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3D-Time-Lapse Analysis of Rab11/FIP5 Complex: Spatiotemporal Dynamics during Apical Lumen Formation

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Summary

Fluorescent imaging of fixed cells grown in two-dimensional (2D) cultures is one of the most widely used techniques for observing protein localization and distribution within cells. Although this technique can also be applied to polarized epithelial cells that form three-dimensional (3D) cysts when grown in a Matrigel matrix suspension, there are still significant limitations in imaging cells fixed at a particular point in time. Here, we describe the use of 3D-time-lapse imaging of live cells to observe the dynamics of apical membrane initiation site (AMIS) formation and lumen expansion in polarized epithelial cells.

Keywords

Apical lumen; Time-lapse microscopy; Matrigel; 3D Tissue culture; Epithelial cell polarity

1. Introduction

Epithelial cells are highly specialized cells that line many tissue cavities and often function as barriers within many organs. Epithelial cells are structurally and functionally polarized to form an apical domain that faces the inter-tissue cavity, or lumen, and a basolateral domain that mediates the anchoring of epithelial cells to each other and to the extracellular matrix [1, 2]. Additionally, epithelial cells also form highly polarized multi-cellular structures, the process that is a key step during tissue morphogenesis and function. The coordinated polarization of multiple epithelial cells and the process of lumen formation during tissue morphogenesis remain to be fully understood and has been the focus of research in many laboratories. There are many pathways of apical lumen formation, which include wrapping, budding, and lumen formation *de novo* from non-polarized progenitor cells [1, 2]. It is now well established that *de novo* lumen formation and epithelial polarization begin at the first cell division and that polarized endocytic transport of apical cargo is crucial to lumen initiation and formation [3]. The budding and transport of these apical vesicles is mediated by the interaction of the small monomeric GTPase, Rab11, with its binding partner FIP5 (Rab11 family interacting protein-5) [4–6]. It was shown that apical lumen formation is initiated at late telophase by targeted transport and fusion of Rab11/FIP5-containing apical endosomes at a specific location between dividing cells, known as the apical membrane

initiation site (AMIS), which is marked by many tight junction proteins including ZO1 and cingulin [3, 7, 8]. The pathways underlying the regulation and dynamics of Rab11/FIP5-endosome transport during epithelial tissue morphogenesis have yet to be determined.

Fluorescent microscopy has proven to be a useful tool in studying the mechanisms and dynamics of lumen formation and many other pathways by allowing visualization of co-localization and distribution of proteins in cells. While imaging of polarized epithelial cells grown on two-dimensional (2D) filters is most widely used, it is not as sufficient for looking at the development of three-dimensional (3D) multicellular structures such as the apical lumen. Instead, epithelial cells can be grown in 3D culture by suspending in Matrigel matrix, which mimics the extracellular matrix and allows formation of 3D cysts with internal apical environments [7, 8]. Although the 3D cysts can be fixed and immuno-labeled, this method limits the viewing of cells at a single time point and thus is not adequate for determining the timing and dynamics of apical lumen formation. These limitations can be overcome with the use of live imaging of tagged-proteins in live cells, which enables the following of a single cell through all of the stages of division as well as lumen formation and expansion.

We have created a protocol for 3D-time-lapse analysis of apical lumen formation using Madin-Darby canine kidney (MDCK) cells [3, 9]. Using this technique we were able to determine that cingulin is one of the first proteins recruited to the AMIS during late telophase, and that the Rab11/FIP5-endosomes are transported after AMIS formation around the midbody [3]. This 3D-time-lapse microscopy approach has greatly expanded our knowledge of the machinery mediating lumen formation by allowing us to elucidate the timing of certain steps in the overall mechanism. Furthermore, this 3D-time-lapse imaging method can now also be used to further expand our understanding of many molecular processes governing epithelial tissue morphogenesis.

2. Materials

3D Tissue Culture

1. Type II Madin-Darby canine kidney (MDCK) cells.
2. MDCK media: Add 50mL fetal bovine serum (FBS) and 5mL penicillin-streptomycin (10,000 U/mL) to 500 mL 1X Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/L glucose, L-glutamine) and filter sterilize.
3. 1X Phosphate buffered saline (PBS).
4. 1X 0.25% Trypsin-EDTA.
5. Matrigel growth factor reduced basement membrane matrix (Corning Life Sciences).
6. Tissue culture (100mm) and 5 cm gridded glass-bottom (35mm) dishes (Ibidi).

Microscopy

1. Inverted Axiovert 200M fluorescent microscope (Zeiss) with 63X oil immersion lens and QE charged-couple device camera (Sensicam).

2. Slidebook 5.0 (Intelligent Imaging Innovations) 3D rendering and exploration software.

3. Methods

Carry out steps 1–8 in a tissue culture hood.

1. Plate MDCK cells on 100 mm tissue culture plate in 10 mL MDCK media and let grow for 24 hours at 37°C (Note 1).
2. One to two hours before plating cells take an aliquot of Matrigel and set in on ice to thaw out. It is very important to keep Matrigel cold even while thawing, since it rapidly solidifies at room temperature.
3. Aspirate media and rinse cells with 10 mL PBS. This is the key step. Leaving some of the serum-supplemented media will inhibit Trypsin and will make very difficult to lift individual MDCK cells.
4. Add 2 mL 0.25% Trypsin-EDTA and let sit at 37°C for 10–15 minutes. MDCK cells are usually difficult to dislodge. Thus, if needed, they can be incubated for 20–25 minutes.
5. Dislodge cells by gently tapping at the side of 100 mm dish. Harvest cells by adding 8 mL MDCK media to plate and transferring cells to 15 ml tube. Sediment cells by centrifugation at 1000xrpm for 3 minutes.
6. Aspirate media and resuspend cells in 1 mL of MDCK media by pipetting up and down 50–100 times using 1 mL blue pipette tip (Note 2).
7. Count cells to determine number of cells/mL and add 20,000 cells (Note 3) to 25 μ L MDCK media in 1.5 mL Eppendorf tube (Note 4).
8. Make a 75% Matrigel solution by adding 75 μ L Matrigel to the 25 μ L cell solution from step 7 (Note 4). Gently mix by pipetting up and down. Immediately plate cell/Matrigel mix by placing a drop onto center of 5 cm gridded glass-bottom dish. This step needs to be done quickly, since Matrigel-cell mixture solidifies readily at room temperature.

¹The plated cells should not be fully confluent since cells need to be in growth phase. Typically we want to have cells at 50–70% confluency after 24 hour incubation. Thus, we usually plate cells at 30% confluency.

²Pipetting up and down many times helps to separate cells. It is crucial for this assay to embed individual cells in Matrigel. Embedding cell clumps will lead to formation of cell aggregates with multiple lumens. We found that pipetting cells 50 times with a 1 mL pipette usually gives a maximum number of individual cells; however, this number may vary. Thus, it is advisable to initially pipette cells varying number of times, while monitoring the efficiency of cell separation.

³The optimum number of cells to plate may vary. Plating too many cells may cause clumping of dividing cells and overlap of fluorescence, making it difficult to distinguish single cells and observe them over time. Plating too few cells will make it more difficult to find single cells that are entering metaphase. We recommend testing a range of 10,000–50,000 cells to find the optimum number of cells for each assay.

⁴The percentage of Matrigel may also vary between 25–80%. The volume of media to suspend cells in and volume of Matrigel added can be adjusted to determine ideal conditions. In lower concentrations of Matrigel, cells may sink through the Matrigel matrix (during process of solidifying the Matrigel) and stick to the bottom of the plate, counteracting the function of the Matrigel to observe the cells suspended in the matrix. If the Matrigel concentration is too high, the cells might not be able to suspend in the matrix and instead would just sit on top, which is again not ideal for 3D imaging.

9. Put cells in the incubator and let solidify in tissue culture incubator at 37°C for 30 minutes.
10. Add 5 mL MDCK media to dish and let cells acclimate for 6–12 hours at 37°C. Typically cells will start dividing within 12 hours after embedding. Imaging them within 6–12 hour time period will ensure that at least some cells will be undergoing first cell division.
11. Mount dish on fluorescent microscope and use a gridded chart to label the location of several individual cells (Note 5), especially those that may be in metaphase (starting division).
12. Adjust focus to set top and bottom of each cell (Note 6) and take initial 0.2 μm -step z-stack images.
13. Repeat imaging according to time frame for desired observation. This will result in taking a mini-z-stack for every time point. Generally, avoid taking more than 50 time-lapse z-stacks (Note 7). To visualize AMIS formation during cell division, we typically use 10 minute time-lapse (Fig. 1). To visualize lumen formation and expansion, we use 30 minute time-lapses (Fig. 2). Finally, if the motility of individual Rab11/FIP5-endosomes needs to be observed, we use 200–500 ms time-lapse. Use grid etched in glass to locate various cells for imaging at different time points.
14. Taking mini-z-stacks at every time point allows the use of post-acquisition image analysis to generate three-dimensional images of MDCK cells at each time point during lumen formation. Alternatively, individual images that best represent the lumen formation dynamics can be selected and displayed/analyzed for every time point.

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⁵Isolated cells will be more ideal for imaging. As nearby cells divide and form cysts, the fluorescence can interfere with the cells you are trying to follow.

⁶To be sure to include the whole cell in the range of imaging from top to bottom, it is best to set the focus a little beyond the point when the cell first begins to blur.

⁷Taking fewer time-points or fewer images in each mini-z-stack will decrease photo-damage to cells. Excessive imaging can be a problem, since mitotic cells are especially sensitive to photo-damage. Shorter exposures will also reduce photo-bleaching. On the other hand, using long time-lapses increases the risk of missing the critical steps of the observation.

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Fig. 1. Live imaging of AMIS formation and lumen expansion in MDCK cells expressing FIP5-GFP. Images were taken every 10 minutes to follow FIP5 transport from the centrosome to the midbody. Shown are the single images taken from the mini-z-stack at selected time points (reproduced from [3] with permission from EMBO Reports).

Fig. 2.

Live imaging of lumen formation in MDCK cells expressing GFP-cingulin (CGN). Images were taken every 30 minutes to follow cingulin concentration at the midbody and expansion of the lumen during cell division. Shown are the single images taken from the mini-z-stack at selected time points (reproduced from [3] with permission from EMBO Reports).