Optical coherence tomography of cell dynamics in three-dimensional engineered tissues

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ABSTRACT

Cell-based engineered tissue models have been increasingly useful in the field of tissue engineering, in *in vitro* drug screening systems, and in complex cell biology studies. While techniques for engineering tissue models have advanced, there have been few imaging technique capable of assessing the complex 3-D cell behaviors in real-time and at the depths that comprise thick tissues. Understanding cell behavior requires advanced imaging tools to progress from characterizing 2-D cell cultures to complex, highly-scattering, thick 3-D tissue constructs. Optical coherence tomography (OCT) is an emerging biomedical imaging technique that can perform cellular-resolution imaging *in situ* and in real-time. OCT, which uses near-infrared laser light, provides deep-tissue imaging up to several millimeters within highly-scattering tissue, thus permitting visualization of changes at depths previously unattainable. In this study, we demonstrate that it is possible to use OCT to evaluate dynamic cell behavior and function in a quantitative fashion in four dimensions (3-D space plus time). We investigated and characterized cell dynamics and processes in deep tissue models, such as cell de-adhesion, cell proliferation, cell chemotaxis migration, cell necrosis, and cell apoptosis. This optical imaging technique was developed and utilized in order to gain new insights into how chemical microenvironments influence cellular functions and dynamics in multi-dimensional models. In addition, by detecting the changes in cell dynamics, effective chemical concentration could be estimated. With high penetration depth and increased spatial and temporal resolution in 3-D space, OCT will be a useful tool for improving our understanding of cell dynamics *in situ* and in real-time, for elucidating the complex biological interactions, and for directing our designs toward functional and biomimetic engineered tissues.

Keywords: OCT, cell dynamics, engineered tissues, non-invasive, three-dimensional

1. INTRODUCTION

Cell activities in three-dimensional (3-D) engineered tissues are of great interest for both basic cell biology research and applications, such as tissue engineering and pharmacological research.[1,2] Invasive imaging methods such as histology and scanning electron microscopy (SEM) are used predominantly to evaluate cell activities and cellular responses to environmental stimuli in engineered tissues. These invasive methods, however, have intrinsic disadvantages. These methods do not permit real-time or dynamic imaging, lack real 3-D information, require long and harsh processing steps at discrete time points, and make structure–function correlations difficult. Consequently, despite a tremendous increase in tissue-engineering research, few have investigated the dynamics of cell behaviors in engineered tissues. The primary limitation has been inadequate imaging technology for high-resolution, real-time, noninvasive imaging deep within highly scattering tissues.

Confocal microscopy has been an important advance in microscopy and has enabled the imaging of intact, optically nontransparent specimens to produce high-resolution (submicron) images of tissue structure with the use of fluorescent probes.[3–5] For a relatively thick specimen (up to several hundred microns), confocal microscopy accomplishes optical sectioning by scanning the specimen with a focused beam of light and collecting the fluorescence signal via a pinhole

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aperture that spatially rejects light from out-of-focus areas of the specimen. Imaging depths, however, are limited to a few hundred microns and exogenous fluorescence probes are usually required for detection, often limiting the long-term viability of the cells being imaged. Multiphoton microscopy, which relies on the simultaneous absorption of two or more near-infrared photons from a high-intensity short-pulse laser (most commonly a mode-locked titanium:sapphire laser) extends the imaging depth of confocal, but still with depth limitations of about 400–500μm.[6,7] Newer technologies for imaging engineered tissues, including high-field strength magnetic resonance imaging and microcomputed tomography, have been pursued for the assessment of cellular structure, with limited success. These techniques, with long data acquisition rates, hazards associated with high-energy radiation, and relatively high costs, are less suitable for both real-time and long-term imaging.[8,9]

Optical coherence tomography (OCT) is an emerging technique that has the potential for overcoming many of the limitations of the current technologies.[10,11] OCT measures the intensity of backreflected near-infrared light. OCT combines the high resolutions of most optical techniques with an ability to reject multiply scattered photons and, hence, image at cellular resolutions (several microns) up to several millimeters deep in nontransparent (highly scattering) tissue. Because OCT relies on variations in indices of refraction and optical scattering for image contrast, no exogenous fluorophores are necessary, enabling cellular imaging within living specimens over time without loss of viability. Near-infrared light is scattered less than visible light. Therefore, the use of near-infrared wavelengths in OCT enables deep imaging penetration within highly scattering tissues. OCT has been applied *in vivo* for imaging the microstructures of different tissues including the eye, skin, gastrointestinal tracts, and neural systems, to name only a few, and is becoming a promising and powerful imaging technology that has widespread applications throughout many fields of biology and medicine.[12–17] However, few studies have investigated the use of the OCT technology for monitoring cell behaviors in the developing *in vitro*-engineered tissues.[18,19]

In this article, we report the use of OCT as a noninvasive imaging modality to explore 3-D cell activities in engineered tissues. OCT is capable of clearly identifying cells, and determining their position, distribution and morphology in 3-D. For the first time, here we investigated and characterized cell dynamics and processes including cell de-adhesion, cell proliferation, cell chemotaxis migration, cell necrosis, and cell apoptosis. This optical imaging technique was developed and utilized in order to gain new insights into how chemical microenvironments influence cellular functions and dynamics in multi-dimensional models. Compared to other microscopy imaging approaches, OCT permits high-resolution, real-time, deep-tissue, 3-D imaging to be performed rapidly and repeatedly over extended periods of time with intact, living specimens. This powerful approach to the noninvasive imaging of the morphology and function of engineered tissues has enormous potential for a wide range of applications ranging from tissue engineering to drug discovery.

2. MATERIALS AND METHODS

2.1 Cell and engineered tissue culture

Engineered tissues are composed of cells, and matrix scaffolds or synthetic polymer scaffolds. In this study, NIH 3T3 cells (American Type Culture Collection [ATCC], Manassas, VA) and macrophages were used. Gel-based engineered tissues were prepared by mixing cell suspension with thawed Matrigel solution (BD Bioscience, Bedford, MA) using 1:1 proportion. Cell and tissue cultures were maintained in an incubator at 37°C and with 5% CO_2. For real-time imaging, tissue culture was performed in a portable microincubator (LU-CPC, Harvard Apparatus, Holliston, MA) that was placed on the microscope stages for *in situ* imaging.

2.2 Cell migration assay

Matrigel-based invasion assays are the most representative method of *in vivo* events. Porous filters were coated with Matrigel layer and placed in a house-made two-chamber system, which is designed like a Boyden migration chamber. Chemoattractant, monocyte chemoattractant protein-1 (MCP-1, Research Diagnostics Inc, NJ), were placed in the lower chamber and macrophages and Matrigel in the upper chamber. Before the migration assay, macrophages were starved in serum-free media for ~2hrs. Then, cell suspension containing $1X10^5$ cells was added on the Matrigel layer in the top chamber. The migration chamber was incubated in a humidified 5% CO_2, 37^0C incubator for 1~2 hrs, before transferring to a microincubator for dynamic imaging under OCT microscope.

2.3 Cell proliferation assay

Engineered tissues made of 3T3 fibroblasts and Matrigel gel were used in the cell proliferation assay. OCT images were used to estimate the cell number increase in 3-D tissues.

2.4 Cell apoptosis assay

Engineered tissues made of 3T3 fibroblasts and Matrigel gel were used in the assay. The addition of an apoptosis inducer on top of the engineered tissue, etoposide or camptothecin (both from BioVision, Mountain View, CA), initialized the cell apoptosis. Three types of apoptosis assays were used. One uses traditional histology method, using 10μm paraffin-embedded tissue sections stained with hematoxylin and eosin (H&E). Another uses Fluorescien-FragEL DNA fragmentation kit (EMD Biosciences, CA) to label the paraffin-embedded tissue section. We performed the assay following the protocol given by the manufacturer. Finally, 3-D OCT images reconstituted from a series of x-z images over y-axis were used to evaluate the progress of cell apoptosis in the engineered tissue.

2.5 Cell necrosis assay

Engineered tissues made of 3T3 fibroblasts and Matrigel gel were used in the assay. To induce cells' to undergo necrosis death, 2% bleach was added on top of the tissue. The engineered tissue was immediately and continuously visualized with an OCT system to track the dynamic process of cell death over the thickness of the tissue.

2.6 Cell detachment assay

The engineered tissue was made by $5x10^4$ 3T3 fibroblasts seeded in a 3-D calcium phosphate scaffold (BD Bioscience, Bedford, MA). After cultured for two days, the tissue was immersed in the solution of .25% trypsin/EDTA. The engineered tissue was immediately and continuously visualized with OCT system to track the dynamic process of cell detachment from the scaffold.

2.7 Optical coherence tomography

OCT imaging was performed on engineered tissues. Our fiber-based OCT system used a Nd:YVO_4-pumped titanium:sapphire laser as a broad-bandwidth optical source that produced 500mW of average power and approximately 90fs pulses with an 80MHz repetition rate at an 800nm center wavelength. Laser output was coupled into an ultrahigh-numerical-aperture fiber (UHNA4, Thorlabs, Inc.) to spectrally broaden the light from 20nm to more than 100nm, improving the axial resolution of our system from 14μm to 3μm. The reference arm of the OCT interferometer contained a galvanometer-driven retroreflector delay line that was scanned a distance of 2mm at a rate of 30 Hz. The sample arm beam was focused into the tissue by a 12.5 mm diameter, 20 mm focal length achromatic lens to a 10μm diameter spot size (transverse resolution). The 12mW beam was scanned over the engineered tissue with a galvanometer-controlled mirror. The envelope of the interference signal was digitized to 12-bit accuracy.

2.8 Three-dimensional data reconstruction

Three-dimensional images were reconstructed with SliceDicer (Pixotec, Renton, WA) and Amira (Image System, Minnetonka, MN).

3. RESULTS AND DISCUSSIONS

3.1 Cell migration

Cell migration is a fundamental function of normal cellular processes, including embryonic development, angiogenesis, wound healing, immune response, and inflammation. Boyden chamber assay using microporous membrane inserts is the most widely accepted assay for cell migration and invasion. In order to facilitate the dynamic imaging of cells, we fabricated a Boyden-chamber-like system and made it adaptable to a microincubator sitting under an OCT imaging microscope. Migration was measured in chemotaxis assay where the cells move towards a chemoattractant, MCP-1. Figure 1 demonstrates the 3-D positional changes of the cells in the tissue over the time. Cell positions at a time were labeled with a specific color. Cell migration direction and velocity can be easily obtained from OCT images. Current methods of analysis of cell migration and invasion are time-consuming and tedious, involving cotton swabbing of non-migrated cells on the top side of insert, manual staining and counting.

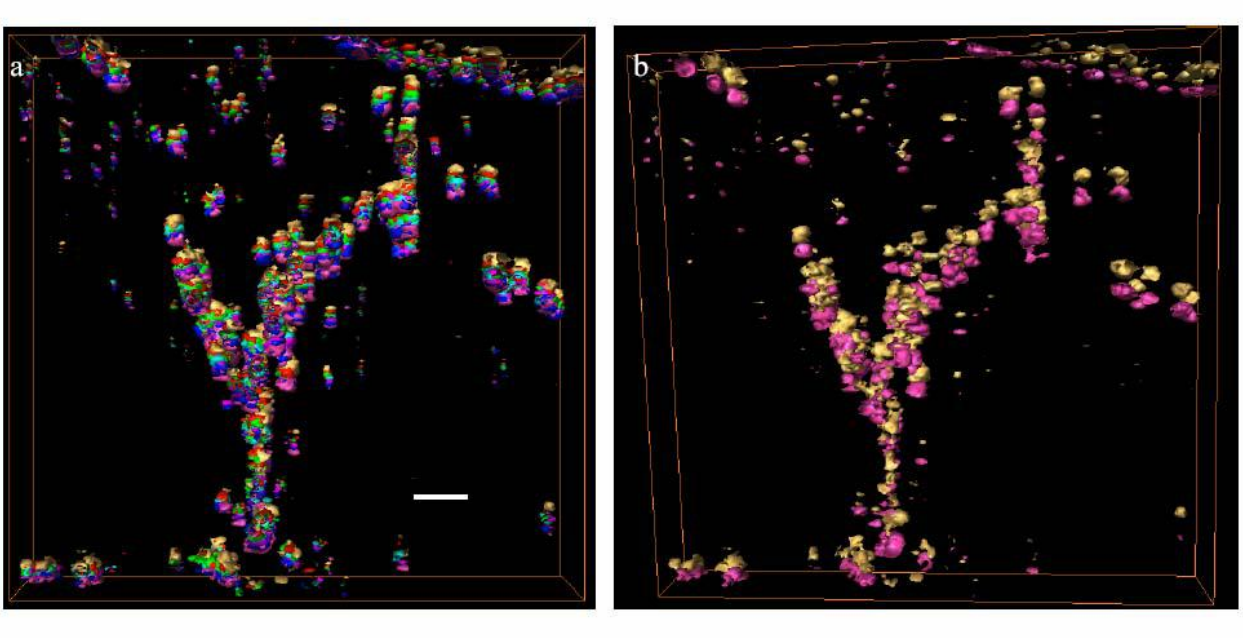

Figure 1. OCT images demonstrate cell migration in 3-D space (a) Cells at different points of time are labeled with different colors. The interval time is 10min. (b) Image shows the position change of cells after 50min. Scale bar is 100μm.

The migration assay based on the non-invasive optical imaging tool provides a quick and efficient system for quantitative determination of factors on cell migration. Furthermore, chemoattractants are potential therapeutic target for intervention in wound healing, cancer, and inflammation. It is possible to use OCT tool to evaluate the performance of various chemoattractants.

3.2 Cell proliferation

Figure 2 illustrates OCT images used in tracking cell proliferation in the 3-D tissue. Compared to the cells cultured on a monolayer, where the cells have minimal migration resistance, cells seeded in 3-D matrix might face higher resistance to migration. Thus, in contrast to a homogeneous proliferated cell monolayer, the engineered tissue is comprised of cell aggregates after days of proliferation. Cells divide and form aggregates at their original sites. In 3-D OCT image, the cell number increase in a 3-D tissue is demonstrated by the increasing size of the cell aggregates.

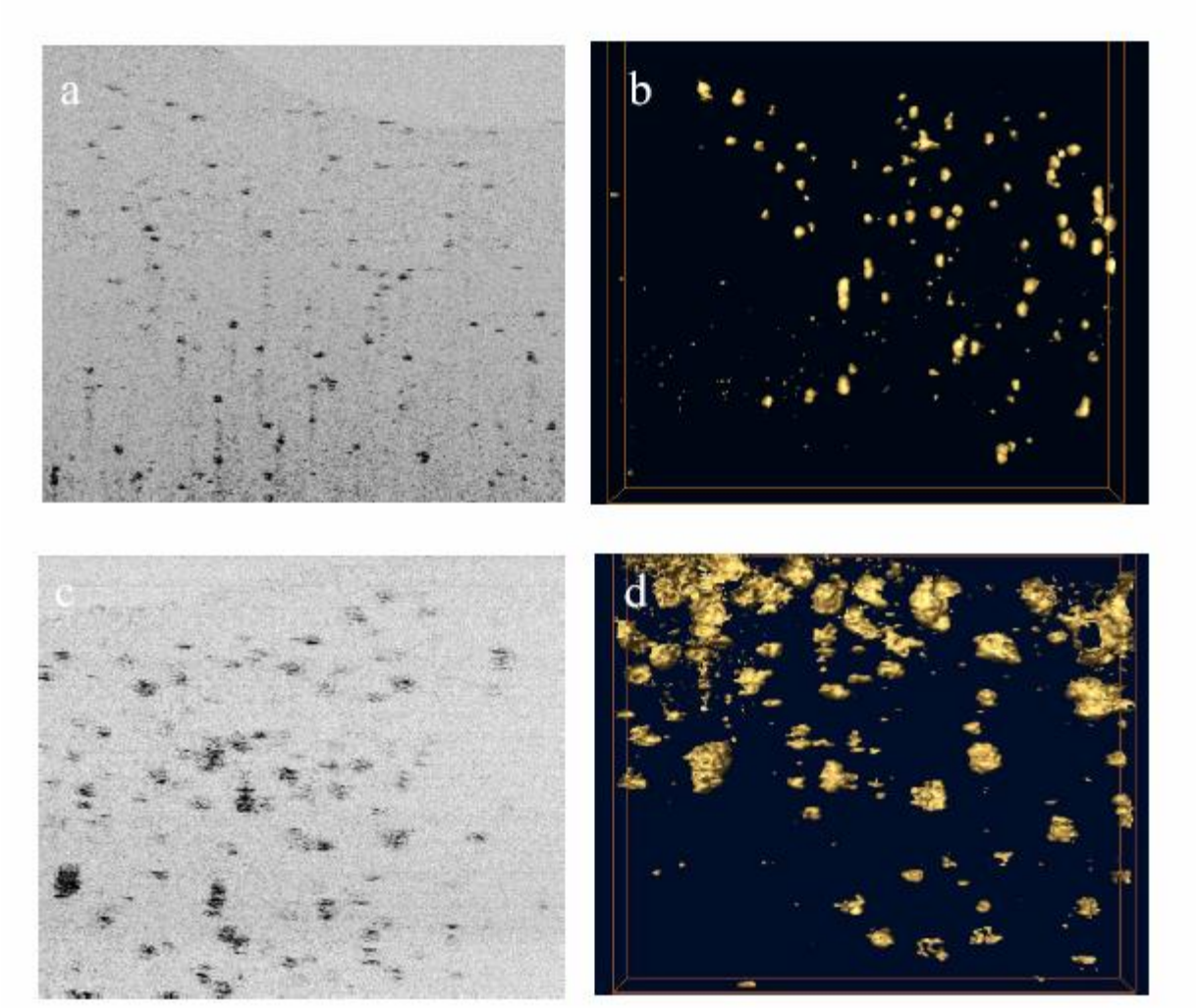

Figure 2. Cell proliferation in 3-D engineered tissues. Figures a-b are the x-z image and 3-D reconstructed image of cells in engineered tissues on the first day of culture. Figures c-d are the x-z image and 3-D reconstructed image of cells in engineered tissues on the fifth day of culture.

3.3 Cell necrosis

Necrosis is death due to unexpected and accidental cell damage. A number of toxic or physical events can cause necrosis: toxins, radiation, heat, trauma, lack of oxygen, etc. Necrotic cells first swell as they begin to die. Eventually holes appear in the plasma membrane so that the membrane become permeable and intracellular materials spill out. The necrotic cells will induce an inflammatory reaction. Existing methods are limited to detect the dynamic process of cell necrosis. We demonstrate that OCT can be used to monitor the fast progress of cell necrosis in a 3-D tissue. Figure 3 demonstrate the process of cell necrosis in an engineered tissue. The speed of necrosis spread, which is related to the diffusion rate of the chemical toxin – bleach, can be determined from the figures. Furthermore, figure 3 h-i illustrate the necrosis process of an individual cell, from a swelling cell to the stage where cellular contents are released.

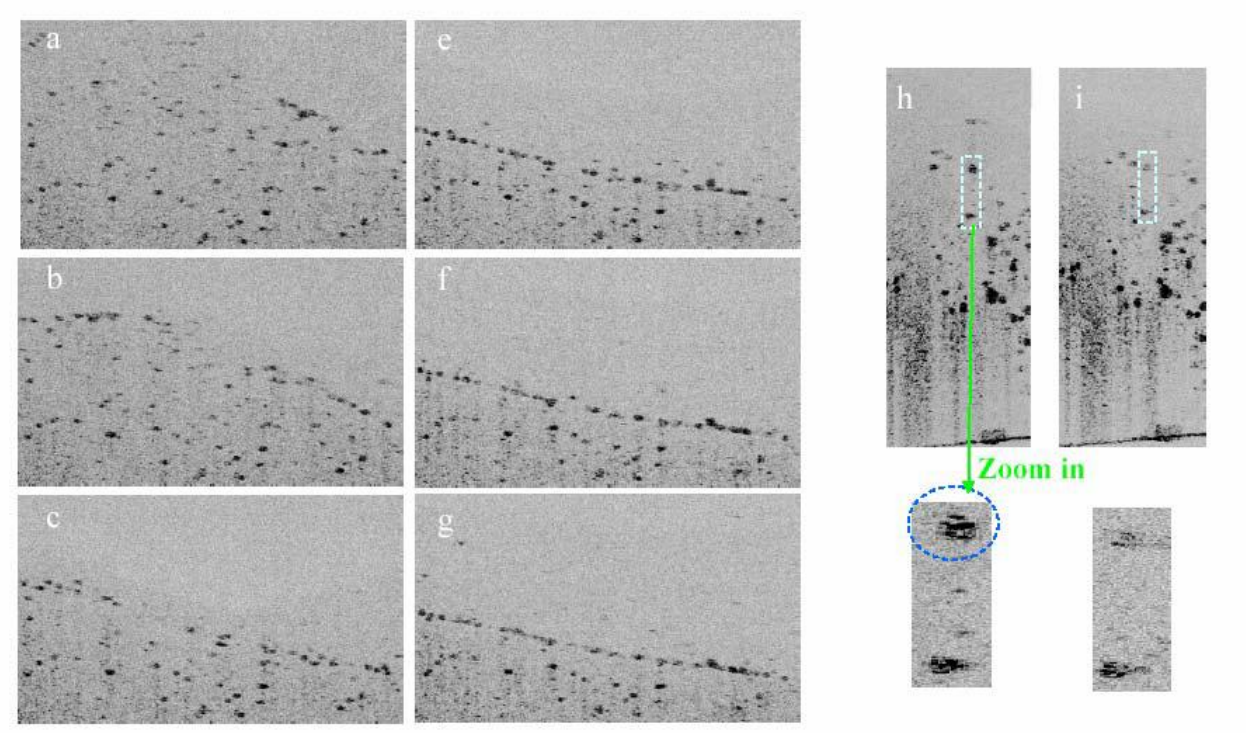

Figure 3. The progress of cell necrosis in the engineered tissue. Figures a-g illustrate the cell responses to the bleach over the time. The time interval between consecutive images is 5 minutes. Figures h and i illustrate the response of an individual cell to the bleach. The images were take with 2-minute interval.

3.4 Cell apoptosis

Programmed cell death is an important mechanism in both development and homeostasis in adult tissues for the removal of either superfluous, infected, transformed or damaged cells by activation of an intrinsic suicide program. One form of programmed cell death is apoptosis, which is characterized by maintenance of intact cell membranes during the suicide process. Key criteria for determining whether a cell is undergoing apoptosis include distinct morphological changes in the appearance of the cell – cell shrinkage, as well as alterations in biochemical and molecular markers, e.g. chromatin condensation and DNA fragmentation.[20] In contrast to necrosis, which is a form of cell death that results from acute tissue injury, apoptosis is carried out in an ordered process that generally confers advantages during an organism's life cycle. During apoptosis, cellular contents are not released and inflammation does not occur**.**

Traditional methods to detect cell apoptosis in a tissue involve a long and invasive process of embedding and sectioning a tissue sample. Using OCT to dynamically monitor the changes of the 3-D cell morphology in engineered tissues, we demonstrate a non-invasive method for detecting cell apoptosis and for evaluating the efficiency of an apoptosis inducer or chemical that is suspect to induce cell apoptosis. Figure 4 compares the OCT method with histology methods for the detection of cell apoptosis. We used apoptosis inducer solutions of two concentrations, one with 50X dilution factor and the other with 1000X dilution factor. Dilution factor indicates the concentration of the apoptosis inducer by the ratio it is diluted to. For example, 50X dilution factor means that apoptosis inducer, e.g. Camptothecin, is diluted with culture media with a proportion of 1:50. The higher the dilution factor, the lower the apoptosis concentration. Histological images (H&E stain) illustrate the differences in cell

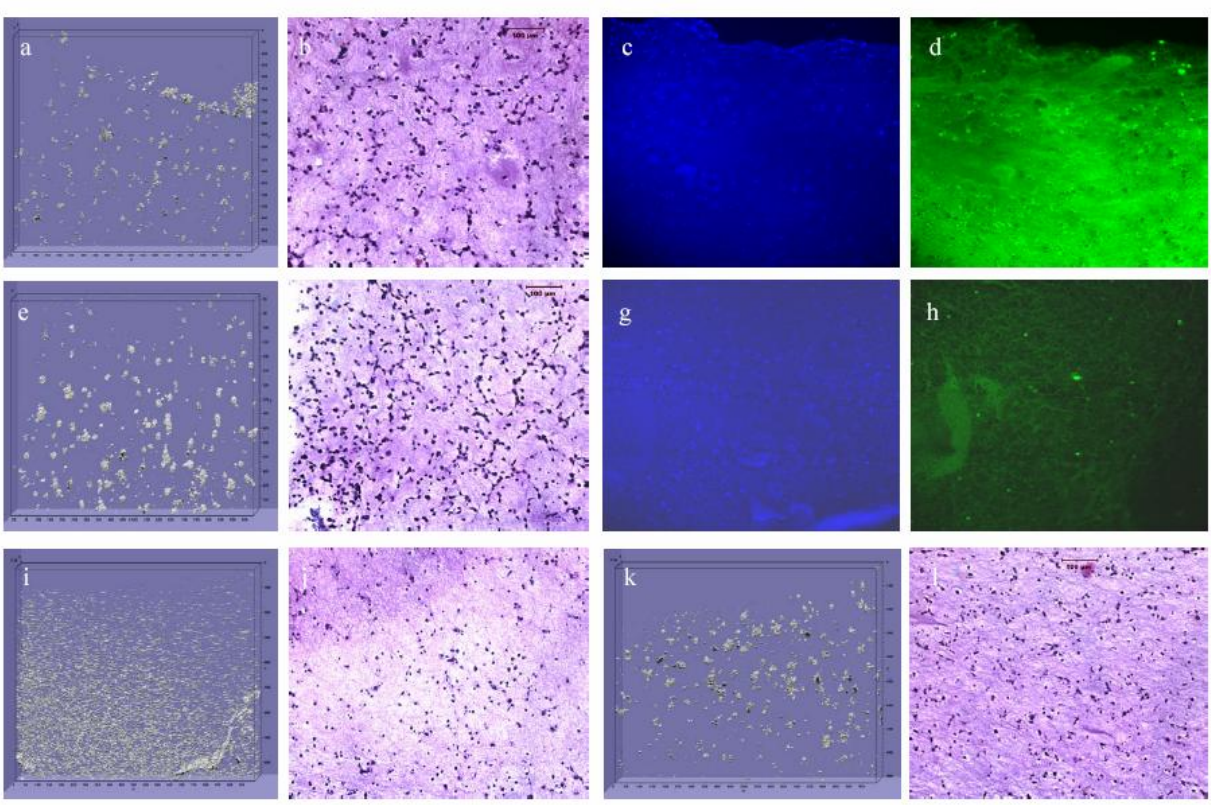

Figure 4. Evaluation of cell apoptosis with different optical tools: (a, e, i, k) OCT 3-D images (x=z=2mm, y=0.1mm); (b, f, j) Histological images using H&E stain; and (c, d, g, h) Fluorescent images using Fluorescien-FragEL kit. The blue fluorescence shows the total cell population by DAPI-labeled nuclei. The green fluorescence shows the apoptotic cells. (a-h) cell apoptosis testing after 20hr culture with apoptosis inducer; (i-l) cell apoptosis testing after 40hr culture with apoptosis inducer. Different concentrated apoptosis inducer solutions were used in the experiment: (a-d, i, j) 50X dilution factor (e-h, k, l) 1000X dilution factor.

differentiations in two culture conditions. Living cells are characterized with elongated cell morphology, while dying cells are round. Fluorescien-FragEL DNA fragmentation kit detects cell apoptosis by observing the oligonucleosomal DNA fragments. The 3'-OH ends of DNA fragments generated in response to apoptotic signals are fluorescently labeled (Figure 4d, h). The resultant fluorescent images demonstrate the percentage of the apoptotic cells out of the total cell population. OCT images demonstrate the changes of 3-D cell morphology in tissue, which can be used to differentiate the normal and apoptotic cells. Due to the cell shrinkage in response to apoptotic signals, the apoptotic cells display smaller 3-D cell volume than the normal cells. Finally, figure 4i shows the fragmentation of the cell into small apoptotic bodies. The cell undergoing apoptosis shows a similar characteristic morphology that is seen under a light microscope. Light microscope has shown that the apoptotic cell first becomes smaller and round (circular), and then the cell breaks apart into several vesicles called apoptotic bodies. Different imaging modalities show similar results on the percentage of apoptotic cells. As far as we know, this is the first time to demonstrate the application of OCT non-invasive tool in the evaluation of the efficiency of the apoptosis inducer.

3.5 Cell Detachment

Engineered tissues were trypsinized with 0.25% trypsin/EDTA at room temperature. Figure 5 shows the process of cell detaching from the calcium phosphate scaffold. In response to the trypsin-EDTA, layers of cells were gradually de-adhered from the 3-D scaffold over the time, which was observed from the density changes in the tissue. In summary,

OCT provides a powerful tool to detect 3-D cell dynamics in a tissue, which sometimes can not be provided by other imaging tools.

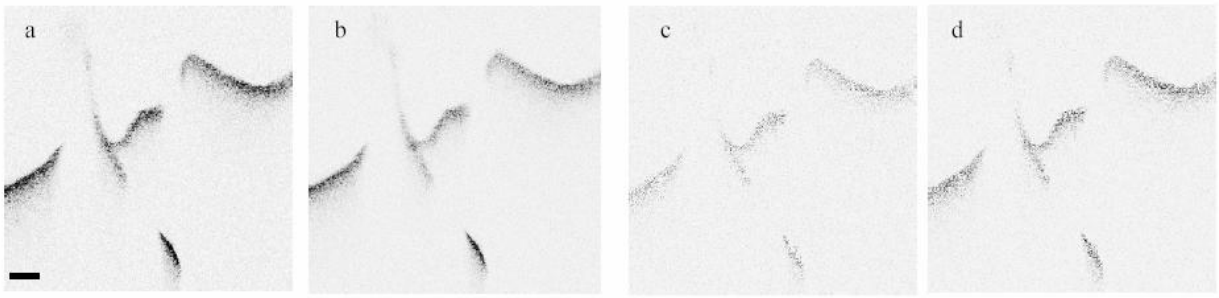

Figure 5. The process of cells detaching from scaffold over the time. Engineered tissues were trypsinized with 0.25% trypsin/EDTA. Images were taken after tissues were soaked in the trypsin solution for 10min (a) and 16min (b). Before 10min, there were little changes in the images. Due to small differences among the images taken in the cell de-attachment process, images were viewed by subtracting one image from the image that were taken after 10min culturing in trypsin (a). Therefore, figures c-h were images resulted from subtracting images at the time points of 14min(c) and 16min(d) from the image at 10min. Scale bar showing 200μm.

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