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Non-invasive continuous imaging approaches to evaluate tissue structure and development

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Abstract. As the field of tissue engineering advances toward fully functional organoids and artificially generated tissues, methods to evaluate the condition of the cultured tissue from the beginning to the end continuously grow more importance. The structure and developmental status of tissues in vivo reveal considerably more information than current approaches to discrete analysis like histology. Optical imaging modalities present a promising future regarding its ability to unveil morphological and biochemical changes of the tissue in a continuous manner without compromising its viability. Therefore, in this paper, several popular and emerging methods include Fluorescence Lifetime Imaging, Hyperspectral Imaging, Optical Coherence Microscopy, Light Sheet Fluorescence Microscopy, Near-infrared II Fluorescence Imaging, Mueller-matrix imaging are discussed with regards to their advantages and limitations in tissue engineering research. Although these approaches allow long term monitoring of developing tissue in vivo, they are not suitable in all conditions and lack spatial resolution and depth penetration. The potential of optical imaging on tissue structure and interactions can be further realized by improving upon fixed sample imaging techniques and utilizing multimodal systems.

Keywords: In-vivo imaging, fluorescence microscopy, optical microscopy, continuous monitoring, tissue engineering.

1. Introduction

In any research on tissue engineering, understanding how tissue structure is formed in the culturing process or how the morphology of cells changes during pathogenesis is vital. The mid-phase structure and morphology of tissue can indicate crucial information regarding the disease state in pathology or the effect of the microenvironment on tissue maturation. Currently, tissue structures are analyzed at discrete time points by histology that destroys the tissue in the end [1]. This severely limits the amount of information extracted from the tissue and is often expensive to use. In addition, histology requires extensive fixing and slicing of the sample, which demands adept understanding to the sample tissue in order to extract useful information. Any mistakes during one of these steps will waste the otherwise viable tissue. The current direction of tissue engineering has shifted from the traditional two-dimensional culturing to three-dimensional paradigm where tissues can be better influenced by factors like gravity and microcirculation that existed in physiological conditions [2]. Thus, to better observe how tissue develops or changes in depth continuously, not just by limiting the tissue surface in various conditions, techniques in optical imaging are utilized. Among the present methods that are capable of continuous,

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in-depth imaging, there are two schools of approach: fluorescence-based imaging and pure optical imaging. This review will introduce techniques that entail these two approaches about their principles and respective advantages and limitations.

2. Fluorescence imaging

2.1. Fluorescence lifetime microscopy

The basic principle of fluorescence imaging follows: a fluorescence molecule is in its ground state at first; when a certain wavelength of light excites the molecule to its excited state, light of a longer wavelength will be emitted as the electron falls back to its ground state. As stated in the name of this imaging modality, Fluorescence Lifetime Microscopy (FILm) focuses on the lifespan of a fluorescence molecule. Differ from regular fluorescence imaging, where the intensity of the fluorescence is the target parameter to measure. FILm instead measures the period of time when the fluorescence molecule is able to emit light for a single excitation [3]. FILm also detects endogenous autofluorescence, which is different compared to traditional fluorescence microscopy, where artificial fluorescence proteins are conjugated with the target molecular for labeling. It is able to sense the change in the cellular environment by the duration of fluorescence.

FILm demonstrates its unique abilities for in-vivo, long-term evaluation in the context of tissue engineering. Since it detects the illumination of endogenous fluorophores, there is no need for genetic editing of cells. In the context of making a tissue-engineering based product, evaluation of its biochemical and mechanical properties must be done without introducing external factors. As FILm is able to target a versatile range of fluorophores like NAD, collagen, elastin, and amino acid tyrosine, it is suitable for detecting structural abnormalities and biochemical phenomenon [3]. In the study conducted by Haudenschild et al., FILm is used to detect changes of collagen crosslinking and proteoglycan concentration in engineered cartilage construct during a four week period of time [4]. The later histology and mechanical testing of cartilage reflects strong correlations between FILm findings and actual structural properties [5]. FILm is also applied by Karrobi et al. to trace the metabolic activities of cancer cell in a 3D spheroid setting [6]. It allows the generation of a long-term profile of cellular activity and microenvironment, which would otherwise be impossible to keep track of in a 3D model by regular fluorescence microscopy.

2.2. Light sheet fluorescence microscopy

Light sheet fluorescence microscopy (LSFM) is one type of conventional fluorescence microscopy. It excites fluorophores in target cells and collects the light emitted from them. LSFM has a unique advantage compared to other fluorescence microscopy that its illumination lens and detection lens are separate and orthogonal to each other. This allows for less exposure to the specimen, specific focal plane illumination, and faster detection speeds [6]. These traits of LSFM make it a great tool for imaging in depth, rapid activities like neuron activation and cardiac events.

Although to achieve the best spatial resolution, tissue clearing and sample fixing are still largely used in LSFM applications, in vivo imaging of organs is possible. Weber et al. used LSFM to investigate the developmental process of zebra fish's heart [7]. Weber's team trapped the zebra fish's embryo in agarose gel in a cylindrical container. Segmented images of zebra fish's heart in vivo are captured and later converted into a 3D model. However, the spatial resolution of the beating heart is poor because of its movement in all axes. Several solutions are proposed to increase the image's resolution: slowing down the heartbeat, referring the image to a static model of the heart, or synchronizing the image with a specific phase of the cardiac cycle. Yang et al. improved the imaging speed of LSFM by using a dual illumination and dual slit gating methods to increase the frame rate twice and filter the excessive fluorescence outside of the focal plane [8]. The high throughput capacity of LSFM also makes it an excellent candidate for live organoid imaging. By incorporating a novel culturing well (JeWell) design, Beghin et al. the imaging system can record 300 organoid per hour [9]. The heterogeneity nature of the

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organoid system is well captured by multi-channel fluorescence detection and content in the image can be used to train neural networks for automated pipeline of cell proliferation tracking.

2.3. Infrared fluorescence microscopy (NIR II fluorescence microscopy)

Another type of fluorescence Microscopy is to utilize fluorophores that emit light beyond the visible spectrum. Infrared Fluorescence microscopy has advanced from detecting what is called the first biological window (650 nm-950 nm) to the second biological window (1000 nm-1700 nm), where the optical probe is able to achieve better tissue penetration and less absorption [10]. Compared to visible spectrum fluorescence probe, NIR II fluorescence microscopy is able to image structure centimeters beneath tissue surface [11]. However, infrared probes, typically used in large animal models, are concerned with their biotoxicity and thermal effects. The current research focus is on brightness, retention time, and stability of NIR II probes by manipulating its energy transfer method, molecular composition, and size [12-14]. Multiple studies have demonstrated the potential of NIR II in detecting macroscopic organ changes in rodent models. By introducing the fluorescence dye in mice's system, Liu et al. successfully visualized hydrodynamics in mice's curable system after stroke and cancer metastasis from lymphnodes [13]. NIR II probes can also be expressed by mice via genetic editing as shown by Chen et al. [14]. Without external injection, they are able to monitor liver regeneration and pancreas pathogenesis.

3. Optical microscopy

3.1. Optical coherence tomography microscopy

Fluorescence is not the only option for deep tissue imaging. Imaging modalities that only employ illumination light is also a viable approach. Optical Coherence Tomography(OCT) Microscopy is one of them. The working principle behind OCT is analogous to ultrasound. The scattered light bounced off from the tissue is collected and compared to the reference light split from the original illumination light [15]. The low temporal coherence light source allows the image signal only from the target depth to form constructive interference, thus filtering the unwanted noise. Light on the infrared spectrum is used for maximum depth penetration [16].

Without any fusion of fluorescence marker, Yamanaka et al. demonstrate section image of a pig's thyroid gland up to 1.8mm depth using OCT microscopy [17]. This study is able to achieve 3.4 and 3.8 μm in regards to the axial and lateral resolution. In 2011, combining a board light bandwidth(800±150 nm laser-generated supercontinuum) and a common reference arm, using SD-OCT microscopy, Liu et al. were able to refine the resolution of OCT to 1μm [18]. By analyzing reflection intensity, the team is able to identify interactions between endothelial cells, immune cells, smooth muscle cells in cardiovascular disease models. OCT microscopy is also applied extensively to measure the extent of scarring in the center of corneal tissues [19, 20]. One of the insufficiencies of OCT image is that it is monochromatic like ultrasound. Therefore, differentiation between the image content is based on intensity, size, and shape. Holmes et al. invented a solution to separate the moving cells from its immobile scaffold structure through the optical signals' fluctuation from cell motions [21].

3.2. Mueller-matrix Imaging

Another distinct physical property that light possesses is its polarization. Mueller-matrix Imaging is capable of detecting the change of polarization of light and differentiate different structures [22]. Materials that is anisotropic, which means can alter light polarization differently depending on incoming light's direction and polarization, can be mapped spatially by Mueller Matrix imaging. Various biological components are anisotropic in this respect: actin, tubulin, cytoskeleton, microfilaments, microtubules, elongated macromolecules, proteins, biopolymers, nerve fibers, and collagen fibrils [23].

A typical design of MM microscope put the sample between the source light and detection lens. In this case, the polarized light detected travels through the entire sample. Since biological samples are heterogeneous, how polarization of light changes is not due to a single factor. One of the most used

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methods to interpret data from Mueller Matrix image is the Lu-Chipman decomposition methods [24]. The effects of various entities can be deconstructed and compared to the optical properties of known tissue [25]. Using MM microscopy, full developmental stage of zebra fish can be recorded without introducing multiple labels for different organs [26]. A similar approach is also applied to evaluation of stem cell therapy. The wood group reported visualization of collagen and cellular reorganization from stem cell therapy in cardiac infraction sites [26]. This method can also be applied to samples in vivo: Jütte et al. is able to characterize the healing process of skin wounds by homogeneity of the image revealed by MM microscopy [27]. However, the penetration depth of MM microscopy is less than ideal. Although images can be captured from 1mm thick specimen, it is not used to created 3d reconstruction of the sample tissue.

3.3. Multi-modal imaging and expectations

Currently, various modalities of imaging techniques are being developed for the in-depth examination of tissues. However, no one tool is capable of investigating macroscopic, microscopic, structural, or chemical components in the context of tissue engineering at the same time. Therefore, it is necessary to incorporate multiple imaging modalities to capture the full picture. In the Haudenschild study where cartilage construct is evaluated [4], the structural and mechanical properiy of the construct is measured and connected through four methods: FILm, ultrasound backscatter microscopy (UBM), histology, and mechanical testing. FILm is able to differentiate the maturation of chondrocytes, and this finding is confirmed via the void detected by UBM. Destructive methods like histology and mechanical testing then can correlates the data. The end goal of all the non-contact, continuous, deep tissue monitoring during tissue engineering process is that observation and subsequent modification can be made without the demise of the target tissue. To evaluate a tissue construct or a organoid model, two aspects are of the utmost importance: morphology and cellular interaction. The basic structural information can be revealed by Meuller Matrix Microscopy, OCT microscopy, and NIR II microscopy. Each capable to provide deeper penetration than the previous one while losing image resolution. Cellular interactions can then be recorded either by FILm or LSFM. By combining the information registered above, the entire 4D biomechanical profile of the tissue can be established which may give rise to better understanding of the development and pathogenesis of tissue.

4. Conclusion

This review has summarized some of the most prevalent image modalities that are used for continuous, deep-tissue monitoring. Two categories of modalities are suitable for such kinds of purposes: fluorescence microscopy and optical microscopy which rely on temporal resolution or polarization of light. FILm utilizes the sensitivity of fluorophores reflected in its fluorescence lifetime to microenvironments for detection of subtle changes; LSFM has a wide view which allows it to image and model the movement of an entire organ; NIR-II microscopy can achieve the best tissue penetration and reflects the macroscopic changes otherwise hide underneath the skin in animal models; OCT microscopy is suitable for 3D reconstruction of bulky tissues and recorded their morphology; and Mueller-matrix microscopy provide excellent differentiation detection at the boundary of heterogeneous tissue type while also require no external modification for prolong term of monitoring.

Although many of the above modalities can generate detailed information regarding the state of the tissue, none of them encompasses all aspects. As the field of tissue engineering advances from fundamental research to practical tissue construct product development, multimodal, non-contact, continuous, deep tissue imaging, accompany with confirmation form destructive methods are necessary to establish 4D model of the respective tissue. In this case, less samples will be wasted for examination. It is also easier to directly observe the tissue than dissecting the tissue for staining, which requires more understanding of its condition. To achieve this level of modeling, the current methods still lack tissue penetration depth and biotoxicity concerns from external fluorescence markers. More imaging modalities that can produce better spatial temporal resolution but requires sample fixing and tissue clearing should be modified towards in vivo, continuous approaches.

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