Optimized Staining Methods of Intracellular Calcium for RAW264.7 Cells

Jingzhi Zhang[1], Ailing Yang[1], Yan Gao[2], Bo Huo[2*]

[1]Biomechanics Lab, Department of Mechanics, School of Aerospace Engineering, Beijing Institute of Technology, Beijing 100081, P. R. China
[2]Institute of Artificial Intelligence in Sports, Capital University of Physical Education and Sports, Beijing 100191, P.R. China

Abstract: Monitoring intracellular calcium ion levels accurately and efficiently is crucial for gaining a profound understanding of cellular functionality. Inappropriate staining conditions may result in deviations in fluorescent signals, which affects the accuracy and repeatability of experimental outcomes. To measure the concentrations change of Ca^{2+} accurately, immunofluorescent staining of intracellular calcium is an essential process. In order to solve the previous dyeing problem of our research group, we designed and performed an experimental scheme. The results indicated that a staining duration of 30 minutes with a dye concentration at a 1/1000 dilution ratio consistently yielded the most reliable and stable staining outcomes. The results of this study have established the foundation of the future experiments and may be further helpful to provide a valuable foundation for the development of therapeutic strategies targeting bone diseases.

1. Introduction

Bone is a dynamic system that responds to mechanical forces by continuously adjusting its structure through bone resorption and formation processes. Beginning with a group of scholars represented by the German orthopedist Wolff in the late 19th century[1], it has been found that the human body's movement imposes mechanical loads on the bones, and when the position and direction of the loads change, the bone structure will be adaptive remodeling, which is also known as Wolff's law of bone remodeling[2]. Bone remodeling is ultimately mediated by bone cells: osteoclasts and osteoblasts are the cells which carry out the resorption and formation of bone, respectively[3].

Numerous studies have focused on the response of bone tissue cells, including mesenchymal stem cells, osteoblasts, and osteoclasts, to mechanical stimuli such as pressure, fluid shear stress, and substrate stretching. When bone tissue experiences mechanical loading, it undergoes deformation, leading to the movement of interstitial fluid within the bone's cavities. This fluid flows across the surfaces of trabeculae or lacunae, exerting fluid shear stress (FSS) on the osteoclasts or osteoblasts. FSS turns out to be a direct factor to stimulate the growth of bone tissue and maintain the balance of bone remodeling, and has been recognized as the most important signal for bone tissue cells to feel mechanical stimulation. Our previous study have shown that osteoclast precursor cells have unique sensitivity to gradient fluid shear stress.

As one of the most versatile secondary messengers in cellular signaling, calcium ions (Ca^{2+}) regulate numerous aspects of cellular functions[4], including proliferation. Specifically, the distribution of intracellular calcium influences critical cellular behaviors such as migration, which is essential for the dynamic process of bone remodeling. Earlier research indicates a significant correlation between intracellular calcium dynamics and cellular migration patterns. For instance, there is a gradual increase in calcium levels from the front to the rear of migrating cells over longer periods, alongside brief calcium flickers exhibiting front-to-rear polarization[5-6]. Alterations in calcium signaling pathways can lead to dysregulation of bone cell activity, contributing to the pathophysiology of conditions such as osteoporosis, and other metabolic bone diseases.

Osteoclasts, which are specialized multinucleated cells, play a pivotal role in the resorption of various mineralized tissues including bone, dentin, and mineralized cartilage[7-8]. Compared with the research of osteoblasts and bone formation, the mechanism of the migration and localization of osteoclast precursors targeting the position of bone resorption are less well studied[9]. Our future research may contribute to a deeper understanding of the cellular/molecular mechanisms behind phenomena such as bone loss in microgravity or disuse conditions, sports injuries, and sports rehabilitation.

To study the intracellular Ca^{2+} distribution of osteoclast precursor RAW 264.7 cells, a Ca^{2+} indicator is needed for the experiments[10]. Therefore, we have to determine the suitable staining condition for our following experiments. In the present study, we used Oregon Green™ 488 BAPTA-1, AM to stain the intracellular Ca^{2+}, and the suitable condition of immunofluorescent staining was studied.

* Corresponding author: huobo@cupes.edu.cn

2. Materials and methods

2.1 Cell culture

In this study, osteoclast precursor RAW264.7 cells (Guangzhou Huatuo Biotechnology Co., Ltd, China) were maintained at 37 ℃ and 5% CO_2 in an incubator (Thermo Fisher Scientific, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 unit mL^{-1} of penicillin (Sigma, USA), and 100 unit mL^{-1} of streptomycin (Sigma, USA) .The medium was changed every 48 h. Culture RAW264.7 cells to 80% confluency before passage.

Table 1. Characteristics of calcium and nuclear indicators

Dye	Excitation	Emission	Color
Oregon Green	494 nm	523 nm	Green
Rhod-2 AM	552 nm	581 nm	Red
Hoechst 33342	350 nm	461 nm	Blue

2.2 Immunofluorescent staining imaging

In order to avoid the issue that calcium dye may uptake into organelles and determine the better condition when using intracellular Ca^{2+} staining dye Oregon Green(Invitrogen, USA), we have tested different conditions at different dyeing time(10 min, 20 min, 30 min) and dyeing concentration (dilution ratio:1/200, 1/500, 1/1000, 1/2000, 1/3000), but the concentration of Rhod-2 AM (for a fluorescent mitochondrial(represents for organelles) Ca^{2+} indicator, Abcam, England)and Hoechst 33342 (nuclear staining, Invitrogen, USA) remain the same. RAW264.7 monocytes were seeded on glass bottom of cell culture dishes (Nest, China) with 20 mm in diameter at a density of $1\times10^4/cm^2$. Cells were covered with medium solution containing 1 μM Hoechst 33342 dye, 4 μM Rhod-2 AM dye and Oregon Green (corresponding dilution). Then cells were incubated at 37 ℃ away from light for corresponding time. Following staining, the dye solution was discarded, cells were washed three times with PBS and following the addition of fresh medium, they were observed and captured under a confocal fluorescence microscope (NIS-Elements C2, Nikon) with a 100× objective at 3 different laser excitations (Table 1). 20-30 cells were quantified at each experiment.

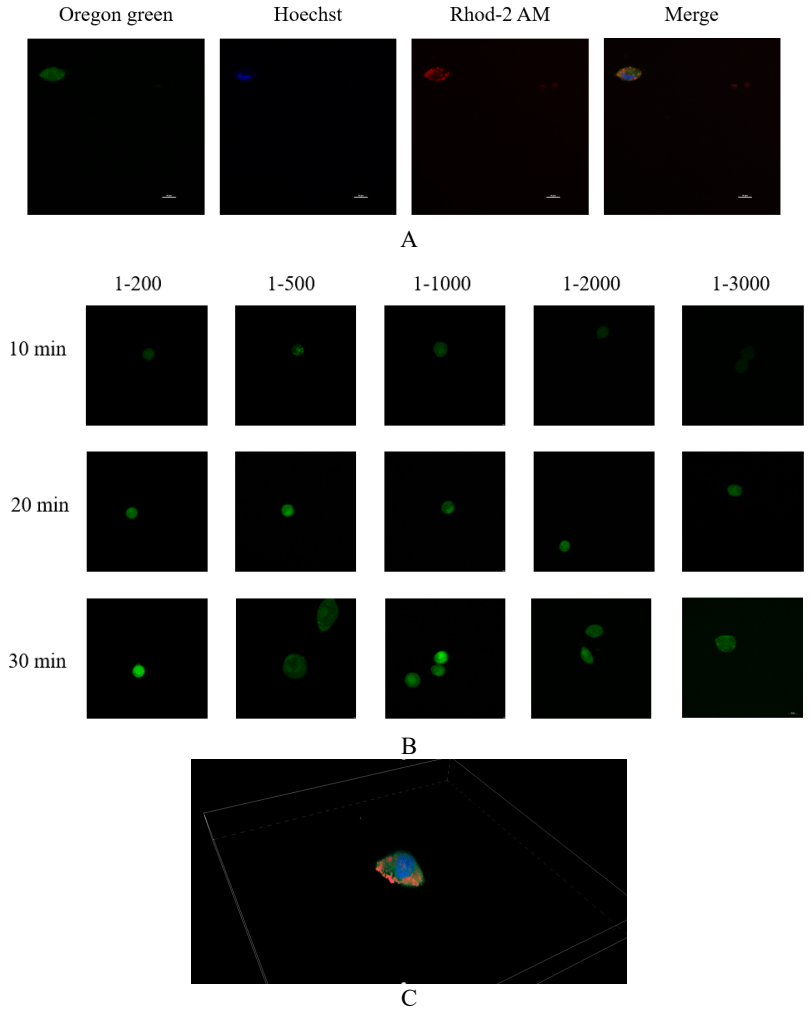

Figure 1. Immunofluorescent staining for RAW264.7 cells. (A) Typical images of cells stained with Rhod-2 AM, Oregon green and Hoechst; Scale bar, 10 μm. (B) Different intracellular Ca2+ example images under different staining situations; Scale bar, 10 μm. (C) A three-dimensional image acquired from Z- series direction.

2.3 Mean fluorescent intensity measurements of RAW264.7 cells.

The mean fluorescent intensity was further quantified using ImageJ software. We defined that MG stands for the mean fluorescent intensity measured under FITC channel's image and MR represents mean fluorescent intensity of ROI (particles recognized under TRITC channel & excluded nuclear regions (DAPI)) which was measured under FITC channel's image (Fig. 1A) and it also reflects the colocalization relationship between calcium in cytoplasmic and organelles. We defined the normalized ratio as MR/ MG, the relative fluorescence intensity, so that the effects of different concentration and time of dying can be eliminated. The normalized ratio also reflects the level that calcium dye uptake into organelles. Figure. 1B shows the outcomes of Ca^{2+} staining under corresponding situations. In addition, a three-dimensional image of cells is captured layer by layer in Z direction. (Fig. 1C).

2.4 Statistical analysis

The data were shown as mean±standard deviation (SD). Statistical differences among group means were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Unless specified, data were presented as mean±SD. Each experimental group was replicated at least three times. A P-value of less than 0.05 was indicated statistically significant.

3. RESULTS

Our study systematically examined the effects of varying staining conditions on the immunofluorescent detection of intracellular calcium in RAW264.7 cells, using Oregon Green™ 488 BAPTA-1 AM as a calcium indicator. By adjusting staining durations (10, 20, 30 minutes) and dye concentrations (1/200, 1/500, 1/1000, 1/2000, 1/3000 dilutions), we aimed to optimize the protocol for clear and accurate visualization of intracellular calcium distributions and to avoid the issue that calcium dye may uptake into organelles as much as possible.

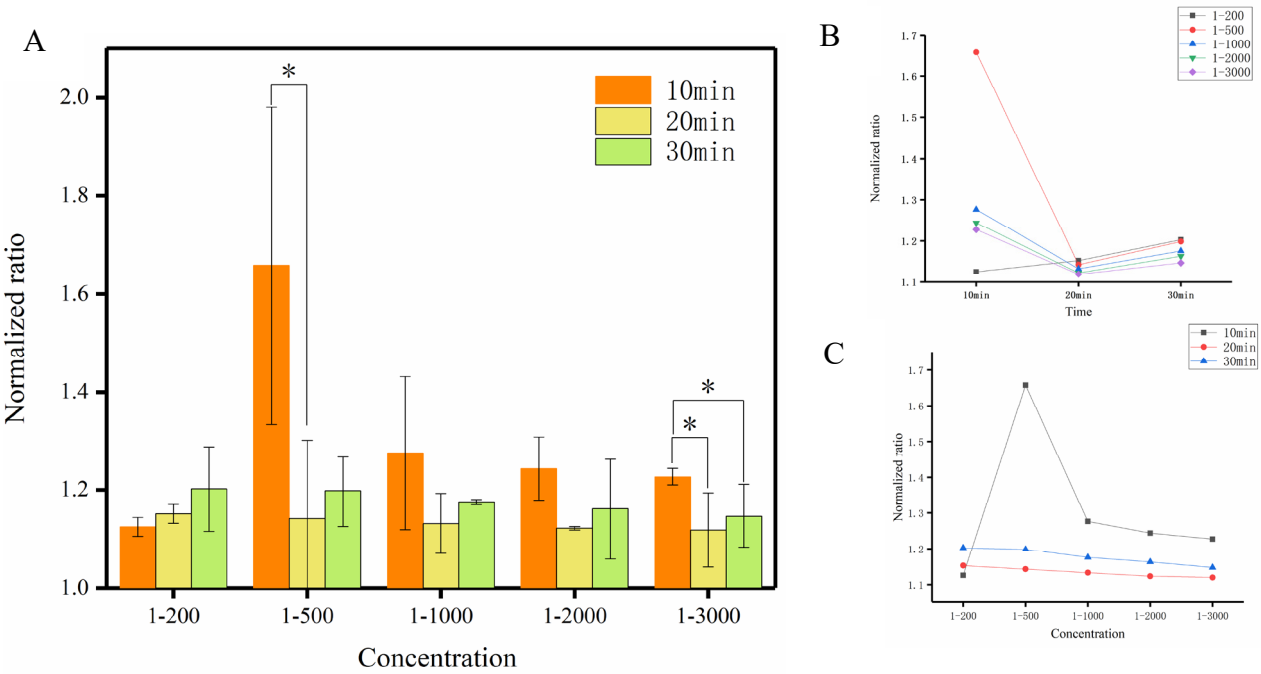

Figure 2. Normalized ratio of mean fluorescent intensity. (A) Overall histogram schematic diagram of different staining situations. *P < 0.05. Error bars are standard deviations. (B) Line chart using time as the horizontal axis. (C) Line chart using concentration as the horizontal axis.

3.1 Optimization of Staining Conditions

The normalized ratio of mean fluorescent intensity (MR/MG) was utilized to evaluate the efficacy of each staining condition. This approach allowed us to assess the extent of dye uptake into organelles, minimizing potential artefacts in calcium detection. Figure. 2A shows an overall decrease in normalized ratio as the concentration is lower each duration. The trend of change in normalized ratio is more pronounced (Figs. 2B and 2C). The normalized ratio (1/1000 dilution) was not statistically significant between 20 min and 30 min duration and 30 min duration revealed a better confidence interval, suggesting minimal error in the data. Our results indicated that a staining duration of 30 minutes with a dye concentration at a 1/1000 dilution ratio consistently yielded the most reliable and stable staining outcomes (Fig. 3). This condition facilitated a balanced dye uptake, enhancing the visibility of intracellular calcium while limiting nonspecific

organelle staining, which is helpful to solve the previous dyeing problem.

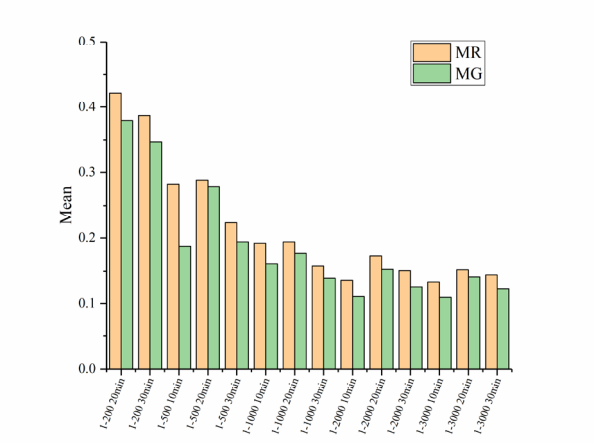

Figure 3. Absolute mean fluorescent intensity of MG and MR, respectively.

3.2 Intracellular Calcium Visualization

Under optimized conditions, we observed distinct patterns of intracellular calcium distribution. The fluorescent intensity measurements revealed significant variations across different experimental setups, underscoring the importance of methodological precision in calcium imaging studies. The three-dimensional image of cells, acquired from Z-series direction (Fig. 1C), provided further insights into the spatial distribution of calcium within the cell, highlighting the potential areas of active calcium signaling related to cell migration and mechanotransduction processes.

4. Conclusions

The findings of our study underscore the critical role of precise staining protocols in the immunofluorescent detection of intracellular calcium in osteoclast precursors. By establishing an optimized staining condition of 30 minutes duration at a 1/1000 dilution ratio for Oregon Green™ 488 BAPTA-1 AM, we have determined the methodology for studying calcium dynamics in RAW264.7 cells. This optimized protocol not only ensures accurate and reproducible measurements of intracellular calcium but also lays the foundation for our future research into the mechanism of osteoclast precursors migration under gradient fluid shear stress.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 12072034(BH)).

References

1 Wolff, J. Das Gaesetz der Transformation der Knochen. Berlin: Hirchwild (1892).

2 Roux, W. Gesammelte Abhandelungen uber die Entwicklungsmechanick der Organismus. Leipzig: Wilhelm Engelmann (1895).

3 Weivoda, M.M., Chew, C.K., Monroe, D.G., et al. “Identification of osteoclast-osteoblast coupling factors in humans reveals links between bone and energy metabolism,” Nat. Commun. 11 (2020).

4 Erkhembaatar, M., Gu, D. R., Lee, S. H., et al. “Lysosomal Ca^{2+} Signaling is Essential for Osteoclastogenesis and Bone Remodeling,” J. Bone Miner. Res. 32(2), 385-396 (2017).

5 Wei, C., X, Wang, et al. “Calcium flickers steer cell migration,” Nature, 457(7231), 901-905 (2009).

6 Wang, S. R., Sun, Q., Zhao, Y., et al. “Distribution of intracellular calcium during flow-induced migration of RAW264.7 cells,” Mechanobiology in Medicine, 1(2), 100012, (2024).

7 McDonald, M. M., Khoo, W. H., Ng, P. Y., et al. “Osteoclasts recycle via osteomorphs during RANKL-stimulated bone resorption,” Cell, 184(5), 1330-1347 (2021).

8 Henriksen, K., Bollerslev, J., Everts, V., et al. “Osteoclast activity and subtypes as a function of physiology and pathology--implications for future treatments of osteoporosis,” Endocr. Rev.32(1), 31-63 (2011).

9 Gemini-Piperni, S., Milani, R., Bertazzo, S., et al. “Kinome profiling of osteoblasts on hydroxyapatite opens new avenues on biomaterial cell signaling,” Biotechnol. Bioeng. 111(9), 1900-1905 (2014).

10 Islam, M. S. Advances in Experimental Medicine and Biology: Calcium Signaling, Springer Nature Switzerland AG, 1131 (2020).