

Applying stereology to measure thickness of the basement membrane zone in bronchial biopsy specimens

To the Editor:

Investigations of airway remodeling require quantitative methods that emphasize sampling and sources of bias. Stereology provides these methods and is particularly valuable for the measurement of thickness and surface area, because these measures are biased if made naively from tangential sections through tissue.¹ Stereologic protocols introduce random tissue orientation (isotropic, uniformly random [IUR] orientation) so that tangential cuts occur with a known distribution and can be handled systematically.^{2,3} IUR sections from small clinical samples can be made by using a spherical mold termed the *isector*.⁴ Here we describe a stereologic approach to measurement of thickness of the basement membrane (BM) zone in bronchial biopsy specimens. We measured BM zone thickness in 2 protocols: (1) we applied the stereologic approach in biopsy specimens from asthmatic and healthy subjects and (2) we compared the stereologic approach to the sampling strategy and handling of orientation described in 2 previously published methods.^{5,6} For protocol 1, we analyzed biopsy specimens from 7 healthy subjects (mean $\pm$ SD FEV₁, 98.0% $\pm$ 6.6%) and 7 asthmatic subjects (FEV₁, 75.0% $\pm$ 14.1%) requiring only inhaled β -agonists for asthma control. For protocol 2, we analyzed biopsy specimens from 8 asthmatic subjects aged 31 $\pm$ 6 years (7 women) with mild asthma (FEV₁, 84% $\pm$ 17% predicted; PC₂₀, 1.3 $\pm$ 2.8 mg/mL) requiring only inhaled β -agonists.⁷ All subjects had signed a consent form approved by the Committee on Human Research at the University of California, San Francisco. Six to 8 endobronchial biopsy specimens were obtained per subject and paraffin embedded. For the stereologic approach, we individually embedded 6 biopsy specimens in a spherical 4-mm isector mold (Stereological Research Laboratory, University of Aarhus, Aarhus, Denmark), rolled these spheres on a bench top, and then re-embedded the 6 spheres for each subject together in a single larger conventional mold. We stained 3- μ m sections with Gomori's trichrome (Fisher Scientific, Pittsburg, Pa) and used a light microscope (Olympus BX50; Olympus Optical Co GMBH, Munich, Germany) equipped with a mechanical stage linked to a computer

(IBM 300PL Personal Computer; IBM, Hampshire, United Kingdom) running Computer Assisted Stereology Toolbox (C.A.S.T.-Grid) software (Olympus Danmark A/S, Ballerup, Denmark). After outlining the BM zone with a 20 $\times$ lens (total magnification, 860 $\times$), we systematically sampled more than 100 high-power fields (total magnification, 4300 $\times$) from 6 biopsy specimens by using a random start. To identify measurement sites, we superimposed a line grid (3 parallel lines) onto each randomly selected high-power field. We measured BM zone thickness by using the orthogonal intercept method,³ measuring the length of a line extended perpendicularly from the epithelial surface across the thickness of BM zone at each intercept of the line grid and basal lamina (Fig 1). We made an average of 170 measurements for each subject and calculated the arithmetic and harmonic mean thickness by using formulas that account for tangential cuts³: Arithmetic mean layer thickness = $\pi/4 \times$ Arithmetic mean of orthogonal intercepts; Harmonic mean layer thickness = $8/3\pi \times$ Harmonic mean of orthogonal intercepts, where harmonic mean = number of measurements/sum of the reciprocal of orthogonal intercept lengths.

In protocol 2, we compared measurements made by using stereology to measurements made in similarly stained sections by using the sampling and orientation methods reported by Roche et al⁶ (five measurements from a single biopsy specimen for each subject, choosing good epithelial orientation and minimum thickness of fibrosis for sites of measurement) and Sullivan et al⁵ (random start with measurements at 20- μ m intervals at a perpendicular angle to the basal lamina for a total of $\sim$ 40 measurements). After using biopsy specimens embedded in conventional molds for these 2 methods, we re-embedded the biopsy specimens in the isector mold for the stereologic approach. Each protocol was applied twice, and the coefficient of error for repeat measurement for each subject was calculated by using the squared mean of variances for each application (STATA 5.0; Stata Corporation, College Station, Texas).

In protocol 1, the thickness of the BM zone was significantly greater in asthmatic than in healthy subjects (arithmetic mean $\pm$ SD, 4.3 $\pm$ 1.0 vs 3.2 $\pm$ 0.4 μ m, respectively, $P = .01$ and harmonic mean, 3.6 $\pm$ 0.7 vs 2.4 $\pm$ 0.4 μ m, respectively, $P = .002$), but the variability of orthogonal intercept lengths was similar in both groups as judged by the mean variance (7.7 vs 9.3 in healthy and asthmatic subjects, respectively; not statistically significant by F test with 6 degrees of freedom). These results suggest that BM zone thickening in asthma is uniform and argue against abnormally thin regions in the BM zone in chronic asthma.

In protocol 2, the measurements made by using stereology were approximately 30% smaller than measurements made with 2 previously published methods, and the reproducibility of stereology was superior (Table I). The smaller values obtained with stereology might be attributable in part to the bias of tangential cutting associated with traditional methods. However, the use of a correction factor ($\times \pi/4$ [arithmetic mean] or $\times 8/3\pi$ [harmonic mean]) did not completely account for lower measurements that remained

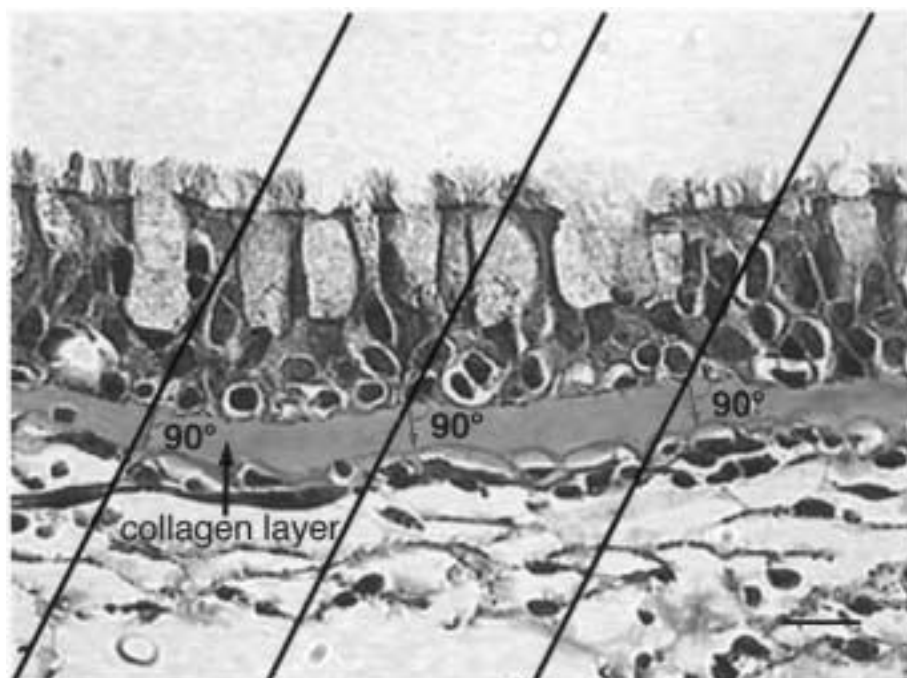


FIG 1. The orthogonal intercept method for measurement of BM zone thickness. Bar = 20 μ m.

TABLE I. Measurements of BM zone thickness in biopsy specimens from 8 asthmatic subjects by using 2 previously published methods (methods A and B) and the isector-based method (method C)

	Method A	Method B	Method C
Thickness (μ m)	6.21	6.60	4.63
Mean coefficient of error for repeat measurements	0.30	0.19	0.06*

Method A refers to the method previously published by Roche et al.⁶

Method B refers to the method previously published by Sullivan et al.⁵

Coefficient of error is defined as SEM/mean.

* $P < .001$ vs method A and method B by F test.

~11% smaller when the correction factor was not used. The difference might reflect the greater number of biopsy specimens studied in the stereologic approach. Notably, there was significant variability between biopsy specimens (CV, 0.31) and within biopsy specimens (coefficient of variation, 0.50), suggesting that adequate sampling procedures are necessary regardless of the method applied.

Using the isector added approximately 15 minutes to the embedding protocol, but it took only 45 minutes to make more than 150 measurements across the 6 biopsy specimens for each subject. The isector generates IUR sections that allow correction for tangential cutting. Furthermore, IUR sections allow measurement of surface area of the basal lamina, which might be a more invariable reference space than volume of submucosa, particularly when there is submucosal edema. From these observations, the application of stereology appears to be more precise than

previously described methods for quantifying the thickness of the BM zone, has greater reproducibility, and allows unbiased estimation of surface area as well as thickness. The improved precision relates to incorporation of sampling methods that could well be applied to advantage in other methods (multiple fields from multiple biopsy specimens). The need for multiple fields is increasingly recognized, but our data also document significance between biopsy variability, which can only be addressed by the use of multiple biopsy specimens.

Ronald E. Ferrando, MA^a

Jens R. Nyengaard MD, DMSc^b

Steven R. Hays, MD^a

John V. Fahy, MD^a

Prescott G. Woodruff MD, MPH^a

^aCardiovascular Research Institute and

Division of Pulmonary Medicine

Department of Medicine

Box 0130

University of California, San Francisco

505 Parnassus Avenue

San Francisco, CA 94143

^bStereological Research and

Electron Microscopy Laboratory University of Aarhus

Denmark

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