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von Kossa Staining of Osteoclast Resorption on BD BioCoat™ Osteologic™ Discs

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Introduction

The BD BioCoat™ Osteologic™ Bone Cell Culture System consists of sub-micron synthetic calcium phosphate thin films coated onto various culture vessels. This system has been used as an alter-native method for compound screening for direct assessment of osteoclast¹⁻⁴ and osteoblast activity *in vitro*. The thin film design permits easy and reliable quantification of results.

The von Kossa method for staining mineralized tissue has been in use since the 19th century to visualize tissue in histological preparations. The method is well known in the field of bone research as a method for staining mineralized tissue, mainly calcium. Ironically, the technique was originally used to visualize pure calcium phosphates in tissue, which stained yellow to brown. The characteristic black staining reaction of biological mineral deposits, a reduction to metallic silver, was considered undesirable and required that the sample be kept in the dark to avoid this reaction. The reduction of silver phosphate was later found to be a result of the organic component of bone. Various versions of the method have now been altered to deliberately reduce the silver to the black metallic form using photographic developers.

Outlined here is a modern method to demonstrate the utility of von Kossa staining as a method to visualize the osteoclast resorption pits on BD BioCoat Osteologic Discs and MultiTest Slides. The results can then be quantified using various software programs that calculate spot density or area. This method can also be applied to bone nodule formation assays using osteoblast cells.

Method

This procedure was performed on a BD BioCoat Osteologic Disc cultured with rat bone marrow cultures as described in the BD BioCoat Osteologic Bone Cell Culture System User Guide [F00B017]. Osteoclast cultures were stained at day ten following the removal of cells with bleach.

von Kossa stain for bone nodule formation and contrast stain for resorption on BD BioCoat Osteologic Discs

1. For analysis of osteoblast activity (bone nodules), fix cells with 10% formalin in PBS or 5% glutaraldehyde in PBS for 30 minutes
 - For analysis of osteoclast resorption activity, go to Step 3
 - For a negative control, stain a disc incubated in the absence of cells
2. Wash 3x with DI water
3. Stain with fresh 5% silver nitrate for 30 minutes
4. Wash at least 3x with DI water

Very important: Wash silver nitrate out completely to prevent false positive. Residual silver nitrate forms brown to black precipitate at edge of staining dish if not completely rinsed.
5. Develop with fresh 5% sodium carbonate in 25% formalin
 - Develop up to five minutes for mineral and matrix staining in bone nodule assay
 - Develop 5-60 seconds for osteoclast resorption of thin film material (black)
6. Wash 3x with DI water
7. Fix with 5% sodium thiosulphate for two minutes
8. Wash 3x with DI water

Results

For osteoblast assays, which generate discrete bone nodules, nodules containing calcium mineral stain black. When less developed nodules are present the matrix will stain yellow to brown.

The negative control disc will stain with von Kossa to a light or dark gray depending on staining time. The film appears tan to brown when viewed on a low power microscope with back light illumination (*Figure 1*).

For osteoclast resorption of the film, it is desirable to maximize staining for contrast purposes. View discs with standard light illumination on regular or inverted microscope. Resorption pits will appear clear through illuminated slide with contrasting brown to black background (*Figure 1*). Partially resorbed film may appear as a thinner "beach front" that stains yellow to light brown.

Simple Calculation

Using a digital camera, a selected area of the BD BioCoat Osteologic Disc was photographed and converted to a tif image for manipulation in Adobe® Photoshop® (Adobe Systems, Inc.). Maximizing brightness and contrast controls, the image was enhanced and then inverted to yield the negative image (*Figure 2*). Using the histogram function in Adobe Photoshop set to cover half of the gray scale, the percentile of pixels in this range represents 5.2%. This represents the total resorbed area.

Simple Calculation (continued)

In the following method the file was then uploaded to a density program to quantify pit numbers and area. Using AlphaEase™ Version 3.3 (Alpha Innotech Corp.) total resorbed area was calculated to be 4.6% over the same gray scale range and the total number of resorption pits was 331.

Although on a small scale the same process could be expanded to include the entire BD BioCoat™ Osteologic™ Disc.

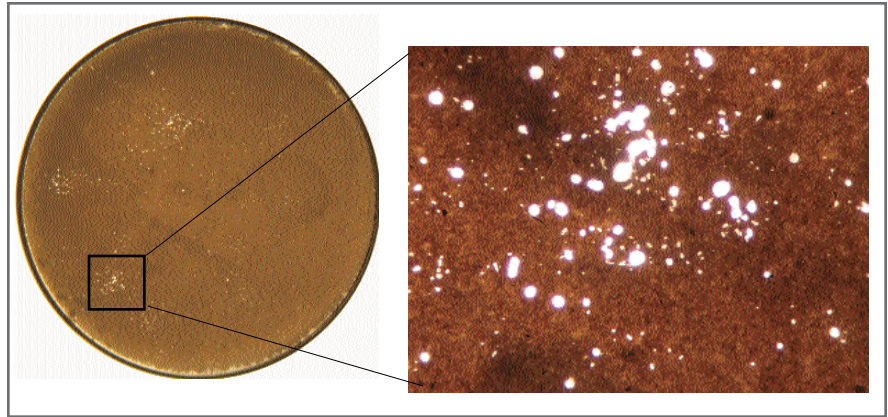


Figure 1: von Kossa stain of BD BioCoat™ Osteologic™ Disc following ten-day culture with rat bone marrow cell washouts. Back illuminated for photography. Enlarged area shows resorption pits. Background stained tan to dark brown.

References

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4. Schneider, M.A., et al., ASBMR 18th Annual Meeting Abstract (1996).

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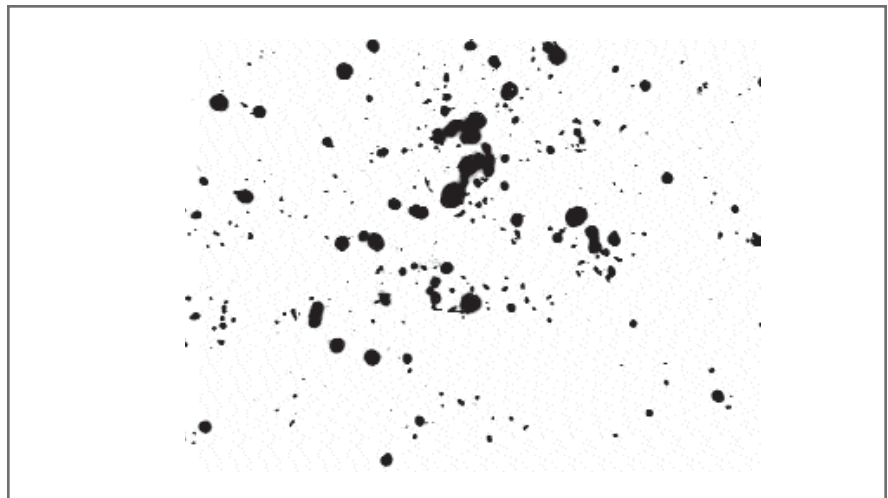


Figure 2: Photo negative image of resorption area for image analysis.

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