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Isolation of fallopian tube epithelium for assessment of cilia beating frequency (CBF)

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Abstract

The fallopian tube epithelium (FTE) plays a critical role in reproduction and the genesis of ovarian cancer. The FTE columnar cells present with hair-like structures named “cilia” that are required for normal FTE function. Impairment of ciliary motion can lead to infertility, and is influenced by hormonal signaling and endocrine disrupting compounds is critical for understanding FTE physiology and pathology. In this protocol, we describe methods for isolating human fallopian tube, oviduct (murine equivalent of fallopian tube) epithelium and ovaries. In addition, we describe methods for imaging and measuring cilia beating frequency using high-resolution time-lapse imaging.

Keywords

fallopian tube epithelium; ovulation; cilia beating frequency; ovary; oviduct

1. Introduction

The human fallopian tubes play a critical role in reproduction. The fallopian tubes are the site of fertilization and are also involved in the transport of the sperm, egg, and embryo. Tubal secretions are rich in glycoproteins and growth factors that regulate fertilization and embryo development (1–3). Ciliary movement is pivotal to the fallopian tubes function (2), and when ciliary motion is disrupted thorough trauma to the tubal epithelium by sexually transmitted infections, pelvic inflammatory disease, or environmental insults, fertility is also affected (4–8).

Ovarian hormones regulate the tubal epithelium during the menstrual cycle (9, 10) and impact the tubal epithelial structure and the expression of cilia genes (2, 11). Estrogen stimulates ciliogenesis and increases cilia beating frequency while high levels of progesterone cause deciliation and decrease beating frequency (12, 13). Elevated testosterone also regulates cilia gene expression and motion in human fallopian tube epithelium (14). Human fallopian secretory epithelial cells are a source of origin of high-grade serous ovarian cancer, and the outgrowth of the secretory population, as compared to the ciliated cells, is thought to be an early event in tumorigenesis (15, 16). However,

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the relationship between ciliogenesis and ovarian cancer development is not known. Some studies suggest that improper cilia function may impede clearance of the fallopian tube epithelium, causing accumulation of oxidative stress (17) that may lead to DNA damage and tumorigenesis. Therefore, studying cilia movements is pivotal to understand fallopian tube normal function and pathogenesis. Herein we describe a method for isolation and *ex vivo* study of fallopian tube epithelium.

2. Materials

2.1 Cell Culture

1. Mice CD1 or any strain of interest (N=6 per condition/treatment), (see Note 1).
2. Fallopian tube tissues from patients (N=6 per condition/treatment).
3. 70% ethanol.

2.2 Media

1. Ovary and oviduct dissection media: Leibovitz media with 1% penicillin/streptomycin solution.
2. Murine oviduct epithelial (MOE) cells media: α-MEM with ribonucleosides, deoxynucleosides, supplemented with 2mM L-glutamine, 10% fetal bovine serum, 2 μg/ml epidermal growth factor (EGF), ITS (5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite), 1 mg/ml gentamicin, and 18.2 μg/ml β-estradiol, 0.1% penicillin/streptomycin solution (all final concentrations) (18).
3. Fallopian tube dissecting media: DMEM/F-12 medium supplemented with 1% penicillin/streptomycin, 50 mg/L gentamicin, 1% FBS.
4. Growth media: 50% αMEM, 50% F-12, supplemented with 0.3% BSA, 1 mg/ml bovine fetuin, 5 μg/ml insulin, 5 μg/ml transferrin and 5 μg/ml selenium, 10 mIU/ml follicle stimulating hormone (FSH), 1% penicillin/streptomycin solution, 50 mg/L gentamicin (see Note 2).

2.3 Equipment and plates

1. Spinning disk inverted microscope, with a 100X silicon oil objective, incubation chamber, camera, and software capable of capturing fast time lapse images.
2. Matek P35G-1.5–14-C dishes.
3. ImageJ-Fiji Software.
4. Laminar flow hood.
5. Temperature-controlled centrifuge.
6. Dissecting microscope.
7. Inverted tissue culture microscope to check tissue health and cilia pulsing.
8. Coverslips.

9. 10 cm plates (to dissect tissue in).
10. 12 well plate.
11. Millicell 0.4 μm pore.
12. Tweezers.
13. Surgical scissors
14. Scalpel.

3. Method

3.1 Isolation of fallopian tube epithelium

1. Obtain human fallopian tubes from the hospital (see Note 3) after approval by the institutional review board (IRB) of the university of interest.
2. Wash the fallopian tubes in 70% ethanol and place in 10-cm dish containing 10 ml of warm dissecting media (n=6 tissues from 6 different patients total, obtained at different times and processed separately, are recommended for sufficiently powered studies, although more may be necessary depending on the perturbation of interest).
3. Open the fallopian tube by inserting scissors in the tube. Opening the tube will expose the lumen. Once the lumen is exposed, the epithelium will become visible and can be removed with tweezers and surgical scissors (Fig. 1).
4. Cut the epithelium from the muscular wall and connective tissue, which is the compact and hard tissue that was originally the external part of the tube.
5. Make sections of the epithelium of similar size, about 2×2 mm, using scissors.
6. Flatten the biopsies and position them on 0.4-μm pore Millicell inserts trans ells (3 biopsies per well).
7. Place the trans-wells in 12 well plates containing 600 μl of growth media to create an air-liquid interface (no liquid is added inside the transwell). The FTE preparation will look transparent at this time (Fig. 1). The day after check under an inverted microscope for cilia pulsing using a 10x or 20x objective to make sure that the tissue had healthy epithelium. Treatment can be added in the bottom well, such as hormones or small molecules inhibitors of the pathway of interest (see Note 4). Cilia can be measured as described in Subheading 3.3 and 3.4
8. Every other day, remove 200 μl of growth media and replace with fresh media. The collected media can be used for ELISA, Western blots or frozen.
9. Maintain in a humidified atmosphere containing 5% CO_2 at 37°C

3.2 Isolation of murine ovaries and oviducts

1. Obtain IACUC approval for animal studies.
2. Sacrifice female mice at 16 days of age (n=6 mice per condition or treatment).

3. Make an incision at the base of the abdomen and remove the reproductive tract (Fig. 2).
4. Isolate murine oviducts and ovaries using a dissecting scope and carefully remove, bursa, uterus, and fat pads (19).
5. Make a cut between the ovary and the oviduct and slide the ovary out of the bursa, which is a membranous sac surrounding the ovary.
6. Separate the uterus from the oviduct. The oviduct is connected to the uterus but looks convoluted as compared to the uterus that it straight.
7. Transfer the ovaries and the oviducts into a 10-cm dish containing 12 ml of ovary and oviduct dissecting media.
8. Cut the ovaries in half and put two halves into a transwell (see Note 5). Transfer the transwells into wells of a 12-well plate containing 600 μl of growth media to create an air-liquid interface (no liquid is added inside the trans-well).
9. Cut the oviduct longwise, flatted them and move them into a transwell (one oviduct per transwell). Transfer the transwells into wells of a 12 well plate containing 600 μl of MOE media to create an air-liquid interface (no liquid is added inside the trans-well). The oviduct preparation will look really transparent at this time (Fig. 2). This tissue will bear cilia beating that can be measured as described in 3.3 and 3.4.
10. The ovaries and the oviduct can be positioned on the same transwell or separate transwells (see Note 6). When together, secretion from one tissue will influence the other tissue response. Treatments can be added in the bottom well, such as hormones or small molecule inhibitors of the pathway of interest (see Note 4).
11. Maintain in a humidified atmosphere containing 5% CO_2 at 37°C.

3.3 Capturing Cilia beating

1. Turn on the spinning disk inverted microscope and camera as indicated by the manual (see Note 7).
2. Remove neutral density (ND) filters from transmitted light path.
3. Select the 100x silicon oil objective and bright field.
4. Turn on incubated chamber and place lens heater on objective. Equilibrate to 5% CO2 and 37°C.
5. Add silicon oil on the objective and transfer the oviduct or human fallopian tube epithelium sample from the transwell to Mattek dish, add a drop of media and position a coverslip on top of the sample and add weight to flatten the tissue (see Note 8).
6. Add silicone immersion oil on the objective and place the Mattek dish with the sample on stage.
7. Focus to detect the epithelium.

8. Perform Kohler alignment for proper condenser alignment in an area away from the sample.
9. Select the microscope capture settings for diascopic imaging.
10. Crop the camera field of view to 100×100 pixels. In camera setting select 2–3ms/frame.
11. Acquire images using fast time lapse (memory capture). Capture a total of 5,000 frames.

3.4 Measuring Cilia beating frequency

1. Use image acquisition software to open the image and determine frames per second from the number of frames captured and elapsed time (we obtain these values by clicking image properties).
2. Close the image acquisition software and open Fiji/ImageJ (can be downloaded for free at https://imagej.net/Fiji).
3. Open the same image file previously opened with the acquisition software by using Fiji and a tool bar will appear.
4. From the tool bar, select the fifth box from the left, which is a line, and drop down to select segmented line.
5. Draw a segmented line midway between the base and the ends of the section of cilia. Double click to end the segment (Fig. 3).
6. From the top of screen, go to Image and select “stacks” first, and then select “reslice”.
7. A window will appear. Click ok for the following settings: output spacing to 1,000, avoid interpretation, rotate 90 degrees.
8. A kymograph will be generated. Zoom the kymograph to View 100% to increase the size of the picture.
9. Draw a single line on the kymograph from one peak to the next peak of the waveform (Fig. 3).
10. From Analyze, Select Measure and report the length of the line that is the distance between two peaks.
11. Cilia beating frequency (CBF) is calculated as following: frames per second/length of the line between two peaks.
12. Record the CBF and analyze four additional movies (5 total) from separate fields for the same patient specimen and average the CBF values.
13. Tissue sections from three different patients are cultured, treated, and analyzed the same way to obtain three independent experiments for statistical analysis based on the experimental design (comparisons of interest, etc).

4. Note

1. For our studies we use 16-days-old mice because we want pre-pubertal mice that we induce to ovulate in vitro. However, older mice can be used to address different questions.
2. Growth media can be made in advanced with all ingredients except fetuin and FSH. Store at 4°C for up to one month. Fetuin and FSH are added fresh to an aliquot of the media needed.
3. Human fallopian tubes are transported on ice from the surgery site and the epithelium can be isolated the same day or kept at 4°C for up one day. The epithelium should feel very soft; however, some patients may have a dry and hard epithelium that is not good for isolation and may not be suitable for cilia beating. Having the surgical indication may help to identify samples that have viable and healthy epithelium. However, depending on the scientific question, normal versus diseased fallopian tube may be chosen.
4. Estrogen (1nM0 increases CBF, whereas progesterone (10nM) and testosterone (2nM) reduce CBF. Basal CBF observed with 0.1nM estrogen stimulation is about 20 Hz.
5. Ovaries isolated from mice need to be used the same day. The ovaries are soon transferred from dissecting media into trans-well containing growth media at the bottom well.
6. When co-culturing ovary and fallopian tube or oviducts, keep in mind that the ovary is viable for about 8–10 days. After that time, the structure of the ovary collapses and does not look very tight. The Fallopian tube alone should remain viable for 14–20 days. The murine oviduct and ovary can be kept in MOE media for experiments lasting less than 24h.
7. After treatments of the epithelium with hormones or small molecules, the tissue is transported to the spinning disk microscope for capturing videos on a heat block pre-heated at 37°C. Cilia movements depend on temperature and therefore the tissue needs to be kept at 37°C before and during image capturing.
8. We have found that a washer works well to weigh down the tissue.

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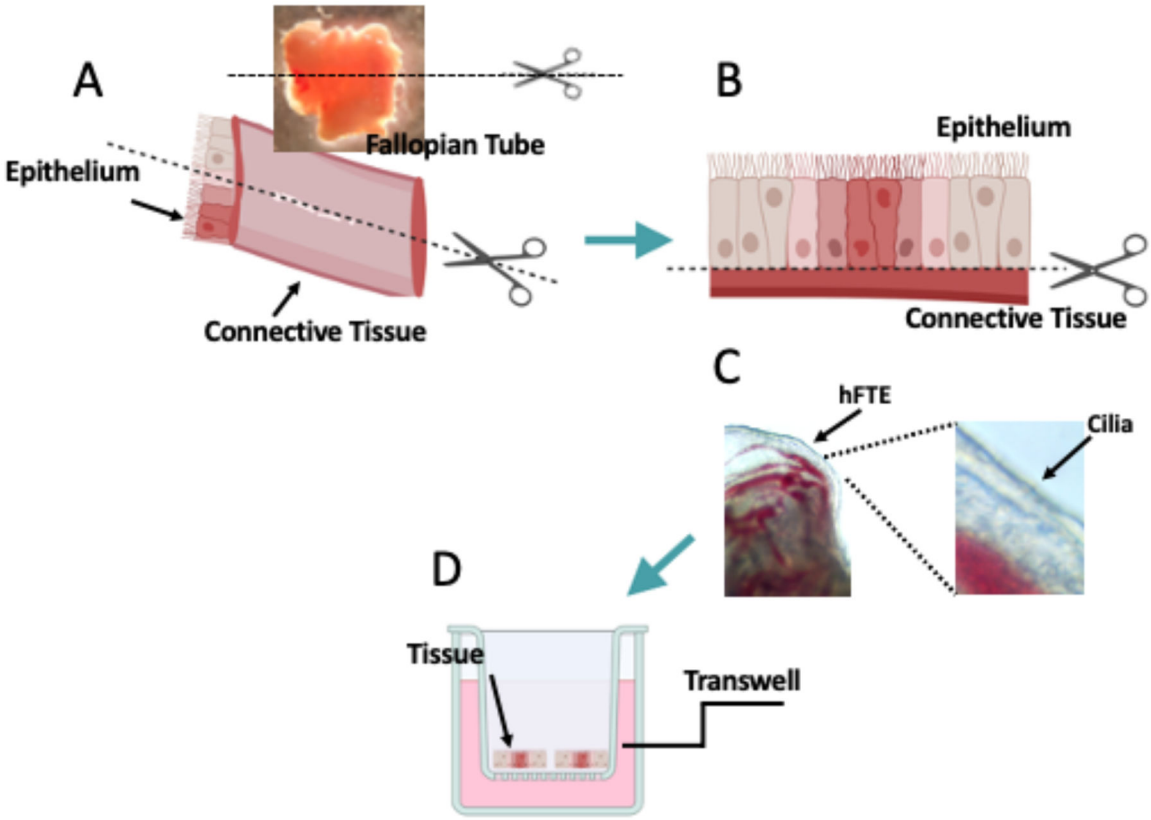

Figure 1.

Isolation of human fallopian tube epithelium (hFTE). A) The tube is opened to expose the epithelium. B) The epithelium is excised from the connective tissue. C) The epithelium is cut into small 2×2mm sections. D) The epithelium is transferred into trans-wells.

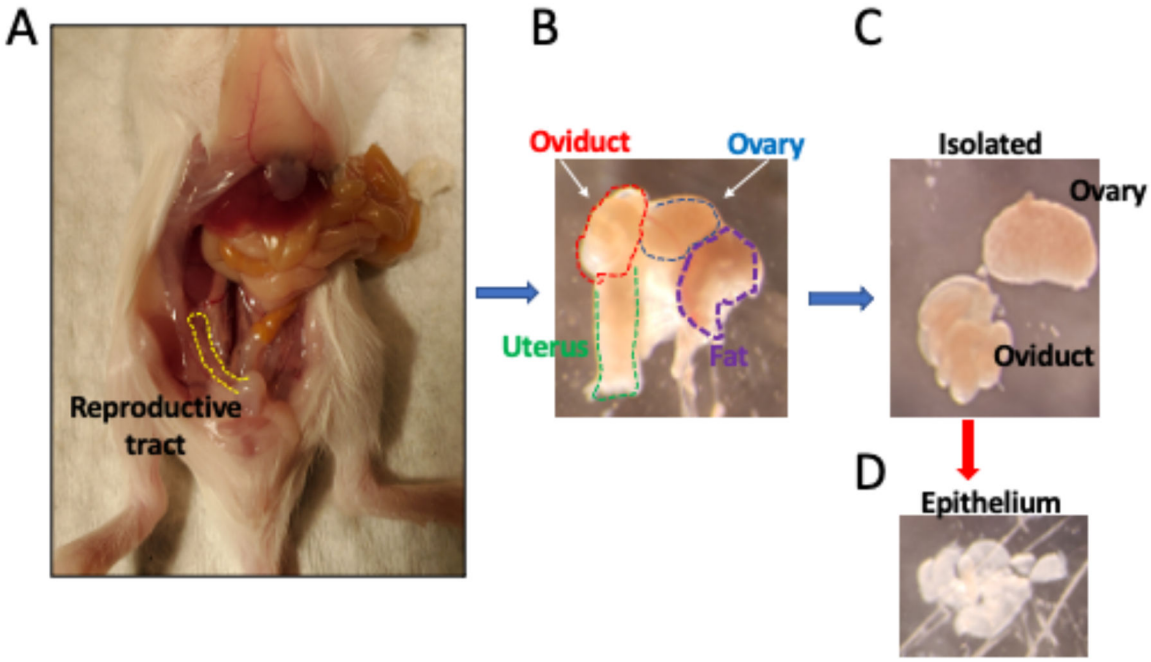

Figure 2.

Isolation of mouse oviduct and ovary. A) Murine reproductive system extending from the cervix to the kidney. B) Isolated reproductive tract with oviduct, ovary, uterus and fat pad under the dissecting microscope. C) Image of oviduct and ovary isolated. D) Flattened epithelium isolated from the oviduct.

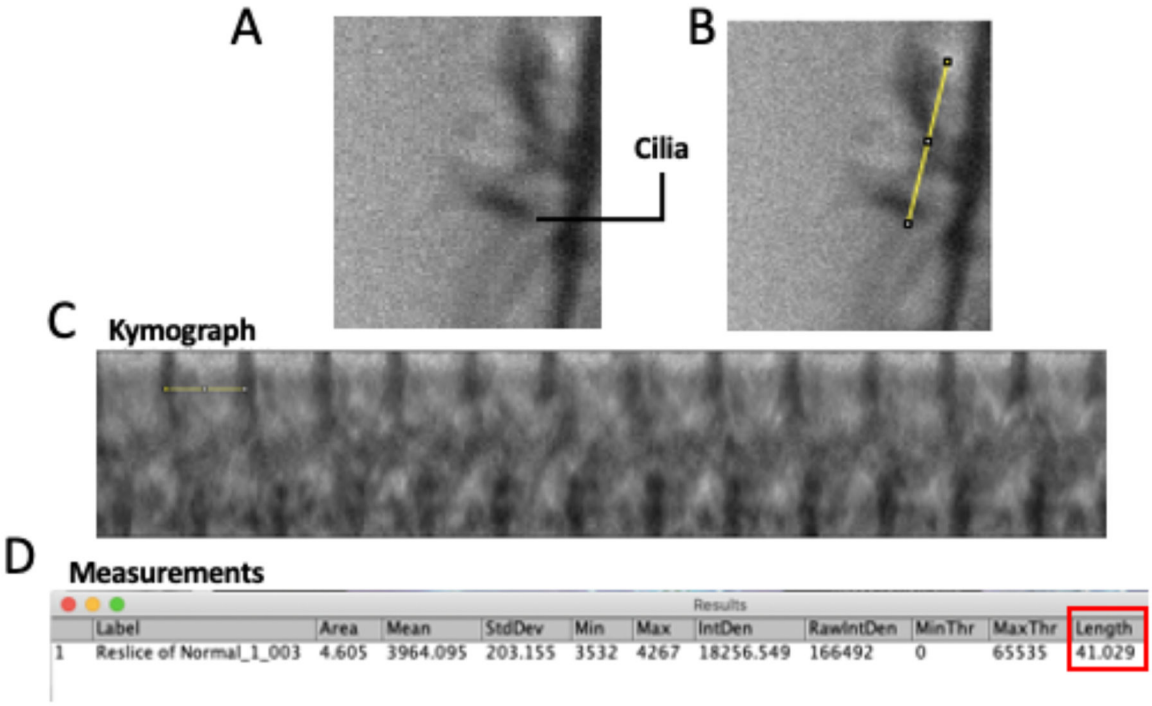

Figure 3.

Kymograph and measurements. A) Images showing cilia. B) Magnification of cilia with selection at the base of the cilia to generate the kymograph. C) Representation of waves over time, or kymograph is shown. The line drawn between two consecutive picks of a waveform is shown in yellow. D) Measurement obtained by selecting analyze and measure. In a red box is shown the length that is the crucial value required for CBF calculations.