Published in final edited form as:

J Struct Biol. 2007 June ; 158(3): 293–306.

Analysis of the orientation of primary cilia in growth plate cartilage: a mathematical method based on multiphoton microscopical images

Maria-Grazia Ascenzi^a, Michelle Lenox^b, and Cornelia Farnum^b

^aDepartment of Orthopaedic Surgery, Biomechanics Research Division, University of California, Los Angeles, California

^bDepartment of Biomedical Sciences, Cornell University, Ithaca, New York

Introduction

Bone elongation in children occurs through the process of endochondral ossification in cartilaginous growth plates at the ends of long bones. Clonal expansion of stem cells results in columns of chondrocytes whose spatial position within the growth plate mirrors their differentiation stage: cellular proliferation, cellular enlargement (hypertrophy), and cellular apoptotic death followed by replacement of bone on the previously calcified cartilaginous matrix (Figure 1). The extent of bone elongation achieved depends on the kinetics of chondrocytic activity at each stage of differentiation, and on the rate of regulated transitions between stages. A complex interplay of genetic and epigenetic factors (e.g., endocrine, paracrine, autocrine, nutritional, biomechanical) influences postnatal longitudinal bone growth, acting primarily at the cellular level through differential effects at specific phases of chondrocytic development and maturation. For recent reviews, see Farnum and Wilsman, 2001 and 2002.

Observed originally in rabbit kidney cells (Zimmerman, 1898), the primary cilium has been suggested to constitute a regular structural feature of virtually all eukaryotic cells within both vertebrates and invertebrates, most characteristically at the incidence of one per cell (for a website on the primary cilium, see: <http://members.global2000.net/browser/cilialist.html>). The axonemal structure of primary cilia is characterized by nine doublet microtubules that extend through the axonemal length (Singla and Reiter, 2006). The monocilia of the nodal cells in the embryo show dynein arms which are hypothesized to generate a characteristic propeller-like movement (Tabin, 2006; Hirokawa, 2006). In general, the primary cilia, that are not nodal, lack dynein arms and are considered to be non-motile in the sense that they lack the ability to generate either a propeller-like movement as for the nodal cilia or a waveform characteristic of the motile cilia whose axonemal core consists of two central microtubules (Bisgrove and Yost, 2006), such as those found in cells of the airway epithelium.. Primary cilia have been observed in the cells of multiple connective tissues including osteoblasts (Tonna and Lampen, 1972); osteocytes (Federman and Nichols, 1974), odontoblasts (Garant et al., 1968), ligament fibroblasts (Bray et al., 2005), meniscal fibroblasts (Le Gaverand et al., 2001), periodontal cells

Corresponding author: Maria-Grazia Ascenzi, PhD, Department of Orthopaedic Surgery, Rehabilitation Bldg #22-69, 1000 Veteran Avenue, University of California, Los Angeles, CA 90095, Tel: 310/825-6341; fax: 310/825-5290, e-mail: mgascenzi@mednetucla.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Beersten et al., 1975), adipocytes (Geerts et al., 1990), and in chondrocytes of articular (Wilsman, 1978) and elastic (Cox and Peacock, 1977) cartilage.

Recent papers recognize primary cilia as sensory organelles for detection and transmission of signals from the extracellular environment to the cell, essential for tissue homeostasis and function (Pazour and Witman, 2003; Whitfield, 2003; Davenport and Yoder, 2005; Schneider et al., 2005; Olsen, 2005). In connective tissues the cilium projects into the extracellular matrix (ECM) and is closely associated with the Golgi apparatus of the cell. Given the highly anisotropic organization of most connective tissues, it has been suggested that the primary cilium may act as a mechanosensor to the local biomechanical environment, and may be significant in the establishment of cellular orientation and directed secretion of ECM components from the Golgi apparatus (Quarby and Parker, 2005). Poole et al. (1997, 2001) demonstrated that the degree to which the primary cilium extends into the ECM, and whether its axoneme is straight or bent, is variable in articular chondrocytes, and that the configuration of the cilium relative to the chondrocyte changes as fluid flow in the environment changes. Poole has hypothesized that the chondrocytic primary cilium acts as a probe of the ECM and, because of its close association with the Golgi and the microtubule organizing center of the cell, is a key player in establishing cellular shape (Poole et al., 1985, 1997, 2001; Badano et al., 2005). A similar hypothesis has been proposed for the primary cilium in osteoblasts (Wheatley et al., 1996; Quarles, 2005). A hypothesis that the primary cilium is the osteocyte's strain-rate sensing flowmeter unites mechanical and fluid-flow sensory functions (Whitfield, 2003). Attractive as these hypotheses are, they are very difficult to test in the living animal.

If the primary cilium of connective tissue cells is a sensory organelle involved with receiving biomechanical signals that result in directed secretion of the surrounding ECM, one could hypothesize that the orientation of the cilium in three dimensional space should be consistent with the orientation of the cell itself (i.e. the long axis of the cell on longitudinal sections), or of the orientation of the cells within the tissue (i.e. the long axis of the macroscopic bone). The growth plate is a particularly appropriate connective tissue to investigate this hypothesis since cellular profiles and their orientation have been studied in growth plate cartilage using stereologically based approaches, and it is clear that the long axis of the cell relative to the long axis of the bone changes as chondrocytes progress from proliferation through their terminal differentiation characterized by cellular enlargement during hypertrophy (Farnum et al., 1990; Breur et al., 1991; Hunziker et al., 1987; Buckwalter et al., 1986; Hunziker and Schenk, 1989; Wilsman et al., 1993; Cruz-Orive and Hunziker, 1986).

The purpose of the current study was to develop an experimental technique for analysis of the orientation of the cilium in the growth plate through a new application of mathematical concepts originally developed by Euler to describe the orientation of a segment in a three dimensional space (Euler L., 1755). Adaptation of the classical concepts to the specifics of the biological context is a necessary, important and not obvious step, requiring knowledge of both the mathematical concepts and the biological specifications of the tissue of interest.

The mathematical methodology described in this paper is part of the rapidly growing field of mathematical methods that are developed on images of a given specimen for the purpose of modeling the specimen through the two-step process of data collection from images and consequent computational algorithms on collected data. Angenent et al. (2006) have recently emphasized how this major impetus for new algorithms in signal and image processing has stemmed from the last decade's advent of a variety of faster, more accurate and less invasive imaging devices. All such mathematical algorithms lead to interactive procedures. That is, in each case there is a human user in the loop who is the ultimate judge of the tuning of parameters. There is a major need for further mathematical techniques and modifications of previous approaches that lead to more automatic, and easier-to-interpret, imaging.

The mathematical application described here assesses cellular orientation with respect to the direction of bone elongation and ciliary orientation with respect to cellular orientation in a three dimensional environment. Rapid cold precipitation-based fixation is used to minimize possible artifactual post-mortem alterations of ciliary orientation with the ultimate goal of assessing ciliary orientation *in situ*. The mathematical method is applied to serial optical sections of growth plate chondrocytes captured by multiphoton microscopy (MPM), after immunohistochemistry to demonstrate acetylated- α -tubulin in the ciliary axoneme. The combined application of the experimental and mathematical methods yields results that support the high anisotropy among the distinct zones of the growth plate that are identified by the organization of chondrocytes in relationship to the long axis of the bone. Such anisotropy is well characterized by the orientation of the ciliary axoneme with respect to the orientation of the chondrocyte. Having developed this methodology, we are now in a position to analyze ciliary orientation in three dimensional space using versatile coordinate systems applicable to a wide variety of connective tissues. The methodology developed is applicable to achieve three-dimensional reconstruction from z-stacks obtained by any microscopical modality (confocal microscopy, transmission electron microscopy), in addition to MPM.

Materials and Methods

Introduction to the methodology

Three different kinds of factors (Table I) potentially affect the reliability of any methodology that has as its goal the description of the primary cilium of a cell in three dimensional space. Specifically for growth plate cartilage, the first is a set of technical factors that includes: i) the choice of tissue's fixation method; and ii) the implications of the optical resolution of MPM. These factors will be analyzed in the Discussion.

The second set of factors is relevant primarily to the steps of the image analysis. For the consideration specifically of growth plate cartilage these include: i) compensating for orientation of the sample relative to the optical plane of the microscope, given that it is not possible to control the exact positioning of the specimen on the microscope's stage; ii) idealization of the shape of the ciliary axoneme; iii) idealization of chondrocytic shape in both two- and three-dimensions; iv) utilization of biologically meaningful reference axes with respect to which angles are measured (potentially either the long axis of the chondrocyte or the direction of elongation of the bone); and v) consideration of the significance of the angle of the ciliary axoneme relative to the associated centriole. Since these factors all are included in steps of the actual image analysis algorithm, they are discussed in the image analysis subsection of the Materials and Methods, and in the Discussion.

The third set of factors relates directly to the reliability of the methodology specifically in relationship to repeatability, given the series of judgments required. In this paper we are proposing the development of an approach to analysis of three-dimensional ciliary orientation in growth plate cartilage, and are not purporting to provide data demonstrating the degree of consistency of ciliary orientation in growth plates in general. Therefore, we are interested in the consistency of output data performed by different individuals on the same data set. Our level of resolution of interest is $\pm 30^\circ$ on each of the xy- and yz- planes. Although at first pass this may seem a coarse level of resolution, it is one that is biologically meaningful if it could be measured consistently, given the panoply of biological variables involved. This point will be analyzed in the Discussion.

Immunocytochemistry

The methodology of Ward et al. (2003) and Chi et al. (2004) was modified for immunocytochemistry of acetylated- α -tubulin, thought to be more abundant in stable

microtubules (Piperno and Fuller, 1985). Male Wistar rats three to four weeks of age were used for this study. The animals were kept under routine housing conditions, and all procedures were approved by the Institutional Animal Care Committee. For each collection, the rat was euthanized with an overdose of pentobarbital delivered by intraperitoneal injection. The hindlimb was disarticulated at the coxofemoral joint and all muscle rapidly removed. The distal limb was isolated by disarticulation at the femoro-tibial joint; the proximal half of the tibia was isolated and immediately placed in 4°C methanol. All remaining steps to isolate growth plate slabs were performed in 4°C methanol.

To isolate thin sections of the proximal tibial growth plate, the tibial section was cut sagittally, bisecting it in the anterior-posterior plane. Each half was then placed on its cut side, and further cut into 0.5-1.0mm thick pieces, including all tissue from the articular surface to the metaphyseal bone. For some sections, the epiphyseal and metaphyseal bone areas were removed essentially in their entirety, leaving slices of full thickness proximal tibial growth plate; for others, approximately 1mm of metaphyseal bone and all of the epiphysis including the articular cartilage were left intact. Fixation continued in cold methanol for three hours.

The sections were washed four times in 0.1M phosphate buffered saline (PBS) at 25°C. Incubation with the primary antibody (monoclonal anti-acetylated- α -tubulin, Sigma, St. Louis, MO, T6793) was at 1:100, first for two hours at 25°C, and then overnight at 4°C. Following four washes in PBS, incubation with the fluoresceinated secondary antibody (goat anti-mouse IgG (H+L), Molecular Probes, Eugene, OR) was at 1:100, again for two hours at 25°C, and then overnight at 4°C. Four rinses in PBS were made and the growth plate pieces were stored at 4°C in PBS before imaging.

Multiphoton microscopy (MPM)

The multiphoton microscope used has been described in detail previously, including all settings for detection of fluoresceinated probes (Kloppenburger et al., 2000; Zipfel et al., 2003a and 2003b; Farnum et al., 2006). Relative to confocal microscopy, second harmonic generation of collagen under MPM improves visualization of the cell within the matrix (Williams et al. 2005). Growth plate specimens were placed in a concave well on a glass slide and mounted in Vectastain (Vector labs, Burlingame, CA) for imaging. A Zeiss 20X/0.75NA dry objective was used. After initial scanning for orientation, z-series were imaged at 2 μ m intervals with an optical thickness of 1.8 μ m to depths as great as 200 μ m. Given that the maximal diameter of a chondrocyte in the z-direction measures approximately 20 μ m, up to ten consecutive columns of chondrocytes could be analyzed through the depth of the slab of cartilage. This stack of images was the source of cellular and ciliary profile images used to develop the mathematical model.

Image analysis

The vector graphic editor XaraX1 software (XaraX Co, London) allowed measurements of details of cells and cilia from each image of each z-stack. The intraobserver and interobserver errors relative to the measurements' collection were assessed on the images relative to five chondrocytes by means of power analyses (Moore and McCabe, 1989). These sources of errors are entered in Table I and will be explored in the Discussion. Maple software (Waterloo, Inc.) was programmed to correct the small tilt of the specimen under MPM when the z-stack was recorded. The rotation by the appropriate angle was applied to re-establish the orientation of the chondrocytic columns within the bone *in vivo* prior to isolation, since precise positioning of the specimen on the microscope's stage is not possible. This means that columns of chondrocytes were analyzed as they are positioned in the growth plate *in vivo*, parallel to the direction of growth.

Algorithms were developed to process data to yield ciliary orientation in three dimensional space with respect to chondrocytic orientation, expressed through tables of calculated entities and graphs, and based on the following assumptions. The ciliary axoneme was modeled as a curve, which is consistent with its profile observed in two-dimensional images, considerably longer than wide. Chondrocytes were modeled as ellipsoids. Multiple recent studies have used stereological approaches to analyze volume and shape changes that growth plate chondrocytes undergo as they differentiate from proliferative cells to hypertrophic cells (Farnum, 1990). Consistent findings from these studies demonstrate that, on histological sections, chondrocytic profiles in the proliferative cell zone have an axial ratio of approximately 0.2-0.3 height to width as measured in the direction of growth, changing to an axial ratio of 1.0 or greater than 1.0 in the hypertrophic cell zone. Considered in three dimensions, the change is from a cell that is significantly wider than tall to one that is either round or even taller than wide. These studies also indicate that the two-dimensional profiles of chondrocytes in all zones are best modeled as ellipses, with a change in direction of the long axis of the ellipse relative to the long axis of the bone during differentiation (Buckwalter et al., 1985).

The major axis of the chondrocyte modeled as an ellipse on each section was chosen as the most meaningful reference to measure the orientation of the angles of the ciliary axonemes. An alternative reference is the long axis of the associated centriole. Analysis of available TEM images from a variety of tissues, including articular cartilage and growth plate cartilage, has demonstrated that this angle varies from cell to cell, although the significance of this is not understood (Poole et al., 1985,1997,2001). However, because the centriole cannot be resolved by MPM, the angle of interest in this study was defined as that between the ciliary axoneme and the long axis of the chondrocyte. The assumption is being made that, if the angle of the axoneme relative to the centriole changes significantly, this would be reflected as a change of the angle of the axoneme relative to the axes of the cell. At this time there are no data in the literature to either support or refute the validity of this assumption.

Transmission electron microscopy (TEM)

The image by transmission electron microscopy presented in this paper refers to observations made in a previous study of the distal radial growth plate of four-week-old minipigs (Farnum and Wilsman, 1987). Briefly, growth plates were rapidly collected following euthanasia by pentobarbital overdose. Trimming to slabs to approximately 1mm × 1mm × 3mm was done in the primary fixative of 2% glutaraldehyde/ 2% paraformaldehyde in 0.1M cacodylate buffer with 0.7% ruthenium hexamine trichloride (RHT). RHT has been shown to optimally preserve the ultrastructure of growth plate chondrocytes by stabilizing the interface between the chondrocytic plasma membrane and the surrounding pericellular matrix (Hunziker et al., 1983). Primary fixation continued for two hours and was followed by two hours of secondary fixation in 1% osmium tetroxide in 0.1M cacodylate buffer, also containing 0.7% RHT. For some collections potassium ferrocyanide was included in both the primary and secondary fixatives, also to enhance preservation of the interface of the plasma membrane and the ECM (Farnum and Wilsman, 1983). Processing included rapid dehydration in graded alcohols through propylene oxide, followed by infiltration and embedment in epon-araldite. Blocks were polymerized at 60°C for three days. No decalcification procedures were used. Sections approximately 60nm thick were collected on 1mm and 2mm formvar-coated grids, stained with uranyl acetate-lead citrate, and viewed on a Philips 410 electron microscope at 60kV.

Results

Imaging the primary cilium in growth plate chondrocytes by TEM and MPM

Figure 2a demonstrates the typical appearance of a primary cilium from growth plate cartilage using transmission electron microscopy. The ciliary axoneme, seen here only as an initial

grazing section, projects into the surrounding ECM. Arrowheads indicate the extent of the axonemal profile seen on this section. The basal body of the cilium is the electron dense section within the cytoplasm from which the axoneme projects (long arrows). The cilium and its associated centriole (seen here in transverse section) are found in that region of the cytoplasm where Golgi stacks are numerous (short arrows).

Figures 2b and 2c show images of the primary cilium of growth plate chondrocytes as visualized by MPM. Figure 2b demonstrates two chondrocytes with the axonemes of the primary cilia projecting toward each other. The curved outline of the cellular profiles is seen, contrasting to the sharply defined straight cilium. In the field of cilia shown in Figure 2c, it can be seen that the length of the ciliary profile in any given section is variable, reflecting its orientation into the z-plane. In the three chondrocytes in the column at the right, the cilium appears as a round dot, indicating that its primary orientation is in the z-plane. In cells in columns on the left, a fuller extent of the axonemal length is seen, indicating orientation primarily in the xy-plane. In Figure 2c, double arrows on one cellular profile indicate Golgi stacks, which stained positively with this antibody. Their form and location can clearly be distinguished from that of the cilium.

Figure 3 presents seven frames taken at 2 μ m intervals and reconstructed as serial sections. In two chondrocytes the cilium comes prominently into view and then disappears again. The mathematical analyses for assessment of three-dimensional orientation of the cilium were made through these types of z-stacks.

Step 1 in development of the mathematical method: morphometry

The z-stacks at hand refer to a 328.7 $\times$ 438.3 $\times$ 79.5 μ m specimen at 309x magnification. Each stack consisted of 40 to 60 tiff images obtained with a plane of focus of thickness either 1 μ m or 1.5 μ m and a gap between adjacent images of 0.5 μ m. Each of the tiff images was imported into XaraX1 software. The flow chart (Figure 4) shows the steps to be described here. A reference system was chosen for each image (Figure 4a of flow chart and Figure 5a). Consistently through the stack, the origin of an xy-coordinate system was placed at the bottom left corner of each image with the x-axis parallel to the specimen width and the y-axis parallel to the specimen length. The z-axis paralleled the direction of the specimen thickness. The origin of the z-axis was placed at the level that corresponds to the level of the bottom surface of the original specimen. The direction of the z-axis was determined by the right-hand rule, that is opposite to the increasing scan number.

The cells that appeared with their cilium on at least three consecutive images were consecutively numbered by their first appearance as the z-stack is examined from top to bottom. Each image was analyzed from top to bottom and from left to right. On each image, an ellipse was adapted to each optical section of each visible cell and the major and minor axis of each ellipse was marked (Figure 4b). A segment was then overlapped to each visible fluorescent detail that was interpreted as a cilium (Figure 4c). Figure 5b shows the ellipse and segment for cell #1 on the first scan on which the cellular optical section is visible. Consistently through the images on which a cell and cilium appeared, the colors red, orange, yellow, green, blue, navy, and violet were employed consecutively. Figure 5c shows ellipses and segments relative to the 4 images on which cell #1 and its cilium appeared.

The XaraX1 file was calibrated in terms of microscope's magnification in order to measure details of each image in real microns. Angles were measured in degrees with respect to the horizontal x-axis. The first and last scan numbers on which the cell appeared with or without its cilium was recorded. On each image on which the cell appeared with its cilium, the x and y coordinates of the ellipse center were recorded, and the major axis length, the major axis angle and the minor axis length were measured (Figure 4d). The drawn segment length and

angle were measured. The x and y coordinates of the initial and final points of segment were recorded (Figure 4e). Table II reports the data for cell #1.

The robustness of the morphometric method was analyzed through consideration of intraobserver and interobserver errors. The magnitude of these errors was assessed for each entity that appears listed in the first column of Table II on the images of five chondrocytes. We present the calculation of errors concerning the data relative to the cilium length on image #5 (Table III).

To assess the magnitude of the intraobserver error, the length of the cilium on each image was measured seventeen times by each of two observers. Seventeen iterations afford sufficient data to consider their distribution. Because the distribution is free of outliers and marked skewness, the t-procedures can be applied to compute the power of the mean to detect actual ciliary length. Because the precision with which we measured lengths equals $\pm 0.005\mu\text{m}$, the probability of the mean to provide the actual length results equal to 0.90 for observer 1 and 0.70 for observer 2. If either mean separately does not reflect the actual length, the error equals at most (max-min)/min, that is 0.06/7.50 for observer 1 and 0.05/7.48 for observer 2. The intraobserver error for a unique measurement was found at most equal to 1%.

In the analysis of the interobserver error between two independent observers, neither the mean of the differences of corresponding measurements nor the difference of the means provides a strong power for the actual prediction of two length measurements, one length by observer 1 and one length by observer 2. In fact the highest power is provided by the mean of the differences of corresponding measurements, on the order of 55%. This is because the means of each of the two data sets are very close in value to each other while the ranges of each of the two data sets differ relatively more from each other. Nevertheless, the error of a unique measurement is at most equal to either the difference of the two largest measurements (0.07) divided by the smallest length (7.48) if the measurements of the two sets are considered paired, or to the largest measurements' difference (0.08) divided by the smallest length (7.48) if the measurements of the two sets are considered unpaired. In either case, the ratios equal 1%. The interobserver error for a unique measurement on five chondrocytes was found at most equal to 1%. On the basis of the small magnitude of the above-analyzed morphometric errors, performance of one measurement by a single observer was deemed appropriate.

Step 2 in development of the mathematical method: algorithm

The collected data became the input of the automated mathematical program written with Maple software. Algorithms that employ Maple's built-in functions conduct the following computations:

- The general orientation of cells in two-dimensions was computed as the average angle of the ellipses' axis whose orientation is closer to the x-axis orientation (Figure 4f). The orientation of cellular columns was measured as 180 degrees minus the general orientation of cells (Figure 6a). The value was 24.65 degrees for the stack shown in Figure 6a.
- All the images of the z-stack and all morphometric data were rotated by 180 degrees minus the angle that measured the general orientation of cells (Figure 4g) to display cells, cilia and their adapted ellipses and segments according to the orientation of the original specimen before isolation (Figure 6b). Hence, the new y-axis parametrized the longitudinal position along the direction of bone elongation. In particular, the new y-coordinate of the center of each cell parameterized the level of the cell within the differentiation cascade from proliferative to hypertrophic.

- The eccentricity of each ellipse was computed (Figure 4h) by means of the formula $1-(b/a)^2$, where a is the major axis and b is the minor axis of the ellipse. The eccentricity parameter varied therefore between 0 and 1. It equals zero when b equals a , that is when the ellipse is a circle. As the ellipse moves away from the circular shape and becomes flatter, a assumes values increasingly larger with respect to the value of b , the fraction b/a decreases towards the 0 value, and the eccentricity value approaches 1. In addition, equations of ellipses were computed from the data collected (Figure 4i). Each cellular profile drawn on an image (Figure 7a) was matched by the plot (Figure 7b) of the corresponding ellipse equations. Further, the segment that models the cilium was plotted with the respective cell in order to model the cilium optical section together with the section of the cell.
- Because the orientation of any chosen segment in three-dimensions is characterized by two independent angles, θ and φ (Figure 8), these two angles were computed for all meaningful segments. For each image on which a given cell appeared, the angle was computed between the segment that models the cilium and the major axis of the ellipse that models the cellular optical section. The average of such angles for a given cell is referred to as the θ -angle of the cilium (Figure 4j). The length of the cilium was computed as the distance between the first and the last centroid of segments that model the cilium on the images on which the cilium appeared (Figure 4j).
- An example of a set of ellipses relative to any given cell is depicted in Figure 9 inside a green box whose dimensions are proportionate to the dimensions of the original specimen. For each cell, the ellipsoid was constrained to the space between the first and the last scan on which the cell appeared. Further, the z -coordinate of the ellipsoid's center was chosen half-way between the middle of the first plane of focus and the middle of the last plane of focus on which the cell profile appeared. Because the ellipses' centers are not perfectly linearly aligned, a line is fitted to the ellipses' centers to provide the orientation of the ellipsoid with respect to the z -axis. The equation of the ellipsoid was computed (Figures 4k and caption to Figure 10).
- The ellipsoid represented the three-dimensional model of the cell (Figure 4l). The three-dimensional reconstruction of a given cilium (Figure 4l) was obtained by interpolating with segments the set of centroids of each of the segments that modeled the ciliary images (Figure 10).
- The angle of the ellipsoid major axis with respect to the z -axis was computed (Figure 4m). The orientation of the cilium within the chosen three-dimensional reference system was described in terms of the vector that passes through any two subsequent points that were computed as centroids of segments that model the cilium on all the images on which the cilium appeared. Also, the angle between the z -axis and segment that modeled the cilium was computed. Then, the two angles were added to obtain the angle between the segment that modeled the cilium and the cellular axis. Such angle is named the φ -angle of the cilium (Figure 4n).

Step 3 in development of the mathematical analysis: output

For each cell, the cellular longitudinal position, the cellular eccentricity, the cilium length and the cilium angles θ and φ with respect to cell orientation are shown in Table IV. Because the orientation of the cilium is described by the two angles θ and φ , we call the overall employed method *ciliary $\theta\varphi$ -algorithm*. The method was developed on a stack of images relative to a growth plate specimen from one rat and tested on a second growth plate specimen from a second rat.

Discussion

MPM, paired with the mathematical *ciliary $\theta\phi$ -algorithm*, allowed assessment of the three-dimensional orientation of primary cilia associated with chondrocytes of the growth plate. The strengths of this modeling method are its accuracy and flexibility. Its accuracy derives from small intraobserver and interobserver errors. Accuracy further derives from the property of mathematics to describe complex biological systems and from the thorough consideration of all details of the application. The flexibility comes from the lack of pre-prepared tools that might limit the rendering of the model. It is the flexibility of the modeling method that will allow application to the study of the primary cilium of cells in connective tissues of different composition and micro-geometries, such as tendon, annulus fibrosus, meniscus, ligament, cartilage, or bone.

Previous analyses of ciliary spatial orientation

In the past ten years, there has been a significant increase in understanding the role of the primary cilium as a sensory organelle in epithelial cells throughout the body, with the greatest breakthroughs coming from discovering abnormalities of the primary cilium linked to specific diseases, such as polycystic kidney disease (PKD) in young children (for a recent review, see Pan et al., 2005). Using transgenic mice models that mimic PKD, the specific abnormalities of the cilium at the molecular level have been extensively characterized, together with the role of the cilium in signal transduction pathways (Davenport and Yoder, 2005; Vogel, 2005; Goldstein et al., 2006). Equally crucial to the understanding of the mechanisms that link abnormal ciliary function to development of cysts in PKD have been analyses of the orientation of primary cilia as they project into the renal tubule luminal space, and how this orientation is altered in the disease state (Fischer et al., 2006).

In epithelia, the primary cilium of each cell projects into the lumen of the organ or to the surface of a monolayer culture. In this superficial position, its presence and orientation can be analyzed by light microscopical techniques following experimental manipulation. Models have been generated to present diagrammatically the response of the primary cilium of renal epithelial cells to fluid flow. The cilia show passive bending, which then initiates signaling cascades that involve molecules such as Wnt and Hedgehog (Corbit et al., 2005; Germino, 2005; Huangfu et al., 2005; Liu et al., 2005; Marshall and Nonaka, 2006; Davis et al., 2006; Michaud and Yoder, 2006).

Similar modeling of the position of the cilium in three-dimensional space has been generated for monocilia associated in the ventral node of embryos undergoing gastrulation. These specialized cilia have been shown to generate a leftward movement of fluid, called nodal flow, which is essential for generating left-right asymmetry of organelle development from the previously symmetrical left-right body axis (Buceta et al., 2005; Hirokawa et al., 2006). Monocilia, unlike the primary cilia of epithelia cells, can actively generate motion. Nonetheless, a key to the understanding of their function has been the development of models that allow visual presentation of their tilted position relative to the posterior end of the node as laterality is established (Hirokawa et al., 2006; Nonaka et al., 2005). Such studies describe ciliary dynamics in terms of the two angles that determine the cilium's orientation in a three-dimensional reference system. Equations describe ciliary movement in terms of time (Okada et al., 2005; Nonaka et al., 2005; Tanaka et al., 2005), and faster and slower movements of the cilium are described as a function of the distance from the cell's surface (Buceta et al., 2005). These studies demonstrate the potential significance of understanding the orientation of the ciliary axoneme in three-dimensional space.

In contrast to what is understood about orientation of primary cilia in epithelial cells and nodal cells, there is almost nothing except anecdotal information about the orientation for the primary

cilium of cells in connective tissues. This is at least partly due to the increased complexity of analyzing the cilium as it projects into the ECM, rather than into the lumen of a tubular organ. Multiple observations of the position of the ciliary axoneme relative to the chondrocytes in articular cartilage *in situ* have confirmed that its orientation is variable, ranging from paralleling the long axis of the cell, to projecting directly into the ECM, or found invaginated for a significant distance along the cellular plasma membrane (Poole et al., 2001). A TEM study that examined the orientation of the ciliary basal body to the centriole by serial section analysis of equine articular cartilage demonstrated that essentially all cilia project away from the articular surface. No consistency of orientation relative to the alignment of the cell was demonstrated (Wilsman and Farnum, 1986).

Comparison with other methods of assessing chondrocytic shape and tissue anisotropy

Growth plate chondrocytes are aligned in columns that spatially represent the temporal differentiation cascade of each individual chondrocyte. During this differentiation cascade, chondrocytes complete multiple cellular cycles. Their post-proliferative terminal differentiation is characterized by a significant volume increase during hypertrophy. A critical concept in understanding how longitudinal growth is achieved during the differentiation cascade is that, as cells hypertrophy, they undergo a regulated shape change, and the orientation of the long axis of the cell changes relative to the long axis of the bone. Proliferative cells that had an average height of approximately 10µm in the direction of growth, become hypertrophic cells with an average height of 25-30µm in the direction of growth. The sum of each cell's incremental height change times the number of cells turned over in a day is the single most significant variable accounting for the amount of growth achieved by a given growth plate (Breur et al., 1991;Farnum, 1994;Farnum and Wilsman, 2001 and 2002).

Multiple stereological-based approaches have been used to understand and model this important shape change of growth plate chondrocytes during the differentiation cascade. Chondrocytes show an elliptical profile in sections with non-zero departure from a circular profile (Buckwalter et al., 1985;Farnum, 1994). Observations of chondrocytic shape reported in the current study are confirmed by calculations of the degree of eccentricity on the multiphoton images (Table IV). Such calculations follow the decreasing values of eccentricity computed by Buckwalter et al. (1985) from the proliferating to the hypertrophic zone.

Buckwalter et al. (1985) applied methods based on equations involving the number of intersections of cellular profiles with a grid of parallel lines that form specific angles with the long axis of the cells. They quantified the shape of cellular profiles and the orientation distribution of cells relative to each other on images of groups of cells. Their approach to quantification referred to each cell in reference to the other cells in the group, while the methods reported here quantified the characteristics of each cell *per se*. Here, only after each individual cellular assessment had been made, were the single cell characteristics compared with the characteristics of the other cells.

An advantage of the sequential images captured by MPM through multiple parallel optical planes in the current study is the elimination of any three-dimensional shape assumption to construct three-dimensional shape from a two-dimensional image. The eccentricity parameter that we utilized measured the deviation of the cellular profile, modeled as an ellipse, from a circle with respect to the long axis of the bone. Proliferative zone cells had the most eccentric profiles and the highest degrees of orientation. Cells of the lower hypertrophic zone had the least eccentric profiles and the lowest degree of orientation.

Recent progress has been made in developing tools for three-dimensional visualization of contour and surface from sequential images obtained on multiple parallel optical sections (see for instance, Kutsuna and Hasezawa, 2005;Eliceiri and Rueden, 2005). Volume rendering and

surface modeling of the single cell requires the semi-automatic selection of contour on two-dimensional images. The separate collection of measurements and their mathematical manipulations are usually conducted by semi-automatic programs written specifically to meet the specific requirements of the study. In general, the three-dimensional visualization of contour and surface from sequential images obtained on multiple parallel optical sections is part of the current effort in the field of mathematical methods to model a given specimen through the two-step process of data collection from images and consequent computational algorithms on collected data. Angenent et al. (2006) find that the semi-automatic nature of such mathematical methods struggles to find a balance between the manual and the automatic contributions. The morphometric portion of our mathematical method has so far been kept as manual to develop an understanding of the imaged details at hand and to provide high accuracy. Such a manual approach is necessary for a future automation of the step that keeps the same order of accuracy.

Assumptions of the model and potential limitations

The three-dimensional reconstruction of the cilium approximated the cilium as a bent curve (Poole et al., 1985). The angles that the cilium forms with respect to the cellular axis were computed in terms of the orientation of the axis that passes through the points of any two subsequent centroids of segments that model the ciliary two-dimensional images. Nevertheless, the modeling can be improved by employing B-splines (Piegl and Tiller, 1997) to interpolate the centroids by a smooth curve and compute the tangent lines to such curve, for a continuous description of the cilium's angle along the length of cilium.

Optimally, imaging should be conducted on non-fixed living tissue *in vivo*, but such imaging is unlikely for connective tissues in the near future. For this study, cold methanol was chosen as a precipitation-based fixative of the tissue because its fixation is rapid and because proteins are precipitated in place without cross-linking. Therefore the potential for antibody penetration into the tissue and exposure of the primary antibody to the epitope of interest is maximized. As a trade off for rapidity and ability of the antibody to penetrate, this type of fixation sacrifices the quality of the morphological image. In fact, because methanol fixation is based on precipitation of proteins, methanol may deform the tissue, and in particular may deform chondrocytic shape and/or ciliary axonemal shape. Despite these potential limitations, the judgment was made that cold methanol was the fixative of choice given that the primary obstacle that needs to be overcome by pre-embedment immunocytochemistry is antibody penetration, which is particularly difficult in connective tissues with a dense extracellular matrix. Currently we have no independent way to assess whether this method of fixation caused deformation of axonemes. However, given the rapidity of fixation, it is unlikely that the ciliary axoneme radically changed position within the chondrocyte, as for example changing the side or octant of the cell from which it projected, in this study of *ciliary orientation* as opposed to a study of ciliary morphology. Thus a choice was made for a fixative that was consistent with obtaining the level of resolution that we sought in terms of measurement of the directionality of the projection of the axoneme, while in keeping with the realistic possibility of having the large molecular weight antibodies penetrate into the tissue for several hundred microns.

A potential limitation of the study refers to the resolution of the MPM with respect to z-axis. In fact, the lens employed displays a "point" as a Gaussian ellipse with a lateral width of 0.5 and an axial length of 1.8 (Zipfel et al., 2003a). Therefore a point looks like an ellipse of approximately 0.5 μ m in the x- and y- directions and of approximately 1.8 μ m in the z-direction and the center of mass of the ellipse coincides with the original point. Therefore, the portion of the cilium within an optical section becomes blurry at the specific scale of the Gaussian ellipse. The orientation of the imaged cilium, and therefore the measurements of the θ and ϕ angles, is unaltered even though the cilium's appearance is blurry. Because the employed

resolution associated a thickness of 1.8 μ m to the plane of focus, it was impossible to know at which level within the plane of focus the end points of the cellular axis and of the cilium were located. Therefore, an error is produced in the assessment of the cellular axis' and cilium's length in the z-direction. The error was at most equal to the sum of the thickness of the plane of focus, for each of the planes of focus relative to endpoints. The Gaussian ellipse also gave rise to a partial overlapping of the ciliary images (Figure 5c). While the optical resolution does not lead to a change of orientation of the imaged cilium, it does change the error of measurement of orientation. The error in this measurement is determined by many imaging factors such as the photon shot noise, background in the image and any pixilation of the structure (Thompson et al., 2002) Lateral localization is typically in the 30nm range for test bead systems and axial localization is expected to be four-fold reduced.

In conclusion, because the potential experimental and morphometric errors (see Table I for a summary) discussed have a small effect on the ciliary orientation, the results constitute a highly reproducible outcome at a level of resolution that permits an experimentally rapid assessment of the orientation of the axoneme of primary cilia associated with cells of the growth plate. It then provides a mean of achieving a quantitative assessment of the three-dimensional spatial orientation of the primary cilium of cells in connective tissues where cellular and matrix organization are highly anisotropic. This methodology will allow us to explore additional topics such as the percentage of the cells that have a cilium in an actively dividing population, and whether, after division, there is a change in the position of the cellular axis with respect to the longitudinal axis of the bone or with respect to the cilium. This *ciliary $\theta\phi$ -algorithm* provides an important analytical methodology for studying the primary cilium in development, growth and homeostasis of the body's skeletal framework.

Acknowledgements

The authors thank Rebecca Williams for her expertise in multiphoton microscopy and helpful discussions; Alexandre Lomovtsev for his imaging expertise relative to image analysis; and Duy Linh Tue Phung, Jaskiran Hundal, Steven Wong for image analysis and data collection. This research was partially funded by NIH grant R21 AR053849 to C. E. Farnum.

Bibliography

- Angenent S, Pichon E, Tannenbaum A. Mathematical methods in medical image processing. Bull Amer Math Soc 2006;43:365–396.
- Badano JL, Teslovich TM, Katsanis N. The centrosome in human genetic disease. Nat Rev Gen 2005;6:194–205.
- Beertsen W, Everts V, Houtkooper JM. Frequency of occurrence and position of cilia in fibroblasts of the periodontal ligament of the mouse incisor. Cell Tissue Res 1975;163:415–431. [PubMed: 1201585]
- Bisgrove BW, Yost HJ. The roles of cilia in developmental disorders and disease. Development 2006;133:4131–4143. [PubMed: 17021045]
- Bray RC, Salo PT, Lo IK, Ackermann P, Rattner JB, Hart DA. Normal ligament structure, physiology and function. Sports Med Arthr Rev 2005;13:127–135.
- Breur GJ, VanEnkevort BA, Farnum CE, Wilsman NJ. Linear relationship between the volume of hypertrophic chondrocytes and the rate of longitudinal bone growth in growth plates. J Orthop Res 1991;9:348–359. [PubMed: 2010838]
- Buceta J, Ibanes M, Rasskin-Gutman D, Okada Y, Hirokawa N, Izpisua-Belmonte JC. Nodal cilia dynamics and the specification of the left/right axis in early vertebrate embryo development. Biophys J 2005;89:2199–2209. [PubMed: 16040754]
- Buckwalter JA, Mower D, Schafer J, Ungar R, Ginsberg B, Moore K. Growth-plate chondrocyte profiles and their orientation. J Bone Joint Surg 1985;67-A:942–955. [PubMed: 3894369]
- Buckwalter JA, Mower D, Ungar R, Schaeffer J, Ginsberg G. Morphometric analysis of chondrocyte hypertrophy. J Bone Jt Surg 1986;68-A:243.

- Chi SS, Rattner JB, Malyas JR. Communication between paired chondrocytes in the superficial zone of articular cartilage. *J Anat* 2004;205:363–370. [PubMed: 15575885]
- Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate smoothened functions at the primary cilium. *Nature* 2005;437:1018–1021. [PubMed: 16136078]
- Cox RW, Peacock MA. The fine structure of developing elastic cartilage. *J Anat* 1977;123:283–296. [PubMed: 870470]
- Cruz-Orive LM, Hunziker EB. Stereology for anisotropic cells: application to growth cartilage. *J Microsc* 1986;143:47–80. [PubMed: 3761364]
- Davenport JR, Yoder BK. An incredible decade for the primary cilium: a look at a once-forgotten organelle. *Am J Physiol Renal Physiol* 2005;289:1159–1169.
- Davis EE, Brueckner M, Katsanis N. The emerging complexity of the vertebrate cilium: new functional roles for an ancient organelle. *Devel Cell* 2006;11:9–19. [PubMed: 16824949]
- Eliceiri KW, Rueden C. Tools for visualizing multidimensional images from living specimens. *J Photochem Photobiol* 2005;81:1116–1122.
- Euler, L. *Institutiones Calculi Differentialis cum Eius Usu in Analysi Finitoum ac Doctrina Serierum*. St. Petersburg Royal Academy of Sciences; Saint Petersburg: 1755.
- Farnum, CE.; Wilsman, NJ. The Growth Plate. In: Shapiro, IM.; Boyan, BD.; Anderson, HC., editors. *Chondrocyte kinetics in the growth plate*. 54. IOS Press; Washington D.C.: 2002. p. 245-257.
- Farnum, CE. Differential growth rates of long bones. In: Hall, B., editor. *Bone: Development and Differentiation*. 8. CRC Press Inc.; Boca Raton: 1994. p. 193-222.
- Farnum CE, Lenox M, Zipfel W, Horton W, Williams R. In vivo delivery of fluoresceinated dextrans to the murine growth plate: imaging of three vascular routes by multiphoton microscopy. *Anat Rec* 2006;288A:91–103.
- Farnum CE, Turgai J, Wilsman NJ. Visualization of living terminal hypertrophic chondrocytes of growth plate cartilage *in situ* by differential interference contrast microscopy and time-lapse cinematography. *J Orthop Res* 1990;8:750–763. [PubMed: 2201757]
- Farnum CE, Wilsman NJ. Pericellular matrix of growth plate chondrocytes: A study using post-fixation with osmium-ferrocyanide. *J Histochem Cytochem* 1983;31:765–775. [PubMed: 6841972]
- Farnum CE, Wilsman NJ. Morphologic stages of the terminal hypertrophic chondrocyte of growth plate cartilage. *Anat Rec* 1987;219:221–232. [PubMed: 3425941]
- Farnum CE, Wilsman NJ. Converting a differentiation cascade into longitudinal growth: stereology and analysis of transgenic animals as tools for understanding growth plate function. *Curr Opin Orth* 2001;12:428–433.
- Farnum, CE.; Wilsman, NJ. Chondrocyte kinetics in the growth plate. In: Shapiro, IM.; Anderson, HC.; Boyan, BC., editors. *The Growth Plate*. IOS Press; Amsterdam, The Netherlands: 2002. p. 245-257.
- Federman M, Nichols G. Bone cell cilia: Vestigial or functional organelles? *Calc Tissue Res* 1974;17:81–85.
- Fischer E, Legue E, Doyen A, Nato F, Nicolas J, Torres V, Yaniv M, Pontoglio M. Defective plan cell polarity in polycystic kidney disease. *Nat Genet* 2006;38:21–23. [PubMed: 16341222]
- Garant PR, Szabo G, Nalbandian J. The fine structure of the mouse odontoblast. *Archs Oral Biol* 1968;13:857–876.
- Geerts A, Bouwens L, Wisse E. Ultrastructure and function of hepatic fat-storing and pit cells. *J Electr Micr Tech* 1990;14:247–256.
- Germino GG. Linking cilia to Wnts. *Nat Genet* 2005;37:455–457. [PubMed: 15858588]
- Goldstein B, Takeshita H, Mizumoto K, Sawa H. Wnt signals can function as positional cues in establishing cell polarity. *Dev Cell* 2006;10:391–396. [PubMed: 16516841]
- Hirokawa N, Tanaka Y, Okada Y, Takeda S. Nodal flow and the generation of left-right asymmetry. *Cell* 2006;125:33–45. [PubMed: 16615888]
- Huangfu D, Anderson KV. Cilia and hedgehog responsiveness in the mouse. *Proc Nat Acad Sc USA* 2005;102:11325–11330. [PubMed: 16061793]
- Hunziker EB, Schenk RK. Physiological mechanisms adopted by chondrocytes in regulating longitudinal bone growth in rats. *J Physiol* 1989;414:55–71. [PubMed: 2607442]

- Hunziker EB, Herrmann W, Schenck RK. Ruthenium hexamine trichloride (RHT)-mediated interaction between plasmalemmal components and pericellular matrix proteoglycans is responsible for the preservation of chondrocytic plasma membranes in situ during cartilage fixation. *J Histochem Cytochem* 1983;31:717–727. [PubMed: 6341460]
- Hunziker EB, Schenk RK, Cruz-Orive LM. Quantitation of chondrocyte performance in growth plate cartilage. *J Bone Joint Surg Am* 1987;69:162–173. [PubMed: 3543020]
- Kloppenborg P, Zipfel WR, Webb WW, Harris-Warrick RM. Highly localized Ca(2+) accumulation revealed by multiphoton microscopy in an identified motoneuron and its modulation by dopamine. *J Neurosci* 2000;20:2523–2533.
- Kutsuna N, Hasezawa S. Morphometrical study of plant vacuolar dynamics in single cells using three-dimensional reconstruction from optical sections. *Microsc Res Tech* 2005;68:296–306. [PubMed: 16315234]
- Le Graverand MPH, Ou Y, Schield-Yee T, Barclay L, Hart D, Natsume T, Rattner JB. The cells of the rabbit meniscus: their arrangement, interrelationship, morphological variations and cytoarchitecture. *J Anat* 2001;198:525–535. [PubMed: 11430692]
- Liu A, Wang B, Niswander LA. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli factors. *Development* 2005;132:3103–3111. [PubMed: 15930098]
- Marshall WF, Nonaka S. Cilia: tuning in to the cell's antenna. *Curr Biol* 2006;16:R604–R614. [PubMed: 16890522]
- Michaud EJ, Yoder BK. The primary cilium in cell signaling and cancer. *Cancer Res* 2006;66:6463–6467. [PubMed: 16818613]
- Moore, DS.; McCabe, GP. *Introduction to the Practice of Statistics*. Freeman and Co.; New York: 1989.
- Nonaka S, Yoshida S, Watanabe D, Ikeuchi S, Goto T, Marshall WF, Hamada H. De novo formation of left-right asymmetry by posterior tilt of nodal cilia. *PLoS Biol* 2005;3:1–6.
- Okada Y, Takeda S, Tankada Y, Izpisua-Belmonte JC, Hirokawa N. Mechanism of nodal flow: a conserved symmetry breaking event in left-right axis determination. *Cell* 2005;121:633–644. [PubMed: 15907475]
- Olsen B. From the editor's desk. *Matrix Biol* 2005;24:449–450. [PubMed: 16226676]
- Pan J, Wang Q, Snell WJ. Cilium-generated signaling and cilia-related disorders. *Lab Invest* 2005;85:452–463. [PubMed: 15723088]
- Pazour GJ, Witman GB. The vertebrate primary cilium is a sensory organelle. *Current Opin Cell Biol* 2003;15:105–110.
- Piegl, L.; Tiller, W. *The NURBS book*. Springer-Verlag; Berlin, Germany: 1997.
- Piperno G, Fuller MT. Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J Cell Biol* 1985;101:2085–2094. [PubMed: 2415535]
- Poole CA, Flint MH, Beaumont BW. Analysis of the morphology and function of primary cilia in connective tissues: A cellular cybernetic probe? *Cell Motil* 1985;5:175–193. [PubMed: 4005941]
- Poole CA, Jensen CG, Snyder JA, Gray CG, Hermanutz VL, Wheatley DN. Confocal analysis of primary cilia structure and colocalization with the golgi apparatus in chondrocytes and aortic smooth muscle cells. *Cell Biol Int* 1997;21:483–494. [PubMed: 9451805]
- Poole CA, Zhang ZJ, Ross JM. The differential distribution of acetylated and deacetylated alpha-tubulin in the microtubular cytoskeleton and primary cilia of hyaline cartilage chondrocytes. *J Anat* 2001;199:393–405. [PubMed: 11693300]
- Quarles LD. Preliminary evidence that PKD1 function as a mechano-sensor in bone. 35th Intern Sun Valley Workshop *Skel Tissue Biol* 2005:61–62.
- Quarby LM, Parker JDK. Cilia and the cell cycle? *J Cell Biol* 2005;169:707–710. [PubMed: 15928206]
- Schneider L, Clement CA, Teilmann SC, Pazour GJ, Hoffmann EK, Satir P, Christensen ST. PDGFRα signaling is regulated through the primary cilium in fibroblast. *Curr Biol* 2005;15:1861–1866. [PubMed: 16243034]
- Singla V, Reiter JF. The primary cilium as the cell's antenna: signaling at a sensory organelle. *Science* 2006;313:629–33. [PubMed: 16888132]
- Tabin CJ. The key to left-right asymmetry. *Cell* 2006;127:27–32. [PubMed: 17018270]

- Tanaka Y, Okada Y, Hirokawa N. FGF-induced vesicular release of Sonic hedgehog and retinoic acid in leftward nodal flow is critical for left-right determination. *Nature* 2005;435:172–177. [PubMed: 15889083]
- Thompson RE, Larson DR, Webb WW. Precise nanometer localization analysis for individual fluorescent probes. *Biophys J* 2002;85:2775–2783. [PubMed: 11964263]
- Tonna EA, Lampen NM. Electron microscopy of aging skeletal cells. I. Centrioles and solitary cilia. *J Gerontol* 1972;27:316–324.
- Ward CJ, Yuan D, Masyuk TV, Wang X, Punyashthiti R, Whelan S, Bacallao R, Torra R, LaRusso NF, Torres VE, Harris PC. Cellular and subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia. *Human Molec Genet* 2003;12:2703–2710. [PubMed: 12925574]
- Wheatley DN, Wang AM, Strugnell GE. Expression of primary cilia in mammalian cells. *Cell Biol Int* 1996;20:73–81. [PubMed: 8936410]
- Whitfield JF. Primary cilium-is it an osteocyte's strain-sensing flowmeter? *J Cell Biochem* 2003;89:233–237. [PubMed: 12704786]
- Williams RM, Zipfel WR, Webb WW. Interpreting second harmonic generation images of collagen I fibrils. *Biophys J* 2005;88:1377–1386.
- Wilsman NJ. Cilia of adult canine articular chondrocytes. *J Ultrastruct Res* 1978;64:270–281. [PubMed: 712881]
- Wilsman, NJ.; Farnum, CE. Three dimensional orientation of chondrocytic cilia in adult articular cartilage; *Proc 32nd Orthop Res Soc, New Orleans*; 1986. p. 486
- Wilsman, NJ.; Farnum, CE.; Breur, GJ.; Leiferman, EM. Spatial arrangement of growth plate chondrocytes and a proposed functional unit as represented by an “average equivalent column”; *Proc 39th Orthop Res Soc, San Francisco*; 1993. p. 694
- Vogel G. Betting on cilia. *Science* 2005;310:216–218. [PubMed: 16223997]
- Zimmerman KW. Beitrage zur kenntniss einiger drusen und epithelien. *Entwicklungsmech* 1898;52:552–706.
- Zipfel WR, Williams RM, Webb WW. Nonlinear magic: multiphoton microscopy in biosciences. *Nat Biotechnol* 2003a;21:1369–1377. [PubMed: 14595365]
- Zipfel WR, Williams RM, Christie R, Nikitin AY, Hyman BT, Webb WW. Live tissue intrinsic emission microscopy using multiphoton-excited native fluorescence and second harmonic generation. *Proc Natl Acad Sci USA* 2003b;100:7075–7080. [PubMed: 12756303]

Figure 1.

(a) The proximal (single arrow) and distal (double arrow) growth plates of the tibia of a four-week-old rat are cartilaginous discs, each located between the epiphyseal bone (“e”), and the metaphyseal bone (“m”), at each end of the tibia. (b) This microradiograph shows bone elongation which occurs during the differentiation cascade of chondrocytes in the proximal tibial growth plate of a four-week-old rat. This 1µm-thick section was stained with methylene blue/azure II to demonstrate the morphology of cells and matrix. The anisotropic arrangement of chondrocytes is demonstrated by the columns of cells, which are a spatial representation of the temporal differentiation of individual chondrocytes. Cellular division is restricted to the proliferative zone (“p”); terminal differentiation is characterized by a significant increase in cellular volume, together with a shape change in the hypertrophic zone (“h”). The death of the terminal chondrocyte occurs just above the metaphyseal bone (“m”).

Figure 2.

The images depict ciliary orientations that are consistent with the way they were found in the growth plate and the way that images of growth plates are oriented by the regular convention, that is, with the epiphyseal bone at the top and the metaphyseal bone at the bottom. (a) This TEM micrograph of the primary cilium shows a hypertrophic cell in the distal radial growth plate of a four-week-old minipig. The axonemal profile (short arrowheads) projects into the extracellular matrix, and appears as an extension of the more electron dense basal body, which lies within the cellular cytoplasm (long arrows). The centriole appears in transverse section. The Golgi stacks (short arrows) are numerous in the region of the centriole. (b) The profiles of two early hypertrophic cells by MPM, whose ciliary axoneme of each cell fluoresces green, demonstrating reaction with the acetylated-α-tubulin antibody. (c) A field of cells and their associated primary cilia are shown at slightly lower magnification. The Golgi stacks (double arrows) are reactive as well with the acetylated-α-tubulin antibody. Because the Golgi stack is positioned entirely in the cells, the Golgi stack is clearly differentiated from the ciliary axoneme of the same cell. The cilium profile at the bottom right of the image appears almost punctate, indicating that the axoneme is essentially projecting in the z-direction of the z-stack.

Figure 3.

This series of seven consecutive images belongs to a z-series taken at 2μm intervals by MPM. Note that the ciliary axoneme is clearly viewed in two chondrocytes through three sections. This type of images was the input for the morphometry and the subsequent mathematical algorithm.

Figure 4.

The flow chart lists the steps applied to the z-stack for morphometry and subsequent mathematical algorithm.

Figure 5.

(a) An xy-reference system is chosen for each image, 309x : the origin of the axes is placed at the lower left corner of the image; the x-axis parallels the horizontal side of the image and the y-axis parallels the vertical side of the image. (b) This is an enlargement of ellipse and segment for cell #1 in (a) relative to the first scan on which the cellular optical section was visible. (c) Ellipses and segments relative to the four images on which cell #1 appeared.

Figure 6.

(a) The axis perpendicular to the cell general orientation (segmented) on the xy-plane was determined. (b) All images were rotated to restore the orientation of the image to the orientation of the specimen prior to isolation from tibia. Here the y-axis represents the direction of growth. The y-value measures the position of the cell within the specimen and was called “position” parameter.

Figure 7.

(a) The ellipses relative to cell #1 were obtained by plotting the equations obtained from the morphometric data. For example, the red ellipses is the plot of $x = -23.80\cos(\theta) + 1.23\sin(\theta) + 54.67$, $y = -1.57\cos(\theta) - 18.56\sin(\theta) + 76.03$, $z = 70.75$, for $0 \leq \theta \leq 2\pi$. The graphs matched well the elliptical cellular profiles which were originally drawn on the microscopical images (b).

Figure 8.

For a segment (colored in grey), the Eulerian angles θ and φ are marked with respect to the xyz-reference system. Usually, $0 \leq \theta \leq 2\pi$ and $0 \leq \varphi \leq \pi$. Here $0 \leq \varphi \leq 2\pi$ was chosen to assess orientation along which the cilium is pointing away from the cell within the four quadrants.

Figure 9.

(a)-(d) show from four different view points the sets of ellipses that describe the cellular profiles obtained from the experimental images of the specimen, represented by the green box. In (d) the ellipses are distanced from each other for detailed visualization.

Figure 10.

The three-dimensional model of cell #1 with its cilium (in black) is viewed from two different angles (a and b). The pink ellipsoid is the plot of the equations $x=26.61\cos(\theta)\sin(\phi)+1.26\sin(\theta)\sin(\phi)-1.19\cos(\phi)+57.67$, $y=1.76\cos(\theta)\sin(\phi)-19.06\sin(\theta)\sin(\phi)-0.08\cos(\phi)+77.03$, $z=-4.22\cos(\theta)\sin(\phi)+7.55\cos(\phi)+68.55$, for $0\leq\theta\leq2\pi$, $0\leq\phi\leq\pi$. The red lines are indicative of the middle plane of the first and the last scans on which the cell appeared without cilium. The projection of the pink ellipsoid on the xy-plane in (b) matches the two-dimensional ellipses in Figure 7.

Table I

Sources of potential errors are here summarized.

First set	Fixation	structural preservation
	Multiphoton microscopy	Gaussian ellipse
		photon shot noise
		pixilation of structure
Second set	Image analysis	orientation of images
		idealization of axonemal shape
		idealization of chondrocytic shape
		use of meaningful reference
		angle between axoneme and centriole
Third set	Morphometry	intraobserver error
		interobserver error

Table II

Cell #1 appeared on image #3-9 with or without appearance of cilium. Here are the cell and cilia measurements for each of the images #5 through #8 on which they both appear. Lengths are measured in microns and angles in degrees. Cilium length (cilL), cilium angle (cilA) with respect to the x-axis; planar coordinates of endpoints P1 (P1x, P1y) and P2 (P2x, P2y) of the segment modeling the cilium; planar coordinates for the center of the ellipse that models the cell (Cx, Cy); ellipse's major axis angle (MaL) with respect to the x-axis; the length of the ellipse's minor axis (maL).

Image #	5	6	7	8
cilL	7.50	9.20	10.10	9.10
cilA	65.63	65.43	74.32	73.35
P1x	99.60	99.40	98.70	100.20
P1y	55.00	54.30	55.20	56.40
P2x	102.70	103.20	101.40	102.80
P2y	61.90	62.60	65.00	65.10
Cx	81.40	85.60	87.20	88.40
Cy	46.30	45.90	44.10	44.40
MaL	47.70	49.10	45.70	45.50
MaA	159.13	159.13	159.14	159.13
maL	37.20	38.20	37.10	35.40

Table III

For the purpose of computing intraobserver and interobserver errors, all the entities listed in Table I were measured 17 times on five chondrocytes with cilium by two observers. For the cilium length on scan #5, each of the measurements numbered (Meas #) were taken by observer 1 (Obs 1) and observer 2 (Obs 2) independently. The absolute value of each difference of paired measurements (AbsD) is listed. Minimum (min), maximum (max), mean, standard deviation (stdev) were computed for measurements of observer 1 and 2 separately.

Meas #	Obs 1	Obs 2	Abs(diff)
1	7.53	7.51	0.02
2	7.56	7.52	0.04
3	7.55	7.51	0.04
4	7.53	7.50	0.03
5	7.50	7.52	0.02
6	7.51	7.52	0.01
7	7.51	7.51	0.00
8	7.54	7.51	0.03
9	7.51	7.48	0.03
10	7.54	7.51	0.03
11	7.52	7.51	0.01
12	7.52	7.50	0.02
13	7.54	7.52	0.02
14	7.51	7.48	0.03
15	7.52	7.49	0.03
16	7.53	7.50	0.03
17	7.53	7.53	0.00
min	7.50	7.48	0.00
max	7.56	7.53	0.04
mean	7.526	7.507	0.023
stdev	0.020	0.019	0.012

Table IV
By decreasing cellular longitudinal position (CeLp), cellular eccentricity (CeE), ciliary length (CiL), and ciliary angles (θ) and (φ) with respect to the cell orientation are listed for each numbered cell (NuCe).

CeLp (μm)	CeE	CiL (μm)	θ (deg)	φ (deg)	NuCe
421.03	0.75	6.44	302.31	39.60	7
288.29	0.49	4.97	101.35	147.37	9
283.01	0.94	5.21	122.63	235.11	2
247.00	0.65	11.46	275.42	354.58	4
243.97	0.72	11.12	302.40	57.02	8
234.06	0.52	12.82	32.98	48.30	5
185.29	0.86	4.56	41.70	327.69	6
155.99	0.86	7.56	214.89	327.60	3
76.79	0.39	6.24	102.21	168.65	1