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Optical Projection Tomography Imaging of Single Cells in 3D Gellan Gum Hydrogel

B. Belay[1], J.T. Koivisto[1,2], K. Vuornos[2], T. Montonen[1], O. Koskela[1], M. Lehti-Polojärvi[1], S. Miettinen[2], M. Kellomäki[1,2], E. Figueiras[3] and J. Hyttinen[1]

[1] BioMediTech Institute and Faculty of Biomedical Sciences and Engineering, Tampere University of Technology, Tampere, Finland
[2] BioMediTech Institute and Faculty of Medicine and Life Sciences, University of Tampere, Tampere, Finland
[3]International Iberian Nanotechnology Laboratory, Braga, Portugal

Abstract— **3D cell culturing has become attractive in biology and tissue engineering laboratories as it mimics the natural environment for the cells to grow, differentiate and interact in all directions. To study cells and cellular interactions within 3D, cell culture requires a non-invasive, non-toxic, and high resolution imaging technique. The existing imaging techniques face challenges to image cells in 3D macro-scale environment because of the sample size, photo-bleaching or resolution requirements. Optical projection tomography (OPT) is a non-invasive 3D imaging technique for samples in the range of 1-10 mm. It works in both emission and transmission modes for fluorescence and bright-field imaging, respectively. Here, we demonstrate the use of OPT for imaging of cells and cellular materials in 3D gellan gum hydrogel. Fluorescence projection images showed alive and dead human lung fibroblast cells encapsulated in hydrogel. The mineralized extracellular matrix secreted by the human adipose stem cells in the hydrogel was evenly distributed throughout the sample and analyzable in 3D volume.**

Keywords— **Optical projection tomography, fluorescence, hydrogel, 3D imaging, mesenchymal cell culture**

I. INTRODUCTION

Tissue engineering (TE) and disease modeling requires a way to step up from 2D flat surface to more biomimicking 3D cell culturing [1]. In 3D, the cells have more homogenous environment, more degrees of freedom for growth and more possibilities to interact with the surrounding matrix [1]. Mesenchymal cells are suitable for the development of *in vitro* cell culture models, notably the multipotent human adipose stem cells (hASCs) with good capacity for osteogenic differentiation [2] are potential for the development of 3D hydrogel culture for a bone TE *in vitro* model.

Hydrogels, hydrophilic polymers with very high water content, are promising candidates as 3D cell culture biomaterial, as they mimic closely the natural extracellular matrix. One promising hydrogel for TE is gellan gum (GG), a bacterial polysaccharide with simple ionotropic gelation in mild conditions, enabling 3D cell encapsulation [3].

Visualizing cells within 3D biomaterial require imaging techniques with good spatial resolution. Several imaging modalities have been applied to image 3D tissue engineered constructs. They are based on the required functional information and penetration depth into the tissue [4]. Multiphoton microscope has been a useful tool for functional imaging of tissue with high resolution. However, it can only image tissues with few hundred micrometers of depth. Imaging depth is still the major challenge in imaging thicker scaffolds [4]. An X-ray-based micro computed tomography (CT) provides good resolution. It is best suited for 3D imaging of calcified tissues such as bone. Contrast enchanced X-ray micro CT was also applied for 3D imaging of rat retina and provided good contrast [6]. But due to the high energy of X-ray radiation, it is unsuitable for imaging living cells.

OPT is a non-invasive optical tomographic imaging technique used to analyze small biological samples in the range of 1 – 10 mm [7-9]. It is an optical analogue of X-ray CT. The specimen is rotated through a series of angular positions while suspended in an index-matching liquid, and projection image is captured at each orientation. Standard reconstruction algorithms such as filtered back projection (FBP) are used to reconstruct the 3D image from projection images [8].

OPT has been widely applied in developmental biology for gene expression studies of small animal embryos such as mouse, and zebrafish [7,9]. Another suitable application of OPT is for characterization of tissue engineered constructs based on hydrogel biomaterials, which have refractive index similar to water [8]. It has been used for 3D characterization of hydrogels and for studying mass transport in hydrogel without the use of optical clearing [8,10].

In this study, OPT was used for the first time for tomographic imaging of cells inside 3D hydrogel cell culture. Fluorescence projection images were used for cell viability study. Bright-field tomographic images were obtained for morphological analysis of cells in hydrogel.

II. Materials and Methods

A. Hydrogel Cell Culture

The commercial human lung fibroblast cell line WI-38 (passage 21-23) (Culture Collections, Public Health England, United Kingdom), applicable for first stage biocompatibility testing, were expanded and cultured in Nunc T75 culture flasks (Thermo Fisher Scientific, USA) with Dulbecco's Modified Eagle Medium/Ham's Nutrient Mixture F-12 (DMEM/F-12 1:1; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (South American origin, Biosera, Finland) and 0.5% Penicillin/Streptomycin 100 U/mL (P/S; Thermo Fisher Scientific) for Live/Dead® imaging study.

The human adipose stem cells (hASCs) were obtained from subcutaneous adipose tissue of a female donor in surgery at the Tampere University Hospital with the patient's written informed consent, in accordance with the Ethics Committee of the Pirkanmaa Hospital District's, Tampere, Finland, ethical approval R15161. The hASCs were isolated as reported [2].

The gellan gum (GG; Gelzan CM; Sigma-Aldrich) solution of 5 mg/mL and bioamine spermidine trihydrochloride (SPD; BioXtra; Sigma-Aldrich) of 1.0 mg/mL and 0.5 mg/mL concentrations were dissolved in 10% (w/v) sucrose in distilled water. GG was crosslinked with 16% (v/v) SPD of 0.5 mg/mL concentration for WI-38 and 1.0 mg/mL for the hASCs. For the fabrication of 3D cell-hydrogel cultures, WI-38 and hASCs were resuspended into GG solution heated to +37ºC, and manually mixed with the crosslinker solution to allow immediate gelation and cell encapsulation. After 10 min gelation, medium was added on top of the hydrogels.

B. Fluorescence Staining

Live/Dead® (Thermo Fisher Scientific) cell viability assay was optimized for cytocompatibility testing. Staining solution with 0.1 µM ethidium homodimer-1 and 0.4-0.6 µM calcein acetoxymethyl ester was prepared in phosphate buffered saline, as instructed by the kit. A puncture hole was made in the middle of the cell-laden hydrogel sample to enhance stain diffusion throughout the hydrogel. The staining solution was added to the puncture hole and the sample was incubated for at least 30 min at +37ºC. The stained hydrogel sample was punctured with a 2.1 mm outside diameter fluorinated ethylene propylene (FEP) tube to push the cell-laden hydrogel into the tube for imaging.

The mineralization of hASC secreted extracellular matrix was chemically induced with a previously reported osteogenic medium optimized for hASC osteogenic differentiation [2] containing DMEM/F-12 1:1, 5% human serum (Biowest, France), 1% L-glutamine (GlutaMAX; Thermo Fisher Scientific), and 1% P/S 100 U/mL, and supplemented with 5 nM dexamethasone (Sigma-Aldrich, USA), 250 µM L-ascorbic acid 2-phosphate and 10 mM β-glycerophoshate (Sigma-Aldrich). For the mineralization assay, the hASCs encapsulated in 3D hydrogel were stained with the OsteoImage assay according to manufacturer's protocol (Lonza, Switzerland) at 29 days. Briefly, the cells were fixed with paraformaldehyde (Sigma-Aldrich) and the hydroxyapatite residues were stained with the OsteoImage Staining Reagent.

C. OPT System

The imaging was performed with an in-house-built OPT setup [8,10]. The schematic of the OPT setup is shown in the Fig.1. The samples (S) were fixed inside FEP tubes (Adtech Polymer Engineering, United Kingdom.) and immersed in a transparent cuvette filled with distilled water. For each sample, 400 projection images were acquired over a full rotation of 360 degrees with a 0.9-degree step size.

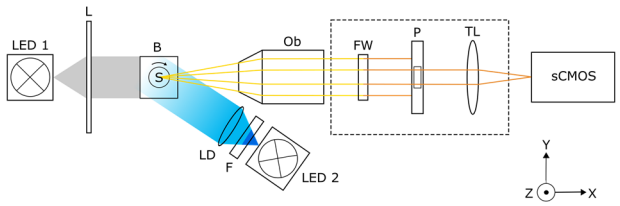

Fig.1 Schematic diagram of the OPT setup: samples inserted in FEP tubes are rotated in the rotation stage (S) inside an index matching water bath (B). A white light (LED 1) and a telecentric lens (L) are used for bright-field imaging in transmission OPT mode. Fluorescence illumination is done with 470 nm wavelength LED (LED 2), a bandpass filter (F) and collimated with a lens with diffuser (LD) in emission OPT mode. The detection system consists of objective lens (Ob), a rotating filter wheel (FW) filter for a band-pass filter (used only for fluorescence imaging), a pinhole (P), a tube lens (TL), and a sCMOS camera.

For bright-field imaging of the samples, OPT was used in transmission mode. The sample was illuminated by a white light source (LED 1) and a telecentric backlight illuminator (L, Edmund,USA). After passing through the sample, the light beam was collected by a 5x infinity-corrected objective lens (Ob, Edmund, USA), an iris diaphragm (P, Thorlabs, USA) and a tube lens (TL, Mitutoyo, USA). An image is captured by sCMOS camera (ORCA-Flash 4.0, Hamamatsu, Japan). The variation in light attenuation between the cells and the hydrogel structure allows to visualize the distribution of the cells in each projection image. As these projections are reconstructed, a 3D image is obtained.

For imaging the sample fluorescence, OPT with epi-illumination was deployed in emission mode. The samples were prepared and stained based on previously stated procedures. A collimated LED (LED 2) with nominal wavelength of 470

nm (M470L2, Thorlabs) used to excite the samples. Each projection image was acquired with 3 s exposure time. In live/dead stain imaging, the emitted light was collected by a 5x objective lens after passing through a band-pass filter with 520 nm/623 nm nominal wavelength (FW, Edmund, USA). For mineralization study, the OsteoImage™ mineralization sample was imaged with excitation/emission bandpass filters of nominal wavelength 470 nm/520 nm, respectively.

D. Tomographic Reconstruction and Image Processing

Bright-field projection images were preprocessed with homomorphic filter to correct inhomogeneous illumination. Fluorescence projection images where brightness adjusted for better contrast, and median filtered used to remove noise. Volumetric 3D reconstructions were computed in MATLAB (MathWorks) using a filtered backprojection algorithm with center of rotation correction [8]. Visualization in 3D was made using Avizo 9.2 (FEI Visualization Sciences Group).

III. Results and Discussion

Two sample types, one with human lung fibroblast cell, and another with self-derived human adipose stem cells in gellan gum hydrogel were imaged using both transmission and emission OPT modes. The bright-field OPT imaging allowed a general view of the cell distribution in 3D hydrogel culture (Fig. 2a-c;3a-c). Puncturing of the cell-laden hydrogel with the FEP tube did not affect cell morphology visibly; therefore, the sample preparation method was valid for OPT imaging of 3D hydrogel cell cultures.

The 3D reconstructed bright-field OPT images showed the tightly rounded cell morphology and the uniform cell density of both WI-38 and hASCs encapsulated in GG hydrogel (Fig. 2a;3a). Due to the limited depth of field of the microscope objective lens, cells that are out of focus are blurred image in the bright-field and fluorescence projection images (Fig. 2c,d;3c). This affected the tomographic reconstruction images shown (Fig. 2a;3a). The reconstruction will be further improved in a future publication by extending the depth of field using multi-focal imaging.

The Live/Dead® assay used for the fibroblasts allowed clear detection of individual alive and dead cells (Fig. 2d-f). The advantage of OPT over conventional fluorescence microscopy systems is it allows to analyze cell viability in a 3D sample in the mesoscopic range. The OPT imaging would also allow more in-depth analysis of cell morphology in the Z-direction. Our results showed rounded morphology of cells evenly distributed throughout the sample. The hydrogel material used for cell encapsulation here is at a developmental stage [3]; more elongated fibroblast morphology is expected with protein functionalized GG in future studies.

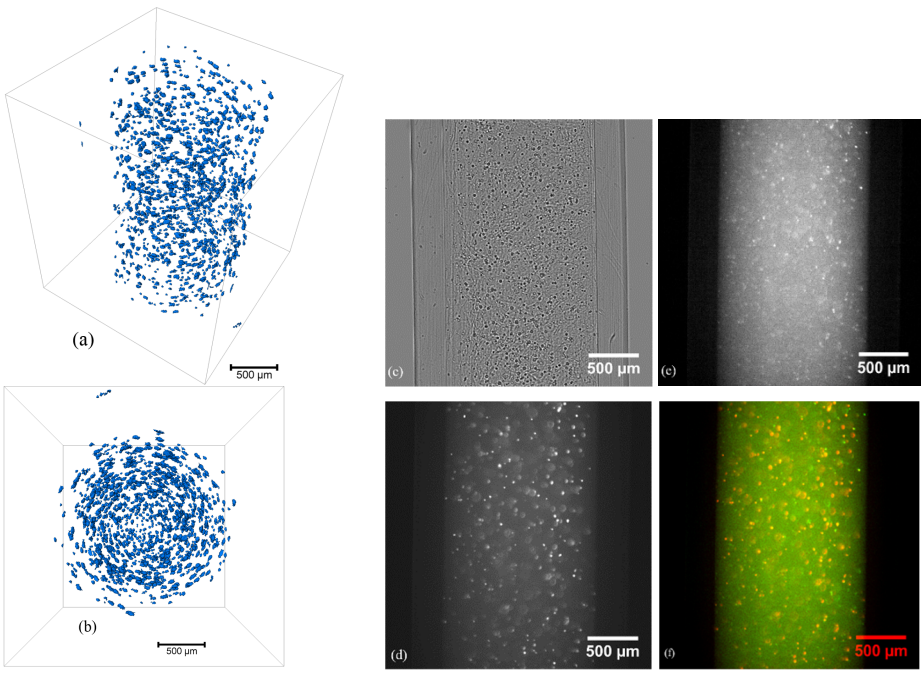

Fig.2 OPT images of fibroblast cells in GG hydrogel (a) 3D reconstruction from bright-field projection images, (b) top view of (a), (c) bright-field projection image, (d) fluorescence projection image of dead stained fibroblast cells, (e) fluorescence image of live stained fibroblast cells, (f) composite image of (d) & (e).

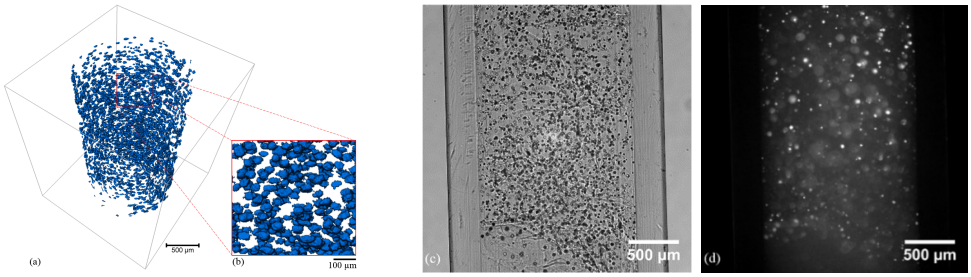

Fig. 3 OPT images of hASCs in GG hydrogel (a) 3D reconstruction from bright-field projection images, (b) close-up view of (a), (c) bright-field projection image, (d) fluorescence projection image hydroxyapatite stained hASCs.

The fluorescence OPT images of the stained hydroxyapatite residues (Fig. 3d) attested the osteogenic differentiation of the hASCs under osteogenic stimulus at 29 days. The general aspect of the stained granular hydroxyapatite deposited in the hASC extracellular matrix was clearly visible throughout the 3D hydrogel matrix compared to previously published hydroxyapatite staining results in hydrogel [11]. Thus, the fluorescence OPT imaging confirmed the mineralized matrix formation, and the osteogenic differentiation of the hASCs encapsulated in GG hydrogel.

IV. Conclusions

In this work, we have demonstrated that OPT enables tomographic imaging of single cells in 3D GG hydrogel. OPT has an advantage over the existing conventional optical imaging methods for 3D cell culture studies as it allows to image cells in macro-scale environment. It provides morphological and functional information about the cells. OPT offers an efficient and quick non-destructive imaging method to image and analyze cell distribution and matrix formation in 3D hydrogels. The ability to reconstruct the whole 3D volume of a hydrogel sample with good visibility of cells opens new possibilities to analyze cell cultures.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Author: Birhanu Assefa Belay
Institute: BioMediTech
Street: Lääkärinkatu 1
City: Tampere
Country: Finland
Email: birhanu.belay@tut.fi