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Technical Note

Polychrome labeling of bone with seven different fluorochromes: Enhancing fluorochrome discrimination by spectral image analysis

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Abstract

Bone formation and remodeling in vivo can be assessed by polychrome labeling using calcium-binding fluorescent dyes. The number of fluorochromes, however, limits this technique due to the fact that with increasing number, fluorescent spectra inevitably overlap, which makes discrimination more difficult. In order to enhance discrimination, we performed spectral image analysis. Non-critical size defects of the femur of male Wistar rats served as a model for bone formation. Eight different fluorochromes (calcein blue, xylenol orange, calcein, alizarine complexone, doxycycline, rolitetracycline, hematoporphyrin, and BAPTA) were administered sequentially subcutaneously every third day starting at day 4 after surgery. Following, bone specimen were embedded in methylmethacrylate and analyzed by spectral image acquisition using a Sagnac type interferometer (ASI, Israel).

Seven of the eight applied fluorochromes could be resolved using spectral image examination. With BAPTA, we present a new fluorochrome suitable for bone labeling. Due to the superior sensitivity of the spectral image acquisition, the thickness of the bone sections could be reduced so that 5 μm thick sections could be analyzed. Spectral decomposition and subsequent linear unmixing allows depiction of each individual fluorochrome without interference of any other, enabling a reliable and superior morphometric analysis of labeled regions.
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Keywords: Polychrome labeling; Fluorochromes; BAPTA

Introduction

For several decades, the standard technique to analyze bone formation in vivo has been polychrome fluorescent labeling of bone using calcium-binding fluorochromes that are deposited at the site of active mineralization [1–4]. The different fluorochrome colors give sequential information regarding accretion and direction of bone formation. Due to the fact, however, that only a limited number of overlapping fluorescent colors can be distinguished by the naked eye or by conventional imaging systems, the number of time intervals normally available for investigation is restricted to four [5,6]. To overcome this limitation, we adapted the technique of spectral image acquisition, recently introduced for multicolor immunofluorescence of stem cells [6], to the analysis of polychrome fluorochrome labeling of bone. The first aim of our technical approach was to enhance the discrimination between different fluorochromes to better facilitate morphometric analysis. The second aim was to expand the number of fluorochromes and thereby the number of investigation time intervals for bone research in order to gain more detailed information of bone growth and regeneration.

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Materials and methods

Fluorochromes

Eight fluorochromes were used for this study: calcein blue, xylenol orange, calcein, alizarine complexone, doxycycline, rolitetracycline, hematoporphyrin, and a new fluorochrome BAPTA (1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid) which has not been established as a fluorescent marker of bone formation yet. All fluorochromes were purchased from Sigma-Aldrich (Munich, Germany). Details of the fluorochromes are given in Table 1. Before use, all fluorochromes were adjusted to pH values of 7.2 and sterilized by filtration.

Bone specimen

Four 12-week-old male Wistar rats weighing approximately 450 g were used in this study. Non-critical size defects (drill holes of 0.32 cm diameter), which included both lamellar and woven bone regions, were set unilaterally in the femur condyle. Post-operatively, the eight abovementioned fluorochromes were administered sequentially every third day starting at day 4 by subcutaneous injection. In order to test whether very similar fluorochromes could be discriminated, two additional rats received either only doxycycline or rolitetracycline for recording the respective pure reference spectra. On day 28, rats were sacrificed by an intraperitoneal injection of an overdose of pentobarbitone sodium. Immediately thereafter, the femurs (exarticulated in the hip and knee joint) were fixed in methanol for 4 days at 4°C, kept in acetone for 2 days, and then block-embedded in methylmethacrylate over 12 days. For further analysis, each specimen was then cut into 100 μm thick sections perpendicular to the bone defect using a saw microtome (Leitz, Wetzlar, Germany). In addition, 5 μm thick sections were cut from the same specimen in the same direction using a special microtome (Jung, Leipzig, Germany). For histological orientation, 100 μm sections were stained with hematoxylin–eosin.

Fluorescence microscopy

Bone sections were analyzed using a fluorescence microscope (Axiophot 2, Zeiss, Germany) with a XBO 75 xenon short-arc lamp. Appropriate fluorescence filter sets (a long pass emission filter (filter #01, Zeiss, Germany), a filter for red (#15, Zeiss) and green fluorescence (#09), a triple band pass filter set for green, red, and infrared (SKY, Applied Spectral Images (ASI), Israel) were used to excite all fluorochromes at their optimal wave length. Fluorescence microscopic images were acquired using a Sagnac type interferometer (SpectralCube SD-200, ASI, Israel) as well as a conventional digital camera with a fluorescence mode as control (Cybershot DSC S 75, Sony, Japan) under identical conditions form identical bone regions.

Spectral image analysis

Details of the principle of the Sagnac type interferometer have been published in previous papers [6,7]. Briefly, the fluorescence emission spectra passing through the barrier filter are split in the interferometer by a dichromatic filter in opposite directions and recombined by reflecting mirrors at the exit with an optical path difference (OPD). The OPD arises from the different optical paths traveled by the two beams in the beam splitter for non-zero angles. Applying the Fourier technique, the visible spectral region is analyzed by synchronizing the recording of successive charge-coupled device (CCD) frames with the steps of the motor used to rotate the collimated beam, so that the instantaneous OPD is known for every pixel in every recorded frame. This technique provides demarcation of wavelength ranges of 10 nm and less [7]. For image analysis, SpectraView Software (ASI, Israel) enabled linear unmixing based on decomposition of the image in its pure spectral components. The data acquisition time for each spectral image was dependent on the field of view and ranged between 120 and 150 s.

Results

Spectral image analysis

By spectral image acquisition and subsequent linear unmixing, seven of the eight applied fluorochromes could be differentiated and specified (Fig. 1): calcein blue, xylenol

Table 1
Details of the applied fluorochromes including day of application, source, dosage based on bodyweight, as well as spectral specification and references

Fluorochrome	Day	Specification	Dosage sc [mg/kg bw]	Max. excitation [nm]	Max. emission [nm]	References
Calcein blue	4	M1255	30	373	420–440	[12]
Xylenolorange	7	227854	90	440/570	610	[12]
Calcein	10	C0875	15	494	517	[13]
Alizarin complexone	13	A3882	30	530–580	624–645	[12]
Doxycycline	16	D9891	50	390–425	520–560	[14]
Rolitetracycline	19	R2253	25	390–425	520–560	[15]
Hematoporphyrin	22	H5518	300	530–560	580	[5,16,17]
BAPTA	25	A4926	75	200–325	410–550	

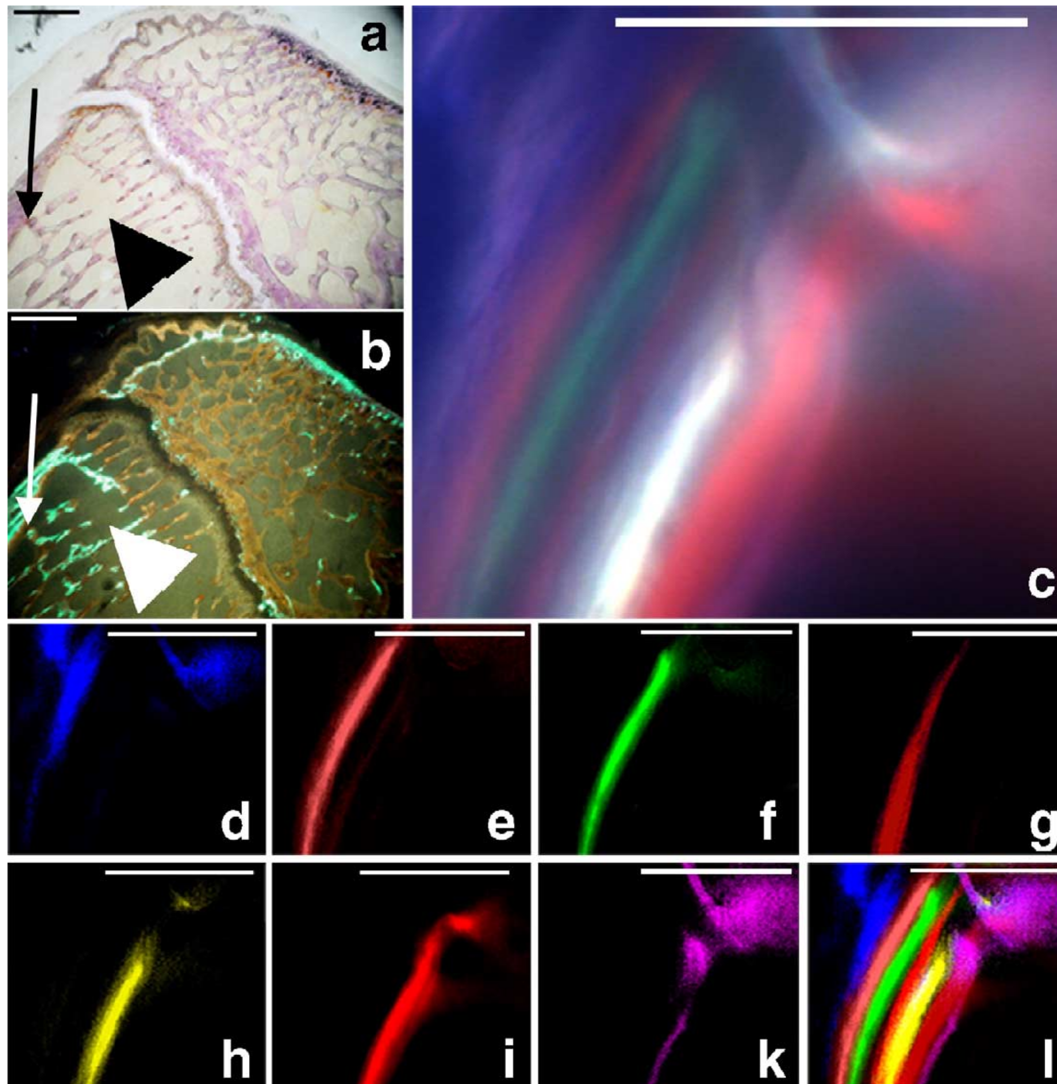

Fig. 1. (a) Hematoxylin–eosin staining of the rat femur condyle (scale bar = 1 mm, 100 μm thick bone section). Complete bony consolidation already occurred after 4 weeks. (b) Fluorescence image of the identical area using a filter set for green and red fluorescence (SKY, ASI, Israel). Big arrows mark the direction of the consolidated drill hole, narrow arrows indicate the magnified area of pictures c–l. (c) Spectral image of an eight-color fluorescent labeling of bone using a long pass emission filter (#01, Zeiss, Germany) (scale bar = 50 μm). (d–l) Single fluorochrome depiction after linear unmixing: (d) calcein blue, (e) xylenolorange, (f) calcein, (g) alizarin complexone, (h) doxycycline/rolitetracycline, (i) hematoporphyrin, (k) BAPTA, and (l) digital overlay of d–k (all scale bars = 50 μm). Note that no discrimination of the two applied tetracyclines was possible in (h).

orange, calcein, alizarine complexone, doxycycline/rolitetracycline, hematoporphyrin, and BAPTA.

However, by spectral image analysis, doxycycline and rolitetracycline revealed nearly the same fluorescent spectra with peaks at 527 and 530 nm, respectively, and could not be discriminated in the polychrome application.

In contrast, no single depiction of the different colors (in particular, the three different red fluorochromes as well as yellow and green) was possible using different filter sets and a digital camera only.

Sensitivity

Microscopic fluorescence investigation of the bone sections revealed a superior sensitivity of spectral image acquisition regarding color discrimination and brightness compared with conventional systems. Consequently, the thickness of the bone sections could be reduced to 5 μm.

Moreover, the sensitivity of the spectral image analysis enabled an investigation of fluorescence after 6 months. While six different fluorescent bands (xylenolorange, calcein, alizarine complexone, rolitetracycline/doxycycline, hematoporphyrin, and BAPTA) were visible by spectral analysis, only four bands (xylenolorange, calcein, rolitetracycline/doxycycline, and BAPTA) could be detected using appropriate filter sets with the naked eye or conventional systems (Fig. 2).

In spite of the prolonged exposure time for spectral imaging, no photo bleaching of the applied fluorochromes was observed during image acquisition. Fluorochrome

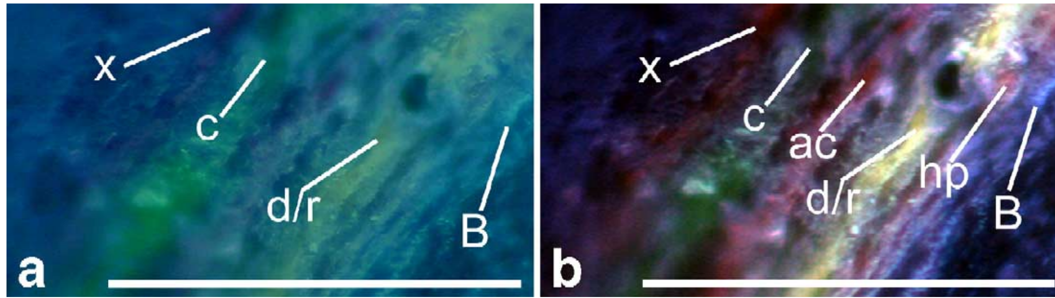

Fig. 2. 5 μm thick bone section of the same area as in Fig. 1 by (a) conventional photography and (b) spectral image acquisition 6 months after embedding of the bone specimen. (a) Only four bands were distinguishable in the conventional image and fluorescence of xylenolorange was hardly detectable. (b) Except for calcein blue (due to fading after 6 months), all fluorescent bands were visible by spectral image analysis (scale bars = 50 μm).

fading was observed for calcein blue after 6 months (specimen kept in dark at room temperature).

BAPTA

With the calcium chelator BAPTA, we present a new fluorochrome for in vivo labeling of bone. Spectral analysis revealed a wide-ranging emission spectrum with a peak at approximately 512 nm and a broad shoulder towards the blue spectrum resulting in a light blue fluorescence using a long pass emission filter necessary to optimally excite this fluorochrome. The light blue fluorescence of BAPTA was clearly distinguishable from calcein blue revealing a narrow peak at 426 nm (Fig. 3). No photo bleaching of BAPTA was observed during the investigation. No fading of BAPTA fluorescence was found after 6 months.

Discussion

The technique of polychrome fluorochrome labeling is used in order to investigate bone formation and remodeling processes in vivo at different time intervals [2,8,9]. Due to the fact, however, that clear discrimination of even four fluorescent spectra is not always possible by the naked eye or by conventional imaging methods (despite of using different filter sets) [6], this technique is limited in certain aspects. Four fluorochromes, meaning four investigation time points, are not sufficient to address detailed questions of bone regeneration or osseointegration processes properly. With our technical approach, the number of different fluorochrome markers could be increased to seven, leading to more detailed analysis of sequential bone formation. Further, by linear unmixing, single fluorochrome depiction and thus a more precise analysis of fluorescence location, especially in areas where fluorochromes overlap, is now possible. Using appropriate software, a superior morphometric quantification of the labeled bone area can be performed. The limitation of our approach was reached in the discrimination of the two applied types of tetracycline revealing spectral peak differences of only 3 nm.

Because conventional methods do not allow visual depiction of overlapping fluorescent colors (especially red, orange, and yellow spectra), it so far was necessary to keep certain time intervals between the applications of the single fluorochrome markers (normally one week or more) [2]. Considering the fact that crucial steps in bone remodeling take place within the first few days, a shorter period between the application of the markers is of high importance in bone research [9,10]. This is evident in particular for osseointegration of implants or for healing of small bone defects. Our technique allows fluorochrome analysis with a time interval of only 3 days.

A further benefit of the presented technique is the superior sensitivity. On the one hand, this may be used to reduce the dosage of the applied fluorochromes, which could be of particular interest in the case of hematoporphyrin, because of its known toxicity [5]. On the other hand,

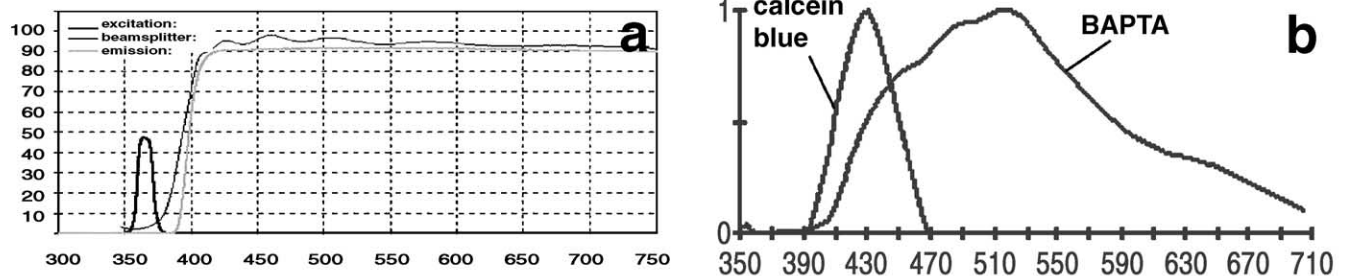

Fig. 3. (a) Details of the long pass emission filter set (#01, Zeiss, Germany), with excitation at wavelength 365 nm, emission at 380-nm, and beamsplitter at 390. (b) Details of the applied spectra of calcein blue and BAPTA.

much thinner bone sections can be investigated, leading to more detailed analysis of bone formation. In this study, 5 μm thick bone sections gave sufficient fluorescent signal for 6 fluorochromes, even after 6 months. In addition, due to the higher sensitivity, fluorescent analysis is possible over a longer period than by conventional analysis systems.

With BAPTA, a calcium indicator useful for in vivo applications [11], we present an additional fluorochrome suitable for the labeling of bone which has not been used for this purpose before. Its light blue emission spectrum gives a sufficiently intense fluorescent signal that is clearly distinguishable from calcein blue or calcein without spectral color discrimination. Because no fading or photo bleaching was found, this fluorochrome might serve as a substitute for calcein blue for which these phenomena have been described and observed in this study.

In conclusion, using spectral image analysis, we were able first to enhance fluorochrome discrimination, second to increase the number of investigation time intervals, third to analyze thinner bone sections, and fourth to extend the period for fluorescence analysis. This methodological progress may provide a basis for further investigations into the details of bone formation and regeneration.

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