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High-Speed Digital Imaging of Ependymal Cilia in the Murine Brain

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Abstract

The development and health of mammals requires proper ciliary motility. Ciliated epithelia are found in the airways, the uterus and Fallopian tubes, the efferent ducts of the testes, and the ventricular system of the brain. A technique is described for the motion analysis of ependymal cilia in the murine brain. Vibratome sections of the brain are imaged by differential interference contrast microscopy and recorded by high-speed digital imaging. Side views of individual cilia are traced to establish their bending pattern. Tracking of individual cilia recorded in top view allows determination of bend planarity and beat direction. Ciliary beat frequency is determined from line scans of image sequences. The capacity of the epithelium to move fluid and objects is revealed by analyzing the velocity of polystyrene beads added to brain sections. The technique is useful for detailed assessment of how various conditions or mutations affect the fidelity of ciliary motility at the ependyma. The methods are also applicable to other ciliated epithelia, for example, in airways.

I. Introduction

Ciliated epithelial cells line the surface of the ventricular system of the brain. Aqueducts and foramina connect the paired lateral ventricles in the cerebrum and the midline third and fourth ventricles in the midbrain and cerebellum, respectively. The ventricular system is filled with cerebrospinal fluid (CSF), a watery fluid (0.8 mPa·s viscosity at 37 °C; (Bloomfield et al., 1998) produced by the choroid plexuses, specialized regions of the ventricles. The CSF drains into the subarachnial space and into the spinal cord. Overproduction of CSF, failure to absorb it, or the blockage of its flow through the ventricular system causes hydrocephalus, an accumulation of fluid in the brain. The ependymal cilia move the CSF, but their contribution to the bulk flow of this fluid is limited. Nevertheless, impaired ciliary motility causes hydrocephalus in mice and other small mammals (Banizs et al., 2005; Ibanez-Tallon et al., 2004; Lechtreck et al., 2008; Sapiro et al., 2002; Zhang et al., 2007) and significantly increases the chance of hydrocephalus and ventriculomegaly in humans (Afzelius, 2004; Ibanez-Tallon et al., 2004). A plausible explanation is that ciliary motility is required in mice to keep the interventricular channels open, and contributes to keeping them open in humans, especially during the rapid postnatal growth of the brain (Ibanez-Tallon et al., 2004). Ciliary beating also has been implicated in neuronal guidance (Clarke, 2006; Sawamoto et al., 2006). Juvenile myoclonic epilepsy has

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Supplementary materials Supplementary Movie 1. Ependymal cilia in side view. Cilia were recorded at 200 fps; the movie is displayed at 8x reduced speed corresponding to 25 fps. See Fig. 2 A for still images of this movie.

been linked to altered ciliary motility, suggesting that defects in ciliary beating can result in neurological diseases (Ikeda et al., 2005; King, 2006; Suzuki et al., 2009).

The efficiency of cilia-based transport depends on the viscosity of the surrounding medium and on ciliary length, beat frequency, bending pattern, and coordination. Most cilia and flagella have a high beat frequency of up to 90 Hz (15 - 40 Hz for airway and ependymal cilia of mice, 40 - 60 Hz for sea urchin spermatozoa or *Chlamydomonas*). Therefore, high-speed imaging is required to reveal ciliary bending patterns and aberrations of these patterns. This is now generally achieved by high-speed digital imaging, in which a sequence of digital images is captured by a camera and recorded directly to a computer. The images can then be analyzed one by one or combined to create a digital video as desired.

Rates of up to 500 images/second have been used to analyze ciliary and flagellar movements of single cells. These include sea urchin and mammalian sperm (Ishijima, 1995a; Ishijima, 1995b; Ishijima and Witman, 1987), *Leishmania major* (Gadelha et al., 2007), *Tetrahymena thermophila* (Wood et al., 2007), and *Chlamydomonas reinhardtii* (Ruffer and Nultsch, 1998), free swimming or captured on micropipettes. Beat patterns also have been analyzed for cilia of airway epithelial cells using tissue samples such as brushings (Chilvers and O'Callaghan, 2000; Chilvers et al., 2003) or lung slices (Delmotte and Sanderson, 2006), or using cultured ciliated epithelial cells (Sutto et al., 2004). The techniques used have been described in several methods-oriented publications (Ishijima, 1995a; Ishijima, 1995b; Sanderson and Dirksen, 1985; Sanderson and Dirksen, 1995). In contrast, only a few studies have analyzed ependymal cilia *in vivo* using tissue preparations such as ventricular brushings (Ibanez-Tallon et al., 2004) and primary cell cultures (Weibel et al., 1986). As a result, the motility and bending pattern of ependymal cilia are less well analyzed. In this chapter we describe techniques for high-speed digital imaging and analysis of ciliary motility of the ependyma in brain slices.

II. Materials and Equipment

A. Materials

1. Animals: mice, mutant and wild-type litter mates, preferably between p5 and p8 (animals should be analyzed before hydrocephalus develops to avoid distortion of data by secondary effects).
2. Euthanasia: sodium pentobarbital (50 mg/ml Nembutal sodium solution), syringe, needle.
3. Tissue preparation: scissors, forceps, spatula, razor blades, superglue (Quick Bond Aron Alpha CE-471, Electron Microscopy Sciences), Petri dishes.
4. Observation chambers: custom coverslip support (see Figure 1C), coverslips, silicone grease, polyester mesh (500 micron), polyethylene tubing.
5. Fluid flow: polystyrene beads (0.5 μ m in diameter, Sigma-Aldrich).

B. Solutions

1. Hanks' Balanced Salt Solution (Invitrogen) supplemented with 25 mM Hepes, pH 7.4.
2. Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, penicillin, and streptomycin.

C. Equipment

1. vibratome (OTS-4000, Electron Microscopy Sciences). 2. microscope (Olympus IX71 inverted microscope). 3. objective (60x, NA 1.2, water immersion).
2. camera (TM-6740, Pulnix, 640×480 pixels, 200 images per second, coupled with a frame grabber (DVR Express, IO industries) linked to a computer hard-drive array).
3. optional: zoom adaptor (Nikon).
4. digital image acquisition software (Video Savant V4, IO Industries). (The equipment we used is shown in brackets).

III. Methods

A. Tissue preparation

Inject mice intraperitoneally with a lethal dose of pentobarbital (0.5 mg/g body weight). Remove the skin from the head and open the skull from the base using scissors. Remove the brain by inserting a spatula below the brain from the back and wash brain in HBSS. Trim the brain using razor blades. For observation of cilia in the third ventricle in side view, trim the brain for coronal sectioning by cutting approximately in the middle between the olfactory bulbs and the Colliculus posterior (line 1 in Fig. 1A). To fasten the brain for vibratome sectioning, remove the cerebellum with a second cut parallel to the first one (Fig. 1A, line 2) and place the brain with this side down into a small drop of superglue on the specimen holder. Gently press down the brain with a spatula until the glue has polymerized. Process the brain accordingly for other views of the ventricular cilia.

B. Sectioning and examination of sections

Place the specimen holder into the vibratome reservoir filled with HBSS. Section 130- μ m slices using a blade speed at a dial setting of ~ 3 and a blade advance setting of 0.2 – 0.5. Prior to observation of cilia at high magnification, it is useful to first locate the ciliated epithelium in sections (Fig. 1B) using an inverted microscope at low magnification. To do this, carefully transfer sections to a drop of HBSS in a glass-bottom culture dish (MatTek Corporation, MA) using a spatula. Then, transfer suitable sections to a coverslip (we use 45×50 mm, number 1) supported on a custom-built Plexiglas support (Fig. 1C). Use a syringe to apply two lines of silicon grease as spacers, and carefully lower a second coverslip (we use 40×20 mm, number 1) onto the bottom coverslip to form a chamber; fill the chamber with HBSS (Fig. 1C,D). For prolonged observation, a polyester mesh shim should be placed around the section to minimize pressure onto the tissue, and the buffer should be replaced regularly by removing buffer from one side and adding fresh buffer to the other side of the chamber. Usually, we try to analyze sections within 30-60 min after sacrificing the animal, but sections also can be stored in culture medium at 37 °C. After 24 hours the ciliated epithelium appeared intact and cilia beat vigorously but sections became sticky and more difficult to handle.

C. Imaging

Image acquisition will vary with each microscope. Good optics, Koehler illumination, and adjustment of differential interference contrast are required. An objective with a relatively long working distance facilitates the examination of thicker tissue slices. In our hands, cilia are easier to observe at the surface of the section, but ciliary motility is usually better in the middle of the section where the tissue is less affected by the sectioning. Top views and front views of cilia can be obtained in brain sections cut horizontally approximately at a level connecting the top third of the olfactory bulbs to the middle of the cerebellum or by sagittal

sectioning close to the midline (Fissura longitudinalis cerebri). To visualize the fluid flow generated by the cilia, polystyrene beads can be added to one side of the chamber and moved near the section by removing fluid from the other side. Because the beads are rapidly captured by the cilia, start the recording as soon as the first beads reach the ciliated surface (Fig. 2C). Often, floating cell debris is sufficient to determine the velocity of the fluid flow. The software Video Savant provides the ability to record extended image sequences that are only limited by the size of the array hard drive. Image sequences can be analyzed within Video Savant using custom scripts or archived in a number of other file formats for analysis using other programs. Sample videos are available in the supplementary materials and from <http://jcb.rupress.org/cgi/content/full/jcb.200710162/DC1> (Lechtreck et al., 2008).

D. Data analysis

1. Bending pattern. To determine the bending pattern, follow an individual cilium recorded in side view through consecutive images (Fig. 2A). We use a graphics tablet (Wacom) and the Adobe Illustrator paintbrush tool (B), which somewhat smoothes the line, to track individual cilia through one beat cycle (Fig. 2B). Tracking of individual cilia recorded in top view will allow determination of whether the beating is planar (Fig. 2E). Alternatively, a running average can be generated using ImageJ; planar movements will generate a straight line, rotating cilia will generate circles.
2. CBF. A line scan is usually sufficient to determine the beat frequency. Top views of beating cilia are well suited for this analysis, but side or front views also can be used. Cilia passing through the line will generate a regular pattern on the scan (Fig. 2C and F). ImageJ or Scion Image with the appropriate plug-in ("muleplekymogram" for ImageJ) are examples of programs useful for generating line scans. Delmotte and Sanderson (Delmotte and Sanderson, 2006) describe an alternative method to determine the CBF based on digital videos.
3. Coordination. Top views will reveal if the direction of beating is similar for cilia in a tissue sample (Fig. 2E). Cilia moving with metachrony will generate diagonal lines on line scans of side and front views (Fig. 2F).
4. Video editing. In addition to the use of Video Savant software to create digital videos, images can be saved as individual files, e.g. in Tiff format. These can be opened in Adobe Photoshop and rotated, cropped, adjusted, labeled, and saved using the 'Actions' command, which ensures that all pictures of a stack are manipulated identically. QuickTime and other programs can be used to generate movies from the image sequences.

IV. Discussion

The above technique allows monitoring of ciliary motility in thick sections, which preserves tissue structure better than other techniques. In previous studies, tissue brushings obtained from mouse brain generated fluid flow at an average of 22 $\mu\text{m/s}$ at room temperature (Ibanez-Tallon et al., 2004); velocities of $\sim 28 \mu\text{m/s}$ were recorded in the lateral and forth ventricles using pre-warmed medium (Banizs et al., 2005). By comparison, the fluid flow observed above the ciliated surface of the lateral and third ventricles of our vibratome sections had a velocity of 80-100 $\mu\text{m/s}$ (Lechtreck et al., 2008), indicating a better preservation of ciliary motility. We analyzed cilia at ambient temperature, which is probably why the CBF was below that reported in other studies ($\sim 18 \text{ Hz}$ compared to $\sim 40 \text{ Hz}$ in rats) (Mönkkönen et al., 2008). The CBF of airway cilia almost doubles when the temperature is increased by 8-10 $^{\circ}\text{C}$ (Delmotte and Sanderson, 2006).

V. Summary

Vibratome thick sections of the brain allow analysis of ependymal cilia from wild-type and mutant animals. Depending on the direction from which the cilia are viewed, various parameters (CBF, bending pattern, beat plane, coordination, and fluid flow) can be easily analyzed. The technique is useful to determine the effect of certain mutations on the motility of ependymal cilia and for physiological studies of wild-type cilia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Tissue preparation for *in vivo* imaging of ependymal cilia

A. Dorsal view of a murine brain. Trim along lines 1 and 2 and glue section plane 2 of brain onto the specimen holder of the vibratome. **B.** A coronal section cut approximately in the plane indicated with a dashed line (3) in panel A. The third (1) and the lateral (2) ventricles are marked. **C.** Coverslip chamber consisting of a Plexiglas support (1) containing a milled groove (2) fitting a 45 × 50-mm coverslip (3). The section is placed between this and a second coverslip (4) using silicon grease (5) as spacers and to seal the sides of the chamber. Optional: A shim cut from polyester mesh (6) is placed around the section to keep it in place and prevent damage from pressure. A polyethylene tube (ID 0.045", OD 0.062") is attached to one side of the chamber for removal of fluid (7). For constant flow, a second tube can be attached to the opposite side of the chamber. To analyze cilia-generated fluid flow, beads are added to the edge of the coverslip opposite the outflow tube. Removal of fluid via the tube (we use a vacuum pump to accomplish this) then moves the beads to the section. **D.** Side view of the observation chamber.

Fig. 2. Motion analysis of ependymal cilia

A. Bending pattern. A series of images from a recording of ependymal cilia and the corresponding tracings of an individual cilium (marked with arrowheads). The time in msec is indicated. **B.** Various phases of the power stroke (left side) and recovery stroke (right side) of the cilium traced in part A. **C.** CBF. Left side: Image from a movie showing the line used to generate a line scan. Right side: Line scan corresponding to 1 second (200 frames) of recording showing a regular pattern of diagonal lines (arrowheads) formed by the moving cilium. The CBF was estimated to be ~15 Hz. **D.** Fluid flow. Two images spaced 100 ms apart; three polystyrene beads marked with arrowheads have moved about 12 μm . **E.** Upper panel: Video image showing cilia from above (top view). Bottom panel: 8-frame image average of the same region showing parallel lines (arrowheads) indicating that the cilia beat in parallel planes. **F.** Left panel: Image showing cilia in front view. Note the sequential positions in the beat cycles of neighboring cilia, which results in metachronal waves (arrowheads). The line used to generate the linescan shown in the right panel is indicated. Right panel: Line scan showing regular diagonal lines (arrows) indicating metachrony of the cilia. Part F of this figure is reprinted from (Lehtreck et al., 2008) with permission of the journal.