Calcif Tissue Int (1982) 34:439–448

Calcified Tissue International

Clinical Investigations

A New Semiautomatic Method for Quantitative Static and Dynamic Bone Histology

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Summary. A new semiautomatic technique combining advantages of the manual and fully automatic methods is described for obtaining quantitative static and dynamic histologic data of bone. The hardware consists of a photomicroscope, digitizing platen, digitizer, plotter/printer, floppy disc drive, and computer. The microscope is equipped with a drawing tube through which the image of the digitizing platen is projected over the optical field. The investigator selects and traces all histologic structures to be measured by moving a cursor on the digitizing platen which is visible by its projection over the histologic field. The results on accuracy and static and dynamic precision of this method show that static and dynamic parameters of bone are obtained with a degree of error (<20%) well within the acceptable range for biologic measurements. Comparison of this method with the grid technique according to Merz and Schenck showed that for almost all micromorphometric parameters comparable absolute data are obtained. Due to the higher precision of our method, however, the number of optical fields evaluated in obtaining these comparable data could be reduced to 25% of the number of fields evaluated by the Merz and Schenck technique. The time requirements for quantitative evaluation of a histologic slide of bone by our technique are 40–50 min; 20–25 min is needed for quantitative evaluation of osteocytes.

Key words: Bone — Histomorphometry — Osteocytes — Bone dynamics — Histology.

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In most metabolic bone diseases the response of bone to pathogenic stimuli is limited, and various histologic bone parameters are altered mainly quantitatively [1]. Therefore, quantitative evaluation of bone histology is required for assessment of metabolic bone diseases. Micromorphometry is particularly required in conditions with incipient and subtle changes. Also, whenever sequential evaluation of changes is needed or information on dynamic parameters of bone is desired, quantitative evaluation becomes essential.

Two major approaches have been used for quantitation of pathologic bone changes. Based on the principles of geometrical probability [2], which permits deduction of three-dimensional structures from their two-dimensional images, integrating grids are projected over the histologic structure. The fraction of points overlying a structure is considered to represent an unbiased estimate of the volume fraction, i.e., volumetric density of the particular structure; therefore, volume fractions are obtainable by the point counting procedure. Surface measurements are determined by counting random intersections between sampling lines and the histologic feature. The method of Merz and Schenk [3] utilizes these principles and is most widely employed for manual quantitative bone histology. Since this method represents a statistical approach, and in addition a certain intraskeletal microheterogeneity exists, a rather large number of microscopic fields has to be evaluated in order to obtain an acceptable precision [4, 5]. This obviously necessitates a rather large expenditure of time.

The second approach employs an image analyzing computer to obtain quantitative evaluation of histologic sections of bone [6]. The microscopic image is projected onto a black and white monitor using a video camera, and a large number of picture points is analyzed according to their "gray values." The use of the image analyzing computer drastically

0171-967X/82/0034-0439 $02.00

reduces the time requirements for quantitative evaluation of bone slides when compared to manual methods. However, at present analysis of cellular and dynamic parameters of bone is not feasible with the image analyzing systems. This is due to lack of ability of the computer to recognize individual cells and to discriminate sectioning and staining artifacts from histologic features to be measured. In addition, the purchase price of such automatic image analyzing systems may strain the budget of many laboratories.

This report describes a new semiautomatic technique that combines some of the advantages of both the manual and the fully automatic methods.

Method

The semiautomatic method utilizes a Zeiss photomicroscope III (Carl Zeiss, Inc., New York, New York) equipped with a drawing tube (camera lucida) through which the image of the digitizing platen is projected over the optical field, thereby preserving the optical resolution of the microscope. The digitizing platen is connected with a digitizer (HP 9864A, Hewlett-Packard, Loveland, Colorado), plotter (HP 9862A), printer (HP 9866A), floppy disc drive (Infotex FD-30A, Infotex Systems, Anaheim, California), and calculator (HP 9830A). (A more advanced version of the system is now routinely produced as a package under the name "Osteoplan according to Malluche" by Carl Zeiss, Inc., New York, New York.)

In our experience, this arrangement is superior to an outward projection of the histologic image possibly because it preserves the optical resolution of the microscope. The investigator selects and traces all histologic structures to be measured by moving a cursor on the platen which is visible by its projection over the histologic field under evaluation. The optical field is determined by a square placed on the tablet. The size of this square is adjustable by changing the position of the prism within the drawing tube. We used a square of 0.25 mm^2. As individual microscopic structures are measured, the values of perimeters and areas are transmitted to the digitizer, xy-plotter, and computer. The measuring routines consist of tracing and circuiting trabecular bone, osteoid, and endosteal fibrosis; counting osteoclasts and osteoblasts; and tracing surface fraction of osteoblasts, osteoclasts, and inactive Howship's lacunae. These routines are performed on 3 μm thick undecalcified bone sections stained according to Masson Goldner's technique [7]. The measurements can be performed at any chosen magnification. The presented histologic data were obtained at a magnification of 200× for static and dynamic parameters of bone and a magnification of 787× for quantitative parameters of osteocytes. Dynamic histomorphometric data and mean wall thickness were measured from 7 μm thick unstained sections, which were cut immediately consecutive to the corresponding stained sections.

The program package utilized for quantitative histologic parameters consists of three main programs:

1. Micromorphometric parameters of bone as evaluated under light microscopy (LM).
2. Dynamic parameters of bone remodeling as evaluated under fluorescent light and phase microscopy (FL).
3. Quantitative analysis of osteocytes (OCY).

A detailed description of the program package and its utilization is reported elsewhere [8]. In Table 1, all quantitative parameters of bone structure, bone formation, and bone resorption are listed. All parameters are printed after a preselected number of fields has been evaluated. In addition, standard deviation and standard error of the mean and ranges of the individual results are printed.

Using phase contrast microscopy, mean wall thickness is measured by tracing endosteal surfaces and cement lines of completed osteons (Fig. 1). After these measurements, the incandescent light source is blocked off and tetracycline double labels are traced under fluorescent light using the same cursor illuminated by a fine red light source in its center. Both measurement routines are done on 7 μm thick unstained sections cut directly after the section used for light microscopical evaluation. The data obtained in this manner are stored and combined with results from light microscopy for calculation of dynamic parameters of bone remodeling as shown in Table 2. The combination of phase contrast microscopy with fluorescent light microscopy allows evaluation of tetracycline uptake, status of mineralization, and mean wall thickness of individual osteons within the same optical field.

Quantitative parameters of osteocytes are obtained by tracing individual osteocytic lacunae. Separate evaluation is made for lacunae occupied by osteocytes and for void lacunae. The quantitative osteocytic parameters calculated from measurements are given in Table 3. Histograms of areas and elliptical ratios of osteocytic lacunae are plotted to separate osteocytic subpopulations. The program allows evaluation of a histologic field regardless of whether bone matrix fully occupies the histologic field under consideration. This permits reliable calculation of volumetric density and surface density of osteocytic lacunae.

During the evaluation of each histologic slide, it is at the discretion of the investigator to have all evaluated structural and cellular parameters of bone graphically reproduced by means of a plotter. This documentation is desirable for teaching purposes and whenever permanent records of the evaluated area are required.

If an unexpected event forces the investigator to stop evaluation of the slide before all the preselected fields have been examined completely, the data can be stored without calculation of statistics. As soon as the remaining data are supplied at any time thereafter, the entire computational process is completed. If any part of measured and stored data are needed at later times, a program overlay allows retrieval of the desired information from storage disks.

The accuracy and precision of the method were evaluated by determining the inter- and intraobserver errors of the various static, cellular, and dynamic parameters of bone histology. These evaluations were made on stained and unstained histological sections of undecalcified bone which were prepared as described previously [9]. The bone biopsies were obtained within 12 h after sudden or violent death of 11 normal American individuals who did not have evidence of metabolic bone disease. The bone samples were obtained using an identical surgical procedure as routinely performed in patients [10]. An electric drill was used providing samples of 0.5 cm diameter and 4 cm length [10].

Criteria used for histologic interpretation are identical to those published elsewhere [1, 11].

To determine the intraobserver error, the same slides were read twice by the same investigator and the interval between the two readings ranged between 1 week and 3 months. The mean difference and the coefficient of correlation between the two readings were calculated. The interobserver error was evaluated

Table 1. Quantitative parameters of bone structure, bone resorption, and bone formation obtained under light microscopy

Abbreviation	Parameter	Unit of measure
VV	Volumetric density of bone	
	(Volume trabecular bone/volume total bone)	mm^3/cm^3
SV	Surface density of bone	
	(Surface trabecular bone/volume total bone)	mm^2/cm^3
D-TRAB	Mean trabecular diameter	microns
V-VOS	Volumetric density of osteoid	
	(Volume of osteoid/volume total bone)	mm^3/cm^3
VVO	Relative volumetric density of osteoid	
	(Volume of osteoid/volume trabecular bone)	mm^3/cm^3
S-VOS	Surface density of osteoid	
	(Surface of osteoid/volume total bone)	mm^2/cm^3
SVO	Relative surface density of osteoid	
	(Surface of osteoid/volume trabecular bone)	mm^2/cm^3
S-VOSB	Surface density of osteoid-osteoblast interface	
	(Surface osteoblastic interfaces/volume total bone)	mm^2/cm^3
S-VOB	Relative surface density of osteoid-osteoblast interface	
	(Surface osteoid-osteoblast interface/volume trabecular bone)	mm^2/cm^3
OS %	Fraction of osteoid seams	
	(Surface osteoid/surface trabecular bone × 100%)	%
OB %	Fraction of osteoid-osteoblast interface	
	(Surface osteoblastic interface/surface trabecular bone × 100%)	%
OBR %	Relative fraction of osteoid-osteoblast interface	
	(Surface osteoid-osteoblast interface/osteoid surface × 100%)	%
S	Mean thickness of osteoid seams	μm
OBI	Osteoblastic index	
	(Number of osteoblasts/unit field area)	$\#/mm^2$
S-VOCL	Surface density of bone-osteoclast interface	
	(Surface full osteoclastic lacunae/volume total bone)	mm^2/cm^3
S-VHL	Surface density of Howship's lacunae	
	(Surface void osteoclastic lacunae/volume total bone)	mm^2/cm^3
OCL %	Fraction of filled osteoclastic lacunae	
	(Surface full osteoclastic lacunae/surface trabecular bone × 100%)	%
HL	Fraction of void osteoclastic lacunae	
	(Surface void osteoclastic lacunae/surface trabecular bone × 100%)	%
L-TOT	Fraction of total resorptive lacunae	
	(Surface full and void lacunae/surface trabecular bone × 100%)	%
OCL-R	Corrected fraction of filled osteoclastic lacunae	
	(Surface full osteoclastic lacunae/mineralized trabecular surface × 100%)	%
OCI	Osteoclastic index	
	(Number of osteoclasts/unit field area)	$\#/mm^2$
VV-FIB	Volumetric density of fibrosis	
	(Volume of fibrosis/volume total bone)	mm^3/cm^3
V-FIB	Relative volumetric density of marrow fibrosis	
	(Volume of fibrosis/volume nontrabecular bone)	mm^3/cm^3
SV-FIB	Surface density of fibrosis	
	(Surface of endosteal fibrosis/volume total bone)	mm^2/cm^3
FIBR	Fraction of endosteal fibrosis	
	(Surface of endosteal fibrosis/surface trabecular bone × 100%)	%

by having two or three different investigators independently evaluate the same slides. The overall mean value of the histomorphometric parameters of bone obtained by the three investigators was calculated, and the deviation of the individual values from the overall mean value was determined for each observer and for all observers together.

To establish the optimal number of microscopic fields to be measured, the changes in coefficients of variance with increasing number of evaluated optical fields were calculated [12].

Results

Static Parameters of Bone Structure, Formation, and Resorption

Data on intra- and interobserver error are given in Tables 4 and 5, respectively. There were only small

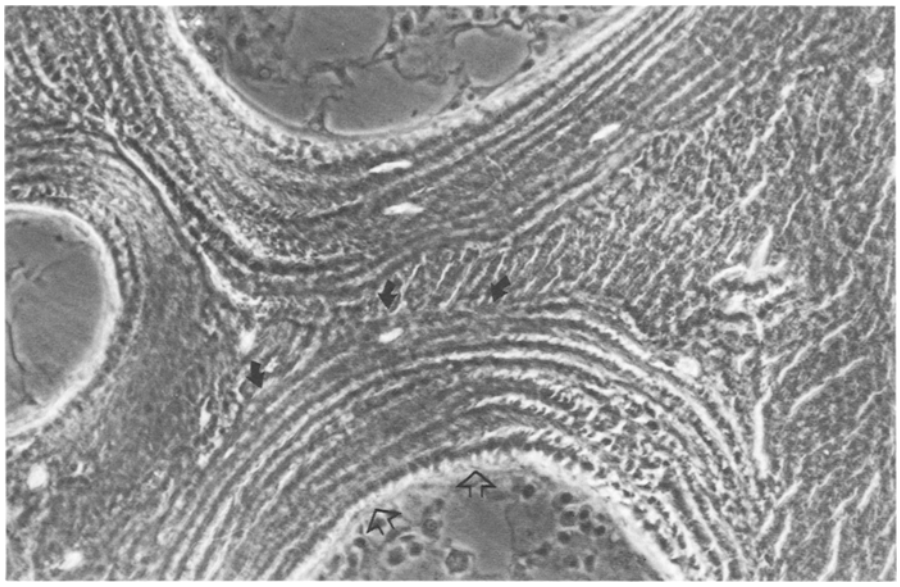

Fig. 1. Photomicrograph of an unstained, undecalcified section of bone. Thickness of section is 7 μm viewed under phase contrast microscopy. Magnification 140×. Cementing line indicated by solid arrows and trabecular surface indicated by open arrows

intraobserver errors (0.5–6.1%) in structural parameters of bone. Noncellular parameters of bone resorption showed higher degrees of variation (9.0–11.9%) than cellular resorptive parameters (6.3%). Cellular parameters of bone formation were obtained with a higher degree of variance than noncellular parameters of bone formation and with a similar variance as noncellular parameters of bone resorption.

There was remarkably little interobserver variation in structural parameters of bone even when obtained by three different observers at different times demonstrating the accuracy of the method. The mean deviation of structural parameters of bone ranged from 0.7% to 7.1%. Parameters of bone resorption were obtained with a mean interobserver error of 6.2% to 9.8%. The largest difference was found for inactive osteoclastic resorption and number of osteoclasts per unit volume bone. Quantitative parameters of bone formation were measured with a higher degree of interobserver difference than resorptive parameters, but even the largest mean difference did not exceed 13.4%. Upper limits of intraobserver errors were within the lower range of variations found between different observers.

Dynamic Parameters of Bone Remodeling

Intraobserver errors of two investigators were not different statistically and did not exceed 8.2 ± 2.9 and 8.2 ± 1.7 for mean distance between two tetracycline labels (MDU) and mean wall thickness of osteons (MWT), respectively. Mean differences between the two observers, i.e., interobserver errors, were 12.2 ± 2.4% for MDU and 6.4 ± 1.8% for MWT (Table 6).

Evaluation of 50 microscopic fields at a magnification of 200× was sufficient to obtain a coefficient of variance of 7% and 6% for MWT and MDU. Measurements of additional microscopic fields yielded relatively small further improvements of coefficients of variance (Table 7).

Micromorphometric Evaluation of Osteocytic Lacunae

Mean differences in measurements of area of osteocytic lacunae obtained by the same two investigators at different times were identical (4.8 ± 0.6%). Surfaces of osteocytic lacunae were obtained even with smaller degrees of intraobserver error (2.8 ± 0.4% for observer A and 2.6 ± 0.3% for observer B). Interobserver variations were 10.8 ± 1.3% for area of osteocytic lacunae and 5.00 ± 0.6% for surfaces of osteocytic lacunae (Table 6).

In order to obtain micromorphometric parameters of osteocytes with coefficients of variation of less than 20%, 100 fields of 174 individual osteocytic lacunae (787×) must be measured. When mean area of osteocytic lacunae alone is considered, measurements of 100 lacunae are adequate to obtain data with a coefficient of variance of less than 10% (Table 7).

Comparison with Graticule Method

The manual method according to Merz and Schenk is the most widely employed graticule technique for

Table 2. Quantitative dynamic parameters of bone remodeling obtained under phase contrast and fluorescent microscopy

Abbreviation	Parameter	Unit of measure
MD-U	Mean distance between double labels	μm
AR/Y	Appositional rate (Mean distance/labeling interval)	mm/year
AR/D	Appositional rate (Mean distance/labeling interval)	μm/day
LAB-OS	Fraction of labeled osteoid seams (Mean label length/mean trabeculum length)	
LAB-TS	Fraction of labeled trabecular surface (Mean label length/mean trabeculum length)	
BFR T-S	Bone formation rate, tissue level—surface referent (LAB-TS * AR/Y)	$mm^3/mm^2 \times$ year
BFR BMU-S	Bone formation rate, BMU level—surface referent (LAB-TS * AR/Y / OS)	$mm^3/mm^2 \times$ year
BFR C-S	Bone formation rate, cell level—surface referent (AR/Y)	$mm^3/mm^2 \times$ year
BFR T-V	Bone formation rate, tissue level—volume referent (Mean trab length * BFR TS)/mean trabecular area	$mm^3/mm^3 \times$ year
BRR T-S	Bone resorption rate tissue level—surface referent (-BFR TS)	$mm^3/mm^2 \times$ year
BRR BMU	Bone resorption rate BMU level—surface referent (BRR TS/L-TOT)	$mm^3/mm^2 \times$ year
BRR T-V	Bone resorption rate tissue level—volume referent (-BFR TV)	$mm^3/mm^3 \times$ year
LRR	Linear rate of resorption (BRR BMU/L-TOT)	mm/year
MWT	Mean wall thickness	μm
SIGMA F	Osteon formation period (MWT/(AR/Y)	years
SIGMA R	Osteon resorption period (L-TOT * SIGMA F)/OS%	years
SIGMA	Osteon remodeling period (SIGMA F + SIGMA R)	years
BFR/OBLAST	Bone formation rate per osteoblast (BFR CS * length double labels)/#OBL	mm/cell $\times$ year
BRR/ARS	Bone resorption rate per active unit resorption surface (BRR TS/OCL %)	
BRR/OCLAST	Bone resorption rate per osteoclast (BRR/ARS * OCL% $\times$ mean trab length)#OCL	mm^2/cell $\times$ year
MIN LAG T	Mineralization lag time (Osteoid seam width/(AR/D))	days

Table 3. Quantitative parameters of osteocytic lacunae obtained under light microscopy

Abbreviation	Parameter	Unit of measure
A O_{cy} F	Mean area of osteocytic lacunae filled with cells	μ2
ER O_{cy} F	Mean elliptical ratio of osteocytic lacunae filled with cells (short axis diameter/long axis diameter of filled osteocytic lacunae	
SS O_{cy} F	Mean specific surface of osteocytic lacunae filled with cells (Surface of filled osteocytic lacunae/area of lacunae)	1/μ
OI O_{cy} F	Index of osteocytic lacunae filled with cells (Number of osteocytic lacunae filled with cells/unit area trabecular bone)	#/mm^2
VV O_{cy} F	Volumetric density of osteocytic lacunae filled with cells (Volume of osteocytic lacunae filled with cells/volume trabecular bone)	mm^3/cm^3
SV O_{cy} F	Surface density of osteocytic lacunae filled with cells (Surface of filled osteocytic lacunae/volume trabecular bone)	mm^2/cm^3
A O_{cy} V	Mean area of void osteocytic lacunae	μ^2
ER O_{cy} V	Mean elliptical ratio of void osteocytic lacunae (Short axis diameter/long axis diameter of void osteocytic lacunae)	
SS O_{cy} C	Mean specific surface of void osteocytic lacunae (Surface of void osteocytic lacunae/area of lacunae)	1/μ
OI O_{cy} V	Number of void osteocytic lacunae/unit area trabecular bone	#/mm^2
VV O_{cy} V	Volumetric density of void osteocytic lacunae (Volume of void osteocytic lacunae/volume trabecular bone)	mm^3/cm^3
SV O_{cy} V	Surface density of void osteocytic lacunae (Surface of void osteocytic lacunae/trabecular bone volume)	mm^2/cm^3

obtaining quantitative bone data. In order to evaluate the differences in the results between the semiautomatic technique and the graticule method, the same observer read the same 11 slides using both methods. Results and differences of micromorphometric parameters are given in Table 8. When 200 optical fields were evaluated manually and 50 fields semiautomatically at the same magnification, the only significant differences were fraction of osteoid-osteoblast interface, surface density of bone, and mean trabecular diameter.

Changes in coefficients of variance obtained with increasing number of evaluated fields by the graticule technique according to Merz and Schenk and our technique are given in Table 9. Structural parameters of bone are obtained with the same variance at 50, 70, and 100 optical fields using both methods.

However, cellular parameters of bone resorption and formation are measured with a consistently lower coefficient of variation using our technique. Parameters of osteoclasts which have the lowest degree of accuracy when measured by the Merz and Schenk technique are obtained by our method with

Table 4. Intraobserver error of static micromorphometric parameters of bone structure, formation, and resorption obtained by the semiautomatic technique

	Mean value	Mean difference	% Difference
VV	228.3	1.2 ± 3.1	0.5
SV	2984.2	56.7 ± 48.3	1.9
V VOS	4.4	0.3 ± 0.2	6.8
S VOS	370.9	31.9 ± 16.9	8.6
$\bar{S}$	11.5	0.7 ± 0.8	6.1
S VOB	141.5	17.1 ± 15.8	12.1
OI	0.4	0.04 ± 0.01	10.0
S VOCL	25.0	0.7 ± 6.0	2.8
S VHL	236.4	28.1 ± 23.5	11.9
D TRAB	283.9	8.8 ± 4.6	3.1
OCL %	1.6	0.1 ± 0.1	6.3
HL %	11.4	0.3 ± 0.7	2.6
OB %	3.8	0.3 ± 0.6	7.8
OS %	7.5	0.3 ± 0.9	4.0

[a] Mean difference ± SE of 2 readings of 11 slides by the same investigator. Values are given as absolute numbers
[b] Coefficient of correlation between 2 readings of 11 slides by the same investigator

Table 6. Intra- and interobserver error of measurements under fluorescent and phase contrast light used for calculations of dynamic parameters of bone formation and resorption and intra- and interobserver error of measurements of area and interobserver error of measurements of area and circumference of osteocytic lacunae under bright field light microscopy

	Intraobserver error (%)		Interobserver error (%)
	A[a]	B[b]	
MWT	8.2 ± 1.7	6.9 ± 0.9	6.4 ± 1.8
MD_u	4.7 ± 1.4	8.2 ± 2.9	12.2 ± 2.4
Area of osteocytic lacunae	4.8 ± 0.6	4.8 ± 0.6	10.8 ± 1.3
Surface of osteocytic lacunae	2.8 ± 0.4	2.6 ± 0.3	5.0 ± 0.6

[a] A = observer 1
[b] B = observer 2

Table 5. Interobserver error of static micromorhometric parameters of bone structure, formation, and resorption obtaineded by the semiautomatic technique

	Overall mean value	Deviation from overall mean value (%) A[a]	B[b]	C[c]	Mean deviation (%)
VV	237.1	3.7	2.8	6.9	4.5
SV	3048.5	3.6	0.2	3.4	2.4
V VOS	3.5	8.2	8.2	0.0	5.6
S VOS	290.8	0.9	0.1	1.0	0.7
$\bar{S}$	10.0	8.6	11.6	10.0	7.1
SV OB	59.6	7.5	14.0	18.6	13.4
OI	0.2	5.7	11.8	11.8	9.8
S VOCL	24.2	3.7	11.4	6.4	7.2
S VHL	263.1	9.9	4.5	6.1	6.8
D TRAB	309.4	7.2	2.6	10.6	6.8
OCL%	0.8	8.4	7.8	2.5	6.2
HL%	8.7	13.9	5.9	8.4	9.4
OB%	2.1	5.0	15.4	17.4	11.6
OS%	9.6	3.1	1.0	3.2	2.4

[a] A = observer 1
[b] B = observer 2
[c] C = observer 3

Table 7. Relation between increasing number of optical fields and coefficient of variation of measurements of dynamic parameters of bone and quantitative parameters of osteocytes

Fields (*N*)	10	20	50	70	100
MD-U and MWT[a]	12	17	32	49	57
Osteocytic lacunae[b]	11	26	95	123	174
CV_{MD}[c]	0.13	0.10	0.07	0.06	0.05
CV_{MWT}[d]	0.11	0.09	0.06	0.05	0.05
$CV_{A_{ocy}}$	0.25	0.17	0.10	0.08	0.07
ER_{ocy}	0.22	0.15	0.10	0.08	0.06
SS_{ocy}	0.49	0.36	0.30	0.25	0.20
OI_{ocy}	0.57	0.39	0.24	0.22	0.16
VV_{ocy}	0.50	0.40	0.26	0.24	0.18
SV_{ocy}	0.53	0.38	0.24	0.22	0.16

Correlation coefficients expressed as fractions
[a] Number of evaluated double-labeled appositional sites (MD) and osteon walls (MWT)
[b] Number of evaluated osteocytic lacunae
[c] Coefficient of variation of tetracycline double labels as evaluated under fluorescent light (200×, field size 0.25 mm²)
[d] Coefficient of variation of mean wall thickness of osteons evaluated under phase contrast light (200×, field size 0.25 mm²)
[e] Coefficient of variation of quantitative parameters of osteocytes (787×, field size 0.016 mm²)
Abbreviations used: See legend to Tables 2 and 3

Table 8. Comparison of histomorphometric parameters of bone structure, resorption, and formation obtained by the graticule method according to Merz and Schenk and the semiautomatic technique

	Merz and Schenk	Semi-automatic	Difference[a] (abs.)	Difference[a] (%)
VV	246.8	246.1	0.7	0.3
SV	3,698.6	2,939.6	759.0[b]	22.9[b]
V VOS	3.4	3.8	0.4	11.1
S VOS	299.8	288.3	11.5	3.9
$\bar{S}$	13.0	11.0	2.0	16.7
S VOB	34.7	55.3	20.6	45.8
S VOCL	22.5	24.2	1.7	7.3
S VHL	371.7	290.4	81.3	24.6
D TRAB	268.1	332.2	64.1[b]	21.4[b]
OCL%	0.7	0.9	0.2	25.0
HL%	11.0	10.0	1.0	9.5
OB%	1.0	1.9	0.9[b]	62.1[b]
OS%	8.5	9.9	1.4	15.2

[a] Difference given in absolute numbers and in percent
[b] Level of significance $P < 0.05$
Osteoclastic index was not included in the comparison since this parameter is calculated by the 2 methods using a different reference unit. Abbreviations used for histomorphometric parameters: see Table 1

coefficients of variance of less than 50% of those obtained by the method of Merz and Schenk (CV 11.1 vs 25.4 for OCL at 50 fields and 9.0 vs 17.5 at 100 fields; Table 9).

Time Requirements

The time required to read a slide using the described semiautomatic method depends on the experience of the investigator and the degree of pathologic changes of the bone specimen. The average quantitative evaluation of 50 optical fields at a magnification of 200× could be completed in 40 to 50 min, whereas in our hands evaluation of 250 optical fields at the same magnification of 200× using the Merz and Schenk technique required 4–5 hs. Using our method it took 20-25 min to complete evaluation of 100 fields (787×) containing 150–200 osteocytes.

Discussion

There are several advantages of the semiautomatic method described in this report. It provides quantitative histologic data of bone with an acceptable degree of precision, biasedness, and thus accuracy. The time required for arriving at such data is markedly reduced. The utilization of a computer for the calculation of all micromorphometric parameters provides additional substantial time savings since raw data storage and conversion is done concurrently with measurement routines and permits immediate retrieval of the final micromorphometric results after completion of data input. The use of floppy disks for storage of data and derived statistics allows easy comparison of newly obtained results with appropriate control data and assessment of variations of quantitative bone data with therapy, time, or any other variable [4, 8]. The integration of a plotter in this system permits recording of permanent graphs of the histologic fields evaluated. This documentation proved to be advantageous for record keeping, comparison, and teaching purposes. The discriminating input of an investigator allows recognition of sectioning and staining artifacts. This enhances accuracy of measurements obtained with the semiautomatic techniques as compared to fully computerized methods.

Two factors influence accuracy of any method utilized for obtaining biologic measurements [13]: unbiasedness and precision. Unbiasedness refers to the tendency to arrive at the true or correct value. Precision refers to the degree of spread of a series of observations. Thus measurements may be unbiased but imprecise or precise but biased. The term *accuracy* encompasses both biasedness and precision. Accurate measurements are both unbiased and precise. We evaluated precision and biasedness, thus accuracy, of our method by analyzing intra- and interobserver error. The results on intraobserver error of our method show that there is a surprisingly small inherent error.

Table 9. Coefficients of variance for static parameters of bone structure, formation, and resorption obtained from evaluation of 50, 75, and 100 optical fields (magnification 200×)

	50		70		100	
Fields	A	B	A	B	A	B
VV	1.9 ± 0.4	1.5 ± 0.3	1.5 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.5 ± 0.3
SV	3.2 ± 0.9	1.7 ± 0.5	2.5 ± 0.5	1.3 ± 0.3[a]	2.0 ± 0.3	1.2 ± 0.2[a]
V VOS	5.1 ± 1.6	5.6 ± 0.8	3.7 ± 0.9	4.7 ± 0.9	3.7 ± 0.5	4.7 ± 1.1
S VOS	6.6 ± 1.1	7.1 ± 1.1	5.3 ± 0.7	6.0 ± 0.9	4.7 ± 0.7	6.2 ± 1.1
$\bar{S}$	4.6 ± 1.3	6.0 ± 1.1	3.6 ± 2.2	5.4 ± 1.1	3.3 ± 0.6	5.2 ± 1.3
S VOB	19.4 ± 6.5	12.0 ± 1.4[a]	15.4 ± 1.9	10.2 ± 0.8[a]	18.2 ± 2.0	8.9 ± 1.5[c]
OI	18.7 ± 3.3	10.1 ± 0.9[a]	20.2 ± 3.7	9.6 ± 0.8[b]	16.0 ± 2.5	8.4 ± 0.8[b]
S VOCL	25.5 ± 2.4	10.9 ± 0.8[d]	22.5 ± 3.0	9.9 ± 1.0[e]	17.6 ± 2.4	8.9 ± 0.8[c]
S VHL	6.4 ± 1.2	5.1 ± 1.2	5.6 ± 1.2	4.7 ± 0.6	4.0 ± 0.8	4.3 ± 0.7
D TRAB	1.7 ± 0.5	2.1 ± 0.4	1.6 ± 0.3	1.8 ± 0.2	1.3 ± 0.2	1.8 ± 0.3
OCL %	25.4 ± 2.6	11.1 ± 0.8[d]	23.2 ± 3.5	9.9 ± 1.0[c]	17.5 ± 2.2	9.0 ± 0.8[c]
HL %	5.5 ± 1.1	5.4 ± 1.2	4.5 ± 1.0	4.9 ± 0.6	3.6 ± 0.6	4.2 ± 0.6
OB %	20.3 ± 2.9	12.6 ± 1.5[a]	9.6 ± 1.9	10.5 ± 0.7[d]	18.0 ± 1.8	9.8 ± 1.1[c]
OS %	6.0 ± 1.0	7.0 ± 1.0	5.6 ± 2.0	6.6 ± 0.9	4.3 ± 1.8	6.3 ± 1.0

Values are given as % ± SE. A = Graticule technique according to Merz and Schenk; B = Semiautomatic technique according to Malluche et al.

[a] $P < 0.05$

[b] $P < 0.02$

[c] $P < 0.005$

[d] $P < 0.001$

Abbreviations used for histomorphometric parameters: see Table 1

Interobserver error comprises the sum of variances introduced by individual observer bias, methodologic error, and interindividual observer differences. Our results on interobserver error show that micromorphometric parameters of bone are obtained with an acceptable degree of variation. Cellular parameters of osteoblasts are subjected to the widest range of variance. This might be a consequence of the known difficulties in defining distinct classification criteria for "active" osteoblasts, "inactive" osteoblasts, and "endosteal lining cells." The differences between intra- and interobserver error of structural parameters of bone and cellular parameters of osteoclasts were small. This demonstrates that even in the hands of different investigators histologic features of bone are measured with similar absolute results. Hence it is possible to compare and/or pool with a high degree of confidence data obtained using our method in different laboratories.

Detailed dynamic parameters of bone formation and resorption could hitherto not be obtained in our laboratory employing manual or statistical approaches because of unacceptable time demands on personnel (>5 h/slide) and a low degree of accuracy of the results (coefficient of variance >20%). The described technique allows quantitative parameters of bone formation and resorption to be obtained with a coefficient of variance of 6–7% from evaluation of not more than 50 optical fields at a magnification of 200×. The small variance of results obtained from evaluated labeled appositional sites (Table 7) points to a relatively constant rate at which bone is laid down or mineralized. This method also allows the evaluation of quantitative dynamic parameters of bone formation and resorption at the cellular level, basic muticellular unit (BMU) level, and tissue level [14]. This is of importance for interpretation of static quantitative bone parameters. For example, total number of osteoclasts might be increased at the tissue level because of decreased activity at the cellular level. This information is relevant for understanding of pathogenetic processes and therapeutic decisions.

In addition to the gathering of static micromorphometric data and dynamic information on bone remodeling at different levels, the described method provides a reliable tool for quantitative evaluation of osteocytes which was previously done for the most part by means of mathematical approximations [15–17]. Our system allows direct measurement of osteocytic lacunae with a coefficient of variance of 7–10%. The changes in coefficients of variance with increasing number of evaluated optical fields call for measurements of at least 170 osteocytic lacunae or 100 optical fields at a magnification of 787× in order to obtain an acceptable coefficient of variance of less than 20% [13] for all quantitative osteocytic parameters. The introduction of "elliptical ratio" of osteocytes represents an objec-

tive approach to distinguish "small inactive osteocytes" [18], which are situated in flattened ellipsoid lacunae, from enlarged "active osteocytes" seen in oval lacunae. The criterion of irregularity of lacunar walls, which in our hands proves to be a rather subjective parameter, thus becomes no longer an essential means for recognizing osteocytic activity. The routine plotting of histograms of data obtained for area of osteocytic lacunae and elliptical ratio of osteocytic lacunae further allows differentiation between subpopulations of osteocytes. Studies on bone response to physiologic and pharmacologic agents demonstrated the usefulness of such quantitative data on osteocytic parameters [19, 20].

The micromorphometric results obtained from identical slides employing our technique and the method of Merz and Schenk show that for almost all micromorphometric parameters comparable absolute data are obtained. Due to the higher precision of our method, however, the number of fields evaluated by our technique in obtaining these comparable data was 25% of the fields evaluated by the Merz and Schenk technique. The significant differences between results of both methods for osteoid/osteoblast interface might be due to intraobserver variation or methodologic differences. The significant difference in surface density of bone and its reciprocal parameter mean trabecular diameter, however, most likely occurs because our method measures actual surfaces and areas whereas the graticule technique obtains random hits and intersections for area and surface measurements, respectively.

The improvement in coefficients of variance with which cellular parameters of bone resorption and formation are obtained allows reduction of the number of fields evaluated by our technique as compared to the graticule method. Based on the results of coefficients of variance for osteoclastic parameters, which show a relatively large range of variance, the number of fields required to be evaluated can be reduced by 50% when our method is employed. The further benefit of substantially lower interobserver error of our method as compared to the Merz and Schenk technique [5, 21] justifies a further reduction in the number of evaluated fields without jeopardazing the validity of the obtained results. For practical purposes, evaluation of 50 microscopic fields at a magnification of 200× obtains data with an overall coefficient of variance of less than 20%, which is within acceptable standards for biologic measurements [13].

The described method should facilitate more accurate and faster collection of scientific histologic data of bone while providing a basis for critical interpretation, comparison, and exchange of data obtained by investigators at different times or in different laboratories.

Acknowledgments. We thank Dr. Randall Manaka for assistance in the statistical evaluation, Ms. Susanne Barragan for technical assistance, and Ms. Dorothy Bodjanac and Ms. Joann Little for their secretarial help.

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