DEVELOPING METROLOGY FOR TISSUE ENGINEERING: COLLINEAR OPTICAL COHERENCE AND CONFOCAL FLUORESCENCE MICROSCOPIES

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ABSTRACT

We present a novel application of optical coherence microscopy (OCM) by combining it with confocal fluorescence microscopy (CFM) to gather simultaneous structural and functional information on tissue engineered medical products (TEMPs) in a registered fashion. In this work, we describe the collinear OCM and CFM instrument. We demonstrate the utility of this dual-mode technique for TEMPs by comparing the images of fluorescently stained osteoblasts cultured in a polymeric TEMP for 21 days and 10 weeks.

1. INTRODUCTION

Tissue engineering is an emerging interdisciplinary field that has evolved to address the dire need for compatible, replacement organs and tissues in light of the shortages of transplantable organs and the problems associated with biomaterial implants. The term “tissue engineering” is defined as: “The application of principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue function.” [1]

Tissue engineered medical products (TEMPs) often consist of a three-dimensional synthetic scaffold that provides form and foundation for cells as they produce the tissue of interest. Successful TEMPs will allow cell infiltration, and foster proliferation and differentiation within the scaffold. More specifically, we are seeking relationships between such structural parameters as pore volume, size distribution, tortuosity and curvature and cell behavior. Creating an “optimum” TEMP relies on an intimate knowledge of how the cells react deep within the scaffold to a variety of external influences.

To this end, we are developing an imaging technique to probe cell response. More specifically, we are using collinear optical coherence microscopy (OCM) and confocal fluorescence microscopy (CFM) to examine the influence of scaffold structure on cell function. We use the OCM modality to collect information on scaffold structure and tissue deposition which complements the functional imaging aspect through traditional confocal microscopy of stained cells. OCM is an interferometric technique that uses both confocal and coherence gating mechanisms for stray light rejection. [2] With this approach, we are able to address two issues related to imaging of TEMPs: turbidity and resolution. In this work, we present the design and characterization of our collinear OCM/CFM system. We demonstrate the utility of this dual-mode technique for TEMPs by imaging fluorescently stained osteoblasts cultured in a polymeric TEMP.

2. EXPERIMENTAL

OCM/CFM Instrumentation

Figure 1 shows a schematic of the collinear OCM/CFM system. The OCM system consists of a broadband, 35 nm FWHM (full width at half maximum), superluminescent diode near infrared (NIR) polarized source (Optiphase, Van Nuys, CA) [3] centered at 1.31 μm with a maximum output of 10 mW. The detection system is a polarization-maintaining, polarization-sensitive fiber-optic coherence domain reflectometer built by Optiphase (Van Nuys, CA) with a core fiber diameter of 6.6 μm. Although we can gather both s and p polarization images, in this work they are virtually identical. Thus, we monitor the s polarization only. In the sample arm, the NIR light from a single mode, polarization-maintaining fiber is launched into a bulk optic system via a 0.55 NA collimating lens. The remainder of the optical train for the OCM component consists of a variable neutral density filter, a cold mirror, a 3:1 expanding telescope, and a 1.3 NA Epiplan-Neofluar oil-immersion objective (Zeiss, Germany). Reflected light is returned to the single mode fiber, which acts as a confocal aperture for the detection system. The reference arm of the interferometer is driven by piezoelectric modulators at 600 Hz and filtered at a Doppler frequency of 705 kHz. The sensitivity of the system is 95 dB.

The CFM instrument (Fig. 1) is comprised of an air-cooled Omnichrome argon ion laser (Melles Griot,

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Carlsbad, CA) at 488 nm. The laser light is modulated at 1.5 kHz, and then sent through a bandpass filter and a dichroic beam splitter before reaching the cold mirror where it is combined with the NIR beam. Because we use a refractive 100X (1.3 NA) Epiplan-Neofluor oil immersion objective (Carl Zeiss, Inc., Thornwood, NY), the focal points of the 488 nm and NIR beams are separated by (5.1 ± 0.1) μm [4], with the visible light focused closer to the objective. This effect is accounted for during the image registration process by in upward shift of the OCM images. The fluorescence signal propagates back through the cold mirror to the dichroic beam splitter where the excitation line is filtered out. Confocal gating of the fluorescence signal is accomplished in the usual way by focusing the collected light through long pass filter (>500 nm) and then through a 10 μm pinhole. The fluorescence signal was detected using a photo-multiplier tube (Oriel, Stratford, CT) and lock-in amplifier (Stanford Research Systems, Sunnyvale, CA).

Imaging is performed simultaneously for the OCM and CFM channels in the following manner for synchronization of stage movement, modulator, and data acquisition: The computer generates a pulse to start the x stage movement. (The x, y, and z stages are from Applied Scientific Instruments, Inc., Eugene, OR and are designed for 250 nm repeatability.) As the x stage is moving, the x stage encoder generates pulses are sent to the arbitrary waveform generator (ARB). It is the ARB that sends one pulse to the computer to initiate data collection and a triangular waveform to the modulator for every x position. Typical modulator scans are 2.5 mm, which aids in compensating for the optical pathlength mismatch of the confocal and coherence gates as we image into the sample. Details about other approaches for compensating for optical pathlength mismatch can be found elsewhere. [5] Repeating a process of scanning the sample in the x direction and subsequently stepping in y allows us to create two-dimensional images with a pixelation rate of 600 Hz. Stepping in the z direction after each x-y scan is accomplished by the motorized z-axis stage.

Scaffold Preparation

Details about the scaffold fabrication process can be found elsewhere. [6] The poly(ε-caprolactone) (PCL) scaffold had a bi-continuous morphology and was comprised of semi-crystalline PCL which had 0.50 mass fraction porosity and >65 % crystallinity. [7] The PCL scaffold was cultured with fetal chick osteoblasts for either 21 days or 10 weeks, stained with nuclear fast red [8], and bathed in a standard refractive index fluid (SPI Supplies, n_D = 1.526) for imaging. This approach of using fixed samples that are cultured at various time intervals and immersed in oil is a prelude to in-vitro imaging of live cells in biological media.

3. RESULTS AND DISCUSSION

Figures 2 and 3 show the OCM (A.) and CFM (B.) images of PCL samples cultured for 21 days (Fig. 2) and for 10 weeks (Fig. 3). For comparison, a representative scanning electron micrograph can be found in previous work. [9] The images are taken 48 μm below the surface in Fig. 2 and 66 μm below the surface in Fig 3. The image depth was calculated by scaling the distance moved by the stage by the refractive index (n_D) of the sample, (1.50 ± 0.02). The average refractive index of the sample was measured using procedures in the paper by Bouma et al. [10] Care was taken to check that the PCL did not autofluoresce under these conditions. The granularity exhibited in the OCM images of PCL is derived from the polymeric crystallites.

In Figs 2 and 3 there is very good comparison between the features detected with OCM and with CFM. According to the CFM data, the cells line the pore walls in most places. In fact, the CFM image in Fig. 3 shows a pore completely filled with cells, as indicated by the arrow. In this scaffold cultured for 10 weeks, cells are detected at common locations in both the OCM and CFM images, showing the potential for the OCM alone as a way to monitor proliferation. It is the crystalline structure in the scaffold itself that makes delineation of scaffold/cell boundary very difficult, especially in TEMPs where the cells are sparsely populating, as in Fig 2. The solution to this hurdle would be to use scaffold material with either no structure or structure on a very different size scale than the cells, such as amorphous polymers.

4. CONCLUSIONS

We have constructed a collinear optical coherence and confocal fluorescence microscope to noninvasively gather complementary information on TEMPs. We have used the OCM modality to image the microstructure in a PCL scaffold. The CFM channel showed the stained osteoblasts lining the pore walls in most instances. Cells in the 10 week sample were also detected by the OCM, showing the potential for detecting cell proliferation without the need for cell staining. This approach works best with TEMPs that themselves exhibit little structure in the scaffold material. Collinear OCM/CFM has the highest potential impact for imaging hydrated scaffolds whose structure would be altered by analysis using traditional techniques that require dehydration of the scaffold.

5. ACKNOWLEDGEMENTS

We gratefully acknowledge David Hermreck, program manager in the Information Technology and Electronics Office of the NIST Advanced Technology Program, for intramural funding of this work.

5. REFERENCES

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[3] Identification of a commercial product is made only to facilitate experimental reproducibility and to adequately describe experimental procedure. In no case does it imply endorsement by NIST or imply that it is necessarily the best product for the experimental procedure.
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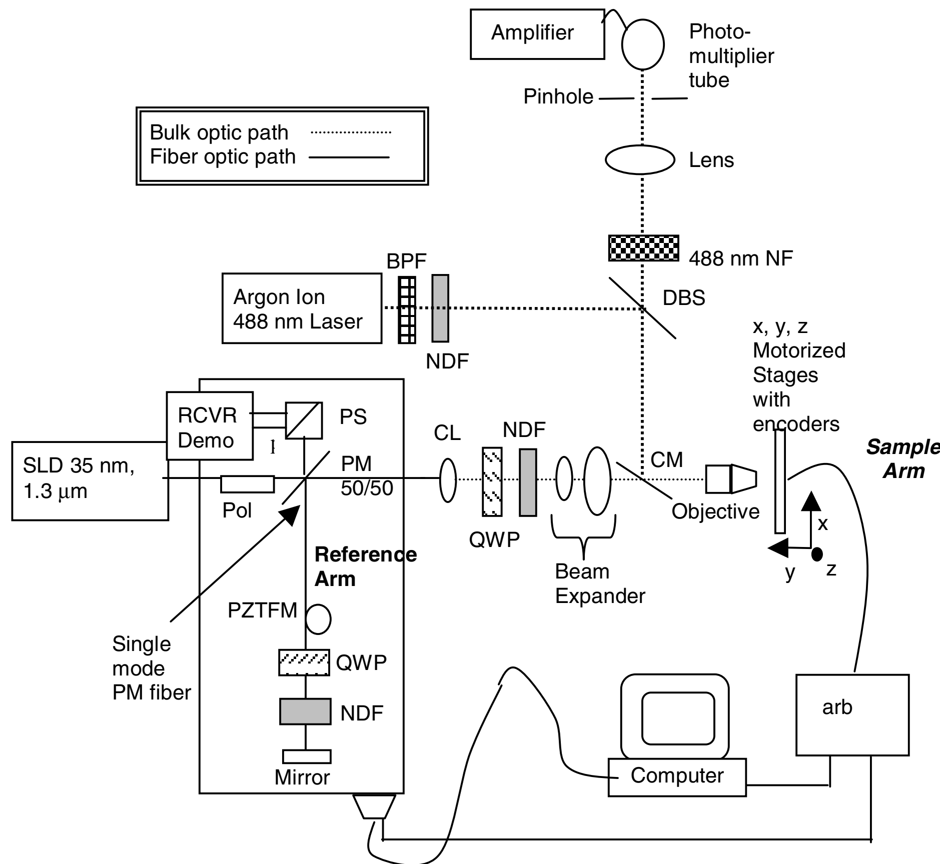

Figure 1: Experimental apparatus for collinear OCM/ CFM. ARB: arbitrary waveform generator; BP: band pass filter; CL: collimating lens; CM: cold mirror; Demod: Demodulator; SOAS: semiconductor optical amplifier source; DBS: dichroic beam splitter; LP: Long pass filter; NDF: Neutral density filter; NF: Notch filter; PM: Polarization maintaining 50/50 coupler; Pol: In-line polarizer; PS: Polarization splitter; PZTFM: Piezoelectic fiber modulator; RCVR: Receiver; QWP: Quarter wave plate.

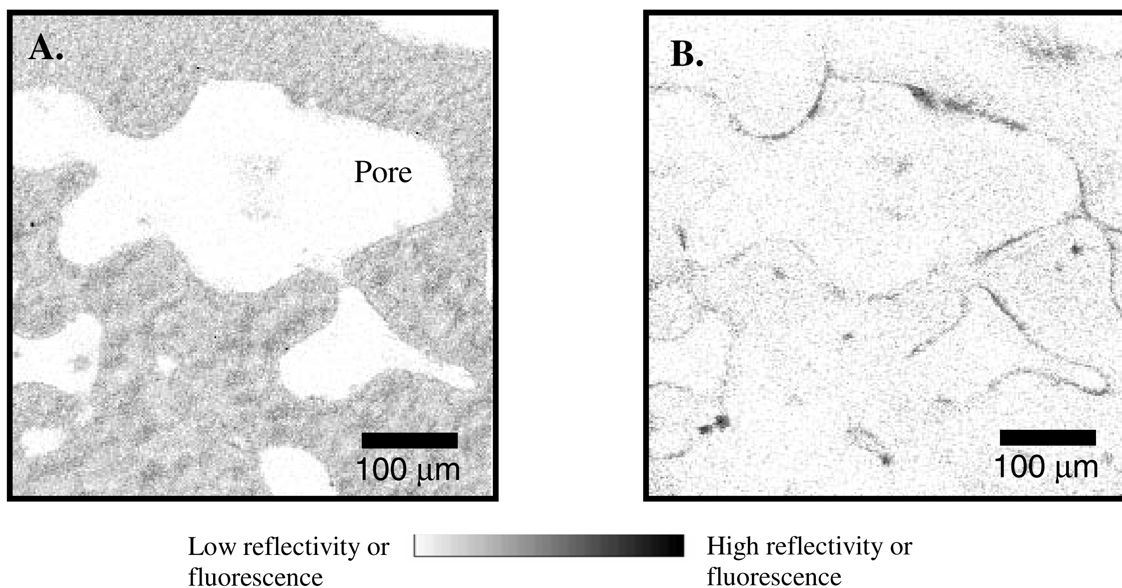

Figure 2: OCM (A.) and CFM (B.) images about 48 µm below the surface of the PCL scaffold cultured for 21 days with osteoblasts then stained. The image size is 500 x 500 µm.

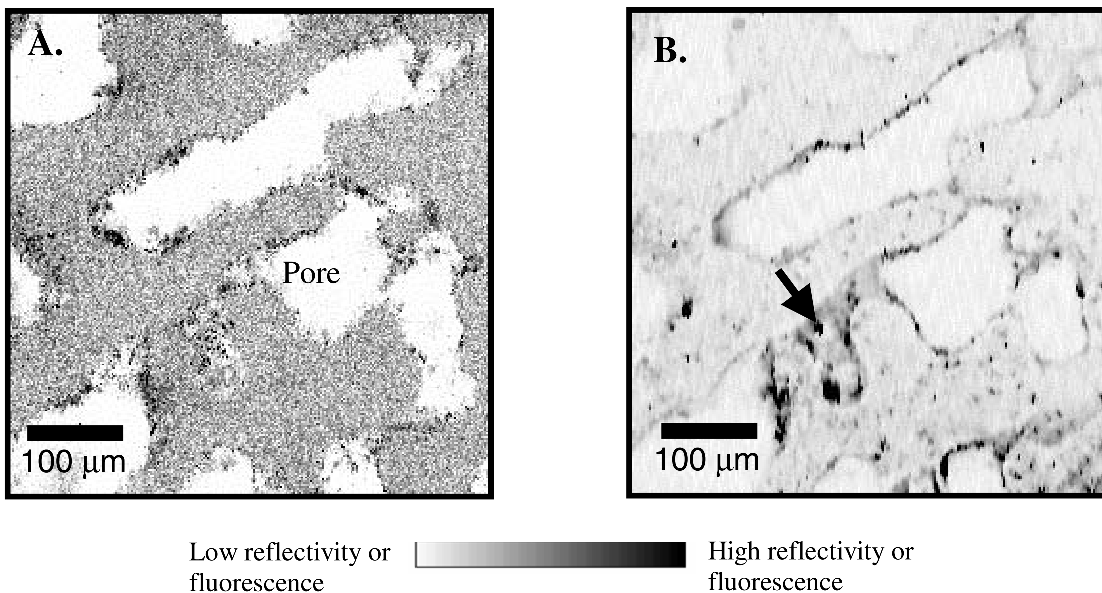

Figure 3: OCM (A.) and CFM (B.) images about 66 µm below the surface of the PCL scaffold cultured for 10 weeks with osteoblasts then stained. The image size is 500 x 500 µm.