CHAPTER SIX

Probing Ciliogenesis Using Micropatterned Substrates

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Abstract
The primary cilium is a biomechanical sensor plugged in at the cell surface. It is implicated in the processing of extracellular signals and its absence or malfunctioning lead to a broad variety of serious defects known as ciliopathies. Unfortunately, the precise mechanisms underlying primary cilium assembly and operation are still poorly understood. Molecular dynamics and intracellular morphogenesis are easier to study in cell culture than in tissues. However, cultured cells are usually nonciliated and the empirical methods that are used to induce ciliogenesis in these cells have variable efficiencies. In addition, these methods require cells to be cultured at high density, which is not convenient for further automated image analysis. Here, we describe a method to induce and modulate ciliogenesis in mammalian cells in culture that is compatible with...
high-throughput imaging and analysis. Surface micropatterning is used to normalize cell shape and actin network architecture. In these conditions, the deprivation of growth factor induces ciliogenesis in individual single cells. The manipulation of cell-spreading area is used to modulate the proportion of ciliated cells. The manipulation of cell adhesion geometry is used to orient the position of the primary cilium. The spatial disposition of cells on a regular array offers a simple way to perform automated image acquisition. In addition, the regular cell shape is convenient to perform robust and automated image analysis to quantify the presence and location of primary cilia. This method offers a new way to study ciliogenesis in automated and high-throughput assays.

1. INTRODUCTION

The primary cilium is a microtubule-based hair-like antenna that projects from the surface of most quiescent mammalian cells. Numerous reports in the past decade have revealed that the primary cilium is a key biomechanical cell sensor implicated in major cell functions such as growth, polarization, and differentiation (Gerdes, Davis, & Katsanis, 2009). Although many recent studies have led to the identification of a large number of molecular players involved in primary cilium assembly and function, the actual mechanisms supporting these processes are still largely unknown (Garcia-Gonzalo & Reiter, 2012; Ishikawa & Marshall, 2011). Therefore, it is useful to investigate these basic principles in cultured cell in vitro, where cells are amenable to precise genetic, physical, and biochemical manipulations and observation. Later on, it will be possible to specifically investigate how the principles identified in cultured cells apply to cell functions in tissues and living animals.

Assembly of the primary cilium (known as ciliogenesis) can be induced in cultured cells. It depends on two main parameters. First, cells have to exit the cell cycle (Plotnikova, Golemis, & Pugacheva, 2008; Pugacheva, Jablonski, Hartman, Henske, & Golemis, 2007), and second, they have to be spatially confined (Alieva & Vorobjev, 2004; Wheatley, Feilen, Yin, & Wheatley, 1994). In some cell types, both parameters may be directly related, and cell cycle exit can be triggered by cell confluence. In others, cell cycle exit must be externally induced by the removal of growth factors from the culture medium. In both cases, the degree of confluency strongly affects the rate of ciliogenesis. Since it is difficult to precisely control this parameter in cultured cells, broad range of ciliogenesis rate has been reported for given cell types. This variability is somehow limiting the possibility to study the fine mechanism regulating ciliogenesis.
Here, we first describe a method to control individual cell spreading on micropatterned surfaces coated with extracellular matrix (ECM) (Fig. 6.1A) and then describe how these cells can be automatically recorded and analyzed. ECM micropatterns provide a reliable way to control cell shape and also offer the possibility to manipulate numerous processes involved at various stages of ciliogenesis. Indeed, the spatial distribution of cell adhesions constitutes the structural basis for the assembly of cell cytoskeleton, and thereby, it directs cell compartmentalization. Variations in the size and the shape of the cell adhesion pattern have been shown to finely modulate and orient actin network architecture, the spatial distribution of microtubules,

Figure 6.1 Array of ciliated single cells. (A) Array of fibronectin-coated micropatterns (left) on which individual RPE1 cells are confined (right). (B) Actin (green), nucleus (blue), and primary cilium (decorated with antibodies against acetylated tubulin, red) in a single cell confined on a 750-μm² micropattern.
intracellular trafficking, cell polarization, cell division, and, in a less direct but still reliable way, cell differentiation (Théry, 2010). Ciliogenesis involving many of these structures and intracellular processes is also strongly modulated by cell adhesion pattern (Pitaval, Tseng, Bornens, & Théry, 2010). Most cells that are spatially confined on small micropatterns assemble a primary cilium on their dorsal surface (Fig. 6.1B). On the opposite extreme, cells that are extensively spread on large micropatterns cannot assemble a primary cilium although they have left the cell cycle. Variations in the degree of cell spreading results in variations in ciliogenesis rate (e.g., from 80% to 20% in RPE1 cells) as well as in the length of the primary cilium (from more than 5 μm in confined cells to less than 2 μm for the few cilia observed in spread cells) (Pitaval et al., 2010).

In addition to the control of ciliogenesis, micropatterned surfaces offer the possibility to automatize image acquisition, thanks to the predictable cell positioning on the regular array of micropatterns (Fig. 6.1A). They also facilitate image analysis, thanks to cell shape normalization. Here, we describe how classical motorized microscopes can be employed to perform a quantitative measurement and fine description of the various steps of ciliogenesis, notably the positioning of the basal body and the elongation of the primary cilium.

2. MICROPATTERNED SUBSTRATES FABRICATION

Several micropatterning methods have been developed to control cell adhesion, including photolithography (Guillou et al., 2008), microcontact printing (Quist, Pavlovic, & Oscarsson, 2005; Ruiz & Chen, 2007), or photo-patterning (Azioune, Storch, Bornens, Théry, & Piel, 2009; Kaneko et al., 2011). After testing most of them, we concluded that deep-UV patterning (Azioune, Carpi, Tseng, Théry, & Piel, 2010) was the most efficient and easiest to install in any laboratory without numerous dedicated equipment or specific know-how. It is relatively fast (less than 2 h) and based on commercially available products. It offers a reproducible protein grafting on the micropatterned regions and a highly repellent coating around them. Here, we not only describe the complete microfabrication protocol (Fig. 6.2) but also suggest some commercial alternatives to circumvent some of the steps requiring specific and costly equipment. The simplest alternative is to order micropatterned glass slides from dedicated companies (Cytoo, France).

Required materials and equipment
- Quartz Photomask (Toppan Photomask, DeltaMask)
- Glass coverslips
Figure 6.2  Surface micropatterning. Steps 1a and 1b are two alternatives. Step 1a illustrates glass slide coating with a thin layer of polystyrene (PS), whereas step 1b illustrates the use of a bare glass slide. Step 2 illustrates slide activation by exposition to oxygen plasma (or deep UV). Step 3 shows how to lay upside down the activated slide on a polylysine–polyethylene glycol (PLL–PEG) solution. Steps 4a and 4b are two alternatives. Step 4a shows vacuum-induced contact between the photomask and the pegylated slide with a homemade mask holder. Step 4b shows capillarity-induced contact between the photomask and the pegylated slide with a water drop. Step 5 shows how to lay upside down the micropatterned slide on a fibronectin/fibrinogen solution. Step 6 shows the final result after rinsing.
- Phosphate-buffered saline (PBS+/−) (Invitrogen, Gibco ref 14040-091)
- PLL(20)-g[3.6]-PEG(2) (Surface Solutions, Switzerland) (stock solution at 1 mg/ml in 10 mM HEPES buffer, pH 7.4)
- Isopropanol
- Acetone
- Ethanol 96%
- UV ozone cleaner (e.g., UVO cleaner Jelight ref 342-220 or Heraeus Noblelight GmH ref NIQ 60/35 XL longlife lamp, λ=185 and 254 nm, quartz tube, 60 W)
- Fibronectin (Sigma F1141) or other ECM
- Alexa Fluor 647 human fibrinogen conjugate (Invitrogen ref F35200)

Facultative materials and equipment
- Software for designing micropattern features (L-edit, Cadence, or Clewin) (facultative)
- Plasma cleaner (PDC-32G, Harrick) (facultative)
- Spin coater (WS-400-6NPP-LITE Laurell Technologies Corporation) (facultative)
- TI Prime (MicroChemicals) (facultative)
- Hot plate (facultative)
- 1% Polystyrene (178890250, ACROS Organics) dissolved in toluene (facultative)
- Homemade vacuum mask holder (facultative)
- Filtered airflow (facultative)

2.1. Designing features of micropatterns

Several softwares can be used to design micropatterns. Like many drawing software, they record the graph coordinates. Using elementary drawing tools, you can draw any shape of interest and then duplicate that shape on a regular array. The final file has then to be exported in a specific format either CIF or GDSII. Such file format can then be used by the photomask manufacturer to direct an e-beam to physically draw the shapes and remove the thin chromium layer lying on a 5-mm-thick quartz plate. Usually, photomask manufacturers can also draw themselves your micropattern in their dedicated softwares in order to convert your manual or schematic drawing into a GDSII or CIF files (additional cost has to be foreseen).

The efficacy of the micropatterned slides to normalize cell phenotypes depends on three key micropattern features: the micropattern size, shape, and the step between them.
The right micropattern size depends on cell type and on the observed phenotypes. In frequent cases, micropatterns are used to normalize and observe cell internal organization. The cell “body plan” is easier to visualize in 2D and simpler to quantify with basic (but robust) image analysis procedures when cells are as spread as possible. It is therefore necessary to find the largest area cells can spread on. However, when the area is too large, cells can move on the micropattern and adopt different shapes from one micropattern to the other. This would result in a loss of cell normalization. The right spreading area for observation of internal cell organization in 2D is thus the largest spreading area that can be fully covered by more than 90% of the cells. The right micropattern size for most cell types is between 500 and 1500 μm². Most epithelial cells such as MCF10A, MDCK need to be plated on small micropatterns about 500 μm². Most fibroblasts such as NIH3T3 require larger micropattern sizes around 1500 μm². However, some cell types such as embryonic fibroblast can require much larger areas (up to 10,000 μm²). Importantly, it should be noted that, contrary to the above example, the right micropattern size to induce ciliogenesis is not the one allowing the largest spreading area but on the opposite it should confine cells on small adhesive regions.

The right micropattern shape also depends on the observed phenotype. In most cases, such as the one studied here, a simple disc is sufficient to normalize cell shape and define a basal-like adhesive ventral surface and an apical-like nonadhesive dorsal surface (Pitaval et al., 2010). Asymmetric geometries such as pac-man or crossbow-shaped micropatterns force the cells to spread upon adhesive and nonadhesive regions. This will induce distinct organization of the actin network, mainly stress fibers above nonadhesive regions and dense and branched polymerizing filaments above adhesive regions. The respective locations of these two types of actin-based structures bias the planar cell symmetry and orient cell polarity (centrosome–nucleus axis as well as internal traffic) in the adhesion plane (James, Goluch, Hu, Liu, & Mrksich, 2008; Schauer et al., 2010; Théry et al., 2006). This orientation can result in a more normalized internal architecture since cells receive spatial information not only along the Z axis but also in the XY plane.

The right distance between the micropatterns depends on cell ability to generate membrane protrusions and contact adjacent micropatterns, which is something that should be avoided. Epithelial cell types appeared to be less efficient than fibroblasts in this distant spatial exploration, so they could be disposed on a dense array with smaller steps in between micropatterns. As a rule of thumb, impose at least one cell diameter in between two
micropatterns. Most often a distance of 100 μm is sufficient to prevent all types of cell–cell interactions or bridges in between the micropatterns. It is convenient to use the same interpattern distance for all mask designs in order to apply the same automatized image acquisition procedures for all conditions (see below).

Finally, it should be noted that one can easily get lost while moving the sample on the stage microscope and looking at the cell array. Visible marks can facilitate cell location appreciation. For example, replacing every 10 rows and columns of micropatterns by large adhesive bars defines a square working area of 100 micropatterns, the size of which fits with the observation field of a 10 × objective.

2.2. Micropatterned slides manufacturing

This step consists in coating a glass slide with a cytophobic polymer (that prevents protein adsorption and cell attachment) and locally destroying this polymer to allow protein and cell adhesion. Poly(ethylene–glycol) (PEG) has proven to be a very efficient cytophobic polymer which generates a water solvation layer preventing protein access to the underlying surface. The use of a copolymer made of a backbone of polylysine (PLL) with PEG side chains is very convenient to graft PEG chains on a negatively charged surface using the positive charges of the PLL (Heuberger, Drobek, & Spencer, 2005). Contrary to most PEG functionalization protocols, the use of PLL–PEG circumvents the need to control sensitive chemical reactions such as the ones involved in silanization, for example. PLL–PEG can adsorb on negatively charged surface in a few minutes in aqueous solution. PEG chains can then be locally oxidized in various ways in order to destroy their conformation and water solvation properties. Here, we describe the use of surface exposure to deep UV light through an optical mask. The exact effect of UV-generated ozone on PEG chain is not clear, but the consequence is that proteins can then adsorb on the exposed regions. All these steps can be performed on bare glass slides. Our experience revealed that a precoating of the glass slide with a thin layer of polystyrene (PS) improved protein adsorption of the UV-exposed regions. But this is a facultative step that, although easy to perform, can be ignored by those who do not have access to a spin coater.

2.2.1 PS coating

This step is not critical but can be used to improve protein and cell adhesion on the micropatterns (Fig. 6.2–1a) compared to bare glass (Fig. 6.2–1b).
1. Wash the glass coverslips (20 mm × 20 mm) with ethanol 96% and dry them with filtered airflow or let them dry under a hood.
2. Put a coverslip on the spin coater, cover it with TI prime and spin it for 30 s at 3000 rpm.
3. Bake the coverslip at 120 °C for 2 min on a hot plate.
4. Put the coverslip back on the spin coater, cover it with the 1% PS solution, and spin it for 30 s at 1000 rpm. Toluene will evaporate immediately.

PS-coated slides can be stored at room temperature.

2.2.2 PLL-g-PEG coating

1. The PS-coated slides should then be exposed to oxygen plasma for 30 s at 30 W (Fig. 6.2–2). Alternatively, for those who do not have access to a plasma generator, PS-coated slides can be exposed to the lamp-generating deep UV for 2 min. Glass slides without the PS coating can also be exposed either to oxygen plasma (2 min at 100 W) or deep UV (10 min).
2. The slides are then incubated with PLL-g-PEG at 0.1 mg/ml in 10 mM HEPES (pH = 7.4) at room temperature for 30 min. To spare reagents, it is possible to put a 100 μl PLL–PEG droplet on a parafilm and place the slide on top of it (the oxidized side of the slide should face the droplet) (Fig. 6.2–3).
3. Slowly lift off the coverslip to ensure complete PLL-g-PEG solution dewetting. Do not rinse it. Remove the last drop of PLL-g-PEG on the slide edges with kimwipes or gentle airflow.

Pegylated coverslips can be stored at 4 °C for 1 week.

2.2.3 Surface patterning

Place the UV ozone cleaner under a chemical hood because of ozone production.

1. Switch on the UV ozone cleaner (or deep UV light) for 10 min before using in order to warm the lamp and work with a reproducible amount of UV.
2. Clean the photomask with acetone to remove organic residues and then with isopropanol to remove inorganic residues and acetone traces.
3. Dry the photomask with filtered airflow.

It is crucial to create an intimate contact between the slide and the mask. This can be achieved either by using a dedicated mask holder in which the vacuum is used to press the slides onto the mask (Fig. 6.2–4a) or by simply using the capillary force of water to stick the slide to the mask (Fig. 6.2–4b). Here are described the two possibilities:
The vacuum mask holder is a plate (larger than the mask) with holes through which the vacuum is applied.

4. Slides are placed on this plate and covered with the mask; the pegylated side of the slides should face the chromium side of the mask. The vacuum is open to aspirate the mask onto the slides, which are now pressed between the mask and the plate (Fig. 6.2-4a).

5. The sandwich is placed in the UV ozone cleaner and exposed to deep UV for 2 min at about 5 cm from the lamp.

6. Switch off the vacuum and separate the plate and the mask. Detach cautiously the coverslips from the mask. Special care must be taken at this step in order not to damage the chromium layer on the mask. A plastic micropipette can be used to lift up the coverslip. Do not use metallic tweezers. The vacuum can also be used to aspirate the slides and detach them from the mask without using any tweezer. Switch to step 7.

For those who cannot manufacture their own mask holder, it is possible to use a water drop.

4. Place the pegylated side of the coverslip in contact with the chromium-coated side of the photomask with a drop of water (for a 20-mm coverslip, use a drop of 1.5 μl of water on the mask). Formation of air bubbles must be prevented (Fig. 6.2-4b).

5. The sandwich is placed in the UV ozone cleaner and exposed to deep UV for 2 min at about 5 cm from the lamp.

6. Detach cautiously the coverslips from the mask. Special care must be taken at this step in order not to damage the chromium layer on the mask. Add water around the slide and wait until it is lifted up. Do not use a metallic tweezers.

7. Incubate the coverslips with PBS during 10 min to induce PEG chains swelling before the protein coating step. Note that when removing the slide from the PBS, it is now possible to observe the micropatterned regions during water dewetting. It is a good sign of experimental success.

8. Incubate the micropatterned slides with the fibronectin solution (Fibronectin at 25 μg/ml and Alexa647-Fibrinogen at 10 μg/ml in PBS) for 30 min at room temperature (Fig. 6.2-5). Avoid longer incubation time since it would result in protein background out of the micropatterned regions. To spare fibronectin solution, it is possible to put a 100-μl droplet on a parafilm and place the micropatterned slide on top of it (the micropatterned side of the slide should face the droplet).

9. Rinse with PBS and check on the microscope for the specific grafting of fluorescent proteins on the micropatterns (Fig. 6.2-6).
3. CELLS PLATING ON MICROPATTERNED SLIDES

Here, we describe how to culture immortalized human retinal pigment epithelial (RPE1) cells, plate them on micropatterns, and then starve them in order to induce their ciliogenesis while they are attached on and therefore normalized by the micropattern. Required materials and equipment

- hTert-RPE1 (CRL-4000, ATCC)
- Phosphate-buffered saline (PBS+/+) (14040-091, Invitrogen Gibco)
- Trypsin–EDTA (0.25%) in HBSS without Ca\(^{2+}\)/Mg\(^{2+}\) (25300-054, Invitrogen Gibco)
- Thymidine (T1895, Sigma–Aldrich)
- Growth medium: DMEM/F12 (31331, Invitrogen Gibco) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 U/ml streptomycin.
- Assay medium: DMEM/F12 (31331, Invitrogen Gibco) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin (no serum).

3.1. Cell culture

The protocol has been established for human telomerase-immortalized retinal-pigmented epithelial cells (hTERT-RPE1), which is an appropriate model to study ciliogenesis since this cell type is actually ciliated in the retina where they form a thin monolayer as they do in culture. RPE1 are not transformed and they conserved their contact inhibition properties as well as the checkpoints for cell cycle progression.

hTERT-RPE1 can be grown in DMEM/F12 medium according to classical cell culture practices. Noteworthy, it is important to prevent the cells from reaching confluence. The reasons are not well known, but the consequence is a significant reduction of primary cilium incidence upon serum starvation in the following experiments. Therefore, it is necessary to check regularly for their ciliogenesis capacities by plating them at high density, starving them for 24 h once they reached confluence, and revealing their cilia with an immunostaining against acetylated tubulin (see below).

3.2. Cell synchronization (facultative)

RPE1 cells ability to exit cell cycle in response to serum starvation depends on their position in the cell cycle (Pitaval et al., 2010). Cells that are in early G1 when the serum is removed exit the cell cycle straight away. Cells that are
anywhere else in the cycle are committed to division although growth factors have been removed. They keep on progressing through the cell cycle until they divide. Right after division, daughter cells are in early G1. They can now exit the cell cycle and enter the ciliogenesis process. On micro-pattern, this latter scenario would be a matter of concern since micropatterns would be occupied by pairs of daughter cells rather than by a single cell, which is a requisite for cell phenotype normalization by cell shape control. Therefore, it is useful to synchronize cell cycles in order to plate cells in early G1 and obtain a maximum incidence of single ciliated cells. To this end, we performed a double thymidine block. But it should be noted that cell synchronization is facultative and performed here to increase the proportion of isolated ciliated cells on micropattern.

Start the procedure at the end of your working day in order to perform the blocking steps overnight and obtain cells ready to be released in the morning.

1. End of day 1: Remove the culture medium of a 75-cm² flask in which cells are about 40% of confluence.
2. Add 12 ml of prewarmed growth medium and 600 µl of thymidine at 100 mM (in order to induce a final thymidine concentration of 5 mM). This excess of thymidine with respect to the other nucleotides induces a feedback inhibition of DNA synthesis that blocks cells that are in S phase.
3. Keep the flask in the incubator for 16 h. Cells that were not in S phase when thymidine has been added progress through the cell cycle and become blocked at the G1/S transition.
4. Beginning of day 2: Remove the medium and wash cells twice with warmed PBS +/+/ for 5 min to remove thymidine excess.
5. Add new prewarmed growth medium. The G1/S block is released and cells can progress through S phase. Cells synchrony is not optimal since released cells are at various stages in the 2-h-long S phase.
6. Keep the flask in the incubator for 8 h.
7. End of day 2: Remove the culture medium and add 12 ml of prewarmed growth medium with 600 µl of thymidine at 100 mM (in order to induce a final thymidine concentration of 5 mM).
8. Keep the flask in the incubator for 16 h. All cells will progress through the cell cycle and become blocked at the G1/S transition.
9. Beginning of day 3: Add new prewarmed growth medium. The G1/S block is released and cells can progress through S phase. Cells are now well synchronized.
10. Keep the flask in the incubator for 10 h. During this period, cells will progress through S, G2, and M phase and enter the G1 phase.

11. End of day 3 (which will be a long working day): There are still many round cells in mitosis at this step. It is, however, necessary to perform this step now to make sure that cells that went through mitosis already will not have time to progress too far in the G1 phase. Shake off the mitotic cells and remove culture medium to get rid of them. Detach and plate the cells on the micropatterned slide.

Note that this synchronization procedure is quite long and impose a 12-h-long working day during which cells are released from the G1/S block in the morning, collected in early G1 10 h later to start a 1-h-long plating procedure on the micropatterned slide. Alternatively, it might be convenient to use a Cdk1 inhibitor (RO-3306) in order to block cells at the G2/M transition in a single-step protocol (Vassilev, 2006) and obtain cells in early G1 earlier in the working day. However, we did not have the opportunity to test it on RPE1 cells.

### 3.3. Cell plating and serum starvation

This step is critical since it will define the rate of micropattern occupancy by single cells and therefore the number of exploitable cells per condition. Plating too many cells would result in aggregation of several cells per micropattern preventing cell analysis. Plating too few cells would result in single cell occupancy but few measurements. The best procedure to obtain high occupancy rate is to plate many cells and wash them before they could all attach to the micropattern.

Serum starvation will be performed once the cells are spread onto the micropattern. Indeed, resuspending cells in the medium without serum and plating them on the micropattern in this medium would promote their attachment out of the micropatterned regions. Serum starvation induces a short-term cell response in which actin dynamics is modified. In these conditions, cells can spread without much adhesion to the substrate. It is therefore preferable to plate cells in serum-containing medium and switch the medium once the cells are confined on their micropatterns.

1. Immerse the micropatterned slide in PBS for 10 min in a Petri dish.
2. Aspirate the PBS and plate about 10,000 cells/cm² in growth medium (roughly 100,000 cells for a 35-mm round Petri dish).

Cell adhesion on micropatterned slides is longer than on classical Petri dish. A consequence is that, large volumes, in which temperature
changes induce fluid convection, promote floating cell aggregation at the dish center, whereas in small volume, cells move less and attach uniformly all over the slide. So the optimal volume to plate cells should be as small as possible (1 ml for a 35-mm round Petri dish).

3. 30 min after plating: Check on the microscope that cells have started to spread on micropatterns. If the number of attached cells is sufficient, remove the floating cells by gently aspirating the medium with a pipetman mounted with a 1-ml tip on one side of the dish while adding some new medium on the other. Do not use vacuum-based aspiration. Indeed, a strong aspiration could induce a complete medium dewetting (due to PEG physicochemical properties) and dry cell to death.

4. Put the cells back in the incubator for 1 h so that they have to complete their spreading.

5. Aspirate the growth medium with a pipetman mounted with a 1-ml tip and add 2 ml of the assay medium (without fetal calf serum).

6. Put the cells back in the incubator for 24–48 h.

4. CELL FIXATION AND IMMUNOFLUORESCENCE

Primary cilia are thin tubular structures protruding out of the cells. Therefore, they could be easily cut or damaged during fixation. Methanol fixation, for example, is based on the substitution of water by alcohol. It involves fast and violent liquids exchange, which can detach some of the primary cilia. Formaldehyde fixation preserves cilia but does not systematically preserve antigen immunogenicity. We found that fixation with a mix of acetone and methanol preserves cilia and as well as cilia- and basal body-associated antigens immunogenicity (Fig. 6.3).

Required materials and equipment
- Methyl alcohol (414814, Carlo Era)
- Acetone (32201, Sigma–Aldrich)
- BSA 1.5% powder (A9647, Sigma–Aldrich)
- Mouse Acetylated tubulin (T7451, Sigma–Aldrich)
- Rabbit Gamma tubulin (ab11317, Abcam)
- Cy3-conjugated goat anti-mouse (115-165-146, Jackson Immunoresearch)
- 488 Alexa-conjugated goat anti-rabbit (111-485-045, Jackson Immunoresearch)
- Hoechst 33342 solution at 2 mg/ml (62249, ThermoScientific)
4.1. Cell fixation

During this step, as well as during medium switching after cell plating, care should be taken not to induce medium dewetting and cell drying.

1. Prepare a methanol/acetone solution (1/1) and keep it at $-20 \, ^\circ \text{C}$ for few hours.

2. Do not wash the slide with PBS prior to cell fixation. Aspirate gently the medium with a pipette in the corner. Add quickly 2 ml of cold methanol/acetone solution in the corner and not directly on the slide.

3. Rotate gently the dish and keep it at $-20 \, ^\circ \text{C}$ for 5 min.

4. Wash twice in PBS.

Figure 6.3 Primary cilium staining. Images show basal body and primary cilium immune-stainings with antibodies against gamma tubulin (green) and acetylated tubulin (red), respectively. A large majority (60–80%) of single RPE1 cells confined on 750-$\mu \text{m}^2$ micropattern (gray) are ciliated.
4.2. Immunostaining

Fixed cells are preincubated with BSA in order to reduce nonspecific antibodies adsorption and fluorescence background. Primary antibodies directed against acetylated and gamma tubulin must be added successively.

Blocking
1. Incubate cells with a 1.5% BSA solution in PBS (filtered through 0.22-μm filters) for 30 min.

Primary antibodies
2. Incubate cells with mouse antibodies against acetylated tubulin (1/10,000 dilution with 1.5% BSA in PBS) for 1 h at room temperature.
3. Rinse with PBS.
4. Incubate cells with rabbit antibodies against gamma tubulin (1/2000 dilution with 1.5% BSA in PBS) for 1 h at room temperature.
5. Rinse with PBS.

Secondary antibodies
6. Incubate cells with Cy3-conjugated goat anti-mouse and Alexa488-conjugated goat anti-rabbit diluted (1/500 dilution for each in 1.5% BSA in PBS) for 45 min at room temperature in the dark.
7. Rinse with PBS.
8. Incubate cells in Hoechst solution at 0.2 mg/ml in PBS for 10 min.
9. Rinse with PBS.
10. Place the slide on a microscope slide with an antifading mounting medium (20 μl of Mowiol). The slides can be stored several weeks at 4 °C and several months at −20 °C.

5. AUTOMATED IMAGE ACQUISITION

Cell arrays are particularly convenient for automated high-magnification image acquisition of single cells (Fig. 6.1A). It simply requires the estimation of the angular tilt between the cell array and the stage axes. The columns and rows of the cell array are then scanned automatically after calibration of the XY steps for the motorized stage. Since the automated acquisition of four color-Z-stacks at each position of the array could be quite long and inefficient considering that many micropatterns contain no cell or several cells, a preanalysis is made to count nuclei and acquire image of single cells only.

Required materials and equipment
Motorized upright microscope equipped with a motorized XY stage (BX71, Olympus)
CCD camera (HQ2, Ropper Scientific)
100× oil objective (Plan Apo, Olympus)
Piezoelectric ceramic for fast objective displacements along the Z axis (Physics Instruments)
Image acquisition and device monitoring software (Metamorph, Molecular Devices)
Journals for automated acquisition (see supplementary files, http://www.elsevierdirect.com/companions/9780123979445) (Metamorph, Molecular Devices)

Calibration of cell array scanning
1. Run the journal and specify the number of rows and columns.
2. Move the stage and record/enter the position of three corners of the array. Using these coordinates, the journal will estimate the angular tilt and the exact positions of all micropatterns.

Images acquisition
3. At each position, the journal first uses the plugin “autofocus software” to find the optimal focus for the micropattern based on the contrast of Alexa657-fibrinogen fluorescence signal.
4. Z-stack in the blue channel is recorded and the number of nuclei is determined using “Metamorph Integrated Morphometry Analysis.” The size and area of detected object are compared to preregistered value to test whether they can be considered as a nucleus.
5. If one single nucleus is detected, z-stacks (11 planes separated by 1 μm) are acquired and saved for all fluorescence channels. Otherwise, the stage moves to the next micropattern position.

6. AUTOMATED IMAGE ANALYSIS

Automated image analyses are usually sophisticated procedures in which the first major hurdle is the detection of cell boundaries. Here, it is not necessary since automated acquisition has been limited to single cell only. The second hurdle is usually in the use of spatial references to calculate the coordinates of intracellular compartments. Here, the reference axes are defined by the micropattern geometry. As illustrated in two examples, the automated quantification provides fast and reliable quantifications of ciliogenesis rates as well as positions of primary cilia.

Required materials and equipment
- ImageJ (National Institute of Health: http://rsbweb.nih.gov/ij/)
- Plugins included in the Fiji package (http://fiji.sc)
– “Template Matching” plugin (sites: https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin)
– Nucleus–centrosome detection plugin (http://www.elsevierdirect.com/companions/9780123979445)

Image processing
1. Z-stacks of all channels were projected into a single plane.
2. Projections were merged into a composite image, and all composite images were combined into a composite image stack (hyperstack).
3. All micropatterns (and therefore all cells) of this hyperstack were not perfectly aligned. Their displacements with respect to a reference micropattern were calculated using the custom written “Template Matching” plugin. All channels were automatically corrected for the same displacement as the one calculated for the micropattern.

6.1. Organelle detection on images
Cells sometimes contain several bright spots in the centrosome or cilium fluorescence channels. However, usually only one large gamma tubulin dot is positioned above, below, or very close to the nucleus. Our strategy is to detect the nucleus first, to search for the centrosome close to nucleus boundaries, and then to search for the primary cilium in contact with the centrosome (Fig. 6.4).

1. Run the plugin and define a “best value” for nucleus size and circularity. The macro then selects the nuclei channel, filters the imager with a “mexican hat” function (Sage, Neumann, Hediger, Gasser, & Unser, 2005), applies an automatic threshold (Kapur, Sahoo, & Wong, 1985), binarizes the image, creates a mask, and runs the “analyze particle” ImageJ plugin to store the outline of each nucleus in Image J’s ROI Manager (Fig. 6.4). If no nucleus is detected, the macro lowers the threshold prior to binarization. If visual inspection reveals that the automatically detected outline is not correct, it is possible to change and save it manually.

2. Centrosome size and circularity have to be predefined within the macro code. The macro then selects the centrosome channel, filters the image with a “mexican hat” function (Sage et al., 2005), applies an automatic threshold (Kapur et al., 1985), and binarizes the image. The macro imports the nucleus ROI list and searches for a spot of the right size and circularity in a region slightly larger than the nucleus contour. Centrosome and satellites or dusts may both be detected. If more than two particles are detected, the contour is progressively reduced until only a single particle is detected (Fig. 6.4).
3. The macro then uses the nuclei/centrosome ROI list to search for a strong signal around the detected centrosome in order to detect the primary cilium (Fig. 6.4).

Quantification of organelle positions

1. The detection of cilia is directly used to calculate the proportion of ciliated cells. Thereby, it is possible to detect any effect due to cell geometry (Fig. 6.5A) or gene-product silencing by siRNA (not shown) on ciliogenesis.

2. In the global table containing organelle position for each cell, lines corresponding to nonciliated cells are ignored.
3. The coordinates of the nucleus–centrosome vector are calculated by subtracting nucleus coordinates to centrosome coordinates (Fig. 6.5B). This vector indicates the orientation of the internal cell polarity and notably the preferential direction for the internal traffic toward the primary cilium. It appeared to depend on the geometry of cell shape and the spatial distribution of cell adhesion (Fig. 6.5B) as it was also the case for nonciliated cells (Théry et al., 2006).

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Figure 6.5 Ciliogenesis rate and cilium position analysis. (A) Illustration of the reduced rate of ciliogenesis and length of the primary cilium in spread cells compared to confined cells. (B) Quantification of basal body positioning, with respect to the nucleus, in ciliated cells on disc-shaped and crossbow-shaped micropatterns. The asymmetry of the crossbow biased the orientation of the nucleus centrosome vector in the adhesion plane. Adapted from Pitaval et al. (2010).


