Ependymal cell differentiation, from monociliated to multiciliated cells

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Abstract

Primary and motile cilia differ in their structure, composition, and function. In the brain, primary cilia are immotile signalling organelles present on neural stem cells and neurons. Multiple motile cilia are found on the surface of ependymal cells in all brain ventricles, where they contribute to the flow of cerebrospinal fluid. During development, monociliated ependymal progenitor cells differentiate into multiciliated ependymal cells, thus providing a simple system for studying the transition between these two stages. In this chapter, we provide protocols for immunofluorescence staining of developing ependymal cells in vivo, on whole mounts of lateral ventricle walls, and in vitro, on cultured ependymal cells. We also provide a list of markers we currently use to stain both types of cilia, including proteins at the ciliary membrane and tubulin posttranslational modifications of the axoneme.

INTRODUCTION

During development, ependymal cells derive from embryonic neural stem cells called radial glial cells (RGCs) (Spassky et al., 2005). During this differentiation process, a large number of centrioles (>50) assemble, migrate apically, and anchor at the plasma membrane, where they mature to become the basal bodies of motile cilia. The coordinated beating of these multiple motile cilia assures the flow of the cerebrospinal fluid (Zappaterra & Lehtinen, 2012). Defective ependymal cells cause major neurological disorders (Sharma, Berbari, & Yoder, 2008) including hydrocephalus, one of the most common developmental abnormalities of the brain, which is associated with a high rate of morbidity and mortality (Banizs et al., 2005; Ibanez-Tallon et al., 2004). Conversely, ependymal cells may also be recruited to form neurons after ischemia (Carlen et al., 2009).

The differentiation of RGCs into ependymal cells provides a simple experimental system to study both primary and motile cilia, as well as the transition between these two. Primary and motile cilia are structurally different. Primary cilia are composed of nine radially organized microtubule doublets (9 + 0), whereas motile cilia contain an additional central microtubule doublet (9 + 2) plus dynein arms that generate the shearing force needed for motility (Marshall & Kintner, 2008). Both types of cilia also differ by the kinds of receptors on their membrane and the posttranslational modifications of their microtubules.

In this review, we describe protocols for staining differentiating ependymal cells (1) on whole mounts of lateral ventricular walls and (2) on isolated primary culture cells. In addition, we provide a nonexhaustive list of cilia markers of primary or motile cilia or both.

1. METHODS

1.1 DISSECTION OF LATERAL VENTRICULAR WALLS FOR WHOLE-MOUNT STAINING

Ependymal cells differentiate progressively from the caudo-ventral to the rostro-dorsal part of the lateral walls between postnatal day 0 (P0) and P15 (Figure 1; Spassky
et al., 2005). The age at which the animals are sacrificed is therefore determined by the stage to be observed.

1.1.1 Materials
- Ca\(^{2+}\)/Mg\(^{2+}\) free phosphate buffered saline (PBS)
- Mayo and Spring scissors, Ultra-fine Micro Knife, and Dumont forceps #5 (Fine Science Tools) precleaned with soap and 70% ethanol
- Stereomicroscope
- Dissection glass dish covered with silicon

1.1.2 Procedure
The different steps of dissection are illustrated in Figure 2.
- After cervical dislocation, the head of the animal is opened with mayo scissors and the skin peeled off with a forceps. To remove the skull, incise at the lambda point (Figure 2(A), arrowhead), cut along the sagittal and interparietal suture with spring scissors and pull off the parietal and interparietal bones with a forceps (Figure 2(A), dashed lines). To avoid damaging the lateral walls located close to the surface of the anterior part of the telencephalon, do not insert the scissors deeply, and only make movements toward the exterior. Insert the forceps underneath the brain to cut the optic nerves. Carefully remove the brain from the cranial cavity and cover it with sterile PBS in the dissection dish. The following steps are performed under a stereomicroscope.
- With an Ultra-fine Micro Knife, cut along the longitudinal fissure of the brain to separate the two hemispheres (Figure 2(B), double arrow 1). Cut diagonally deep

![Figure 1](image-url)
underneath the cortex along the superior colliculus to separate the forebrain from the midbrain and the cerebellum (Figure 2(B)–(C), double arrows 2 and 3). To maintain the brain, the forceps can be inserted into the cerebellum.

- Place the brain with the cortex facing down. To maintain the half-brain, the forceps can be inserted above the corpus callosum and into the thalamus without damaging the lateral wall (Figure 2(D), dots). Cut the most caudal part of the half-brain. Insert the scalpel in the space between the cortex and the hippocampus (Figure 2(D) arrowhead). Cut the remaining caudal link between them by pulling the scalpel away from the tissue. This cut releases the medial wall and the hippocampus, which can then be pulled away from the lateral wall toward the anterior part of the telencephalon. Cut on each side to separate the lateral wall from the medial wall (Figure 2(E), arrowheads). This must be done with care because the two walls are connected; the cuts should be superficial to avoid damaging the lateral wall. This creates a pit between the lateral and medial walls where another cut can be made (Figure 2(F), dashed line). The forceps can be inserted into the olfactory bulb and above the corpus callosum in order to maintain the structure while cutting (Figure 2(E), dots).

- Finally, to allow better access by fixatives and antibodies, cut the cortex at the interface between the corpus callosum and the lateral wall and remove the thalamus (Figure 2(G), dashed line). Avoid touching, even gently, the underside of the lateral wall. Usually, the choroid plexus peels off while the thalamus is being cut. If not carefully removed, it can decrease the penetration of fixative.
and antibodies. The lateral wall is underlined with a white-dashed line in Figure 2(H).

Another description of the dissection of the lateral ventricular wall, with a video, has been published (Mirzadeh, Doetsch, Sawamoto, Wichterle, & Alvarez-Buylla, 2010).

1.2 EPENDYMAL CELL CULTURE

We usually use animals from P0 to P3 for these cultures. In our hands, younger animals give faster-growing and better-differentiating ependymal cells. After setting up the culture, the cells first expand until they reach confluence (around 4 to 5 days). They are subsequently plated for the second step in which they differentiate and grow multiple motile cilia (reaching 70–80% ependymal cells within 15 days, Figure 3).

1.2.1 Materials

- Poly-L-Lysine (Sigma) at 40 μg/mL, sterile H2O
- Hank’s solution: 1X Hank’s balanced salt solution (Gibco), 10 mM Hepes (Gibco), 0.075% sodium bicarbonate (Gibco), 1% Penicillin/Streptomycin (10,000 Units; Gibco, P/S)
- Enzymatic digestion solution: 30 μL of Papain (stock 2× crystalline suspension, Worthington), 15 μL of DNAse I (stock 1% in water; Worthington), 24 μL of L-Cysteine (stock 12 mg/mL in water; Sigma) in 1 mL of DMEM/Glutamax, 1% Penicillin/Streptomycin (10,000 Units; Gibco, P/S)

FIGURE 3 Differentiation of radial glial cells into ependymal cells in primary culture.

(A) Schematic representation of coverslips with a drop of cells in the middle at different stages of differentiation (days after serum withdrawal, from D0 to D12). (B) Representative images of the ciliary axoneme in the middle of the cell drop labeled with the glutamylation marker GT335 at different stages of differentiation, as in A. Scale bar = 5 μm.
• Stop solution: 1 mL of trypsin inhibitor (Stock 10 mg/mL trypsin inhibitor Worthington, 500 μg/mL BSA in PBS), 200 μL of DNase I (stock 1%; Worthington) in 10 mL of L15 (Gibco).
• L15 (Gibco), DMEM/Glutamax, decomplemented Fetal Bovine Serum (FBS), Penicillin/Streptomycin (P/S)
• Absolute ethanol, acetone
• Trypsin-EDTA, Ca2+/Mg2+ free-PBS
• 12 mm diameter round glass coverslips, 25-cm² flasks, 24-well plate

1.2.2 Procedure
The protocol is given for one newborn mouse telencephalon and should be adjusted to the number of animals.

• Coat a 25-cm² flask with Poly-L-Lysine for 1 h up to overnight at 37 °C. Rinse 3× with sterile water and let the flask dry under a sterile hood for at least 1 h. Flasks can be kept dried for one month in the refrigerator.
• Prepare and filter 1 mL of enzymatic digestion solution.
• Dissect the mouse telencephalon as described in Section 1 (step 1 to 3) in cold-filtered Hank’s solution in a sterile dissecting Petri dish. In addition, cut the olfactory bulbs and peel off the meninges at the surface of the telencephalon using a sharp forceps. With a small knife, cut the dissected telencephalon into pieces small enough to pass through a 1-mL pipette tip. Transfer the Hank’s solution and the pieces of telencephalon to a 15 mL Falcon tube using a 1-mL tip and centrifuge 1 min at 110 g.

Note 1: Dissection should be rapid and the dissected telencephalon kept on ice to minimize tissue damage.

Note 2: Up to six telencephala can be pooled together.
• Remove the supernatant and add 1 mL of the enzymatic digestion solution per brain. Incubate 45 min to 1 h at 37 °C.
• Prepare and filter 10 mL of stop solution. This volume can be used for up to four telencephala.
• Centrifuge 1 min at 110 g. Remove the supernatant and add the stop solution
• Centrifuge 1 min at 110 g. Remove the supernatant and add 10 mL of L15 to rinse. This volume can be used for up to six telencephala.
• Centrifuge 1 min at 110 g. Remove the supernatant, add 1 mL of L15, and carefully dissociate the cells mechanically with a 1-mL tip (do not pipette the cells more than 10 times). This volume can be used for up to six telencephala.
• Centrifuge 7 min at 110 g. Remove the supernatant and add 3 mL of DMEM/ Glutamax-10% FBS-1% P/S. Inoculate in the coated flask.
• Renew the media the following day.
• When the cells reach confluence, usually 4–5 days after inoculation, close the flask tightly and shake it at 250 rpm overnight at room temperature to remove weakly attached cells (mainly differentiated oligodendrocytes and neurons).
• Coat precleaned coverslips with Poly-L-Lysine at 40 μg/mL, in a 24-well plate for at least 1 h at 37 °C. Rinse 3× with sterile water and let the coverslips dry under the hood for at least 1 h.

Note 1: We usually clean the coverslips with a 20-min acetone bath, two 20-min baths in absolute ethanol, and dry/sterilization at 220 °C for 7 h.

Note 2: One mouse brain suffices for 12 coverslips.

• Remove the media from the flasks. Rinse with sterile Ca^{2+}/Mg^{2+}-free PBS. Add 1 mL of trypsin-EDTA solution and incubate for less than 5 min at 37 °C. After shaking briefly, the cells should float. Add 1 mL of decomplemented FBS. Count the cells with a hemocytometer. Centrifuge the cells 7 min at 110 g.

• Resuspend the cells in DMEM/Glutamax with 10% FBS and 1% P/S at a concentration of 2 × 10^5 cell per 20 μL. Carefully drop 20 μL of the cell suspension onto a coated, dried coverslip without touching the coverslip. Since the coverslip is dry, the drop should not spread. Incubate 1 h in a cell incubator to allow the cells to adhere at high density before adding 1 mL per well of DMEM/Glutamax, 10% FBS, 1% P/S.

• The following day (Day 0), rinse and incubate the cells in DMEM/Glutamax, 1% P/S without FBS. The culture is now mainly composed of ependymal progenitor cells that will progressively differentiate into ependymal cells (Figure 3).

1.3 IMMUNOCYTOCHEMISTRY OF Cilia
1.3.1 Fixation
Dissected lateral walls or cells on coverslips are fixed and rinsed in 6-well and 24-well plates, respectively. The fixation protocol depends on the marker to be analyzed. For example, proteins linked to the ciliary membrane are often sensitive to detergent and methanol fixation, whereas the antigens of proteins located at the base of cilia are often masked by paraformaldehyde (PFA) fixation (Figure 4). The accessibility of antibody epitopes can vary depending on the fixation. Therefore, different fixation protocols should be tested for new markers (See Figure 5 for antibodies tested in our laboratory). We usually perform immunofluorescence on the lateral wall just after fixation, and coverslips can be kept in PBS in the refrigerator for several days.

1.3.1.1 Paraformaldehyde fixation
Dissected lateral walls or cells on coverslips are immersed in 4% PFA in BRB80 (80 mM K-Pipes, 5 mM Na-EGTA, 5 mM MgCl₂ pH 6.8) for 20 min and 6 min, respectively. However, depending on the antibodies used, the fixation time may need to be adapted from 6 min at room temperature to overnight at 4 °C. Longer fixations facilitate dissection of the lateral wall prior to mounting. Many antibodies that do not work after PFA fixation might work if the sample is treated with detergent prior to fixation and if the fixation time is reduced. The detergent partly removes the cytoplasmic protein pool and increases accessibility of some antibodies. Different detergents can be used, such as Tween 20 (0.5–1%), NP40 (0.1–0.5%),
and Triton-X100 (0.1–1%) in BRB80. Except when labeling membrane proteins, we routinely use the strongest detergent, Triton-X100, to remove most of the cytoplasmic protein pool. Examples of immunofluorescence with different detergent pretreatments are presented in Figure 4(A) for a surface membrane protein enriched on cilia (CD24, (Mirzadeh, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008)). The dissected lateral wall or the cells on coverslips are then transferred into PBS.

1.3.1.2 Methanol fixation
Dissected lateral walls or cells on coverslips are rapidly immersed in fresh–20 °C methanol and kept at this temperature for 10 min. They are then transferred to PBS. This fixation procedure causes rapid dehydration and precipitation of proteins that often deforms tissues and, in our hands, produces curled cilia. Curling can be avoided by detergent treatment with 0.1% Triton-X100 in BRB80 for 1 min prior to fixation (Figure 4(B)).

1.3.2 Immunofluorescence
1.3.2.1 Materials
• Saturation solution: 10% FBS, 0.1% Triton-X100, and PBS
• Rinsing solution: 0.1% Triton-X100, PBS

FIGURE 4 Examples of fixation.
(A) Examples of short paraformaldehyde (PFA) fixations (6 min) of dissected lateral ventricle walls with different detergent pretreatments (1 min). Walls were stained for centrins (recognizing basal bodies, Red, 20H5) and a cell surface marker that is enriched in ependymal cells (Green, CD24). (B) Examples of different fixations of ependymal culture cells, with or without a 1-min 0.1% Triton-X100 detergent pretreatment, and subsequent staining for glutamylated tubulin (GT335). Scale bar = 5 μm. (See color plate)
### 1. Methods

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Antibody reference (subclass/dilution)</th>
<th>Fixation (Detergents)</th>
<th>Staining</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Signalling or membrane protnes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ad13b</td>
<td>UC Davis/NIH NeuroMab Facility #75-287 clone N21dQ66 (IgG2a/1:500)</td>
<td>PFA (NO)</td>
<td></td>
<td>Caspary et al. (2007)</td>
</tr>
<tr>
<td>ACIII</td>
<td>SantaCruz Sc588 (Rabbit 1:500)</td>
<td>PFA or MetOH (OK, 0.1%triton)</td>
<td>No</td>
<td>Bishop et al. (2007)</td>
</tr>
<tr>
<td>CD24</td>
<td>BD Biosciences #557436 CloneM1/69 (Rat 1:200)</td>
<td>PFA (NO)</td>
<td>Rarely</td>
<td>Calacora et al. (1996) Mirzadeh et al. (2008)</td>
</tr>
<tr>
<td>CD133/ Prominin1</td>
<td>eBiosciences #14-1331 (Rat 1:100)</td>
<td>PFA (NO)</td>
<td>Rarely</td>
<td>Mirzadeh et al. (2008)</td>
</tr>
<tr>
<td><strong>Posttranslational tubulin modifications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Acetylation</td>
<td>Sigma T6793 Clone 6-1181 (IgG2a 1:2000)</td>
<td>PFA or MetOH (OK, 0.1%triton decrease cytoplasmic microtubule staining)</td>
<td></td>
<td>LaDizet and Piperno (1991) Piperno and Fuller (1985)</td>
</tr>
<tr>
<td>Glutamylation</td>
<td>Adipogen #AG-20B-0020 Clone GT335 (IgG1 1:1000)</td>
<td>PFA or MetOH (OK, 0.1%triton)</td>
<td></td>
<td>Wolff et al. (1992)</td>
</tr>
<tr>
<td>Polyglutamylation</td>
<td>Adipogen #AG-28B-0030 purified antibody polyE</td>
<td>PFA or MetOH (OK, 0.1%triton)</td>
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<td>Rogowski et al. (2010) Shang, Li, Gorovoy (2002)</td>
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<td>Monoglycylation</td>
<td>Millipore #AB9277 Clone TAP952 (IgG1 1:1000)</td>
<td>PFA or MetOH (OK)</td>
<td>Rarely</td>
<td>Bre et al. (1995) Callen et al. (1994) Levilliers, Fleury, Hill (1985)</td>
</tr>
<tr>
<td>Detyrosination Polyglutamylation</td>
<td>Synaptic Systems #30201 Clone 126 (IgG1 1:1000)</td>
<td>? or MetOH (OK, 0.1%triton)</td>
<td></td>
<td>Rüdiger et al. (1995) Warn, Harrison, Planques, Robert-Nicoud, Weihland (1990)</td>
</tr>
<tr>
<td>Detyrosination</td>
<td>Millipore #AB20201 polyclonal antibody (Rabbit 1:1000)</td>
<td>PFA or MetOH (OK)</td>
<td></td>
<td>Patrue-Lafanechere et al. (1994)</td>
</tr>
<tr>
<td>(\Delta 2) tubulin</td>
<td>Millipore #AB3203 polyclonal antibody (Rabbit 1:1000)</td>
<td>PFA or MetOH (OK)</td>
<td></td>
<td>Patrue-Lafanechere et al. (1994)</td>
</tr>
</tbody>
</table>

**FIGURE 5** Table of currently used cilia markers with representative image. Fixations used in the representative images are in bold.

Scale bar = 5 \(\mu m\).
• Slides, 24 × 40 mm coverslips
• Fluoromount G (SouthernBiotech)
• Small spoon to manipulate the dissected lateral wall

1.3.2.2 Procedure
• Prior to immunostaining, dissected lateral walls or cells on coverslips are incubated 1 h in the saturation solution.
• We typically incubate the samples 2 h at room temperature with the primary antibodies. However, overnight incubation at 4 °C may improve staining for some markers. Incubation with secondary antibodies is performed for 1 h.
  Note: Each dissected lateral wall is incubated with the primary and secondary antibodies in the saturation solution in a 2-mL Eppendorf tube (volumes should be adapted to the age of the animals: 100 µL for P0 to 400 µL for adult telencephalon) and on Parafilm for the coverslips in a humidified chamber (45 µL are sufficient for one coverslip).
• Lateral walls or cells are rinsed after the primary and secondary antibodies for at least 2 × 5 min in the rinsing solution in 6- or 24-well plates, respectively.
  Note: Manipulation of the dissected lateral walls should be kept at a minimum to avoid damage. We usually use a small spoon to transfer the wall to the 2-mL Eppendorf tube and then pour off the wall from the 2-mL tubes into the rinse solution in a 6-well plate.
• Dissected lateral walls should be further dissected prior to mounting. Cut the cortex around and underneath the lateral wall. Care should be exercised, since the wall is curved and is not at the same depth in the rostral and caudal parts; the rostral part should be undercut first then the caudal part. Forceps can be inserted into the remaining thalamus in order to maintain the structure while cutting. Finally, the thalamus should be cut away. Transfer the lateral wall in a large drop (approximately 100 µL put on the coverslip using a 1-mL tip) of Fluoromount onto a slide. We usually put two dissected lateral walls on one slide. Cover the samples with the coverslip. Make sure that the amount of mounting media is sufficient for the surface of the coverslip, otherwise the sample might be damaged by the pressure generated while covering it.
• The cells on coverslips are quickly rinsed in water to remove salts and mounted on a drop of Fluoromount with the cells face down on the slide.
• Allow the Fluoromount to dry before observation.

1.3.3 Cilia markers
Different markers recognize primary and/or motile cilia, notably, antibodies that recognize proteins linked to the ciliary membrane or various posttranslational modifications of microtubules that are enriched in the axoneme. In the following section, we will describe a few markers that we recently used (see also Figure 5 for references, fixation procedures and antibody concentrations).
1.3.3.1 Signalling and membrane proteins

It is well established that primary cilia are sensory organelles in which signalling molecules are concentrated (Valente, Rosti, Gibbs, & Gleeson, 2013). However, the kinds of the signalling molecules in cilia vary according to cell type. In the laboratory, we currently use two different antibodies against signalling molecules to label the primary cilia of RGCs:

- **Arl13b** (ADP-ribosylation factor like 13b), a small GTPase of the Arf/Arl family that specifically associates with the ciliary membrane through a palmitoyl moiety (Cevik et al., 2010; Duldulao, Lee, & Sun, 2009; Horner & Caspary, 2011). We observe Arl13b mostly on primary cilia, however, residual staining of motile cilia is sometimes visible. Interestingly, it has been shown that ciliary membrane and Arl13b staining may remain at the basal body after axoneme resorption during the cell cycle (Paridaen, Wilsch-Brauninger, & Huttner, 2013).

- **Adenylate cyclase III** that converts ATP to cAMP is present on RGC primary cilia (Bishop, Berbari, Lewis, & Mykytyn, 2007).

Several studies have identified receptors on motile cilia; for example, the bitter taste receptor T2R on airway epithelial cells (Shah, Ben-Shahar, Moninger, Kline, & Welsh, 2009) or progesterone (Natu et al., 2009; Teilmann, Clement, Thorup, Bykov, & Christensen, 2006), estrogen (Shao et al., 2007), and interleukin-6 (Shao et al., 2009) receptors on oviduct epithelium. Motile ependymal cilia can also be enriched in receptors such as platelet-derived and epidermal growth factor receptors (Danilov et al., 2009), and in signalling molecules such as polycystin-1 (Wodarczyk et al., 2009) or Vangl2 (Guirao et al., 2010). However, we usually stain the motile cilia with the pan-ependymal surface marker CD24.

- **CD24**, a glycosylphosphatidylinositol-anchored membrane protein attached to the exterior leaflet of the plasma membrane, is enriched at the surface of ependymal cells and therefore along the ciliary membrane, thus allowing the detection of motile cilia (Calaora, Chazal, Nielsen, Rougon, & Moreau, 1996; Mirzadeh et al., 2008). Rare long-primary cilia can sometimes be observed with this antibody. Coupled to a fluorophore, this antibody can be used for video microscopy experiments.

- **CD133** (also named prominin1) is a multipass membrane protein proposed to play a role in the organization of the apical membrane and bind cholesterol. Like CD24, it is enriched at the surface of ependymal cells including along motile cilia (Pfenninger et al., 2007). It might also label primary cilia and/or the cell surface of neural stem cells (Coskun et al., 2008).

1.3.3.2 Posttranslational microtubule modifications

Microtubules are the structural basis of the ciliary axoneme (Satir & Christensen, 2007). A particularity of axonomal microtubules is the large number of posttranslational tubulin modifications; thus, many antibodies that specifically detect those
modifications have been used as cilia markers. A more detailed analysis of the dynamics of posttranslational tubulin modifications in ependymal cells indicates that the presence of these modifications on ciliary axonemes depends on the developmental stage, i.e., the maturity of the cilia (Bosch Grau et al., 2013); thus, the antibodies should be chosen with care.

The tubulin-modification marker, most commonly used for ciliary staining, is the antibody 6-11B-1 (LeDizet & Piperno, 1991; Piperno & Fuller, 1985), which specifically detects the acetylation of lysine-40 of alpha-tubulin. Though the modified residue lies inside the assembled microtubules, the antibody nicely labels acetylated microtubules in fixed cells. Primary cilia are also completely labeled, indicating that the fixation procedure might partially damage axonemal microtubules giving access to the previously hidden epitope for this antibody. On motile cilia fixed with PFA, however, 6-11B-1 strongly labels the tip of the cilia; labeling decreases toward the base of the cilia. This effect is more pronounced in aged cilia and becomes problematic for the labeling of motile cilia in the ventricle walls of adult mice. This can be circumvented by fixation with cold methanol after Triton-X100 treatment, which allows staining of the entire length of primary and motile cilia (Figure 6(A)).

All other tubulin-modification markers used to label cilia detect post-translational modifications in the carboxy-terminal tails of tubulin, such as (poly)glutamylation, (poly)glycylation, detyrosination, and Δ2-tubulin. Since the carboxy-terminal tails

FIGURE 6 Examples of immunostaining of posttranslational tubulin modifications.
(A) Immunostaining of tubulin acetylation (6-11B1) in PFA or triton-MeOH fixed cells.
(B) Triple Immunostaining of tubulin polyglutamylation (green, polyE), monoglycylation (red, TAP952) and acetylation (white, 6-11B1) in differentiating ependymal cells; monoglycylation on primary or motile long cilia is shown. These antibodies can be labeled simultaneously thanks to their different mouse subclasses (IgG1 and IgG2b). Scale bar = 5 μm. (See color plate)
are situated outside the assembled microtubules (Janke & Bulinski, 2011), they are freely accessible for the antibodies.

Tubulin glutamylation is detected with the antibody GT335 (Wolff et al., 1992), which recognizes the branch point of the glutamate-side chain independently of its final length. Therefore, this antibody is a rather broad marker of tubulin glutamylation and polyglutamylation and nicely labels primary as well as motile cilia at all developmental stages (Figure 1 and 2). More recently, an antibody that labels long carboxy-terminal glutamate chains has been used successfully to detect polyglutamylation of microtubules (polyE), and is thus even more specific for primary and motile cilia since axonemes are modified by long glutamate-side chains. The antibody shows some sensitivity to the fixation method used (Magiera & Janke, 2013), but once the experimental conditions have been determined, the antibody is a nice cilia marker. For example, fixation with Triton-X100 and methanol allow robust staining, although methanol fixation sometimes lead to spotty labeling with this antibody. PolyE also visualizes the progression of glutamate-side chain elongation during axonemal ageing: a gradient of immunoreactivity is observed from the base to the tip of the cilia (Figure 6(B)).

Glycylation and polyglycylation have so far been detected exclusively on ciliary axonemes and seem to be particularly concentrated in motile cilia. Antibodies against these modifications are thus ideal markers of motile cilia, although with some limitations. The antibody TAP952 specifically and selectively detects monoglycylation, i.e., the addition of a single glycine residue onto its modification site—a glutamate residue within the primary sequence of tubulin. Thus, in contrast to GT335 for glutamylation, TAP952 cannot detect glycylation once the chains become elongated. Longer glycine chains are detected by two other antibodies: AXO 49 and polyG, which specifically recognize carboxy-terminal glycine chains with more than three or four glycine residues, respectively. No antibody exists so far for the detection of glycine side chains with two residues. In contrast to glutamylation, glycylation is generated after ciliary assembly. Monoglycylation, detected with TAP952, appears on long cilia, whereas polyglycylation, detected with polyG, appears only on long mature cilia about one month after assembly of the motile ependymal cilia (Figure 6(B); Bosch Grau et al., 2013). Primary cilia are only slightly labeled with TAP952; only long primary cilia can be seen on developing ventricle walls. It thus appears that TAP952, a good marker of mature motile cilia, does not efficiently stain primary cilia.

Detyrosinated tubulin and Δ2-tubulin also accumulate in axonemal microtubules; thus, antibodies specific for those modifications (anti-detyr-tubulin and anti-Δ2-tubulin polyclonal antibodies, Millipore) can be used as markers of both primary and motile cilia. The generation of Δ2-tubulin is a follow-up modification of detyrosination and might thus appear slightly later than detyrosination. However, Δ2-tubulin has an important advantage: it is scarcely found on cytoplasmic microtubules of ciliated cells.

Finally, the monoclonal antibody 1D5 is a very good cilia marker; however, this antibody detects two distinct tubulin modifications and can therefore not been used
to draw conclusions on the modification status of the observed cilia. 1D5 is specific to C-terminal sequences of two glutamate residues; it thus labels detyrosinated tubulin as well as polyglutamylated tubulin, both of which are enriched in cilia.

Thus, due to the large spectrum of posttranslational tubulin modifications on ciliary axonemes, many tubulin-modification-specific antibodies can be used as cilia markers. However, it should not be forgotten that each of these modifications plays a functional role in cilia. Furthermore, not all of the modifications are present on all subtypes of cilia, and are not necessarily labeled with equal intensity over the whole length of the ciliary axoneme. We therefore recommend testing different combinations of antibodies to assure reliable labeling of cilia under specific experimental conditions.

CONCLUSION

In this chapter, we have described the protocols currently used to stain different types of cilia in differentiating ependymal cells in vivo or in vitro. We also gave an overview of the different markers we use to recognize primary or motile cilia, with their specificities and fixation requirements. The ependymal system is unique in that it allows analysis of the transition between primary and motile cilia in vivo as well as in vitro.

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CHAPTER 2 Ependymal cell differentiation


