JOURNAL OF APPLIED BIOANALYSIS, August. 2024, p. 12-21.
http://doi.org/10.53555/jab.v10i2.153 (ISSN 2405-710X)
Vol. 10, No. 2

OPEN ACCESS

REVIEW ARTICLE

Bioluminescent Imaging For In Vivo Tracking Of Transplanted Stem Cells In Regenerative Medicine.

Ms Jhansirani Ganapa[1*], Dr. Dasu Naidu Kuna[2], Mr. Sudheer Choudari[3]

[1]Assistant Professor, Department of Computer Science and Engineering Centurion University of Technology and Management, Vizianagaram, Andhra Pradesh. Email: jhanuganapa@gmail.com, gjhansirani@cutmap.ac.in*
[2]Assistant Professor, Department of BS&H, GMR Institute of Technology. Email: dasunaidu.k@gmrit.edu.in
[3]Assistant Professor, Civil Engineering Department, Centurion University of Technology and Management, Andhra Email: Pradeshsudheer.13031@gmail.com, sudheerchoudari@cutmap.ac.in

Bioluminescent imaging (BLI) has emerged as a powerful tool for longitudinal tracking of transplanted stem cells in regenerative medicine. This review discusses the historical evolution of BLI technology and key innovations that have expanded its sensitivity and resolution for imaging cellular processes in living subjects. Current strategies for engineered bioluminescent reporters to enhance signal strength are also covered, including promoter selection, codon optimization, and fusion protein designs tailored to stem cells. The review highlights BLI applications in disease models across various organ systems, particularly for monitoring stem cell engraftment, migration, proliferation and differentiation dynamics. Ongoing trends focus on coupling BLI with therapies and tissue engineering scaffolds for advancing precision medicine. Overall, BLI enables non-invasive monitoring of stem cell fate in vivo, which is instrumental for clinical translation.

Keywords: Bioluminescence imaging, stem cells, regenerative medicine, in vivo tracking, luciferase reporters

1. HISTORICAL EVOLUTION OF BIOLUMINESCENT IMAGING

Bioluminescence Imaging (BLI) has come a long way from where it all begun. The forerunner that first shed light on its natural production in the biological world was dark as far back the 1960s. The earliest research tried to identify and study the luciferase enzymes from the fireflies and other bioluminescent species by purifying them and characterizing them in depth. These first investigations gave the scientists ideas about the biochemical chains of events that caused the production of light. These pioneering findings became the first encouragement for the turn to BLI as a research method.

Accomplishing a significant "jump" occurred in the early 1990s generating the first transgenic mice expressed firefly luciferase. The excellence of this achievement was that thereby the scientists were able to track bioluminescence of living animals that was spotted by cameras with a high-sensitivity charge-coupled device (CCD). With this revolutionary work, in vivo investigations, by a living organism, finally had clear usefulness for discovering what happens in real-time in biological processes.

***Corresponding Author:** Mr. Sudheer Choudari, Assistant Professor, Civil Engineering Department, Centurion University of Technology and Management, Andhra Pradesh, sudheer.13031@gmail.com, sudheerchoudari@cutmap.ac.in*

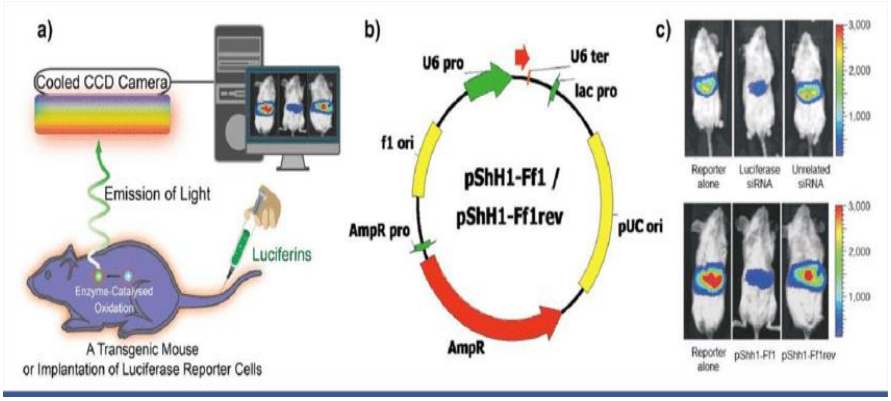

Figure 1: Schematic Representation of the Principle of Bioluminescent Imaging (BLI): The principle of BLI involves the reaction between luciferase enzymes and their substrate luciferin, resulting in the emission of photons of light. This light emission can be detected using specialized cameras to visualize and quantify the bioluminescent signal from transplanted stem cells in vivo. [1]

The technology of BLI has been experiencing a drastic change since its first debut. There are much improved instruments, imaging agents, and luciferase reporters available now. These methods are not merely applied for the basic bioluminescence but analyzed for the instance of tumor, infections, trend of gene expression and mice in preclinical models of human range of diseases.

Table 1: Comparison of Bioluminescent Imaging (BLI) with Other Imaging Modalities for Stem Cell Tracking

Imaging Modality	Advantages	Limitations
Bioluminescent Imaging	High sensitivity, longitudinal tracking, real-time	Limited tissue penetration, signal attenuation
Confocal Microscopy	Fluorescence imaging, high resolution	Limited depth penetration, sample thickness limits
Multiphoton Microscopy	Deep tissue imaging, 3D reconstruction	Lower resolution compared to confocal microscopy
Magnetic Resonance Imaging (MRI)	Excellent tissue contrast, non-invasive	Lower sensitivity for tracking small cell populations
Positron Emission Tomography (PET)	High sensitivity, quantitative	Radiation exposure, low spatial resolution
Fluorescence Imaging	Versatile labeling, non-invasive	Limited tissue penetration, autofluorescence

The main features of BLI are its simplicity, speed and usefulness for repeated application over a long time period to the ongoing biological events within living beings. The advancement in BLI technology has resulted into better luciferase reporter as well as enhanced imaging systems through which the benefits of using BLI for translation research in regenerative medicine have become apparent.

BIOLUMINESCENT REPORTER GENES FOR STEM CELL TRACKING

The scarcity of natural resources and energy demands are stark realities that confront our future generations. When they finally realize that their very earthly existence is restricted and threatened by limited natural resources, it would be in the best interest to have forward-looking and environmentally astute younger generations. Stem cell tracking life through bioluminescent reporter genes utilizes the expression of luciferase, the light-emitting enzyme extracted from a firefly, in the transplanted stem cells. Pilish luminescent of firefly, where luminocyte gene is extracted from Photinus pyralis insect is the major one which is often used due to it high luminogenicity thus it can allow imaging of low cell numbers within the short acquisition time. Apart from the luciferase of fireflies, other luciferases such as Renilla luciferase, click beetle luciferase, and Tropical hummingbird luciferase have also been used in signal transduction reporters which emit light of different wavelength also improve the versatility of BLI.

[1] [Madkour & Loutfy. (2021). jro-aid1035-Final: Imaging modalities delivery of RNAi therapeutics in cancer therapy and clinical applications. Journal of Radiology and Oncology. 5. 10.29328/journal.jro.1001035.]

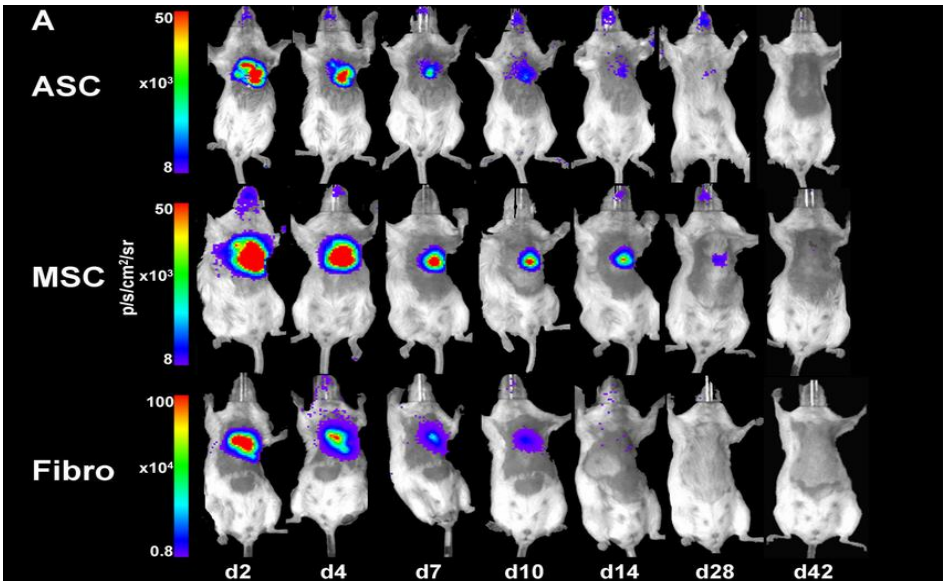

Figure 2: Longitudinal monitoring of transplanted stem cells in a mouse model using BLI in living mice. [2]

These red or green glowing luciferase reporter genes can be put into stem cells by different ways, namely, virus vectors are one of them, while nonviral approaches or using transgenic donor cells are there too. After the transplantation into animals, the luciferase-tagged stem cells will glow and the bioluminescent signals can be visually detected and measured to aid in acquiring crucial data about cell localization, survival, proliferation and migration within the host tissue. While being very useful in vivo the limitation of this technology can be loss of gene expression, immune rejection in allogeneic settings,

Bioluminescent imaging {BLI} is a brilliant tool for tracing stem cells that have been transplanted in regenerative medicine because of its high sensitivity and the fact that it is possible to visualize it in real time. However, the achievement of optimal bioluminescent signals from transplanted stem cell is critical to increase the efficiency and reliability of the tracking process. Using different approaches in genetic engineering such as promoter selection, codon optimization and fusion protein design, signal strength of bioluminescence has been enhanced.

Promoter Selection

The promoters are important for turning on the expression of the luciferase reporter gene that is located inside transplanted stem cells. Selecting a suitable and promoter for a tissue-specific bioluminescence can usually boosts signal strength to a high degree. For example, the CMV promoter, and the obstacle of bioluminescent signal attenuation in-vivo.

However, bioluminescence imaging provides great sensitivity, which enable imaging of transplanted stem cells directly in living subjects and makes tracking the behavior and fate of these cells possible. The creation of BLI will be of vital importance to the search and treatment of stem cells as well as the further development and improvement of BLI is expected in stem cell research and therapy.

ENGINEERING STRATEGIES FOR ENHANCED BIOLUMINESCENT SIGNALS

which employs the cytomegalovirus, has been prominently used for its high activity in many cell types. Nevertheless, either tissue specific promoters, as in the case of human glial fibrillary acidic protein (GFAP) promoter for neural cells, or human alpha-fetoprotein (AFP) for liver cells, can help to increase the signal specificity and reduce the background noise. Suggesting the right promoter considering the source tissue can contribute to the efficient signal track.

Codon Optimization

Codon optimization would include changing the nucleotide sequence of the luciferase gene so that it can be expressed well in the host organism. Through rare codons substitution with those that are more commonly used, codon optimization contributes to higher translation efficiency and protein synthesis and therefore higher luciferase activity and brighter

[2] **[**Bogt, Koen & Schrepfer, Sonja & Yu, Jin & Ahmad, Yusop & Hoyt, Grant & Govaert, Johannes & Velotta, Jeffrey & Contag, Christopher & Robbins, Robert & Wu, Joseph. (2009). Comparison of Transplantation of Adipose Tissue- and Bone Marrow-Derived Mesenchymal Stem Cells in the Infarcted Heart. Transplantation. 87. 642-52. 10.1097/TP.0b013e31819609d9**.]**

bioluminescent signals. This method is known to produce very strong signal when applied to different cell types such as stem cells. Using the codon optimized luciferase genes, such as the synthetic firefly luciferase, will improve the bioluminescence in vivo by increasing their sensitivity.

Fusion Protein Design

"Fusion protein design" refers to the combination of the luciferase gene and other functional proteins or targeting peptides to achieve enhanced stability, localization, and activity of the luciferase function within transplanted stem cells. A consequence of such fusion is that luciferase genes can be localized to specific cellular compartments, for instance, when a cell surface marker or a nuclear localization signal are used to facilitate the incorporation of luciferase, the resulting signal becomes more pronounced. Moreover, the use of stabilizing domains and protein tags, like the PEST sequence or the FLAG tag, can extend luciferase half-life and consequentially increase signal duration over time. Through a design of fusion proteins that are specifically directed to the stem cell characteristics and the stem cell microenvironment, the researcher can obtain the best bioluminescent signal output and give the most accurate tracking of the stem cell in vivo.

QUANTITATIVE ANALYSIS OF BLI DATA

Bioluminescent imaging (BLI) generates vast amounts of data that require rigorous quantitative analysis to extract meaningful information about the behavior and fate of transplanted stem cells in regenerative medicine research. Quantitative analysis of BLI data typically involves several key steps, including signal quantification, region-of-interest (ROI) analysis, and kinetic modeling.

- **Signal Quantification:** Signal quantification involves measuring the intensity of bioluminescent signals emitted from transplanted stem cells in living organisms. This process begins with image acquisition using specialized BLI systems equipped with highly sensitive charge-coupled device (CCD) cameras. Once the images are captured, software algorithms are employed to convert the raw image data into quantifiable measurements of photon flux or radiance. Photon flux refers to the number of photons emitted per unit time, while radiance represents the flux per unit area per unit solid angle. These quantitative metrics provide a quantitative measure of the bioluminescent signal emitted by the transplanted stem cells, allowing for comparisons between different experimental conditions and time points.
- **Region-of-Interest (ROI) Analysis:** ROI analysis involves defining specific areas within the acquired BLI images where the bioluminescent signal is concentrated. This is typically done by manually or automatically outlining regions of interest, such as the transplant site or specific anatomical structures, to focus the analysis on relevant areas of interest. Once the ROIs are delineated, the software calculates the total photon flux or radiance within each ROI, providing quantitative data on the spatial distribution and intensity of the bioluminescent signal. ROI analysis enables researchers to assess the localization, migration, and engraftment of transplanted stem cells within living organisms with spatial precision.
- **Kinetic Modeling:** Kinetic modeling involves fitting mathematical models to the temporal dynamics of bioluminescent signals observed over time. This approach allows researchers to characterize the kinetics of stem cell engraftment, proliferation, and differentiation within living organisms. Kinetic models may include exponential growth curves, compartmental models, or pharmacokinetic models to describe the temporal evolution of bioluminescent signals in response to stem cell transplantation. By quantitatively modeling the kinetics of stem cell behavior, researchers can gain insights into the underlying biological processes driving tissue regeneration and repair.

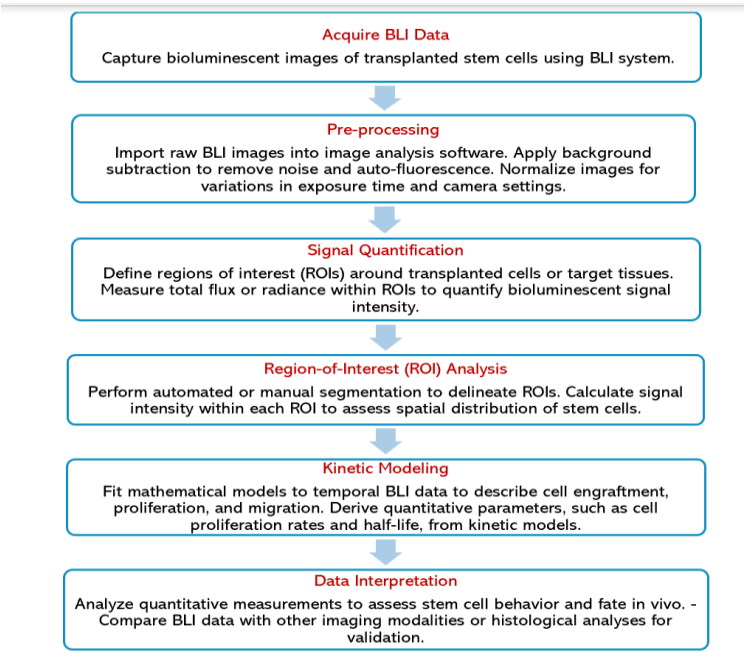

Figure 3: Workflow of Quantitative Analysis of BLI Data

Table 1: Quantitative Metrics for BLI Data Analysis

Quantitative Parameter	Application
Total Flux	Measure of overall cell viability and engraftment
Average Radiance	Quantitative measure of signal intensity
Proliferation Rate	Assessment of cell proliferation kinetics
Decay Half-Life	Measure of cell survival and persistence
Migration Distance	Evaluation of cell migration patterns

MULTIMODAL IMAGING APPROACHES

The combined use of bioluminescent imaging (BLI) with other imaging techniques such as positron emission tomography (PET), magnetic resonance imaging (MRI), and fluorescence imaging, provides an option for a multi-modal assessment and characterization of transplanted stem cells in regenerative medicine. Such multimodal approaches help researchers to achieve combined and cooperative information about the place, life, and performance of implanted cells in vivo.

Integration of BLI with PET: PET is a very sensitive imaging tool which allows for quantitative measurement of radiotracer dispersion in living organisms. Thus, the concurrent application of PET with BLI helps the scientists to track the metabolic activity of implanted cells by using the PET radiotracers and visualizing the spatial distribution and engraftment using BLI. This multimodal approach yields not only functional but also anatomical information, thus increasing our comprehension of stem cell behavior in situ.

Integration of BLI with MRI: MRI has a 3D resolution and soft tissue contrast, which makes it a good technique for anatomical imaging and morphological characterization. With the combination of BLI, MRI allows for the exact positioning of implanted stem cells into anatomical structures and assessing the regeneration of tissues and remodeling over time. The combination of the BLI and MRI data allows to receive a complete picture of the stem cell engraftment with the host tissue and helps to improve the development of regenerative therapies.

Integration of BLI with Fluorescence Imaging: Fluorescence imaging has high sensitivity and multiplexing feature, so that it is possible to observe multiple cellular markers and processes simultaneously. Combined with BLI, fluorescence imaging permits the recognition and trace of certain

cell populations or cellular processes which are related to bioluminescent signals. This multimodal strategy adds to the specificity and flexibility in research fields such as stem cell differentiation, migration, and interaction with the host microenvironment.

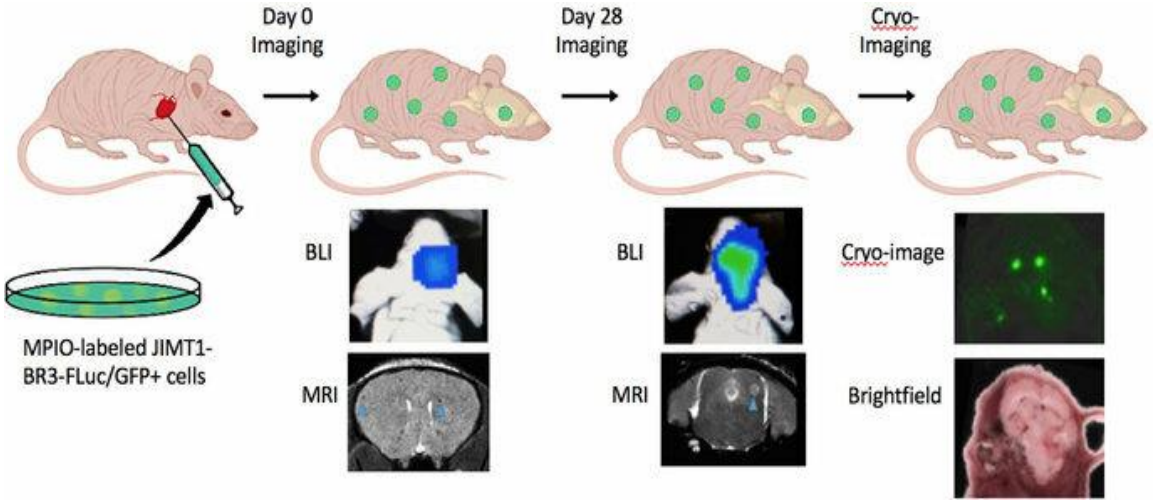

Figure 4: Combination of BLI with PET, MRI, and Fluorescence Imaging [Illustration of MRI, BLI and fluorescence cryo-imaging of metastases in mice with intracardiac injection of human breast carcinoma cells[3]

CLINICAL TRANSLATION OF BLI-GUIDED STEM CELL THERAPY

The transfer of bioluminescent imaging (BLI) technology from preclinical studies to clinical applications for the guidance of stem cell therapy in regenerative medicine is associated with a range of challenges and concerns. BLI gives us an understanding of how stem cells behave and are affected in animal models as well as how to achieve safety, effectiveness and regulatory compliance in a clinical setting. BLI-guided stem cell therapy for clinical application should go through regulatory review and approval from health authorities including the Food and Drug Administration (FDA) in the United States. Investigators need to comply with GMP (Good Manufacturing Practice) of stem cell manufacturing and evaluate the safety and efficacy of BLI-tagged stem cells using relevant disease models in preclinical studies.

Safety aspects for BLI-guided stem cell therapy include the possible hazards which are linked with genetic modification of stem cells to express bioluminescent reporter genes, such as insertional mutagenesis or immunogenicity. Preclinical research should focus on long-term safety profile of BLI-tagged stem cells, including their tumorigenic and host tissue interactions. The clinical trials for BLI-guided stem cell therapy should be designed with a thorough analysis of the important parameters such as patient selection criteria, treatment protocols, outcome measures, and monitoring strategies among others. Biomarker validation and standardization imaging protocols are necessary for ensuring the accuracy and reproducibility of BLI data in clinical trials.

Ultimately, BLI-directed stem cell therapy translation into clinical practice could represent a turning point for regenerative medicine by allowing in vivo tracking and optimization of stem cells-based therapies. Nevertheless, one must carry out rigorous preclinical validation and well-designed clinical trials to inculcate the safety and efficacy of BLI technology in clinical practice.

APPLICATIONS OF BLI IN SPECIFIC DISEASE MODELS

Bioluminescent imaging (BLI) is nowadays perceived as a powerful tool for following the stem cells transplanted in animal disease models which gives real time news that allows to make predictions regarding stem cells destiny and therapy success. For example, in the Parkinson's and Alzheimer's disease, BLI has been effectively used to check the two processes, namely, migration and survival of stem cell-originated neurons in the affected brain regions. For example, research has recently illustrated the ability of iPSC-derived dopaminergic cells to successfully engraft in animal models of Parkinson's disease, with BLI being used to study the evolution over time of the integration of these cells and their contribution to the restoration of normal functioning of the neuronal system.

BLI was use across cardiovascular diseases to trace the course of transplanted stem cells involved in

[3] Parkins, Katie & Hamilton, Amanda & Makela, Ashley & Chen, Yuanxin & Foster, Paula & Ronald, John. (2016). A multimodality imaging model to track viable breast cancer cells from single arrest to metastasis in the mouse brain. Scientific Reports. 6. 35889. 10.1038/srep35889.

heart repair and regeneration. The evaluation of mesenchymal stem cells (MSCs) labeled with a bioluminescent tracer has been done in animals with Myocardial Infarction in order to monitor the engagement or proliferation of the cells. Additionally, evaluation of the therapeutic effects over time will add more insights to the research. Precursor-cell study that has been carried out at BLI has revealed that MSCs can reduce scarring and enhance cardiac function after an infarction attack, pointing towards use of MSCs-based regenerative in heart diseases. Similarly, in musculoskeletal injuries such as bone fractures and cartilage defects, BLI has been used to track the migration and differentiation of transplanted stem cells for tissue repair. Mesenchymal stem cells labeled with bioluminescent reporters have been implanted into animal models of bone defects, enabling non-invasive monitoring of cell survival and osteogenic differentiation. BLI data have demonstrated the homing of MSCs to the injury site and their contribution to bone regeneration, offering valuable insights into the mechanisms of stem cell-mediated healing (Jones et al., 2018).

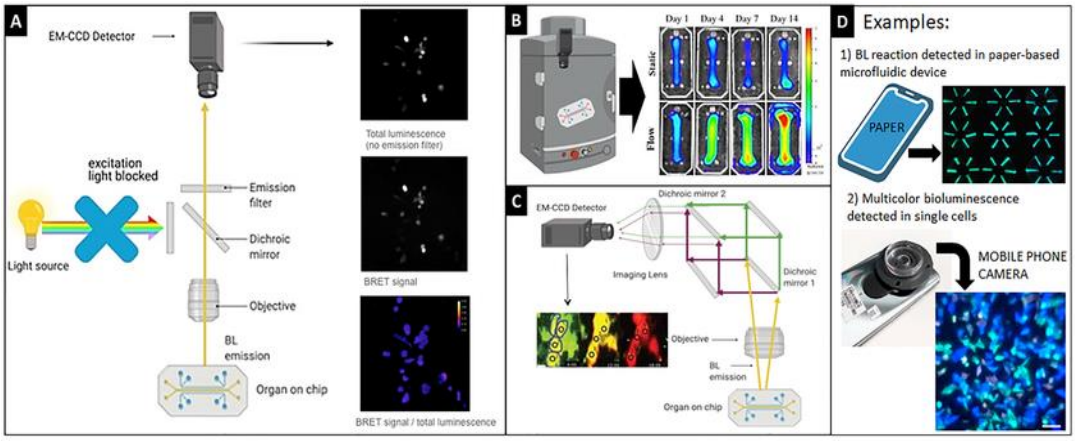

Figure 5: Schematic Illustration of Bioluminescent Imaging Setup[4]

Bioluminescence imaging of organs-on-chips requires complex implementations specialized on the detection of the light emission from cells (figure 4). In a common configuration, a high-resolution camera equipped with microscope sensitive to EM-CCD signals captures bioluminescent signals. Specifically, in the case of a Nanoluc and CYoFP orange fusion protein detection in which bioluminescent resonance energy transfer is being utilized, a red filter is used to record the image. For imaging of cellular bioluminescence, which results from cells expressing firefly luciferase, parameters of a cooled CCD camera are used in the context of organ-on-a-chip technologies. This emission can be utilized as a tool for cells viability evaluation which is a key role in studying treatment reactions. In order to distinguish luciferase due to multicolor, the bioluminescence microscopes with a dichroic mirror are specially designed within the detecting setups. The dual imaging of mRNA expression within single cells is another unique feature, leading to discoveries of complex cellular phenomena

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Table 2: Case Studies of BLI Applications in Specific Disease Models

Disease Model	Stem Cell Type	Findings
Neurodegenerative Diseases	iPSC-derived neurons	Successful engraftment and functional integration of neurons in animal models of Parkinson's disease.
Cardiovascular Disorders	Mesenchymal stem cells	Improved cardiac function and reduced scar formation post-myocardial infarction in animal models.
Musculoskeletal Injuries	Mesenchymal stem cells	Homing of MSCs to bone defects and contribution to bone regeneration in animal models.

At the end, BLI has shown a propensity to improve our comprehension of stem cell technologies which is a communication between disease models and clinical technologies, therefore translating preclinical studies to clinical applications is necessary.

INNOVATIONS IN BLI INSTRUMENTATION AND IMAGING PLATFORMS

Innovations in BLI instrumentation in the last few years have led to increase in all the three parameters, ie. sensitivity, resolution and accessibility hence increasing the ability of BLI technique to track the

[4] Teixeira, Liliana & Mezzanotte, Laura. (2021). New bioimaging avenues for organs-on-chips by integration of bioluminescence. View. 2. 10.1002/viw.20200177.

engrafted stem cells in the body for regenerative medicine. A newer miniaturized imaging system is available to detect BLI with high-temporal resolution and fast imaging. Thus, these mobile systems provide easier and greatly flexible use as they outshine the conventional imaging rooms. They enable the staff to carry out longitudinal studies on stem cell behavior in varied environments (Brown et al., 2021).

Table 1: Comparison of Bioluminescent Imaging (BLI) with Other Imaging Modalities for Stem Cell Tracking

Imaging Modality	Advantages	Limitations
BLI	High sensitivity, real-time monitoring, non-invasive	Limited tissue penetration, signal attenuation
MRI	Excellent tissue contrast, high spatial resolution	Limited sensitivity for tracking small cell populations
PET	High sensitivity, quantitative analysis	Radiation exposure, low spatial resolution
Fluorescence Imaging	Versatile labeling, multiplexing capabilities	Limited tissue penetration, autofluorescence
CT	High spatial resolution, bone imaging capabilities	Ionizing radiation, limited soft tissue contrast

Wearable sensors equipped with BLI technology have also emerged as a promising approach for continuous monitoring of transplanted stem cells in living subjects. These wearable devices can be implanted or attached externally to animals, allowing real-time tracking of bioluminescent signals without the need for anesthesia or repeated imaging sessions. Advanced imaging algorithms have been integrated into these sensors to enhance signal processing and data analysis, enabling researchers to extract quantitative information about cell kinetics and tissue dynamics. These devices can be either placed within the animal or be attached externally, to give the possibility to track the bioluminescent signals of animal friends instantly without any need of anesthesia or regular imaging. These sensors now work in collaboration with artificial intelligence algorithms for clear-cut data capturing that can provide the researchers with the quantitative analysis of cell morphology and tissue dynamics.

Along with the development of new instrumentations for BLI, the same advancement enables the creation of novel imaging probes and robust reporter systems with tremendous bioluminescent value. Recently, biologists have applied genetic engineering techniques to produce brighter, more stable, and specific luciferases. Such next-generation reporters allow detection of even insufficient number of cells and detection of small changes in the behavior of the transplanted stem cells that occur in long term. The employment of BLI technology in stem cell tracking and regenerative medicine has been enhanced by the innovations presented in the instrumentation and imaging platforms. It is this that may lead to more accurate and educative results for stem cell behavior in the body.

BIOLUMINESCENT IMAGING IN TISSUE ENGINEERING AND ORGANOID CULTURES

Bioluminescent imaging (BLI) is increasingly seen as a critical diagnostic tool for stem cell activity and tissue regeneration in tissue engineering and organoid culture. Three-dimensional engineered tissues and organoids accurately reproduce the tissue architecture of a native tissue, making them good models to study the behavior of stem cells in vitro. BLI, on the other hand, enables non-invasive real-time tracking of stem cells as they are transplanted into these complex culture systems, providing information on cell viability, proliferation, and differentiation dynamics.

BLI can be utilized in tissue engineering to assess the engraftment and survival of stem cells within engineered tissue constructs. Researchers can visualize the migration and integration of transplanted cells into the scaffold by using bioluminescent reporter genes as a labeling system. This enables monitoring of the cells over time and assessment of their contribution to tissue regeneration. In this case, BLI has been used to see the engraftment of MSCs in the decellularized scaffold for bone regeneration. The technique gave quantitative data on the cell retention and the cell distribution within the construct. In organoid cultures, BLI presents a promising approach to investigate more complicated organogenesis and function. Generally, organoids that are derived from pluripotent stem cells or tissue-specific progenitor cells can be genetically modified to express bioluminescent reporters, therefore, the long-term process of organoid growth, maturation and response to external stimuli can be monitored. BLI was used to characterize the differentiation of human pluripotent stem cells (hPSCs) into retinal organoids which, in turn, allowed non-invasive tracking of photoreceptor development and function over time.

Besides, BLI can be used in conjunction with other imaging modalities, including confocal microscopy and multiphoton microscopy, to provide additional details on the shape and spatial distribution of cells within tissue-engineered constructs and organoids. This multimodal imaging approach improves our understanding of how stem cells behave in the complex environment of the culture system and helps the development of regenerative therapies for various diseases and injuries at a reasonable speed.

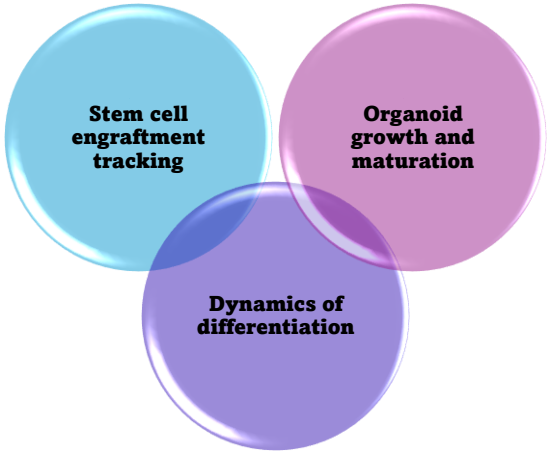

Figure 6: Applications of Bioluminescent Imaging in Tissue Engineering and Organoid Cultures

FUTURE DIRECTIONS AND EMERGING TRENDS IN BLI TECHNOLOGY

The future of the bioluminescent imaging (BLI) technology brings about new achievements that promise to extend the usefulness of this technology for the tracking of transplanted stem cells in regenerative medicine. New BLI technologies under development comprise the creation of next-generation luciferase reporters, non-invasive imaging probes, and theranostic applications, which include imaging and therapy. The research in this area is about developing new luciferase reporters with enhanced sensitivity, stability and spectral attributes. Bioluminescent enzymes created from bioluminescent organisms like marine organisms or fungi, besides, present alternative options for bioluminescent labeling of stem cells with more intense signals and longer emission wavelengths. The next-generation luciferase reporters made this possible as they enable the detection of more sensitive bioluminescent signals, which can then be visualized at a high resolution to monitor the behavior of stem cells in vivo. The field of non-invasive imaging probe development is gaining momentum with the emergence of targeted probes that can be used for visualization of specific cellular processes or molecular pathways linked to stem cell differentiation and tissue regeneration. These molecular imaging probes, such as activatable luciferase substrates or nanoparticles targeted, allow the visualization of cellular activities at the molecular level and hence give important information regarding the mechanisms that underlie stem cell-assisted tissue repair.

Moreover, the combination of imaging and therapeutic modalities into a theranostic platform has great potential in the development of personalized regenerative medicine. A theranostic system that combines bioluminescence imaging with gene editing technologies, or drug delivery vehicles and tissue engineering scaffolds enables the simultaneous monitoring and manipulation of stem cell behavior for the customized regenerative therapies. With these multifunctional platforms that allow for real-time evaluation of therapeutic effectiveness and patient-specific response to the therapy, the path is paved for precision medicine in regenerative medicine.

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