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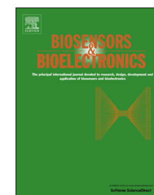

Label-free analysis of prostate acini-like 3D structures by lensfree imaging

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ABSTRACT

We present a lensfree imaging method to analyze polarity in RWPE1 prostate epithelial cells that form polarized acini with lumen under standard tridimensional (3D) culture conditions. The first event in epithelial carcinogenesis is loss of polarity, followed by uncontrolled proliferation leading to metastasis. We demonstrate that it is possible to use optical signatures to discriminate 3D objects with distinct polarities in a large field of view. The three metrics we present here are designed as image processing tools to discriminate acini from spheroids without any 3D reconstruction. To demonstrate that our lensfree imaging platform may be used to study the 3D organization of epithelial cells, we analyzed and quantified the modulation of dynamic processes, e.g., the polarity of acini and the merging of polarized structures, upon transforming growth factor beta-1 (TGF beta-1) addition to the culture media. Hence, coupling lensfree microscopy with 3D cell culture provides an innovative tool to study epithelial tissue morphogenesis in a large field of view and to elucidate the regulation of growth, morphogenesis and differentiation in normal and cancerous human prostate cells. Moreover, such biosensor would be a powerful tool to follow cancer progression and to evaluate anti-cancer drugs.

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1. Introduction

For many decades, cell biology experiments have been based on two dimensional (2D) cell cultures on rigid plastic substrates. Unfortunately, such culture conditions poorly reflect the reality of the cell microenvironment, which is composed of a fibrous network of proteins from the extracellular matrix (ECM) and neighboring cells in a tridimensional (3D) configuration. Cell culture in 3D offers a more realistic model of the in vivo microenvironment that favors polarized cell–cell and cell–ECM interactions (Pampaloni et al., 2007). Therefore, 3D cell culture is becoming routine in cell biology laboratories, and systems enabling the more complex reconstruction of tissues have been proposed (Mailleux et al., 2008; Pearson et al., 2009; Bryant and Mostov, 2008; O'Brien et al., 2002; Zegers et al., 2003). In this paper, we focus on the study of acini, which are the basic 3D structures constituting a secretory epithelium. The integrity of fully differentiated acini is determined by their apical and basal polarities, which are both characterized by specific cell–basement membrane contacts, intercellular junctions and luminal apoptotic cells (Debnath and Brugge, 2005). The maintenance of this polarity is critical for the structure and function of epithelial cells. The mechanisms controlling epithelial polarity and lumen formation are poorly understood (Pearson et al., 2009). Moreover, the disruption of apical polarity and the progressive transformation of acini into spheroids are key events in tumor progression. Therefore, the analysis of cell polarity is critical to assess epithelial development, integrity and homeostasis and to study the pathogenesis of epithelial tumors. To properly analyze polarity, in vitro 3D culture systems are necessary and are of increasing interest for cancer research because they recapitulate in vitro the alteration of the tissue architecture that precedes tumor development. Moreover, tissue architecture and the ECM influence the responses of tumor cells to signals from the microenvironment provided in a 3D context.

The imaging of 3D polarized structures in culture is not developing as fast as the 3D culture systems themselves. Immunofluorescence imaging of apical and basal polarity markers in fixed cells is the classical approach to study epithelial tissue phenotypes (Lee et al., 2007). It provides a way to follow the loss of apical

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polarity that is often described by the redistribution of tight junction proteins away from apical sites and by the filling of the lumen by disorganized cells, indicating that acini are transforming into spheroids (Hebner et al., 2008). However, the immunostaining approach presents some limitations (Yue et al., 2012), one being that it remains expensive because it is time-consuming and costly. In addition, data acquisition from living cells requires the use of transfection to induce the stable fluorescent expression of particular proteins, and the side effects on cellular function have to be considered (Baum et al., 2003). For clinical purposes, those traditional methods are not suited for the rapid and high-throughput counting/imaging of 3D cultures and the high-throughput evaluation of drugs that would cause high-throughput increase of the acinar forming ability of the cells while decreasing their invasiveness.

To overcome the above-mentioned limitations, a new imaging methodology to fully exploit the benefits of 3D cultures is needed. A direct imaging technique would (i) allow the observation of living cells, (ii) not require any staining, (iii) be time-efficient and (iv) enable high-throughput analysis and hence provide satisfactory statistical data.

As reviewed recently in Greenbaum et al. (2012), there is great potential for imaging without lenses, i.e., on-chip cell imaging and lensfree holographic microscopy. The resolution of these techniques is approximately 1 μm and can hardly compete with microscopy, but they offer several other advantages: the field of view (FOV) can cover up to several cm^2, the equipment is mostly compact in size; there is a wide range of readouts ranging from 1 nm to 1 mm; and they are easy to use. The e-Petri dish (Zheng et al., 2011), an on-chip cell imaging platform based on shadow imaging, challenges the superiority of video microscopy for applications such as cell migration assays. It allows continuous monitoring over a large field of view (24 mm^2), enabling the live imaging of thousands of cells simultaneously, but it remains limited to 2D cell culture. Moreover, the system requires a specific cell culture chamber and the customization and skillful integration of a sensor; 3D cell culture imaging with such a device has not yet been demonstrated. The second method, lensfree holographic microscopy, is very efficient for the detection of biological objects over very large FOVs (>20 mm^2) (Bishara et al., 2011; Mudanyali et al., 2011; Allier et al., 2010). Recently, it has been used to detect viruses (Mudanyali et al., 2013), to perform high-throughput 3D tracking of human sperm (Su et al., 2012) and to image microvessels from endothelial cells (Weidling et al., 2012). Little has been done to use lensfree holographic microscopy for monitoring cell culture (Moscelli et al., 2011), although it offers great promise for observing 3D cell cultures and even reconstructed tissues (Weidling et al., 2012; Isikman et al., 2011). Lensfree imaging could be used in difficult cellular assays for the quantification and analysis of complex cellular responses to address fundamental questions in developmental biology and to evaluate the effects of drugs in a physiological context.

Here, we use lensfree imaging to observe 3D structures from epithelial cells. We demonstrate that it is possible to use distinct optical signatures to quickly discriminate 3D objects of different polarities in a large field of view. Each object may exhibit either a well-ordered architecture (acinus) or a disrupted 3D structure with filled luminal space (spheroid). The three metrics we present here, i.e., gray level, Zernike features and the reconstructed holographic amplitude, are designed as image processing tools to discriminate acini from spheroids without 3D reconstruction. To illustrate the capabilities of our lensfree imaging platform, we showed how the 3D microenvironment influences 3D epithelial cell morphology and the merging of a population of cells. More specifically, we studied the effects of transforming growth factor beta-1 on acini size, lumen presence and acini merging, supported by strong statistical data.

This imaging system could be used extensively for studying cell–cell and cell–environment interactions by modulating the biochemical and mechanical stimuli within the ECM. We believe that this imaging system will be a powerful method for the large field analysis of multiple soluble effects, including fundamental and pharmacological research in carcinogenesis.

2. Materials and methods

2.1. Cell lines

RWPE1 and WPE1-NB26 cells were used in this study as models for normal and malignant epithelial acinar morphologies, showing two distinct and well-characterized phenotypes in 3D. The RWPE-1 cell line was obtained from ATCC (CRL-11609). This cell line was derived from non-neoplastic human prostate epithelial cells by immortalization with human papillomavirus (22). The WPE1-NB26 cells were derived from RWPE-1 cells by exposure to a direct-acting carcinogen, N-methyl-N-nitrosourea, and were purchased from ATCC (CRL-2852). The RWPE1 cells mimic normal prostate epithelial cell behavior as characterized by a well-established polarized morphology, whereas the WPE1-NB26 cells represent tumorigenic cells at a highly invasive stage of neoplastic transformation with the loss of acinar morphogenesis.

2.2. Cell culture (2D and 3D) and staining

RWPE1 and WPE1-NB26 cells were both maintained in KSFM (Life Technologies, Carlsbad, CA, ref. 17005-075) supplemented with 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract. The cells were maintained in culture until approximately 70% confluency. For passaging, cells were washed with Dulbecco's Ca^{2+}/Mg-free PBS (D-PBS, Life Technologies, ref. 14190) and incubated with 1 mL trypsin–EDTA (Lonza, Basel, CH, ref. CC-5012, 0.25 mg/mL) for approximately 7 min. The trypsin was neutralized with 2 ml trypsin neutralizing solution (Lonza, ref. CC-5002), and the cells were recovered by centrifugation and counted using a Scepter 2.0 Handheld Automated Cell Counter (Millipore, Billerica, MA, ref. PHCC 20060). For the acinar morphogenesis assay, the RWPE1 and WPE1-NB26 cells were cultured in 3D with KSFM (Life Technologies, ref. 17005-075) supplemented with 50 ng/mL EGF and 2% fetal bovine serum (FBS). The 3D culture was grown in Matrigel (BD Biosciences, San Jose, CA, ref. 356231) according to the top-coat protocol (8). The top-coat assay was preferred to the 3D 'embedded' assay because it requires less time and EHS (Engelbreth–Holm–Swarm Mouse Tumor) and because it facilitates imaging as the colonies are in a single plane (8). Briefly, Matrigel was thawed overnight and poured into 4-well (160 μL of Matrigel, 500 μL of culture media) or 8-well Labtek (90 μL of Matrigel, 250 μL of culture media) plates on ice. For polymerization, Matrigel was incubated for 30 min at 37 °C. Cells were seeded in half the final volume and allowed to adhere for approximately 45 min. The top coat layer containing 8% Matrigel was slowly poured over the attached cells. The culture media was changed every other day. All cells were routinely cultured in a humidified atmosphere with 5% CO_2 at 37 °C. To perform statistical analysis and to determine spheroid-to-acini ratios in the same field of view, a mix of WPE1-NB26 and RWPE1 cells was seeded onto 3D Labtek slide chamber and analyzed. To quantify the effect of TGF beta on acinar formation, we conducted an experiment in which TGF beta was added to RWPE1 3D cell cultures after 3, 5 and 10 days of culture.

After at least 8 days of 3D culture, the cells were fixed with 4% (v/v) paraformaldehyde in PBS for 20 min and washed once with PBS. Actin filaments were stained with TRITC phalloidin (Sigma-Aldrich,

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