Published in final edited form as:
Curr Protoc Cell Biol. 2009 June ; CHAPTER: Unit–4.22. doi:10.1002/0471143030.cb0422s43.

Culturing Three Dimensional MDCK cells for Analyzing Intracellular Dynamics

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Abstract

Epithelial cells grown in three dimensional (3D) cultures of extracellular matrix differentiate into a multicellular structure of polarized cells. This process shares many characteristics with the physiological development of an epithelial tissue and the formation of polarity in epithelial cells. Imaging 3D cultures of polarized epithelial cells is therefore a powerful tool to study epithelial architecture and morphogenesis under close to physiological conditions. The new generation of confocal microscopes allows live cell imaging of fluorescently tagged molecules in these cultures. This opens up new opportunities for studying how molecules behave and are distinguished asymmetrically within a 3D setting. This unit discusses technical aspects for culturing and imaging MDCK 3D culture for both fixed 3D cultures and live cell imaging.

Keywords

3D cultures; MDCK cells; Live cell imaging; MDCK cyst; Epithelial cyst; 3D culture imaging

Introduction

MDCK (Madin-Darby canine kidney) cells embedded in extracellular matrix (ECM) form three dimensional cultures (3D cultures). These cultures are spherical cysts characterized by a hollow lumen surrounded by one layer of polarized cells (Fig 2C). 3D MDCK cultures recapitulate numerous features of epithelial tissues in vivo (O'Brien LE 2002;Debanath J. and Brugge JS, 2005) and therefore provide an ideal model system to study epithelial morphogenesis and polarity under physiological conditions in a tissue culture dish.

Live cell imaging of 3D MDCK cultures using fluorescently tagged proteins provides a unique opportunity to study the intracellular dynamics of proteins in these cells.

This unit describes fluorescence based techniques to image intracellular proteins in fixed and live 3D cultures using confocal fluorescent microscopy. It begins by describing the general requirements for fluorescent imaging of these cultures. Then, a protocol for plating and growing MDCK 3D cultures for both fixed and live cell imaging is provided. Next, an indirect immunostaining protocol is described. A live cell imaging protocol is then discussed, including the optimal settings for imaging different fluorescently tagged proteins and the more advanced live cell imaging techniques of fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP).

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Strategic planning

Culture condition

1. We describe conditions for growing MDCKII cells (herein referred to as MDCK) in 3D cultures. Other MDCK clones may require different conditions for 3D cultures formation.
2. Because MDCK cells may loose their ability to form a 3D culture when they are subjected to repeated trypsinization cycles, it is important to use low-passage cells and to have a large number of frozen aliquots for each cell type.

Fluorescent tags

Imaging a fluorescently tagged protein in live 3D cultures usually requires the protein to be stably expressed in the MDCK cells. This is because it is very difficult to transfect 3D cultures with the conventional transfection reagents (i.e. lipofectamin, fugen6) after the cultures are formed. In addition, transfecting the cells before plating them in collagen is very inefficient because the levels of the over expressed protein usually drop by the time the cultures are formed and ready to be imaged (4–9 days later). To obtain the best high quality images, the protein-of-interest should be tagged with a bright fluorescent protein that is not sensitive for photobleaching, such as EGFP. Moreover, it is best to use a clonal line strongly expressing the fluorescent protein-of-interest. If this is impossible, FACS sorting the cells after transfection to enrich the population of expressing cells is recommended.

Basic Protocol 1

Growing MDCK 3D cultures

To induce 3D culture formation of MDCK cells, extracellular matrix components need to be provided. This protocol will describe plating of MDCK cells in collagen I matrix, which is a major component of the extracellular matrix. Plated in collagen I, MDCK cells form 3D cultures that further differentiate into tubes in the presence of the growth hormone, HGF (O'Brien L.C, 2002). This protocol describes plating of MDCK 3D cultures in membrane filter inserts (Fig 1B), which is best suited for immunofluorescence, or in chamber slides (Fig 1A), which are required for live cell imaging. For immunofluorescence, MDCK 3D cultures may also be plated in chamber slides. However, the quality of the images will be best when the cells are plated in membrane filters due to the smaller distance of the cultures from the cover slip.

Since both protocols are very similar, they will be described as one with specific instructions for each method indicated at the necessary steps.

Materials List

*All solutions and reagents should be sterile.

1. MEM supplemented with 10% FBS, 2mM Glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. For stable cell lines the media should contain 1 mg/ml of G418 or any other antibiotic that was used to generate the cell line.
2. Trypsin- EDTA 0.25%.
3. GlutaMAX stock 200mM (can be replaced with Glutamine 200mM).
4. $NaHCO_3$ stock, 23.5 mg/ml in DDW (store in 4°C).
5. MEM 10X (without glutamine) (store in 4°c).

6. PureCol purified collagen I (INAMED biomaterials, 3 mg/ml) (can be replaced by any highly purified bovine collagen I) (store in 4°c).
7. Nunc Anapore membrane inserts, 0.2μm pore, 10 mm (Nalge Nunc 13935).
8. Lab-Tek Chamber Slide - 4 well (Nalge Nunc 155383).

Laboratory equipment:

1. Humidified 37°C incubator with 5% CO_2.
2. Sterile tissue culture hood.
3. 37°C oven.

Steps and Annotations

1. Preparing the cells:

1a. Grow MDCK II cells in MEM supplemented with 10% FBS, 2mM Glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. When using a stable cell line the additional antibiotic should be added (we use G418 1mg/ml). Maintain cells by splitting them every 3–4 days with Trypsin-EDTA 0.25%. MDCK cells should be splitted 1 day post confluency (100%), after they formed a tightly packed layer of hexagonal cells.

1b. Split a post confluent dish of MDCK II cells 1:10 to a 10 cm dish one day prior to plating day. This step is required in order to make sure that the cells are in their growing and proliferating stage, which is crucial for culture formation.

2a. Prepare a collagen I solution:

	Final concentration	Volume
GlutaMAX (200mM)	24mM	750μl
$NaHCO_3$ (2.35 g/100ml)	2.35mg/ml	625 μl
MEM 10X	MEM 1X	625 μl
Hepes (1M pH 7.6)	20 mM	125 μl
Collagen I	2 mg/ml	4.13ml
Total vol.	--------	6.255ml

Add the reagents listed above to a 15 ml tube and mix by pipeting up and down. Verify the pH by applying 100 μl of the collagen I solution on a pH indicator stripe. pH should be neutral. Store the solution at 4°C to avoid premature polymerization of the collagen. Make a fresh collagen I solution for every experiment.

3. Preparing the plates:

For live cell imaging: pre-coat the chamber slide with 75–100 μl of collagen I solution for each well of a 4 well chamber slide. Make sure the collagen solution completely covers the bottom of the chamber by tapping on the chamber. If tapping does not help, use a pipette tip to spread the solution throughout the well surface. Pre-coating is a critical step for live cell imaging because it reduces the number of cells that adhere to the coverslips and thus don't form a 3D culture. However, pre-coating with too much collagen solution will increase the distance of the 3D cultures from the coverslip, which will dramatically reduce imaging quality due to the thickness of the sample.

Incubate the chamber for 30 min in a 37°C oven (No CO_2).

For immunostaining: Place Nunc membrane filter inserts in a 24 well plate. Pre-coating is not necessary for this procedure.

4. Trypsinize the cells with Trypsine-EDTA (0.25%) and resuspend them in about 10 ml media. Pipette the cells up and down to get a single cell suspension. MDCK cells tend to form cell clumps and therefore it is often necessary to pipet them vigorously to obtain a single cell suspension.

5. Pellet the cells by centrifugation (4 min, 400 rpm, RT). Remove the supernatant and wash again with about 5ml of media. Repeat centrifugation.

6. Remove supernatant and resuspend the cells in 2 ml media. Pipet up and down to avoid cell clumps. This step is critical for getting uniform cultures with a single central lumen. Avoid bubbles.

7. Determine cell concentration (cells/ml) using a hemocytometer.

8. Add cells to the collagen solution to a final concentration of 3×10^4 cells/ml. For live cell imaging, cells can be plated at higher concentrations (up to 6×10^4 cells/ml). When adding the cells, make sure that the volume of the cells does not exceed 10% of the collagen solution volume because the diluted collagen solution will not polymerize properly into a matrix.

9. Plating the cells:

For live cell imaging: Add 300 μl of collagen-cells mixture to a pre-coated single well of a 4 well chamber slide.

For immunostaining: Apply 150 μl of collagen-cell mixture to the center of a membrane filter insert.

In both cases, make sure that that the collagen-cell solution is evenly distributed by gently shaking the dish. Avoid bubbles.

10. Incubate for 30–45 min in a 37°C oven (No CO_2). Collagen I polymerizes best under these conditions. Different temperature or CO_2 levels may cause uneven polymerization that can cause variability in 3D culture morphology in the same dish. It is therefore recommended to place the dish in the 37°C oven as soon as possible and to avoid CO_2.

The time required for polymerization may differ between different batches of purified collagen I. Gently tap the dish to make sure that the collagen has solidified and formed a gel texture.

11. After the collagen has polymerized, add culture media to the plates and place in a 37°C CO_2 incubator.

For chamber slide: add 500 μl to each well of a 4 well plate.

For membrane filter inserts: add 500 μl to the inside of the insert and 1ml to the outside (between the insert and the 24 plate well) (see Fig 1).

12. Maintaining 3D cultures:

For chamber slide: change media every 1–2 days.

For membrane filter inserts: change media every 2–3 days.

A lumen is already observed at day 4 (Fig 2) and cultures continue to develop until day 12. For a more extensive description of culture morphology, see anticipated results.

Basic Protocol 2

Indirect immunofluorence of MDCK 3D cultures

The intracellular localization of proteins in 3D culture may be different than that in other systems due to differences in environmental cues and morphogenetic responses. It is therefore crucial to study the localization of endogenous proteins via indirect IF. Indirect IF also allows checking for correct cell polarity and other morphological features of the 3D cultures prior to performing live cell imaging experiment. Common polarity markers that specifically label certain domains in polarized cells (apical membrane, basolateral membrane, tight-junctions) should be used in the indirect IF analysis (Fig 3 and 4). This permits detection of morphological phenotypes that may have been induced by over expression of the protein-of-interest and evaluation of the quality of the cultures. Importantly, it permits determination of whether the 3D culture is fully polarized. Live cell imaging of 3D cultures that are not fully polarized may lead to misleading results. The most commonly used method for indirect immunofluorescence (IF) of 3D cultures involve whole culture fixation. This approach is less time consuming and avoids extensive culture extraction that may affect culture morphology. This method will be described in detail in this chapter including recommended staining for culture evaluation.

Material list

1. PBS+ (PBS X1 supplemented with Ca^{2+} 0.9 mM and Mg^{2+} 0.5 mM). Store at RT.
2. Collagenase-1 (Type CVII, Sigma C-2799) 1000 U/ml in PBS+ stock. (Store in aliquots at −80°c).
3. Paraformaldehyde (PFA) 4%.
4. Permeabilization solution: 10% FBS, 0.5% Triton X-100 in PBS (make fresh for every experiment. Store at 4°C during the experiment).
5. Primary antibody against protein of interest.
6. Secondary antibody tagged with fluorophore (Alexa dyes and cy dyes are recommended).
7. Fluorescently tagged Phalloidin (Molecular Probes). All fluorescence tags that Molecular probes provide can be used.
8. Hoechst 33258 nuclear dye (Molecular Probes H-3569).
9. Fluoromount-G mounting solution (SouthernBiotech 0100-01).
10. Microscope slides.
11. Circular coverslips 18–25 mm.
12. Nail polish.

Steps and Annotations

1. Collagenase treatment. In order to improve the permeability of the antibody through the collagen matrix a collagenase treatment is needed.

    1a. Dilute an aliquot of collagenase-1 stock solution 1:10 with PBS+.

1b. Wash the cultures (inside and outside the membrane insert) three times with PBS+.

1c. Apply 250 μl of collagenase-1 solution to the inside of the membrane insert and 500 μl to the outside. Incubate 10 min in 37°C. Do not incubate for longer times. Extensive collagenase treatment will solubilize the collagen matrix which will make the sample difficult to work with and may affect culture morphology.

1d. Wash three times with PBS+.

2. Fixation.

2a. Add 4% PFA (250 μl to the inside and 500 μl to the outside). Shake slowly for 30 min at room temperature.

2b. Wash quickly three times with PBS+ followed by 3 wash for 10 min each. Pause point: Fixed cultures can be stored at 4°C in 0.1% PFA for up to 2 weeks.

3. Permeabilization and blocking: add permeabilization solution (250 μl to the inside and 500 μl to the outside) shake slowly for 30 min at RT

4. Primary antibody: dilute primary antibody to the desired working dilution in permeabilization solution. Use at least twice the concentration as that for adherent cells on a coverslip. Add 200 μl to the inside of a membrane insert and 250 μl to the outside. Incubate over night at 4°C. Over-night incubation is usually recommended in order to maximize antibody permeability. Shorter incubation times (shaking 3–4 h in RT) may be possible in some cases. Antibody permeability is a potential problem in whole mount immunofluorescence of 3D cultures since the antibody needs to penetrate the collagen polymers, the cell membrane and to penetrate to the lumen in some cases. It is therefore important to use high antibody concentrations and long incubation times to increase the probability for maximal antibody staining.

5. Wash six times with permeabilization solution. Three quick washes followed by three 10 min washes.

6. Secondary antibody: dilute secondary antibody to the desired working dilution in permeabilization solution. For the Alexa and cy dyes a 1:400 dilution usually gives a strong fluorescence signal that is easily detectible through the collagen layer. Add 200 μl to the inside of a membrane insert and 250 μl to the outside. Incubate 2–3 h shaking at RT.

For nuclear staining, add Hoechst 33258 at a 1:1000 dilution to the secondary antibody mix. Nuclear staining is recommended as an indicator for culture morphology.

Cytoskelaton staining is also recommended as a routine staining at this stage. For actin staining, add a fluorescently tagged phalloidin (Invitrogen) at a 1:300 dilution to the secondary antibody mixture.

7. Wash 6 times with permeabilization solution. Three quick washes followed by three 15 min washes.

8. Mounting:

8a. Apply a large drop of Fluoromount-G mounting solution on a microscope slide.

8b. Cut the bottom of the membrane insert with a steel blade and lift the gel carefully with a fine forceps. The membrane filters are made of glass and therefore cutting them without breaking the glass can be difficult. If the glass breaks, you can still lift the gel off the filter. What is important is to not damage the collagen gel.

8c. Place the gel (with or without the glass filter) on the Fluoromount-G mounting solution drop. Make sure that the top of the gel is facing up.

8d. Cover the gel with a circular cover slip (18–25 cm). Make sure the culture is completely covered with mounting solution. If not, apply some extra mounting solution to the edge of the cover slip. Avoid bubbles at all times. Avoid pressing the cover slip toward the gel.

8e. Wait for a few minutes to allow the mounting solution to spread and remove excess of mounting solution, but be careful not to remove too much. If you are not sure, it is preferable to have some excess left.

8f. Place the sample in a dark place at RT for over-night to allow the mounting solution to dry.

8g. Seal the sample with nail polish.

9. Samples can be stored at 4°C in the dark for at least 6 month.

Imaging setup

MDCK 3D cultures form a three-dimensional hollow sphere composed by a single cell layer. In order to image the entire culture, images from different focal planes should be taken and combined together. This can be done by taking a series of optical sections using confocal microscopy. Z-sectioning through a 3D culture also allows examination of the intracellular localization of proteins in single cells. For this purpose it is best to take an optical z section from the middle of the cyst (where the lumen is the largest). This optical section provides a nice cross section of the polarized cells surrounding the lumen (Fig 4F). In this view it easy to determine whether a protein is localized to the apical, basal or lateral membrane.

One of the difficulties in imaging whole mount 3D cultures is the thickness of the sample and the distance of the cells from the cover slip. This means that the imaging parameters should be adjusted very carefully in order to acquire the best possible images. However, even when the imaging parameters are optimized the image quality will still be lower than imaging adherent cells on a cover slip.

Setting up the microscope

A high resolution confocal microscope should be used for imaging immuno stained 3D cultures (we are using either the Olympus F1000 or the Zeiss 510).

Immunostained 3D culture can be imaged using an oil objective (we are usually using the 40X NA 1.3 Plan NeoFluar or UPlanFI or the 60X / 63X NA 1.4 Plan Apochromat oil objectives). A 40X NA 1.3 objective allows imaging deeper into the collagen and is therefore recommended for imaging cysts that are buried deeper in the collagen. To improve image quality, it is recommended to use a 1 airy unit pinhole size, to image at low speed, to average each pixel at least twice and to increase pixel resolution when necessary (more than 512 × 512).

When detailed intracellular localization is required it is better to zoom in and focus on three to four cells of the culture and to acquire thinner z sections (up to 50% overlap between two z sections).

Imaging using these parameters can be time consuming. To save time, it is usually enough to image only one half of the cyst. If you decide to do so, make sure to image the half that is closer to the cover slip. Another option is to use a spinning disk confocal which enables high speed image acquisition. A spinning disk confocal is also recommended for imaging fine intracellular structures such as microtubules which can be resolved better in this system.

Data analysis

A three dimensional reconstruction of a 3D culture cyst provides detailed information that is not readily detectible from looking at the z sections separately. It is therefore recommended to use a three dimensional rendering software for image analysis of 3D culture cysts (we use the Volocity software, Improvision).

Basic Protocol 3

Live cell imaging of MDCK 3D cultures

Live cell imaging of 3D cultures is a powerful tool to study both the dynamics of cells in a culture during culture morphogenesis and the dynamics of intracellular proteins in a single cells in real time. Live cell imaging can also be used as a more direct way to study protein localization because it gets around the need to use antibodies which sometimes have permeability and specificity issues. This protocol details the culture conditions and the microscopy setups that are best suited for live cell imaging of 3D cultures.

Culture conditions and culture preparation

1. Fluorescently labeled MDCK cells should be plated in a pre-coated chamber slide as described above (Basic protocol 1). Cultures can be imaged from day 1 to day 12 depending on the desired experiment.
2. Labeling the cultures with a plasma membrane dye. Imaging 3D cultures in DIC gives poor images (Fig 5). It is therefore difficult to determine the cell boundaries in a 3D culture and the subcellular localization of the protein of interest in a single cell. Fluorescence labeling of the plasma membrane outlines the boundaries of all cells in the sample and makes it easy to detect 3D cultures cysts, to distinguish between different cells in a cyst and to outline the different membranes of a single cell (apical, basolateral).

   To fluorescently label the plasma membrane of live cells in 3D culture apply 4 μM of FM4-64 dye (Invitrogen) to the culture media and incubate for 1h in a tissue culture incubator (37°C 5% CO_2). The dye will initially label the basolateral membrane and than the apical membrane (60 min later) (Fig 4). During this time the FM4-64 dye will also endocytose. The intracellular staining of the dye is stable for days and therefore this dye can be used for extensive live imaging experiments (up to several days).

Setting up the microscope

Live imaging of collagen embedded 3D cultures is somewhat challenging. Both the thickness of the sample and the collagen itself lead to fluorescence light scattering and therefore high laser intensity is required to acquire an image. Imaging under these conditions makes the specimen sensitive to photobleaching of the fluorescence signal and to culture photodamage. It is therefore very important to optimize the fluorescence parameters prior to

performing a live imaging experiment. These parameters should be adjusted for every experiment depending on the intensity of the fluorescence signal and the condition of the cultures. There are, however, a few general guidelines that can be followed.

1. The microscope: a confocal microscope should be used to image live 3D cultures. For high speed image acquisition it is best to use either a spinning disc confocal or the Zeiss LSM live duo system.
2. Choosing the culture:
   - **2a.** It is best to image only the cysts that are closest to the cover slip. Increasing the imaging distance will greatly reduce image quality and will require higher laser intensities which will lead to extensive photobleaching and photodamage.
   - **2b.** Image the cultures with the brightest fluorescence. This will reduce the laser intensity required for imaging and will therefore minimize photobleaching and culture photodamage.
3. Objective: A water objective (40X or 60X NA1.2) is recommended for high speed image acquisition and for samples that are sensitive for photobleaching. However, in some cases a 63X NA 1.4 or a 40X NA 1.3 are also applicable.
4. Scanning parameters: use line scanning, scan at low speed, average each pixel at least twice and increase pixel resolution when necessary (more than 512 × 512).
5. Z sectioning: setting up the pinhole between 1–1.5 airy units gives best results. When taking a series of z sections make sure the sections overlap.
6. Prolonged imaging: for extended imaging (more than a few hours) it is highly recommended to use an auto-focus or a perfect-focus module to avoid focus shift in time. This will prevent focus drift in time and will allow detection of whole culture movement along the z axis in time.

Basic Protocol 4

FRAP and FLIP in MDCK 3D cultures

Photobleaching techniques are highly useful to study the intracellular dynamics of proteins. In Fluorescence recovery after photobleaching (FRAP), a specific region of interest (ROI) is bleached once with a high laser intensity and the diffusion rate of the protein of interest to this region can be determined. In fluorescence loss in photobleaching (FLIP), the depicted region of interest is repeatedly bleached and the kinetics of fluorescence signal decay from different domains throughout the cell can be measured. The rate of recovery in FRAP and the fluorescence decay pattern in FLIP reflect the subcellular dynamics of the protein of interest therefore providing a direct tool to study protein dynamics in real time (for more information see Chapter 21, Unit 21.1) There are two critical points that should be taken under consideration when applying these techniques to 3D cultures. First, these techniques require exposure of the sample to high laser power for long durations, which can cause extensive photodamage to the cells in the culture and extensive photobleaching of the sample. Second, the 3D culture is a thick specimen and the time it takes to image throughout the z axis greatly reduces time resolution.

To get around these difficulties it is best to use the Zeiss live duo line scanner which is capable of taking hyperfast z sections or a spinning disk confocal. The scanning and photobleaching parameters should also be adjusted to enable efficient photobleaching of the ROI with minimal photobleaching of the overall sample. Extensive photobleaching of the specimen will greatly affect the results of both FRAP and FLIP experiments. It is therefore

very important to measure the change in fluorescence in different parts of the photobleached cells as well as in neighboring cells in every photobleaching experiment (see fig. 6 and 7)

In general, all of the suggestions regarding culture condition, culture preparation and imaging setups listed for live imaging holds here but with some specifications.

Suitable scanning and bleaching parameters for EGFP using the Zeiss live duo line scanner listed below:

1. Objective: The C-Apochromat 63X water objective NA 1.2 is recommended.
2. Scanning parameters: Frame size 1024 × 1024, scan speed between 1–2 frames per second, frame mean 1, pinhole size up to 2 μm.
3. Bleaching parameters: scan speed 6 μsec/pixel, iteration 3X. Low iteration number is crucial to avoid culture photodamage.

Commentary

Epithelial cells serve as a barrier between the interior of the body and the outside world. To accomplish this barrier function, epithelial tissues have developed special characteristics such as polarized morphology, unique cell-cell contacts and intimate interactions with the extra cellular matrix (ECM). MDCK cells have been extensively studied as a model system for epithelial polarity mainly due to their ability to form apical-basolateral polarity in a tissue culture dish. When plated on a permeable filter MDCK cells form a monolayer of polarized cells in which the basal membrane is attached to the filter and the apical membrane faces the culture media (K. Simons and S.D. Fuller, 1985) Although much of our knowledge regarding cell polarity came from these studies, this system is somewhat artificial since it provides a strong cue for polarization which also determines directionality. Plated in collagen, or any other extracellular matrix, MDCK cells form a three dimensional culture composed of polarized cells. In this system, MDCK cells embedded in extracellular matrix organize in a multicellular structure that creates its own lumen. This process recapitulates many of the physiological characteristics of lumen formation during epithelial development and share many morphological similarities to an epithelial tissue (Mcateer J.A, 1986; O'Brien, 2002; Martin-Belmonte F., 2008). This system therefore provides a unique opportunity to study epithelial morphogenesis in a tissue culture dish. Moreover, MDCK 3D cultures also provide a more physiological model system for cell polarity, its regulation and its relationships with the extracellular matrix. Indeed, using this system it was shown that Rac1 determines apical membrane orientation through an affect on the basolateral membrane (O'Brien L.C., 2001) and that CDC42 and aPKC are essential for the formation of both the apical membrane and the inner lumen (Martin-Belmonte, F, 2007)

Plated in 3D cultures, epithelial cells form cysts that are characterized by a spherical epithelial monolayer with a central single hollow lumen (Fig 2c). The epithelial cells surrounding the lumen are polarized with the apical membrane facing the inner lumen. The apical membrane is separated from the basolateral membrane by the tight junction complex (TJ) which also creates a diffusion barrier to the inner lumen (Martin-Belmonte, F, 2008).

3D cultures are also used in cancer research as a tissue culture tool for tumor progression and reversion and many of the current knowledge on 3D cultures came from these studies (Debnath J. and Brugge J., 2005; Schmeichel K.L. and Bissell M.J., 2003). 3D culture development and lumen formation was of focus in these studies and several distinctive stages in culture formation were specified. First, apicobasal polarization is observed and the lumen is filled with unpolarized cells. Second, the nonpolarized cells trapped inside the layer of polarized cells undergo apoptosis. This leads to the formation of a hollow lumen which is

maintained throughout the lifetime of the culture. Apotosis was also been shown to occur during lumen formation in MDCK cultures plated on collagen (Kim M., 2007; Martin-Belmonte F., 2008). However, the preliminary steps of culture formation and the role of apoptosis in culture development are not fully understood.

The protocols describes here are optimized for growing MDCK cells in collagen I matrix. Other extracellular matrixes (such as Matrigel) and other cell lines (such as mammary epithelial cells) can also be used as a 3D culture model system. The advantage of using collagen matrix is the simplicity of the matrix components. However, for some applications using a more complex extracellular matrix is required. A protocol for growing 3D cultures of breast cancer cell lines in Matrigel was recently published by Mina Bissell's group (Lee G.Y, 2007). This protocol is highly recommended when these parameters are desired.

3D cultures can also be used for biochemical analysis. These protocols are however outside the scope of this chapter. An extensively detailed protocol for biochemical analysis of MDCK cells in 3D cultures is described by Keith Mostov's group (O'Brien L.E., 2006) who is one of the pioneers in studying MDCK cells in 3D cultures. This protocol also include plating and immunostaining protocols for MDCK cells in collagen and is recommended as another source of information for issues that might not be covered in this chapter.

Live cell imaging is a powerful technique to study cellular morphogenesis is real time. These techniques, however, have not been applied for 3D cultures formation in the past mainly due to technical difficulties. 3D cultures are a relatively challenging specimen for live cell imaging for several reasons. First, the cultures are embedded in matrix and therefore the distance between the cells and the objective is substantially larger than in adherent cells. Second, the matrix that surrounds the cells scatters the fluorescent light. Third, the culture is a complex and thick organization of cells in all directions (x,y,z) and imaging it in all dimensions is time consuming and therefore gives poor resolution in time. The new generation of high speed confocal micrposcopes provides new possibilities for live cell imaging that can be optimized for 3D culture imaging. Imaging those cultures live can shed light on epithelial morphogenesis, on the involvement of different proteins in these stages and on the intracellular dynamics of proteins during this process.

Critical parameters

There are few parameters that need to be taken under consideration before starting a 3D culture experiment.

1. Before plating cells for 3D culturing the type of desired experiment should be determined. For an immunostaining experiment it is better to plate the cultures on membrane filter inserts and for live cell imaging the culture has to be plated on a chamber slide.
2. For live cell imaging the cells should stably express a fluorescent tagged version of the protein of interest. Moreover, the fluorescent signal should be as strong as possible in order to overcome the difficulties of imaging these samples.
3. The general morphology of the cultures should be estimated before any live cell imaging experiment. This could be done using the FM4-64 membrane dye or by fixing and immunostaining the culture after the imaging experiment. It is critical to determine if the culture has normal morphology (a single hollow lumen surrounded by a monolayer of polarized cells) because altered morphology may affect intracellular dynamics.

4. The overall condition of the cells in the chamber slide well should also be taken under consideration before starting an experiment. Extensive death of neighboring cells, for example, can affect the physiology of a culture with normal morphology in ways yet to be evaluated. This is especially critical in 3D cultures because diffusion inside the collagen polymer is limited and therefore the different cellular factors that are released during cell death will remain in the surrounding area for a long time and will not be washed out easily. It is therefore recommended to image only 3D cultures that are part of a chamber slide that is in good condition.

Troubleshooting

protocol	problem	Possible cause	solution
Growing MDCK 3D cultures (Protocol 1)	Collagen I does not polymerize	pH in not neutral	Check the pH of the Collagen I solution before adding the cells to it. pH should be neutral. Lower pH inhibits polymerization.
		Insufficient incubation time	Increase incubation time in 37°C oven. Different batches of purified collagen I often require different polymerization time.
	No 3D culture cyst formation or altered 3D culture cyst morphology (see anticipated results)	Cells concentration	Plate cells again and make sure to use the recommended concentration. A very low concentration will decrease the probability for 3D culture formation. A too high concentration will lead to cell aggregation and to defects in lumen formation.
		Cells are not in their proliferative state	Make sure the cells you are using for plating 3D cultures do not exceed 70% confluency.
			Thaw a new aliquot of MDCK cells with a low passage number.
		Cells were not in a single cell suspension when plated	MDCK cells tend to form cell clumps. Pipette the cells up and down throughout all cell preparation stages. When counting the cells make sure that they are not in clumps.
		Cells were over trypsinized	Trypsinize cells for the minimum required time. Add an extra wash with culture media before plating the cells to efficiently remove trypsin.
		For cells plated in chamber slides - chamber slide was not coated properly.	Increase the volume of collagen I solution used for pre-coating.
			Make sure the collagen solution covers the entire surface of the chamber slide.
			Make sure the collagen solution used for pre-

			coating has completely polymerized before adding the collgen-cells mixture
Immunofluorescence (protocol 2)	No antibody staining	Permeabilization of the antibody	Antibody permeabiliztion problems can be caused by the collagenase that is used to permeabilize the collagen matrix. Make sure to use a fresh aliquot of collagenase solution at the specified concentration. Try increasing the incubation time by 1–2 minutes. Incubate with the primary antibody for over night. Increase secondary antibody incubation time.
	High background	Insufficient blocking	Increase incubation time with permeabilization solution. Make sure the antibodies are diluted in permeabilization solution. Replace 10% FBS with fish skin gelatin (7mg/ml) in permeabilization solution.
	Antibody staining is not as expected	Detergent in permiabilization solution distorts protein localization	Use saponin (0.025%) instead of Triton X-100 in permeabilization solution.
Live cell imaging (protocol 3)	Unable to focus on cultures in bright field	cells are too far from the coverslip.	Change to a water objective or to an objective with slightly lower NA. Reduce the volume used for precoating of the chamber slide.
	No or very little fluorescence signal	Cells are too far from cover slip.	Try finding a 3D culture cyst that is closer to the cover slip. Change to a 40X NA 1.3 objective. When using a point scanner zoom in to achieve the same magnification.
		Fluorescence signal is quickly bleached	Switch to a water objective. Minimize exposure to light before image acquisition.
		fluorescent signal is masked	Clean both the objective and the coverslip thoroughly before imaging. Make sure to use the immersion oil recommended by the manufacturer.
		Expression level of the fluorescently tagged protein is	FACS sort the cells to enrich the population of high fluorescent cells.

too low

Anticipated results

3D Culture morphology

The overall morphology of 3D culture cysts can be observed by bright field. In our hands, at day 4 a small lumen surrounded by few cells is observed (fig 2A). At day 6 cultures continue to develop and the lumen grows bigger (Fig 2B).

Immunostaining

The following are recommended immunostainings for evaluating culture morphology and polarization.

1. Nuclear staining: if cultures have developed normally expect a single layer circle of nuclei (Figure 3A). Looking for these unique circles is very useful when screening for cultures with normal morphology.
2. Phalloidin staining: actin is highly accumulated on the apical surface of the polarized cells surrounding the lumen (FIG 3B). In addition, a weaker actin staining can also be observed throughout the outline of the cells. Therefore, phalloidin staining is recommended in order to evaluate cell polarity and to determine the boundaries of the cells surrounding the lumen.

A 3D culture is a three dimensional sample that is composed of many cells. Therefore, different optical slices through the z axis will show different population of cells in the 3D culture and different areas of the lumen. A series of different optical sections of a single 3D culture cyst is presented in Fig 4 to demonstrate the different appearances of the cyst in the z dimension.

Live cell imaging

The membrane dye FM4-64 can be used to label the membranes of individual cells in live 3D cultures. This dye can be used at any time point between 30 min to several days depending on the desired experiment. The dye though changes its staining over time and it is therefore important to distinguish between the different labeling patterns. At the first stage the basolateral membrane is stained (about 30 min after adding the dye) (Fig 5 A, C). Later on, this staining is accompanied by an apical membrane staining and an intracellular pool endocytosed dye (about 1.5 h after adding the dye) (Fig 5 B, D). At steady state the apical membrane staining usually remains, the endocytosed pool increases and the basolateral staining disappears.

FRAP of intracellular proteins

After photobleaching a specific ROI, the specified area should be completely dark while the rest of the fluorescence in the culture should not be affected. During recovery (if any) the bleached box should be gradually filled with fluorescence. Fig 6 demonstrates a FRAP experiment of the microtubule +end binding protein Clip170.

FLIP of intracellular proteins

In FLIP experiments the area of the ROI is continuously bleached and the total decay of fluorescence in the cell is monitored. In these experiments the overall fluorescence signal of a protein that has a cytosolic pool should be decreased in time. However, the fluorescence in neighboring cells should remain the same and should be used as a control. Fig 7 represents a FLIP experiment of Clip170.

Time consideration

1. Plating 3D culture: 2–4 h.
2. Culture growth and development - 4–12 days.
3. Immunostaining - 2 days.

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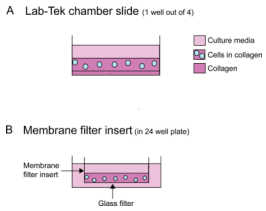

Figure 1.
Schematic representations of 3D culturing of MDCK cells plated in a chamber slide (A) or in a membrane filter insert (B).

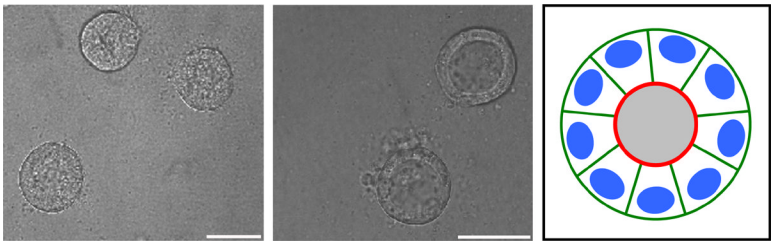

Figure 2.
A DIC image of 3D cultures of MDCK cells plated in a Lab-tek chamber slide at day 4 (A) and day 7 (B). Scale bar = 50 μm. (C) A schematic representation of a 3D culture. Red - apical membrane. Green - basolateral membrane. Blue -nucleus. Grey - lumen.

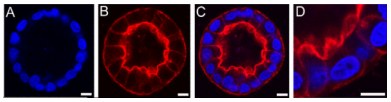

Figure 3.

An immunofluorescent image of a fixed MDCK cyst in a 3D culture at day 5. Nuclei were stained with Hoechst 33258 (blue) (A) and actin was stained with Texas-red phalloidin (red) (B). Images were taken using a 60X oil objective NA 1.4. (D) a zoomed in image of polarized cells in the culture. Scale bar 10μm.

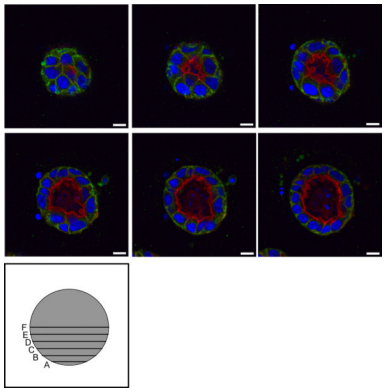

Figure 4.

(A–F) Different optical z sections of an MDCK cyst in a 3D culture from the bottom (A) to the middle (F) of the culture. Culture was fixed at day 5 and stained with Hoechst 33258 (blue), Phalloidin (red) and E-cadherin (green). (G) A schematic view of the different optical z sections shown in A–F.

Scale bar = 10μm.

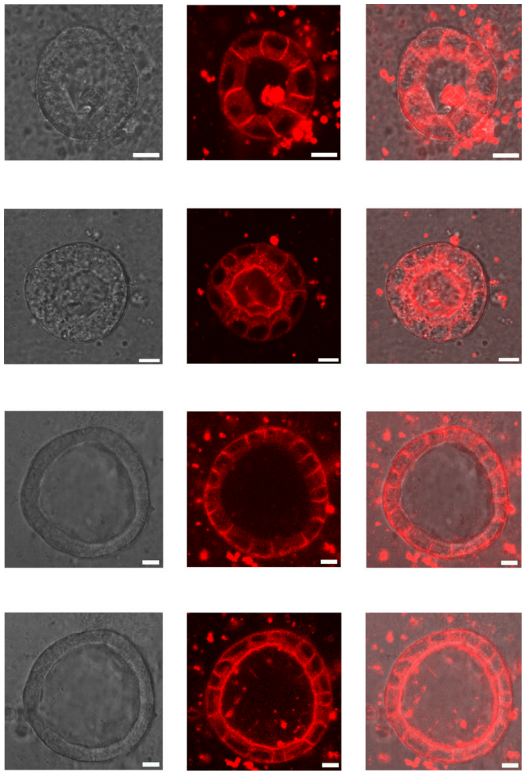

Figure 5.
Live cell imaging of MDCK cysts in 3D cultures using the membrane dye FM4-64. (A–B) cultures at day 4. (C–D) cultures at day 6. (A, C) 30 min after addition of the dye the basolateral membrane is labeled. (B, D) 2 h post dye addition the apical membrane is labeled as well as intracellular endocytic structures. Scale bar = 10μm.

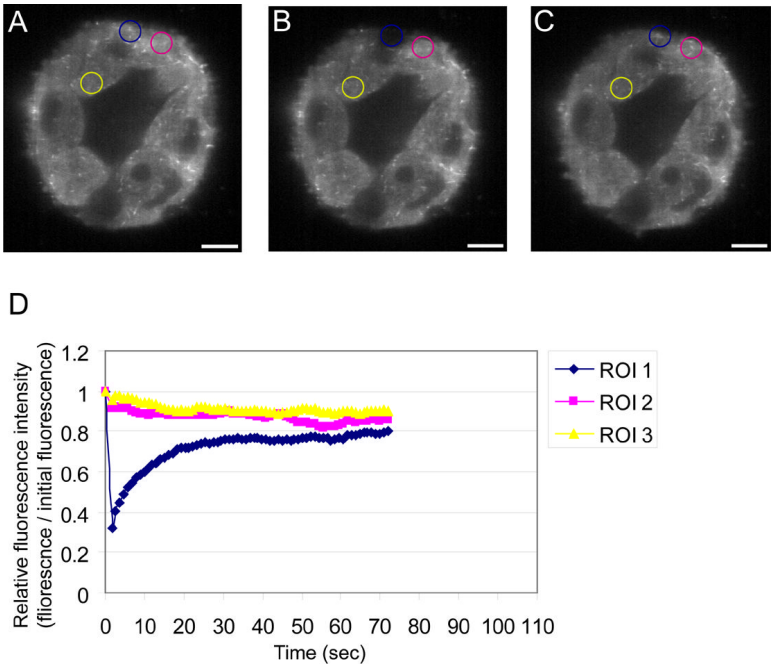

Figure 6.

FRAP analysis of MDCK cells stably expressing the +end binding protein clip170-GFP in 3D cultures. (A) Before bleaching, (B) the first image post bleaching. (C) 25 sec post bleaching the bleached area has recovered. (D) The change in fluorescence intensities over time. ROI 1: the bleached zone, ROI 2: a different region of the same cell. ROI 3: a region from a neighboring cell. The ROIs are depicted in A–C and are color coded according to the graph (D).

Cultures are in day 4. Scale bar = 10um

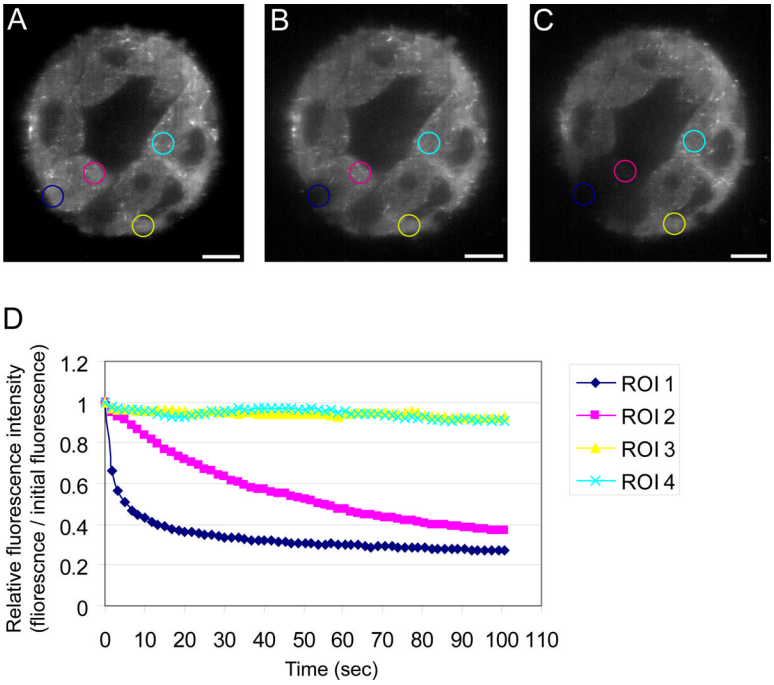

Figure 7.

FLIP experiment of a 4 day old 3D culture expressing Clip170-GFP. (A) Before bleaching. (B) After the first bleaching cycle. (C) After 60 bleaching cycles. (D) The change in fluorescence intensities over time. ROI 1: the bleached zone, ROI 2: a different region of the same cell. ROI 3 and 4: regions from neighboring cells. The ROIs are depicted in A–C and are color coded according to the graph (D). Scale bar = 10μm.