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Obtaining primary osteocytes through murine calvarial fractionation of GFP-expressing osteocytes

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Corresponding Author:	Hideki Kitaura Tohoku Daigaku Sendai, Miyagi JAPAN
Corresponding Author's Institution:	Tohoku Daigaku
Corresponding Author E-Mail:	hideki.kitaura.b4@tohoku.ac.jp
Order of Authors:	ASEEL MARAHLEH
	Hideki Kitaura
	Saika Ogawa
	Wei-Ren Shen
	Jiawei Qi
	Fumitoshi Ohori
	Takahiro Noguchi
	Yasuhiko Nara
	Adya Pramusita
	Ria Kinjo
	Itaru Mizoguchi
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Cover Letter

April 30, 2020

Editor of Journal of Visualized Experiments

Dear Sir/Maʾm:

I herewith attach a revised manuscript JoVE61513 entitled "Obtaining primary osteocytes through murine calvarial fractionation of GFP-expressing osteocytes" which I would like to publish to "**Journal of Visualized Experiments**"section of **Biology**. I appreciate your careful review of the manuscript and editorial decision of publication at your earliest convenience, thank you.

Corresponding author: Hideki Kitaura.

Address: Division of Orthodontics and Dentofacial Orthopedics, Tohoku University,

Graduate School of Dentistry. 4-1, Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan

Tel: +81-22-717-8374

Fax: +81-22-717-8372

E-mail: hideki.kitaura.b4@tohoku.ac.jp

Sincerely yours,

Hideki Kitaura, DDS, Ph.D.

Division of Orthodontics and Dentofacial

Orthopedics, Tohoku University, Graduate

School of Dentistry. 4-1, Seiryo-machi,

Aoba-ku, Sendai, 980-8575, Japan

Tel: +81-22-717-8374

Fax: +81-22-717-8372

E-mail: hideki.kitaura.b4@tohoku.ac.jp

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TITLE:

Obtaining Primary Osteocytes through Murine Calvarial Fractionation of GFP-Expressing Osteocytes

AUTHORS AND AFFILIATIONS:

Aseel Marahleh[1], Hideki Kitaura[1], Saika Ogawa[1], Wei-Ren Shen[1], Jiawei Qi[1], Fumitoshi Ohori[1], Takahiro Noguchi[1], Yasuhiko Nara[1], Adya Pramusita[1], Ria Kinjo[1], Itaru Mizoguchi[1]

[1]Division of Orthodontics and Dentofacial Orthopedics, Department of Translational Medicine, Tohoku University Graduate School of Dentistry, Sendai, Japan.

Corresponding author:

Hideki Kitaura (hideki.kitaura.b4@tohoku.ac.jp)

Email addresses of Co-authors:

Aseel Marahleh (marahleh.aseel.mahmoud.t6@dc.tohoku.ac.jp)
Saika Ogawa (saika.ogawa.a4@tohoku.ac.jp)
Wei-Ren Shen (shen.wei.ren.t5@dc.tohoku.ac.jp)
Jiawei Qi (qi.jiawei.p8@dc.tohoku.ac.jp)
Fumitoshi Ohori (fumitoshi.ohori.t3@dc.tohoku.ac.jp)
Takahiro Noguchi (takahiro.noguchi.r4@dc.tohoku.ac.jp)
Yasuhiko Nara (yasuhiko.nara.q6@dc.tohoku.ac.jp)
Adya Pramusita (adya.pramusita.q6@dc.tohoku.ac.jp)
Ria Kinjo (ria.kinjou.p5@dc.tohoku.ac.jp)
Itaru Mizoguchi (itaru.mizoguchi.c3@tohoku.ac.jp)

KEYWORDS:

Primary osteocyte, Dmp1, SOST, GFP, Fractionation, FACS.

SUMMARY:

This protocol describes the dissection of neonatal dmp1-topaz mouse calvaria and isolation of osteocytes expressing the green fluorescent protein through cell digestion and fractionation, in addition to osteocyte preparation for fluorescence activated cell sorting (FACS).

ABSTRACT:

The osteocyte, once thought to be a passive resident of the bone given the backstage function of sensing mechanical loading, is now brought to the spotlight and has been shown to have multiple major functions like actively modifying the extracellular matrix and forming an endocrine organ with the lacunocanalicular system that encloses it sending messages to distant sites. Owing to the methods that made it possible to test the osteocyte in vitro from isolating primary osteocytes to osteocyte-like cell lines, osteocytes are now experiencing a resounding interest and a surge of knowledge on structure and function. Many aspects of the osteocyte biology and interaction with other molecular components are yet to be discovered. In this protocol, we describe in detail the

efficient isolation of primary osteocytes from dmp1-topaz neonatal mouse calvaria, which express the green fluorescent protein in osteocytes, through cell fractionation and subsequently acquiring cultures of primary osteocytes by FACS.

INTRODUCTION:

Osteocytes are terminally differentiated cells from osteoblastic progenitors that became embedded in their secreted matrix[1]. They are the most abundant and longest-living cells among bone cell populations. They reside within lacunae and have a characteristic stellate morphology with dendrites that extend through channels called canaliculi forming an extensive network of communication and metabolic exchange with their surrounding environment and the bone surface[2]. Osteocytes choreograph both osteoblasts and osteoclasts roles in bone remodeling, they are the primary mechanosensory cells conferring adaptation to mechanical loading[3], are involved in phosphate homeostasis[4] and bone matrix mineralization[5], and together with the lacunocanalicular system, they act as an endocrine organ signaling distant tissues[6].

Osteocytes are situated within a mineralized matrix, which limits accessibility and renders their isolation challenging, thus hindering in vitro investigation. One of the first isolation methods described isolated osteocytes from chicken calvaria using an osteocyte-specific monoclonal antibody (OB7.3)[7] which was later known to be the avian variant of PHEX (PHosphate-regulating gene with homology to Endopeptidases on the X chromosome)[8]. Other researchers used sequential digestion of rat[9] or mouse[10] long bones to obtain osteocyte-rich fractions in which osteocyte purity was reported to be around 70%[9]. Limitations of this procedure include the sub-optimal purity of cultures contaminated with other cell types besides osteocytes and that osteocytes could be potentially overgrown by other cells in culture since osteocytes have lost the ability to divide. These challenges restrict the usability of long term cultures.

To overcome these limitations, different osteocyte cell lines were developed. The MLO-Y4 cell line[11] and the MLO-A5 cell line[12] are notably the most widely studied cell lines which are useful for the study of the early stage osteocytes; however, they are less useful for studying mature osteocyte signaling as they express low levels of sclerostin and FGF23[13], both of which are mature osteocyte markers. Other cell lines including IDG-SW3[14] and Ocy454[15], express high levels of sclerostin and FGF23 and are useful in studying the late osteocyte stage. Cell lines prove to be useful research tools; nevertheless, they do not come without limitations as they do not fully represent the biology of the primary cell. Different cell lines represent different developmental stages of the osteocyte maturity spectrum, and cell lines fail to represent the heterogeneity of primary osteocytes[16,17].

To obtain pure cultures of primary osteocytes, researchers took advantage of the cre mouse model in which the 8-kb dmp1 promoter is used to drive green fluorescent protein (GFP) expression in osteocytes[18,19]. Dual transgenic mice (pOB-Col 2.3- GFP-cyan and DMP1-GFP-topaz) by Paic et al.[19] and dmp1-topaz transgenic mice by Nakashima et al.[20] have been used to obtain osteocyte populations. In which they employed sequential digestion and FACS of osteocytes expressing GFP to acquire cultures of primary osteocytes[19,20]. The direction of cre in the 10-kb

dmp1 reporter mouse Ai9, which activates the tdTomato protein, was shown to be present in osteocytes, osteoblasts, muscles, and cells within the bone marrow. The 8-kb dmp1 promoter had the same expression pattern, but only a proportion of osteoblasts and bone marrow cells expressed the protein, which indicates that the 8-kb dmp1 promoter is more specific[21]. Despite this, results obtained using the 8-kb dmp1 promoter should be interpreted with caution, and gene expression profiles should be routinely carried out using osteocyte vs. osteoblast-specific markers to ensure that the population obtained is of high enough purity.

Osteoclast markers OSCAR and Dcstamp were found in hematopoietic non-depleted vs. depleted osteocyte populations, this finding led the authors to conclude that digests obtained from fractionation of 8-kb dmp1-topaz neonatal calvaria and GFP sorting are contaminated with hematopoietic cells. Contamination with hematopoietic cells could have been mitigated by tightening the GFP sort gate since GFP-positive hematopoietic cells had a reasonably lower GFP intensity than GFP-positive mesenchymal cells (osteocytes)[22].

The methods for studying osteocytes in vitro have contributed to the recent wealth of information on osteocyte biology. However, osteocyte isolation remains a labor-intensive and lengthy procedure with low cell yields. The described method of bone digestion using collagenase and EDTA often up to fraction 8[23], takes several hours in which osteocyte viability is taxed. Researchers have reported using fractions (2–5) for cell sorting[20], and have shown that the expression profile of genes associated with osteocytes versus those of osteoblasts confirms the success of isolating pure osteocyte populations[20]. In this article, we describe the process of obtaining fractions (2–5), and we compare the yield of osteocytes from each fraction starting with fraction 1 through 8 to determine the return of osteocytes in each fraction. We also describe the dissection of newborn dmp1-topaz mouse calvaria and calvarial digestion using collagenase and ethylenediaminetetraacetic acid (EDTA), as well as, preparation of cells for FACS.

PROTOCOL:

All animal procedures and animal care were performed in accordance with Tohoku University rules and regulations.

1. Dissection of newborn dmp1-topaz mouse calvaria

1.1. For this protocol, use 6–7 day old pups of C57BL/6-Tg(Dmp1-Topaz)1Ikal/J mice. Euthanize mice with 5% isoflurane inhalation and then transfer the pups into 70% ethanol.

1.2. Transfer the euthanized pup into a non-treated culture dish.

1.3. Using scissors and tweezers, grab the skin at the base of the skull and make an incision.

1.4. Using the first incision as a starting point, cut on both lateral sides of the skull in front of the ears, remove the skin, and expose the calvaria.

1.5. Hold the head from the nasal bridge and cut the calvaria along the lambdoid suture. Cut along the lateral edges of the parietal bones and extend it to include the frontal part.

1.6. Separate the calvaria from the underlying brain tissue.

NOTE: To ensure a clean bony calvaria with minimal soft tissue attachment do not cut deep into the bone; for reference, one should be able to see the shadow of the scissors beneath the bone.

1.7. Transfer the calvaria into phosphate-buffered saline (PBS, pH 7.4 throughout the protocol). Make sure the concave part is facing upwards.

2. Fractionation of newborn mouse calvaria

2.1. Transfer up to 5 calvariae to a 50 mL conical tube containing 5 mL of 2 mg/mL collagenase solution. Use fresh collagenase solution prepared just before use.

2.1.1 To prepare a fresh collagenase solution, add collagenase powder to isolation buffer (70 mM NaCl, 10 mM $NaHCO_3$, 60 mM sorbitol, 3 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mg/mL bovine serum albumin (BSA), 5 mg/mL glucose and 25 mM HEPES) at 2 mg/mL. Dissolve the collagenase powder on a magnetic stirrer.

2.1.2 Run the collagenase solution through a 0.22 µm sterile filter unit into a 50 mL tube and keep it on ice throughout the protocol.

2.2. Incubate the calvaria at 37 °C for 20 min on a shaker at 300 rpm to obtain fraction 1.

2.3. Discard the digest of fraction 1, wash the calvaria with 5 mL of phosphate buffered saline (PBS) and discard the wash.

2.4. Incubate the calvaria in 5 mL of 5 mM ethylenediaminetetraacetic acid (EDTA) containing 1 mg/mL BSA in PBS, PH 7.4 at 37 °C for 15 min on a shaker at 300 rpm.

2.5. Collect the digest in a 50 mL conical tube. Wash the calvaria with 5 mL of PBS and add the wash solution to the digest.

NOTE: At each point of collecting the digest and washing, pipette the bones in their solution to detach the cells from the bone and to prevent doublets and cell aggregates.

2.6. To obtain fraction 2, centrifuge the digest at 300 x *g* at 4 °C for 5 min. Discard the supernatant, re-suspend the pellet in 8 mL of α-Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin G, and 100 µg/mL streptomycin. Seed on a 10 cm culture dish. Incubate fraction 2 at 37 °C under 5% CO_2.

2.7. Incubate the calvaria in 5 mL of collagenase solution at 37 °C for 20 min on a shaker at 300 rpm.

2.8. To collect fraction 3, repeat steps 2.5–2.6. Incubate fraction 3 at 37 °C under 5% CO_2.

NOTE: At this point, the bones will become smaller, and they will be reduced to fragments. To ensure that no bones get accidentally aspirated into the digests, which leads to cell loss in the process, use a small tip pipette for collecting the digests (1000–200 µL pipette tips).

2.9. Incubate the calvaria in 5 mL of collagenase solution at 37 °C for 20 min on a shaker at 300 rpm.

2.10. To collect fraction 4, repeat steps 2.5–2.6. Incubate fraction 4 at 37 °C under 5% CO_2.

2.11. Incubate the calvaria in 5 mL of 5 mM EDTA containing 1 mg/mL BSA in PBS (pH 7.4) at 37 °C for 15 min on a shaker at 300 rpm.

2.12. To collect fraction 5, repeat steps 2.5–2.6. Incubate fraction 5 at 37 °C under 5% CO_2. Osteocytes can be prepared for sorting on the same day or cultured up to 24 h before sorting.

3. Preparation of osteocytes for fluorescence activated cell sorting

3.1. Aspirate the medium of each cell fraction and wash gently with 10 mL of PBS twice.

3.2. Add 5 mL of 0.5% Trypsin-EDTA in PBS and incubate the cells for 5 min at 37 °C. Add 5 mL of 10% FBS α-MEM and pipette to detach the cells gently. Combine the cells in each fraction by sifting through a 40 µm cell strainer into a 50 mL conical centrifuge tube.

3.3. Wash the cells with 10 mL of PBS and collect by sifting through a 40 µm cell strainer into a 50 mL conical centrifuge tube. Centrifuge the cells at 300 x *g* at 4 °C for 5 min.

3.4. Aspirate the supernatant and add 10% FBS α-MEM to the pellet. Adjust cell concentration to approximately 1 x 10^7 cells/mL.

3.5. Filter the cells in a 35 µm nylon mesh capped tube. The cells are now ready for FACS.

3.6. For this protocol, use a 100 µm size nozzle. The collection fluid should consist of 10% FBS α-MEM. Keep the sample being sorted on agitation and at 4 °C throughout the sort if possible.

3.7. Prior to the sort, optimize gating to remove artifacts and cells by adjusting the side scatter (SSC) area and forward scatter (FSC) area. Eliminate doublets by adjusting SSC-width vs SSC-Height and FSC-area vs FSC-width. GFP-negative osteocytes should be used as a control to adjust the parameters of the sort instrument.

3.8. After cell collection is complete, centrifuge the cells at 300 x *g* at 4 °C for 10 min. Wash cells with PBS. Centrifuge the cells at 300 x *g* at 4 °C for 5 min.

3.9. Re-suspend the cells and adjust the number as desired using 10% FBS α-MEM.

REPRESENTATIVE RESULTS:

The purpose of this protocol is to demonstrate the process of obtaining cultures of primary osteocytes from dmp1-topaz neonatal mouse calvaria through a fractionation process using collagenase to degrade the collagen matrix and EDTA for calcium chelation, after which cells are prepared for FACS to separate osteocytes from other cell populations.

Methods for obtaining primary osteocytes from neonatal mouse calvaria often describe the use of fractions (1–8) for sorting[23]. To test the efficiency of this method, we compared the yield of osteocytes obtained from one dmp1-topaz mouse calvaria, starting from fractions 1 through 8. Fractions 1–8 were sorted separately to determine the percentage and yield of osteocytes from each fraction. After the sort, we cultured osteocytes for 24 h on a 96-well plate to compare the density of the seeded cells. The density of osteocytes in fractions 2–5 is shown to be higher than that of fraction 1, and osteocyte density starts to decrease remarkably in fractions 6, 7, and 8 (**Figure 1A**).

Although the percentage of osteocyte obtained among all fractions is not statistically significant (**Figure 1B**), the density of osteocytes differs dramatically. Researchers[20] have reported the use of fractions 2–5 for isolating osteocytes via FACS, and we show in **Figure 1** that using fractions 2–5 optimizes the process for obtaining osteocytes and decreases the time of the sort.

Figure 2 shows the number of cells and gating strategy practiced to isolate GFP-positive from GFP-negative cells in which cells obtained through fractionation of GFP-negative mouse calvaria were used as control. Osteocytes obtained through this method were analyzed for gene expression of Dmp1 and SOST, which are known osteocytes markers. Dmp1 and SOST mRNA expressions are higher in osteocytes when compared to pre-sort fraction 2, which is known as a high osteoblast fraction (**Figure 3A**). **Figure 3B** shows the morphology of a GFP-positive osteocyte retaining a stellate shape with dendrites extending from the cell body cultured for 24 h on a plastic culture dish post sort.

FIGURE AND TABLE LEGENDS:

Figure 1: Efficiency of osteocyte fractionation. (**A**) Microscopic images of the density of osteocytes from fractions 1–8 seeded on a 96-well plate captured after 24 h of the sort. Fractions 2–5 have a higher cell density than fractions 1, and 6–8. Scale bar = 100 µm. (**B**) Percentage of osteocytes obtained from fractions 1–8 as measured by the flow cytometer software. Results are derived from three separate representative experiments. Data are presented as a mean ± standard deviation.

Figure 2: Isolation of GFP-positive osteocytes via fluorescence activated cell sorting. The upper panel represents cells isolated from GFP-negative C57Bl/6J littermate calvaria as GFP threshold control. The lower panel represents the number of cells and gating control practiced to isolate GFP-positive osteocytes from GFP-negative cells in fraction 2 obtained from dmp1-topaz mouse calvaria.

Figure 3: Osteocyte characterization post sort. (**A**) Relative mRNA expression of Dmp1 and SOST of fraction 2 cells cultured for 24 h and osteocytes cultured for 24 h post sort. Data are presented as a mean ± standard deviation. Statistical differences were detected by using Student's t-test: * $p < 0.05$, ** $p < 0.01$. (**B**) Microscopic image of an osteocyte retaining dendrites extending outwards from the cell body cultured for 24 h post sort. Scale bar = 50 µm.

DISCUSSION:

The first isolated osteocyte was from a chicken calvaria[7] isolated by using (OB7.3) or the aviant variant of PHEX; however, this method is limited by the availability of workable antibodies, as osteocyte-specific antibodies that are also specie-specific have to be manufactured. Researchers used a different modification of the sequential enzymatic process to obtain osteocytes from mouse and rat long bones; the reported purity of these cultures were set at about 70%[9]. The development of the cre mouse model allowed for engineering osteocytes, which express GFP on their surface. This mouse model, along with FACS, was used to obtain pure cultures of primary osteocytes[20].

We omit the use of fractions 1 and fractions 6–8 as little amounts of osteocytes come off in these fractions. Using fractions 2–5 give the best possible yield of osteocytes over the shortest processing time; this limits the handling time of osteocytes and works towards preventing cell death or a possible alteration in cell signaling as a result of the stress the cell is subjected to during fractionation. We also culture osteocytes for 24 h pre-sort, which by default, excludes non-adherent suspension cells (hematopoietic cells) during sort preparation. This minimizes contamination by hematopoietic cells expressing GFP[22]. The workflow provided in this protocol takes advantage of previously published methods[23] and shortens the time of the sort allowing for an efficient and quick osteocyte recovery with minimal contamination.

Critical steps in the protocol include obtaining a clean bony calvaria and trimming soft tissue from the brain or connective tissue attached to the bone to limit the contamination of adherent cells (fibroblast and neural cells). Also, pipetting the cells during steps that include obtaining and washing the digests is crucial, since cell aggregates and doublets are read as waste during sorting and will contribute to a low yield of osteocytes.

Osteocytes can be sorted without prior culture. However, this means that a higher number of cells have to be sorted, increasing the time of the sort and increasing the chance of hematopoietic contamination. This can be mitigated by applying hematopoietic cell depletion pre-sort. However, this is taxing and not recommended for routine and batch laboratory analysis[22]. Trouble may arise while sorting due to the presence of large cell aggregates and doublets clogging

fluid flow of the sort machine. In our protocol, this has not been an issue, but this can be solved by reducing the FBS content of the sort buffer (less than 10%).

This protocol does not come without limitations. This method utilizes mouse osteocytes, which do not entirely resemble human osteocytes. This restricts extending the results obtained by studying murine osteocytes to meaningful clinical outcomes. Protocols for isolation of human osteocytes have been described[24], and researchers are encouraged to use the cell species that best serves their goals. As with other protocols, the quantities of osteocytes obtained using this protocol are limited, and a large number of mice are required for large scale analysis, however, by decreasing the time needed for preparing and sorting osteocytes, higher quantities of cells can be acquired in a single time frame.

Osteocytes obtained through this process can be used for further culture and co-culture, gene expression analysis, downstream analysis of substrate activation/ inhibition, molecular probing, and staining applications. Primary cells can also be used to construct a 3D osteocyte matrix model resembling the native osteocytic environment for the study of mechanotransduction and mechanosensing.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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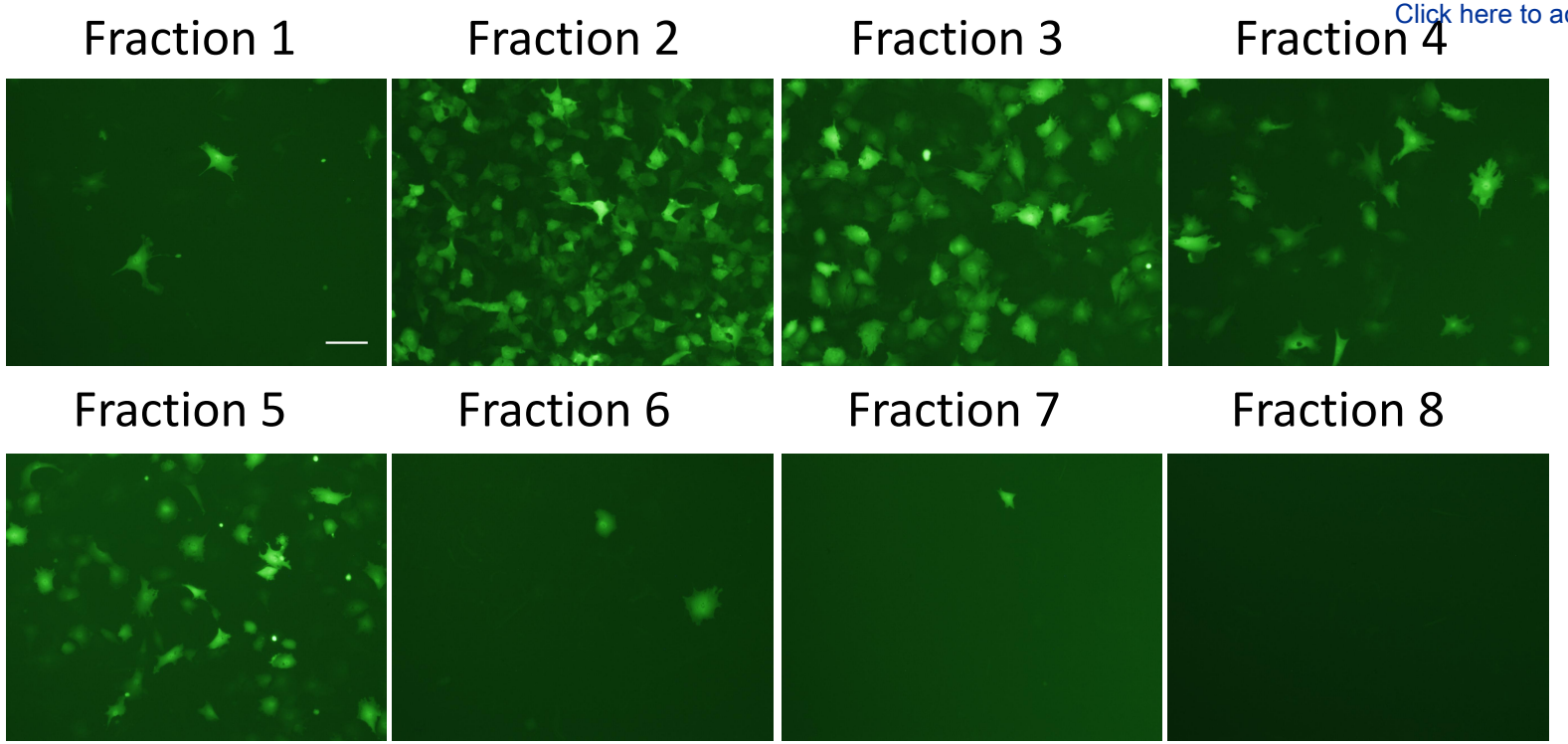

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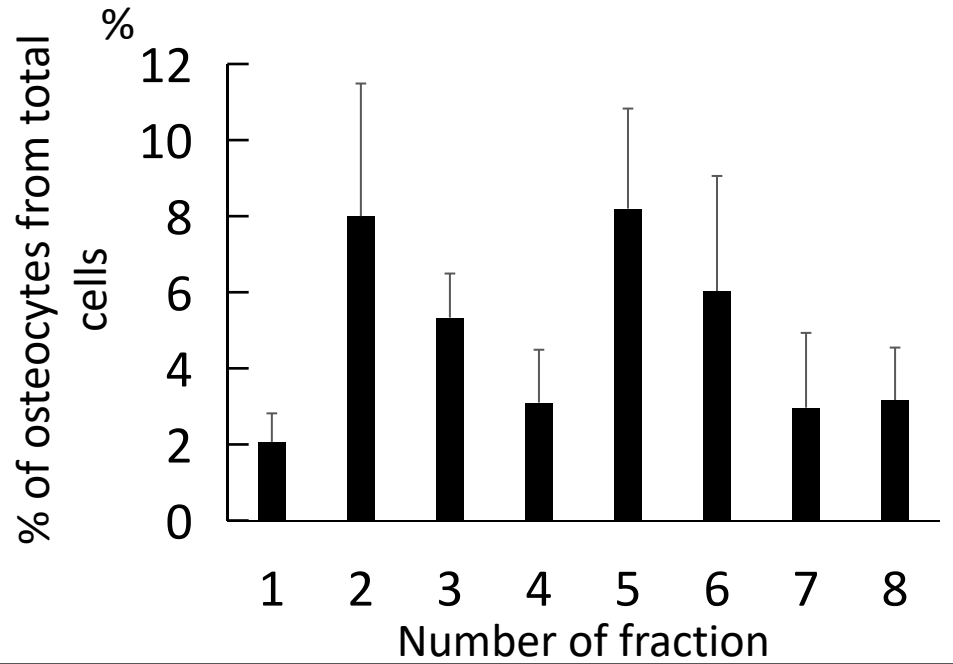

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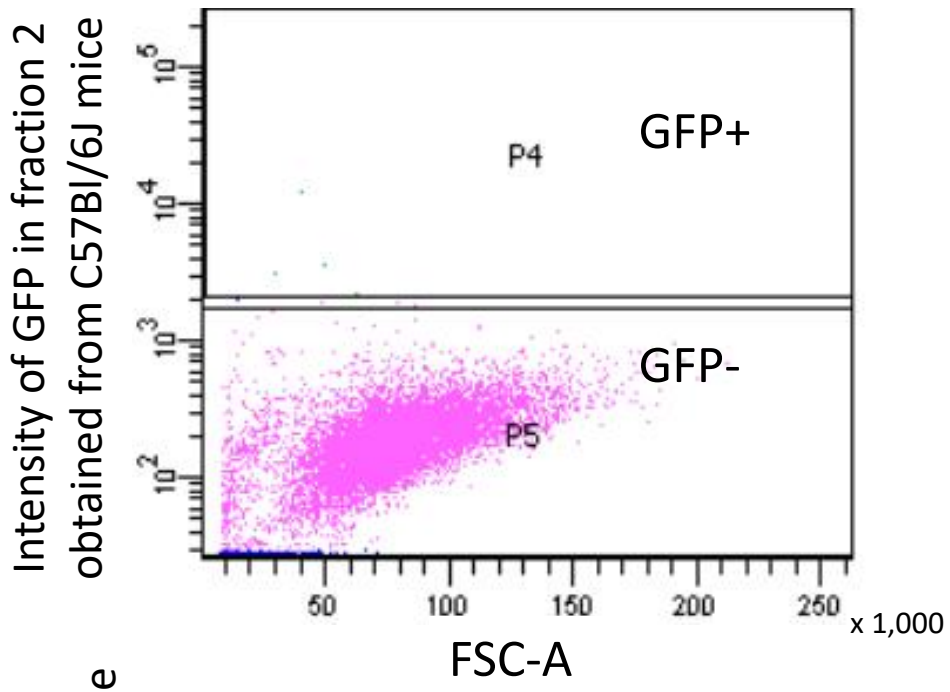

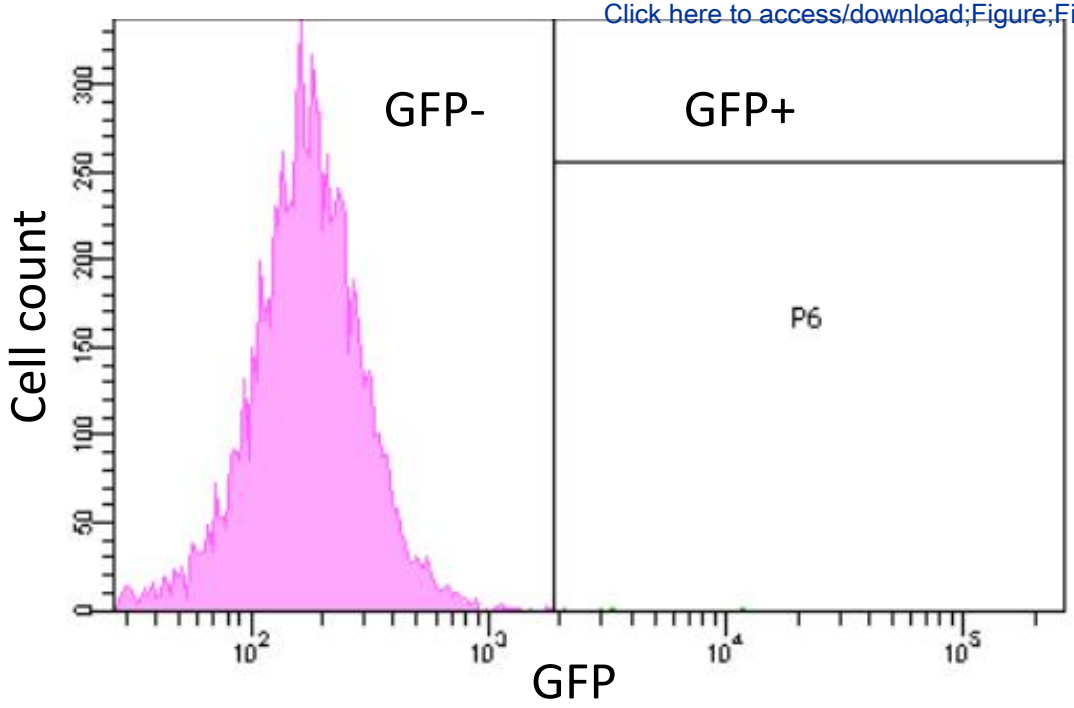

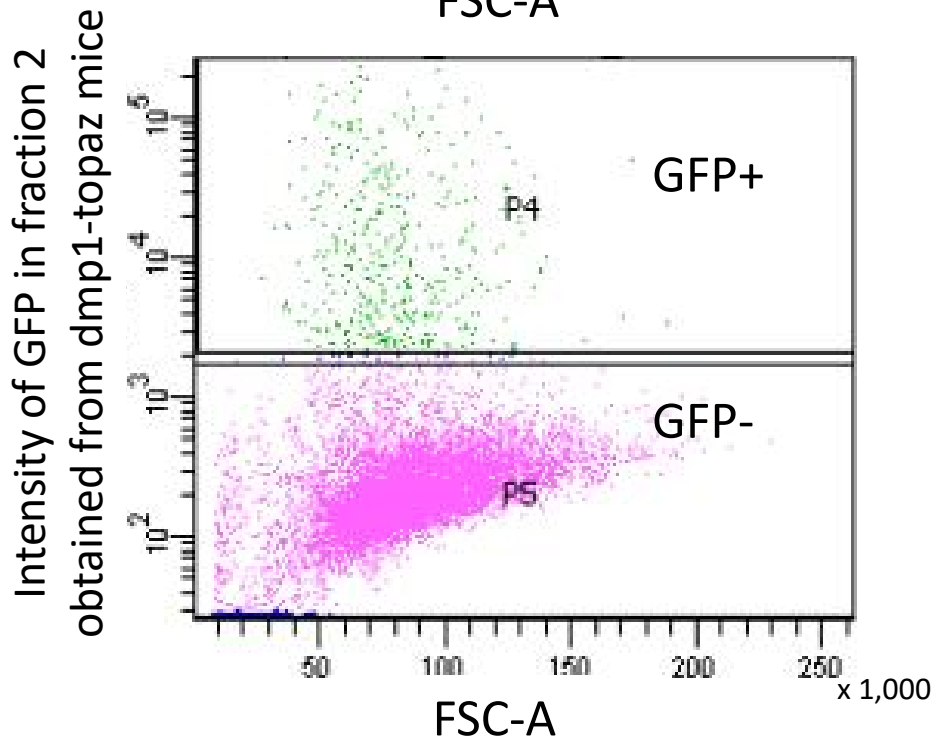

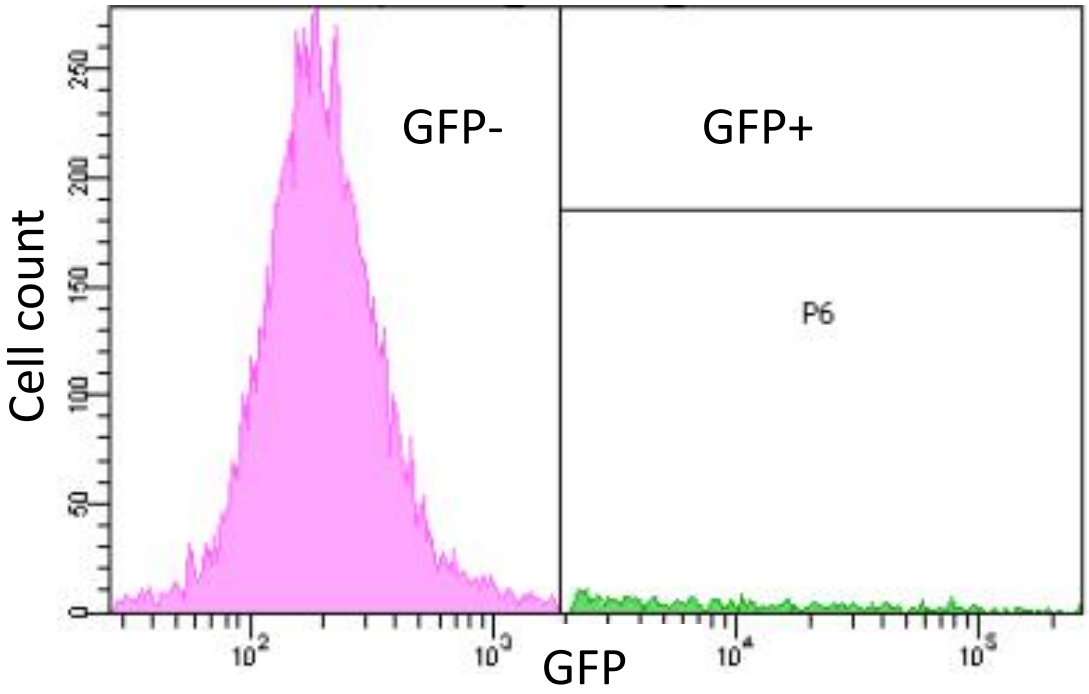

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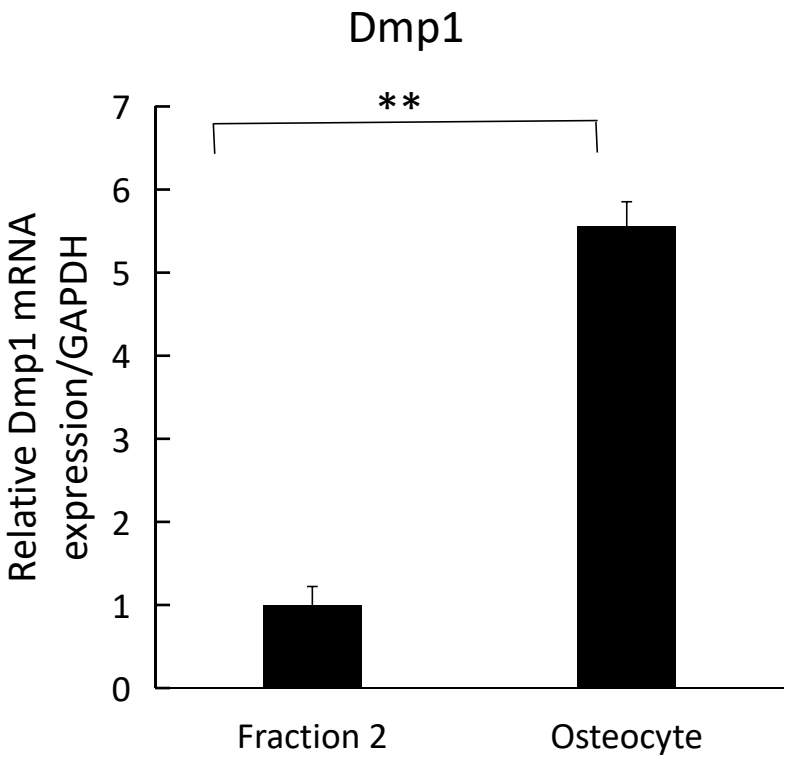
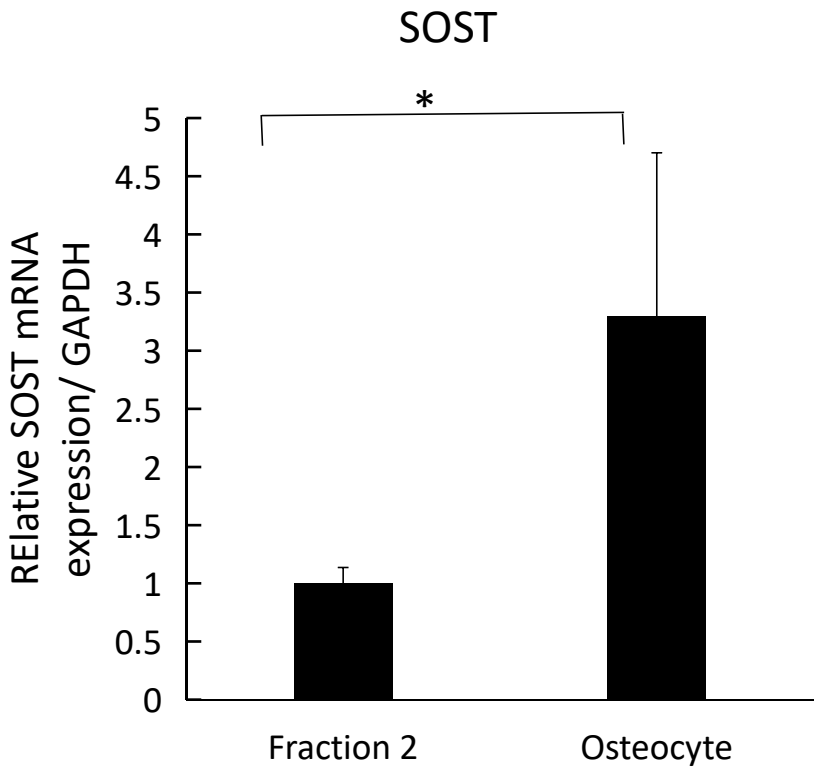
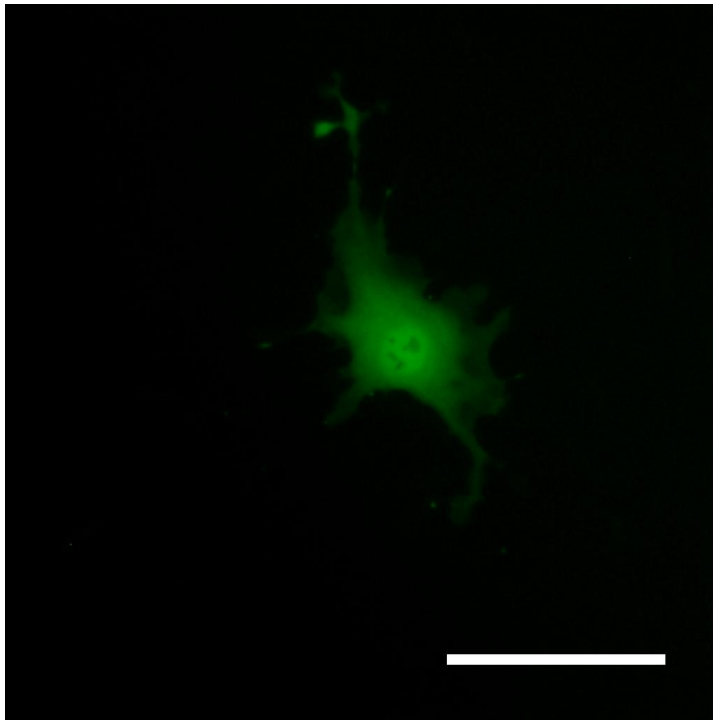
A





B



Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number
BD FACSDiva software	BD Biosciences	
BD Falcon Tube	BD Biosciences	352235
Bovine serum albumin (BSA)	Sigma-Aldrich, MO, USA	
Collagenase	Wako, Osaka, Japan	034-22363
EDTA	Dojindo, Kumamoto, Japan	
FACSAriaTM II	BD Biosciences	
Fetal bovine serum (FBS)	Biowest, Nuaillé, France	
Isolation buffer		
Millex Sterile Filter Unit	Merck Millipore, Ireland	SLGV033RS
Nylon cell strainer	FALCON, NY, USA	
Trypsin-EDTA	Life Technologies, NY, USA	
α-MEM	Wako, Osaka, Japan	

Comments/Description

Data aquisition and analysis
12 x 75 mm Tube with Cell Strainer Cap, 35 µm nylon mesh.

0.2% (w/v), crude collagenase mix sourced from C. histolyticum.
5mM EDTA prepared with 0.1% BSA

70mM NaCl, 10mM NaHCO, 60mM sorbitol, 3mM K2HPO4, 1mM CaCl2, 0.1% (w/v) BSA, 0.5% (w/v) glucose and 2
0.22µm
40µm
0.5% x10. diluted to x1 in PBS
Containing 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 µg/ml streptomycin

:5 mM HEPES

Rebuttal Letter

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May 11, 2020

Dear Editor of Journal of Visualized Experiments:

Thank you very much for your decision letter, dated May 6, 2020, regarding manuscript, JoVE61513 "Obtaining primary osteocytes through murine calvarial fractionation of GFP-expressing osteocytes". The following are our answers to the editor’s comments:

1. We added keywords: SOST, and Fractionation for a total of 6 keywords.

2. Clarify concentrations in g/mL:

-Collagenase solution 0.2% or 2:1 --> 2 mg/mL (step 2.1, 2.1.1)
-BSA 1 mg/mL (step 2.1.1)
-Glucose 5 mg/mL (step 2.1.1)
-BSA 1mg/mL (step 2.4)

3. α-MEM --> α-Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 µg/ml streptomycin.(step 2.6).

4. Fraction 1 is mentioned in step 2.2 and 2.3 and is discarded. Fraction 2 is mentioned in step 2.6 and is seeded.

5. Define all abbreviations.
- FBS: Fetal bovine serum.

6. Mention sorting conditions and gating strategy. Unclear how the sorting is performed.
- Prior to the sort, optimize gating to remove artifacts and cells cells by adjusting the side scatter (SSC) area and forward scatter (FSC) area. Eliminate doublets by adjusting SSC-width vs SSC-Height and FSC-area vs FSC-width.

7.Missing figure. Please include it in your submission.
- Figure 1 is included in the submission.

8. Remove the product name.
- Product name moved to table of content.

9. Mention statistical test used.
- T student test is added.

10. Confusing. Please clarify what is meant.

- Line 273-275: The first isolated osteocyte was from a chicken calvaria[7] isolated by using (OB7.3) or the aviant variant of PHEX; however, this method is limited by the availability of workable antibodies, as osteocyte-specific antibodies that are also specie-specific have to be manufactured.
-The meaning is that it is difficult to come up with antibodies that recognize osteocyte specifically.

11. Ref 24: mention book/chapter title.

- We updated the citation: Bernhardt A, Wolf S, Weiser E, Vater C, Gelinsky M. An improved method to isolate primary human osteocytes from bone. *Biomed Tech (Berl)*. 2020;65(1):107-111.