Chapter 16

3D Tumor Models and Time-Lapse Analysis by Multidimensional Microscopy

Dimitri Scholz and Nobue Itasaki

Abstract

The 3D culture is advantageous in reflecting the in vivo condition compared to the 2D culture; however, imaging 3D-cultured cells may be a challenge due to technical restrictions. Recent development of confocal spinning disc microscope system as well as sophisticated software has enabled us to monitor dynamism of cell movement in multiple dimensions. Here we describe the method for time-lapse imaging of 3D-cultured cancer cells co-cultured with non-cancerous cells and discuss current limitations and future perspectives.

Key words Three-dimensional culture, Time-lapse imaging, Multidimensional microscopy, Live cell imaging, Deconvolution

1 Introduction

The three-dimensional (3D) cell culture system is far more advantageous compare to the 2D culture in reflecting in vivo conditions in many aspects such as cell morphology, movement, cell–cell adhesion, cell polarity, and cancer cell malignancy [1]. The cytoarchitecture, which is critical for the function of epithelial cells as well as for the degree of malignancy of cancer cells, can be reestablished in the 3D culture with acquisition of apical-basal polarity and cell adhesion [2]. Cancer cells grown in 3D also show different metabolic pattern from those grown in 2D [3, 4]. Furthermore, cancer cells display different sensitivity and selectivity to anticancer drugs between 3D and 2D cultures [5–7]. These advantages postulate 3D culture not just a replacement of 2D culture but as a necessary system for in vitro cancer research. Despite the fact that such advantages had already been highlighted more than a decade ago [8], the method has not been accepted as widely as it deserves, partly due to unaccustomed technical requirements. Obtaining good quality of images of 3D materials under the microscope is a particular challenge, which may have hampered a wide usage of 3D culture.

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In this chapter, we describe the method of 3D culture and following time-lapse filming that was employed in our recent studies [4], and concisely discuss other possible approaches. The method of analysing 3D-cultured cells by immunocytochemistry is also mentioned. Our studies are on dynamic interaction of cancer cells with non-cancerous epithelial cells using cell lines [4]. However, the method should be widely applicable to other types of studies such as the use of primary cells, drug test, and personalized medicine.

2 Materials

2.1 Cells

MDA-MB-231, breast cancer cell lines from American Type Culture Collection (ATCC), have been made to stably express green fluorescent protein (GFP). Likewise, Madin-Darby canine kidney (MDCK) cell line is mCherry labeled. While stable fluorescent labels are useful for time lapse imaging, they are not necessary for other purposes such as immunocytochemical or biochemical analyses. Primary cells can also be used. For non-fluorolabeled cells (e.g., primary cells) phase-contrast or differential interference contrast (DIC) allows live imaging. Combining fluorescent imaging with transmission light-based techniques are useful not only for detecting non-labeled cells but also for outlining fluorescent cells (*see* **Note 1**).

2.2 Reagents and Consumables

Before setting up 3D culture, cells can be maintained in 2D as usual. The reagents used for MDA-MB-231 and MDCK cells in this study were DMEM (sigma D5546), fetal calf serum (FCS) (Sigma F7524), Penicillin–streptomycin (Sigma P4333), Glutamax™ (Gibco 35050-038), and 0.25 % Trypsin–EDTA (Gibco 25200-056). For the 3D culture, Geltrex™ reduced growth factor basement membrane matrix (Gibco 12760-021) was used in this study. More widely used matrix, Matrigel [9], is anticipated to work in a similar way.

It is essential to use special imaging-grade dishes or slides with the cover slip-thin (150–190 μm) bottom. We got best results with products from ibidi (Germany), who provide a broad range of cell culture slides, dishes and gadgets (http://ibidi.com/home/). For our 3D time-lapse imaging, ibidi μ-dish (ibidi 81151) or μ-slide (ibidi 80426, 80421) were used. The plate does not need coating as the matrix is used for the 3D culture.

3 Methods

3.1 Cell Culture

The cell lines were cultured in DMEM containing 10 % FCS, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM Glutamax™. All cells were incubated in a 37 °C humidified cham-

ber supplied with 5 % CO_2. The medium was replaced every 3 days and the cells were passaged once they were 80–90 % confluent using trypsin.

3.2 Three-Dimensional Co-cultures

The cells that have been cultured in 2D and reached near-confluent were trypsinized and suspended at 1×10^5 cells/ml in the culture medium. On the desired ibidi μ-dish, Geltrex™ was spread to form a thin layer on the surface and allowed to set in the incubator at 37 °C for 20 min (*see* **Note 2**). Cell dilutions were prepared containing MDA-MB-231 and MDCK cells at a desired ratio (1:1 in this study). The cell mix were then centrifuged at 1000 rpm ($188 \times g$) for 5 min at room temperature. The supernatant was aspirated off and the cells were resuspended in Geltrex™ in each tube. The amount of cells and gels should be optimized beforehand; it may depend on the speed of cell proliferation, length of incubation and purpose of the study. For time-lapse imaging of MDA-MB-231 and MDCK cells after 7–10 days of incubation, 1×10^5 of cells resuspending in 0.1 ml of Geltrex is optimal; however, this might be relatively sparse for other purposes.

The cells and the gel matrix were pipetted up and down to mix well. The mix was then pipetted on top of the already-set Geltrex™. The plate was incubated at 37 °C for 20 min to allow the matrix to set. The culture medium was then added slowly to cover the set-gel. The medium was replaced every 3 days and the cells were grown for 7–10 days. The cells may be grown for at least 14 days.

For immunocytochemical analyses, the above method were applied on the cover slips which had been put in culture wells. For example, 12 mm diameter round cover slips were put in 24-well-plate wells. After the culture, fixing, washing, and immunolabeling can be performed all in the well while keeping the cells in the matrix. Once secondary antibodies were washed off, the cover slip to which matrix containing the cells were attached was taken out of the well and mounted on a glass slide with mounting medium (e.g., Mowiol 4-88, Sigma 81381; Slowfade, Life Technologies S36938).

3.3 Multidimensional Live Cell Imaging

In this study, the Andor Revolution Laser Confocal spinning disc microscope system was used with the inverted Nikon Ti microscope, motorized XYZ Prior stage, and Andor iXon897 EM EMCCD camera operated by Andor IQ2.6 software. The microscope was equipped with a chamber keeping the cells at 37 °C with continuous supply of 5 % CO_2 and humid air. The experimental setup included sequential time-lapse acquisition (every 1–20 min) from 10 to 40 different XY fields, in two fluorescent channels, GFP and mCherry. For each time point and XY location, a *Z*-stack of 9–40 optical sections was acquired (*see* **Notes 3–5**).

If we consider the individual 3D with *Z*-stacks as 3D microscopy, then the time lapse of *Z*-stacks is 4D. When these

are performed at multiple positions of the field, it is 5D. With multiple-channel imaging, it is 6D. We hence use the term "multidimensional microscopy."

3.4 Data Analysis

Data sets resulting from multidimensional acquisition could be huge. *Z*-stacks of 30 layers at each XY position, in two colours at 120 time points (over 24 h) and 40 positions of XY fields, result in 288,000 individual frames. With an Andor EMCCD camera with 512×512 pixels and 14-bit digitalization, the resulted images were mounted in over 100 GB of raw data. It is therefore important to ensure a sufficient storage space available in the computer. During the acquisition, the data streamed directly to the hard drive, which prevented the complete loss of data even in case of power cut. The data were then sorted by the XY field of view and analyzed on a separate working station. We excluded those films where cells traveled outside of the field or went out of focus. Therefore, a multitude of fields are essential for the analysis and statistics. The usual workflow then included the 4D deconvolution (XYZT) for each remaining field of view using the Autoquant 3.0 software. Volume rendering, measurements and export in the form of single shots or movies were performed using Imaris 7.6 software.

It is very important to use a different computer for image analysis because of the following reasons: (a) The analysis may take hours during which the acquisition on the microscope would be prevented. (b) Different requirements have to be applied to those computers; while the "microscope" computer has to allow the fast data stream from the captured data and safe storage, the "off-line" working station has to possess a huge operative memory and a great graphic card.

3.5 The Current Setups, Limitations, and Future Perspectives

The live cell imaging is currently performed with an inverted microscope using transmission light, fluorescent light, or reflected light modes. To detect fluorescence-labeled proteins, detection of the fluorophore is the key part of imaging. Each of individual images is a two-dimensional projection. To acquire information of the third dimension, different approaches are currently used. The epi-fluorescent microscopy delivers blurry images because it captures the image of the whole thickness of the sample. It is therefore useful for acquiring images of thin monolayers of cells, especially under low magnifications. There is no advantage of using confocal microscope over epi-fluorescent one if you employ a 10×–40× lens for monolayer culture of HeLa cells or endothelial cells, for example. To get the volume information from epi-fluorescent image, a Z-stack of images followed by 3D deconvolution is required. In future, rotation of sample followed by tomography-like deconvolution is expected. Another advantageous usage of epi-fluorescent application is a quantitative measurement of

fluorescence such asCa^{++} imaging, because the whole *Z*-range of the object is illuminated and the whole fluorescence is collected by the detector.

In confocal microscopy (single pinhole or spinning disc), the out-of-focus light is blocked by the pinhole(s). The resulting image therefore represents an optical section. This makes confocal images unsuitable for fluorescence measurements. For volume reconstruction, multiple optical sections need to be captured and rendered *in silico*. Spinning disc confocal microscope is best for live imaging of relatively thin samples that do not exceed 30–50 μm in *Z*-dimension. Thicker samples such as 100 μm may be acceptable if they are well-labelled with a high signal-to-noise ratio. Otherwise, cross-talk of pinholes could deteriorate the result. For thicker samples, though, deconvolution is more effective.

If your sample is in the range of 50 μm–2 mm, the multiphoton microscope would be the choice, although microscope manufacturers claim to render up to 8 mm in thickness. If your sample is transparent eggs or embryos sized 100–1000 μm, the best microscopy of choice is Single Plane illumination microscopy (SPIM).

In all cases, the fluorescent microscopy may be combined with the transmission light microscopy (phase of differential interference contrast) to outline the live cells. For imaging metallic nanoparticles, we have developed and recommend to use the reflected mode that is fully compatible with the fluorescence [10].

4 Notes

1. Cell labeling
   To distinguish different cell types under the microscope, the use of cell lines stably expressing fluorescent proteins is the current method of choice. However, establishing coloured cell lines is time-consuming and sometimes not practical, especially for primary cells. To distinguish two cell populations, it is enough to label only one and then combine the fluorescent and transmission light microscopy. As such, distinguishing three different cell types requires two different labelings. We have not been confronted yet with a task to co-culture more than three different cell types. However, contemporary imaging technologies would allow differentiating three or more fluorescent proteins in the live mode.
2. 3D culture thickness
   It is best not to make a thick matrix for the optical reason. Nonetheless, the first bottom matrix layer is important for the 3D culture, because cells would stick to the bottom glass without it thus resulting in the 2D culture.

3. Parameter setting
   At the beginning and/or end of filming, snapshots may be taken with a larger number of *Z*-stack layers. If there are too many Z-stack layers during the film capture, it would take long time to scan all layers. The optimal *Z*-step should be 2–3 times smaller than the *Z*-resolution of the objective lens (Nyquist theorem). Further practical advice is available online: http://www.svi.nl/NyquistCalculator. Surely, a compromise between spatial and temporal resolution as well as sensitivity of the detector has to be found for each particular experiment. The same rule applies for XY-resolution, although we had less choice for it. For our biological system we used the 20×/NA0.70 PlanApo objective lens that covers a reasonably wide XY field while obtaining a good resolution.

   Different from a classical singe pinhole confocal microscope, the spinning disc pinholes are not flexible but are optimized for NA1.4 lenses. Nonetheless, we obtained decent confocal images with 20×/NA0.7 and even with 10×/NA0.45 lenses.

   For multipositional acquisition from a multi-well dish for many hours, only dry objective lens, not immersion oil lens, is possible to use, as immersion oil would be lost during the repetitive movement of the stage. A long-hour usage of oil objective lens may become possible if immersion oil is continuously supplied. Such a system with a nozzle for water immersion is already available for some Olympus microscopes, which opens a possibility in the development of similar nozzle systems on the oil or silicone objective lenses in the future (Figs. 1 and 2).

4. Acquisition protocol
   It appears to be better to scan all *Z*-stacks in one channel then scan again in another channel, rather than scanning all channels on one *Z* plane and moving to the next plane, because it takes shorter for the hardware to change the focal plane (less than 1 ms) than to change the channel (approximately 70 ms in our case).

5. Time-lapse interval setting
   Time lapse of every minute provides films of smooth movement of cells, however, other parameters such as the number of XY positions may need to be compromised. For example, in the condition of ten XY positions with 25 *Z*-stacks and two channels each, it takes 4 min 30 s for one round of scan in our setup; hence it was not possible to take time-lapse filming with an interval shorter than that. Time lapse of every 5 min is reasonably acceptable in detecting the cell movement. Different time-lapse intervals can be compared in our published literature [4].

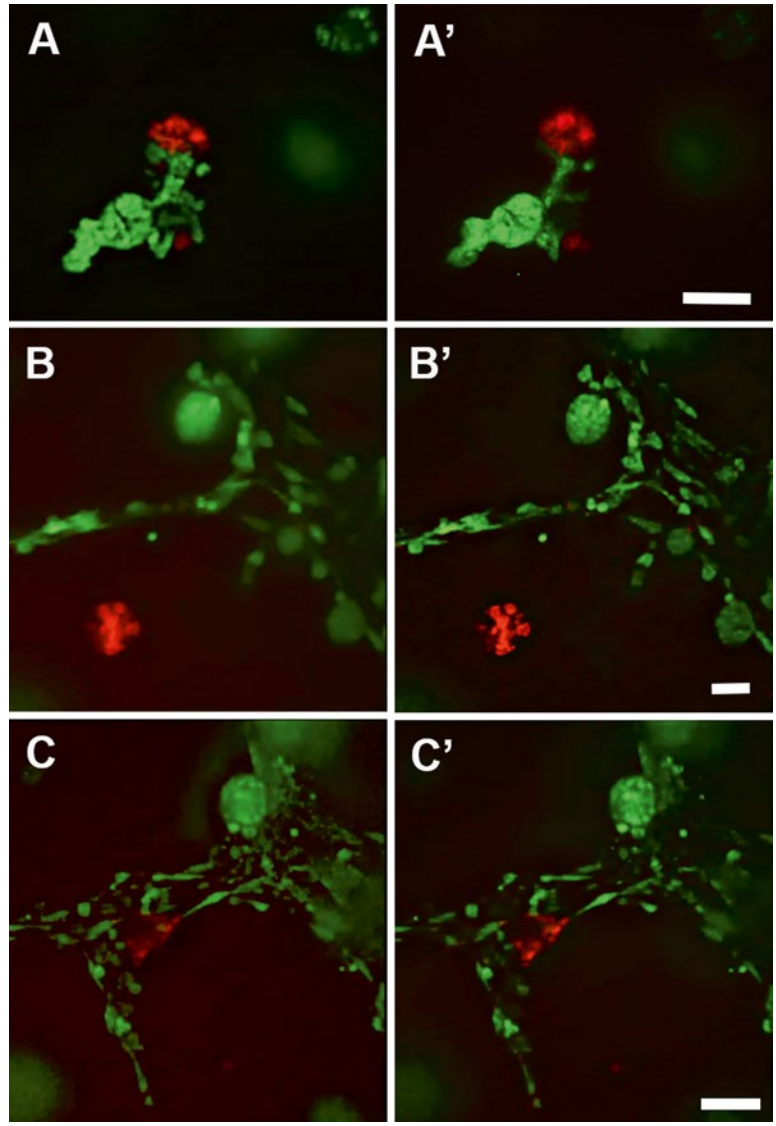

Fig. 1 The effect of deconvolution. Three examples of before and after deconvolution of snapshot images. A–C are snapshots of live images of 3D cultures whereas A′–C′ are deconvolved versions of A–C, respectively. Scale bars; A and C, 50 μm; B, 30 μm

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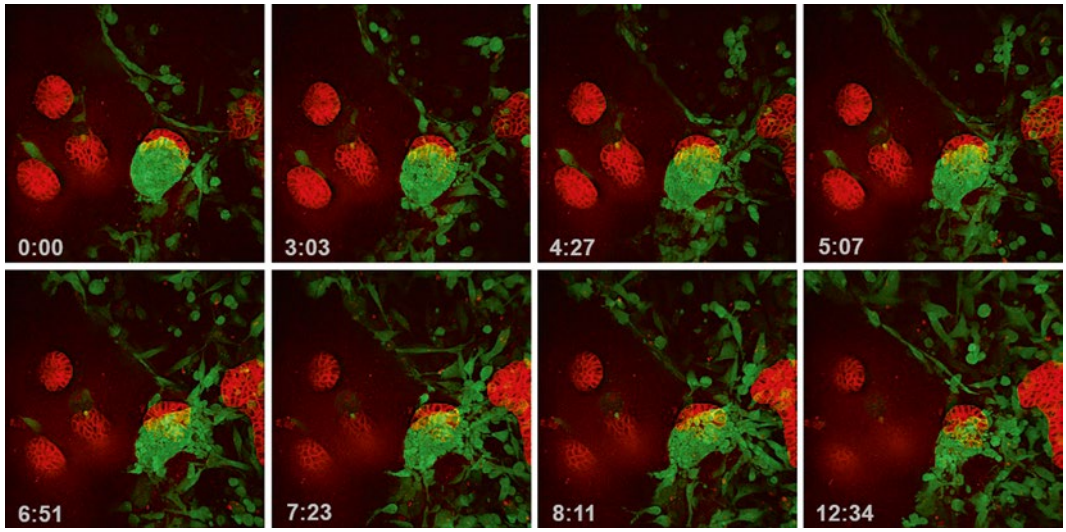

Fig. 2 Snapshots of a time lapse movie of 3D co-culture of MDA-MB-231 (*green*) and MDCK (*red*) cells, showing dynamism of MDA-MB-231 cells and rather static MDCK cells. The images were captured every 5 min at 25 *Z*-planes ($\Delta Z = 1.5$ μm; total *Z* range, 37.2 μm). Total duration of filming was 12 h 45 min. The image data were deconvolved

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