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Non-invasive monitoring of cell metabolism and lipid production in 3D engineered human adipose tissues using label-free multiphoton microscopy

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ABSTRACT

Non-linear optical microscopy methods can characterize over time multiple functional properties of engineered tissues during development. Here, we demonstrate how the combined use of third-harmonic generation (THG) and two-photon excited fluorescence (2PEF) imaging can provide direct quantitative biomarkers of adipogenic stem cell differentiation and metabolic state, respectively. Specifically, we imaged over nine weeks silk scaffolds embedded with human mesenchymal stem cells and exposed to either propagation (PM) or adipogenic differentiation media (AM). THG was employed to visualize the formation of lipid droplets. 2PEF was used to assess the metabolic state of the cells through the redox ratio defined based on the endogenous FAD and NADH fluorescence. The redox ratio of cells in the AM scaffold was significantly lower than that in the PM scaffold during week 5 and 9, and correlated with significant increases in lipid-to-cell volume ratio, and number and size of lipid droplets in the AM scaffold. These findings indicate that the decrease in redox ratio during adipogenic differentiation is associated with fatty acid synthesis and lipid accumulation. Our methods therefore enabled us to identify and measure dynamic correlations between lipid droplet formation and cell metabolic state, while providing insight on the spatial heterogeneity of the observed signals.

1. Introduction

Loss of adipose tissue due to trauma or disease is characterized not only by a loss of tissue volume, but also by a loss of tissue function. Over the last few decades, it has become clear that in addition to acting as a storage system for fat and energy, adipose tissue serves a key role in the endocrine system [1] and disruptions in signaling functionality can result in diabetes, cardiovascular disease and hypertension [2–4]. Therefore, the development of quantitative methods to assess adipose tissue function is of direct relevance not only for tissue engineering applications in the context of tissue replacement, but also for improved understanding of numerous diseases and the effects of corresponding treatments. Non-invasive, optical imaging modalities are well-suited for this purpose as they can be implemented in multi-modal imaging platforms that simultaneously provide functional, morphological and biochemical information about natural and synthetic biomaterial scaffolds, extracellular matrix components (such as collagen and elastin) and cells. Such images can be acquired based entirely on endogenous sources of contrast, thus, obviating the need for any processing steps that can in principle introduce artifacts or prevent dynamic monitoring of the sample.

In recent years, microscopic imaging modalities that rely on non-linear light–matter interactions have been increasingly employed to characterize the morphology and function of cells and other tissue components. Such methods include two-photon excited fluorescence (2PEF) [5–12], second-harmonic generation (SHG) [6,12–15], third-harmonic generation (THG) [16,17], coherent anti-Stokes Raman scattering (CARS) [18–20] and stimulated Raman scattering (SRS) [21] microscopic imaging. These approaches typically employ near-infrared wavelengths for sample illumination and achieve deeper penetration than confocal microscopy. In addition, the non-linear nature of the optical interactions

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dictates that they occur only at the focus of the beam, minimizing photodamage and photobleaching during scanning of 3D volumes, which is important both for tissue viability and for enabling optimal detection of endogenous signals that are generally weak.

2PEF is particularly well-suited to assess cell function and morphology, using natural sources of fluorescence such as nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavin adenine dinucleotide (FAD), keratins, lipofuscin, retinol and porphyrins [22,23]. A number of investigators have developed approaches to quantify the signal emanating from NADH and/or FAD as a measure of the metabolic status of the cell, but also to assess some morphological aspects, such as nuclear to cytoplasmic ratio and mitochondrial organization [24–27]. Both lifetime fluorescence [9,28–30] and traditional intensity based [5,6,10,24,25] 2PEF measurements have shown promise as methods that can provide quantitative biomarkers of stem cell differentiation. For example, we recently demonstrated that dynamic changes in the redox fluorescence ratio, defined as the ratio of intensities corresponding to FAD/(NADH + FAD), can be correlated to specific adipogenic differentiation stages of adipose derived stem cells over the course of six months [10]. However, to assess differentiation in that case, it was necessary to sacrifice scaffolds at distinct time points and perform invasive immunohistochemical and PCR-based assays. Thus, direct correlations between the metabolic status of a cell and its level of differentiation as assessed by lipid droplet formation were not possible.

Third-harmonic generation (THG) is an additional contrast mechanism [31] that is compatible with 2PEF imaging. THG imaging is usually performed using an excitation wavelength in the 1.0–1.7 μm excitation range, and signal is detected at one third of this wavelength. Differing from SHG, it does not require molecular asymmetry and can be produced by any medium. However, in biological THG imaging no signal is generally observed from homogeneous media, as a consequence of destructive interference resulting from the axial phase shift (the Gouy shift) experienced by the excitation beam near its focus [31]. When the beam is focused at the interface between two media (a) and (b), the signal approximately scales as $|\alpha_a - \alpha_b|^2$, where $\alpha = \chi^{(3)}/n_{3\omega}\,(n_{3\omega} - n_{\omega})$, $\chi^{(3)}$ is the third-order non-linear susceptibility, $n_{3\omega}$ is the refractive index at the harmonic frequency, and $(n_{3\omega} - n_{\omega})$ is the refractive index dispersion [16]. This mechanism produces contrasted images where optical in inhomogeneities of size comparable to the beam focus are highlighted on a dark background. In particular, a strong signal is observed at the interface between the (aqueous) cytoplasm of cells and a lipidic or absorbing organelle a few 100 s of nm in size [16]. Recent studies have shown that THG can be used to visualize the morphology of unstained tissues [32–36], and in particular to detect lipid structures [16,37–39]. Importantly, THG imaging is best achieved with focused infrared femtosecond pulses, and is easily combined with 2PEF imaging [16,35,40,41].

Therefore, one primary goal of this study was to develop a method for studying correlations between lipid droplet formation and cell metabolism in differentiating engineered tissues over extended time periods, based on combined THG and 2PEF measurements. We examined silk scaffolds embedded with mesenchymal stem cells that were exposed either to propagation or adipogenic differentiation media, and acquired THG and 2PEF images from the same scaffolds at 2, 5 and 9 weeks following the induction of differentiation. We developed an image processing workflow to identify cells and lipid droplets, and to extract quantitative metrics associated with the redox ratio, lipid content, lipid droplet number and lipid droplet size in each cell included in the analysis. Through this cell-by-cell quantification approach, we also assessed potential correlations between the metabolic and lipid droplet readouts within individual tissues. Collectively, the label-free biomarkers described in this study can be used to assess adipogenic differentiation in engineered tissue constructs, and more generally, could serve as measures of adipose tissue function for disease diagnostics and drug screening efficacy.

2. Materials and methods

2.1. 3D adipose tissue engineering

Hexafluoro 2 propanol (HFIP) silk solution was used to generate silk scaffolds based on methods established in previous studies [10,42,43]. Scaffolds measured 8 mm in diameter and 0.6 mm thick; pores were created using 200 μm diameter salt crystals. Human mesenchymal stem cells (hMSCs) were seeded in the scaffolds and two experimental groups were cultured, each with different media reported previously to either induce adipogenic differentiation (DMEM plain, high glucose, with L-glutamine: 434 mL, 0.1 mM Non-essential amino-acids: 5 mL, 1% Pen-Strep: 5 mL, 10% FBS: 50 mL, 50 μM Indometacin, 1000x stock solution: 500 μL, 0.5 mM Isobutyl Xanthine 100x stock solution: 5 mL, 1 μM Dexamethasone: 500 μL, 5 μg/mL insulin: 250 μL) or allow the hMSCs to propagate undifferentiated (DMEM plain, high glucose with L-glutamine: 440 mL, 1% Non-essential amino-acids: 5 mL, 1% Pen-Strep: 5 mL, 10% FBS: 50 mL, 2 ng/mL bFGF: 10 μL) [43]. The hMSC-seeded scaffolds were placed in standard 12-well plates and maintained in culture within a standard 37 °C, 5% CO_2 incubator.

2.2. Two-photon excited fluorescence and third-harmonic generation image acquisition

A schematic of the experimental arrangement is shown in Fig. 1. The principles of operation and relevant excitation/emission wavelengths are shown in Fig. 1a, while Fig. 1b illustrates the optical setup. Imaging was performed on a custom-built laser scanning microscope incorporating a femtosecond Titanium:Sapphire (TiS) laser (750–980 nm, 80 MHz, Chameleon, Coherent Inc., CA, USA), an optical parametric oscillator (1180 nm, 80 MHz, KTP-OPO, APE, Germany), galvanometer mounted mirrors (GSI Lumonics, USA) and a water-immersion objective (40× 1.1NA Zeiss, Germany). Ti:S and OPO powers were controlled with wave plates and polarizers, and beams were combined using a dichroic mirror (1000-DCXR Chroma). Signals were detected in the epi- and trans-directions by photomultiplier modules (SensTech, UK) and lab-designed counting electronics. Scanning and acquisition were synchronized using lab-written LabVIEW software and a multichannel I/O board (PCI-6115, National Instruments, USA). Fluorescence was collected in the backward (epi) direction using a dichroic mirror (695dcxru, Chroma, USA) and 2PEF signals were directed toward two independent detectors using a dichroic mirror (Semrock). THG was collected in transmission.

Two 2PEF and one THG image stack were acquired sequentially with 750, 860 and 1180 nm excitation, respectively. Detection was done on separate channels equipped with 450/80, 582/75 and 377/50 nm filters, respectively (Semrock, USA). Fig. 1c shows typical 2PEF and THG images. 2PEF signal detected from hMSCs at 450 nm with 750 nm excitation emanates primarily from NADH, while the 2PEF emission at 582 nm using 860 nm excitation is attributed mostly to FAD [6,10,23]. Voxel size was typically 0.6 × 0.6 × 2 μm^3. Voxel dwell time was approximately 5 μs per image channel. Image volume thickness varied between typically 30 μm and 100 μm. Image stacks of AM- and PM-seeded scaffolds were recorded at three time points after seeding: weeks 2, 5, and 9.

2.3. Tissue mounting for longitudinal observations

To ensure sterility and cell viability for the duration of the experiment, scaffolds were placed prior to each imaging session in between two sterile coverslips of a heated confocal imaging chamber (model RC-30 WA, Warner Instruments LLC, Hamden CT), while immersed in media (Fig. 1b). The temperature was maintained at 37 °C throughout imaging using a temperature controller (model TC-344B, Warner Instruments LLC). Images from both experimental groups were then analyzed to assess tissue development.

2.4. Image processing: spatial isolation of cells and lipid droplets

Cells and lipid droplets were identified and segmented using algorithms written in Matlab software (Mathworks, Natick, MA). Fig. 2 illustrates the image processing approach we developed to measure cell and lipid droplet characteristics. Fig. 2a shows representative initial images, where the THG image (blue) was overlaid with the NADH (green) and FAD (red) 2PEF images. For the initial part of the analysis consisting of cell border extraction, we found it difficult to develop a fully automated algorithm that yielded accurate results for all image stacks. The strong endogenous silk fluorescence compared to the weak cellular fluorescence was one of the reasons that prevented consistent outlining of cell borders in a manner that truly represented the shape of the cells. We therefore isolated individual cells from each other