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Review

Quantitative *in situ* fluorescence imaging to unveil the morphological and functional heterogeneity of osteocytes

Ji-Won Lee [a], Tadahiro Iimura [a,b,c,*]

[a] Division of Bio-Imaging, Proteo-Science Center (PROS), Ehime University, Ehime 791-0295, Japan
[b] Artificial Joint Integrated Center, Ehime University Hospital, Ehime 791-0295, Japan
[c] Translational Research Center, Ehime University Hospital, Ehime 791-0295, Japan

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ABSTRACT

Background: With colorful and high-contrast images, fluorescence imaging has long been a powerful tool used to confirm existing hypotheses or ideas suggested by other experimental approaches.
Highlight: Since data acquired by fluorescence imaging contain numerical values for spatio-temporal information as well as fluorescence intensities, quantitative fluorescence imaging is considered a robust tool that can provide novel insights by combining spatio-temporal information and quantitative analysis.
Conclusion: This short review presents the applications of quantitative fluorescence imaging in histological bone sections and its contribution to osteocyte biology. In osteocyte biology, fluorescence imaging has provided novel evidence on osteocytes being a heterogenic population both morphologically and functionally. Current direction and future applications of quantitative fluorescence imaging are also discussed in this review.

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1. Introduction

Fluorescence imaging represents a considerable breakthrough in biomedical science by facilitating new ways of observing molecular and cellular function [1,2]. Though fluorescence imaging is widely used as a tool to confirm research hypotheses by high resolution and colorful images, the technology is advantageous in that quantitative data can be derived from the acquired images. There is no doubt that histological analysis has long been a powerful and informative approach providing spatial information in tissues; however, such analyses often lack quantitative information. The quantification of bio-molecules by biochemical and molecular biological assays is a common approach; however, these techniques do not provide spatial information since data regarding only the average levels of molecules of interest in tissue extracts are obtained. Fluorescence imaging with quantitative analysis is considered a compatible method by which to obtain spatial and temporal data as well as functional quantification. In this short review, we introduce the general methodology of quantitative

* Corresponding author at: Division of Bio-Imaging, Proteo-Science Center (PROS), Ehime University, Shitsukawa, Toon-City, Ehime 791-0295, Japan.
Tel.: +81 89 960 5507; fax: +81 89 960 5052.
E-mail address: iimura@m.ehime-u.ac.jp (T. Iimura).

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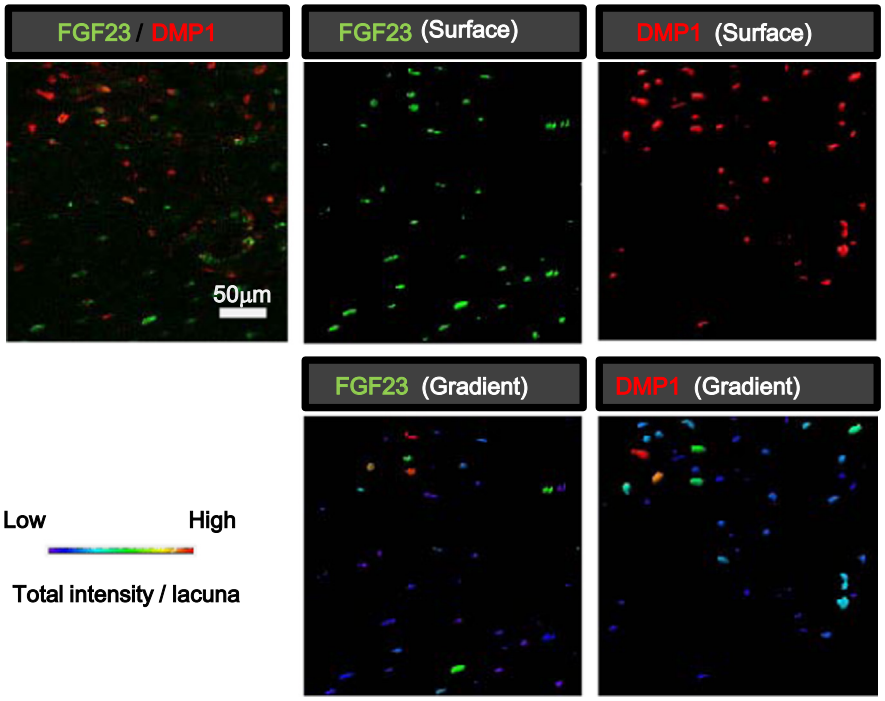

Fig. 1. Application of three-dimensional fluorescence morphometry to double immuno-staining reveals distinct spatial distributions and relative expression levels of FGF23 and DMP1. Confocal 3D-reconstituted images of the confocal z-series slices obtained from the sectioned femurs of 16-week old rats double immune-stained with fluorescently labeled FGF23 (green) and DMP1 (red) antibodies obtained from the femurs of 16-week old rats are shown. Scale bar: 50 μm. Surface rendering images of FGF23 (green), DMP1 (red), and merged signals are shown (upper middle, right, and left panels, respectively). Relative expression levels of FGF23 and DMP1 are demonstrated by graded colors (lower panels). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

fluorescence imaging and discuss its applications by reviewing our recent findings in osteocyte function.

2. Advantages of quantitative analysis of tissue sections by fluorescence imaging

In general, conventional and descriptive microscopic observation can provide limited information: for example, the presence or absence of particular molecules or phenomena and their localization can be ascertained from such analyses. Compared to such methods, fluorescence microscopy exhibits significantly improved resolution and enables investigators to easily binarize fluorescence signals, thereby allowing functional and more quantitative localization to be assessed. On the other hand, it is possible with fluorescence microscopy to select images for analysis that are not necessarily representative of the entire tissue or cell population, thereby generating unreliable or inaccurate data. Since cell populations are diverse within tissues and can be functionally heterogeneous in the same population, a cell selected for analysis may confirm a particular hypothesis which other findings have disproven or support contradictory idea, and thus such analyses may mislead scientific interpretation of collective data. To eliminate such inaccuracies, there is therefore an increasing demand for phenomena of interest to be demonstrated more broadly such as at tissue or organ level with high resolution images prior to conducting detailed, local analyses.

Fluorescence microscopy relies on charge-coupled devices (CCD) or photomultipliers (PMT) that capture visual information in a numerical form. Reliable quantitative data can thus be extracted from acquired fluorescence images and provide new ways in which molecular and cellular function can be analyzed to support qualitative findings. Single digitalized fluorescence images are composed of a number of pixels that contain numerical information regarding color (wavelength), fluorescence intensity, and spatial position. In principal, therefore, it is possible to carry out quantitative analyses of labeled molecules or cellular functions in a spatial manner, which in turn facilitates comparative analyses of numerical data sets and subsequent statistical analyses. These technical aspects of fluorescence microscopy can be advantageous in understanding biomedical phenomena in a comprehensive manner from tissue to subcellular levels.

3. Quantitative fluorescence imaging in osteocyte biology

Our research group has established several applications of quantitative fluorescence imaging in histological bone sections (Fig. 1). By measuring three-dimensional distributions and intensities of fluorescence signals in osteocytic lacunae, several unprecedented findings have been reported.

After simple tissue staining of frozen bone sections by fluorescent dies specific for nuclear DNA and cellular f-actin, three-dimensional quantitative analyses enabled measurements of the volumes, surface areas, and shapes of nuclei and cells to be carried out, as well as for the osteocytic cellular process and their branching points to be quantified [3]. These analyses revealed distinct shapes of osteocytes and the complexities of osteocytic process networks between flat and long bones, suggesting distinct functions and mechano-adaptations of bone due to different mechanical loading levels.

Sclerostin is a hallmark protein of osteocytes, which negatively regulates proliferation and differentiation of osteoblast, thus regulating bone formation [4–8]. Mechanical loading negatively regulates the expression of sclerostin and therefore, this molecule bridges mechanical loading to bone and bone metabolism [9–11]. Imaging of whole bone sections by confocal image tiling and successive three-dimensional fluorescence morphometry assessing the distribution of sclerostin in osteocytic lacunae has revealed the increasing expression of sclerostin during postnatal