Mitotic orientation in three dimensions determined from multiple projections

Matthew Bjerknes and Hazel Cheng
Department of Anatomy, University of Toronto, Toronto, Ontario M5S 1A8 Canada

ABSTRACT The three-dimensional orientation of mitoses in mouse small intestinal crypts of Lieberkuhn was determined from multiple projections of the mitotic figures in whole mounts of isolated intestinal crypts. We found evidence of a significant orientational bias for mitoses whose daughter cells would be added along the long axis of the crypt, and thus conform to the maintenance of the cylindrical shape of the intestinal crypt. However, we also observed many mitoses whose progeny must be rearranged if the simple cylindrical shape of the intestinal crypt is to be maintained. Our results indicate that the ultimate behavior of progeny cells and hence of local tissue form may not strictly depend on the orientation of mitosis. The methods presented may also be used in the study of mitotic orientation in other tissues.

INTRODUCTION

The orientation of each mitosis in a tissue may influence the direction of growth in a tissue and the relations of neighboring cells. As a result, it has often been proposed that mitotic spindle orientation is an important determinant in morphogenesis. With the exception of cleavage patterns in early development (Balinsky, 1970), little is known in animals about the three-dimensional distribution of mitotic spindle orientation in tissues, its relationship to the tissue form, or the mechanisms that determine orientation. We have developed simple methods to measure three-dimensional orientation of mitotic figures in tissue and used them to determine the distribution of mitoses in the crypt of Lieberkuhn of the small intestinal epithelium.

The small intestinal crypt is a good system to investigate mitotic orientation. Crypt geometry is simple, roughly a cylinder with a hemispherical base, and the crypt is a site of active cell proliferation. We found clear orientational biases of mitoses within the tissue (mitoses tend to run approximately parallel to the crypt lumen) but mitoses with almost every orientation were also observed, including those which would tend to thicken the epithelium. The significance of these observations is discussed.

MATERIALS AND METHODS

We reduce the problem of the orientation of mitosis to that of the orientation of the mitotic spindle by taking advantage of the classic observation that in animal cells the plane of cytokinesis is perpendicular to the long axis of the spindle (Morgan, 1927; Wilson, 1928; Kawamura, 1977; Rappaport and Rappaport, 1985; Rappaport, 1985; Mabuchi, 1986; Bjerknes, 1986). Thus our problem is to determine the relative placement of the poles of the mitotic spindle in three-dimensional space. Our solution is to stain a block of tissue for DNA by Feulgen. This allows the chromosomes of cells in late anaphase or early telophase to be used as indicators of the orientation of the mitotic spindle. A mitosis is located and the position of the poles in the two-dimensional image (a projection) is noted. Then the tissue is rotated about a known axis by a predetermined amount and the position of the poles recorded for the new orientation. After this process is repeated a number of times, sufficient data exists in the multiple projections to calculate the positions of the poles in three dimensions.

Data collection

Intact crypts were isolated from mouse jejunum by EDTA perfusion (Bjerknes and Cheng, 1981; it is not known whether EDTA treatment has any effect on mitotic orientation), fixed in ethanol–acetic acid, 3:1, stained with Feulgen, dehydrated through graded alcohols into xylene, and then into microscope immersion oil. The crypt is then sucked into a fine capillary tube filled with immersion oil. The capillary tube is mounted in the center of a brass octagonal cylinder with a hole drilled through its center (the tube holder is much like a pencil with the capillary tube representing the pencil lead). This simple device allows the tissue to be rotated about a known axis under the microscope in precise 45 degree increments. The crypts are examined at 1,000× with oil-immersion objective and condensor. Because the refractive index of the glass capillary tube and the immersion oil are similar, distortion is minimal over the central portion of the tube where observations are made. We used a drawing tube attached to the microscope to record each projection. A line was drawn through the center of the crypt lumen and a dot was placed on the poles of mitotic figures in late anaphase or early telophase. All such mitoses found in the cylindrical portion of the crypt were used. Each mitosis was recorded at five different positions. The five projections were combined into a least squares estimate of mitosis orientation in space (Sutherland, 1974).

Data analysis

A point in three-dimensional space is conveniently represented by a vector,

$$\mathbf{X} = \begin{pmatrix} x \\ y \\ z \end{pmatrix}.$$

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In the discussion that follows, the long axis of the capillary tube is taken to lie along the z-axis. If the object containing the point $\mathbf{X}$ is rotated about the z-axis by an amount θ, then the new position of $\mathbf{X}$ will be

$$\mathbf{X}' = \begin{pmatrix} x\cos\theta - y\sin\theta \\ x\sin\theta + y\cos\theta \\ z \end{pmatrix},$$

which is compactly expressed in matrix notation as

$$\mathbf{X}' = R\mathbf{X},$$

where R is the matrix,

$$R = \begin{pmatrix} \cos\theta & -\sin\theta & 0 \\ \sin\theta & \cos\theta & 0 \\ 0 & 0 & 1 \end{pmatrix}$$

and $R\mathbf{X}$ represents matrix multiplication.

The process of recording a projection of the image of the point under the microscope corresponds to multiplication of the position vector $\mathbf{X}$ by the matrix

$$P = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix}.$$

Thus, the projection of the point will be found in position

$$X^* = P\mathbf{X} = \begin{pmatrix} x \\ 0 \\ z \end{pmatrix}$$

on the piece of paper where the drawings are made. By our definition, the z-axis corresponds to the long axis of the capillary tube (about which rotation occurs), and the x-axis is perpendicular to the z-axis. If the tissue is then rotated about the z-axis, the new position projected on the paper is $\mathbf{X}^* = PR\mathbf{X}$. If we define a new matrix

$$T = PR = \begin{pmatrix} \cos\theta & -\sin\theta & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix},$$

then the position of the projected point after rotation by θ_n is

$$\mathbf{X}_n^* = \begin{pmatrix} x_n \\ 0 \\ z_n \end{pmatrix} = T\mathbf{X} = \begin{pmatrix} \cos\theta_n & -\sin\theta_n & 0 \\ 0 & 0 & 0 \\ 0 & 0 & 1 \end{pmatrix} \begin{pmatrix} x \\ y \\ z \end{pmatrix}.$$

The vector $\mathbf{X}^*$ is the raw data (the tracings of a projection). What we must do is estimate x, y, and z (the true position of the mitotic spindle pole) given a set of measurements of $\mathbf{X}_n^*$, one for each θ_n. This is equivalent to solving an overdetermined set of linear equations, $A\mathbf{X} = B$ for $\mathbf{X}$, where

$$A = \begin{pmatrix} \cos\theta_1 & -\sin\theta_1 & 0 \\ 0 & 0 & 1 \\ \cos\theta_2 & -\sin\theta_2 & 0 \\ 0 & 0 & 1 \\ & \vdots & \\ \cos\theta_n & -\sin\theta_n & 1 \\ 0 & 0 & 1 \end{pmatrix}$$

and

$$B = \begin{pmatrix} x_1 \\ z_1 \\ x_2 \\ \vdots \\ x_n \\ z_n \end{pmatrix}.$$

The usual procedure for solving this equation is to use the least-squares best estimate of the solution, $A^TA\mathbf{X} = A^TB$, where A^T is the matrix transpose of A. Thus,

$$\mathbf{X} = (A^TA)^{-1}(A^TB),$$

which, after some algebra, yields,

$$\hat{x} = \frac{cd - be}{ad - b^2} \quad \hat{y} = \frac{bc - ae}{ad - b^2} \quad \hat{z} = \frac{\sum_{i=1}^{n} z_i}{n}, \tag{1}$$

where $a = \Sigma_{i=1}^{n} \cos^2\theta_i$, $b = \Sigma_{i=1}^{n} \cos\theta_i \sin\theta_i$, $c = \Sigma_{i=1}^{n} x_i \cos\theta_i$, $d = \Sigma_{i=1}^{n} \sin^2\theta_i$, and $e = \Sigma_{i=1}^{n} x_i \sin\theta_i$, and $\hat{x}$, $\hat{y}$, and $\hat{z}$ are the least-squares estimates of x, y, and z.

Substitution of the five measured projections from each mitosis into Eqs. 1 gives a least-squares estimate of the true position of the point in space. In the ith projection, measurements of x_i and z_i were taken by placing the center of the coordinate system at the point halfway between the mitotic spindle poles and aligned so that the z-axis was parallel to the crypt lumen.

The approximate rotational symmetry of the crypt about its lumen was used to place all mitoses on a common coordinate system for comparison (Fig. 1). The coordinate system is placed at the center of the mitotic figure with the z-axis running towards the crypt top parallel to the crypt lumen, the x-axis is perpendicular to the z-axis and points towards the center of the lumen, and the y-axis is normal to the x- and z-axes. The y-z plane is therefore tangential to the surface of the crypt and normal to the x-z plane. Projections of 271 mitoses from 73 crypts isolated from three mice were determined. The data were combined using the common coordinate system and the three-dimensional distribution of mitotic orientation in mouse small intestinal crypts thus determined.

Statistical tests

The data obtained with the methods used can be represented as pairs of antipodal points on a sphere. Thus, for example, a mitosis running

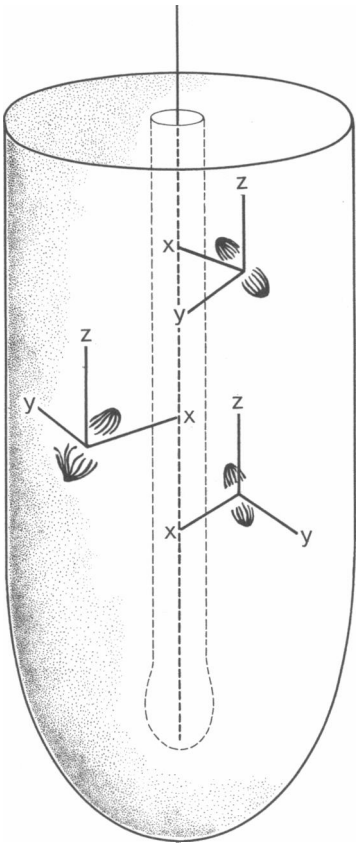

FIGURE 1 Schematic drawing illustrating the convention used to place all mitotic figures in a common coordinate system. The coordinate system is placed at the center of the mitotic figure with the z-axis running parallel to the crypt lumen, the x-axis pointing towards the center of the lumen, and the y-axis normal to the x- and z-axes (the y-z plane is tangential to the surface of the crypt).

parallel to the crypt lumen (the z-axis) may be represented by the poles of the sphere, whereas a mitosis orientated so that it runs parallel to the x-axis may be represented by a pair of points on the equator of the sphere. The representation of the results as points on a sphere is useful because a number of statistical tools have been developed to handle such data (Mardia, 1972). Of particular interest are tests for axial bias and axial symmetry. Axial bias is a tendency for mitoses to orient in a particular direction, whereas a test for axial symmetry would explore the degree to which the mitotic figures were uniformly distributed about that axis.

Briefly, the moment of inertia of the orientation data was determined. The eigenvalues and eigenvectors of the moment of inertia matrix were calculated and used to test for uniformity and rotational symmetry of the distribution of mitotic orientation (Mardia, 1972).

RESULTS

The three-dimensional orientation of 271 mitoses from the cylindrical portion of jejunal crypts is displayed as stereo pairs in Fig. 2. Fig. 2 *a* shows a view of the mitoses as seen from a point on the positive z-axis looking towards the origin. In other words the viewer is looking down onto the mitoses from the top of the crypt. It is evident that fewer mitoses are oriented in the vicinity of the x-z plane (also compare Fig. 2, *b* and *c*, noting that *c* appears flattened relative to *b*). Fig. 2 *b* shows a view of the mitoses as seen from a point on the positive x-axis looking towards the origin. Thus the viewer is looking at the mitoses from the crypt lumen with the y-z plane tangential to the surface of the crypt and the x-y plane forming the "floor." It is evident that no mitosis is oriented in the vicinity of the horizontal x-y plane. Fig. 2 *c* shows a view of the mitoses as seen from a point on the positive y-axis looking towards the origin. Thus the viewer is looking at the mitoses from the outside of the crypt with the y-z plane tangential to the crypt surface and normal to the x-z plane. Again there is no mitosis oriented in the vicinity

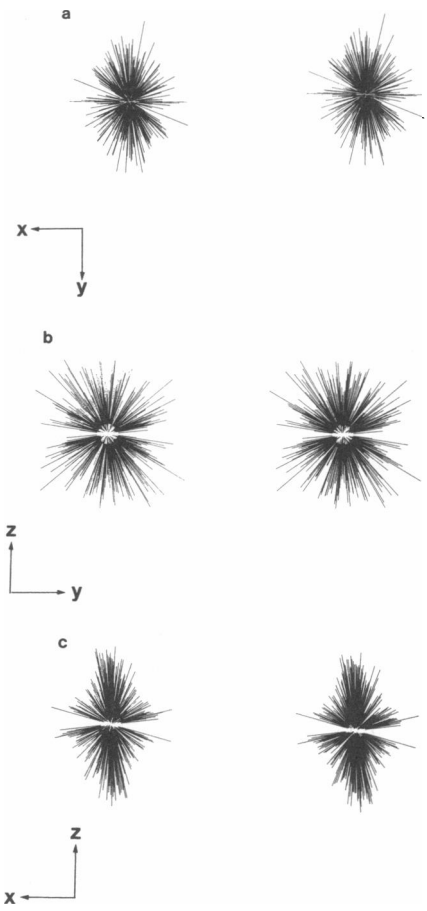

FIGURE 2 Stereo pairs illustrating the orientation of mitotic figures in crypts of the mouse small intestinal epithelium. The coordinate system is as described in Fig. 1. Each line represents the orientation of the poles of a mitosis. (*a*) View from a point on the positive z-axis and looking towards the origin. (*b*) View from a point on the positive x-axis. (*c*) View from a point on the positive y-axis.

of the horizontal x-y plane. Many of the mitoses appeared to be oriented in the vicinity of the vertical y-z plane.

Statistical tests give evidence of axial bias, i.e., the distribution is not uniformly distributed about the x, y, z-axes, with the preferred direction approximately parallel to the z-axis. The moment of inertia matrix was

$$T = \begin{pmatrix} 48.67 & 4.106 & 3.415 \\ 4.106 & 91.61 & 5.966 \\ 3.415 & 5.966 & 130.7 \end{pmatrix},$$

with eigenvalues $t_1 = 48.2$, $t_2 = 91.0$, $t_3 = 131.8$, and eigenvectors $t_1 = (0.99, 0.09, 0.03)$, $t_2 = (0.08, 0.98, -0.15)$, and $t_3 = (0.05, 0.15, 0.99)$. As mentioned above, the x, y, and z axes correspond to directions running from the center of the mitosis towards the lumen, tangential to the crypt surface, and parallel to the crypt lumen, respectively (Fig. 1). Thus our data show that significantly more mitoses in the intestinal crypt are oriented in a direction approximately parallel to the crypt lumen (tilted ~8 degrees relative to the lumen to be exact; $p < 0.005$, $x^2 = 96.8$, $dF = 5$). A test for rotational symmetry about the preferred axis gives evidence for a bias toward the plane tangential to the surface of the crypt, i.e., the y-z plane ($p < 0.01$, $x^2 = 37.0$, $dF = 5$). These results are visually evident in Fig. 2, particularly if one compares Fig. 2, *b* and *c*. In the figure, mitoses are seen to avoid the horizontal plane, i.e., the x-y plane, whereas the distribution is denser for mitoses oriented roughly parallel to the crypt lumen in the y-z plane.

DISCUSSION

The intestinal crypt consists of a single layer of epithelial cells arranged in an approximate cylinder surrounding a lumen. If crypt cells were incapable of significant rearrangement after mitosis, then maintenance of normal crypt morphology would require that the vast majority of new cells be added along the long axis of the crypt. At first sight, the summary statistics of the three-dimensional orientation of mitoses in the crypt appear to conform to such a basic plan. There is a significant orientational bias for mitoses oriented along the long axis of the crypt (z-axis, Fig. 2 *c*) and lying tangential to the epithelium. Because the plane of cytokinesis is normal to the axis of the mitotic spindle (Morgan, 1927; Wilson, 1928; Rappaport and Rappaport, 1985; Rappaport, 1985; Kawamura, 1977; Mabuchi, 1986; Bjerknes, 1986), the daughter cells from these mitoses would be added along the long axis of the crypt. Equally interesting is the fact that no mitoses were found oriented along the plane normal to the crypt lumen, i.e., near the horizontal x-y plane (Fig. 2 *b* and *c*). The presence of orientational biases (mitotic orientation biases have also been noted in the early chick embryo; Stern, 1979) in the distribution of mitoses in the intestinal crypt indicates that the process governing the orientation of the mitotic apparatus is not entirely random. In other words, the biases indicate the presence of some forces acting to orient the mitotic spindle. This may be related to mitotic cellular shape (Bjerknes, 1986).

Things are not quite this simple however. In the intestinal crypt many mitoses were observed which would, under this simple view, either increase the thickness of the epithelium or increase the crypt radius (Fig. 2). Thus it would seem likely that crypt cells must also be capable of altering their placement relative to each other sometime after completion of mitosis. Reorientation of the progeny cells of mitoses has been reported during development of lepidopteran scales (Lawrence, 1973) and milkweed bug hairs (Lawrence, 1966a,b).

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