Fluorescent Cell Imaging in Regenerative Medicine

Supplementary Issue: Image and Video Acquisition and Processing for Clinical Applications

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ABSTRACT: Fluorescent protein imaging, a promising tool in biological research, incorporates numerous applications that can be of specific use in the field of regenerative medicine. To enhance tissue regeneration efforts, scientists have been developing new ways to monitor tissue development and maturation in vitro and in vivo. To that end, new imaging tools and novel fluorescent proteins have been developed for the purpose of performing deep-tissue high-resolution imaging. These new methods, such as intra-vital microscopy and Förster resonance energy transfer, are providing new insights into cellular behavior, including cell migration, morphology, and phenotypic changes in a dynamic environment. Such applications, combined with multimodal imaging, significantly expand the utility of fluorescent protein imaging in research and clinical applications of regenerative medicine.

KEYWORDS: fluorescent protein, tissue engineering, regenerative medicine, microscopy, live-cell imaging

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Introduction

In recent years, the field of regenerative medicine has shown great advancements in many fronts.[1] Such advancements include tissue engineering of biomimetic constructs for organ replacement, the ability to derive patient-specific pluripotent stem cell populations through reprogramming technology, and the capability to precisely edit the genome using CRISPR/Cas9 and other developing technologies. In many of these applications of regenerative medicine, there is a growing need for noninvasive techniques to monitor tissue development and maturation. Some imaging capabilities have already found widespread applications in cell biology research and medicine[2] and include medical imaging, such as magnetic resonance imaging (MRI) and X-ray, and optical imaging, such as bioluminescence and fluorescent imaging. While most medical imaging tools provide with imaging depth of several centimeters, a major limitation is their lack in cellular-level resolution data.[3] Live-cell fluorescent imaging using fluorescent proteins (FPs) and dyes overcome some of the limitations of medical imaging techniques, offering high resolution (submicron) and functional reporters.[4] Noninvasive live-cell fluorescent imaging techniques provide comparable metrics to standard invasive tools such as quantitative polymerase chain reaction and immunohistochemistry, while still having an imaging penetration depth of up to several millimeters. Bioluminescent imaging relies on luminescent proteins and external chemical stimuli to provide highly sensitive quantification of individual cells with low background noise, yet it has a limited spatial resolution compared with fluorescent imaging. This has resulted in relatively limited applications for in vivo tracking of cells, including cancer and stem cells.[5,6] Another limitation of this technique is the requirement for the delivery of the bioluminescent substrates that are relatively short-lived in vivo. The significant advances in our understanding of cell and tissue biology facilitated by these imaging tools have driven the development of new fluorescent imaging tools and reporters to gain further insights into dynamic cell behavior in the context of new regenerative medicine technologies. These new live-cell imaging tools aim to overcome many of the limitations of current tools to provide novel capabilities to researchers, including improvements in spatial resolution, temporal resolution, and signal-to-noise ratio, while maintaining nontoxic physiological conditions. Furthermore, more specific applications such as use in in vitro bioreactor systems or in vivo animal models may have additional requirements such as greater imaging depth, dynamic range, and sensitivity. Deep-tissue imaging remains a significant challenge for fluorescent imaging due to absorbance and scattering, which results in limited capability to perform in vivo imaging since imaging penetration depth remains in the range of 1–2 mm. In an attempt to accommodate for these limitations, researchers have been working

on new tools such as multiphoton, light-sheet microscopy, and intravital microscopy (IVM). These tools along with the development of new fluorescent reporters, which provide strong signal and low background noise, are offering the foundations for live-cell and in vivo imaging under the given optical constraints.

Fluorescent Imaging Tools: Device and Protein

The demand for new applications to monitor cell and tissue function and the need for improved techniques for deep-tissue imaging have driven the development of new innovative imaging tools.[7] Since the initial introduction of fluorescent microscopy, advanced platforms created new standards for imaging. These basic improvements include confocal imaging that allowed for three-dimensional (3D) fluorescent image reconstruction, the development of multiphoton microscopy that uses longer wavelength excitation, and a 3D focused point-by-point scan that allows imaging through several millimeters for in vivo experiments. Furthermore, nonlinear microscopy is being used to detect some biomolecules, such as nicotinamide adenine dinucleotide (NAD), and extracellular matrix (ECM) components, such as collagen. Additional imaging platforms that show promising results include light-sheet fluorescent microscopy (LSFM), which uses light perpendicular to the sample to provide with better axial sectioning,[8] and photoacoustic imaging, which provides improved imaging depth through the use of ultrasonic emission to detect the fluorescent signal.[9,10] Notably, IVM, which uses an optical window in animal models,[9] is being used extensively for in vivo experiments to visualize cell and organelle information without the challenges of deep-tissue imaging. Newer developments based on advances in imaging instrumentation include new excitation sources, detectors, and novel optical paths. Some examples of these tools include (a) fluorescent lifetime microscopy (FLIM), which provides with novel ways to differentiate fluorescent targets in noisy background through fluorescent decay profile, (b) fluorescent loss in photobleaching (FLIP), which tracks dynamic movement of fluorescent molecules in the bleached region in cells, and (c) super-resolution techniques such as photoactivated localization microscopy (PALM) that allows enhanced resolution of subcellular domains through identification and localization of spontaneous fluorescent signal using photoswitchable FP (Table 1).

The applications of these tools are also dependent on the recent advances in the development of new fluorescent markers that are compatible with the instrumentation and matched to the specific imaging requirements. Since the initial discovery in 1962 of the green fluorescent protein (GFP)[11] and its subsequent imaging applications, many new FPs have been developed spanning the visible light spectrum, providing new opportunities for multiplexing. Modifications in FP are being used to improve the basic properties of the proteins, including increased photostability, low toxicity, and higher quantum efficiency. Other improvements have arisen through modifications to FP[4] to develop photoactivated (PA) FP used in super-resolution imaging, as well as long Stokes shift FP and far-red FP for improved signal in noisy environment along with improved imaging penetration. FPs have also found widespread application as reporters of genetic activity, serving as a noninvasive indicator of gene expression, suppression, or other genetic interactions. Generally, FP genetic reporters include three main categories as follows: (1) general cell expression, (2) fusion with target protein, and (3) promoter-driven FP expression. Each kind of fluorescent reporter genes has special applications, such as fusion FP sensors for Förster resonance energy transfer (FRET) to detect molecular structure changes, where FPs in close proximity (donor and acceptor) lead to changes in donor photon release.[12] All these markers depend on the development of effective labeling techniques.[4] Vectors with improved integration probability, new gene editing methods, and improved cloning all play a part in the creation of target cell expressing fluorescent reporters. Labeling targets include established cell lines used in in vitro experiments, primary cells, and stem cells applied in vitro, ex vivo and in vivo, as well as applications in transgenic animal expression of fluorescent marker targeting a specific location or function.

Applications for Live-cell Imaging

Fluorescent signal identification has found widespread application for noninvasively detecting cell dynamics within tissues and organs. Cell localization, subcellular colocalization, and morphological changes represent some of the basic outcomes for cellular therapy and tissue engineering. Additionally, promoter-driven fluorescent expression provides a functional aspect to the monitoring application. For example, induced pluripotent stem (iPS) cells have been evaluated for their ability to differentiate to endothelial fate with terminal differentiation detected by fluorescent marker expression.[13] Transplantation of fluorescent labeled iPS cells and pericytes confirmed their ability to form new blood vessels in vivo. In another case, a more complex system was used in which intestinal stem cells were monitored in a fluorescent transgenic mouse model using IVM.[14] By using unique Cre-induced fluorescent targets, lineage tracing was performed to monitor their cell behavior over time and space within the cell niche (crypt), showing dynamic differentiation profile that changed as a function of its position. IVM has also been used to monitor cell dynamics in a mouse model of muscle injury, providing new insights for this in vivo model of muscle regeneration.[15] Researchers monitored satellite cells, observed the formation of new muscle fibers in the form and direction of ghost fibers, and identified the regenerative cell source within the injured fibers. Monitoring dynamic subcellular domains can provide new insights of cell division. Another example includes a study of the asymmetric division

Table 1. Fluorescent imaging tools and applications.

IMAGING TOOL	PRINCIPLE	APPLICATIONS	EXAMPLES
Confocal laser scanning microscopy (CLSM)[28]	One photon absorption with three-dimensional (3D) resolution achieved by pinhole rejecting out of focus light	In vitro 3D cell construct analysis Limited in vivo imaging, only in the ~200 μm depth range and also since it may have phototoxic effects for long exposure to excitation light	Spinal axis degeneration and regeneration in transgenic mouse model[29] Vasculogenesis in 3D scaffold involving endothelial cells and fibroblasts[30]
Multiphoton Microscopy (MPM)[28]	Non linear photon absorption giving less scaterring (vs. one photon) and high resolution in 3D (imaging depth up to 1–2 mm)	3D in vitro and in vivo analysis for fluorescent cells and label free molecules Second harmonic generation (SHG) analyzing ECM proteins (e.g. collagen)	3 photon imaging over intact skull of transgenic mouse brain[20] 3D scaffold remodeling by fibroblasts using label free bio-molecules and ECM collagen structure[31]
Light sheet fluorescent microscopy (LSFM)[8]	3D imaging using optical 2D lattice layer by layer excitation allowing faster scan, improved resolution, and reduced bleaching and toxicity when compared with confocal	Single-molecule binding kinetics Cell migration and division Embryonic development	Zebrafish embryo development[32] Cancer cell response in 3D collagen matrix including actin protrusion and blebbing[33]
Intravital microscopy (IVM)[9]	Optical window in animal model providing access of various microscopy tools to target tissues	Multiple organ window monitoring with better results with tissues closer to surface and less sensitive to physiological noise (e.g. blood flow) mostly used in transgenic fluorescent mice models	Intestine stem cell dyanmics in niche[14] Muscle progenitor cell regeneration[15] Dorsal skin window to monitor tumor cell phenotype and physiology[34]
Fluorescent Lifetime Imaging (FLIM)[35]	Decay time of fluorescent excitation back to ground state; allowing high sensitivity independent of fluorescent intensity	FRET for identifying cell-interaction with environment Label free measures including oxygen, and bio-molecules such as NAD/NADH indicating metabolism	Cell mechanotransduction and stem cell differentiation[21] Stem cell metabolism dynamics[25]
Fluorescent loss in photobleaching (FLIP), and fluorescent recovery after photobleaching (FRAP)[36]	Tracking dynamic movement of fluorescent molecules adjacent to bleached fluorescent region and the recovery of bleached regions	Analyzing cell structure dynamic such as membrane, endoplasmic reticulum (ER)	Age depended stem cell asymmetric division[17] Morphogen gradient formation[37]
Photoactivated localization microscopy (PALM)[38]	Super-resolution through identification and localization of spontaneous fluorescent signal using photo-switchable FP	Observing sub-cellular structural changes with resolution beyond diffraction limit (200 nm) such as chromatin, and cytoskeleton	Polymerase II clustering used for gene regulation[39] Nano organization in integrins and talin in focal adhesion (FA) sites[40]

of stem-like epithelial cells.[16] Novel labeling with PA-GFP fused to the cell mitochondria provided a new way to monitor this specific organelle over a period of time. Ultraviolet light activation facilitated the identification of older and newer (nonactivated) organelles. This study provided new observations of the asymmetric distribution of older and newer mitochondria by which stem cells could maintain better function after following cell division. In another study using FLIP, researchers were able to identify the differences in fluorescent molecule distribution after cell division between older and younger neural stem cells in vitro, observing a loss in the ability to undergo asymmetric multiplication in older cells.[17] Morphogenesis studies[18] are also providing insights of whole organism embryo development through animal models in zebrafish, fruit fly, and mouse.[19] Such models have resulted in a new understanding of multiple organ development as a function of signaling pathways and physical forces. The push for greater penetration depth in imaging has resulted in innovations such as three-photon imaging for imaging red fluorescent protein (RFP) neural cells through the intact skull of a mouse.[20]

Changes in fluorescent signal can be further utilized in providing molecular real-time data. Such information can be applied for real-time evaluation of cell phenotype and functional state. FPs are being used to measure various molecular processes with fluorescent intensity as the measurable reporter of such an activity. Recent studies now suggest that live-cell imaging data can be comparable with the gold-standard protein and gene molecular biology tools but without the need for sample destruction. One example is the use of a FRET sensor to monitor vinculin structural changes that assist in focal adhesion,[21] capable of identifying cell responsiveness to changes in surface tension. This approach provided a novel mechanotransduction model, which will be valuable in the field of tissue engineering studying how stem cell differentiation is altered as a function of scaffolding material.[22] FRET has also been applied for measuring local adenosine triphosphate (ATP) levels within single cells as a direct measure of cell metabolism and function.[23] Binding ATP subunit was attached to FRET pair, and it showed responsiveness to the dynamic changes in ATP levels. This was used in visualizing the changes in ATP levels in response to glycolysis inhibitors, which can assist

in understanding the impact that cell pathways have on cell energy balance. FPs have also been used in a calcium sensor (GCaMP3) to detect cellular function, following transplantation into an injured organ. The vector was introduced into embryonic stem cells, which were then transplanted in injured guinea pig heart with the goal of restoring function through the engraftment and formation of functional cardiomyoctyes.[24] The sensor produced GFP signal in response to calcium influx and correlated with improved electrocardiogram data when following cells with IVM up to 28 days after transplantation. Lifetime imaging (FLIM) is another tool being used for single-cell information. Using label-free markers of reduced and oxidized nicotinamide adenine dinucleotide (NADH/NAD+) ratio lifetime, scientists were able to detect in vivo changes in epidermal stem cell metabolism in synchronization with the circadian clock, oscillating between oxygenation phosphorylation during the day and glycolysis during the night.[25] These tools are already offering new type of data for the dynamic cell response to a complex environment. Fluorescent reporters have already provided new information about cell–cell and cell–ECM interactions, cell division dynamics, and new functional assays related to cell biophysics and metabolism. All these assays offer better ways to assess regenerative medicine approaches and improve outcomes.

Future Directions

New developments in both optical tools and novel FP offer great promise for the future, providing an unprecedented understanding of the dynamics of cellular function in the context of tissue regeneration and regenerative medicine. Improved understanding of photophysical properties can assist in gaining new capabilities for deep-tissue imaging using lifetime imaging, photoacoustics, light-sheet microscopy, or photoactivated FP. The general trend is advances in deep-tissue imaging with maintenance of high temporal and subcellular resolution, along with multiplexing capabilities for multiple target imaging through spectral imaging. Ideally, fluorescent molecular tomography, which is being used extensively in cancer research,[26] could greatly benefit from improved higher resolution. Overall, the attempt to break the boundaries of the trade-off between imaging depth and resolution is bound to continue. Multimodal imaging will likely bring additional progress to the field. By combining the strength of different imaging modalities, imaging data can complement each other with structural and functional information. Such tools include micro computed tomography (micro-CT), magnetic resonance imaging (MRI), positron emission tomography (PET), optical coherence tomography, and bioluminescent imaging. Some research is already being done using multiple tools such as the use of fluorescence, MRI, and bioluminescence imaging (BLI) to identify cell fate over time.[27] Bridging the gap between new fluorescent reporters and the enabling imaging technologies will widen the application of fluorescent imaging. With better understanding of FP effects and function, we envision these tools to be extensively used in translational medicine. While FPs are not likely to be used in patients in the near future, they can still be used in ex vivo testing for drug discovery, fluorescent dyes in wearable monitoring tools, and new medical imaging tools to assess cell level information for tissue function.

Author Contributions

Wrote the first draft of the manuscript: ES. Contributed to the writing of the manuscript: ES, GN, YZ, SM, and SS. Agree with manuscript results and conclusions: ES, GN, YZ, SM, and SS. Jointly developed the structure and arguments for the paper: ES, GN, YZ, SM, and SS. Made critical revisions and approved final version: ES, GN, YZ, SM, and SS. All authors reviewed and approved of the final manuscript.

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