Published in final edited form as:

J Microsc. 2018 April ; 270(1): 41–52. doi:10.1111/jmi.12648.

Imaging analysis of the interface between osteoblasts and micro-rough surfaces of laser-sintered titanium alloy constructs

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Abstract

Previous work using focused ion beam (FIB) analysis of osteoblasts on smooth and micro-rough Ti surfaces showed that the average cell aspect ratio and distance from the surface are greater on the rough surface. In order to better interrogate the relationship between individual cells and their substrate using multiple imaging modalities, we developed a method that tracks the same cell across confocal laser scanning microscopy (CLSM) to correlate surface micro-roughness with cell morphology and cytoskeleton; scanning electron microscopy (SEM) to provide higher resolution for observation of nano-roughness as well as chemical mapping via energy dispersive X-ray spectroscopy; and transmission electron microscopy (TEM) for high resolution imaging. FIB was used to prepare thin sections of the cell-material interface for TEM, or for three-dimensional electron tomography. Cells were cultured on laser-sintered Ti-6Al-4V substrates with polished or etched surfaces. Direct cell to surface attachments were observed across surfaces, though bridging across macro-scale surface features occurred on rough substrates. Our results show that surface roughness, cell cytoskeleton and gross morphology can be correlated with the cell-material cross-sectional interface at the single cell level across multiple high resolution imaging modalities. This work provides a platform method for further investigating mechanisms of the cell-material interface.

Second Abstract – Lay Description

The success of biomaterials starts with the cell-biomaterial interface. At this interface, cells attach to proteins that adsorb onto the biomaterial surface. Factors including surface roughness, wettability and chemistry can all affect cell attachment, morphology and growth or differentiation. Although studies have shown that aggregate cell response to biomaterials can also predict implant success or failure in the body, little is understood about individual cell behavior. Viewing cell morphology at the macro-, micro- and nano- scales requires different imaging modalities, which

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makes it challenging to track the behavior of one single cell. This study introduces scalable methods for viewing the cell-surface interface across multiple imaging modalities for a single cell.

Keywords

Material characterization; cytoskeleton; surface roughness; titanium; biomaterials; morphology

Introduction

Numerous studies have examined the morphology of osteoblast lineage cells on biomaterial surfaces (Lai *et al.*, 2015, Zinger *et al.*, 2005, Zhao *et al.*, 2007). When grown on smooth substrates like tissue culture polystyrene (TCPS) or smooth titanium (Ti), cells exhibit a flattened and spread morphology (Zhao *et al.*, 2007). However, scanning electron micrographs of osteoblasts grown on microtextured Ti substrates indicate that the cells become more rounded with filopodia extending as anchors out from the cell body to pits on the surface (Lai *et al.*, 2015). Focused ion beam (FIB) milling and scanning electron microscopy (SEM), combined with three-dimensional image reconstruction confirmed that osteoblasts on grit blasted/acid etched Ti surfaces had an elongated morphology with reduced cell area, increased cell thickness, and more contact points compared to cells grown on machined Ti surfaces.

While this approach can provide correlations between the number of and distance between surface attachments obtained by cross sectional analysis and quantitative cell morphology obtained by top-down SEM images and with gene expression, it is necessary to average the morphological correlations over different experiments due to the inability to ensure that specific cells are being assessed via the different imaging modalities. While averaged correlations may be suitable for homogenous cell populations, it can introduce error from other experimental variables. Thus, correlation across the sample or even a specific cell is much more useful for analyzing cell-material interactions.

Correlative light and electron microscopy (CLEM) has been used in many fields including animal and plant biology, ophthalmology and neuroscience (Rizzo *et al.*, 2016, Schumann *et al.*, 2014, Hogstrom *et al.*, 2016). Many of the first applications of high resolution correlative microscopy were in cellular structural biology (Zhang, 2013, Polishchuk *et al.*, 2000). Early work employed gridded coverslips, pre- and post-etched sample symbols and even nail polish to serve as fiducial markers for identifying the same physical location (van Rijnsoever *et al.*, 2008, Spiegelhalter *et al.*, 2010, Colombelli *et al.*, 2008). Advancements in hardware and software have now lead to automating this process, with sample holders compatible across multiple imaging modalities (Arnold *et al.*, 2016, Lidke & Lidke, 2012).

Correlative microscopy became more accessible after methods for correlating images with fiducial markers were introduced in ImageJ, a publicly available free software (Keene *et al.*, 2014). it was possible to observe bone tissue around titanium dental implants using light microscopy, SEM and transmission electron microscopy (TEM), but the tissue-implant interface was only observable in light microscopy (Tirre *et al.*, 2010). There was clear sample deterioration after processing for SEM, and implants had to be completely removed

for TEM analysis. TEM images were viewed and referred back to the previous sample area for correlation, rather than areas being pre-determined prior to analysis. Earlier “correlative” light and TEM studies imaged over 2000 TEM lamellas to achieve a comprehensive understanding of the tissue, without any direct information on the tissue-implant interface because implants were removed prior to TEM sectioning (Steflik *et al.*, 1992a). Another semi-correlative study attempted to understand the bone-implant interface by creating light and TEM sections from the same implant, but did not analyze the same section at the same location with both microscopes (Steflik *et al.*, 1992b). Thus, correlative analysis of the tissue-material interface across multiple spatial scales remains a challenge.

High resolution analysis of the biology-material interface is limited by sample preparation and correlation across multiple spatial scales. TEM investigations of the cell-surface interface have mostly been performed on silicon, a popular sensor material that can also be removed by etching or freeze fracture after fixation and embedding the cell monolayer in resin (Wrobel *et al.*, 2008, Hanson *et al.*, 2012). However, removal of the substrate also limits additional TEM diffraction or chemical analyses, which can provide insight into preferred substrate areas of cell attachment. Pioneering work on focal adhesions and the cell-material interface used correlative microscopy of cultured cells on electron-microscopy grids (Patla *et al.*, 2010, Sartori *et al.*, 2007). While useful for mechanistic and structural studies, these surfaces possess neither the chemistry nor the topography of clinically relevant biomaterials. To facilitate clinically relevant studies on the cell-material interface, versatile and high resolution sample preparation techniques must be employed.

FIB has commonly been employed for materials science applications, most recently as a powerful imaging and sample preparation technique for biological specimens (Narayan *et al.*, 2014). Multiple studies have used FIB to examine cross sections of biological samples, using different sample preparation techniques, each with its own set of limitations. Wierzbicki *et al.* used FIB milling to investigate the cell-material interface of fibroblasts cultured on glass slides with submicron topography (Wierzbicki *et al.*, 2013). Samples were stained and coated with resin to facilitate FIB milling and viewing of cellular components; however, processing with resin prevented top-down SEM imaging and morphological observations of the cell and surface. We have used an alternate approach, analyzing cell volume and attachment parameters by FIB milling serial cross sections of osteoblasts on smooth and clinically relevant micro-rough titanium substrates (Lai *et al.*, 2015). In both examples, the observations are limited by the need to assess average culture behavior, particularly on complex topographies where cells exist in a variety of habitats.

In this study, we present the first correlative light and electron microscopy analysis of osteoblasts on a clinically relevant, optically opaque biomaterial. We provide examples of multi-scale analysis and flexibility across multiple modalities, each providing unique information about the cell-material interface.

Materials and Methods

A diagram of all steps and options for correlative analysis described in this study is presented in Figure 1.

Surface manufacturing

Substrates were disks 15mm in diameter and 1mm in height, which were laser sintered from Ti-6Al-4V powder as described previously (Cheng *et al.*, 2014). Smooth surfaces were polished with aluminum oxide sandpaper (Norton Abrasive, Paris, France). Surfaces were etched for 90 minutes in a 10% solution of 1:1 maleic acid and oxalic acid (Sigma-Aldrich, Missouri, USA) in distilled water to achieve mesoscale roughness. Surfaces with hierarchical roughness were additionally blasted with calcium phosphate (proprietary, AB Dental, Ashdod, Israel) and acid etched to produce micro-roughness, and then acid etched to achieve mesoscale roughness followed by pickling to produce nano-roughness, as previously described (Cheng *et al.*, 2014).

Cell culture

Calvarial osteoblasts were isolated from SD-Tg(UBC-EGFP)2BalRrrc transgenic rats (Rat Resource and Research Center, Columbia, Missouri, USA) that express ubiquitous enhanced green fluorescent protein (EGFP) under the human ubiquitin-C promoter with the woodchuck hepatitis virus posttranscriptional regulatory element (WRE). Osteoblasts were isolated using a previously published explant technique (Olivares-Navarrete *et al.*, 2010, Bellows *et al.*, 1986). GFP-osteoblasts were plated on disks in 24-well plate at a density of 30,000 cells/cm² (60,000 cells/well). Full medium (DMEM +10% FBS + 1% PenStrep) was changed 24 hours after plating. Medium was aspirated 48 hours after plating. Wells were rinsed twice with 1mL of pre-warmed 1XPBS, which was then aspirated. Cells were fixed with 1mL of 4% paraformaldehyde in 1xPBS for 15 minutes, then rinsed with 1mL 1xPBS. In order to observe actin filaments and nuclei, cells were incubated in 500μL 1xPBS with 1:80 phalloidin 594 (Alexa Fluor 594 Phalloidin 300 units, Thermo Fisher, Waltham, MA, USA) and 1:1000 Hoechst (Hoechst 33342 10 mg/mL, Thermo Fisher) for 20 minutes in the dark, respectively. Cells were rinsed again three times with 1xPBS.

Sample fixation

Ti-6Al-4V disks were carefully mounted on 22×22mm glass coverslips (Zeiss, Oberkochen, Germany) with epoxy (Epoxicure 2 epoxy resin and hardener, Buehler, Lake Bluff, Illinois, USA). Epoxy resin was mixed with hardener at a ratio of 4:1. Pressure was applied on the edges of samples to secure them to the glass slide, and samples were allowed to dry overnight to allow the epoxy to cure. A small drop of epoxy was placed at one corner of the glass slide as a marker for orienting the sample during analysis.

Surface roughness and fluorescence imaging

Laser confocal microscopy (Zeiss LSM 710) was used to analyze surface roughness and image GFP fluorescence of cells on the surfaces. Samples were mounted onto a Shuttle and Find sample holder (Zeiss), with orientation within the sample holder noted by the epoxy location. Three-point calibration was performed for each sample and imaging location coordinates were stored within software for automated recall during electron imaging. During confocal imaging, samples were wetted with phosphate buffered saline (PBS). A coverslip was secured to the sample with tape, making sure the tape only covered the edges of the coverslip and did not obstruct the sample view. Low magnification z-stacks were

taken using a 20x (Plan-Apochromat 20x/0.8 M27, 0.8 numerical aperture, 45.06mm parfocal length), 0.1µm step size, 0.6 zoom and 0.79µs pixel dwell time. High magnification collapsed z-stacks shown in figures were taken using a 40x objective (LD Plan-Neofluar 40x/0.6 M27, 0.6 numerical aperture, 45.06mm parfocal length), 0.1µm step size, 0.6 zoom and 1.58µs pixel dwell time. Four separate tracks were used with standard automatic settings applied consistently for all samples, with a 1 AU pinhole size for imaging. Surface roughness was characterized at 405nm in reflection mode, cell GFP was imaged at 488nm, Hoechst staining for the nucleus was imaged at 405nm, and phalloidin staining for actin was imaged at 594nm. Surface roughness was characterized using a 20x objective and analyzed using ZEN Blue software (Zeiss) with a bandpass filter wavelength of 100µm. Average surface roughness (R_a) was analyzed on three regions per sample, with at least two samples per group. Surface roughness values are reported as average $\pm$ standard deviation.

Preparation for electron microscopy

Samples were dehydrated in increasing concentrations of ethanol for 2 hours each: 15%, 30%, 45%, and then at least 1 hour each in 60%, 75%, 90%, 100%. Samples were immersed twice more in fresh 100% ethanol for at least 1 hour, then exchanged in 1:1 100% ethanol and hexamethyldisilazane (HMDS) for 30 minutes in a fume hood. Samples were transferred to 100% HMDS for 30 minutes twice, then transferred to a vacuum dessicator to dry for at least 24 hours prior to electron microscopy.

Energy dispersive x-ray spectroscopy

Samples analyzed with EDX were not sputter coated prior to imaging. Regions of interest (ROI) previously characterized with LCM were relocated after stage calibration using the ZEN Shuttle and Find software package (Zeiss) in the Zeiss Auriga SEM/FIB system. EDX was performed with at a working distance of 9.5mm accelerating voltage of 15kV. EDX maps were performed at a magnification of 260X. Prior to analysis, EDX was calibrated on pure copper tape and aluminum substrates.

Scanning electron microscopy

Prior to scanning electron microscopy, samples were platinum-sputtered at 35µA for 90 seconds. Previously characterized regions of interest were located using the Shuttle and Find sample holder with electron microscopy adaptor in the Zeiss Auriga Zeiss FIB/SEM system. LCM and SEM correlative images were overlaid in Shuttle and Find software. Images were taken at a working distance of 4mm and accelerating voltage of 4kV.

Focused ion beam milling

FIB milling was conducted on a TESCAN LYRA 3 FEG-SEM/FIB system (Brno, Czech Republic) with a working distance of 9mm and 55° tilt. Regions of interest characterized previously by LCM and SEM were located using the Shuttle and Find system using a Zeiss Auriga Zeiss FIB/SEM (Zeiss). To locate regions across multiple SEM and FIB systems without Shuttle and Find, large “X” markers were FIB milled onto some samples. A layer of platinum (Pt) with a thickness of approximately 1µm was deposited at 200pA, 30kV and with a 100µm aperture at the location of interest to provide mechanical stability during FIB

milling. Initial milling was performed using a fast stair rectangle template at 5 μ A and 30kV in front of and behind the Pt-deposited region of interest to expose the interface. Polishing was performed at 1 μ A and 30kV to thin sections between 500nm-1 μ m, and the remaining side area attached to the substrate was milled away. A “U” cut was milled around the sides and bottom of the thin section to prepare for removal, leaving a small area attached for stability. The thin section was attached to a tungsten nano-manipulator using platinum deposition. The thin section was then attached to the TEM grid with Pt deposition on both sides of the sample, and the area attached to the nano-manipulator was removed by milling. Final polishing was performed on thin sections while attached to the TEM grid. This consisted of an initial milling decreasing from 1nA to 200pA to 100pA and at 30kV to mill sections to a thickness of approximately 200nm. Secondary polishing was performed at 100pA to 50pA and at 10kV to further decrease sample thickness to approximately 100nm. Final polishing to prepare for TEM was performed at 20pA and at 5kV and decreasing to 1.5kV to limit sample damage. Samples for electron tomography (ET) were only milled to 200–300nm in thickness.

Thin section contrast staining

Sections were stained to enhance contrast prior to further electron microscopy. Staining was conducted automatically using the Leica EM AC20 (Leica, Wetzlar, Germany). Samples were double contrast stained for 20 minutes in 0.5% uranyl acetate, followed by 30 minutes in 3% lead citrate.

Transmission electron microscopy (TEM)

TEM was conducted with a Hitachi H7650 system (Hitachi, Tokyo, Japan) at 80kV and a Zeiss Libra 120 system (Zeiss) at 120kV.

Electron tomography (ET)

ET was conducted with a Titan Krios system (FEI Company, Oregon, USA). The sample holder and section were cooled with liquid nitrogen before transferring to the microscope. Images were taken with a 300kV accelerating voltage using a Falcon direct electron detector at 29kX or 75kX magnification. Manual tracking was used to center each image. The sample tilt angle ranged from $\pm 58^\circ$, at 2° when less than 20° , and at 1° for greater angles. Images were taken with exposure time of 1 second, dose of 1.03 electrons per square angstrom ($\text{\AA}^2$) and pixel size of 2.88 $\text{\AA}$ at 29kX magnification and 1.11 $\text{\AA}$ at 75kX magnification. Tomographic reconstruction was conducted using IMOD software (University of Colorado at Boulder, USA) (Kremer *et al.*, 1996)

Results

Confocal imaging of GFP-cells showed a semi-confluent culture with heterogeneous cell morphology (Figure 2A). Cells were elongated and appeared to be nestled between surface features, with filopodia extending out to anchor the cell to the surface. SEM of the same location demonstrated that surface roughness was additionally punctuated by Ti-6Al-4V particles that were partially sintered (Figure 2B, C). Higher magnification confocal (Figure 2D) and SEM (Figure 2E) images were correlated (Figure 2F) to show greater detail of the

cell and surface. Using this method, we were able to observe specific cell morphology corresponding to surface features.

Confocal imaging of GFP cells (Figure 2G) and SEM images of the same location (Figure 2H) on unsputtered surfaces were able to assess cell morphology on rough surfaces (Figure 2I). A ROI was then selected in SEM (Figure 2J) to yield a high magnification light-electron correlated image (Figure 2K). This image showed that cells were generally elongated on the rough surface, but were rounder when attached to specific surface features. Additional analysis of material chemistry using the EDX feature in SEM identified carbon content on surfaces, which correlated with the spatial positioning of cells (Figure 2L).

While cell morphology of GFP cells could be seen on CLSM and SEM, correlation only provided limited additional information compared to using either method alone. However, by combining the two images it was possible to see how cell morphology related to the material surface. Moreover, LCM enabled identification of internal components of the cell, which were then correlated with SEM top-down images. GFP cells (Figure 3A) stained for actin (Figure 3B) and nuclei (Figure 3C) exhibited aligned actin fibers and normal distribution of nuclei within the cells. High magnification confocal images (Figure 3D) were correlated with SEM images (Figure 3E) to produce an overlay image (Figure 3F) which distinguished individual cells on the polished surface. Analysis of surface roughness using LCM enabled us to assess multi-scale roughness (Figure 3G), which showed that the surface possessed a relatively homogeneous micro-roughness with a z-range within 70µm. The same region of interest was correlated with fluorescent cells on the same surface (Figure 3H). This provided quantitative information about surface micro-roughness for a typically qualitative SEM image. The images showed a homogeneous distribution of cells attached on rough surfaces, though with less confluence than on polished surfaces.

SEM was also used to further evaluate nano-roughness. Smaller regions of interest were magnified to create correlative LCM-SEM image overlays (Figure 3I), with progressive high resolution magnification to desired ROIs (Figures 3J, 3I) in SEM. Using this approach, high magnification SEM images of cell filopodia were imaged on surface nano-features (Figure 3L). Osteoblasts cultured on rough surfaces appeared to exhibit more filopodia than on smooth surfaces. At high magnification, filopodia were observed spreading over the surface nano-roughness, while still adhering to the curvature of the micro-roughness.

The correlative imaging approach was used successfully to examine the interface between osteoblasts and Ti-6Al-4V surfaces produced by laser sintering, a form of additive manufacturing. Smooth surfaces possessed an average surface micro-roughness (R_a) of 0.92 ± 0.3 µm, and rough surfaces possessed a roughness of 7.6 ± 1.1 µm. On smooth surfaces, the location of GFP osteoblasts (Figure 4A) correlated with that of EDX carbon mapping (Figure 4B). Confocal images were overlaid on SEM images at the same location (Figure 4C) to produce a correlative image (Figure 4D). A ROI (Figure 4D) was located with the FIB detector at a 55° tilt (Figure 4E) and a layer of Pt was deposited across the region to be milled (Figure 4F) to provide mechanical stability during milling. Trenches were milled around in front of and behind the section (Figure 4G). A “U” cut was milled around the section (Figure 4I) before attaching to the nano-manipulator (Figure 4I) and milling away

the remaining section attached to the substrate. The section was attached to the TEM grid (Figure 4J) and final polishing was performed to prepare thin sections with approximately 100nm thickness (Figure 4K). Final lamellas were stained and imaged with TEM to observe the cell-material interface (Figure 4L). Sections were thin enough to view differences between the bottom patterned Ti-6Al-4V substrate, the lighter-colored cell on top of the substrate and the opaque Pt deposited on top of the cell.

After the proof of concept was completed on smooth surfaces, more clinically relevant micro-rough surfaces were used. On rough surfaces, GFP osteoblasts (Figure 5A) and SEM images of the same location (Figure 5B) were used to create a correlated overlay image (Figure 5C). A ROI (Figure 5C) was located with the FIB detector (Figure 5D) and a thin section was milled (Figure 5E). Final milling was performed after the thin section was attached to the TEM grid (Figure 5F). After staining, cellular components could be observed, but the section was too thick to observe the titanium substrate in TEM (Figure 5G). Higher magnification TEM images showed multiple layers and significant biological sample damage in the form of white semi-circular holes (Figure 5H, 5I). Unidentified cell organelles were observed as a result of contrast staining in the form of darker oval shapes in the cell. In addition, direct cell attachment was observed on the surface that followed the nano-scale surface contours.

To combine the entire process from CLSM to electron tomography, GFP osteoblasts were additionally permeabilized and stained for actin and nucleus on rough surfaces (Figure 6A). This showed a heterogeneous cell morphology across the surface. Confocal images were correlated with SEM of the same location (Figure 6B) to produce an image overlay (Figure 6C). This correlation revealed a morphological preference for cells attaching to various surface features. Cells attached on a micro-scale surface feature tended to bridge across the feature, either onto another adjacent feature or onto the bulk surface below. Where there were no adjacent surface features, cells would spread and cover the entire surface feature. Cells attached on the bulk substrate exhibited a smaller but still elongated morphology. A ROI (Figure 6C) was located with the FIB detector, and platinum was deposited at the location to be milled (Figure 6D). A thin section was milled (Figure 6E) and the final section, approximately 300nm in thickness, was attached to the TEM grid (Figure 6F). High voltage electron tomography was used to image the 3D volume of interest (Figure 6G), which was rotated to view depth of the sample and provide higher contrast at certain locations (Figure 6H). A volume of interest was selected at the cell-material interface, and an additional high magnification tomography analysis was used to observe the interface. Individual planes are shown that span through the reconstructed tomogram thickness (Figures 6I–L). Though sample damage was observed (thinner or nonexistent portions of the cell were lighter or white in color, respectively), high magnification tomography was still able to reveal structural changes in cellular organization at the interface (Figures 6I–L, middle portions). Because of increased sample thickness, the Ti-6Al-4V surface was opaque (Figures 6I–L, bottom).

Discussion

Our study demonstrates that single-cell correlative analysis can be achieved across multiple imaging modalities. This workflow is especially attractive because it overcomes previous limitations in surface and cell imaging for opaque materials. Initial quantitative surface roughness analyses at the micro-scale can be combined with high resolution imaging of individual filopodia on nano-rough surfaces. For titanium substrates with hierarchical surface roughness, these correlations can provide a glimpse into structural and biological mechanisms regulating osteoblastic differentiation and cell-material interactions. For other biomaterials, this method can be used to elucidate cell preference for specific surface structural or chemical features. This correlative platform method can also be enhanced for future “smart” material analyses.

Because this study highlighted different examples to show versatility and a concept of our novel correlative methods, we did not focus on one particular variable. The most obvious application of this method would be to correlate staining for focal adhesion proteins with attachment morphology and substrate topography. This could be done with the combined use of fluorescent staining and nanoparticle tags that would be observable in both light and electron microscopy (Takizawa *et al.*, 2015, Glenn *et al.*, 2012). In this example, we can also envision a use for EDX chemical mapping in locating metallic nanoparticles within the cell that correspond to surface roughness features. Advancements in high resolution characterization technology may also provide a biochemical map of single cells, which could be correlated with material and morphological information (Wu & Singh, 2012, Swain & Stevens, 2007). While we can correlate TEM interface images with an individual cell or even its fluorescently imaged cytoskeletal structure in this study, we cannot definitely identify proteins to correlate with sites of attachment. We chose to section at edges of the cell to focus on these sites of attachment, which also explains the low cell height and lack of major organelles, such as the nucleus, in our TEM images.

From top-down qualitative images from LCM and SEM, it is clear that morphological differences exist between cells grown on smooth or rough substrates. While exact cell type and surface chemical composition varied from a previous study in our lab that quantified these differences, our results still corroborate that osteoblasts are rounded and more spread out on smooth titanium substrates, and elongated on rougher substrates (Lai *et al.*, 2015). This comparison can be observed directly when comparing Figures 3F and 3I (images are presented at the same magnification). These morphological changes on smooth versus rough surfaces are correlated with the degree of osteoblast differentiation (Hyzy *et al.*, 2016).

Cross sectional images of osteoblasts in SEM and TEM after FIB milling showed a much thinner osteoblast cross section on smooth compared to rough Ti-6Al-4V surfaces. While osteoblasts on polished smooth surfaces had a cross sectional thickness of approximately 100nm, osteoblasts on micro-rough surfaces had a thickness of approximately 500nm to 1µm, depending on the location of sectioning within the cell. This observation was consistent with previous quantification of FIB-milled osteoblast cross sections on smooth and micro-rough titanium surfaces, which showed that cross sectional osteoblast thickness was much higher for cells cultured on rough titanium surfaces compared to on smooth

surfaces (Lai et al., 2015). An enhanced presence of cell filopodia was also observed on micro-rough surfaces compared to on smooth surfaces, and cross-sectional images indicated that these projections fully engulfed the surface nano-features. Other studies have also reported a correlation of increased filopodia with enhanced osteoblastic differentiation on rough titanium surfaces with increasing nanotube diameters (Oh *et al.*, 2009). Our study suggests that while surface micro-roughness may be responsible for osteoblastic differentiation and maturation, nano-topography can be important for cell attachment and motility.

However, while our previous study noted that cells would “tent” over micro-rough surface features, in this study we observed a differential morphological preference of cells that was feature specific. Cells on partially sintered micro-particles would either tent across to another adjacent particle or the underlying surface, or wrap around the particle almost completely. We believe the site of initial cell attachment as well as the size and spacing between surface features may affect its decision to spread across the feature or remain covering the feature. We have shown this size-specific effect on cell bridging previously, where cells remained within 100µm diameter cavities but would anchor to adjacent cavities when they were reduced in diameter to 30µm (Zinger et al., 2005). Our laser sintered particles ranged between 25–45µm in diameter with variable spacing between partially sintered surface particles, and this accordingly resulted in a differential response in cell morphology. These observations indicate that cell attachment and morphology are sensitive to distinct micro- and nano-scale surface features.

Sample preparation is very important when imaging at the nano-scale. We chose GFP-cells to optimize the correlative approach because it did not require permeabilization of the cell membrane to stain for cytoskeletal components, which would compromise high resolution electron microscopy analysis. However, we still observed artefacts in the cell membrane. While HMDS has been shown to induce less cell shrinkage than critical point drying, research has also shown that increasing HMDS exposure time correlates with increased cell shrinkage (Katsen-Globa *et al.*, 2016). In addition, handling of samples for confocal imaging, including mounting and creating an orientation marker using epoxy resin and securing a coverslip with PBS for better optical resolution, may affect cells. Future work should include optimization of fixation and processing protocols to decrease these sample artefacts.

While providing a unique way to observe biological cross sections, FIB milling is still a destructive technique (Narayan et al., 2014). We chose to use FIB as a sample preparation technique rather than an imaging modality. This technique provides the flexibility to choose between traditional TEM and electron tomographical analysis of cross sections, depending on section thickness. In addition, samples for TEM could be analyzed multiple times (before and after staining, or for chemical or diffraction analyses) for future studies. This is a significant improvement from traditional TEM sectioning, which requires removal of the implant even when sectioning with diamond knives in an ultramicrotome (Steflik *et al.*, 1994).

While ET can be useful for resolving thicker sections, it is also much more time consuming and very data intensive. A high resolution analysis of a nanometer-scale sample can easily take over 24 hours and require over 4 terabytes of data (Thompson *et al.*, 2016). Even a “slice and view” automated FIB milling and viewing technique of a $90\mu\text{m} \times 32\mu\text{m} \times 2\mu\text{m}$ volume can take over 24 hours, though this may be preferable since processing and reconstruction can be completed in the same system (Bushby *et al.*, 2011). Our method allows the user to analyze across different imaging modalities that would otherwise be incompatible. For example, correlative cryo-EM may require a cryo-light or focused ion beam milling electron microscope to preserve sample temperature (Sartori *et al.*, 2007, Hayles *et al.*, 2007, Rigort *et al.*, 2012). Transportation or reanalysis of samples then also becomes a challenge. By fixing and sectioning samples at room temperature, the user can choose between FIB sectioning, SEM viewing, traditional TEM imaging or ET, without having to keep the sample vitrified during the entire process.

Final milling of thin sections to the desired thickness was challenging due to the inhomogeneous nature of the samples. Limited resolution of the cell-material interface in these sections may have been an intrinsic limitation of the TEM due to section thickness. A curtaining effect can be observed even after fine milling in Figure 6F. While a thicker platinum deposition of approximately $2\mu\text{m}$ has been shown to decrease curtaining effects, the already existing rough topography of our samples will inadvertently introduce artefacts from an uneven disintegration of platinum during milling (Hayles *et al.*, 2007). Another way to enhance FIB milling is by ultra-thin resin embedding of the sample, which provides mechanical stability during sectioning (Belu *et al.*, 2016). Studies have shown 3D reconstruction of FIB milled cells with resolution as great as 3nm using resin embedding (Narayan *et al.*, 2014). However, even a thin film of resin will obstruct nanotopographic features of the cell and substrate surface, so this method is recommended only when correlating between confocal and TEM, without consideration of top-down SEM imaging of surface topography.

The applications of this work are vast. First, our study shows the feasibility of evaluating the cell-material interface on almost any biomaterial, regardless of its optical properties. Second, this technique opens the door for dynamic and single cell analyses on these materials, which can provide insight into adhesion, migration and differentiation of wild type or compromised cells. Additional correlative analyses such as AFM, XPS or Raman spectroscopy could provide an even more comprehensive understanding of the surface topography and chemistry or cell differentiation profile.

Conclusion

We present a correlative microscopy method that spans multiple imaging modalities, allowing multi-scale spatial analysis of the same cells on clinically relevant biomaterial surfaces. Using this method, we evaluated osteoblast morphology and interaction with smooth and micro-rough, laser sintered Ti-6Al-4V surfaces. This platform method can be used to further understanding of the cell-material interface and enhance design of future biomaterial surfaces. Development of these methods can provide insight into cell-specific interaction mechanisms with different materials.

Acknowledgements

The authors would like to acknowledge Professor Rong Wang at the State Key Laboratory of Tribology and Tsinghua University for assistance with FIB milling and Professor Kelly Dryden at University of Virginia for assistance with electron cryo-tomography. The authors would also like to thank Professor Jun Xu at Peking University for guidance on FIB milling, and Professor XueMei Li at Peking University and Professor Massimo Bertino and Kenneth Kane at Virginia Commonwealth University for assistance with TEM. Professor Dmitry Pestov at the Virginia Commonwealth University Nanomaterials Core Characterization Facility provided guidance on sample preparation and helped in troubleshooting multiple imaging modalities. Laser sintered substrates were generously donated by AB Dental (Ashdod, Israel). Caroline Bivens was responsible for the artwork in Figure 1.

Research reported in this publication was supported by AB Dental and by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR052102. AC is a National Science Foundation Graduate Fellow and was supported by the Whitaker International Program. Electron cryo-tomography was conducted at the Molecular Electron Microscopy Core facility at the University of Virginia, which is supported by the School of Medicine and NIH grants for the Titan Krios (S10-RR025067) and Falcon II direct detector (S10-OD018149). HC acknowledges support from the National Basic Research Program of China (2012CB933903).

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Figure 1. Schematic of correlative microscopy workflow. (A) Cells are cultured on a clinically relevant biomaterial of interest, or an implant is placed in vivo. The cells or tissue are fixed and fluorescently stained for proteins of interest before (B) fluorescence and 3D z-stack imaging in LCM. After dehydration, samples are ready for (C) chemical analysis in EDX. Samples are sputter coated to increase conductivity for (D) SEM high resolution correlative imaging and FIB milling of cross sections. Cross sections are stained for (E) high resolution imaging at the biology-material interface in TEM.

Figure 2. Correlative light and electron microscopy (CLEM) of rat GFP calvarial osteoblasts on laser sintered Ti-6Al-4V substrates. Osteoblast were plated on surfaces for 24 hours and imaged with (A) laser confocal microscopy and (B) scanning electron microscopy. (C) GFP fluorescence was superimposed on the correlated scanning electron micrograph, with a region of interest (ROI) indicated within the red dashed lines. This ROI was enlarged to show the (D) GFP fluorescent osteoblasts, (E) electron micrograph of the surface roughness and (F) correlated. Osteoblasts were plated on surfaces for 72 hours and imaged with (G) confocal and (H) scanning electron microscopy and (I) correlated. An ROI indicated within the red dashed lines was imaged with (J) SEM to produce a (K) correlated light and electron micrograph. Samples were not sputtered prior to SEM, allowing for (L) EDX analysis of carbon content (in purple), which correlated with the presence of cells.

Figure 3.

Correlative light and electron microscopy of GFP osteoblasts with cytoskeletal staining after 24 hours on smooth and rough Ti-6Al-4V substrates. Cells plated on smooth surfaces were imaged with LCM and show (A) GFP of the entire cell, (B) the actin cytoskeleton and (C) cell nuclei. (D) All three fluorescent tracks were merged with the corresponding (E) scanning electron micrograph in a (F) correlative image. LCM was used to analyze (G) surface micro-roughness of rough Ti-6Al-4V substrates before (H) fluorescence imaging was performed of the cell (green), actin (red) and nucleus (blue). The ROI indicated within the red dashed lines was chosen for (I) correlation with SEM. Each ROI indicated within the red dashed lines in (I, J, K) was imaged at higher magnification in (J, K, L), respectively. A

high magnification image obtained with SEM shows the (L) surface nano-roughness that isn't detectable from the micro-roughness map obtained by LCM alone.

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Figure 4.

Complete correlative light and electron microscopy of osteoblasts on smooth sintered surfaces. (A) LCM of GFP osteoblasts plated on smooth Ti-6AL-4V surfaces, (B) chemical mapping performed in EDX, (C) SEM micrograph after platinum sputtering and (D) a correlated light-electron image. The region of interest indicated within the red dashed lines (E) was identified with the focused ion beam detector at a 52 degree tilt, and a red dashed line indicates the location to be prepared for TEM analysis. (F) Platinum was deposited atop the location to be milled to provide mechanical stability during milling, and (G) a section approximately 500nm thick was milled. (H) The perimeter was milled around the thin section to prepare for detachment, (I) the section was attached to a nanomanipulator by platinum deposition before the remaining edge was milled away. (J) The section was attached to a TEM grid by platinum deposition and (K) final milling was performed to reduce section thickness to less than 100nm. (L) TEM image shows the cell-material interface with high resolution.

Figure 5.

Complete correlative light and electron microscopy of osteoblasts on rough sintered surfaces. (A) LCM of GFP osteoblasts plated on smooth Ti-6AL-4V surfaces, (B) SEM micrograph after platinum sputtering and (C) a correlated light-electron image. The region of interest indicated within the red dashed lines (D) was identified with the focused ion beam detector at a 52 degree tilt, and a red dashed line indicates the location to be prepared for TEM analysis. (E) A section approximately 1µm thick was milled. (F) The section was attached to a TEM grid by platinum deposition and final milling was performed to reduce section thickness to less than 200nm. TEM images shows the cell-material interface at (G) lower and (H, I) higher magnification with sample damage induced by the milling process.

Figure 6.

Complete correlative light microscopy and electron tomography of osteoblasts on rough sintered surfaces. (A) LCM of GFP osteoblasts plated on smooth Ti-6AL-4V surfaces stained for actin (red) and nucleus (blue), (B) SEM micrograph after platinum sputtering and (C) a correlated light-electron image. (D) The region of interest indicated within the red dashed lines was identified with the focused ion beam detector at a 52 degree tilt, and platinum was deposited on the area to be milled. (E) A section approximately 1μm thick was milled. After attachment to TEM grid, (F) final milling was performed to reduce section thickness to less than 400nm. The region of interest indicated within the red dashed lines was identified as a 3D volume (G) in electron tomography, and (H) could also be tilted for depth perspective. The volume of interest indicated within the red dashed lines was imaged

using electron tomography at high magnification to show (I-L) changes in the cell-material interface at different depths of the z-stack.

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