Advances in optical imaging of dynamic three-dimensional engineered tissues

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Abstract: Recent advances in OCT and multiphoton microscopy have enabled nondestructive monitoring of cell dynamics and distributions in 3-D engineered tissues. Dynamic cell processes including migration, proliferation, and mechanical restructuring are observed during engineered tissue development.

OCIS codes: (170.4500) Optical coherence tomography; (170.6900) Three-dimensional microscopy; (170.1790); (190.4180) Multiphoton processes; (999.9999) Tissue engineering.

1. Introduction

The field of tissue engineering has emerged over the past decade, driven by a diverse range of clinical needs for replacement of discarded or damaged tissue [1]. Aimed at regenerating the native structural and functional properties of living tissues *in vitro* as well as *in vivo*, engineered tissues are subject to controlled microenvironments for tissue development. Recently, tissue engineering trends have been toward developing 3-D constructs that may be subjected to dynamic external stimuli to modify and control tissue organization and growth. Currently, destructive imaging methods such as histology and SEM are predominantly used in evaluating the development process of engineered tissues and their response to environmental stimuli. These destructive methods, however, intrinsically have many disadvantages: (a) inability to perform real-time, *in situ* or dynamic imaging; (b) lack of true 3-D information; (c) long and harsh processing steps; and (d) difficulty correlating structure-function data. Thus, despite increasing research in tissue engineering, few have investigated the dynamics of cell behaviors and biological interactions in engineered tissues. Therefore, we have developed integrated non-invasive imaging tools, namely optical coherence tomography (OCT), confocal, and multiphoton microscopy (MPM) methods to explore dynamic 3-D tissue microstructure and cell functions in engineered tissues.

OCT is a promising imaging technology that has found wide-spread applications in the fields of biology and medicine [2-4]. With imaging resolutions on the order of 3-5 μm, imaging depths of 1-2 mm in highly-scattering tissues, real-time acquisition rates for volumetric microscopy, and the ability to detect endogenous or exogenous contrast based on index changes in tissue, OCT is ideally suited for nondestructive imaging of 3-D engineered tissues throughout development and even following grafting to a host. To date, there have been few investigations using OCT to monitor *in vitro* tissue cultures [5-8]. Confocal and MPM have been effective techniques for imaging 3-D biological tissues [9]. The fluorescence-based microscopies can detect fluorophore-tagged molecules, such as Green Fluorescent Protein (GFP), and thus provide information on functionality [10]. Coupled with fluorescence-based functional imaging, the structural properties imaged by OCT will help contribute to understanding fundamental biological mechanisms in engineered tissues during development and under various external stimuli.

2. Material and Methods

Representative engineered tissues were constructed, composed of cells and 3-D scaffolds. In our study, NIH-3T3 cells (ATCC) were seeded in varying types of scaffolds including a 3-D porous chitosan scaffold, 3-D matrices of Matrigel™ or collagen, or onto substrates with a 3-D topographic grooved surface. Cells were transfected with GFP-vinculin plasmid (courtesy of Dr Geiger, Weizman Institute, Israel), forming a stable cell line that express GFP-vinculin (a cell adhesion protein). For transfection, $7x10^5$ cells were seeded in a 6-well plate. For each well of cells, 4μg of DNA and 2μl of Lipofectamine 2000 reagent were diluted separately with 50μl of FreeStyle 293 expression medium, then mixed, and cultured for 20min to form DNA-LF2000 complexes. The concentration of cells and DNA was determined in pilot experiments to be optimal for transfection efficiency.

One setup used in this work (Fig. 1) was an integrated OCM-MPM microscope [11]. The light source consisted of a frequency-doubled Nd:YVO_4-pumped Ti:sapphire laser with a center wavelength of 800 nm, a bandwidth of 60 nm, and an average power on the sample of 1 to 5 mW at an 80 MHz repetition rate. To compensate for the pulse lengthening due to the high dispersion of the objective (Olympus, water-immersion, 20x, 0.9 NA), pulses were first pre-compensated using a double-fold prism path. The beam was then guided to a scan head that consisted of two

galvo-controlled mirrors for high speed acquisition, and then matched to the back-aperture of the objective where the beam was focused in the sample. The source functions both as an excitation source for two-photon absorption and as a low-coherence source for OCM. The MPM detection scheme is standard and consists of a PMT (Hamamatsu, H7421-40) working in photon counting mode. The OCM detection scheme is different with respect to the one previously proposed by Beaurepaire, *et al*. [12]. We have implemented a spectral-domain (SD) OCM system, instead of time-domain OCM, with many different advantages. While standard OCM requires two scans to be performed (axial and lateral scanning), the spectral-domain technique can be implemented using only lateral scanning. Moreover, SD-OCM inherently provides direct access to the spectral information for spectroscopic OCM signal analysis. In addition, it has been recently shown [13] that the spectral-domain configuration provides significant advantages in terms of acquisition speed, sensitivity, and simplicity in the acquisition module; benefits that are incorporated into our integrated microscope. We also emphasize that the absence of any moving reference arm in the setup provides an inherent phase stability and makes this modality ideally-suited for the evaluation of the spectral components in the interference pattern. In fact, because different tissue structures and molecules have different spectral absorption and scattering properties, the spectral analysis, combined with the coherence gating, increases the OCM image contrast, with the potential for generating spatial maps of molecules within the sample.

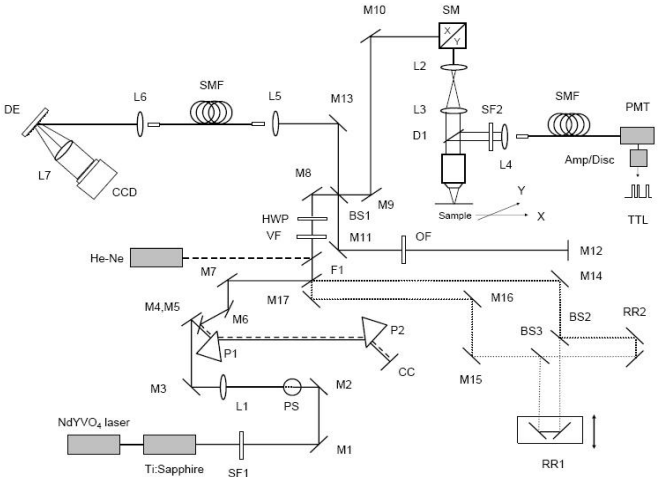

Fig. 1. Schematic of integrated OCM-MPM microscope used for advanced optical imaging of 3-D engineered tissues.

In our setup, light is collimated and dispersed off of a blazed diffraction grating having 830.3 grooves per millimeter. The optical spectrum is focused using a pair of achromatic lenses which have a combined focal length of 150 mm. The focused light is incident on a line-scan camera (L104k–2k, Basler) which contains a 2048-element CCD array of detection elements. This camera has a maximum readout rate of 29 kHz, thus one axial scan can be captured during an exposure interval of 34 μs. Digital processing of the detected signal included a Spline interpolation to make the signal more uniform and a discrete Fourier transform on each set of 2048, 10-bit, values captured by the CCD to transform the signal from the frequency (spectral) domain into the spatial (depth) domain. The scattering amplitudes corresponding to the focus in each adjacent axial scan were subsequently assembled into 2D *en face* images for visualization on a personal computer. Acquisition and visualization of OCM and MPM images was performed simultaneously.

We performed OCT imaging on engineered tissues at varying time-points during culture, and up to 10 days. For real-time *in situ* imaging, cell cultures were maintained in a microincubator (LU-CPC, Harvard Apparatus). For histology, samples were fixed in 3.7% formaldehyde, embedded in paraffin, sectioned into 5μm-ultrathin films with a microtome, and stained with hematoxylin and eosin.

3. Results and Discussion

OCT, confocal, and MPM were used for examining the engineered tissues, and conventional histology was performed on the same specimens. Our OCM-MPM system integrates multiple modalities. Here we show representative images from several ongoing studies. In Fig. 2, single-cell images of cultured fibroblasts are shown. The cells are expressing GFP-vinculin (a cell adhesion protein) and the nuclei are stained with a second dye for specific localization of nuclei relative to the cell and surrounding structures. Spectroscopic analysis of the OCM data is consistent with the MPM information, identifying the locations of the nuclei.

Mechanical forces play a significant role in tissue organization, both in natural developing tissues and in engineered tissues. We have investigated the morphological changes that occur in engineered tissues in response to varying external mechanical forces. Using a 3-D topographically-grooved culture substrate fabricated from a flexible biocompatible polymer (PDMS), cultured fibroblasts were imaged before and after mechanical stretching of the substrates using a commercial Flexcell™ apparatus. Prior to stretch, fibroblast projections between the microgrooves were noted. Following stretch, the fibroblasts became more rounded in appearance and withdrew projections from the grooves. OCT has the potential to monitor many of the depth-resolved mechano-structural

changes that occur deep in engineered tissues, beyond the limits of more conventional light, fluorescence, confocal, and multi-photon microscopy.

Additional technological developments and investigations in tissue engineering have been conducted in our laboratory, including the use of Doppler OCT for characterizing fluid flow in microfluidic vascular models, and the detection of optical scattering changes in electrically-active cultured neurons. To characterize the evolving biomechanical properties of both natural and engineered tissues, we have utilized optical coherence elastography methods and have shown that optical coherence elastography can detect increased stiffness in heterogeneous engineered tissues over time as cells proliferate and increase cell-cell and cell-matrix adhesions [8].

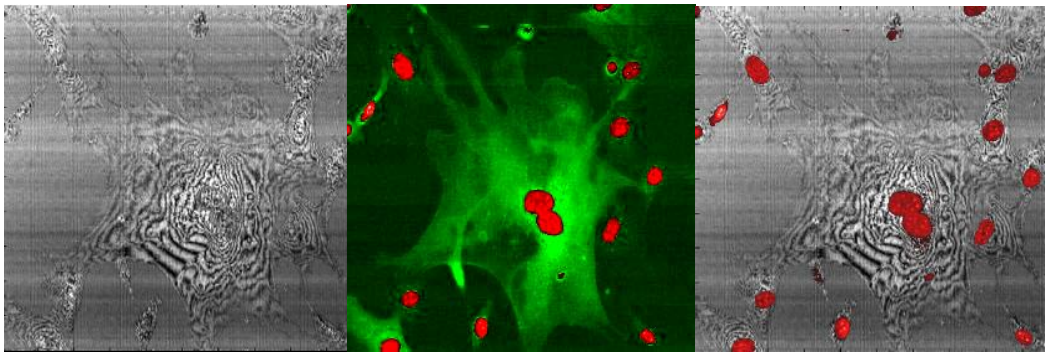

Fig. 2. Integrated OCM-MPM of GFP-transfected fibroblasts. Left: OCM; Middle: MPM of GFP-vinculin (green) and DNA dye (red); Right: fused OCM-MPM (red channel) showing correspondence of nuclei within cells. Image size is 60 x 60 microns.

4. Conclusions

Compared to conventional visualization techniques, there are many advantages of OCT and MPM for imaging 3-D engineered tissues: (a) non-invasive diagnostics; (b) real-time, dynamic, *in situ* imaging; (c) deep penetration depth; (d) functional imaging correlated with structural properties; (e) real 3-D information for evaluating micro-environmental factors; and (f) fast image acquisition without disruption of normal cell processes. The assembly of cells into tissues is a highly orchestrated set of events that require time scales ranging from seconds to weeks and dimensions from 1μm to 10cm. With large penetration depths and increased spatial and temporal resolution in 3-D space, OCT and related advanced optical imaging techniques will be useful modalities for gaining new insights into cell dynamics *in situ* and in real-time, elucidating the complex biological interactions, and directing our designs toward functional, biomimetic, and mature engineered tissues.

Toward this goal, we have developed and applied an integrated microscope that is capable of simultaneous image acquisition from multiple optical imaging modalities. We have highlighted the use of SD-OCM and MPM for the detection of structure and function, respectively. The use of SD-OCM allows for the visualization of background morphology and spectroscopic analysis of tissue composition, while the use of MPM permits the visualization of biological function (in this case, GFP-labelled vinculin). This instrument provides a new investigational tool for the visualization of structure and function in fields such as tissue engineering and tumor biology.

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