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Validation of a simple and fast method to quantify *in vitro* mineralization with fluorescent probes used in molecular imaging of bone

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ABSTRACT

Alizarin Red S staining is the standard method to indicate and quantify matrix mineralization during differentiation of osteoblast cultures. KS483 cells are multipotent mouse mesenchymal progenitor cells that can differentiate into chondrocytes, adipocytes and osteoblasts and are a well-characterized model for the study of bone formation. Matrix mineralization is the last step of differentiation of bone cells and is therefore a very important outcome measure in bone research. Fluorescently labelled calcium chelating agents, e.g. BoneTag and OsteoSense, are currently used for *in vivo* imaging of bone. The aim of the present study was to validate these probes for fast and simple detection and quantification of *in vitro* matrix mineralization by KS483 cells and thus enabling high-throughput screening experiments.

KS483 cells were cultured under osteogenic conditions in the presence of compounds that either stimulate or inhibit osteoblast differentiation and thereby matrix mineralization. After 21 days of differentiation, fluorescence of stained cultures was quantified with a near-infrared imager and compared to Alizarin Red S quantification. Fluorescence of both probes closely correlated to Alizarin Red S staining in both inhibiting and stimulating conditions. In addition, both compounds displayed specificity for mineralized nodules. We therefore conclude that this method of quantification of bone mineralization using fluorescent compounds is a good alternative for the Alizarin Red S staining.

1. Introduction

During osteoblast differentiation collagen is deposited in the extracellular space. When Ca^{2+} and inorganic phosphate accumulate to form hydroxyapatite crystals, the matrix mineralizes to form bone [3,15,18]. *In vitro*, primary bone cells or bone marrow derived cells can be induced to form mineralized matrix when cultured in osteogenic medium [8]. KS483 cells, a murine mesenchymal progenitor cell line, can differentiate into chondrocytes, adipocytes and osteoblasts and are a well-characterized model for bone formation [5,19]. These cells are used for the study of many aspects of bone biology, as well as new drug targets for bone-related diseases [1,4,10].

The quantification of matrix mineralization in cell cultures is one of the most important indicators for successful differentiation. Currently, the most common method for the quantification of mineralization is the Alizarin Red S staining [12]. This staining can be quantified by extraction with hexadecylpyridinium chloride and spectrophotometric analysis [3].

IRDye® 800CW BoneTag™ and OsteoSense® 800 are fluorescently labelled probes with high affinity for bone currently used for *in vivo* imaging. BoneTag is a tetracycline derivate coupled to IRDye 800CW [7], while OsteoSense is based on the bisphosphonate pamidronate with an IRDye78 label [20]. Both compounds have an emission wavelength of 800 nm, reducing auto-fluorescence both *in vivo* and *in vitro* [17].

We now describe a simple and fast method for the quantification of mineralization in cell cultures using BoneTag and OsteoSense. To validate this method, results obtained with these compounds were compared to the well-established method of Alizarin Red S staining in different culture conditions. BoneTag and OsteoSense fluorescence correlated with Alizarin Red S staining quantifications in cultures after incubation with compounds that either stimulate or inhibit osteoblast differentiation and/or matrix mineralization. We therefore show the wide range of possibilities for use of these fluorescent probes for *in vitro* bone research.

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2. Materials and methods

2.1. Cell culture and differentiation

KS483 cells were cultured as described previously [5]. Briefly, cells were cultured routinely in α-MEM (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with penicillin/streptomycin (Sigma–Aldrich, St. Louis, MO, USA), Glutamax (Gibco) and 10% fetal calf serum (FCS; Greiner Bio One, Frickenhausen, Germany). For differentiation assays, KS483 cells were seeded at a density of 9200 cells/cm^2 in 12-wells plates. Every 3–4 days the medium was replaced. At confluence (from day 4 of culture onward) 50 μg/ml ascorbic acid (BDH Prolabo, VWR International, Radnor, PA, USA) and when nodules appeared (from day 11 of culture onward) 5 mM glycerol 2-phosphate disodium salt (βGP; Sigma–Aldrich, St. Louis, MO, USA) was added to the culture medium. When applicable, bone morphogenetic protein 6 (BMP-6; kindly provided by prof. S. Vukicevic, Department of Anatomy, School of Medicine, Zagreb, Croatia) was added at day 4 of culture or parathyroid hormone related protein (PTHrP; Bachem, Bubendorf, Switzerland) was added from day 4 onward.

2.2. Alkaline phosphatase measurements

Medium samples were taken every 3–4 days before medium change. Alkaline phosphatase (ALP) activity was measured by adding 200 μl of 120 nM *p*-nitrophenylphosphate (PNPP, ThermoScientific, Waltham, MA, USA) in 100 mM glycine/1 mM $MgCl_2$/0.1 mM $ZnCl_2$ buffer (pH 10.5) and measured for 10 min using a VERSAmax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. ALP activity was determined as the slope of the kinetic measurement (mOD/min).

2.3. Quantification of mineralization by fluorescence

At 24 h before analysis, the cell cultures were incubated with the calcium binding agent IRDye® 800CW BoneTag™ (BoneTag, Li-COR Biosciences, Lincoln, NE, USA) or the IRDye78 labeled bisphosphonate pamidronate OsteoSense® 800 (Perkin Elmer, Waltham, MA, USA), both at a concentration of 2 pmol/ml (1:10,000 dilution). After incubation the cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% buffered formaldehyde. The fixed cells were scanned with the Odyssey Infrared Imaging System (Li-COR) at a resolution of 42 μm, medium quality and intensity 5.0–6.5. Integrated intensity (counts-mm^2) of each well was calculated by the Odyssey software.

2.4. Quantification of mineralization by Alizarin Red

Mineralized nodules were fixed with 3.7% buffered formaldehyde, stained with 2% Alizarin Red S solution for 2 min and washed 3 times with H_2O. Pictures were taken using a Powershot A650IS Digital Camera (Canon, Ōta, Tokyo, Japan) through a light microscope at 5× magnification. For quantification, Alizarin was removed from the cell layer by overnight incubation with 10% hexadecylpyridinium chloride in 10 mM PO_4 buffer (pH 7.5), and measured at 550 nm on a VERSAmax Tunable Microplate Reader (Molecular Devices) against a range of Alizarin standards (0.005–0.8 μg/ml).

2.5. Statistics

Values represent means ± SD. Differences were tested by two-way (ALP measurements) or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test using Graphpad Prism 5 software (La Jolla, CA, USA). Nonlinear regression was performed for the correlation between Alizarin Red S staining and fluorescent markers. Results were considered significant at $p < 0.05$.

3. Results

3.1. Alizarin staining and BoneTag or OsteoSense fluorescence increase over time during differentiation

To validate the quantification of *in vitro* matrix mineralization using the fluorescent compounds BoneTag and OsteoSense, readouts of these compounds were compared to the well-established method of Alizarin Red S staining in different culture conditions. First, Alizarin Red S staining and fluorescent images were quantified at different time points during differentiation. Fig. 1A shows representative pictures of Alizarin Red S staining, BoneTag and OsteoSense fluorescence at each time point. ALP activity was measured in medium as a measure for osteoblast differentiation and increased over time (Fig. 1B). As shown in Fig. 1C–E, signals from all three readouts increased in intensity as the matrix becomes more mineralized over time. Both BoneTag and OsteoSense displayed slight background fluorescence. However, a significant correlation was observed between Alizarin Red S staining and both BoneTag ($R^2 = 0.73$, $p < 0.0001$; Fig. 1F) and OsteoSense ($R^2 = 0.93$, $p < 0.0001$; Fig. 1G). In addition, both fluorescent readouts appear to be more sensitive for small changes in mineralization than Alizarin Red S, as shown by the nonlinear correlation. Indeed, at day 18 of differentiation both BoneTag and OsteoSense were able to identify an increase in mineralization, while this was only apparent from day 21 with Alizarin Red S staining.

3.2. Correlation between Alizarin staining and fluorescence after modulation of differentiation

To validate BoneTag and OsteoSense quantification further, we examined whether the correlation between Alizarin Red S staining and BoneTag or OsteoSense fluorescence is still present after modulation of osteoblast differentiation. Therefore, KS483 cell cultures were stimulated with BMP-6 and inhibited with PTHrP [6]. BMP-6 stimulated ALP activity and increased Alizarin Red S staining and fluorescence dose-dependently (Fig. 2A-E). PTHrP dose-dependently decreased ALP activity and both Alizarin Red S staining and fluorescence (Fig. 3A–E). Correlation between both BoneTag and OsteoSense and Alizarin Red S staining was significant with $R^2 = 0.92$ and 0.75 ($p < 0.0001$), respectively, for cultures stimulated with BMP-6 (Fig. 2F–G) and $R^2 = 0.84$ and 0.92 ($p < 0.0001$), respectively, for cultures with PTHrP (Fig. 3F and G).

3.3. Specificity of staining for mineralized nodules

Next, we examined the specificity of BoneTag or OsteoSense binding to mineralized matrix by culturing KS483 cells without βGP. Without phosphate, the nodules did not mineralize as shown by the absence of staining in samples without βGP (Fig. 4A). ALP activity after stimulation with BMP-6 but without βGP is slightly increased compared to BMP + βGP (Fig. 4B). Staining of unmineralized nodules was minimal, even after stimulation with BMP-6 (Fig. 4A). Quantification of staining showed a large difference between conditions +βGP and −βGP in both Alizarin and BoneTag-stained samples. OsteoSense showed some background staining in samples without βGP, especially in the control condition (Fig. 4C–E). Fluorescence microscopy of stained nodules revealed the localization of fluorescence in the mineralized matrix (Supplementary Fig. 1).

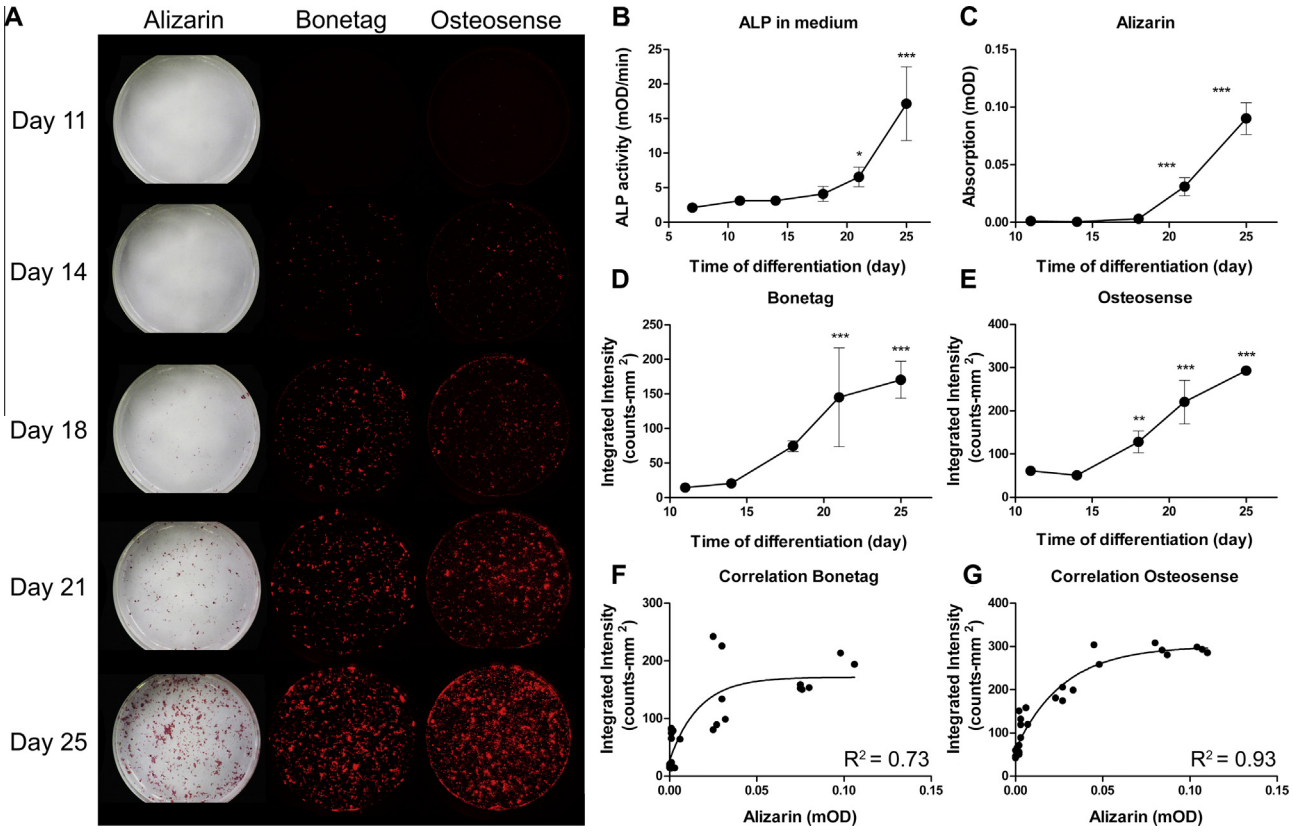

Fig. 1. Comparison of Alizarin Red S staining, BoneTag and OsteoSense in KS483 cell cultures during differentiation. (A) Representative photos of Alizarin Red S staining and BoneTag and OsteoSense fluorescence; (B) measurements of ALP activity; (C) quantification of Alizarin Red S staining; (D) quantification of BoneTag fluorescence; (E) quantification of OsteoSense fluorescence; (F) correlation between Alizarin Red S staining and BoneTag fluorescence. $R^2 = 0.73$, $p < 0.0001$; (G) correlation between Alizarin Red S staining and OsteoSense fluorescence. $R^2 = 0.93$, $p < 0.0001$.

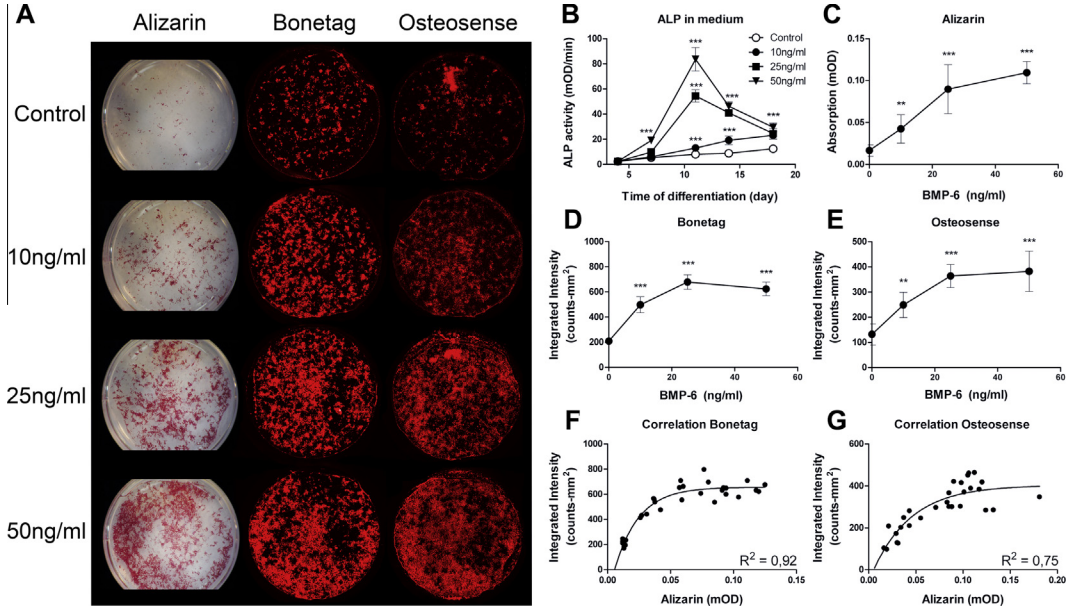

Fig. 2. Comparison of Alizarin Red S staining, BoneTag and OsteoSense in differentiated KS483 cell cultures after stimulation with different concentrations of BMP-6. (A) Representative photos of Alizarin Red S staining and BoneTag and OsteoSense fluorescence; (B) measurements of ALP activity; (C) quantification of Alizarin Red S staining; (D) quantification of BoneTag fluorescence; (E) quantification of OsteoSense fluorescence; (F) correlation between Alizarin Red S staining and BoneTag fluorescence. $R^2 = 0.92$, $p < 0.0001$; (G) correlation between Alizarin Red S staining and OsteoSense fluorescence. $R^2 = 0.75$, $p < 0.0001$.

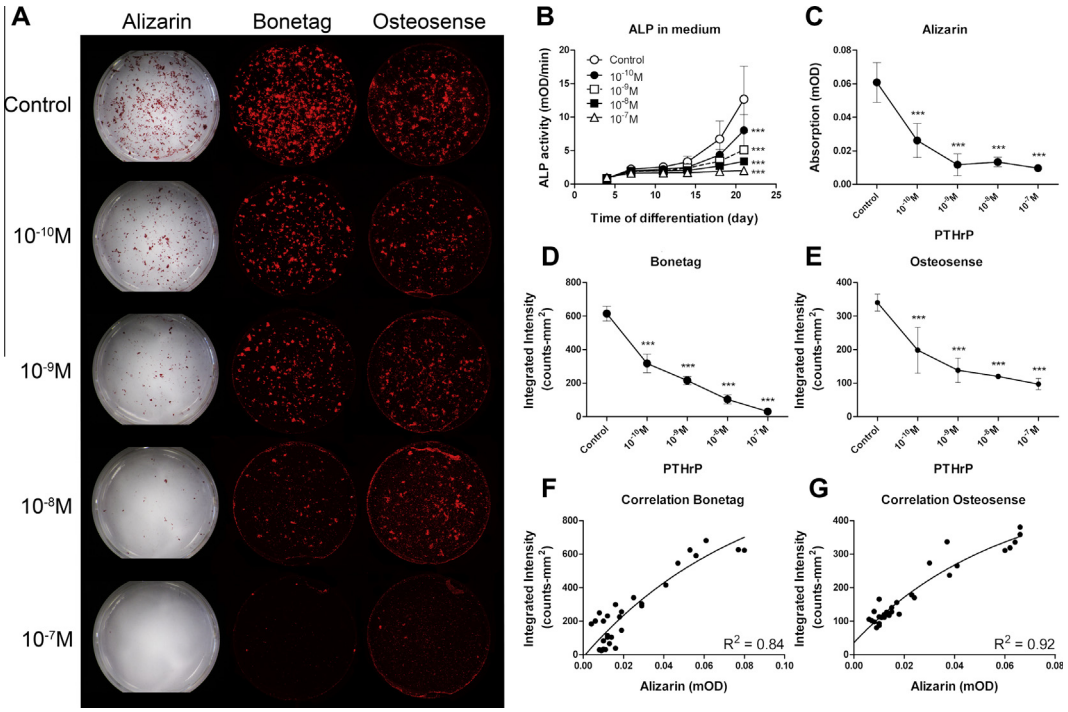

Fig. 3. Comparison of Alizarin Red S staining, BoneTag and OsteoSense in differentiated KS483 cell cultures after inhibition with different concentrations of PTHrP. (A) Representative photos of Alizarin Red S staining and BoneTag and OsteoSense fluorescence; (B) measurements of ALP activity; (C) quantification of Alizarin Red S staining; (D) quantification of BoneTag fluorescence; (E) quantification of OsteoSense fluorescence. (F) correlation between Alizarin Red S staining and BoneTag fluorescence. $R^2 = 0.84$, $p < 0.0001$; (G) correlation between Alizarin Red S staining and OsteoSense fluorescence. $R^2 = 0.92$, $p < 0.0001$.

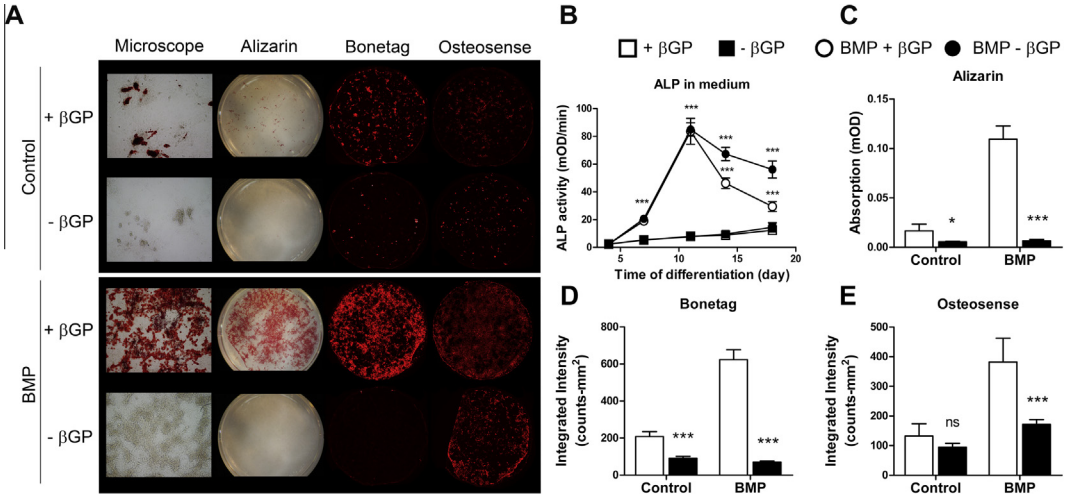

Fig. 4. Comparison of Alizarin Red S staining, BoneTag and OsteoSense in KS483 cell cultures differentiated in the presence or absence of βGP and/or stimulated with 50 ng/ml BMP-6 at day 4 of differentiation. (A) Representative photos of Alizarin Red S staining, light microscope images of alizarin stained samples at 100× magnification, and BoneTag and OsteoSense fluorescence; (B) measurements of ALP activity; (C) quantification of Alizarin Red S staining; (D) quantification of BoneTag fluorescence; (E) quantification of OsteoSense fluorescence. $^{*}p < 0.05$; $^{***}p < 0.001$ vs +βGP.

4. Discussion

The near-infrared fluorescent calcium binding agents BoneTag and OsteoSense are currently used for *in vivo* imaging of bone. To validate these agents for quantification of matrix mineralization in cell cultures, we compared the results of quantification with these compounds to the well-established method of Alizarin Red S staining after modulation of differentiation and matrix mineralization. In stimulating and inhibiting conditions both BoneTag and OsteoSense fluorescence correlated with the quantification of mineralization by Alizarin Red S staining. In addition, similar to Alizarin Red S staining, both fluorescent compounds showed selectivity for mineralized nodules.

Differentiation of osteoblasts and matrix mineralization were increased by culturing in the presence of BMP-6. Increased ALP activity with increasing BMP-6 concentrations is evidence of the stimulation of differentiation of osteoblasts [5,14]. Mineralization of the extracellular matrix increased dose-dependently when the cells were stimulated with BMP-6, which is also reflected by an increase in fluorescent signal of both BoneTag and OsteoSense. On

the other hand, PTHrP decreased ALP activity in a dose-dependent manner. Like PTH, PTHrP signals via the PTH receptor and the cyclic AMP pathway to inhibit osteoblast differentiation and thereby matrix mineralization [2,6,9,21]. Our results show a good correlation between Alizarin Red S staining and BoneTag or OsteoSense fluorescence after modulation with either BMP-6 or PTHrP. While the fluorescent stains were more sensitive to small changes, Alizarin Red S staining could be used in highly mineralized samples.

Specificity for mineralization was tested by culturing KS483 cells in the presence or absence of βGP. ALP activity dramatically increased after stimulation with BMP-6, and then declined after day 11 of culture. However, ALP activity remained high when the cells were cultured without βGP. The difference in staining quantification between conditions +βGP and −βGP in both control and BMP-stimulated conditions indicated that the staining was able to distinguish between mineralized and unmineralized nodules and thus indicates specificity. Like Alizarin Red S, BoneTag displayed low background staining in unmineralized nodules, while OsteoSense staining seemed to have a higher background and could not distinguish between + and −βGP without BMP-6 stimulation. This indicates that BoneTag has a higher specificity to the bone mineral compared to OsteoSense.

Bisphosphonates are compounds that bind with high affinity to bone and are used in the clinic for treatment of osteoporosis because of their ability to inhibit bone resorption by osteoclasts [11]. Because of their high affinity, bisphosphonates have a long half-life when bound to a mineralized matrix [11,13]. OsteoSense is based on the bisphosphonate pamidronate [20], and care must be taken when using this probe in bisphosphonate research, since competition between the administered bisphosphonate and OsteoSense staining may occur. This potential problem is not expected when using BoneTag, which is a tetracylin-based probe [7]. In addition, it is important to note that in our experience OsteoSense is stable for a limited period of time (up to 8 weeks), even when stored at −80 °C (data not shown), while BoneTag storage could be extended to over a year without loss of specificity.

Alizarin Red S is the standard staining for mineralized matrix in bone research. It can be dissolved and quantified by spectrophotometry. There are several advantages of using BoneTag and OsteoSense compared to Alizarin Red S staining:

(1) They are added in the culture medium prior to fixation of the cells, and require no additional washing or staining procedures.
(2) Both BoneTag and OsteoSense are available with fluorescence at 800 and 680 nm. The long wavelength of these probes makes them ideal for use with mineralized matrix as this greatly reduces autofluorescence.
(3) These probes are universal in use for *in vivo and in vitro* bone research as they can also be used for *in vivo* imaging of bone formation. They are commercially available as quality controlled validated batches, reducing variability.
(4) They can be quantified with great sensitivity using the dedicated Odyssey near-infrared imaging system.
(5) Because small amounts of dye are needed for staining, the dyes are very cost-effective; up to 12,000 samples can be stained with a single vial of 24 pmol.

Other options for fluorescent detection of *in vitro* mineralization may be xylenol orange or calcein blue. Wang et al. [16] have for example used these substances to evaluate mineralized nodule formation in living osteoblastic cultures. However, this method was used to count the number of positive nodules, and not to quantify mineralization. Furthermore, these fluorescent dyes emit blue or red light, which is sensitive to autofluorescence of the mineralized matrix.

In conclusion, BoneTag and OsteoSense are good substitutes with many advantages over Alizarin Red S staining. The results of both fluorescent compounds provide good correlation with Alizarin Red S staining in a range of different experiments stimulating and inhibiting differentiation and mineralization. BoneTag is more specific in conditions of low mineralization and can be stored for extended periods of time. The long emission wavelength in the near-infrared range reduces autofluorescence during detection. For both compounds numerous possibilities exist for combination with other fluorescent markers at different wavelengths, such as DNA stains. This opens up many possibilities for quantitative and high-throughput experiments in bone research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.055.

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