Designation: F2997 – 13

Standard Practice for Quantification of Calcium Deposits in Osteogenic Culture of Progenitor Cells Using Fluorescent Image Analysis[1]

This standard is issued under the fixed designation F2997; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice defines a method for the estimation of calcium content at multiple time points in living cell cultures that have been cultured under conditions known to promote mineralization. The practice involves applying a fluorescent calcium chelating dye that binds to the calcium phosphate mineral crystals present in the live cultures followed by image analysis of fluorescence microscopy images of the stained cell cultures. Quantification of the positively stained areas provides a relative measure of the calcium content in the cell culture plate. A precise correlation between the image analysis parameters and calcium content is beyond the scope of this practice.

1.2 Calcium deposition in a secreted matrix is one of several features that characterize bone formation (*in vitro* and *in vivo*), and is therefore a parameter that may indicate bone formation and osteoblast function (i.e., osteoblastic differentiation). Calcium deposition may, however, be unrelated to osteoblast differentiation status if extensive cell death occurs in the cell cultures or if high amounts of osteogenic medium components that lead to artifactual calcium-based precipitates are used. Distinguishing between calcium deposition associated with osteoblast-produced mineralized matrix and that from pathological or artifactual deposition requires additional structural and chemical characterization of the mineralized matrix and biological characterization of the cell that is beyond the scope of this practice.

1.3 The parameters obtained by image analysis are expressed in relative fluorescence units or area percentage, e.g., fraction of coverage of the area analyzed.

1.4 *Units*—The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds

F2739 Guide for Quantitating Cell Viability Within Biomaterial Scaffolds

3. Terminology

3.1 Unless provided otherwise in 3.2, terminology shall be in conformance with F2312.

3.2 *Definitions:*

3.2.1 *mineralized matrix, n*—a calcium phosphate-containing substance produced by cells typically in the osteoblast, odontoblast, and calcifying chondrocyte lineages, which is composed of crystals of calcium phosphate and contains collagen Type I and other non-collagenous proteins.

3.2.2 *osteoblasts, n*—secretory mononuclear cells that will initiate the formation of a matrix containing characteristic proteins, such as collagen, and non-collageneous proteins such as bone sialoprotein and osteocalcin, that will mineralize in the presence of a calcium and phosphate source.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *calcium deposits, n*—a calcium phosphate-containing substance synthesized in cell cultures during mineralization assays; such as, osteoblast differentiation assays, that may have precipitated out of solution rather than being produced by the cells.

4. Summary of Practice

4.1 This practice consists of *(1)* fluorescently staining the calcium deposits in a cell culture using the non-toxic calcium-chelating dye xylenol orange, *(2)* collecting fluorescent microscopy images of the stained samples, *(3)* collecting images of

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.43 on Cells and Tissue Engineered Constructs for TEMPs.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

intensity standards in bead form, and *(4)* conducting image analysis of thresholded images of the standards and the samples to determine area percentage and mean intensity of the stained areas.

4.2 The practice involves the testing and analysis of a fluorescent intensity standard in order to determine standardized image analysis settings for imaging of the calcified cell cultures. The use of a standard allows for the comparison between different samples or different time points. Methods for determining area percentage and mean intensity of the standard and the samples are described.

5. Significance and Use

5.1 *In-vitro* osteoblast differentiation assays are one approach to screen progenitor stem cells for their capability to become osteoblasts. The extent of calcified deposits or mineralized matrix that form *in-vitro* may be an indicator of differentiation to a functional osteoblast; however, gene expression of osteogenic genes or proteins is another important measurement to use in conjunction with this assay to determine the presence of an osteoblast.

5.2 This test method provides a technique for staining, imaging, and quantifying the fluorescence intensity and area related to the mineralization in living cell cultures using the non-toxic calcium-chelating dye, xylenol orange. The positively stained area of mineralized deposits in cell cultures is an indirect measure of calcium content. It is important to measure the intensity to assure that the images have not been underexposed or overexposed. Intensity does not correlate directly to calcium content as well as area.

5.3 Xylenol orange enables the monitoring of calcified deposits repeatedly throughout the life of the culture without detriment to the culture. There is no interference on subsequent measurements of mineralized area due to dye accumulation from repeated application (**1**).[3] Calcified deposits that have been previously stained may appear brighter, but this does not impact the area measurement. Calcein dyes may also be used for this purpose (**1**) but require a different procedure for analysis than xylenol orange (i.e., concentration and filter sets) and are thus not included here. Alizarin Red and Von Kossa are not suitable for use with this procedure on living cultures since there is no documentation supporting their repeated use in living cultures without deleterious effects.

5.4 The test method may be applied to cultures of any cells capable of producing calcified deposits. It may also be used to document the absence of mineral in cultures where the goal is to avoid mineralization.

5.5 During osteoblast differentiation assays, osteogenic supplements are provided to induce or assist with the differentiation process. If osteogenic supplements are used in excess, a calcified deposit may occur in the cell cultures that is not osteoblast-mediated and thus is referred to as dystrophic, pathologic, or artifactual (**2**). For example, when higher concentrations of beta-glycerophosphate are used in the medium to function as a substrate for the enzyme alkaline phosphatase secreted by the cells, there is a marked increase in free phosphate, which then precipitates with Ca^{++} ions in the media to form calcium phosphate crystals independently of the differentiation status of the progenitor cell. Alkaline phosphatase production is associated with progenitor cell differentiation, and is frequently stimulated by dexamethasone addition to the medium, which enhances the formation of calcified deposits. These kinds of calcified/mineral deposits are thus considered dystrophic, pathologic, or artifactual because they were not initiated by a mature osteoblast. The measurement obtained by using this practice may thus result in a potentially false interpretation of the differentiation status of osteoprogenitor cells if used in isolation without gene or protein expression data (**3**,**4**).

5.6 Due to the potential of artifactual calcified deposits during mineralization assays (**2-4**), gene expression analysis or protein analysis techniques demonstrating the RNA message or the presence of osteocalcin and bone sialoprotein are recommended for use in conjunction with the calcified deposit quantification procedure described here in order to confirm the presence of mature osteoblasts that are in the process of secreting a mineralizing matrix.

5.7 The deposition of a mineralized substance in the culture dish does not confirm that the cells being cultured are capable of forming bone *in vivo*.

5.8 The pattern of mineralized matrix deposition in the culture dish will vary depending on the number of times the cells have been passaged (i.e., first passage primary cells versus cells that have been passaged several times, including cell lines). First passage primary cells typically form relatively large nodules of osteoprogenitor cells that differentiate and mineralize, while cells that have been passaged many times lead to the formation of diffuse, dispersed mineral throughout the culture dish. This test method is independent of pattern of mineralization and can be used to analyze mineralized matrix in both primary cells and cell lines.

5.9 Since some cells proliferate slower than others and since some of the cell culture surfaces being tested may affect proliferation of the cells, the data can be normalized to total cell number. Since reduced proliferation typically reduces mineralization, normalization to cell number typically does not influence the outcomes. Total DNA content can be determined as an indirect measure of cell number. There are several commercially available kits for this purpose. Since DNA analysis is a destructive, toxic assay, additional cell cultures must be prepared if this assay is used.

6. Interferences

6.1 Xylenol orange does not photobleach during microscopy nor leach out of the stained mineral with time, and is stable for several months; thus, stained samples can be reanalyzed or analyzed at multiple time points without loss of identified areas due to previous dye application.

6.2 There is no interference on stained area measurements due to repeated application of xylenol orange.

[3] The boldface numbers in parentheses refer to the list of references at the end of this standard.

6.3 The substrate on which the cells are grown can affect the quantitation if non-specific fluorescent dye absorption to the substrate occurs. Tissue culture plastic commonly used for culture of cells does not interfere with this test method, but calcium-containing substrates and scaffolds, such as calcium phosphate or calcium carbonate, will bind the calcium-chelating dye used to identify the cell-produced mineral and cause background fluorescence that will interfere with this test method (**5**). Background values from analysis of xylenol orange-stained substrates that have been exposed to osteogenic medium for the same length of time must be determined and subtracted from the obtained values. All substrates beyond tissue culture plastic should be tested for non-specific dye binding prior to initiating this practice.

6.4 This test method is designed for use with living cells. Dead cells may become calcified and take up the calcium-chelating dyes leading to artifactual mineral deposition. Because the culture medium is changed immediately before imaging to avoid non-specific fluorescence from unbound dye, floating dead cells that may interfere are also removed; however, the user must confirm if the cell cultures are vital to avoid possible misinterpretation of the assay.

7. Apparatus

7.1 *Fluorescent Microscope and Digital Camera:*

7.1.1 A 10× objective is recommended with an additional 10× in the eyepiece that results in 100× magnification in total.

7.1.2 Microscope filter sets specific for the dyes. Xylenol orange has an excitation wavelength of 570 nm and emission wavelength of 610 nm and should be examined with a TRITC (tetramethyl rhodamine isothiocyanate) red filter. The filter set range will be recorded and similar filters can be used as long as there is no bleed through of another fluorophore in the culture which can be detected by imaging control cultures without the xylenol orange.

7.1.3 Camera and image collection software specifications. Digital imaging system which can include either a greyscale monochrome camera or color camera. Images obtained with a monochrome camera will be of better quality. Minimum resolution of 1000 × 1000 pixels, a minimum of 12 bit. The camera and image collection software shall be capable of saving the image in a lossless file format (i.e., tiff file).

7.1.4 Computer with image analysis software.

7.1.4.1 The image analysis can be conducted using a program such as the publically available National Institutes of Health program called ImageJ (http://rsbweb.nih.gov/ij/) to quantify the positively stained areas. ImageJ is image analysis software available through the NIH (http://rsb.info.nih.gov/ij/) and does not require a license to use. It may be utilized on Linux, Mac OS X and Windows. It is widely used and customizable for specific image analysis tasks. Many image file types are compatible with this software including: TIFF, GIF, JPEG, BMP, PGM, FITS, ASCII and DICOM.

8. Reagents and Materials

8.1 Xylenol orange ($C_{31}H_{28}N_2O_{13}SNa_4$) is a fluorochromatic calcium-chelating dye suitable for staining calcified deposits in cell cultures. This compound binds to calcium, allowing the calcified deposits to be visualized. This dye has proven to be reliable for assessing mineralization of osteoprogenitor cultures (**2**). At the concentrations used in this practice guide the dye is safe and non-toxic to cells and can be used without detriment to the cultures enabling the analysis of multiple time points.

8.2 Xylenol orange is commercially available as a powder and should be made into a stock solution using sterile distilled water at 20 mM and filtered through a 0.20 µm filter, protected from light and stored at 4°C for up to three months. It is important to use aseptic technique and sterile reagents since this is an assay on live cultures. The xylenol orange stock solution should be added directly into the cell culture medium at a concentration of 20 µM within the culture well for 12–24 hrs before imaging. It is important to replace the medium with fresh media that does not contain dye prior to imaging to limit background fluorescence. At this concentration the dye will effectively stain an area of mineralized matrix in a similar manner to von Kossa staining which is commonly used in osteogenic cultures (**2**). It is thus important to use xylenol orange at this concentration and not other untested dyes which may reduce or enlarge the mineralized areas from their actual size.

8.3 A fluorescent intensity standard in the form of a suspension of microspheres is used in this standard practice. For xylenol orange, a commercially available fluorescent intensity standard is InSpeck Red (580/605 nm) Kit (6 µm, Cat.# I14787) available from Invitrogen Corp. Standards from other suppliers can be used. For consistency between groups, and between tests conducted on different days or in different labs, the same standards should be used. Each kit includes six separate suspensions of InSpeck fluorescent microspheres with relative fluorescence intensities of 100%, 30%, 10%, 3%, 1% and 0.3%. Beads of each one of the six types have the same diameter (6 µm). Guidance from the manufacturer should be followed with respect to storage and shelf-life.

8.4 *Cell Culture:*

8.4.1 Living cell cultures that have been exposed to mineralizing conditions, such as osteogenic supplements described in references (**3-5**) to induce calcified deposits are needed for this test method.

8.4.2 Cell cultures that have *not* been exposed to mineralizing conditions, but cultured for the same time period, as well as tissue culture dishes containing medium only should also be included as negative controls.

8.4.3 If biomaterial substrates are used, tissue culture wells containing the biomaterial substrates with osteogenic medium should also be prepared as controls and kept in the incubator for the same time as the samples with cells.

8.4.4 Cultures prior to xylenol orange application should be examined prior to commencing this procedure to determine if there is any background from the cells or biomaterials.

8.4.5 Cell culture plates of various sizes can be used. The examples in the appendix describe analysis and imaging of 6 well cell culture plates.

8.4.6 Sample size will depend on the test groups that are specific to a given experiment. Appropriate statistical methods

should be used to determine the number of replicates or cell culture wells used per group.

8.4.7 Additional cell cultures may be required if the imaging data is going to be normalized to cell number, which can be quantified by measuring total DNA content. Total DNA content is a destructive assay and thus requires additional control wells or plates for this purpose.

9. Hazards

9.1 The fluorescent light source on the microscope can cause eye damage. Avoid direct contact with eyes.

9.2 Consult the xylenol orange materials safety data sheet (MSDS) for safe use.

10. Sampling, Test Specimens, and Test Units

10.1 Within each sample to be analyzed, multiple images that form a representative field of view shall be acquired. The cell culture dish should be examined throughout to determine the pattern of mineralized matrix deposition and the presence of cells before beginning to acquire images. A uniform pattern of calcified deposits throughout the cell culture is required in order for the recommended image acquisition procedure to be representative and accurate. Samples for analysis should be restricted to those with intact culture monolayers that are not retracted or otherwise structurally perturbed. The center area of a cell culture plate has the best focus for image collection and ideally should be used. An array of 1 × 10 adjacent images at 100× magnification (10× from the eye piece and 10× from the objective) with the center point of the array in the middle of the cell culture is adequate for a 6-well plate. Linear array image collection should be conducted in relation to culture well dimensions and should not extend beyond the edge of the culture plate. Linear sample array collection is based on previous measurements in human osteoblast progenitor cultures where up to 400 images (20 × 20) at this magnification were obtained without any difference in the value of area% of calcified deposits. Additional images may be obtained and used; however, it is important to randomly select the images and not deliberately select fields with fluorescence. The 10 adjacent images may be stitched together into one image for the image analysis using an automated or manual stitching program. Stitching the images together saves analysis time, but is not required.

10.2 A linear array of images is preferred because it represents a slice of the cell culture dish and thus more broadly samples the potential heterogeneities within the cell culture such as the areas of higher cell density in the center of the well.

10.3 The units of analysis will be area percentage and mean intensity in relative fluorescence units.

11. Calibration and Standardization

11.1 *Fluorescence Reference Standards:*

11.1.1 Fluorescent microspheres that match the excitation and emission wavelengths of xylenol orange and are certified for emission fluorescence intensity and for diameter are available. These shall be used as reference standards to ensure that comparisons can be made between different microscope acquisition systems and different users of the same systems.

11.1.2 It should be determined if there is a linear response to exposure time by varying exposure time and determining intensity and plotting the exposure time vs intensity. This is necessary to ensure that the lamp and imaging settings are being used within the correct range. The data to be used for this standard practice should be obtained in the linear range of exposure time and intensity.

11.1.3 The calcium content is related to the fluorescent area, not the intensity; however, an intensity measurement is important to make in order to confirm that the images are not overexposed.

11.1.4 This practice includes a procedure to measure the area of the standard beads to verify that the area of the standard beads has not been falsely enlarged due to excessive exposure times, incorrect focus or use of the procedure that can artifactually enlarge the bead diameter. The intensity standards and area standards (e.g., recommended concentration of beads to use as a standard) have been selected to match that of typical calcified osteogenic cultures.

11.2 *Image Acquisition Settings and Parameters:*

11.2.1 Exposure times shall be assessed on each sample, on the most fluorescent portion of the well or plate so that the images taken for actual analysis will not be overexposed which can happen if settings were established on less fluorescent areas. If overexposed, the data cannot be analyzed. The range of intensities of the data procured should be spread out over the available intensity values such that a maximum of 1% of the pixels of the image are saturated (in the top bin).

12. Procedure

12.1 *Prepare Samples for Analysis:*

12.1.1 *Test Sample*—Apply the calcium-chelating dye xylenol orange to the cell cultures one day prior to imaging at 20 μM concentration in cell culture medium. Replace the cell culture medium immediately prior to imaging with medium that does not contain dye. Examine the entire sample throughout to confirm the homogeneity of the pattern of mineralization and of the cell culture.

12.1.2 *Fluorescence Standard Sample Preparation*—For a 6-well dish, prepare the standard bead preparation as follows: after shaking to disperse the microspheres in the stock solution, take 300 μL of the 0.3% standard and add it to 2 mL of the medium that the cells are being cultured in. This volume of the XO standard in one well of a 6-well plate has been shown to give comparable percent area results to human osteogenic cell cultures with calcified deposits. The beads do not float, but to increase the speed of settling the plate can be centrifuged.

12.2 *Image Acquisition:*

12.2.1 *Automated Field Collection or Manual Method with Unbiased Field Collection*—Images may be collected using a microscope equipped with an automated stage or may be collected manually. For both automated and manual field collection of a 6-well dish with uniform calcified deposits, a tiled vertical or horizontal assembly of images made up of 10 individual images taken in the center of the well with an approximate overlap of 10% using a 10× objective with a 10× eye piece for a total of 100× magnification is sufficient. Images should be acquired in the center of the cell culture well or plate

where the focus of the camera is best, by moving from one side to the opposite side of the center of the plate. The total area represented by the images must be known in square millimeters because the intensity and area of fluorescently stained calcium deposits will be reported per total image area analyzed. Image acquisition software or manual use of a calibrated ruler within the image area should be used to determine the total area of analysis.

12.2.2 *Focus*—The focal plane should be selected so that the calcified deposits are in focus, rather than the cells, which are typically below the calcified deposits. Focus the sample while the fluorescent lamp is on and illuminating the sample.

12.2.3 Take phase contrast images, as well as fluorescent images, to confirm and provide evidence that a uniform layer of cells is present in the area to be analyzed and that the cell layer has not retracted. This procedure cannot be applied to cells that have retracted over a large area of the cell culture plate.

12.3 *Data Analysis:*

12.3.1 To determine percent area and mean intensity follow the detailed instructions in the appendix.

13. Calculation or Interpretation of Results

13.1 The concentration of the standard beads specified in this practice has been selected to match a well-mineralized two-dimensional culture of human mesenchymal cells cultured in an osteogenic medium. The expected area percentage is 27%. Analysis of the standardized beads alone prior to completing a cell culture experiment is recommended in order to confirm that the procedure has been followed correctly.

13.2 By using standard beads with certified diameter and intensity value it is possible to normalize data from all the samples to the standard beads even if the images are taken at different time points, in different laboratories, or of cells from different donors, or with different differentiation treatments.

13.3 The intensity may have some subtle variations and appear to be brighter in some stained regions due to the three-dimensional nature of calcified deposits in a mineralizing cell culture. Calcified deposits that have been previously stained with xylenol orange may also appear slightly brighter (i.e., have a higher intensity) when a second application of dye is applied within a day or two from the previous application, but this will not affect the area measurement which is most closely associated with calcium content. Typical applications at weekly intervals will not result in noticeably brighter areas.

13.4 Data may be normalized to total DNA content to account for differences in total cell number that can occur due to proliferation differences between test groups and between different experiments and cell types.

14. Report

14.1 The report shall include the cell type, the passage number of the culture, the density of the cells initially seeded, the time point the images were acquired with day 0 as the initial cell seeding day, the type of medium used and the amount of supplements such as serum, amino acids and antibiotics, the type and amount of osteogenic supplements used, the size of the wells, and the portion of the well acquired by images for the analysis.

14.2 The reported values should include the name and version of the image acquisition program used, the microscope and the microscope lamp used, the camera used, the objective used to acquire the images, the pixel dimensions, the number of pixels per image, the name and version of the image analysis program used together with any plugins.

14.3 The reported values should include the average intensity and area percentage of calcified deposits within the cultures, both un-normalized and then normalized to the standard bead values.

14.4 If area and intensity are normalized to ug of DNA, the total DNA content values and the normalized area and intensity values should be included.

15. Precision and Bias

15.1 Round robin studies have not yet been completed to determine the intra- and inter-laboratory variability.

15.2 Sensor linearity is determined within this practice and helps to ensure precision between analyses.

16. Keywords

16.1 bone; calcification; image analysis; mineralization; osteoblast; stem cell; tissue engineering; xylenol orange

ANNEXES

(Mandatory Information)

A1. IMAGE ACQUISITION AND ANALYSIS FOR AREA AND INTENSITY OF FLUORESCENTLY STAINED CALCIFIED MATRIX

INTRODUCTION

The flow chart shown in Fig. A1.1 describes the entire procedure.

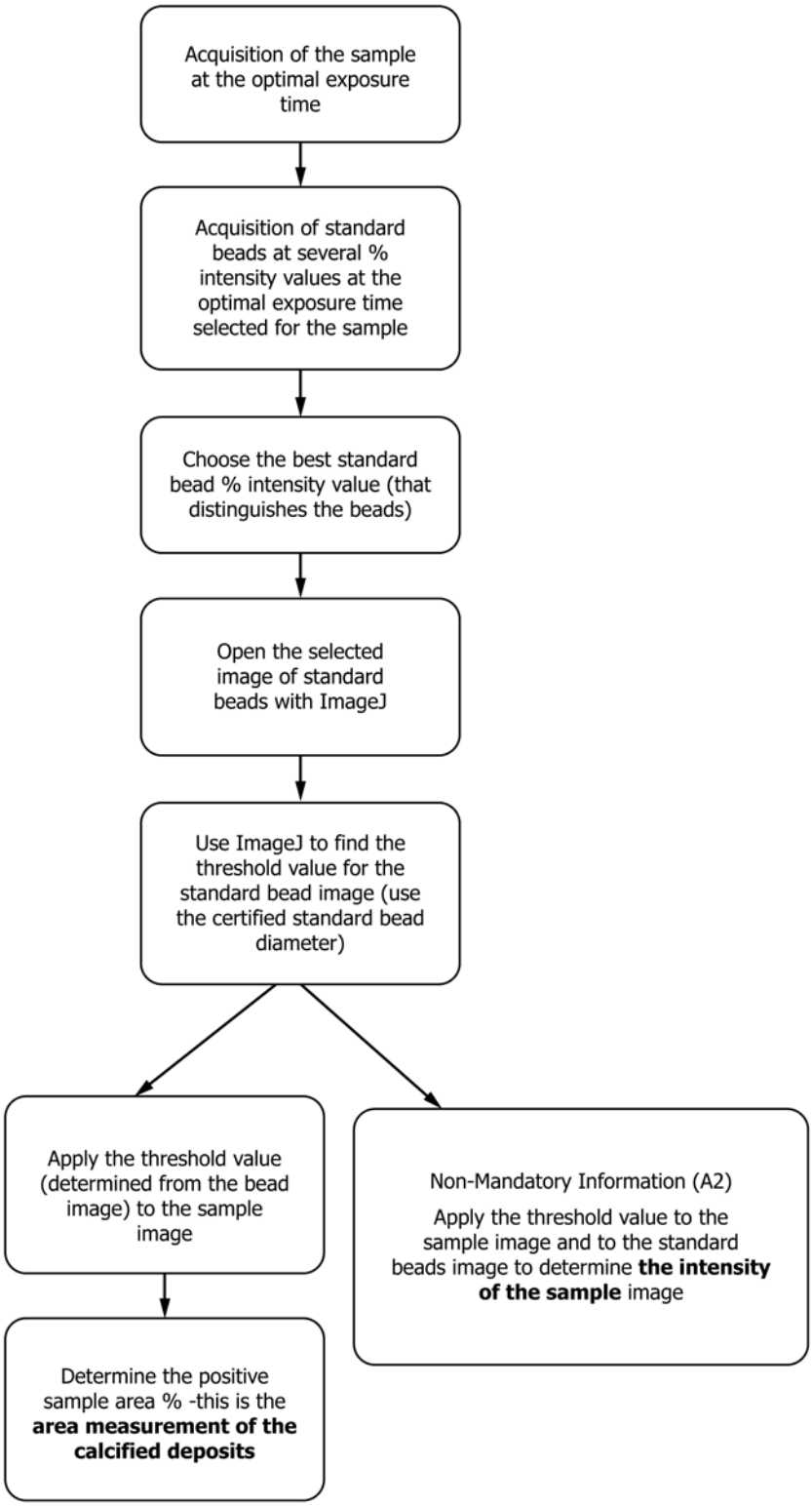


FIG. A1.1 Procedure Flow Chart

A1.1 Sample Acquisition Parameters and Exposure Time

A1.1.1 Samples shall be acquired with a 10× objective and 10× eye piece for 100× total magnification with the following setup:

A1.1.1.1 minimum resolution of the camera (e.g., 1000 × 1000 pixels at minimum 12 bit),

A1.1.1.2 no gain,

A1.1.1.3 no attenuation filters, and

A1.1.1.4 diaphragm of the camera completely open in order to have the maximum intensity and homogeneity of the light.

A1.1.2 *Exposure Time:*

A1.1.2.1 In order choose the appropriate exposure time that maximizes the dynamic range being used for analysis, *for each cell culture sample*, use the histogram of the pixel intensity related to the live image shown by the camera and adjust the exposure time until:

(1) a maximum of 1% of the pixels of the image are saturated, and

(2) the distribution of the values of light intensity are the widest possible.

A1.1.2.2 Following this guidance is the best way to avoid overexposure and associated artifacts. The cell cultures must be imaged before imaging the intensity beads.

A1.1.2.3 If the acquisition software does not have the ability to examine the histogram of pixel intensity, an image should be acquired and then the histogram feature in ImageJ should be utilized in order to check the saturation level of the image pixels, as follows:

(1) Open the image with ImageJ → Image → type → select 8 bit and save the image.

(2) Then go to: analyze → histogram → look at the histogram.

A1.1.2.4 To determine the % of pixels saturated it is also possible to calculate it as follows:

(1) From the menu in ImageJ, select analyze → histogram → list and from the list check how many pixels are at the higher level of intensity (255 is the top level or bin of intensity).

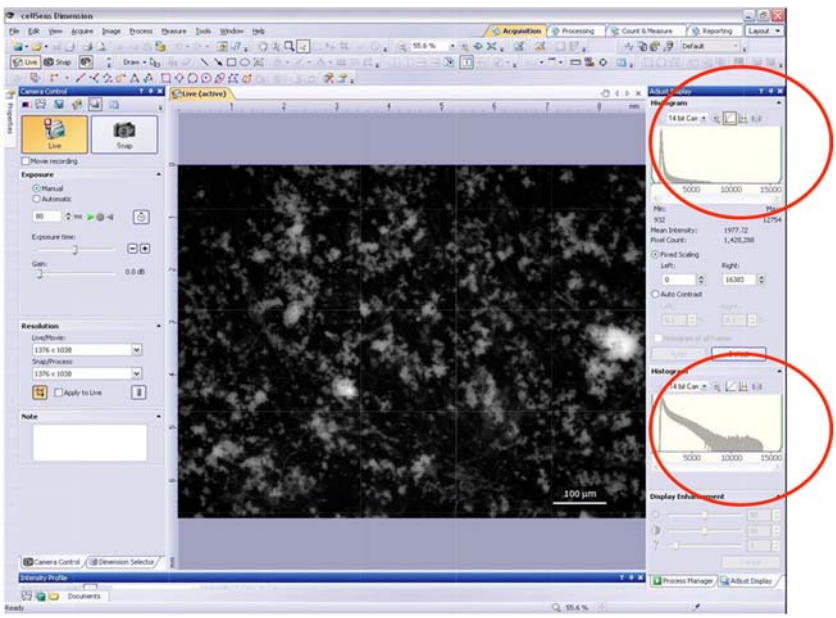
(2) Divide the pixels in this bin by the number of pixels forming the entire image (indicated usually at the top right of the image) and calculate the percentage. This percentage has to be ≤ 1%. The exposure time shall be adjusted until the data is distributed according to this value.

A1.1.2.5 Examples of three histograms as seen within an image acquisition software program for three different exposure times are provided here. Fig. A1.2 is an example of a correctly adjusted exposure time. Fig. A1.3 is an example of an underexposed sample and Fig. A1.4 is an example of an overexposed sample.

A1.1.2.6 Each sample, and its standard, has to be acquired with its appropriate exposure time, chosen independently from the exposure time of other samples. A sample with low mineralization will need a longer exposure time than a sample with high mineralization. However, in any case there is no need to overexpose the low mineralized sample to have a suitably clear image of the mineralization for this analysis. Cameras have sensors that are more sensitive than the human eye and imaging software (e.g., ImageJ) is utilized to analyze the pixel light intensity of each image acquired.

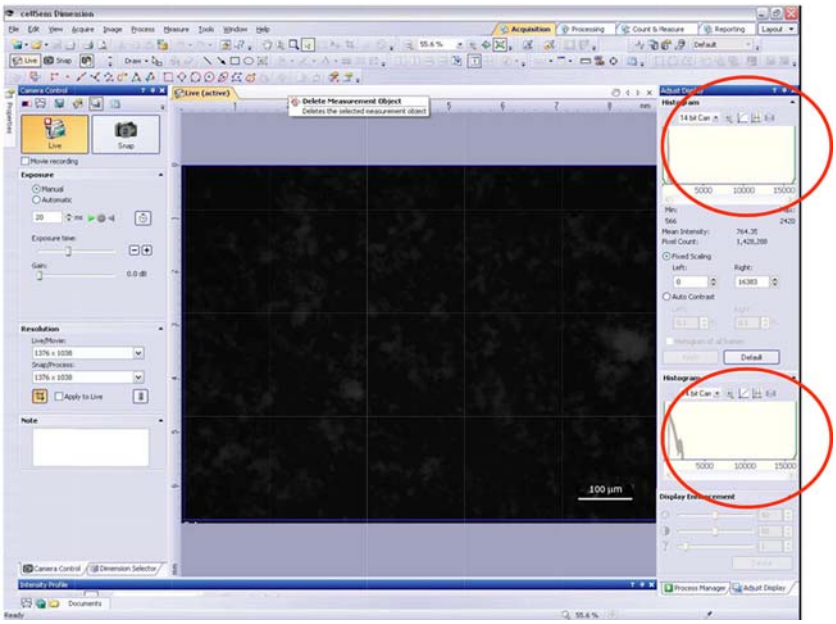
A1.2 Area Measurement of Calcified Deposits

A1.2.1 In order to measure the areas of positive stain within the images, a thresholding algorithm shall be applied to isolate the area to be analyzed. In this standard procedure thresholding is accomplished through the use of the standardized beads as follows. When an optimal exposure time has been selected using the mineralized samples, images of standard beads, at several intensity percentage values, have to be acquired using the same setup at the same exposure time chosen for the sample. From these images, the optimal standard bead intensity values will be those that give a “good image” with the selected exposure time and can be used to threshold the beads by comparing the bead image diameter to the manufacturer’s reported size. A “good image” of the beads is when the beads



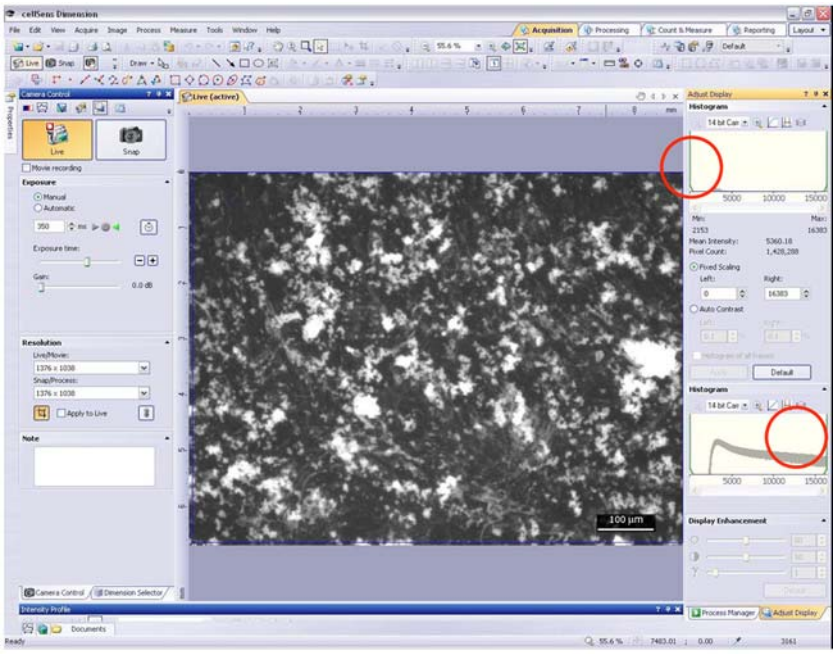
A single, non-tiled image of calcified deposits is shown in the center. On the right side two histograms are shown and circled in red: they indicate the light intensity of the pixels in the image in live mode. The top histogram has a linear scale and the lower histogram has a logarithmic scale. For this exposure time (80 ms), the pixels are not saturated as evidenced by the fact that the data shown in the linear scale does not have a large peak at the end of the data line and that the data in the logarithmic scale does not extend off the graph.

FIG. A1.2 Example of a Correctly Adjusted Exposure Time



Underexposed image shown in the central dark area – histograms of pixel intensity are circled in red. For this shorter exposure time (20 ms), the pixel intensity distribution is very narrow, and the image is very dark. This acquisition time does not make optimal use of the camera dynamic range and is not a good image for analysis of the calcified deposits.

FIG. A1.3 Example of an Underexposed Sample

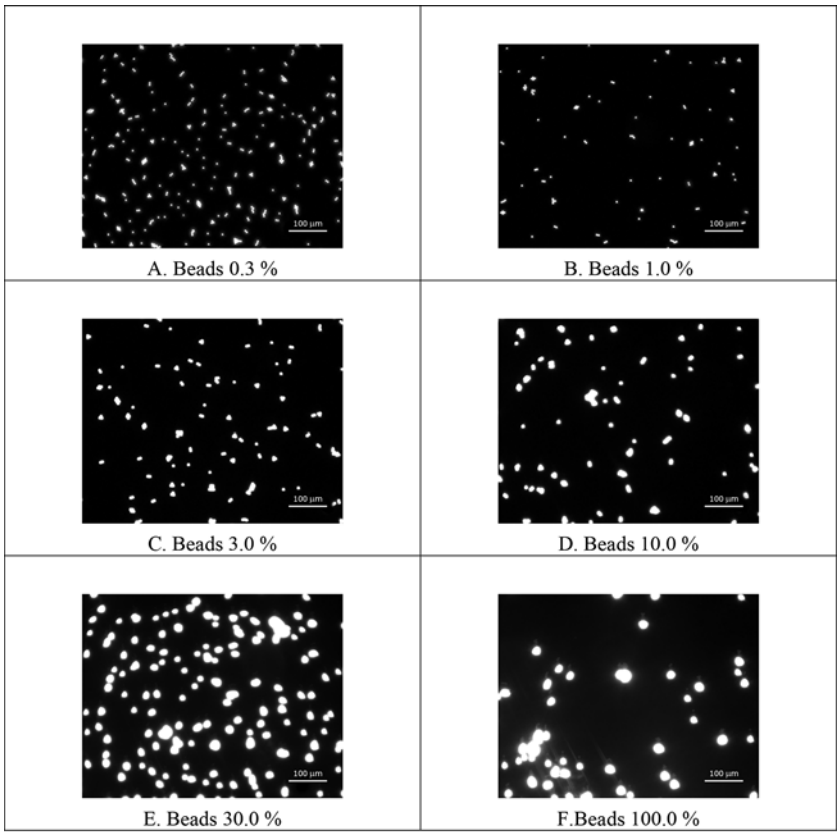


Overexposed image. Areas of exceptional brightness are observed. If the exposure time is too long, more than 1% of the pixels are saturated (as shown clearly in the histogram with logarithmic scale). The red circle is drawn around the line of the graph which does not asymptote to zero indicating there are many high values.

FIG. A1.4 Example of an Overexposed Sample

appear to be clearly distinguishable and in focus. The most appropriate standard bead intensity values have to be selected for each sample. Examples of how to obtain a good image of the beads are included below and also an example of how to threshold the image such that the size of the beads in the image matches the manufacturers reported value. Thresholding in this way minimizes user bias.

A1.2.2 Selecting the appropriate standard. Start with the lower intensity beads (0.3% for the InSpeck Red Beads) until the highest intensity beads (i.e., 100.0%) and use the optimal exposure time selected for the sample (see above) to take the image of the beads (in this example 80 ms). Examples of the images taken for the beads at 80 ms with 10× magnification are shown in Fig. A1.5.



Images of the various possible standard beads. The 0.3% and 1% beads are both suitable for selecting a threshold value that allows the discrimination of background from beads. The best "good images" are those where the beads are clearly distinguishable and in this example are the images of 0.3% and 1% standard beads. In the other images the borders of the single beads are not clear and the bead diameter seems to be wider than the real one. The 0.3% and 1% beads are both suitable for selecting a threshold value that allows the discrimination of background from beads.

FIG. A1.5 Images of the Various Possible Standard Beads

A1.2.3 *Defining the Threshold with Standard Beads and Application to the Image*—For example purposes, the 1% beads were selected to define the threshold. It is necessary to know the pixel dimension (in this case 0.654 µm) and the bead diameter dimension (in this case the manufacturer reported value is 6 µm). Define the threshold value as follows:

A1.2.3.1 Open the beads image with ImageJ.

A1.2.3.2 Zoom in and select a portion of the image so that only 10–15 beads can be seen (it is easier and more suitable than working on the entire image).

A1.2.3.3 Determine the threshold such that the image of the beads is set at the manufacture-reported value.

A1.2.3.4 Apply this threshold to the sample. The area remaining will thus be the positively stained area of the calcium deposits.

A1.2.4 Step-by-step instructions are shown in Figs. A1.6-A1.10.

A1.2.5 The threshold value found in this way shall be applied to the sample image (in this example the value is 27) as follows (Figs. A1.11-A1.15):

A1.2.5.1 Open the sample image.

A1.2.5.2 Click on adjust → threshold, select "Dark background" and "Red".

A1.2.5.3 Set the threshold value to the value selected from the bead image analysis (in this case 27).

A1.2.5.4 Click "apply" to obtain the image in Fig. A1.13 (if needed, click to black&white and again to red to update the image).

A1.2.5.5 Select the image and open the histogram. Click on analyze → histogram (or CTRL+H).

A1.2.5.6 Click on List to see how many pixel compose the background (0) and how many compose the sample (255).

A1.2.6 In this way it is possible to determine the sample area % to be compared with the area % (in pixels) of other samples (in pixels). In the example: the total area comes from the image resolution data but also from the calculation total area (in pixel) = 1023132 + 383848 and area of the sample = 383848, hence area% = area of sample (%) / total area (%) = 383848 / (1023132 + 383848) = 27.3% calcified area.

A1.2.7 The same result will appear automatically by clicking on analyze → set measurement → select area, area portion, limit to threshold. Then click on measure and save the results.

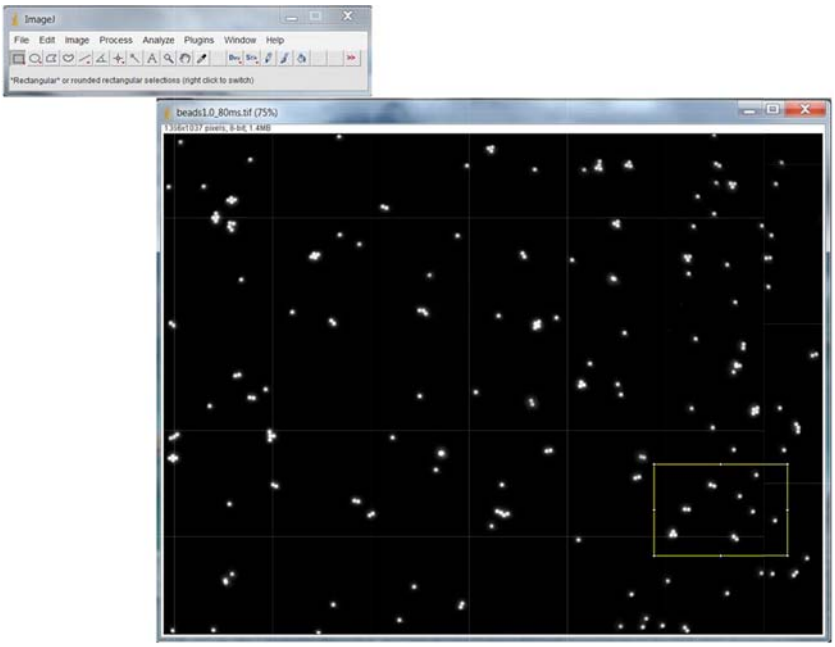


FIG. A1.6 Open an image and select a smaller area to work on at higher magnification

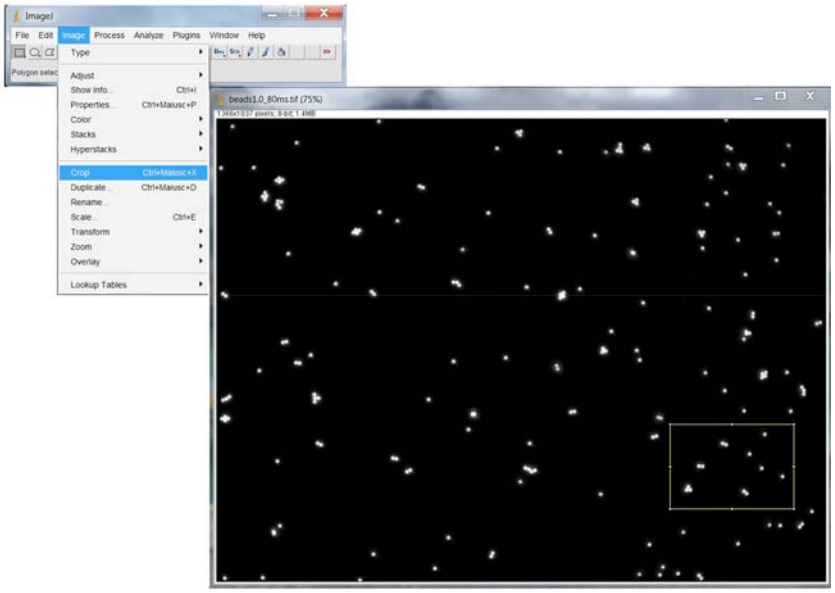
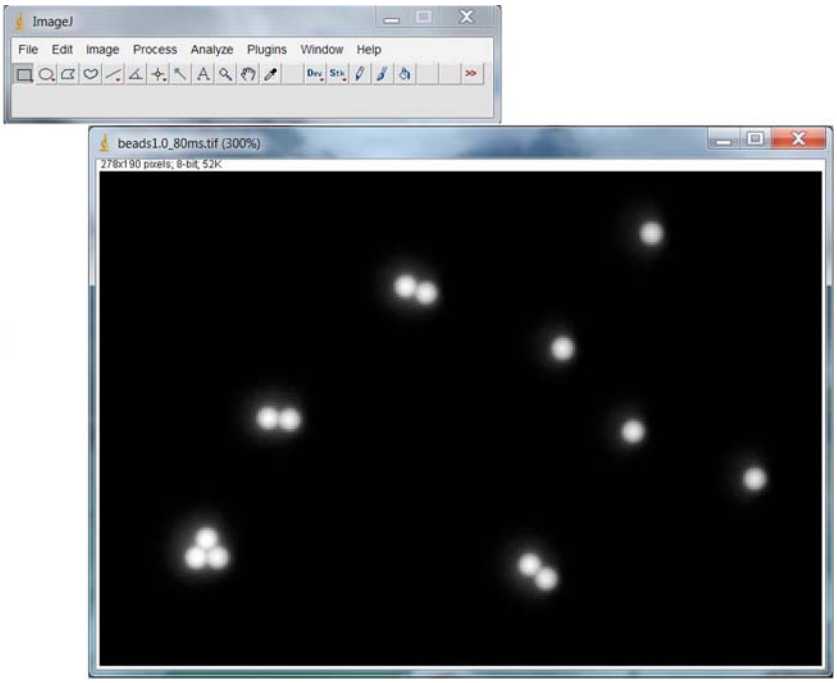


FIG. A1.7 Click on image → crop → select a portion and obtain the cropped image



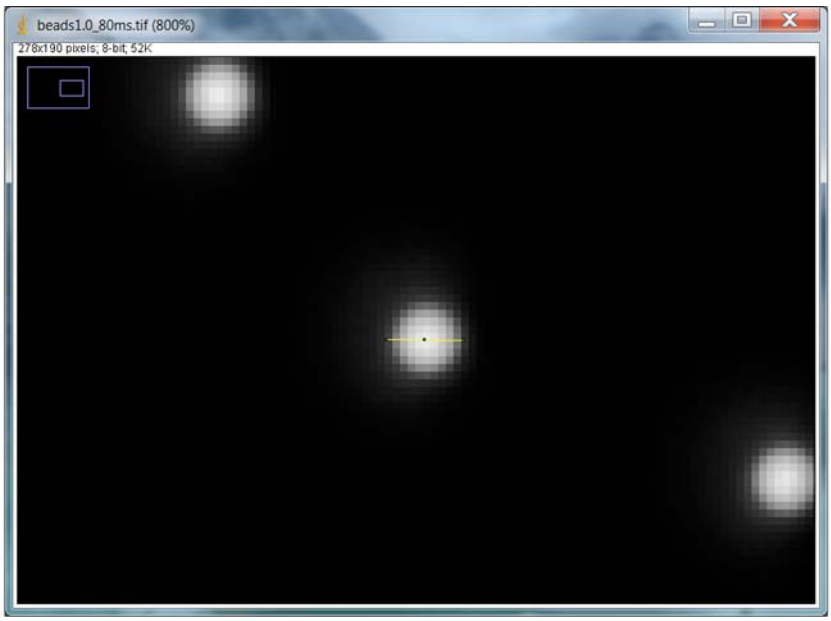
On this cropped image it is easier to identify the edges of the bead.

(1) Zoom in on the cropped image to see in detail one bead (Fig. A1.9).

(2) By knowing the standard bead diameter dimension and the pixel dimension, draw a line with the same diameter dimension (in pixel). This will reduce the error due to distortion of fluorescence.

(3) Track the line from one side of the bead to the other crossing the center (track the diameter).

FIG. A1.8 Cropped Image



(1) Adjust the threshold until obtaining the real diameter: click on Image → Adjust → Threshold, select “Dark background” and “Over/Under” and move the threshold value to obtain the right diameter.

FIG. A1.9 Drawing the line to identify the bead diameter

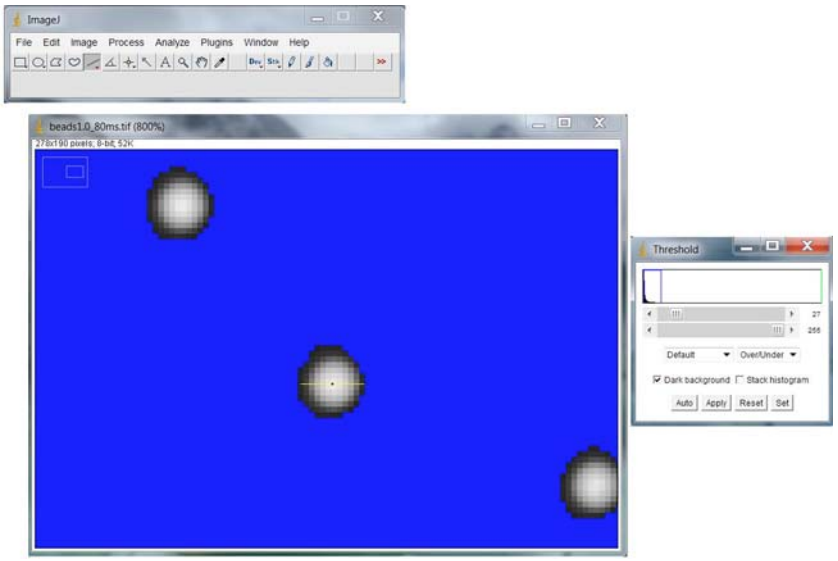


FIG. A1.10 Example of Properly Thresholded Standard Beads

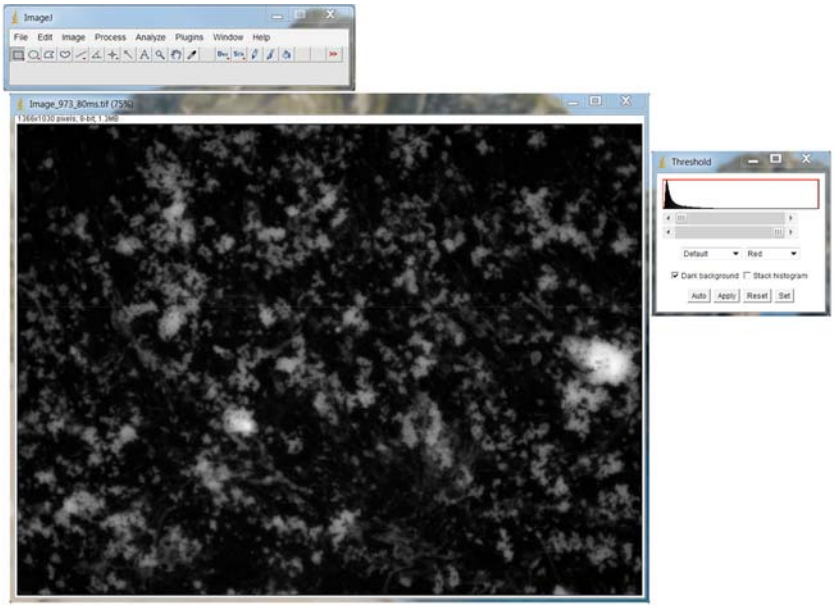


FIG. A1.11 Example of a Sample Image to be Thresholded

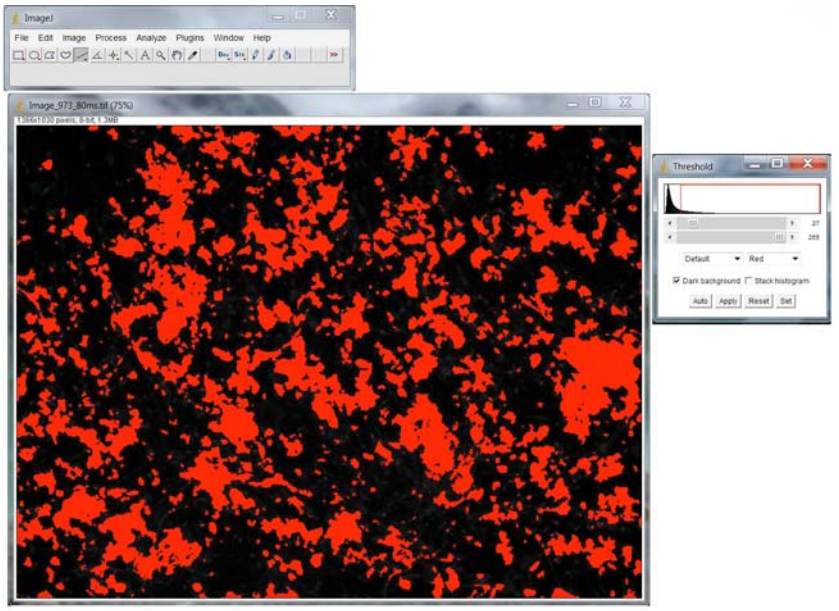


FIG. A1.12 After the threshold has been applied to the sample image

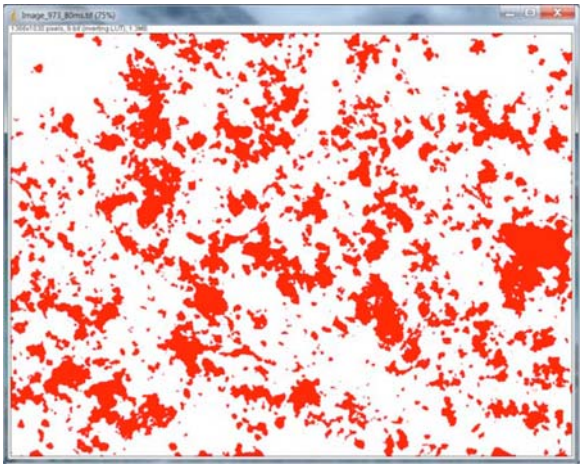


FIG. A1.13 Threshold has been applied and the image area recolored for clarity

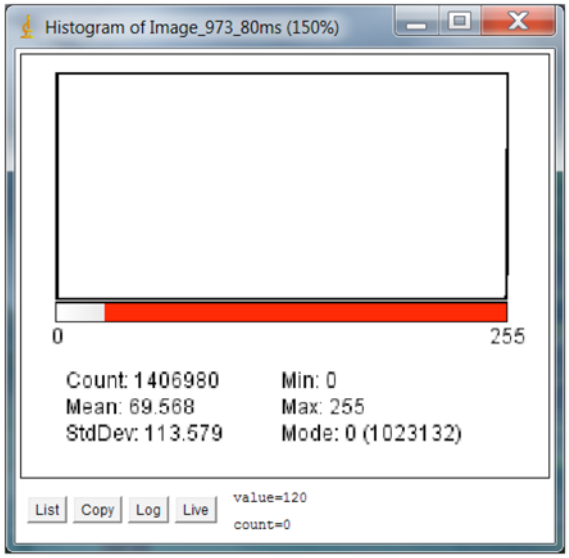


FIG. A1.14 Example of Histogram Analysis for Positively Stained Areas

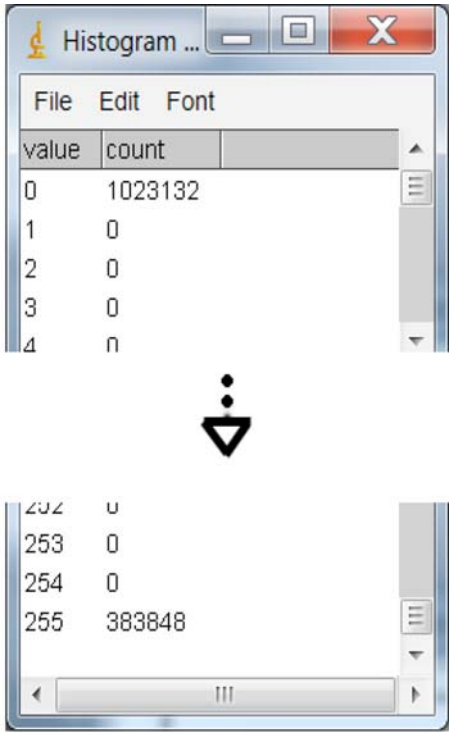


FIG. A1.15 ImageJ Histogram Analysis Window Showing the Pixels Above and Below the Threshold

A2. INTENSITY MEASUREMENT OF THE CALCIFIED DEPOSITS

A2.1 Calcified deposits are three-dimensional and area measurements that could possibly underestimate the differences in calcium deposits between two test groups if one of the groups has extensive 3-D vertical calcified deposits relative to another sample that has flat 2-D deposits. If there are extensive vertical deposits in one group relative to others, as detected by an inability to focus on the perimeter of the calcified deposit, normalized intensity measurements can be made in conjunction with area measurements. Thicker 3-D deposits will have a brighter intensity in the center of the deposit. The measurement of the intensity of the calcified deposits relative to the standardized beads will allow for the variations in calcified deposits to be assessed. Repeated application of xylenol orange at multiple time points should be avoided in this case since there is a retention of xylenol orange staining in the calcified deposits. Since the mineral grows outward from initial focus, the earlier stained deposits may appear brighter than the newly stained periphery and thus give an appearance similar to a thick 3-D deposit.

A2.2 After determining the appropriate exposure time for each sample, as shown above, acquiring the images at the selected exposure time and determining the threshold value for each image (as described above in the "area measurement of calcified matrix" section) use the images of the selected standard beads (in this example 1% beads as described above), and of the samples, to analyze the intensity of the calcified deposits.

A2.3 For each image (samples and standard beads): open the image with Image J → Image → Type → 8 bit and save the image. Then go to Analyze → Histogram → List. Here values of light intensity of the pixels are indicated (first column, ranging from 0 to 255) and in the second column the number of pixels for each intensity level. Limit the intensity measurement to the pixel areas above the threshold as follows (in this example, only the values > 27) and until the last positive value greater than zero (e.g., if between the intensity values of 250 and 255 the number of pixels is 0, do not consider those values). From this subset of pixels calculate the mean intensity and compare this result to the standard beads mean intensity to normalize them to the standard intensity of the beads.

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