Imaging approaches in functional assessment of implantable myogenic biomaterials and engineered muscle tissue

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Abstract

The fields of tissue engineering and regenerative medicine utilize implantable biomaterials and engineered tissues to regenerate damaged cells or replace lost tissues. There are distinct challenges in all facets of this research, but functional assessments and monitoring of such complex environments as muscle tissues present the current strategic priority. Many extant methods for addressing these questions result in the destruction or alteration of tissues or cell populations under investigation. Modern advances in non-invasive imaging modalities present opportunities to rethink some of the anachronistic methods, however, their standard employment may not be optimal when considering advancements in myology. New image analysis protocols and/or combinations of established modalities need to be addressed. This review focuses on efficacies and limitations of available imaging modalities to the functional assessment of implantable myogenic biomaterials and engineered muscle tissues.

Key Words: Imaging, Tissue Engineering, Regenerative Medicine, Implantable myogenic biomaterials, Engineered muscles

Tissue engineering and regenerative medicine are rapidly growing fields of research that aim to use a multi-disciplinary approach to restore or replace tissues that have either been damaged or lost through diseases or trauma. Although strategies for restoring function and structure typically vary considerably, most investigations involve the use of cells, biomaterial scaffolds, and various inducible factors, that are either first incubated in bioreactors or directly implanted in vivo. The complex environments that these components and methodologies generate present unique challenges – not only in generating functional engineered tissue, but in monitoring and assessing outcomes both in vitro and in vivo.

Of the many tissue types under investigation in these fields, the creation of new muscle through tissue engineering represents a promising alternative to the replacement of tissue after either severe damage or degeneration from various myopathies.1,4 Surgical reconstruction in patients with significant muscle tissue loss typically utilizes transferred tissue from local and/or distant sites, which often results in significant local denervation, functional loss, and/or volume deficiency.5,6 The engineering of lost connective, nervous, and muscular tissue on a patient-specific basis has been suggested by many to represent the future of muscle surgical reconstruction.7,9 Likewise, tissue engineering approaches have been suggested for treating musculoskeletal myopathies such as spinal muscular atrophy or Duchenne muscular dystrophy (DMD).10,12 However, a common challenge remains in either approach to muscular regeneration – how best to non-invasively assess the viability and function of engineered or extant muscle tissue, both in vitro and in vivo.

Functional Assessment in Myology

As in every focus of tissue engineering, engineered muscle tissues should exhibit biomimetic functional properties and recapitulate native structure – specifically with regards to densely packed and uniformly aligned myofibers throughout the tissue volume.1 As such, methods for assessing cellular viability, proliferation, biological integration, and/or differentiation are crucial to identify optimal strategies. What is herein broadly referred to as the “functional assessment” of engineered muscle tissue and/or implantable biomaterials depends largely on the methodology employed in the particular investigation. In the case where large volumes of muscle, nervous, and connective tissue must be replaced, regeneration may not be possible by the exclusive transplantation of autologous cells – rather, a bioreactor-based approach
may be prescribed, wherein large-volume tissue formation is initiated and controlled prior to implantation. In contrast, in considering general musculoskeletal degeneration due to a particular myopathy, regeneration might be optimally achieved through the injection of myoblasts or relevant multipotent progenitor cells, both with or without biomaterials and/or therapeutic factors, to stimulate the release of soluble signals, the formation of extracellular matrix (ECM), and/or the incorporation of new tissues. In either approach to regeneration, functional assessment of engineered cells or large-volume tissues remains a crucial step in quantitatively and non-destructively characterizing and monitoring the dynamic and complex interactions of the host site and engineered cells and/or large-volume of muscle tissue. Such assessments are crucial in understanding the optimal methods for rational control of muscle tissue structure and function via complex, temporally-dependent interactions between cells, biomolecules, and engineered scaffolds.

**Traditional Functional Assessments and the Expanding Role of Imaging**

There are many standard analysis methods currently available for tissue engineers to be utilized at both the individual cell and whole-tissue levels, but all of them have their innate limitations (Fig. 1). For example, metabolomlc assays offer only a temporal snapshot of tissue physiological function – an assessment that likewise kills the construct in question. MTT assays can be used as a quick and relatively simple assay for cellular metabolic activity (and, indirectly, viability) but is limited by the use of the tetrazolium dye, as only certain cells can participate in its reduction. Other methods such as fluorescent immunohistological staining or tissue biopsy are labor intensive, invasive, and destructive; thus, multiple timepoints and endpoint studies must be performed and temporal correlation inferred. Rendering engineered cells unrecoverable is of particular concern in cell therapy investigations, where cells and implantable

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**Fig 1.** Types of functional assessment metrics for various tissue engineering studies. A) Proliferation of cardiomyocytes measured by immunohistochemical staining of phosphohistone H3. B) Muscle cells viability measured in cells stained with CellTracker™ Green and propidium iodide showing dying myocytes. C) Periphery of implanted engineered muscle showing neonatal rat satellite cells and their myogenic predifferentiation via f-actin filamentous formation and integration with extant, CD31-labeled endothelial cells. D) CT reconstructions of denervated thigh muscle that was electrically stimulated for growth, highlighting a possible modality for measuring myogenesis in engineered muscle tissue. E) Changes in redox ratios observed in adipogenic differentiation with TPEF along with characteristic, non-autofluorescent lipid droplets.
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Biomaterials are introduced to their hosts and tracked over time to assess both viability and cell fate. To alleviate these concerns, several noninvasive imaging techniques have recently been developed to obtain information on both cell metabolism and fate in engineered tissues. These methods exploit a number of intrinsic cellular phenomena, such as autofluorescence and tissue glycolysis. While many of these methods have shown considerable promise, it is important to comprehensively distill their respective benefits and limitations, as there exists a clear need for the development of an assessment platform that operates in real-time, at relevant tissue depths and in vivo.

High resolution imaging methods that can allow for noninvasive, real-time assessment of cellular and tissue-level function within engineered muscle could provide significant aid to the fields of tissue engineering and regenerative medicine. These methods have readily been identified as a strategic priority in other venues of tissue engineering research, and extant applicable imaging modalities have been identified; however, their employment via standard methods may not be optimal when considering advances in myology research. While researchers continue to address many of these concerns, there is yet relatively little literature offering a comprehensive assessment of modern approaches to noninvasive functional imaging of engineered muscle and myogenic biomaterials. This review presents these modalities to guide and promote current discussion on the development and evaluation of optimal methods, in particular for myology applications.

Fig. 2 Standard destructive methods for the functional analysis of engineered tissue
Two-Photon Excited Fluorescence
As previously mentioned, non-invasive imaging techniques that take advantage of the endogenous fluorophores of cells have been in development for decades.\textsuperscript{31-34} http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3842212/ - R8. Many of these modalities have investigated the quantification of cellular and tissue metabolic state through fluorescent excitation of both the primary electron donor and receptor in cellular respiration: nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), respectively. In its reduced form, NADH may be fluorescently excited at 350 nm (emission maximum at 460 nm), whereas its oxidized counterpart, NAD+, is non-fluorescent.\textsuperscript{35} Likewise, FAD has fluorescent excitation and emission maxima of 450 nm and 535 nm, respectively.\textsuperscript{36} During high tissue metabolic demand, the ratio of mitochondrial NADH to NAD+ decreases, resulting in a markedly reduced fluorescent profile which therein allows for the single-channel (350 nm) assessment of respiration within the mitochondrial matrix space.\textsuperscript{37, 38} Alternatively, both an 350 nm and 450 nm excitation may be employed to obtain an approximation of the mitochondrial oxidation-reduction ratio, or the ratio of the fluorescent intensity of FAD to that of NADH. Two-photon excited fluorescence (TPEF) is a promising method for the imaging of mitochondrial redox ratios \textit{in vivo}, since it offers high resolution (approx. 400 nm) at relatively large tissue depths (approx. 1 mm).\textsuperscript{36, 39, 40} Two-photon excitation occurs when a fluorophore interacts with two photons whose energies are equal to half of the excitation energy of that particular fluorophore. These fluorophores can be the same target probed by single-photon fluorescence.\textsuperscript{41} Both NADH and FAD can be readily excited by two-photon excitation at near infrared wavelengths (650-900 nm). Excitation wavelengths from 710-780 nm results in NADH excitation, whereas oxidized flavoprotein in FAD may be excited from 700-900 nm, and these wavelength ranges are both relatively safe, yet highly penetrative, due to reduced scattering and absorption as the excitation volume is inherently confined by its non-linear dependence on incident beam intensity.\textsuperscript{42-44} As such, many recent investigations have employed TPEF of NADH and FAD to monitor metabolism of many tissue types, including cardiac, corneal, pancreatic, and brain.\textsuperscript{42, 44-52} TPEF is a promising modality for the functional assessment of glycolysis and oxidative phosphorylation in engineered tissues due primarily to its non-destructive use of infrared light, as opposed to UV excitation otherwise inherent to single-photon excited fluorescence. However, despite TPEF being a relatively deeply-penetrating optical method, its maximum penetration depth of 1 mm is its greatest drawback when considering the assessment of deeper tissues.

Photoacoustic Microscopy
Photoacoustic Microscopy (PAM) is a hybrid modality combining principles of ultrasound detection and what is known as the photoacoustic effect. In PAM, pulses of near-infrared light are absorbed rapidly by a tissue and quickly converted to heat which leads to a transient thermoelastic expansion. This expansion propagates as an ultrasonic wave and is eventually detected by an ultrasound transducer.\textsuperscript{53,54} Structural and functional information of various tissue morphologies can be readily determined, as photoacoustic absorption varies strongly with oxyhemoglobin content. As such, many recent studies have successfully resolved both the structure and oxygenation levels of complex tissues and vasculatures down to the capillary level.\textsuperscript{55,56} PAM has been suggested by many for its utility in tissue engineering applications. Due to its inherent ability to detect functional vasculature, PAM has already been used to resolve angiogenesis in tumors: a crucial metric in assessing the biological integration of engineered tissue.\textsuperscript{57-59} Likewise, neovascularization and cellular distribution within implantable biomaterials, such as porous polymer foams,\textsuperscript{56,60} mesenchymal stem cell seeded hydrogels,\textsuperscript{61} and cell-free hydrogels containing the growth factor FGF-2,\textsuperscript{62} have been monitored using PAM. Introducing contrast agents such as gold

| Pathophysiology Target | Radiopharmaceutical |
|------------------------|---------------------|
| Metabolism             | \(^{18}\text{F}-\text{fluorodeoxyglucose (}^{18}\text{F-FDG)}\) |
| Cell Proliferation     | 9-(4-fluoro-\(^{18}\text{F}-3\)-hydroxyethyl)guanine (\(^{18}\text{F-FHB G)}\) |
particles to engineered tissues, biomaterials, or injected cells has likewise proven successful for researchers investigating blood oxygen saturation levels and stem cell trafficking. In general, PAM offers a number of benefits over other functional imaging methods, but its maximum depth of penetration (approx. 3 mm) and its dependency on local laser fluence might make longitudinal studies of deeper tissues prohibitively difficult to perform.

**Fluorescence-Lifetime Imaging Microscopy**

As previously mentioned, improvements in the field of multiphoton microscopy present a great opportunity for both high-resolution and long-term imaging of engineered tissues and biomaterials. Due to both its intrinsic 3D resolution and high depth of penetration, multiphoton microscopy allows for the investigation of a local environment in femtoliter volumes located deep within tissues. Investigations utilizing multiphoton microscopy take advantage of the autofluorescent nature of endogenous fluorophores, such as collagen, elastin, porphyrin, flavin, hemoglobin, serotonin, and as previously discussed, NADH and FAD. However, using emission wavelengths to discriminate between multiple fluorescent species within the same focal volume is majorly limited by their emission spectra overlap. Fluorescence-Lifetime Imaging Microscopy (FLIM) is a promising imaging modality that has elicited many successes in monitoring the differentiation of stem cells. Additionally, FLIM has been shown to have utility in separating NADH and NADPH emission spectra, aiding greatly in the study of the photochemistry of living tissues. While FLIM has shown considerable flexibility and utility in a number of studies in the field of tissue engineering, there is still much discussion regarding the optimum FLIM analysis methodology.

One such method, known as Phasor analysis, has proven its utility as a fit-free and label-free approach to probing cellular environments and identifying biologically relevant details regarding cell fate. Rather than fitting multiple lifetimes of one or multiple species, Phasor analysis represents each lifetime as a vector defined by its modulation and phase—thereby removing the need for a priori knowledge of each species' specific lifetimes. This likewise means that relative concentrations of each species can readily be determined by simply ascertaining the ratio of their phasor representations. This method has recently been employed in characterizing the different metabolic and differentiation states of both C. Elegans and human endothelial stem cells. (see in particular at)

http://www.nature.com/srep/2013/131205/srep03432/full/srep03432.html

However, for species that are close to each other on the phasor diagram, phenomena such as photobleaching and quenching could account for significant error in assessing relative species' concentrations. Nonetheless, as a non-invasive, non-destructive, and label-free imaging modality that can identify cellular differentiation and metabolic states, the phasor approach to FLIM is a promising imaging modality for future muscle tissue engineering applications.

**Optical Coherence Tomography**

Another potential strategy for the real-time, non-invasive, and label-free assessment of the structure and function of engineered tissue is optical coherence tomography (OCT). OCT is an interferometry technique that uses broadband near-infrared light at wavelengths greater than 800 nm, allowing for deeper tissue penetration (approx. 2000 mm) than previously-mentioned near-infrared imaging modalities. OCT has been utilized extensively to monitor cell location, migration, differentiation, and interaction with local biomaterials. Thanks to its near-cellular level resolution, OCT has been employed in investigations that would have otherwise been prohibitively challenging with other imaging modalities, such as the study of structural changes in the retina, the 3D characterization of polyacrylate and chitosan scaffolds, and the kinetics of cell integration into collagen, agarose, and other hydrogels. In addition, OCT has also been utilized to monitor the optical attenuation of cells that were cultured within agarose gels. Other studies have utilized OCT to monitor macroscopic tissue regeneration via such processes as calcification in gelatin scaffolds and the growth of skin models. In many recent studies, OCT has been combined with other imaging or processing techniques to image cells and assess their deposition onto engineered tissue scaffolds in vitro. Additionally, the combination of OCT and Doppler velocimetry has revealed a technique that can image micron-level fluid flow, allowing for noninvasive and real-time imaging of engineered tissue rheological and elastic properties. Another type of OCT, known as Time Domain OCT, has likewise been utilized for the noninvasive imaging of the porosity of polymer foam scaffolds that have been seeded with aggregates of cells. In this investigation, cell proliferation and extracellular matrix deposition could be monitored as a function of the allowable imaging depth and the degree to which porosity could be detected over time. In another type of OCT, whole-field OCT was employed, along with the use of magnetic beads as a contrast agent, to observe changes in cellular morphology within engineered bone tissue in vitro. Likewise, OCT has been shown...
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Fig 3. The potential function of a portable simultaneous PET/MR scanner. A) Theoretical design abstraction of a portable simultaneous PET/MR scanner. B) Example output of a small-animal PET/MR scan. C) Monte Carlo simulation comparing the positron pathlengths in traditional PET (blue) and simultaneous PET/MR (red), illustrating a greatly-increased resolution in PET/MR.

Simultaneous imaging with PET and MRI combined modalities may become a powerful monitoring technique for the functional assessment of engineered muscle tissue – especially in regards to the characterization of engineered muscle glycolysis versus fatty acid metabolism. However, to accurately discern its utility in this regard, further research on the miniaturization of these scanners for use on engineered tissue constructs will be necessary.

Positron Emission Tomography
Position Emission Tomography (PET) is an extremely valuable clinical diagnostic imaging modality that aims to assess physiological function in situ in the human body in a non-invasive manner. PET relies on the emission of a positron from a radioactive pharmaceutical, or radiopharmaceutical, which then decays into two coincident photons that can be collected and traced by imaging equipment. This technique has been especially useful in the diagnosis of many types of cancer, and has likewise shown efficacy in diagnosing hypothyroidism and detecting localized inflammation through mapping the distributions of leukocyte. While many radiopharmaceuticals may be used as Fluorine-¹⁸ positron emitters (Table 1), the most common by far is F-fluorodeoxyglucose (FDG) which, as a functional glucose analogue, may be used as a direct metric for cellular glycolysis – even from extremely low cell concentrations.

PET imaging in accordance with the use of FDG could provide considerably more information when measuring the metabolic capacity of engineered tissue constructs. However, even the smallest commercially-available PET scanners, typically used in nuclear medicine departments in veterinary facilities, are excessively bulky to directly be repurposed for small tissue construct imaging. Additionally, average system resolutions for these scanners are typically on the order of 4.5-5.0mm and would thereby be ineffectual for resolving important details in tissue engineered constructs any smaller than this size. Design proposals therefore must include some method of controlling the resolution loss attributed to positron blur – the governing source blur in PET imaging.

To ameliorate the reduction of resolution from positron blur, simultaneous imaging with PET and magnetic resonance imaging (MRI) have been proposed and have recently been utilized for small animal studies. The presence of a unidirectional magnetic field was
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originally theorized in combination with PET imaging,\textsuperscript{96, 97} but its effects on image resolution have only recently been characterized (Fig. 3).\textsuperscript{13} However, recent advances in the electronics involved with such a system have made it possible to fabricate a working device. In order to construct the proposed device, one would have to take into account issues with function due to the interferences between the PET imaging mechanism and the magnetic field. For instance, PET detectors are typically comprised of scintillation crystals coupled with photomultiplier tubes. The sensitivity of these tubes, along with other electrical and radiofrequency components, towards an magnetic field would be a major constraint, but recently, silicon photomultipliers have been considered for use in combination with magnetic fields present from MRI imaging.\textsuperscript{96-100} These photomultipliers have been shown to be able to resolve most of these issues, as silicon based avalanche photodiodes utilize very short optical fiber bundles, which are not affected by the presence of external electromagnetic field fluxes up to 4.0T.

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Bioluminescent Imaging

Bioluminescent Imaging (BLI) is an imaging technique that relies on emitted light from the enzymatic generation of luminescence in living organisms. BLI typically requires the introduction of the luciferase gene from \textit{Photinus pyralis} into a receptive cell line or tissue. The luciferase and luciferin reaction results in the emission of photons from 500-620 nm (blue-green to yellow-orange) wavelengths, with peak emission at 612 nm at 37°C.\textsuperscript{101,102} However, luciferase from the \textit{Renilla} wildtype emits light from 460-490 nm.\textsuperscript{103,104}

Additionally, small genetic changes in these luciferase reporters’ encoding DNA can be made to adjust their emission wavelengths without significant changes to the biochemical reaction – thereby allowing for the potential to utilize multiple luciferase reporters in the same engineered tissue to simultaneously investigate multiple biological processes.\textsuperscript{45} Aside from transfection, luciferase may be introduced to tissue via simple diffusion – a technique employed by many investigations to characterize perfusion in vascularized tissue constructs or live animal models.\textsuperscript{101,105}

Many studies utilize BLI to track cells following implantation, providing detailed information on cell viability, migration, differentiation, and integration with surrounding scaffolds and/or tissues.\textsuperscript{58,106,107}

Additionally, stem cell viability, migration, and proliferation have been monitored for months using BLI on various engineered polymer scaffolds in both bioreactor and non-bioreactor culture conditions.\textsuperscript{108-110}

The potential for lengthy investigations has given rise to many current projects aiming to monitor temporal changes in not only cytotoxicity, but also cell gene expression, the progression of infection, and the growth and angiogenesis of tumors.\textsuperscript{111} Investigations on the formation of bone in mice in vivo have likewise relied on luciferase transfection to gauge the degree to which bone formation from extant bone and introduced stem cell sources occurred.\textsuperscript{88,106,112-114} BLI has recently become a modality of great interest from within the tissue engineering community – primarily for its noninvasive, non-destructive, and longitudinal imaging of cells and engineered tissue, both \textit{in vivo} and \textit{in vitro}. However, transfection of a luciferase reporter gene is invasive and potentially destructive to target cells and tissues, and spatial information is limited to two dimensions. Current BLI research calls for the development of optimum luciferase transfection or introduction strategies, along with an imaging system that allows for simultaneous 3D spatial acquisition.

Micro X-ray Computed Tomography

Micro X-ray Computer Tomography ($\mu$CT) is another promising imaging modality for the functional assessment of engineered tissue, as it allows for the non-destructive quantification of many aspects of engineered scaffolds, such as porosity, pore size, and interconnectivity.\textsuperscript{115-122} Inferring cellular integration and interaction with its engineered scaffold is made possible in $\mu$CT, as images can be acquired sequentially over time with minimal negative affects to cells from X-ray dose.\textsuperscript{123,124} However, in order to achieve sufficient X-ray absorption (contrast), soft tissues and biomaterials oftentimes need to be freeze-dried or kept under dry conditions instead of standard culture conditions – a notion which can significantly affect certain cell or tissue types. Due to this X-ray absorption issue, $\mu$CT is most commonly associated with the characterization of engineered bone tissue, as the comparatively high X-ray absorption of calcium eliminates the need for freeze-drying in mineralized tissues and hydrogels (Fig. 4).\textsuperscript{125,126} In these types of studies, $\mu$CT can allow the for noninvasive, non-destructive, and label-free assessment of mineralization as a function of time with minimal affect to the tissue or engineered construct being imaged.\textsuperscript{127,128}

In studies involving on-mineralized tissue, many techniques have otherwise been employed to minimize the aforementioned negative affects of conventional $\mu$CT use. For example, contrast agents such as heavy metal, barium sulfate, or Microfil may be used to image anything from 3D scaffold architectures to blood vessel or neural tissue integration and neovascularization within engineered tissues.\textsuperscript{129-134} High resolution $\mu$CT has also been utilized \textit{in vivo} to
identify stem cells that were labeled with magnetic nanoparticles following an intra-arterial transplant. However, the use of contrast agents, dyes, or radioactive labels always results in the destruction of engineered tissue, so functional assessment of engineered constructs or post-transplant engineered tissues is limited to longitudinal studies assessing multiple timepoints from individual subjects or constructs.

Conclusions
The impact that the aforementioned imaging modalities have on muscle tissue engineering is constantly growing. The impressive advances of electron microscopy 3D reconstruction of molecular and supramolecular fine details of muscle fibers are well exemplified in the first issue of the EJTM Special mini-series “News on Muscle imaging”. Further, there is a pressing need for the noninvasive, non-destructive, real-time methods for the functional assessment of cells and tissues. As it currently stands, imaging modalities such as PAM, FLIM, and BLI seem optimally suited for in vitro application due to their depth of resolution limitations, whereas μCT, OCT, TPEF, and PET are making great strides towards being relevant in large-muscle construct or in vivo work. However, promising new analysis methods and/or combinations of these modalities offer exciting glimpses at their future applicability to a wide range of myology studies.

In order to translate the basic experimental results of muscle tissue engineering and regenerative myology into the clinical setting, it is critical that researchers identify and exploit the strengths of each of these imaging modalities. Further optimization of these systems will be pivotal in discerning their respective utility in serving the complex and challenging field of tissue engineering. To progress and replace extant methods for functional tissue assessment, the modern imaging systems will need to be highly accurate, reliable, inexpensive, fast and automated. Ideally, these systems must be flexible in applications with traditional in vitro protocols, emerging bioreactor systems and current in vivo studies.

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Fig 4. μCT image and its corresponding 3D model of an implantable biomaterial scaffold. A) μCT image of an engineered bone construct with B), its corresponding 3D model.
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