International Journal on Oral Health

A Short Review on PEGASOS Tissue Clearing Method and Deep Imaging of Oral and Craniofacial Tissues

Review Article

Volume 4 Issue 1- 2024

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Article History

Received: January 18, 2024 Accepted: January 22, 2024 Published: January 22, 2024

Abstract

Background: The advent of confocal microscopy has revolutionized our ability to visualize entire tissues and organ structures in detail. Despite these advancements, the inherent opacity of tissue samples limits the imaging depth of confocal microscopy to approximately 100 micrometers. To circumvent this limitation, tissue clearing techniques have been developed. These methods employ both physical and chemical treatments to render tissues transparent, thereby reducing light absorption and scattering during image acquisition. When paired with three-dimensional imaging technology, tissue clearing enables the comprehensive visualization of whole tissue structures. Among the most recent advancements in this field is polyethylene glycol (PEG) associated solvent system (PEGASOS), a novel tissue clearing method that shows promise due to its effective clearing properties for both hard and soft tissues.

Objectives: A short literature review was undertaken to summarize current application of PEGASOS tissue clearing method in dental and craniofacial research.

Conclusions: This literature review provides information on the concepts and current application of PEGASOS tissue clearing method and 3-dimentional imaging system, demonstrating that PEGASOS is a superior technique within dental and craniofacial research domains which has the potential to provide 3-dimensional information on both hard and soft tissues of the craniofacial development and homeostasis, however, more research using PEGASOS on the craniofacial tissue regeneration and interaction between biomaterials with tissues are encouraged in the future.

Introduction

The development of craniofacial organs encompasses a highly intricate interplay among bone, cartilage, and soft tissues [1]. This system is further complicated by an elaborate network of blood vessels and neural components. Traditional two-dimensional imaging modalities fall short in capturing the dynamic interactions and spatial configuration of these elements, which are critical for advancing the understanding of craniofacial development and tissue regeneration. The three-dimensional organization of the craniofacial complex is paramount in studies focused on morphogenesis, functional integration, and therapeutic innovation. Therefore, there is a pressing need for imaging technologies that can provide comprehensive insights into the volumetric and relational intricacies of the craniofacial architecture.

In recent years, the evolution of tissue clearing techniques has markedly advanced the field of neuroscience, granting researchers the ability to obtain authentic three-dimensional images of entire organs, such as the mouse brain and heart [2,3]. Initially pioneered in neuroscience for deep imaging, tissue clearing has since expanded its utility to other domains. During the past decade, the introduction of this technique revolutionized the visualization capabilities of researchers, enabling them to explore the complex architecture of various organs in unprecedented detail. Optical tissue clearing methods, which encompass

both solvent-based and hydrophilic reagent-based approaches [4], when integrated with sophisticated labeling techniques and advanced microscopy, facilitate the imaging of three-dimensional microstructures within tissue blocks or whole organs. This includes but is not limited to the brain and spinal cord, allowing for high-resolution observations that were previously unattainable. These methodologies, continually refined and adapted, hold immense promise for elucidating the intricate biological processes underlying craniofacial development and pathology [5].

The PEGASOS tissue clearing method, which is the abbreviation for polyethylene glycol (PEG) associated solvent system, represents a multi-step process designed to render tissue samples transparent [6]. This method involves a sequence of treatments including fixation, decalcification (for hard tissues), decolorization, delipidation, dehydration, and clearing. It is noteworthy that PEGASOS effectively clears nearly all tissue types except for pigmented epithelium. It renders hard tissues such as bone and teeth virtually invisible, thereby providing an unprecedented view of their structure. A significant advantage of the PEGASOS method is the protective properties of the polyethylene glycol in the clearing medium, which preserves endogenous fluorescence for extended periods. This has enabled groundbreaking imaging of intact mouse heads, inclusive of composite structures such as bones, teeth, brain, and muscles. Despite these advancements, the application of tissue-clearing techniques in dental research is still in its infancy. Further exploration and refinement of this method could lead to significant insights into dental biology and pathophysiology.

This article includes a concise literature review focused on the application of the PEGASOS tissue clearing method within the realms of dental and craniofacial research. The review aims to encapsulate the breadth of current methodologies, highlight the innovative applications of PEGASOS, and discuss the methodological nuances that have been reported to influence the outcomes of such studies. By examining the impact of PEGASOS in this specialized field, we seek to synthesize existing knowledge, identify trends in the application of this technique, and provide a critical assessment of its efficacy and utility in advancing the understanding of dental and craniofacial biology.

PEGASOS Tissue Clearing Method Workflow

The PEGASOS (Polyethylene Glycol Associated Solvent System) tissue clearing method encompasses a series of meticulously orchestrated steps to achieve transparency in tissue samples. These steps are fixation, decalcification (specifically for hard tissues), decolorization, delipidation, dehydration, and clearing, as illustrated in Figure 1.

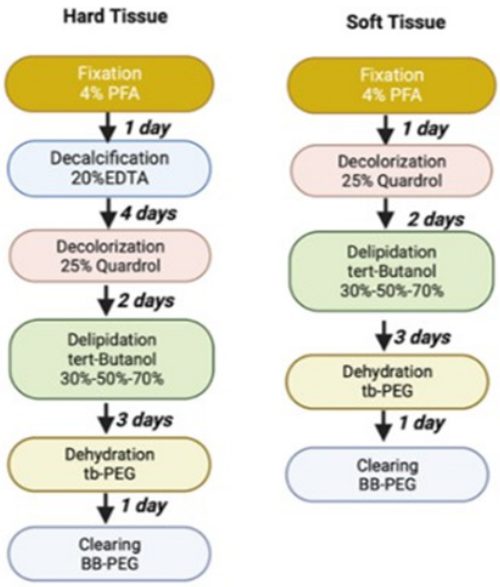

Figure 1: Diagram of PEGASOS tissue clearing method in both hard and soft tissue.

The procedure initiates with 4% paraformaldehyde (PFA) fixation to preserve the tissue structure. Subsequently, a 20% ethylenediaminetetraacetic acid (EDTA) solution is employed for the decalcification of hard tissues. For the crucial step of decolorization, a 25% solution of N,N,N',N'-Tetrakis (2-Hydroxypropyl)ethylenediamine (Quadrol) in water is utilized. Then a combination of 25% Quadrol followed by a 5% ammonium solution, applied sequentially, to achieve optimal decolorization of the samples. Tert-butanol (tB) supplemented with 3% Quadrol for delipidation. tB-PEG reagent for dehydration, which is composed of 75% tB + 22% poly (ethylene glycol) methacrylate (PEGMMA) + 3% Quadrol. For final tissue clearing, the BB-PEG medium, which is composed of 75% benzyl benzoate (BB), 22% PEGMMA, and 3% Quadrol was used [6-8].

Application PEGASOS Tissue Clearing in Postnatal Craniofacial Development

The PEGASOS tissue clearing method, combined with three-dimensional (3D) imaging, has made significant strides in the study of postnatal craniofacial development. Luo et al. have effectively utilized this technique to reveal the intricate spatial relationship between osteogenesis and angiogenesis during the development of craniofacial bone in mice [9]. Through the application of the PEGASOS method, a vivid 3D visualization of these concurrent processes was achieved. Furthermore, the study capitalized on the use of endogenous fluorescence markers like Tdtomato and GFP in transgenic animal models, alongside calcein green labeling, to assess osteogenic activity. Given the relatively thin structure of mouse skulls, the tissue clearing process was expedited to just one week. In instances of calcein-labeled samples, the elimination of the decalcification step further reduced the processing time to a mere 2-3 days-a significant improvement over traditional hard tissue histology techniques.

The research also incorporated the use of mice with Vascular endothelial cadherin (Cdh5) labeling, enabling the observation of the angiogenesis and osteogenesis fronts at cranial sutures. The co-local-

ization of Gli1+ stem cells and CDH5-labeled vasculature, (Figure 2), underscores a specific distribution pattern of Gli1+ cells within the sutures, nestled among blood vessels. Additionally, the heightened activity of calcein staining proximal to sutures delineated these regions as zones of active bone formation.

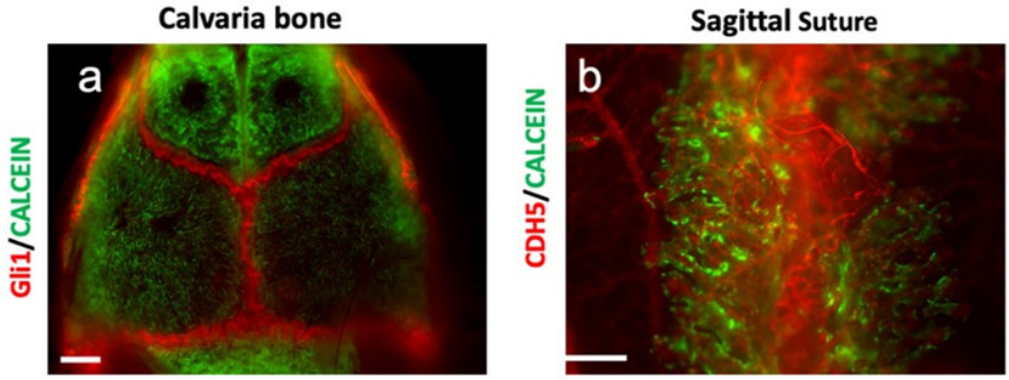

Figure 2: Gli1+ Mesenchymal stem cells and blood vessels in a PEGASOS Tissue cleared calvaria bone. (a) a whole mouse skull analyzed with Zeiss Stereo Microscope at 5X magnification, Gli1+ cells (red signal) indicating the distribution of suture stem cells and calcein green showing the activity of bone mineralization (green signal). (b)Vasculature in sagittal suture of a CDH5-TDT mouse analyzed with Zeiss Stereo Microscope at 11.7X magnification, blood vessels (red signal) and calcein green (green signal), showing the interaction between angiogenesis and osteogenesis. Scale bars in panels a, 1mm; b scale bar, 100 μm.

Application PEGASOS Tissue Clearing in Mouse Teeth

Leveraging transgenic mouse models, such as Tie2-Cre;Ai14 and Cdh5-CreERT2;Ai14, the PEGASOS tissue clearing method has facilitated the three-dimensional visualization of intricate blood vessel networks within the pulp chamber and the periodontal ligament (PDL) space [6]. This advancement was particularly evidenced in the study by Men et al., where the Synapsin-Cre; Ai14 mouse model was employed to delineate the nerve network within the PDL space and pulp chamber [10].

The tissue clearing method has proven to be instrumental in dissecting the neurovascular organization within the PDL tissue and understanding the role and dynamics of Gli1+ cell contributions and turnover in the molar PDL. Furthermore, lineage tracing—when integrated with tissue clearing and 3-D imaging technologies-provides a powerful approach to trace and visualize the spatial distribution of progeny originating from stem cells. This combination of techniques offers a comprehensive tool to elucidate the complex biological processes in dental and craniofacial research, capturing the true spatial context of cellular lineage and tissue organization.

Application PEGASOS Tissue Clearing in Bone-Implant Interface Research

The PEGASOS tissue clearing method, developed by Dian et al. [6], has significantly enhanced the three-dimensional visualization capabilities within craniofacial research. This technique has been effectively applied to delineate blood vessels within the pulp chamber and the periodontal ligament (PDL) space, revealing the intricate vasculature in a comprehensive 3D context.

Yi further extended the use of the PEGASOS method to investigate the implant-tissue interface [11]. This application yielded insights into the contributions of periodontal stem cells to the integration of implants with alveolar bone and provided detailed visualization of the vascular networks involved in the healing processes following implant placement in a mouse model [12]. By integrating tissue clearing with 3D imaging, researchers were able to simultaneously evaluate angiogenesis and osteogenesis, uncovering the dynamic interplay between vascular and bone tissues during regeneration.

Moreover, Stenberg et al. [13] utilized the PEGASOS method to assess the early healing stages around zirconia and titanium dental implants. This study illuminated a more robust vascular response and superior levels of bone formation in the peri-implant area surrounding zirconia implants compared to titanium (Figure 3). These findings underscore the potential of PEGASOS in providing a deeper understanding of biomaterial integration and tissue healing, which are pivotal in the field of dental implantology and craniofacial tissue engineering. The collective research utilizing PEGASOS tissue clearing thus offers a transformative lens through which the spatial and functional complexities of craniofacial development and healing can be examined in unprecedented detail.

3-D deep imaging systems for cleared samples

Whole tissue imaging represents a paradigm shift from conventional two-dimensional approaches, necessitating specific protocols for sample preparation and storage. Once cleared, tissues must be immersed in a chamber filled with the same clearing medium to maintain a consistent refractive index (RI) for accurate imaging. This medium serves as an alternative to traditional oil immersion, essential for maintaining the sample's transparency. The viability of cleared samples is generally limited to one month, with imaging recommended before signal degradation. For optimal preservation, samples should be stored at 4°C.

There are 3 major microscopes used for cleared tissue deep imaging. The confocal laser-scanning microscope (CLSM) is a mainstay in high-resolution imaging, employing a spatial pinhole to eliminate out-of-focus light. Its primary drawback lies in its relatively slow imaging speed. In contrast, two-photon microscopy (2-P) capitalizes on a nonlinear excitation mechanism [14], affording deeper tissue penetration and reduced laser-induced toxicity. It also facilitates second harmonic generation (SHG) imaging, an effect wherein collagen within bones or teeth generates photons at half the excitation wavelength, providing a unique mechanism for visualizing these structures. However, the extended excitation wavelength of 2-P microscopy slightly compromises lateral and axial resolution when compared to CLSM. Light-sheet fluorescent microscopy (LSFM) has emerged as the preferred method for large, cleared samples [15]. LSFM differs markedly from CLSM,

employing a perpendicular laser light sheet to illuminate a thin plane of the specimen, which significantly mitigates phototoxicity and background noise. The principal limitation of LSFM is its comparatively lower resolution. Despite this, its efficiency in imaging large volumes makes LSFM an increasingly popular choice for comprehensive tissue analysis, enabling researchers to capture extensive datasets with reduced sample damage and faster acquisition times.

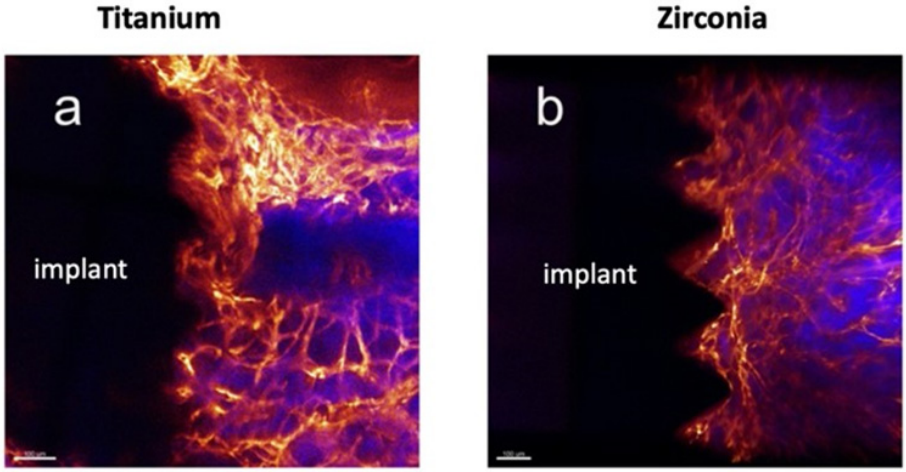

Figure 3: Confocal multi-photon images of bone and blood vessels healing around titanium (a) and zirconia (b) implants. Images were acquired following extraction of maxillary first molars, implant placement, tissue clearing with the PEGASOS technique, a 2-week healing period. Bone, (blue signal) and blood vessels, (gold signal), demonstrated the neovascularization within bone.

Conclusion and Future Perspective

Tissue clearing employs chemical agents to methodically remove water, lipids, and other light-scattering components from biological samples, thereby enhancing the depth of imaging and revealing true three-dimensional structures [16]. This process allows for the visualization of the brain and other tissues in a transparent state, enabling the acquisition of 3D images using confocal or two-photon microscopy without the need for physical sectioning. Consequently, tissue clearing techniques are pivotal for yielding a more comprehensive understanding of tissue architecture and interactions within their native environments.

The PEGASOS method, a notable advancement in tissue clearing, has been successfully applied in studies of cranial development, periodontal stem cell research, and vascular systems. However, its potential in craniofacial tissue regeneration and the elucidation of interactions between biomaterials, resident stem cells, and vasculature remains largely untapped. Utilizing the PEGASOS method to achieve complete tissue transparency, researchers can capture detailed 3D images that can demonstrate the spatial relationships between various tissues, such as craniofacial bone and vasculature during postnatal development. These images are obtained through a variety of labeling strategies, which can significantly aid in understanding the complex processes of osteogenesis.

Future research endeavors will expand the application of the PEGASOS and other similar clearing methods, particularly employing dual labeling techniques to simultaneously visualize the blood vessel system and bone mineralization. This approach promises to open new avenues for studying the intricate dynamics of craniofacial tissue regeneration and the integration of biomaterials with the innate biological systems.

Author Contributions

Y. Deng, contributed to design and data acquisition and analysis, drafted the manuscript; J Li and W Stenberg contributed to data acquisition and interpretation; KKH Svoboda, contributed to data analysis and interpretation, critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. Data available on request from the authors.

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