This report outlines a method for rapid quantification of corneal epithelial cell movement in a block organ culture system designed to model a penetrating or perforating wound of the cornea. Rabbit corneas were secured over a plastic conformer with a chalazion clamp. Two-millimeter, full-thickness corneal specimens were sampled from central and peripheral cornea and placed in tissue culture. The aim of the procedure was to produce full-thickness corneal specimens from well-defined geographic areas of the cornea of reproducible size and shape without undue trauma to the epithelium. The instruments used for measurements were simple, allowing for accumulation of a large number of observations which could be subjected to rigorous statistical evaluation. This method was used to determine if there was a detectable difference in the rate of epithelial cell movement over stromal cut surface of specimens from the periphery of the cornea, versus the rate over specimens from the central area of the cornea. In this block organ culture system the peripheral epithelial cells migrated more rapidly than samples of central epithelial cells ($P < 0.05$). This study shows a simple, rapid method for producing and evaluating block corneal organ cultures and shows that the site of specimen sampling must be controlled in corneal epithelial cell studies employing block organ culture. This method will be useful for in vitro screening of novel pharmacologic agents which have the potential for influencing corneal wound healing prior to evaluating these agents in vivo.

Full-thickness corneal specimens in tissue culture (block corneal organ culture) have been used to study corneal epithelial cell reactions and cell movement as an experimental model of in vivo penetrating or perforating corneal wounds. In this type of tissue preparation, the epithelial cells migrating over exposed corneal stroma (and exposed Descemet's membrane) may be influenced by factors released from damaged corneal stroma and endothelial cells, replicating the microenvironment of partial-thickness (radial keratotomy), full-thickness (penetrating keratoplasty), and traumatic corneal wounds. In this in vitro system, tissue culture media can be modulated to investigate the influence of both endogenous and exogenous agents. In the past, this type of full-thickness organ culture assay has been technically difficult to prepare in a reproducible fashion and to evaluate. Trephine knives have been used to remove corneal specimens of reproducible size. However, because of the corneal curvature, the tissue was cut with the epithelial surface against a cutting block, resulting in a variable degree of mechanical damage to the epithelial cells. Razor blades have also been used to obtain full-thickness corneal organ culture samples; this method, however, sacrifices the precision of reproducible block size and contour in order to ensure uninjured epithelial cells. Evaluation of the extent of epithelial migration in block organ culture preparations has been accomplished by various expensive and time-consuming morphometric techniques with either paraffin- or plastic-embedded histologic specimens. Even these highly precise methods may not be very accurate. The overall pattern of epithelial cell migration is not uniform around the circumference of the cut surface of the organ culture specimen. The leading edge of the migrating epithelial tissue sheet frequently assumes a gently undulating form. Therefore, the extent of migration on the stromal cut surface in one vertical plane of the specimen may vary greatly from that of adjacent planes. To our knowledge, this tissue feature of epithelial cell growth has not been addressed previously.

The new system described in this report was designed to (1) allow for selection of the site of corneal tissue collection relative to the limbus in a precise and reproducible manner; (2) preserve the integrity of the epithelial surface; (3) be technically simple; and (4) allow for efficient evaluation of a large number of specimens.

A recent report has confirmed that the growth potential of dissociated peripheral epithelial cells may be much greater than dissociated central epithelial cells. Thus the site of origin of the corneal block may bias the results of a study that selects corneal epithelium without reference to sampling location. To our knowledge, none of the previous papers using block organ culture systems has indicated the exact geographic source of the corneal tissue. The purpose of this study was to demonstrate the degree of precision of our current method by determining whether there are detectable differences in the rate of epithelial cell spreading or migration from the site of origin among specimens sampled from the central cornea and from the peripheral cornea.
Materials and Methods. All investigations described in this manuscript conform to the ARVO Resolution on the Use of Animals in Research and the University of Minnesota guidelines for animal use.

Five-pound New Zealand white rabbits (Birchwood Farms, Red Wing, MN) of either sex were sacrificed with an intrathoracic injection of sodium pentobarbital solution (Vet Labs, Lenexa, KS). Both globes were removed by sharp dissection, rinsed in Hank's balanced salt solution, and transported in Hank's balanced salt solution with gentamicin (500 /µg/ml). (All biochemicals were obtained from Sigma, St. Louis, MO unless otherwise noted.) The cornea and a portion of the scleral rim was removed with scissors and the entire lens-iris diaphragm, anterior uveal tract, and retinal remnants were removed by blunt dissection. The cornea was placed epithelial side up, on an acetal form (Delrin®, DuPont, Wilmington, DE) designed to conform with the internal contour of the anterior segment and held in place with a 10-mm chalazion clamp (Fig. 1). A 2-mm trephine was used to collect full-thickness specimens either from the peripheral or central portions of the cornea (Fig. 2). The specimens were transferred into a 60-mm Petri dish (no. 1007, Falcon, Oxnard, CA.) containing supplemental hormonal epithelial media (SHEM)³ (Dulbecco's modified Eagle media/Ham's F-12 media with 5% fetal bovine serum, 5 /µg/ml insulin, 100 ng/ml cholera toxin (Behring Diagnostics, La Jolla, CA), 10 ng/ml epidermal growth factor (Behring Diagnostics), 0.5% dimethyl sulfoxide, and 50 /µg/ml gentamicin). The culture dish was incubated at 37°C in humidified air with 5% CO₂.

Samples were fixed in 10% neutral buffered formalin after culture for 18, 20, or 22 hr. The samples were stained with 10% aniline dye in 1% glacial acetic acid for 30 sec, washed twice in 1% glacial acetic acid for 30 sec, and washed in distilled water for 30 sec. The stained specimens were placed on transparent plastic, oriented with the cut stroma down. The leading edge of epithelial cells, which do not stain with aniline dye, could be identified against a background of intensely stained exposed corneal stroma when viewed by oblique and transmitted light of a standard light microscope at 10X magnification (Fig. 3). A reticle image was photographed using direct positive film (Ektochrome), cut out, and placed in one ocular of a standard light microscope. The outer margins of the reticle were superimposed over the anterior and posterior borders of the corneal stroma on the outer edge of one side of the specimen (Fig. 4). The number of the zone containing the leading edge of epithelium was recorded. The variable width of the ruler (reticle

Fig. 1. Ten-millimeter chalazion clamp, top and side views. Acetal was lathed to resemble the internal contour of the rabbit anterior segment to serve as a cutting platform for fresh tissue. The 10-mm chalazion clamp was used to immobilize the fresh tissue over the form. This allowed sampling of tissue with a 2-mm trephine without injuring the epithelium. During the procedure the surface of the cornea was kept moist with topical applications of Hank's balanced salt solution.

Fig. 2. Four 2-mm trephine specimens were collected from the central portion of the rabbit corneas (A). Six to ten trephine specimens were collected from the periphery of the cornea (B).
Fig. 3. Gross 2-mm trephine button of cornea maintained in organ culture for 20 hr. The exposed corneal stroma has been stained with aniline dye. The corneal stroma covered by advancing epithelial cells does not readily pick up the stain although punctate areas of staining are visible on the epithelial surface. Note the variability of the distance between the edge of the anterior corneal stroma (large arrow) and the leading edge of the advancing epithelial cells (small arrows). Measurement of a single plane through this type of specimen would lead to a considerable range of data. A large number of data points is needed to accurately account for this variability. Aniline dye stain. Original magnification ×10.

Image) permitted the observer to measure the proportion of surface covered by epithelial cells. The opposite edge of the specimen was measured in the same manner. The specimen was rotated approximately 45°, and the specimen was remeasured in this new position. By rotating the specimen twice, six measurements were obtained for each tissue block (Fig. 5).

The six values for each specimen were averaged and the results were statistically evaluated using the

Fig. 4. The specimen was aligned so that the top line of the reticle was placed at the anterior border of the stroma (A). The bottom line of the reticle was aligned with Descemet's membrane (B). The number of the zone which contained the leading edge of the epithelium was recorded.
Migrating Epithelium
Exposed Corneal Stroma

Fig. 5. Schematic diagram of measuring system. (A). View of the corneal specimen lying on its stromal surface on the stage of a conventional light microscope. The reticle image is located within one of the oculars of the microscope. (B). View of the epithelial surface of the specimen. Two measurements were made with the specimen in the initial orientation. The specimen was rotated twice to allow for four additional measurements.

Mann-Whitney-Wilcoxon test. Values for U, normalizations of the sum of the ranks associated with the observations of our experimental parameters, were generated using Statworks 1.1 (Cricket Software, Philadelphia, PA) on an Apple Macintosh. Values for z, the standardized score, which expresses in standard deviations the difference between the results and the value expected for the null hypothesis, were obtained with the following equation:

$$ z = \frac{(U - mn/2)\left[12/((mn)(m + n + 1))\right]^{1/2}}{\text{where } m \text{ and } n \text{ are the number of central and peripheral specimens examined, respectively. Values for } P, \text{ the probability that the two populations are the same, were obtained from } z \text{ by using a standard normal distribution table.}}$$

Results. The results of the three experiments examining central versus peripheral corneal epithelial migration are shown in Table 1. The mean numeric values represent the proportion of stroma covered by migrating epithelium, which does not stain with aniline dye. A value of 8 indicates completed migration of epithelial cells over the stroma, while a value of 0 indicates no migration of the epithelium. The Mann-Whitney probability values represent the probability that the two conditions (central and peripheral sample sites) are equivalent in their migratory potential. The experiments were significant ($P < 0.05$) in showing that peripheral epithelium migrated faster over exposed stroma than did central epithelium.

Discussion. Corneal organ culture, as a general procedure, can be subdivided into categories by the method of tissue preparation: cultured corneal tissue with and intact scleral rim;7–11 cultured corneal tissue without an intact scleral rim;12,13 culture of an imprecisely defined large segment of cornea;14 culture of

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<th>Experiment no.</th>
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blocks of tissue; and culture of the entire globe with a passage of access into the anterior chamber. Corneal organ culture methods have been used to investigate embryonic development of the cornea, basic biochemistry of the cornea, corneal storage, corneal infections, corneal epithelial healing, penetrating wound healing, perforating wound healing, endothelial wound healing, and alkali-burned cornea. The method described in this paper employs block corneal cultures that model the early epithelial events of perforating or penetrating corneal wounds caused either by accidental or by surgical trauma.

This method of quantification of epithelial cell movement in an organ culture system has multiple advantages over those previously reported. Specifically, the exact geographic site of tissue sampling can be controlled; the integrity of the epithelial surface is preserved; the technique is simple; and a large number of observations may be made in a short period of time.

Whole specimens prepared by our original method caused a variable degree of damage to the epithelial cells. Therefore, the number of viable cells probably varied from specimen to specimen despite the uniform 2-mm diameter of the specimens. The most likely source of damage was contact of the epithelium with the bottom of the plastic culture dish and incidental shearing forces during sampling with the trephine. The epithelial surface must be cut first, therefore, in order to preserve its integrity. However, if the intact eye is used to produce a tissue sample, only one sample can be collected from each eye; after perforation of the cornea, the globe collapses and thereby makes additional sampling impossible. The corneal tissue must be supported from behind by a material that is rigid enough to allow completion of the trephine cutting without distortion or stretching of the tissue. The material needs to be pliable enough to make up for small degrees of variation from the perpendicular as the trephine engages the tissue. For this study, multiple types of dental impression material were tested to serve as the potential cutting die. Condensation silicone dental impression material was found to be useful for obtaining a true reproduction of the internal contour of the anterior segment; it was not firm enough, however, to allow for distortion-free cutting, with the result that the tissue was displaced toward the cutting material. Other materials proved too hard to allow completion of the trephine cut without compressing the corneal endothelium. Acetal was found to be sufficiently soft to allow the trephine blade to extend into the material far enough to complete the cut without distorting the tissue. Several methods, including complicated vacuum systems, were tried to stabilize the fresh corneal tissue to the cutting platform. However, the most effective method was simple mechanical compression of the limbal tissue with the ring of an unmodified 10-mm chalazion clamp. The orientation of the tissue with this mould–clamp system is controllable to the extent that the trephine can be directed precisely toward the peripheral corneal tissue.

A variable scale ruler was used to measure the proportion of the stromal cut surface covered by migrating epithelial cells. The continuously variable width of the ruler accommodated the variable widths of the specimens. A small number of divisions simplified measuring and allowed for rapid processing of individual specimens. In this way the precision of the final result of any experimental protocol was based upon many replicates measured by the ruler, reducing the overall work needed and reducing variation among different observers.

The statistical method used to evaluate the data was the Mann-Whitney-Wilcoxon test, which functions without the need for paired samples; which requires few assumptions about the distribution of the data; and which is suited to analysis of an experimental condition related to a numeric scale. In these experiments the Mann-Whitney-Wilcoxon test gave slightly more stringent probabilities than the student t-test.

The hypothesis that the stem cell location for corneal epithelium is at the limbus has been supported by the observations that corneal epithelial cells migrate in a centripetal manner and that corneal basal cells are more highly differentiated than are limbal cells. The hypothesis has been confirmed by others using cell culture methods indicating that the cells of the cornea do not all have the same growth potential, or pleuripotentiality. The relative location of cells in the corneal domain is important. It appears as though the cells with the most growth potential are the limbal derived epithelial cells, while the cells in the center of the cornea show a growth potential that is substantially limited. The reason for this difference in growth is not known, but may be that the central cornea cells are more highly differentiated or aged. Therefore, the site of tissue collection from the cornea for organ culture assays must be precise, rather than random, and the method of tissue collection must be reproducible in this regard.

In our studies the epithelium from the peripheral cornea specimens showed a more rapid rate of movement down the native substrate of exposed corneal stromal collagen than did the epithelium from central cornea specimens. Therefore, the site of origin of the specimen appears to be an important factor both in experimental design and in interpretation of data.
For example, in some experiments it may be more desirable to collect samples from the central cornea to represent a more biologically relevant model of aged cells with a limited growth potential.

There remain many practical limitations to the possibility that data derived from block organ culture can be converted into absolute rather than relative values. This method produces a more precise tissue sample than has been previously possible. However, the degree of variability, primarily in tissue preparation (incidental trauma, oblique cutting angle) and the relatively unpredictable stromal swelling, limits the precision of direct measurement. The most reliable data was derived from comparisons with controls within each experiment. A primary consideration was to minimize the effect of this variability by producing a large data base with simple methods for statistical evaluation. Other methods, such as transmission and scanning electron microscopy, may result in more precise analytical measurements once the tissue is sectioned; however, these methods require dehydration during tissue preparation, which introduces an uncontrollable degree of variation and which makes subtle changes impossible to discern. Furthermore, tissue embedment is time consuming and expensive, considering the number of specimens which must be processed for each experiment. Evaluation of the gross fixed specimen as performed in the present study does not introduce any such additional variation due to dehydration.

The method described in this report was designed to provide rapid screening of a large number of agents, such as peptides and growth factors, which have the potential to influence tissue response to injury. Once an active agent is identified, additional data could be obtained with other methods to evaluate dissociated cell populations or healing in vivo. The screening method we have described would aid in minimizing the number of in vivo screening assays that might otherwise be attempted to define novel pharmacologic agents.

Key words: cornea, organ culture, rabbit, epithelial migration, quantification

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