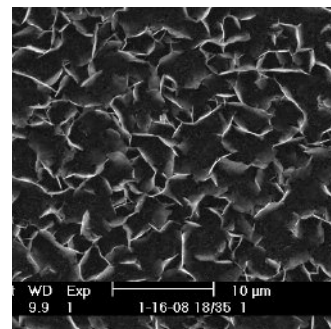


Corning® Osteo Assay Surface: A New Tool to Study Osteoclast and Osteoblast Differentiation and Function



SnAPPShots

A brief technical report
from the Corning
Development Group

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Abstract

Bone is a biomaterial composed of organic and inorganic molecules that continuously remodel to preserve structural integrity and adaptation to stress. Two major types of cells are responsible for this process: the osteoblast and the osteoclast, which synthesize and resorb bone, respectively. A delicate balance between the function of these two cell types is required to maintain proper bone homeostasis. An imbalance between osteoclast and osteoblast activity can lead to diseases, such as osteopetrosis or osteoporosis (1). Corning Osteo Assay Surface multiple well plates can be used to characterize and assay osteoclast-mediated bone resorption, providing a method to study osteoclastogenesis and functionality. Additionally, the Corning Osteo Assay Surface provides a biomimetic surface for osteoblast cell growth and differentiation.

The osteoclast is a multinucleated cell that differentiates from monocytes under the influence of macrophage-colony stimulating factor (M-CSF) and the receptor activator for nuclear factor- κ B ligand (RANKL). After differentiation, the osteoclast polarizes, forming a resorption lacuna between its apical membrane and the mineralized bone surface. Within this space, the osteoclast secretes enzymes and acid. The acidic pH and activated enzymes promote dissolution of the bone mineral matrix (2). Here, we present a synthetic surface for the study of osteoclast, differentiation and pit formation. This document describes methods for the differentiation of both human osteoclast precursor cells and the mouse macrophage cell line RAW 264.7 to osteoclasts. Standard methods for staining of the osteoclast-specific marker tartrate-resistant acid phosphatase (TRAP) and visualization of osteoclast pit formation are employed (3). Additionally,

Corning Osteo Assay Surface multiple well plates can be used to assess cell functional activity in response to drug treatment.

Research related to basic osteoblast biology focuses on cell differentiation and function (4). Osteoblasts are of mesenchymal origin. Several growth factors have been implicated in osteoblast differentiation, including bone morphogenic proteins (BMPs) and Indian hedgehog. Additionally, several hormones are believed to play an important role in bone formation, including estradiol, parathyroid hormone and leptin. Osteoblast differentiation on the new Corning Osteo Assay Surface provides an *in vivo*-like surface for improved osteoblast growth and differentiation. The following describes the application of Corning Osteo Assay Surface for bone nodule formation from differentiated osteoblasts, a key parameter of osteoblast function *in vitro*. These bone nodules on Osteo Assay plates can be visualized by von Kossa and osteocalcin staining.

Understanding the biology of bone disease is of critical importance to the aging population. Due to the difficult nature of the assays involved in studying bone, scientists have long been limited with regard to the methods, available formats and throughput used in this area of research. Corning Life Sciences has developed a new platform to aid researchers with their bone studies that is consistent and repeatable. The Corning Osteo Assay Surface multiple well plates are coated with a proprietary synthetic inorganic bone mimetic, which allows the direct assessment of osteoclast and osteoblast activity *in vitro*. This surface permits easy and reliable quantification of results in a format that is amenable to drug screening, as well as basic research applications.

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Materials and Methods

Human Osteoclast Precursor Cells for Osteoclast Differentiation and Pit Formation Assay

To culture human osteoclast precursor cells, one vial (>1 million cells) of Osteoclast Precursor cells (Lonza Cat. No. 2T-110) was rapidly thawed and washed with Osteoclast Precursor Basal Medium (Lonza Cat. No. PT-8201), which had been supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 units/mL penicillin and streptomycin (Pen/Strep). Cells were counted and viability determined by using trypan blue exclusion.

Primary human osteoclast precursors cannot be passaged. However, the cells can be differentiated into osteoclasts in the presence of RANK ligand (66 ng/mL) and M-CSF (33 ng/mL). Precursor cells grown in the presence of M-CSF but lacking soluble RANK ligand will expand in number but will not differentiate into functional osteoclasts. All cells, including a non-differentiation control, were re-suspended to a final concentration of 50,000 cells/mL, and 200 μ L were plated per well in a 96 well Corning® Osteo Assay Surface plate. Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. After 5 to 7 days of culture, the osteoclasts were identified as large multinucleate cells when visualized by phase microscopy.

Mouse Macrophage Cell Line RAW 264.7 for Osteoclast Differentiation and Pit Formation Assay

Mouse RAW 264.7 cells were obtained from the ATCC (ATCC TIB-71). One vial of cells was quickly thawed and suspended in 10 mL of prewarmed growth medium (GM) (DMEM, ATCC 30-2002) supplemented with 10% FBS and 1% Pen/Strep. Cells were used to seed one T75 Tissue Culture Treated (TCT) flask (Corning Cat. No. 430641) and cultured at 37°C in a humidified atmosphere of 5% CO₂ overnight. The medium was replaced, and cells were cultured for 3 to 5 days until the flask was approximately 85% confluent, replacing the medium every 2 to 3 days.

Cells were detached from the flask using Trypsin/EDTA solution and re-suspended as a single cell suspension. The cells were diluted 1:5 or 1:6 in GM and transferred to T75 TCT flasks. Cells were passaged 1 to 2 times to obtain enough cells for the number of plates to be tested. Cells were passaged a maximum of 4 times, as they lose their ability to differentiate with additional passages.

After trypsinization, the cells were re-suspended in differentiation medium (DM) (MEM-Alpha (Gibco A10490) supplemented with 10% FBS, 1% Pen/Strep, and 50 ng/mL RANK ligand (Sigma R-0525) at a concentration of 50,000 cells/mL. The diluted cell suspension (100 μ L, approximately 5,000 cells per well) was transferred into each well of a 96 well Corning Osteo Assay plate (Corning Cat. No. 3988) to begin the differentiation process. Negative control wells received 100 μ L of DM (without cells) for future staining and pit visualization (see Results and Discussion). Plates were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 7 days with a medium change on day 3 or 4.

Tartrate-Resistant Acid Phosphatase Staining for Differentiated Osteoclast

To analyze for the presence of the osteoclast-specific marker TRAP, a staining kit from B-Bridge International (Cat. No. AK04) was used. Typically, cells were washed with 100 μ L of PBS and fixed for 5 minutes with the fixative reagent at room temperature. The cells were washed three times with distilled water and then stained for 20 to 60 minutes at 37°C with the chromogenic substrate supplied with the kit. Cells were washed with distilled water to stop the reaction when optimum color was achieved. Longer incubations with the stain caused precipitation and were avoided.

Resorption Pit Assay for Osteoclast

To analyze the surface for pit formation, the media was aspirated from the wells on day 7, and 100 μ L of 10% bleach solution was added. Cells were incubated with the bleach solution for 5 minutes at room temperature. The wells were washed twice with distilled water and allowed to dry at room temperature for 3 to 5 hours. Individual pits or multiple pit clusters were observed using a microscope at 100x magnification.

Human Osteosarcoma Cell Line Saos-2 for Osteoblast differentiation and bone nodule formation

Human osteosarcoma cell line Saos-2 cells (ATCC Cat. No. HTB-85) were cultured in growth medium (McCoy's 5A supplemented with 15% FBS and 1% Pen/Strep in a T175 flask (Corning Cat. No. 430641) until confluent. Cells were detached from the flask using Trypsin/EDTA, washed with growth medium and re-suspended at 80,000 cells/mL in growth medium and plated 200 μ L/well in a Corning Osteo Assay 96 well plate. Cells were incubated overnight at 37°C, 5% CO₂ in a cell culture incubator. The following day, growth medium was replaced with differentiation medium (growth medium supplemented with 50 μ g/mL ascorbic acid, 10 mM glycerol phosphate, and 10 nM dexamethasone). Bone nodule formation was observed after 1 week. Cells were cultured in differentiation medium for up to 3 weeks, with media changes approximately every three days.

Von Kossa Staining for Bone Nodule Formation

Osteoblast differentiation is marked by the formation of mineralized nodules. A common method for the detection of these nodules is von Kossa staining. Cells were washed with phosphate buffered saline (PBS) 3 times and fixed with 3.7% formalin in PBS for 45 minutes at room temperature. After washing with water, cells were stained with 5% silver nitrate for an additional 45 minutes at room temperature under a bright light. To stop the silver nitrate development, cells were washed with water and treated with a 5% solution of sodium thiosulfate. Following another water wash and air drying, nodules were visualized as dark staining patches using a light microscope.

Immunohistochemistry Staining for Bone Nodules

Bone nodule formation can be conveniently visualized by using the Lonza OsteoImage kit (PA-1503). Fixed cells were stained with the proprietary bone nodule fluorescent reagent following the manufacturer's protocols.

Results and Discussion

TRAP expression is a standard measure of osteoclast differentiation. TRAP is a di-iron containing metalloenzyme that is expressed in differentiating osteoclasts. TRAP expression is dramatically upregulated during osteoclast differentiation, and is therefore a common histochemical marker for osteoclasts (3). Figure 1A shows TRAP staining of human osteoclast precursor cells that have been stimulated to differentiate in the presence of RANK ligand and M-CSF for 5 days. Figure 1B shows TRAP staining of mouse RAW 264.7 cells, which have been stimulated to differentiate in the presence of RANK ligand for 7 days (5). Both micrographs show a large number of multinucleated differentiated osteoclasts, which are representative of the cells observed on the Corning® Osteo Assay Surface multiple well plate.

In addition to the TRAP assay, another method to evaluate osteoclast differentiation and function is the ability to resorb mineralized matrix and form resorption pits on the surface. Figure 2A shows a typical field with numerous large pits produced by human osteoclast precursor cells on the Corning Osteo Assay Surface after 5 days of differentiation. Figure 2B shows a typical field of pits produced by RAW 264.7 on the Corning Osteo Assay Surface after 7 days of differentiation. Resorption pits are smaller using the RAW 264.7 cell line. However, it is clear that the Corning Osteo Assay Surface is a suitable substrate for the detection of osteoclast functional activity by the resorption pit assay.

Osteoblast differentiation and bone-nodule formation represent counterparts to osteoclast differentiation and bone resorption. As stated above, the balance between these two functions is essential to healthy bones. Osteoblast differentiation is marked by the formation of mineralized nodules (6). A convenient method for the detection of these nodules is von Kossa staining. Figure 3 shows the results of von Kossa staining of Saos-2 cells, which were grown and differentiated for 21 days on the Corning Osteo Assay Surface multiple well plates, compared with cells that were differentiated on a standard TCT plate. The nodules are larger and more easily recognized on the Corning Osteo Assay Surface, compared with the TCT plate, which would be invaluable in a drug screen.

An alternative method for visualizing bone nodule formation is by using the Lonza OsteoImage kit. This kit allows not only for the identification of bone nodules but also the concomitant staining of the nucleus and actin stress fibers, providing even more insight into the viability of the cells (7). Figure 4 shows an overlay of a nodule stained with the nuclear stain DAPI, an actin stain for cytoskeleton and the proprietary OsteoImage bone nodule staining reagent. For the micrograph, Saos-2 cells had been differentiated for 21 days on the Corning Osteo Assay Surface as described above.

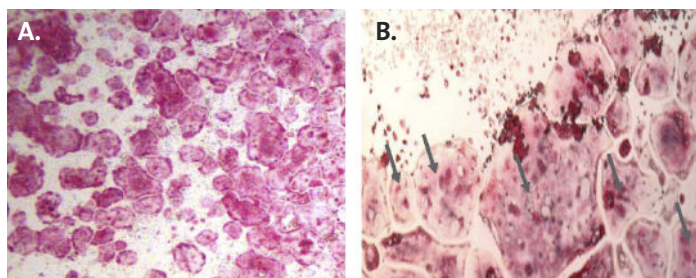


Figure 1. (A) TRAP staining of differentiated human osteoclast precursor cells. (B) TRAP staining of mouse RAW 264.7 cells.

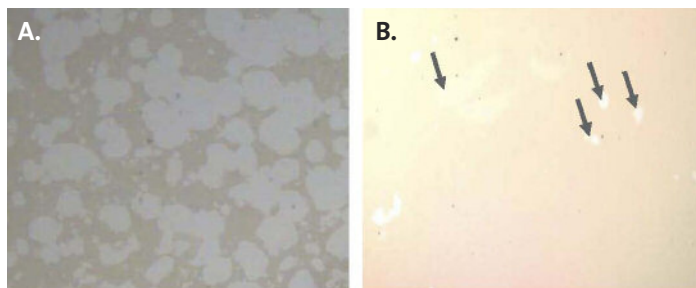


Figure 2. (A) Visualization of resorption pits from human osteoclast precursor cells and (B) mouse RAW 264.7 resorption pits.

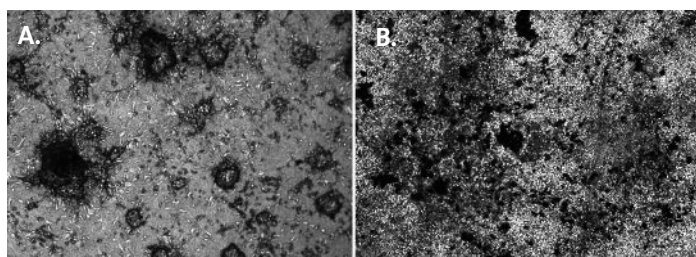


Figure 3. (A) Von Kossa staining of Saos-2 cells differentiated on Corning Osteo Assay Surface compared with (B) cells differentiated on TCT.

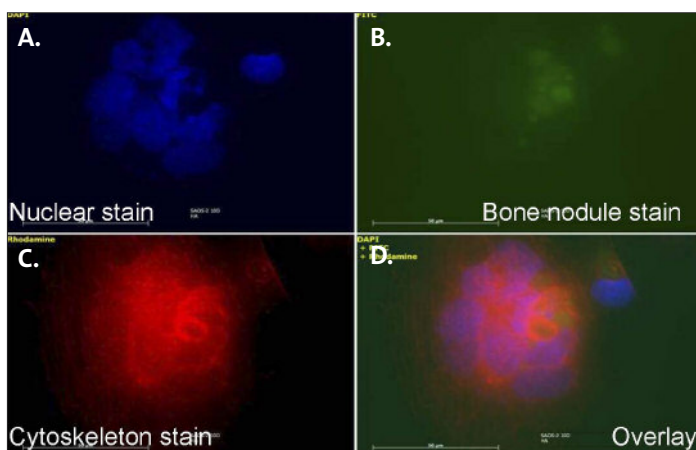


Figure 4. Bone nodule staining of differentiated Saos-2 cells on Corning Osteo Assay Surface plates.

Conclusions

- ▶ The Corning® Osteo Assay Surface is a suitable substrate for the growth and differentiation of primary osteoclast precursors and mouse RAW 264.7 cells into mature osteoclasts.
- ▶ Use of the Corning Osteo Assay Surface results in large numbers of differentiated osteoclasts as measured by the histochemical staining of tartrate-resistant acid phosphatase, a key marker of osteoclast differentiation.
- ▶ The Corning Osteo Assay Surface can also be used to measure the functionality of differentiated osteoclasts by measuring the size and number of resorption pits formed.
- ▶ The Corning Osteo Assay Surface provides a suitable growth and differentiation surface for Saos-2-derived osteoblasts.
- ▶ Bone nodule formation on the Corning Osteo Assay Surface is more organized and distinct, compared to the same cells differentiated on TCT.
- ▶ The Corning Osteo Assay Surface is compatible with immunohistochemical staining techniques, as demonstrated by the use of OsteoImage stain (Lonza) and standard nuclear and actin staining techniques.
- ▶ The Corning Osteo Assay Surface is an ideal substrate for drug discovery in the area of osteoclast differentiation.

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