant after an implantation period of 18 months. A dilemma arises when evaluating a large tissue sample with a wide reaction zone using standard evaluation systems. At lower magnification, a larger area adjacent to the implant border is evaluated within a single microscopic field, but correct cell differentiation is endangered. At high magnification (400 ×) correct cell differentiation is possible but the evaluated area adjacent to the implant border is small and therefore not necessarily representative of the specific sample. The less homogeneous a zone is, the larger the number of fields which must be analysed. A reduction of the number of required fields is to be expected from separate evaluation of zones with clearly differing cellular composition. This type of histomorphometric analysis requires the use of statistical methods to determine the minimal number of microscopic fields to be evaluated. The solution envisaged was the evaluation at high magnification of a minimum number of randomly distributed microscopic fields with precise localisation within the evaluable area.

The literature on histomorphometric analyses of soft tissues does not describe statistical methods for calculating the appropriate number of microscopic fields to be evaluated per sample when taking the heterogeneity of the histological structures into ac-

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count. A formula published by Stein allows the calculation of the number of fields required to reach an error probability of $p < 0.05$ [6–8]. It was thus the goal of the present study to develop a technique for histomorphometry for a situation in which the tissue encapsulating the implant is heterogeneous and larger than the suitable optical field of the microscope.

2. Materials and methods

2.1. Evaluation system

A light microscope with a software-controlled motorized stage is connected to a personal computer. The software permits the definition of one or more polygons by entering the corner points. In the presence of obviously distinguishable tissue layers, the evaluation is started by entering the corner points of these zones at low magnification. For compatibility testing of metallic implants, an inner zone, the area of dense connective tissue with cells and fibers parallel and close to the implant, and an outer zone, the zone of loose connective tissue separated from the implant by the dense area, can be distinguished. Once the coordinates of the corners of these zones are entered, the software calculates the evaluable area of the sample in each zone. A random generator then determines the coordinates of an initial, arbitrary number of randomly distributed microscopic fields without overlap (Fig. 1). The edge effect is taken into account [9]. The software-controlled motorized stage moves automatically to the coordinates of the previously determined microscopic fields with a positioning precision of 0.1 μm. A square grid in the eyepiece facilitates orientation during counting and allows further subdivisions if required. The number of histological structures determined by eye control are counted interactively using the coded keys of the personal computer. The coordinates of each subdivision are integrated into the coordinate system of the whole evaluable area of the sample. Furthermore, the thickness of the two zones is measured, as well as the length of the implant–tissue border line.

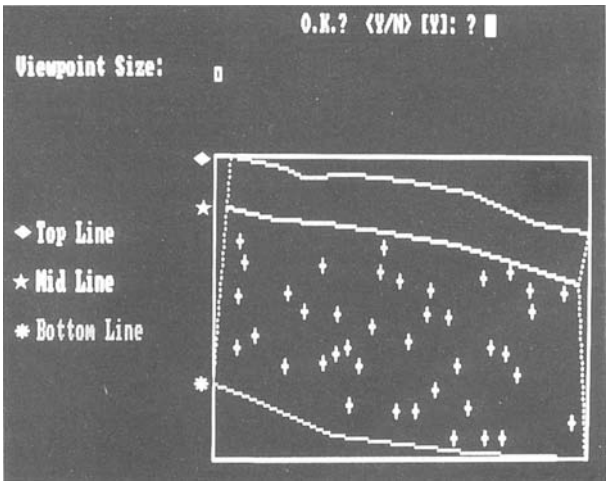

Figure 1 The computer screen is shown after registration of the corner points of the entire evaluable area. This area is interactively subdivided into zones containing obviously differing tissue elements. The small crosses indicate the localization of the centre points of randomly distributed microscopic fields in one of the two subdivisions. The relative size of the microscopic field of view is shown on the top left.

Counting was performed at a magnification of 400× (eyepiece 10×, lens 40×) in a microscopic field of 300 × 300 μm. The following cell types are differentiated according to their morphological structure and staining behaviour [10]: connective tissue cells (connective tissue cells; fibroblasts, fibrocytes), polynucleated cells, macrophages, round cells (lymphocytes, plasma cells), mast cells. Furthermore, the number of blood vessels is counted according to their inner diameter: small vessels (< 20 μm), medium vessels (20–40 μm), large vessels (> 40 μm). Cells which are circulating in blood (polynucleated cells, round cells) are only counted when localized in the tissue or in the vessel wall; they are not counted when situated intravasally. A software package ranks the various cells at selectable class intervals off the material–tissue interface (distance histogram). Data are represented in a three-dimensional graph to visualise the different cell types, their number per mm^2, and their distance to the implant (Fig. 3).

2.2. Histological procedures

To test the method, tissue samples were analysed which originated from five arbitrarily selected patients from an ongoing clinical study in which long bone fractures have been stabilized using an anodized commercially pure Titanium plate [11]. After an implantation period of about 18 months, depending on the site and type of fracture, the implant material was removed. During this procedure the soft tissue covering the plate was harvested. The tissue samples were clipped to a polystyrene block to clearly define contact zone and orientation, and to minimize deformation. Then the specimens were immersed in 4% formalin for fixation, dehydrated (alcohol, xylene), embedded (methylmethacrylate), sectioned at 7 μm, stained (Giemsa) and mounted on glass slides for evaluation in the light microscope.

2.3. Statistical procedures

The number of microscopic fields to be evaluated in each sample was calculated by statistical methods (Stein formula, Fig. 4). This takes the heterogenicity of histological structures into account in order to finally obtain an error probability of $p < 0.05$ for the values of a specific structure. This formula requires that one starts with an arbitrarily chosen number of fields. In all samples, we observed two zones in which the cell concentration and distribution was obviously different. The evaluation, using the Stein formula, was performed for the entire area including both zones, and for each zone separately (Fig. 5).

3. Results

Influence of heterogeneity on the number of fields: for our test samples the Stein formula recommended the evaluation of 400–600 microscopic fields when the

connective tissue cells were evaluated for the entire area, including the two zones with differing aspects. It was no problem to determine a borderline between the two zones, and on average the evaluation of 42 microscopic fields in the inner zone and 56 fields in the outer zone was sufficient to reach an error probability of $p < 0.05$ (Table I). On average it took about 4 h to evaluate the approximately 100 fields of one sample. For other cell types such as round cells, macrophages and mast cells the evaluation of about 1500 microscopic fields would be required to reach an error probability of $p < 0.05$. For polynucleated cells and blood vessels about 10000 microscopic fields would be necessary. These numbers can be used to estimate the heterogeneity of distribution of a specific cell type, but full evaluation of such a large number does not seem justified, for obvious reasons. Therefore, the number of microscopic fields as calculated for the connective tissue cells was also used for the evaluation of other cell types and for blood vessels. This implies that the error probability of $p < 0.05$ is only correct for the connective tissue cells.

TABLE I Number of microscopic fields calculated and evaluated in each sample. The number was calculated according to the Stein formula for an error probability of $p < 0.05$ for the mean values of histological structures. In zone two, the outer zone, a larger heterogeneity of the histological structures was observed, this is reflected in a higher number of microscopic fields for the same error probability

Sample	Microscopic fields per sample			
	Calculated		Evaluated	
	Zone 1	Zone 2	Zone 1	Zone 2
10851	33	68	33	68
10860	38	41	38	43
10920	47	70	47	70
11448	42	57	42	57
11470	51	40	51	42

The total evaluated area, the area actually evaluated as a percentage of the predefined area and the numbers of blood vessels and cells (+ / − SEM) for each sample are listed in Table II and Table III. the mean values (+ / − SEM) of the histological structures for all samples are summarized in Fig. 2. The distance histogram of blood vessels and cell densities is shown in Fig. 3. In the inner zone of the histological structures are arranged in 50 μm class intervals off the tissue–implant interface, for the outer zone they are arranged in 150 μm class intervals.

Inner zone: the cell density for connective tissue cells is significantly higher in this zone as compared to the outer zone. All other cell types have much lower numbers in the zone of dense connective tissue as compared to the loose zone. Blood vessels are rare and only small vessels could be detected. The thickness of the inner zone is slightly different in different patients and has a mean value of 400 μm + / − 30 (mean + / − SEM). Only in some samples foreign body giant cells could be seen at the implant–tissue interface.

Outer zone: the cell density for connective tissue cells is significantly lower as compared to the inner zone. Other cell types like mast cells, macrophages and round cells show a significantly higher value. This phenomenon runs parallel to the higher density of blood vessels. There were more blood vessels in this zone. The distribution of mast cells was homogeneous in the tissue, whereas macrophages could be found in higher numbers near the blood vessels. We found a small number of round cells distributed homogeneously, about 80% being concentrated in clusters

TABLE II The values for all tissue elements in the inner zone (1) and the standard error of the mean are shown for each sample. The evaluable area (mm^2) and the percentage of the area which was evaluated in each sample to obtain an error probability of $p < 0.05$ are listed. The percentage values show a wide range depending on the dimensions and the heterogeneity of the tissue. Abbreviations: small, medium and large blood vessels (SV, MV, LV), connective tissue cells (CTC), polynucleated cells (PC), mast cells (MC), macrophages (MP), round cells (RC)

Reference	Area	%	SV	MV	LV	CTC	PNC	MC	MP	RC
10851	17.2	19	3.3 ± 2.8	0.0 ± 0.0	0.0 ± 0.0	1088 ± 33	0.0 ± 0.0	6.1 ± 2.1	1.7 ± 0.9	77 ± 13
10860	20.3	18	21.6 ± 4.8	0.6 ± 0.4	0.0 ± 0.0	830 ± 134	0.0 ± 0.0	13.0 ± 3.1	5.9 ± 2.3	68 ± 16
10920	21.1	20	3.1 ± 1.1	0.0 ± 0.0	0.0 ± 0.0	765 ± 144	0.5 ± 0.5	1.4 ± 0.8	3.3 ± 1.6	27 ± 6
11448	17.0	22	1.9 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	965 ± 149	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	144 ± 32
11470	16.5	28	32.5 ± 5.4	0.0 ± 0.0	0.0 ± 0.0	891 ± 115	0.0 ± 0.0	14.2 ± 2.9	5.0 ± 1.8	51 ± 11

TABLE III The values for all tissue elements in the outer zone (2) and the standard error of the mean are shown for each sample. The evaluable area (mm^2) and the percentage of the area which was evaluated in each sample to obtain an error probability of $p < 0.05$ are listed. The percentage values show a wide range depending on the dimensions and the heterogeneity of the tissue. Small, medium and large blood vessels (SV, MV, LV), connective tissue cells (CTC), polynucleated cells (PC), mast cells (MC), macrophages (MP), round cells (RC)

Reference	Area	%	SV	MV	LV	CTC	PNC	MC	MP	RC
10851	59.4	6	26.1 ± 5.2	1.4 ± 0.7	0.0 ± 0.0	498 ± 16	0.0 ± 0.0	27.8 ± 3.2	7.2 ± 2.2	77 ± 30
10860	70.5	6	39.3 ± 4.9	2.6 ± 0.9	0.0 ± 0.0	573 ± 37	0.0 ± 0.0	20.2 ± 3.4	20.9 ± 3.8	216 ± 88
10920	38.9	16	26.5 ± 4.4	0.8 ± 0.3	0.2 ± 0.2	514 ± 67	0.6 ± 0.4	15.2 ± 2.9	15.7 ± 2.9	39 ± 8
11448	35.6	14	25.0 ± 5.1	3.3 ± 1.0	0.2 ± 0.2	546 ± 57	0.0 ± 0.0	18.3 ± 3.5	7.4 ± 2.1	302 ± 100
11470	25.4	15	50.0 ± 6.4	6.6 ± 1.4	0.0 ± 0.0	598 ± 32	0.0 ± 0.0	38.6 ± 4.5	20.1 ± 4.1	74 ± 13

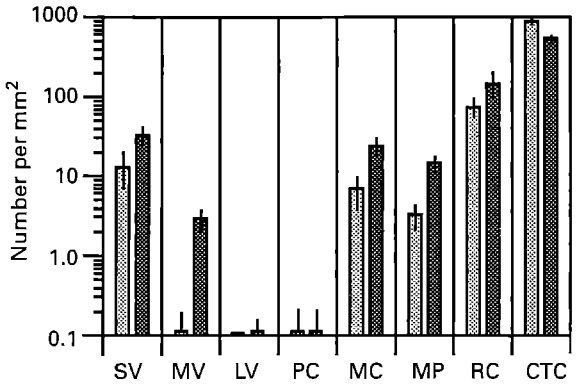

Figure 2 The data (mean + / − SEM) for all histological structures in both zones are shown in a logarithmic scale. The blood vessels and all cell types have higher values in the outer zone (2) except for the connective tissue cells. Abbreviations: small, medium and large blood vessels (SV, MV, LV), polynucleated cells (PC), mast cells (MC), macrophages (MP), round cells (RC), and connective tissue cells (CTC), (▨ zone 1; ■ zone 2).

around the blood vessels. A small number of plasma cells could also be seen within the round cell clusters.

4. Discussion

This technique allows an unbiased histomorphometric evaluation of the tissue surrounding implants. It represents a method of testing the biocompatibility of different materials. The microscopic fields, evaluated at high magnification, are randomly distributed in the tissue sample, which allows the evaluation of large areas distant to the implant–tissue interface. The size of the tissue sample is only limited by the limits of the motorized stage (50 × 70 mm).

Using a grid in the eyepiece of the light microscope, the localisation of the histological structures or degradation particles can be registered in more detail. For purposes of data presentation, we have chosen the number for each histological structure in the two distinguishable zones and a distance histogram at 50 µm classes in the inner zone and at 150 µm classes in the outer zone. The class intervals should be selected according to the heterogeneity of histological structures and the dimensions of the tissue sample. In the evaluation of soft tissue reactions to different implants, a large heterogeneity of histological structures is usually observed.

The question of the number of microscopic fields which have to be evaluated in each sample could not be answered before. By using the Stein formula (Fig. 4) the number of microscopic fields which we have to evaluate in order to obtain an error probability of $p < 0.05$ can be determined. A large heterogen-

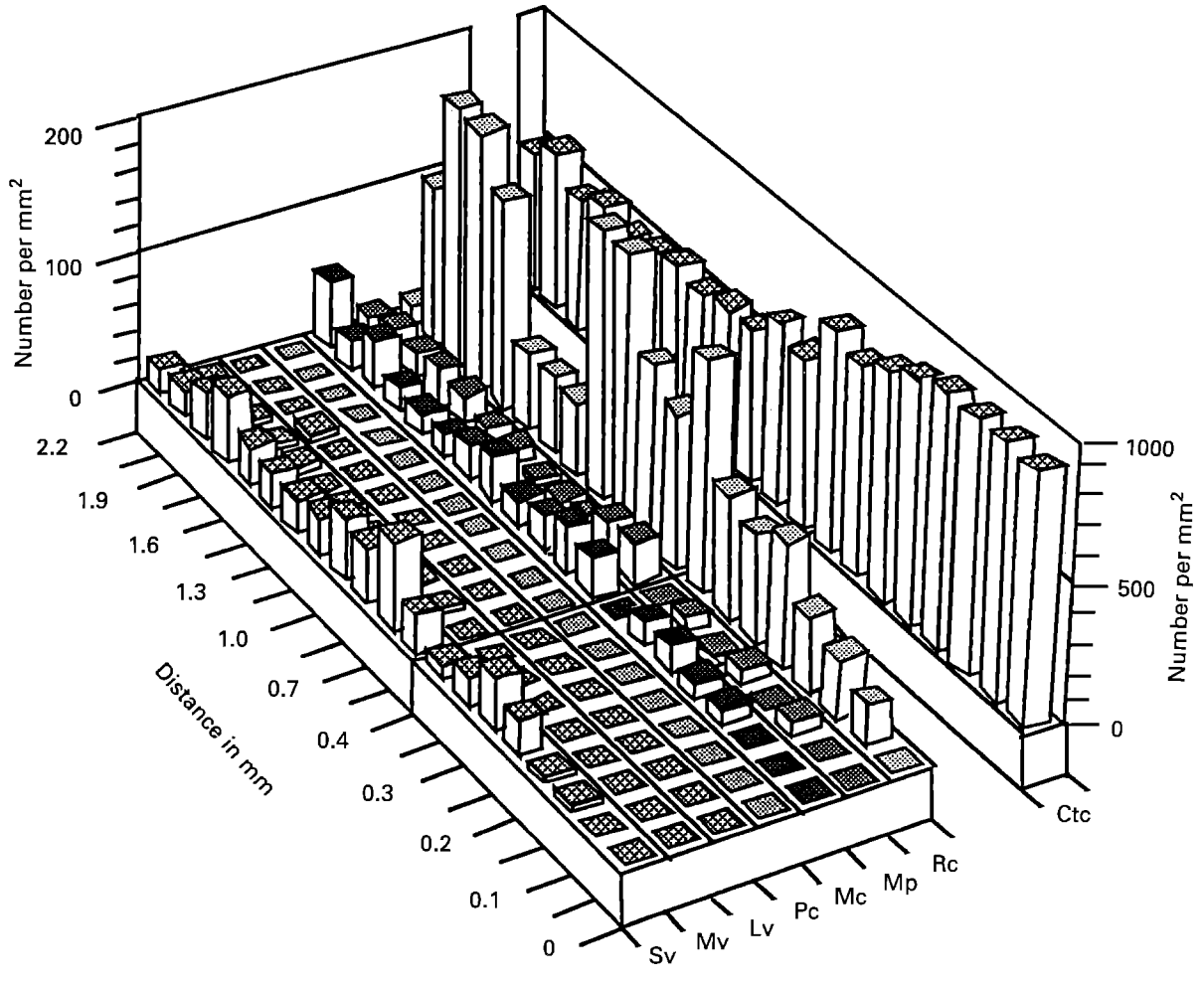

Figure 3 Distance histogram of the two zones. The histological structures in the inner zone (1) are ranged in 50 µm classes off the implant–tissue interface (elements per mm²). Zone one ranges from 0 to 0.4 mm. An increasing density for all histological structures is observed with increasing distance to the implant, except for connective tissue cells, where the density decreases slightly. The histological structures of the outer zone (2) are ranged in 150 µm classes off the implant–tissue interface. Zone two starts at a distance of 0.4 mm where zone one ends. The numbers for small, medium and large blood vessels (SV, MV, LV), polynucleated cells (PC), mast cells (MC), macrophages (MP) and round cells (RC) are much higher than in the inner zone (1), whereas the number of connective tissue cells (CTC) is lower.

Formula of Stein:

$$n = \max\left\{\left(\frac{s^2}{z}\right) + 1, \quad n_0 + 1\right\}$$

$$s^2 = \frac{1}{(n_0 - 1)} \sum_{i=1}^{n_0} (x_i - \bar{x}_{n_0})^2$$

$$z = \frac{l^2}{4t^2}$$

n: number of microscopic fields per zone and sample which were required to obtain an error probability (a) ≤ 0.05

n_0: number of microscopic fields in the initial set which served as a basis to determine n

s: variance of element density obtained from the initial set of fields

z: the density of the student distribution for $n_0 - 1$ degrees of freedom with a confidence interval of the level $1 - a(a = 0.05)$ and a length $l(1 = 5\%$ of $\bar{x})$

$\bar{x}$: the mean of element density in the initial set of fields used to calculate n

l: the length of the confidence interval l is freely choosable, e.g. $l = 5\%$ of $\bar{x}$

t: two-sided student-test value for $n_0 - 1$

Figure 4 The Stein formula used to calculate the number of microscopic fields required to evaluate each sample in order to reach an error probability of $p < 0.05$.

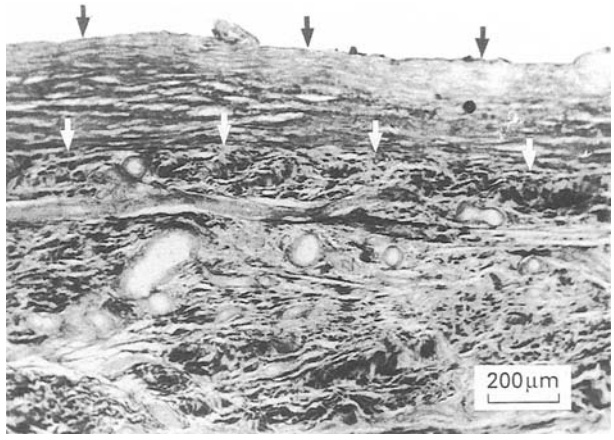

Figure 5 Representative histological section of the soft tissue covering the plate (Giemsa staining). The implant–tissue interface is indicated by black arrowheads. The tissue was subdivided in two zones: area of dense connective tissue with cells and fibres parallel to the implant and zone of loose connective tissue, separated from the implant by zone (1). The border of these two zones is indicated by white arrows.

eity in the tissue sample demands a large number of microscopic fields, whereas in homogeneous tissue a small number of microscopic fields is sufficient to obtain the same error probability. It is therefore useful to subdivide an inhomogeneous area into smaller, more homogeneous compartments in order to drastically reduce the number of microscopic fields without loss of precision. Calculation of the number of microscopic fields which have to be evaluated in order to minimise the error probability seems to be absolutely necessary. Since the materials used nowadays for implants are generally highly biocompatible, only small differences in the histological reaction can be expected. The assessment of small differences can only be improved by using more sensitive evaluation techniques.

The disector technique for counting histological structures [12] was not applied in this study, but there is no problem in combinating it with the technique described here. The eye controlled method described here, with a standard staining method (Giemsa), identification of the histological structures by eye according to their morphology and staining behaviour and computer-assisted recording and evaluation is widely used. The use of such a system is time-consuming and it is sometimes difficult to identify some cell types without special markers (macrophages without phagocysed material, distinction of fibroblasts and fibrocytes). Some cell types are not distinguishable at all without specific markers (T-lymphocytes and B-lymphocytes). It is clear that intra- and inter-observer errors may additionally influence the results, and in comparative investigations this factor has to be taken into account. It seems to be worthwhile, in terms of time saved and more precise identification of cell types, to try the monoclonal antibody staining method [1, 2] in combination with a fully automatic image analysing system. A comparison of the results of the statistically supported, eye-controlled evaluation system with those of a fully automatic system in the same samples is currently in progress.

5. Conclusions

Whenever no specific antibodies are available for the structure to be analysed, the visual control method is to be recommended. The evaluation system described above allows for an unbiased histomorphometric evaluation of the biological reaction to implant materials:

- The microscopic fields are randomly distributed in a sample which can reach large dimensions (limits of the motorized stage).
- Calculation of the number of microscopic fields which have to be evaluated in order to obtain a certain error probablity seems to be imperative.
- The results obtained using this technique are only accurate if applied to homogeneously distributed tissue elements (in this case connective tissue cells).
- The above-mentioned characteristics of this technique (formula of Stein, randomly distributed fields for evaluation, software controlled motorized stage) seem also to be advantageous in combination with other techniques, such as monoclonal antibody staining.

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