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Non-invasive assessments of adipose tissue metabolism *in vitro*

Rosalyn D. Abbott, **Francis E. Borowsky**, **Kyle P. Quinn**, **David L. Bernstein**, **Irene Georgakoudi**, and **David L. Kaplan**

Biomedical Engineering, Tufts University, 4 Colby St. Medford MA 02155

Abstract

Adipose tissue engineering is a diverse area of research where the developed tissues can be used to study normal adipose tissue functions, create disease models *in vitro*, and replace soft tissue defects *in vivo*. Increasing attention has been focused on the highly specialized metabolic pathways that regulate energy storage and release in adipose tissues which affect local and systemic outcomes. Non-invasive, dynamic measurement systems are useful to track these metabolic pathways in the same tissue model over time to evaluate long term cell growth, differentiation, and development within tissue engineering constructs. This approach reduces costs and time in comparison to more traditional destructive methods such as biochemical and immunochemistry assays and proteomics assessments. Towards this goal, this review will focus on important metabolic functions of adipose tissues and strategies to evaluate them with noninvasive *in vitro* methods. Current non-invasive methods, such as measuring key metabolic markers and endogenous contrast imaging will be explored.

Keywords

tissue engineering; non-destructive characterization; optical imaging

Introduction

Adipose tissues not only act as thermal insulators, provide cushion for internal organs, and serve as a reservoir for fat; they also have highly specialized metabolic pathways that regulate energy storage and release affecting local and systemic functions.[32,57] For tissue engineering applications, proper metabolic regulation within adipose tissues is essential to promote and to monitor differentiation and functionality *in vitro* in order to allow extrapolations to *in vivo* scenarios.

Adipose tissue engineering is a diverse area of research where the developed tissues can be used to study normal adipose tissue function, create disease models *in vitro*,[38] and replace soft tissue defects from disease, trauma, or injury *in vivo*.[13] While advances in our understanding of adipose tissue metabolism are significant, new experimental approaches are required to provide insights into mechanisms of energy balance and adipokine (cytokines released by adipocytes) regulation and action.[32] In particular, many diseases, including

Corresponding author: David L. Kaplan, *Address:* 4 Colby St. Medford MA 02155, david.kaplan@tufts.edu, *Phone:* 617-627-3251.

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obesity, dyslipidemia, type II diabetes, thyroid hormone disorders, and different cancers (breast, colon, etc.), disturb normal metabolic regulation in adipose tissues.[28,40,50]

Techniques such as the polymerase chain reaction, immunohistochemistry, flow cytometry, mass spectroscopy, and electron microscopy are all destructive endpoints for cell or tissue analysis that require sacrificing samples to acquire the desired information. Consumption of reagents to maintain the large sample sizes required for these endpoints is not practical due to logistics and cost, especially when cultures are extended for long periods of time. Long term *in vitro* adipose tissue models will likely be required to improve physiological relevance[1] since differentiation *in vitro* can take multiple weeks,[25] and disease mechanisms that affect the adipose tissues develop *in vivo* over weeks to months.[29] Additionally, specimen variability can affect the endpoint readouts of destructive assays that do not enable baseline readings and repeated measurements of the same tissue in culture. By focusing on non-invasive, dynamic measurement systems, cell growth and development can be quantified in the same tissue construct over time,[12,46] minimizing sample reagents, increasing statistical power, and allowing for long term studies. Towards this goal, this review will focus on important metabolic functions of adipose tissues (**Figure 1**) and strategies to evaluate them with non-invasive methods (**Table 1**).

Different metabolically active human adipose tissues

In humans, there are many metabolically distinct adipose tissue depots throughout the body. White adipose tissue is found in visceral, subcutaneous, and intramuscular regions. Visceral white adipose tissue wraps around inner organs and is divided into different regions accordingly: omental (begins near the stomach and spleen and extends into the ventral abdomen), mesenteric (intestine), retroperitoneal (kidney), gonadal (in females it surrounds the uterus/ovaries and in males it surrounds the epididymis/testis), and pericardial (heart).[10] Subcutaneous white adipose tissue is situated beneath the skin predominately in the abdomen and gluteofemoral region (measured by hip, thigh, and leg circumference)[10] Finally, white adipose tissue can be found between and within the skeletal muscles of the body and increases in abundance with age.[34]

Brown adipose tissue is located in the supraclavicular and subscapular region, and is the smallest mass of adipose tissue in the body.[15] Unlike white adipose tissues which store energy, brown adipose tissue dissipates energy by thermogenesis. In addition, brown adipose tissues have a higher uptake (and shorter half-life) of lipids compared to other adipose tissues.[33] They also have a different morphology in which brown adipocytes contain multilocular lipid droplets and a large quantity of mitochondria, while white adipocytes have a large unilocular lipid droplet and a smaller quantity of mitochondria.

Finally, there are specialized adipose tissue depots, mainly the mammary gland and bone marrow, which are functionally distinct from white and brown adipose tissues. Mammary gland adipose tissue is involved in milk production and regulates epithelial cell growth, whereas bone marrow is involved in hematopoiesis and osteogenesis and regulates skeletal metabolism.[48]

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Non-destructive evaluation of triglyceride storage and release

Triglycerides are stored in lipid droplets of adipocytes as an energy resource from two routes: *de novo* lipogenesis (synthesis of fatty acids from non-lipid substrates) or uptake of free fatty acids. White and mammary adipose tissues are capable of *de novo* lipogenesis; however, uptake of free fatty acids is the main source of triglyceride storage.[11,32,52] While triglyceride storage cannot be quantified directly without cell lysis, the levels can be monitored nondestructively using different imaging techniques (see section on tracking cells non-invasively over time with optical imaging).

Lipolysis breaks down lipid stores by the hydrolysis of triglycerides into free fatty acids and glycerol. This process is important to monitor, as it plays a large role in energy balance. Free fatty acids can either be transported to the mitochondria for β-oxidation (providing cellular ATP), enter the nucleus (acting as ligands for nuclear hormone receptors and regulating gene transcription), or released from the cells (where it fatty acids are used as fuel and signaling molecules for tissues).[26] While free fatty acids can be reutilized by adipocytes, glycerol cannot and is a more accurate measurement of lipolysis.[59] Both fatty acid and glycerol assay kits are commercially available and rely on spectrophotometric measurements. These assays make it possible to monitor lipolysis out to extended time points without putting additional stress on tissue constructs or cells,[2,8] since fatty acids and glycerol can be collected in culture media.

Byproduct monitoring of fatty acid oxidation

Fatty acids oxidation allows for the production of ATP to fuel metabolic activity.[16] Fatty acid oxidation can be measured non-invasively by adding a low concentration of [^{14}C] palmitic acid to the culture media for a brief period, and measuring the production of radio-labeled CO $_{62}$.[2] In comparing the oxidation rates observed in human brown and white adipose tissues, brown adipose tissues are able to use fatty acids as an energy source more efficiently. This method has been used to quantify fatty acid oxidation in human white and brown progenitors.[62] Given this assays non-destructive nature, it may be used for repeated metabolic analysis of the same tissue construct.

Tracking glucose metabolism non-invasively

In response to insulin stimulation, adipocytes translocate glucose transporter type 4 (GLUT4) to the plasma membrane, resulting in increased glucose uptake. Glucose transport can be evaluated in a number of destructive ways using radioactive glucose analogs (e.g., 2-deoxy- glucose, 3-O-methyl glucose, [3-^{3}H]-glucose) and evaluating the cell lysate.[5] However, for nondestructive purposes, indirect measurement of glucose metabolism can be determined by tracking glucose levels in the supernatant over time to indicate glucose consumption.[39] Once glucose is taken up by the adipocytes, it is metabolized to either lipids (~20% de novo lipogenesis), lactate (~70%) or carbon dioxide (~10%) end products.[35] Therefore, another indirect measurement of glucose metabolism involves measuring lactate released into the media, which can be measured with spectrophotometric kits[5]

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Non-destructive evaluation of thermogenesis

In brown adipose tissue, thermogenesis is characterized by high levels of cellular catabolism and thus oxygen consumption.[27] Therefore, assessing the metabolic capabilities of *in vitro* adipose cultures is important for brown adipose tissue engineering,[56] and if replicated, could provide a way to combat obesity.[55] One technique used to monitor this thermogenic process *in vitro* requires the use of a commercially available probe that exhibits fluorescence upon oxygen depletion. In order to ensure the availability of a finite amount of oxygen, the cells are covered by a non-toxic mineral oil,[56] making the addition of chemicals or compounds and repeated measures on the same samples more difficult.[49] Commercially available devices such as extracellular flux analyzers, have also been used to evaluate mitochondrial respiration and are label free and non-destructive[21,49] and have been used to determine thermogenesis in human brown adipose progenitors.[62]

Adipokine secretion for non-destructive monitoring of differentiation and function

As an endocrine gland, adipose tissue secretes pleiotropic adipokines that regulate appetite, insulin sensitivity, angiogenesis, blood pressure, and immune responses.[36] Non-destructive analysis techniques used on adipose tissues and their isolated cell types often rely on the collection of supernatant which contains adipokines for monitoring adipogenic differentiation [58]. These proteins also provide important insight into obesity and other diseases involving white adipose tissue. Leptin and adiponectin are two commonly studied proteins that play an important role in metabolism.[4,6,36] Leptin negatively regulates body weight and food intake through receptors in the brain, and is increased in obesity.[4] Adiponectin is secreted by adipocytes into the circulation and acts predominantly on the skeletal muscle and liver (causing an increase in fatty acid oxidation and reduction in liver glucose synthesis), and is reduced with insulin resistance and obesity.[4] Other adipokines, like tumor necrosis factor alpha, produce inflammation in adipose tissues. Because these proteins are more prevalent in obese individuals, chronic inflammation has been linked to obesity. This observation indicates that inflammatory adipokines may play a role in diseases associated with obesity, like type 2 diabetes, metabolic complications, and atherosclerosis.[30] Adipokines can be detected in the supernatant using enzyme-linked immunosorbent assays. This method has been used to quantify leptin release at extended time points up to 6 months.[8,60] Additionally, cytokine protein arrays can be used with a variety of proteins as a more general, less quantitative screening of protein secretion.[7]

Adipokine secretion can be monitored to track adipogenic differentiation and to evaluate the accuracy of long-term *in vitro* models in replicating obesity and its associated complications. While analysis of extracellular concentrations of proteins provides an overall view of the metabolic function of the tissue, it does not provide a spatial map of development within the tissue. To assess variability within tissue constructs, optical imaging can be utilized to quantify the morphological and biochemical heterogeneity of tissues.

Tracking cells non-invasively over time with optical imaging

Non-destructive, optical imaging modalities are ideally suited for tracking adipose tissue engineered constructs over time (**Table 2**). They concurrently provide information on the scaffold, matrix organization (collagen and elastin) and cell morphology and functionality.[12]

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For further background, the basis for various analytical imaging methods are reviewed elsewhere.[22,23] By taking advantage of the inherent optical properties of cells and tissues, optical imaging of adipose tissue does not necessarily require additional cellular tagging to assess cell function and tissue organization. However, when necessary, non-toxic tags can supplement label-free imaging to provide additional information.

For imaging human derived stem cells in 3D scaffolds, the use of two-proton excited fluorescence (TPEF) has been shown to be effective in assessing naturally fluorescent endogenous markers.[12,46,47,61] Natural markers include nicotinamide adenine dinucleotide (phosphate) (NAD(P)H), flavin adenine dinucleotide (FAD), keratins, lipofuscin, retinol, and porphyrins.[12,43,63] When specifically considering mesenchymal stem cells, an optical redox ratio of FAD/(FAD+NADH) autofluorescence intensity provides information on the stage of adipogenic differentiation (Figure 2).[46] Since TPEF has the ability to provide optical tissue sections at depths up to 500 microns, individual cells can be tracked over a period of time within the 3D environment. TPEF-derived optical redox ratios have been used to track human adipose derived stem cell (hASC) proliferation and adipogenic differentiation in silk protein scaffolds for over six months.[46] This approach has been supplemented by a perfusion bioreactor system to analyze *in vitro* adipose tissue structure and function under flow as well. [61] Third harmonic generation (THG), coherent anti-stokes Raman scattering (CARS), and stimulated Raman scattering (SRS) are additional non-linear optical methods that can be used to characterize lipid droplet dynamics and provide context for TPEF-based metabolic outcomes.[12,20,42,44,53] THG in particular has been paired with redox ratio outcomes to evaluate hASC differentiation during 3D *in vitro* development.[12] Furthermore, the use of fluorescent lifetime imaging (FLIM) can be used in conjunction with TPEF microscopy to help discriminate naturally fluorescent endogenous markers and provide additional context to redox ratio based outcomes.[18,22] A variety of visual markers of differentiation can also be evaluated with microscopy techniques, such as CARS, THG, or phase contrast imaging. For instance cell volume, lipid volume per cell, lipid volume normalized to cell volume, lipid droplet count per cell, and average droplet size per cell can all be quantified.[12] In addition, mitochondria can be quantified and visualized in live cell cultures noninvasively,[47,51] which is particularly relevant for brown adipose tissues which have a greater volume of mitochondria.

Both SRS and CARS can be used to identify droplets via C-H bond vibrations, and also may offer insights into more subtle differences in the chemical composition of droplets [20,37,42,44,53] . One of the benefits of SRS is that it lacks a non-resonant background signal often found in CARS[37,45,53], but there are more technical challenges to implement a SRS system, and consequently it is not available through commercial vendors. The practical implementation of CARS is a little more straightforward, and it has recently become commercially available. THG does not have the molecular specificity of SRS and CARS; since a signal is produced at any interface with distinct optical properties [12,19,54] (e.g. the edge of a lipid droplet). The implementation of THG requires only a single pulsed laser, resulting in a simpler, more cost effective approach however.

Biocompatible, non-toxic labeling of hASCs has been implemented to supplement label-free imaging.[12,24,47] Non-toxic gold nanorods were used to label and image hASCs using

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spectroscopic photoacoustic imaging.[41] In addition, 20 nm gold nanospheres were used to nontoxically tag hASCs and were imaged via ultrasound and photoacoustic methods and tracked *in vitro* for over two weeks.[14] The use of ultrasound in conjunction with photoacoustic imaging has also been used to collect data on the quantitative factors related to hASC growth and development when implanted *in vivo*. [14,41] Ultrasound and spectroscopic photoacoustic imaging were used to examine injured tissue recovery,[41] and to track the progress of stem cell therapies in the subcutaneous adipose tissue.[41]

Additionally, imaging using fast spin echo based magnetic resonance can be used to quantify and distinguish different types of adipose tissues *in vivo*[9] and has been shown to be an effective imaging technique *in vitro*. [3] Other *in vivo* imaging modalities, such as CT-volume data, which is used to evaluate body fat,[31] could be important for assessing tissue engineering constructs that have been implanted *in vivo* for regenerative applications. In the future, the use of *in vivo* techniques to assess *in vitro* cultures will be an important step towards correlating clinical outcomes with *in vitro* tissue models.

While imaging has many advantages, there are some challenges that should be considered. For instance, some scaffolds auto-fluoresce, which can interfere with the collection and isolation of signals from other fluorophores. Silk scaffolds, in particular, can be excited and emit fluorescence due to tyrosines, tryptophan, and cross-links.[24] To address these issues, algorithms and techniques such as linear discriminant analysis can be implemented to differentiate between cells and silk fluorescence.[12,46,61] Phototoxicity can also be a problem of extended imaging, and can be countered by changing media supplements and decreasing exposure times.[17] Finally, the penetration depth of high-resolution optical imaging approaches (TPEF, CARS, SRS, THG) is typically limited to 100-200 microns in lipid rich tissues because of the highly scattering nature of lipid droplets. However, this depth is usually sufficient for studying most 3D *in vitro* adipose tissue models.

Conclusions

As the need for *in vitro* adipose tissue engineering increases, further advances in nondestructive analyses will be required to minimize sample sizes and evaluate the same tissue construct throughout culture time. Current non-destructive methods of evaluating differentiation and functionality, such as optical imaging, are important areas of research that allow multiple time points to be taken on the same tissue sample. This non-destructive approach enables tracking of tissue features relative to baseline measurements, which helps account for variability that may exist among tissue samples. Moving forward, it will be essential to focus on non-invasive methods for analyzing long-term changes in architecture and metabolism in adipose tissue.

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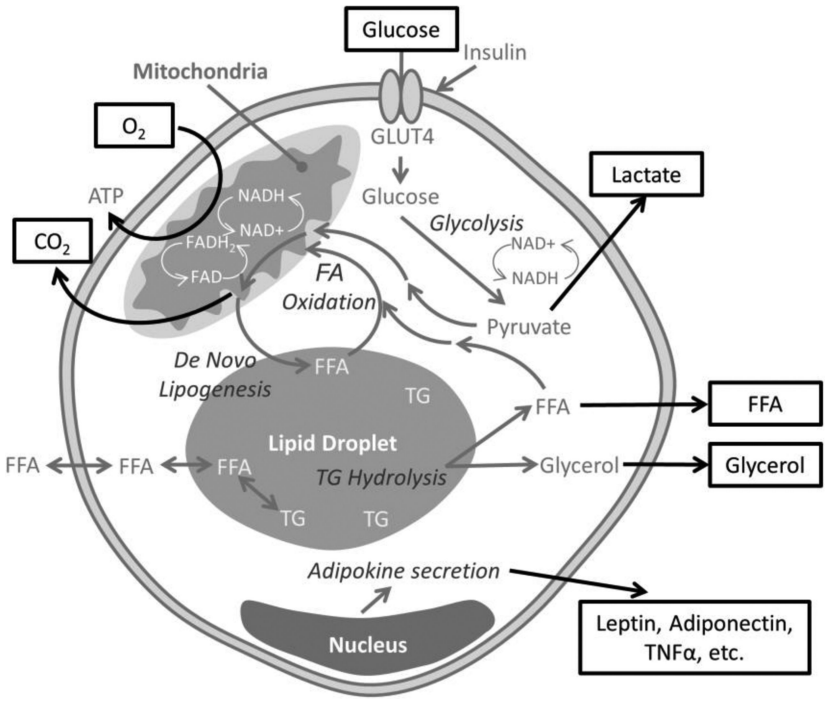

Figure 1.

Schematic representation of an adipocyte's metabolism, where factors that can be evaluated non-invasively are outlined. In response to insulin stimulation, adipocytes translocate glucose transporter type 4 (GLUT4) to the plasma membrane, resulting in glucose uptake. Therefore, extracellular changes in glucose levels can be monitored. Once glucose is taken up in the cell, it is metabolized to the final end products of: lipids, lactate (released from the cell) or carbon dioxide (CO_2, released from the cell). Triglycerides (TG), are stored in lipid droplets (which can be visualized by microscopes) as an energy resource, from two routes: synthesis of fatty acids from non-lipid substrates (de novo lipogenesis) or uptake of free fatty acids. In times of fasting, triglycerides are hydrolyzed (lipolysis) into glycerol and free fatty acids (FFA). Fatty acids (FA) can be secreted, oxidized in the mitochondria resulting in CO_2 as an end-product, or enter the nucleus. Glycerol is secreted. Throughout all of these metabolic cycles, different adipokines are expressed, resulting in secretion of proteins that can be monitored.

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Figure 2.

A sample redox ratio image of differentiating adipocytes at 4 weeks. The differentiating cells have a lower redox ratio compared to the stem cells, which have high redox ratios. On a heat map the blue represents a low redox ratio while red represents a high redox ratio. The scale bar is 50 μm.

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Table 1

Summary of non-destructive *in vitro* assays for studying metabolism in adipose tissues

Metabolic Activity	Technique	Reference
Lipolysis	Colorimetric/Fluorometric quantification of glycerol, FFA	(22, 23)
Fatty Acid Oxidation	Quantification of radio-labelled CO_2 from uptake of [^{14}C] palmitic acid	(24, 25)
Glucose consumption	Quantification of glucose/lactate levels	(26, 27)
Thermogenesis	Measurement of oxygen consumption	(25, 30)
Adipokine secretion	ELISA or protein array of adipokines	(23)

Abbreviations: FFA = free fatty acids, ELISA = enzyme-linked immunosorbent assay

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Table 2

Non-destructive imaging techniques for adipose tissue engineered models

Technique	Label	Measurement	Reference
TPEF, FLIM	None	NADH and FAD (endogenous contrast)	(11, 43, 53)
THG, SRS, CARS	None	Lipid droplet morphology and content (endogenous contrast)	(12, 47-50)
Ultrasound and photoacoustic imaging	Gold Nanomarkers	Location of tagged cells	(54, 55)
MRI	None	Distinguishes fat versus water based tissues due to differences in relaxation times	(56, 57)

Abbreviations: TPEF = Two-Photon Excitation Fluorescence, THG = Third Harmonic Generation, MRI = Magnetic resonance imaging