Method Article

Video-based calcification assay: A novel method for kinetic analysis of osteogenesis in live cultures

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A B S T R A C T

Traditional methods of quantifying osteoblast calcification in culture require the use of calcium sensitive dyes, such as Arsenazo III or Alizarin Red S, which have been successfully used for decades to assess osteogenesis. Because these dyes elicit a colorimetric change when reacted with a cell lysate and are cytotoxic to live cells, they forfeit the ability to trace calcification longitudinally over time. Here, we demonstrate that image analysis and quantification of calcification can be performed from a series of time-lapse images acquired from videos. This method capitalizes on the unique facet of the mineralized extracellular matrix to appear black when viewed with phase contrast optics. This appearance of calcified areas had been previously documented to be characteristic to the formation of bone nodules in vitro. Due to this distinguishable appearance, extracting the information corresponding to calcification through segmentation allowed us to threshold only the pixels that comprise the mineralized areas in the image. Ultimately, this method can be used to quantify calcification yield, rates and kinetics facilitating the analyses of bone-supportive properties of growth factors and morphogens as well as of adverse effects elicited by toxicants. It may also be used on images that were acquired manually.

- The method is less error-prone than absorption-based assays since it takes longitudinal measurements from the same cultures
- It is cost effective as it foregoes the use of calcium-sensitive dyes
- It is automatable and amenable to high-throughput and thus allows the concurrent quantification of multiple parameters of differentiation

Specifications table

Subject Area:	Agricultural and Biological Sciences
More specific subject area:	In vitro osteogenesis of stem cells
Method name:	Video-based calcification assay
Name and reference of original method:	Morphometric image analysis
	zur Nieden NI, Price FD, Davis LA, Everitt RE, Rancourt DE. Gene profiling on mixed embryonic stem cell populations reveals a biphasic role for beta-catenin in osteogenic differentiation. Mol Endocrinol. 2007;21(3):674-85.
	zur Nieden NI, Davis LA, Rancourt DE. Comparing three novel endpoints for developmental osteotoxicity in the embryonic stem cell test. Toxicol Appl Pharmacol. 2010;247(2):91-97.
Resource availability:	N/A

Method details

The aim of the current method is to add to the growing body of work regarding image analysis of a sequence of micrographs in an automated manner to analyze biologically relevant events in stem cells as they undergo osteogenic differentiation. The specific stem cell culture system used here were human embryonic stem cells (H9, WiCell) and human induced pluripotent stem cells of the Riv4 line, generated by the University of California Riverside's Stem Cell Core Facility [1,2].

Both cell lines were maintained undifferentiated on Matrigel (BD Biosciences) treated 6-well tissue culture plates in mTeSR 1 medium (Stem Cell Technologies) as feeder-free cultures at 37°C with 5% CO_2. Colonies were passaged every 5 days at a 1:6 ratio using accutase treatment (2– 4 min at room temperature) and a cell scraper to dislodge cell clumps from the plastic. Cell banks of karyotyped cells were generated, and cells only used within passages 3–10 after thawing. To prepare cells for image acquisition, cells were passaged into 48-well plates as described above and let grown for 5 days in mTeSR1. Differentiation was then initiated without changing the culture plates by switching to Dulbecco's Modified Medium (DMEM, Gibco Thermofisher, Waltham, MA) supplemented with 15% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, selected batch), 1% non-essential amino acids (NEAA; Gibco), 1:200 penicillin/streptomycin (Gibco), and 0.1 mM β- mercaptoethanol (Sigma-Aldrich) as described [2]. Cells were either differentiated in these conditions throughout (non-osteogenic) or osteogenesis was induced by supplementing the medium with 10 mM β-glycerophosphate (Sigma), 50 μg/ml ascorbic acid (Sigma-Aldrich), and 50 nM 1,25(OH)$_2$ Vitamin D_3 (Calbiochem, MilliporeSigma, Burlington, MA). Throughout the differentiation, culture plates were incubated inside the Nikon Biostation CT, a hybrid and automated incubator that contains a phase contrast microscope and maintains the culture at 37°C with 5% CO_2. Phase contrast images were taken every 12 h for a period of 20 days from 10 separate areas within the culture plate. Images were assembled to create time-lapse videos (supplemental video 1).

Time lapse videos were separated into a series of images and segmentation techniques applied to segment calcified areas from the individual phase contrast images using Matrix Laboratory (MatLab) program as described below. Remaining pixels were counted to quantitatively represent the degree of calcification from each image. To determine the calcification rate the amount of calcified pixels from each image at a given time (t_x) were subtracted from the amount of calcified pixels at t_0 and divided by the hours of elapsed time. All video bioinformatic experiments were run in biological triplicate and video bioinformatic assessment was performed from a total of 30 areas within three independent culture wells. For comparative purposes, quantification of calcium using Arsenazo III was performed according to published protocols [3] on an n of 5. Statistical assessment was performed with a t-test when appropriate (http://www.graphpad.com/quickcalcs/ttest1/) or One-Way ANOVA with Holm-Sidak
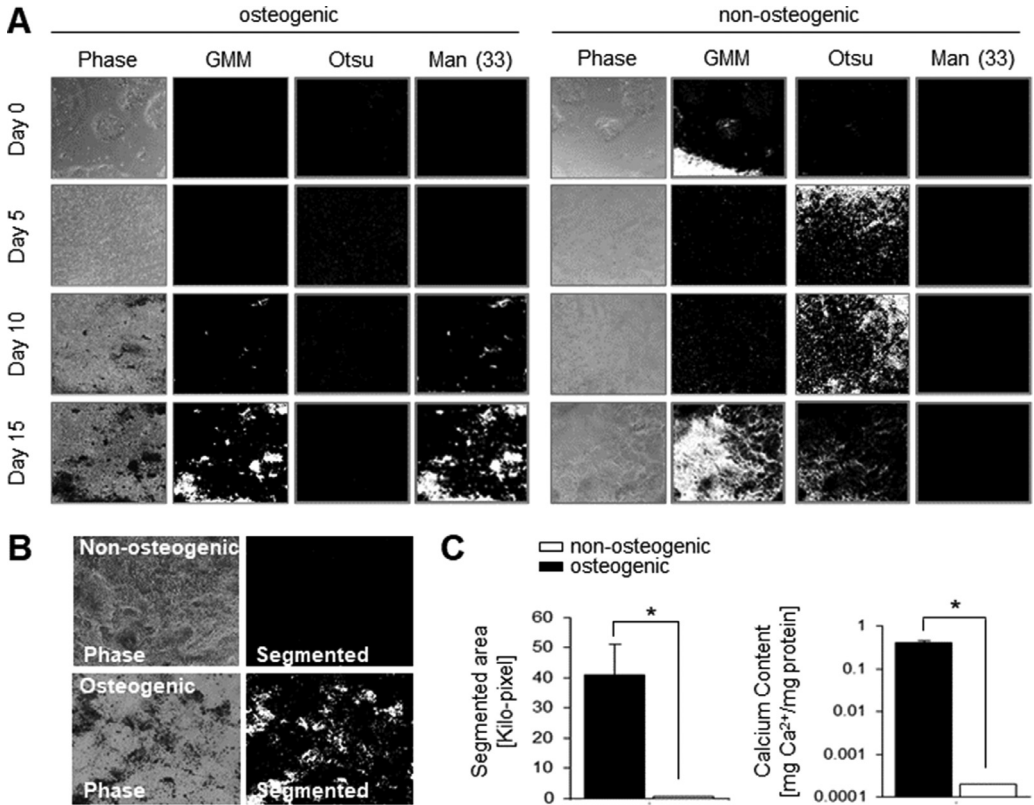
Fig. 1. Development of an image-based kinetic calcification assay. (A) Calcified matrix from phase contrast micrographs of 20-day-old osteogenic and non-osteogenic (control) cultures was segmented by thresholding pixels according to three different segmentation techniques based on the last image in the sequence and then applied to all images from earlier time points. Only the manual thresholding was successful in capturing the calcified areas only. (B, C) Measurement of pixels (≤ 33) in images of d20 cultures (B) representing calcified areas, n = 3 biological replicates (10 technical replicates ea) $\pm$ SD were compared to results of an Arsenazo III-based Ca^{2+} assay [3] (C) from separate samples, but on the same differentiation day. $^*p < 0.05$, student's t-test. GMM, Gaussian mixture model; Man (33), manual with a threshold of 33.

posthoc test when multiple groups were compared (SigmaPlot). A P-value below 0.05 was considered significant.

Method validation

The last collected phase contrast micrographs (d20) of differentiating H9 human embryonic stem cells were processed to segment out the calcified regions of interest from the background. In an 8-bit image, all pixels within the matrix range from 0 to 256 value of pixel intensity with 0 being defined as the darkest shade of black and 256 representing the brightest shade of white, allowing us to segment areas of calcification from phase contrast images using thresholding.

Initially, Gaussian Mixture Model and Otsu Thresholding segmentation techniques were applied to create an automated threshold to isolate calcified areas from images (Fig. 1A). These methods rely on finding threshold values that separate two Gaussian curves, however most of the images produced by the machine contained too many gray areas resulting in a singular Gaussian curve. Due to this computational error, areas in control cultures that were not calcified were called as calcified and those that were calcified in the osteogenic cultures were not called. To eliminate this error, a manual

threshold limit was then identified. This was accomplished by segmenting the same d20 image with different pixel intensity value thresholds and comparing the resulting segmentation to phase contrast images by eye, to validate that the segmentation would only pick up the dark osteogenic clusters. We have previously identified these dark areas to stain positively with Alizarin Red S, to be immune-positive for an antibody against osteocalcin, a protein uniquely associated with bone tissue, and dissolvable in ethylenediaminetetraacetic acid [2,4–6]. The same threshold was then applied to a d20 non-osteogenic image to confirm it did not pick up false positive pixels. The application of this manual threshold on images from day 20 of differentiation led to the retention of pixels that made up the calcified areas on the micrographs only (Fig. 1B). When comparing to images from the control cultures, application of this segmentation method resulted in little to no extracted data (Fig. 1A), correctly representing the absence of black deposits in phase contrast. Grey coloration, which appear in areas where cells grow in a three-dimensional manner, are correctly disregarded by this thresholding, as they do not represent calcification. These correctly segmented remaining pixels were then quantified to represent the degree of calcification (Fig. 1B). The image-based quantification showed a ~20-fold increase in calcium content between osteogenic and the non-osteogenic control cultures displaying a similar trend in calcification as was found with the reagent-based Arsenazo III calcium assay (Fig. 1B).

Pixel intensity versus pixel count

We next evaluated whether the assessment of pixel intensity rather than using an average pixel count increased the sensitivity of our method by using a human induced pluripotent stem cell line (Riv4) (Fig. 2A), which had known deficits in osteogenic lineage differentiation efficiency [2]. When evaluating H9 cells and Riv4 cells (Fig. 2B,C), only the pixel count picked up the difference between the non-osteogenic and osteogenic cultures, not the average pixel intensity. Additionally, only the pixel count showed the reduction in calcification in the Riv4 line. This suggested that our approach of using pixel counts was more sensitive as opposed to quantifying calcification based on changes in pixel intensity. When we charted the pixel count at each gray value intensity against its corresponding pixel intensity (Fig. 2 D,E), it became apparent that H9 cells not only calcified more, but also more intensely compared to Riv4 cells, a feature that was equally well detected by the calcification rate (Fig. 2F). Thus, these data show that the image-based calcification assay was sensitive to detect differences in calcification, which is relevant to the comparison of different stem cell lines, differentiation conditions and toxicological studies.

Calcification rate

To assess differentiation kinetics even further, we next determined the calcification rate of the cultures from the time-lapse video, a property that is not readily available from the classic reagent-based method of quantifying calcium in culture. First, segmentation was applied to all images in the kinetic sequence (supplemental video 2). Then, the calcification rate was deduced from the calcium content in pixels measured at t_x from t_0 and divided by the hours passed. This calculation resulted in the number of pixels added every hour. Based on this calcification rate, we could determine that the amount of calcification added to the extracellular matrix of the H9 cells accelerated as time progressed (Fig. 2F), while calcification in Riv4 cells stalled around d10. Thus, this study has shown that the image-based calcification assay can be used to successfully characterize the differentiation potential associated with different cell lines.

Additional information

Disadvantages of currently used methods

Classical methods of calcium quantification often fail to properly identify or quantify osteoblasts once mineralization, a final step in their genesis, has begun. For example, generating single cell suspensions for flow cytometry is hindered by the very fact that the cells of interest are deeply
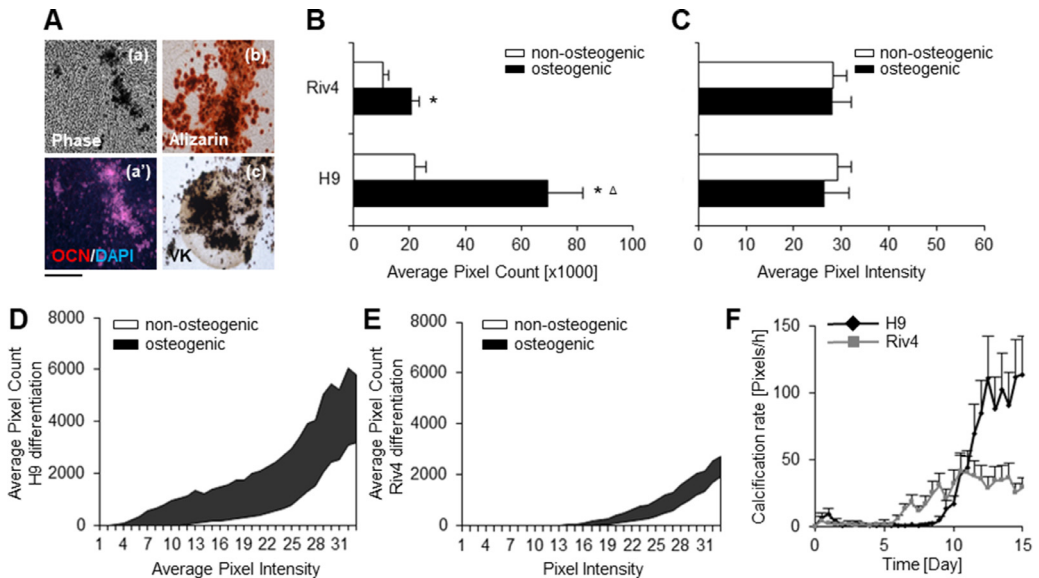
Fig. 2. Pixel count is a more representative measurement compared to pixel intensity after manual threshold segmentation. (A) Osteoblast presence was verified on day 20 after differentiation induction by immune reactivity against an anti-OCN antibody antibody ((a) and (a')). Calcification was also identified based on Alizarin Red S (b) and von Kossa (vK) staining (c). Scale bar = 20 μm. (B) Comparison of calcification in H9 human embryonic stem cells and Riv4 human induced pluripotent stem cells based on pixel counts at d15 of differentiation against a non-osteogenic culture. $*$ $p < 0.05$, One Way ANOVA versus non-osteogenic, $^{\Delta}$ $p < 0.05$, One Way ANOVA versus Riv4, n=3 biological replicates (10 technical replicates ea) ± SD. (C) Comparison of calcification properties based on differences in pixel intensities found in d15 cultures. (D, E) Differences in calcification between osteogenic and non-osteogenic cultures derived from H9 human embryonic stem cells (D) and Riv4 human induced pluripotent stem cells (E) by charting the pixel counts for each pixel intensity value (area under the curve). (F) Calcification rate as deduced from average pixels added per hour, n = 3 biological replicates (10 technical replicates ea) ± SD. Positive error bars (standard deviation) shown only for better graph readability.

embedded in hydroxyapatite preventing required separation of cells and matrix. Thus, to evaluate the success of a given differentiation, maturation of osteogenic cultures is typically assessed by qualitative staining for protein biomarkers found exclusively in the bone matrix, such as osteocalcin (OCN) [7,8]. The ability of osteoblasts to calcify the extracellular matrix can also be confirmed by calcium specific stains, such as von Kossa [9,10]. Quantitative methods include measuring calcium levels from osteogenically induced cultures through a reagent-based assay utilizing either fluorescent (i.e. calcein, coelenterazine, dehydrocalcein fluo-3, fura-2, indo-1 and rhod-2) or absorbent calcium sensitive dyes (e.g. Arsenazo III, 2,2'-bisbenzenearsonic acid, Alizarin Red) that are excellent at detecting and quantifying intracellular and matrix-bound calcium [3,10–12]. As the latter category quantifies the amount of deposited calcium, these dyes can be used to characterize the success of osteogenesis between different sources of stem cell lines [13,14], and the effects of growth factors [15], morphogens [16,17], toxicants [18,19], or chemicals [15,20] on osteogenesis.

However, because of their cytotoxic nature, traditional quantification of calcium with dyes are typically terminal experiments. For example, Arsenazo III has been identified to inhibit calcium transport and ATP hydrolysis [21]. Therefore, such an in vitro measurement of calcium is only a snap-shot of how much calcification there is at the time the sample is measured. Any time-course assay used to assess the calcium deposition in live cultures hence requires measuring different samples at different stages of the calcification process. The technique is thus inherently variable and, consequently, prevents the observation of calcification as a complete process from a single live sample. Thus, novel methods for quantifying calcium from live cultures without destroying them are of great importance for studies involving developing osteoblasts. Our lab has used the physical black

appearance of the calcified matrix in culture from a time course of still bright-field images to quantify the amount of calcium deposited by embryonic stem cells as they differentiate and when exposed to xenobiotics [22–26]. However, the quantification of these traits requires a great deal of man power and time to accomplish satisfactory comparisons [27]. Thus, image analysis if performed is often used on a small scale using a limited number of images to process.

Advantages of the video bioinformatic approach

To overcome the limitations of obtaining quantifiable data from single images alone, we exploit here the signature black appearance of calcified matrix in such developing cultures to kinetically and longitudinally analyze calcification from images using image analysis software. Extracting the information corresponding to a calcified area through segmentation allowed us to threshold only the pixels that comprise the mineralized areas in the image. Therefore, the image-based calcification assay does not require the addition of calcium sensitive reagents, which could be toxic to live cultures [28]. In doing so, the time-lapsed image-based calcification assay that is presented in this study analyzes the complete calcification process as a whole from a single culture measured longitudinally over time.

During the development of this image-based calcification assay, we characterized a novel kind of calcification measurement: the calcification rate, which is defined as the amount of calcium – in the form of pixels corresponding to segmented calcium – added into the matrix over time. This endpoint is important, since calcium deposits may need time to build up and their presence requires the prior existence of cells capable of forming the matrix. Hence the calcium amount that is present at a given time point is a function of osteoblasts previously found in the culture. The calcification rate determined that H9 human embryonic stem cells exhibited a greater, yet delayed level of calcification than Riv4 human induced pluripotent stem cells and that the osteoblasts derived from H9 cells had a faster calcification rate than the Riv4 cells. This suggested that even though H9-derived osteoblasts emerged later, they compensated by having a faster rate of calcification resulting in more calcified matrix in culture by the end of the study. The calcification rate also discovered the specific time window the developmental progression diverged between the two cell lines and facilitates more targeted follow-up studies into the underlying reason for this difference at preceding time points.

Considerations when applying video bioinformatic-based calcification assays

One drawback of the developed assay is that it is not able to discern why calcification is increased or reduced. For instance, a decrease in calcification could be a function of less osteoblasts secreting the same amount of matrix per cell, or the same amount of cells secreting less matrix. In this however, the new method is similar to any other methods that quantify calcification. Nonetheless, the assay is able to pick up differences in calcification between two or more sample groups, which may be a useful endpoint in identifying molecules that are pro-osteogenic and in the screening of bone toxicants.

An additional consideration is the inherent variability of calcification yield between different experiments, as is shown in Fig. 3. While this variability may seem to be a function of the image-based assay, the same variability is noted when performing absorption-based assays with calcium-sensitive dyes or when examining gene expression patterns associated with osteogenesis [2]. Hence, we conclude that this variability appears to be grounded in the fact that pluripotent stem cells are capable of differentiating into all three germ layers and will do so, even under selection pressure exerted by 'induction protocols' such as the one used herein. Such induction protocols will select for a specific cell type, but only rarely eradicate all other alternative cell fates. Similarly, promoters necessary for specific differentiations may be hypo- or hypermethylated under specific culture conditions [2] or their methylation pattern may change with time in culture preventing the successful differentiation into the specific lineages they usually regulate.

Notably, this experimental variability can be contained by including a non-treated condition, which to normalize calcium content of all experimental conditions to. Previous studies successfully applied this normalization when assessing the bone-anabolic effect of Wnt ligands, signal transmitter agonists and antagonists as well as the toxic effects of drug compounds and environmental chemicals[22-26].
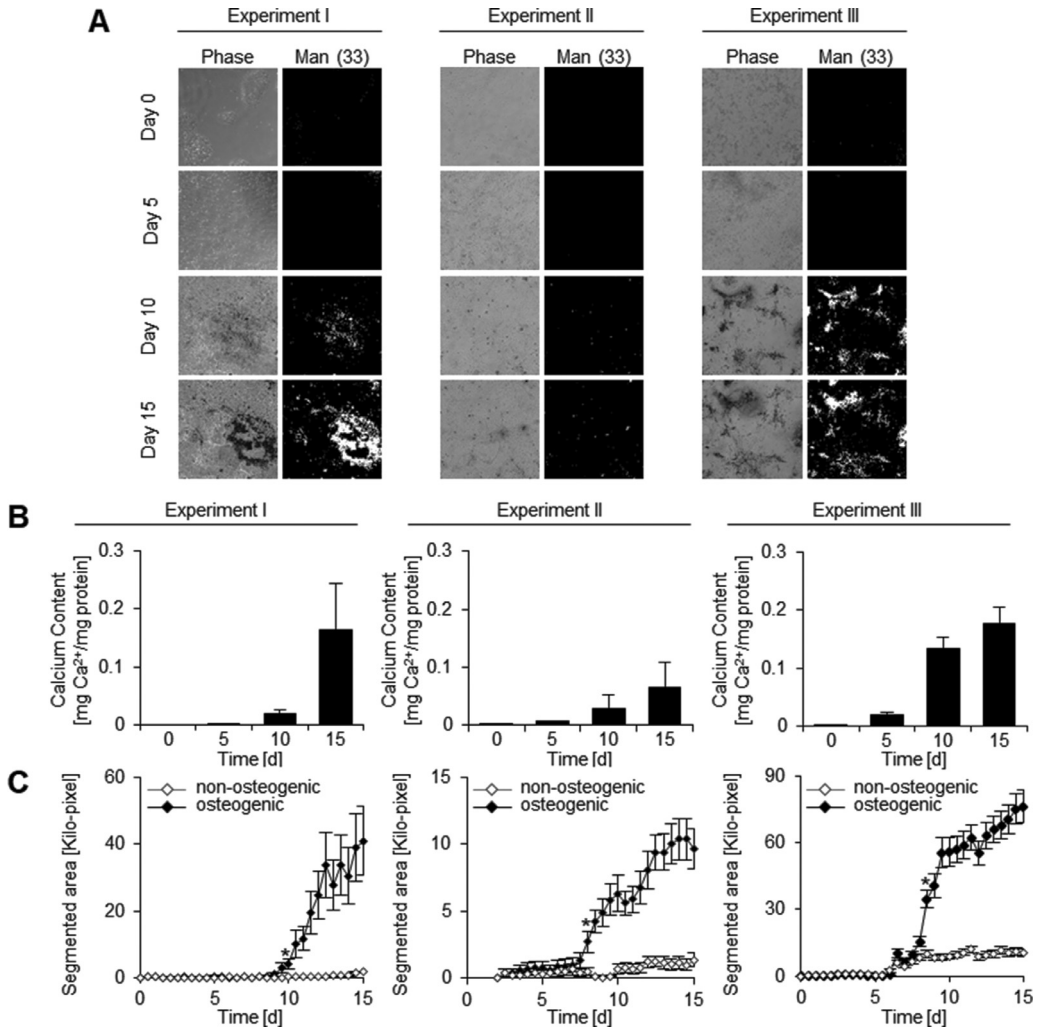
Fig. 3. Segmentation and image analysis for three independent experiments. (A) Photomicrographs taken over time. Segmentation was performed manually with a threshold of 33 based on the last image in the sequence. (B) Calcium was measured with Arsenazo III from 5 independent experiments and normalized to the total protein content. (C) Images were acquired every 12 h and segmented areas that represented calcification determined in kilo-pixel. Each data point represents 30 individual measurements. * $p < 0.05$ shown for the first time point that calcification was significantly up-regulated over the non-osteogenic control. Man, manual.

While we have not specifically determined limits of detection, they may be determined correlatively from current and previously published data. For example, the earliest time point that the segmentation method can detect differences in osteogenic cultures versus control cultures was determined to be d10 (Fig. 3). On this day, the absorbance-based calcium assay determines the calcium concentration to be 10.41 microg/mg total protein [2]. In contrast, on day 5, when the calcium concentration is around 1.2 microg/mg total protein [2], the image segmentation technique cannot distinguish between the control and the osteogenic cultures, but neither can the absorbance-based assay.
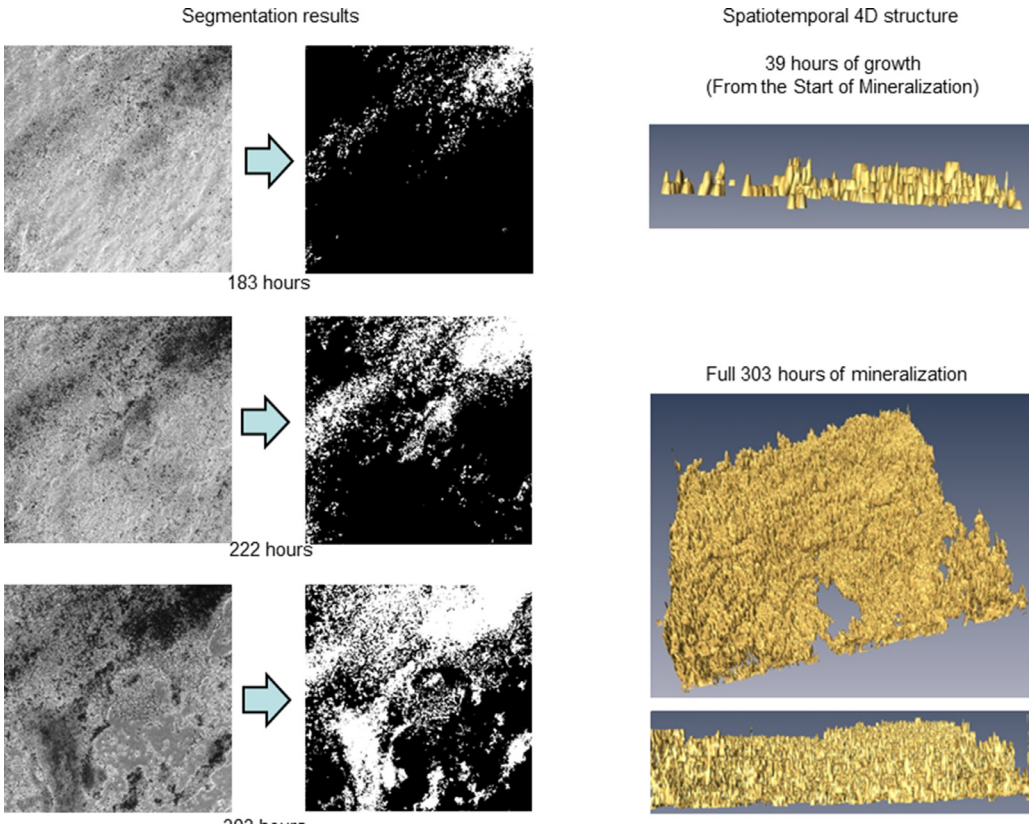
Fig. 4. Spatiotemporal rendering of segmentation results over time.

In turn, we may have encountered an upper limitation of the technique in the later days of the differentiation (i.e. d15-20), when the curves plateau. This could occur for example because calcium deposition happens in a three-dimensional manner. While this may not matter in some applications of this new technique, for example when measuring on early differentiation days at which this plateau is not yet reached, the image technique could be exploited to represent a three-dimensional view of calcium deposition over time (spatiotemporal view), which circumvents the issue of plateauing (Fig. 4). In this technique, the newly added deposit between two time points is calculated and additively superimposed over the image from the previous time point.

While not all laboratories are equipped with an automated image taking system, such as the one used in this study, the image analysis described here may also be executed on serial images acquired with a regular microscope. However, when using a manual approach, it is important to photograph the same region of the culture well as time progresses, which will succeed by utilizing a microscope equipped with a coordinate system and a micro positioner. Similarly, in vitro calcification is a feature not only unique to osteogenically differentiating pluripotent stem cells, but also for example to calvarial cell lines, mesenchymal progenitors and stem cells, and adipose-derived stem cells [29-35]. The described assay could therefore be useful in a whole host of studies that need to assess the osteogenic capacity of such stem cells and, or importantly, a whole range of different chemicals, constituents of tobacco or other [6], that are suspected of inducing developmental toxicity in the osteogenic lineage.

Declaration of Competing Interests

The Authors confirm that there are no conflicts of interest.

Acknowledgements

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2021.101265.

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