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TITLE:

A Lab-On-A-Chip Platform for Stimulating Osteocyte Mechanotransduction and Analyzing Functional Outcomes of Bone Remodeling

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SUMMARY:

Here, we present protocols for analyzing bone remodeling within a lab-on-a-chip platform. A 3D printed mechanical loading device can be paired with the platform to induce osteocyte mechanotransduction by deforming the cellular matrix. The platform can also be used to quantify bone remodeling functional outcomes from osteoclasts and osteoblasts (resorption/formation).

ABSTRACT:

Bone remodeling is a tightly regulated process that is required for skeletal growth and repair as well as adapting to changes in the mechanical environment. During this process, mechanosensitive osteocytes regulate the opposing responses between the catabolic osteoclasts and anabolic osteoblasts. To better understand the highly intricate signaling pathways that regulate this process, our lab has developed a foundationary lab-on-a-chip (LOC) platform for analyzing functional outcomes (formation and resorption) of bone remodeling within a small scale system. As bone remodeling is a lengthy process that occurs on the order of weeks to months, we developed long-term cell culturing protocols within the system. Osteoblasts and osteoclasts were grown on functional activity substrates within the LOC and maintained for up to seven weeks. Afterward, chips were disassembled to allow for the quantification of bone formation and resorption. Additionally, we have designed a 3D printed mechanical loading device that pairs with the LOC platform and can be used to induce osteocyte mechanotransduction by deforming the cellular matrix. We have optimized cell culturing protocols for osteocytes, osteoblasts, and osteoclasts within the LOC platform and have addressed concerns of sterility and cytotoxicity. Here, we present the protocols for fabricating and sterilizing the LOC, seeding

cells on functional substrates, inducing mechanical load, and disassembling the LOC to quantify endpoint results. We believe that these techniques lay the groundwork for developing a true organ-on-a-chip for bone remodeling.

INTRODUCTION:

Bone is a highly dynamic tissue that requires intricate coordination among the three major cell types: osteocytes, osteoblasts, and osteoclasts. Multicellular interactions among these cells are responsible for the bone loss that occurs during paralysis and long-term immobility and for the bone formation that occurs in response to growth and exercise. Osteocytes, the most abundant bone cell type, are highly sensitive to mechanical stimuli applied to the bone. Mechanical stimulation alters osteocyte metabolic activity and leads to an increase in key signaling molecules^{1,2}. Through this process, known as mechanotransduction, osteocytes can directly coordinate the activities of osteoblasts (bone forming cells) and osteoclasts (bone resorbing cells). Maintaining bone homeostasis requires a tight regulation between bone formation and bone resorption rates; however, disruptions in this process can result in disease states such as osteoporosis or osteopetrosis.

The complexity of interactions between these three cell types lends itself well to investigation utilizing microfluidic and lab-on-a-chip (LOC) technologies. To that end, our lab has recently established proof of concept of a LOC platform for analyzing bone resorption and formation (functional outcomes) in the bone remodeling process. The platform can be used for the study of cellular interactions, altered loading environments, and investigational drug screening. In recent years, various microfluidic devices have been developed for investigating the molecular signaling pathways that regulate bone remodeling; however, many of these systems quantify remodeling through indirect markers that are indicative of functional activity³⁻⁷. An advantage of our system is that it can be used for direct quantification of functional outcomes. Bone remodeling is a long-term process. As such, direct quantification of bone resorption and formation requires a culturing system that can be maintained for a minimum of several weeks to months⁸⁻¹¹. Thus, when developing the LOC platform, we established long-term culturing protocols necessary for formation and resorption and have maintained cells within the system for up to seven weeks¹¹. Additionally, we incorporated appropriate culturing substrates for both cell types into the platform; osteoclasts were cultured directly on bone, and osteoblasts, which are known to be plastic adherent, were cultured on polystyrene discs. Further, we addressed issues concerning sterility, long-term cytotoxicity and chip disassembly for remodeling analysis^{11,12}.

The LOC platform can also be used to induce osteocyte mechanotransduction through matrix deformation. A 3D printed mechanical loading device was developed to pair with the LOC and apply a static out of plane distention to stretch the cells¹³. To accommodate this mechanical load, the depth of the well within the LOC was increased. This small scale, simple mechanical loading device can be easily produced by labs with limited engineering experience, and we have previously shared drawings of the 3D printed components¹³. In the current work, we demonstrate some of the novel techniques necessary for the successful use of the LOC. Specifically, we demonstrate chip fabrication, cell seeding on functional substrates,

mechanical loading and chip disassembly for remodeling quantification. We believe that the explanation of these techniques benefit from a visual format.

PROTOCOL:

1. Chip mask preparation

NOTE: Steps 1.1 – 1.3 only need to be performed once upon initial receipt of the chip mask. They ensure the mask does not bow during use. The design of the microfluidic masks was previously described^{11,14}. Masks were designed in-house and commercially fabricated using high resolution stereolithography (**Figure 1A**).

1.1. Cover the top surface of the mask with plastic sheeting to protect this surface from adhesive. Secure the chip mask to an equally sized acrylic sheet using spray adhesive. Clamp the pieces together overnight to allow the adhesive to fully cure. After the adhesive is dry, remove the plastic sheeting from the top of the mask.

1.2. Attach the bottom of the acrylic sheet to a 3D printed leveling box (**Supplementary Figure 1, Supplementary Files 1-4**) using double-sided tape. Press firmly to ensure a tight bond.

1.3. Seal any small openings near the detachable wall of the leveling box with a waterproof sealant. Allow the sealant to cure for 24 h.

1.4. Clean the surface of the mask with 70% ethanol (EtOH). Place the leveling box with the desired chip mask in the oven. Use a digital protractor to ensure the top of the mask is level. Adjust the leveling screws if necessary.

2. PDMS fabrication

NOTE: A shallow-well (1 mm) chip design is used for functional activity (formation and resorption) assays, and a deep-well (10 mm) chip design is used for mechanical loading studies. The bottom of the deep-well is formed by attaching a separate thin PDMS membrane (**Figure 1B**).

2.1. Lid layer (Layer 1)

2.1.1. Combine 63 g of PDMS prepolymer and 6.3 g of curing agent (10:1 ratio) in a plastic cup. Mix thoroughly with a disposable cell spatula and degas in a vacuum desiccator for 30 min.

2.1.2. Slowly pour mixture into the prepared leveling box. Let the PDMS sit for 30 min and then bake at 45 °C for 18 h.

2.1.3. Loosen the edges of the PDMS with a tapered laboratory spatula and remove the polymer sheet from leveling box.

2.1.4. Cut individual lids to size (70 mm x 34 mm) using a scalpel and a 3D printed template.

2.1.5. Punch access holes through each lid with a biopsy punch (1 mm diameter).

2.1.6. Surface clean the lids with packaging tape.

NOTE: If the lids are not being used immediately, wrap each in packaging tape and store at room temperature (RT).

2.2. Well and microchannel layer (Layer 2)

2.2.1. Combine the PDMS prepolymer and curing agent (10:1 ratio) in a plastic cup. The shallow-well design requires 43 g of prepolymer and 4.3 g of curing agent, and the deep-well design requires 227 g of prepolymer and 22.7 g of curing agent. Mix the polymer vigorously and degas for 30 min.

NOTE: This assumes a mask dimension of 152.4 mm x 152.4 mm.

2.2.2. Slowly pour mixture over appropriate pre-leveled mask. Let the PDMS sit for 30 min and then bake at 45 °C for 18 h.

2.2.3. Loosen the edges of the PDMS with a tapered laboratory spatula and carefully peel the polymer away from the mask. Use a scalpel and 3D printed template to cut out individual chips.

NOTE: For loading studies it is important that the chip dimensions (70 x 34 mm) are precise and that the well is located in the center of the chip.

2.2.4. Surface clean the PDMS with packaging tape.

NOTE: If the chips are not being used immediately, wrap each chip in packaging tape and store at RT.

2.3. Thin PDMS membrane (Layer 3)

NOTE: This layer is only used for the deep-well design.

2.3.1. Add 12.7 g of PDMS prepolymer and 1.3 g of curing agent (10:1 ratio) to a plastic cup. Mix vigorously and degas for 30 min.

2.3.2. Slowly pour polymer into prepared leveling box and thoroughly scrape plastic cup to remove as much PDMS as possible.

2.3.3. Use cell spatula to spread PDMS over entire surface.

NOTE: If the polymer is not manually spread out, surface tension will be sufficient to prevent the polymer from forming a uniform sheet.

2.3.4. Let the PDMS sit for 30 min and then bake at 45 °C for 18 h.

2.3.5. Loosen the edges of the PDMS with a tapered spatula and carefully remove the PDMS sheet from the leveling box. Cut individual membranes that match the dimensions of layer 2.

2.3.6. Measure the membrane thickness at the center of the membrane using calipers. Discard any membranes that are outside of the desired thickness (0.5 mm \pm 0.1 mm).

2.3.7. Carefully clean membranes with packaging tape and place on a piece of paraffin film.

NOTE: If the membranes are not being used immediately, cover the top of the membrane with packaging tape and store at RT.

3. Functional activity substrates

NOTE: Polystyrene discs and bone wafers must be attached to the bottom of wells that will be used for osteoblast and osteoclast cultures, respectively.

3.1. Polystyrene discs (**Figure 1C**)

3.1.1. Place masking tape on the back side of a tissue culture treated polystyrene coverslip. Cut circular discs from the coverslip using a sharpened cork-borer (5.4 mm diameter). Submerge the discs in 70% EtOH and leave overnight.

3.1.2. Gently scrub the top surface of the disc with a cotton tipped applicator soaked in 70% EtOH. Ensure that the outer edge of the disc is thoroughly cleaned.

3.1.3. Using two pairs of forceps, hold the disc and remove the masking tape backing. Place the disc treated side down and clean the back side with a cotton tipped applicator.

3.1.4. Dip the wooden end of a cotton tipped applicator into a degassed mixture of uncured PDMS and place a small amount of the polymer on the bottom of the desired well.

NOTE: Remaining uncured PDMS can be stored at -20 °C.

3.1.5. Place the polystyrene disc, treated side up, into the well and gently press down on the disc with a cotton swab. Ensure that no uncured PDMS comes in contact with the treated side of the disc.

NOTE: If PDMS does come in contact with the treated surface of the disc, remove the disc from the well and repeat steps 3.1.4 and 3.1.5 with a new disc.

3.1.6. Let the chip sit on a level surface for 30 min and then bake at 65 °C for 4 h.

3.2. Bone wafers

3.2.1. Use forceps to place a bone wafer (6 mm diameter, 0.4 mm thick) on the bottom of a 100 mm dish. Hold the wafer steady with the forceps and gently etch an 'X' on the back of the wafer with a scalpel.

NOTE: During the imaging process, the 'X' is used to distinguish between the back of the wafer and the surface on which cells were seeded.

3.2.2. Use the wooden end of a cotton tipped applicator to add a small amount of uncured PDMS to the bottom of the desired well. Place the bone wafer, marked side down, into the well and use a cotton tipped applicator to press the wafer down.

3.2.3. Let the chip sit on a level surface for 30 min and then bake at 65 °C for 4 h.

4. Chip assembly and sterilization

4.1. Activate the surfaces of layer 1 and layer 2 with a plasma cleaner for 30 s using a medium radio frequency (RF) power setting (equivalent to approximately 10.2 W).

4.2. Align the access holes in layer 1 with the microchannels in layer 2 and firmly press the two layers together.

4.3. For the deep-well design, repeat step 4.1 with layer 2 and layer 3. During the plasma treatment, use double-sided tape to attach the paraffin film of layer 3 to a flat surface.

4.4 Use a scalpel to trim off excess material from the PDMS membrane. Carefully peel away the sheet of paraffin film from the bottom of the chip

4.5. Bake the chip at 65 °C for 10 min to increase bond strength between the PDMS layers.

4.6. Insert angled dispensing tips (18 Gauge, 0.5 in, 90°) into the access holes in the lid. Secure the dispensing tips to the lid with a two part epoxy. Use a micropipette tip to apply the epoxy around each dispensing tip.

4.7. After the epoxy has fully cured, surface clean the chip with 70% EtOH and place in a biosafety cabinet. Perform all subsequent steps within the biosafety cabinet.

4.8. Connect a 5 mL syringe to the dispensing tips with sterile silicone tubing (1/32" ID, ~10 cm in length) and fill the entire chip with 70% EtOH for at least 30 s. For the shallow-well design,

administer all liquids with a syringe pump set to a flow rate of 4 mL/h. For the deep-well design, administer all liquids by slowly dispensing the syringe by hand.

4.9. Remove the EtOH from the chip and sterilize the chip with UV light overnight.

4.10. Wash the chip 3 times with dH₂O. Fill the chip with dH₂O, remove tubing from the dispensing tips and incubate for at least 48 h at 37 °C.

5. Mechanical loading device assembly

NOTE: The design and fabrication processes for the 3D printed mechanical loading device (**Figure 2A-C**) were previously described and all design files for printed components have been previously provided¹³.

5.1. Autoclave all components of the loading device for 30 min at 121 °C.

NOTE: To avoid warping of printed components, wrap each piece individually in foil and place on a hard flat surface during the autoclaving process. Metal hardware can be wrapped together. Complete all subsequent steps within a biosafety cabinet.

5.2. Place a compression spring around the shaft of the platen and insert the platen into the central hole on the bottom of the base (**Figure 2D**).

5.3. Attach the dial block to the bottom of the base using four self-tapping screws.

5.4. Place a second compression spring around the central screw. Insert the screw into the hexagonal-shaped hole in the bottom of the dial and screw the assembly into the threaded hole in the center of the dial block.

5.5. Screw four male-female standoffs into the bottom of the base.

5.6. Remove slack from the device by turning the dial counterclockwise until the top of the platen is below the top of the base. Then slowly turn the dial clockwise until the top of the platen is level with the top of the base.

6. Experimentation

NOTE: Protocols for functional activity experiments were previously provided^{11,12}.

6.1. Loading studies (**Figure 3**)

6.1.1. Following step 4.9, use a 5 mL syringe to remove dH₂O from the deep-well chip. Coat the bottom of the well with 200 µL of 0.15 mg/mL type I collagen (CTI) in 0.02 M acetic acid for 1 h.

6.1.2. Rinse the chip three times with Dulbecco's phosphate-buffered saline with calcium and magnesium (DPBS++).

6.1.3. Place chip into the chip holder and seed with MLO-Y4 osteocytes at a density of 2×10^4 cells/mL in minimum essential alpha medium (MEM α) supplemented with 5% calf serum, 5% fetal bovine serum, and 1% penicillin/streptomycin.

6.1.4. Remove the tubing from the dispensing tips and place the chip into a deep-well culture dish (150 mm x 25 mm). Incubate cells at 37 °C and 5% CO₂ for 72 h.

6.1.5. Attach sterile tubing to dispensing tips and use a 5 mL syringe to remove spent culture medium from the chip. Slowly dispense in fresh culture medium to refill the chip.

6.1.6. Place the chip holder into the rectangular inset on the top of the loading device base. Feed the tubing through the slots located on the loading device lid and secure the lid to the base with four pan head screws and hex nuts.

NOTE: To ensure that the lid remains level, first secure two screws that are located diagonally from one another before securing the remaining two screws.

6.1.7. Apply load to the cells by turning the dial clockwise until the desired platen displacement is reached.

NOTE: The device is designed so that one rotation of the dial equates to a platen displacement of 1 mm. The strain field generated on the top of the PDMS membrane was previously modeled as a function of platen displacement using finite element analysis (FEA)¹³.

6.1.8. Place the loading device into an empty P1000 micropipette tip box. Incubate the cells with the applied load for 15 min.

NOTE: The loading time period used here serves as an example. Alternative loading times can be used.

6.1.9. Following incubation, remove the load from the cells by turning the dial counterclockwise until the platen returns to the original starting position. Remove the lid from the device and place the chip holder into the deep-well culture plate. Incubate the cells for a 90-min post load recovery period.

NOTE: Again, the recovery time period used here serves as an example. Alternative recovery times can be used. For long term studies, feed cells every 72 h.

6.1.10. Use a 5 mL syringe to remove the conditioned medium from the chip.

NOTE: This medium can be saved and stored at -80 °C.

6.1.11. Remove the chip from the chip holder and use a tapered spatula to break the bond between the PDMS lid and well layer.

NOTE: Cellular assays can now be performed following any protocol established for a cell culture plate.

REPRESENTATIVE RESULTS:

The shallow-well configuration can be used for analyzing functional activity of osteoblasts and osteoclasts. Bone formation via osteoblasts and resorption via osteoclasts requires culturing times on the order of several weeks to months. Bone formation from MC3T3-E1 pre-osteoblasts was quantified using alizarin red and von Kossa stains^{11,15}. At day 49, the average surface area stained with alizarin red was $10.7\% \pm 2.2\%$ (mean \pm standard errors of the mean)¹¹. The average surface area stained with von Kossa was $6.4\% \pm 1.6\%$ ¹¹. **Figure 4A** shows typical formation results from osteoblast cultures at day 49 stained with alizarin red and von Kossa. Bone resorption from RAW264.7 pre-osteoclasts was quantified using toluidine blue staining^{11,15}. At day 30 the average surface area stained with toluidine blue was $30.4\% \pm 4.5\%$ ¹¹. **Figure 4B** shows typical bone resorption results from osteoclast cultures stained with toluidine blue at day 30. Scanning electron microscopy was performed to verify the presence of resorption pits. Typical results are shown in **Figure 4C**. These results demonstrate that cells within the device remain viable and functionally active for at least seven weeks.

A 3D printed loading device was designed and fabricated to accommodate the deep-well chip configuration. Together, this system can induce osteocyte mechanotransduction by stretching the cells via a static out-of-plane distention. The 48 h incubation of the chip described in step 4.9 has proven to be a critical process for maintaining cell viability and typical morphology. **Figure 5A** shows representative images of MLO-Y4 osteocytes at 72 h seeded in chips with and without this incubation period. During bouts of loading, osteocytes were exposed to a strain gradient induced on the PDMS membrane on which the cells were seeded (**Supplementary Video 1**). The equivalent strains generated during this process were modeled with FEA¹³ and the average equivalent strain produced on the top of the PDMS membrane was determined. **Figure 5B** shows the relationship between average equivalent strain and platen displacement for values between 1 and 2 mm. A representative heat map of the induced strain gradient is shown in **Figure 5C**. In this example, a platen displacement of 1.5 mm generated an average equivalent strain of 12.29% on the top of the membrane. This model also demonstrates that the strains induced near the center of the well are relatively low and gradually increase radially outward, with maximum strains generated directly above the outer edge of the platen. Following loading, cell viability was analyzed with lactate dehydrogenase staining and the annexin V and dead cell assay^{14,16}. Typical results are shown in **Figure 5D,E**, respectively.

FIGURE LEGENDS:

Figure 1: Microfluidic device. (A) Fabricating and leveling the chip mask. (Left) Schematic of the deep-well chip mask that was designed in-house using CAD software. (Middle) Image of the deep-

well chip mask that was commercially printed using high-resolution stereolithography. (Right) The mask is placed within a 3D printed leveling box to ensure the mask remains level during the casting of layer 2 of the chips. (B) Schematic sketches of the shallow-well and deep-well designs of the device. (Top) The shallow-well design was used for analyzing functional activity (formation and resorption) of osteoblasts and osteoclasts. This configuration is formed from two PDMS layers. A functional activity substrate was secured to the bottom of the culture well prior to sealing the layers together. Polystyrene discs and bone wafers were used for osteoblast and osteoclast cultures, respectively. (Bottom) The deep-well design was used for applying mechanical load to osteocytes. This configuration consists of three PDMS layers. The bottom of the culture well is formed by the deformable PDMS membrane (layer 3). (C) Fabrication steps for the polystyrene disc used for osteoblast cultures. The back of a tissue culture treated coverslip was marked with masking tape. Individual discs were cut out with a cork-borer. The disc was cleaned with ethanol and a cotton swab. The tape backing was removed and disc was attached to the bottom of the PDMS culture well.

Figure 2: Design and assembly of the mechanical loading device. (A) Image of the assembled mechanical loading device. When coupled with the deep-well design of the PDMS chip, the loading device stretches cells by applying an out of plane distention to a deformable membrane. (B) Exploded-view of the device. All parts shown in blue were 3D printed with a heat resistant polylactic acid filament. All hardware is stainless steel. (C) Screw jack mechanism of the loading device. Rotation of the central screw (orange) pushes the platen (green) upward through the base. The upward movement of the platen deforms the PDMS membrane of the deep-well chip on which the cells have been seeded. (D) Assembly process of the loading device.

Figure 3: Mechanical loading experiment. All liquids were administered and removed from the chip using a 5 mL syringe connected to the access tubing. A critical step in this process is the 48 h incubation with sterile distilled water prior to cell seeding. Without this incubation cells showed low viability and atypical morphology.

Figure 4: Typical functional activity results. (A) Typical formation results stained with alizarin red (left) and von Kossa (right) from induced MC3T3-E1 osteoblast cultures at day 49. Whole disc images measure 5.4 mm in diameter. (B) Typical osteoclast resorption results from RAW264.7 preosteoclasts induced with receptor activator of nuclear factor kappa-B ligand (RANKL). Cells were cultured on bone wafers and stained with toluidine blue at day 30. (C) Typical scanning electron microscopy images verifying the presence of resorption pits on bone wafers. The scale bar in the top image represents 200 μm and scale bar in the bottom image represents 50 μm .

Figure 5: Effects of mechanically induced strain on osteocytes. (A) Images of MLO-Y4 osteocytes at 72 h in the deep-well chip fabricated with (left) and without (right) a 48 h incubation with distilled water prior to cell seeding. (B) Finite element analysis was used to model the average equivalent strain generated on the top of the deformable PDMS membrane based on the displacement of the loading device platen. Results from displacements between 1.0 mm and 2.0 mm are shown. (C) Heat map of the modeled strain gradient induced on the PDMS membrane for a platen displacement of 1.5 mm. (D) Typical results of a lactate dehydrogenase stain of drug

induced osteocytes that were stretched using a 1.5 mm platen displacement. A lighter cell staining is observed near the outer edge of the well, which corresponds to the location of higher strain values indicative of cell damage and/or death. (E) Representative flow cytometry results of load-induced apoptosis indicated by an annexin V and dead cell assay.

Supplementary Figure 1: 3D printed leveling box with detachable wall.

Supplementary File 1: Leveling box CAD file 1.

Supplementary File 2: Leveling box CAD file 2.

Supplementary File 3: Leveling box CAD file 3.

Supplementary File 4: Leveling box hardware list.

Supplementary Video 1: Mechanical loading device.

DISCUSSION:

This article describes the foundations for fabricating a bone remodeling LOC platform for culturing osteocytes, osteoclasts, and osteoblasts. By altering the depth and size of the well within the chip, multiple configurations were developed for stimulating osteocytes with mechanical load and quantifying functional outcomes of bone remodeling (**Figure 1B**).

During chip assembly, optimizing the plasma oxidation protocol was critical for eliminating leakage concerns. We found that exposing PDMS surfaces to 30 s of oxygen plasma generated using a medium RF power setting (step 4.1), equal to approximately 10.2 W, was sufficient for creating a strong bond between the layers. The integrity of the bond decreased when longer exposure times or a higher RF power setting were used. This is consistent with previous reports that noted an overexposure of PDMS to oxygen plasma increased surface roughness and reduced adhesiveness^{17,18}.

Additionally, maintaining high cell viability and typical cell morphology was critically dependent on the PDMS curing process. The PDMS polymer consists of crosslinked dimethylsiloxane oligomers. This crosslinking process is both time and temperature dependent; however, even with extensive curing the polymer fails to fully polymerize¹⁹. The remaining oligomers have been shown to leach out of the bulk polymer into surrounding cell culture medium and have even been found in the membranes of cells grown on the polymer surface²⁰. Several groups have reported cytotoxic effects from non-crosslinked PDMS oligomers²¹⁻²³. To address this concern in our system, we optimized the PDMS curing process. Although the curing rate for PDMS is temperature dependent, the material properties of our chip masks restricted our curing temperature to 45 °C. As such, we determined that the chips should be baked for a minimum of 18 h. Further, we have found that incubating chips with dH₂O for at least 48 h prior to cell seeding (step 4.9) was necessary to reduce cytotoxic effects. **Figure 5A** shows MLO-Y4 osteocytes cultured in chips with and without this incubation period.

The deep-well configuration, which was designed to accommodate mechanical load application, requires that the chip be fabricated from three separate layers. Unlike the shallow-well configuration, fabricating the well and membrane portions as a single piece for the deep-well configuration caused the membrane to warp. This may be due to a difference in curing rates between the thick and thin portions of the chip. To overcome this issue, the membrane layer was constructed separately and bonded to the bottom of the chip following the curing process. To generate a separate membrane layer with uniform thickness, it is critical that the leveling box be completely level prior to adding the PDMS (step 1.4). As such, the use of a digital protractor is recommended to ensure a high degree of accuracy. Additionally, during step 2.3.2, it is critical to remove as much PDMS from the plastic cup as possible. Inconsistencies at this step will lead to high deviations in membrane thicknesses, and ultimately high deviations in the average substrate strain used to induce osteocyte mechanotransduction.

Our bone remodeling platform provides a high degree of versatility. This system can be used to investigate a variety of factors that regulate bone remodeling, such as load-induced mechanotransduction, multicellular signaling, inflammation, or drug effects. For example, the system was used to analyze the combined effects of mechanical load and inflammation on bone remodeling in a drug induced environment¹⁴. Mechanical load was applied to bisphosphonate-treated osteocytes. Protein analysis of the conditioned medium revealed a significant increase in the levels of leptin and osteonectin and a significant decrease in the levels of CCL21 and CD36 when compared to osteocytes that were not mechanically loaded¹⁴.

The protocols presented here provide a foundation for culturing each cell type within the LOC as well as methods for inducing mechanical load and quantifying functional activity. Moving forward, we are working towards a true bone organ-on-a-chip. Additionally, we believe that using our system to develop mathematical modeling systems could greatly enhance our understanding of the complex multicellular signaling processes that regulate bone remodeling^{24,25}.

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DISCLOSURES:

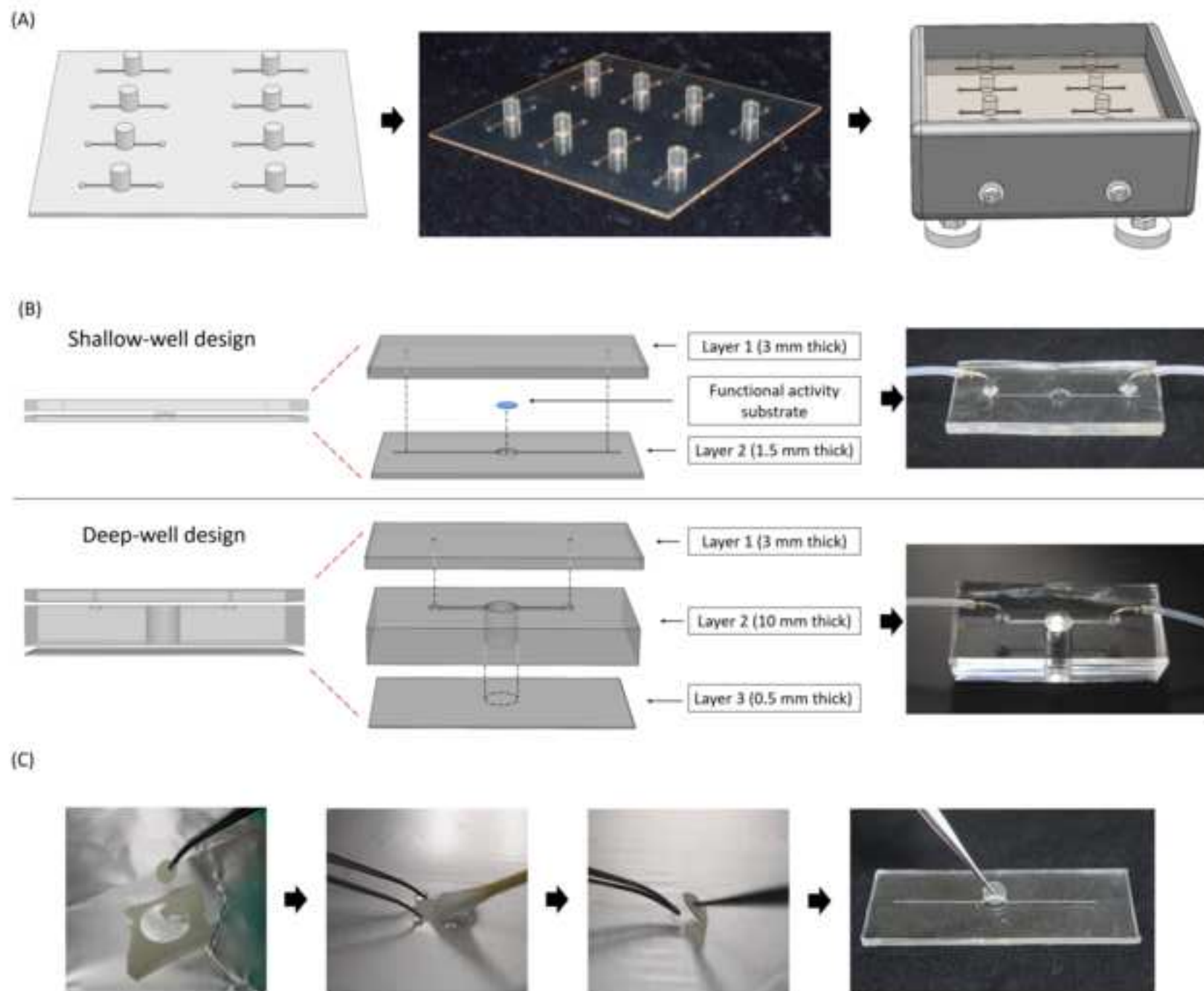
The authors have nothing to disclose.

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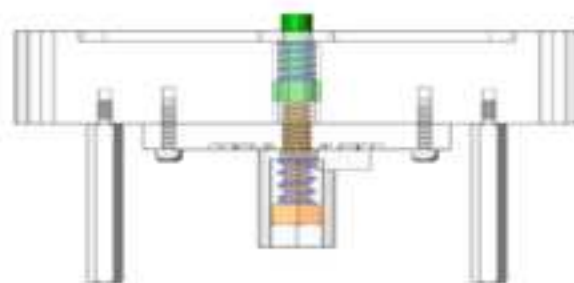
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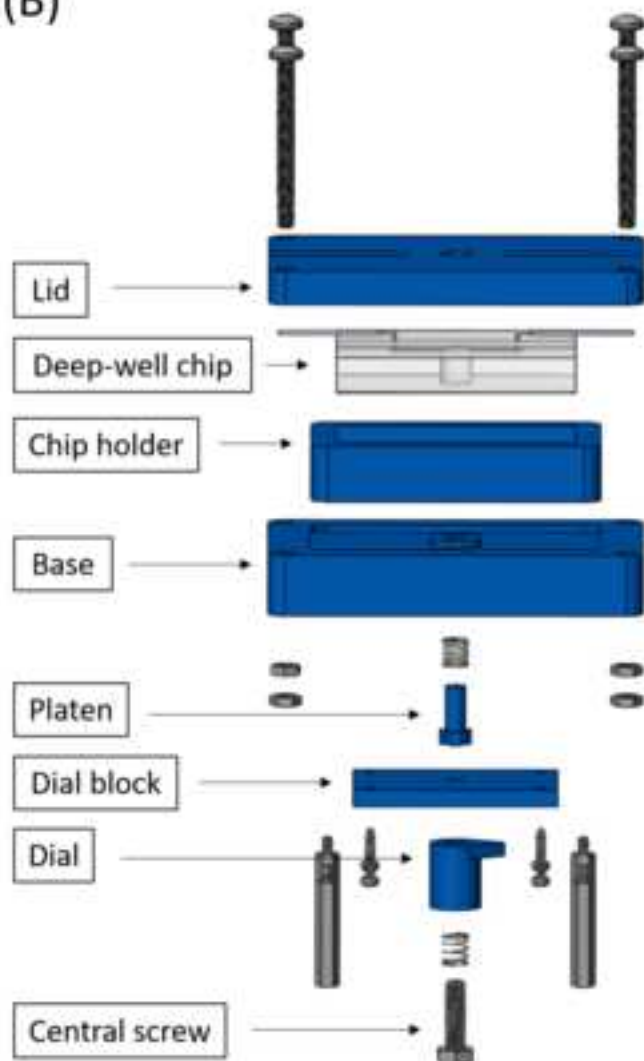
(A)



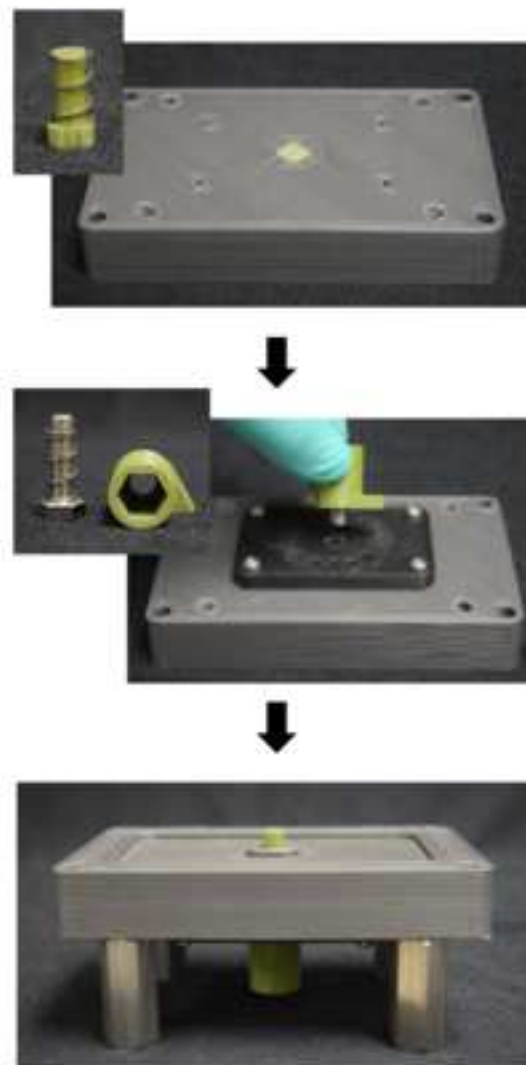
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(B)



(D)



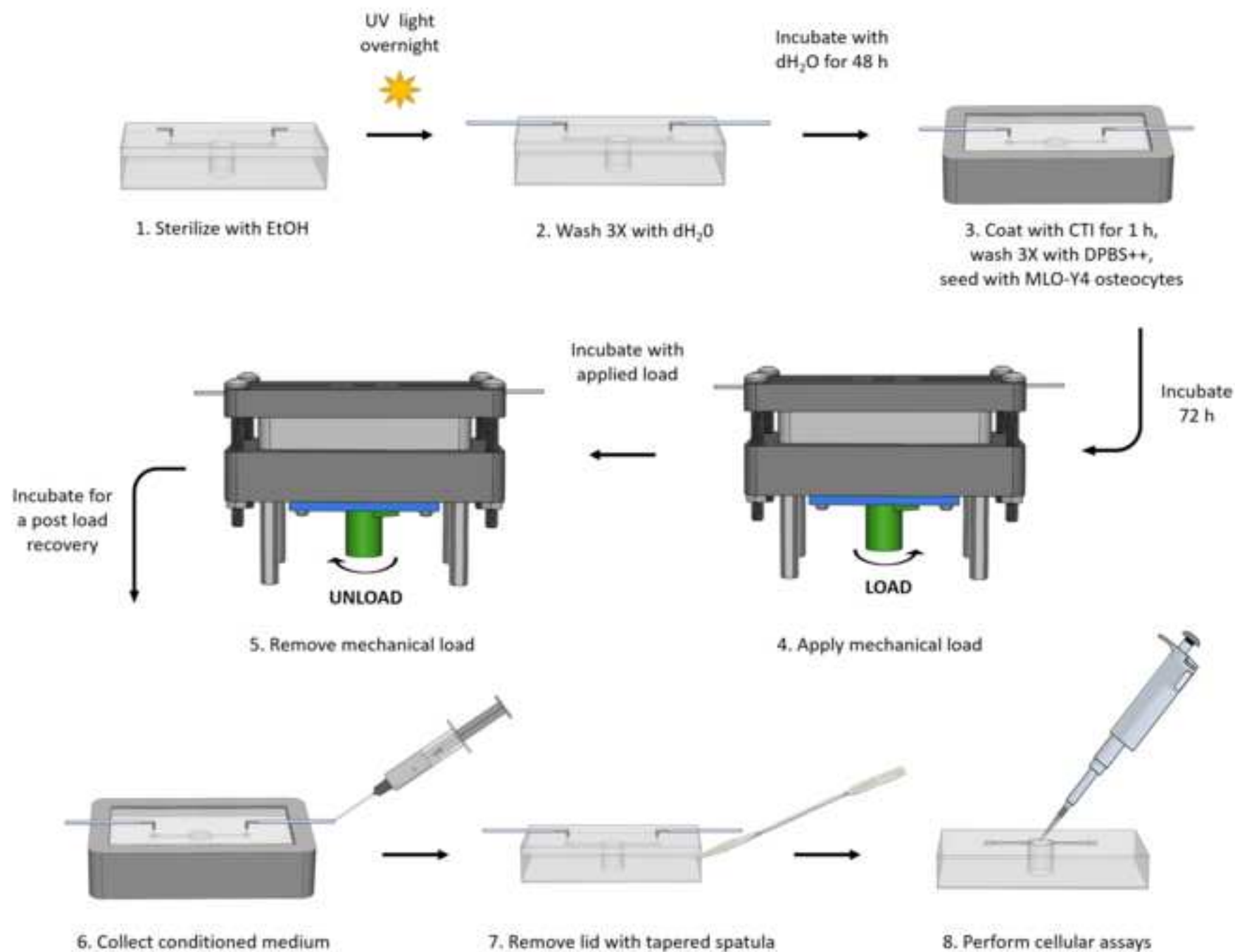
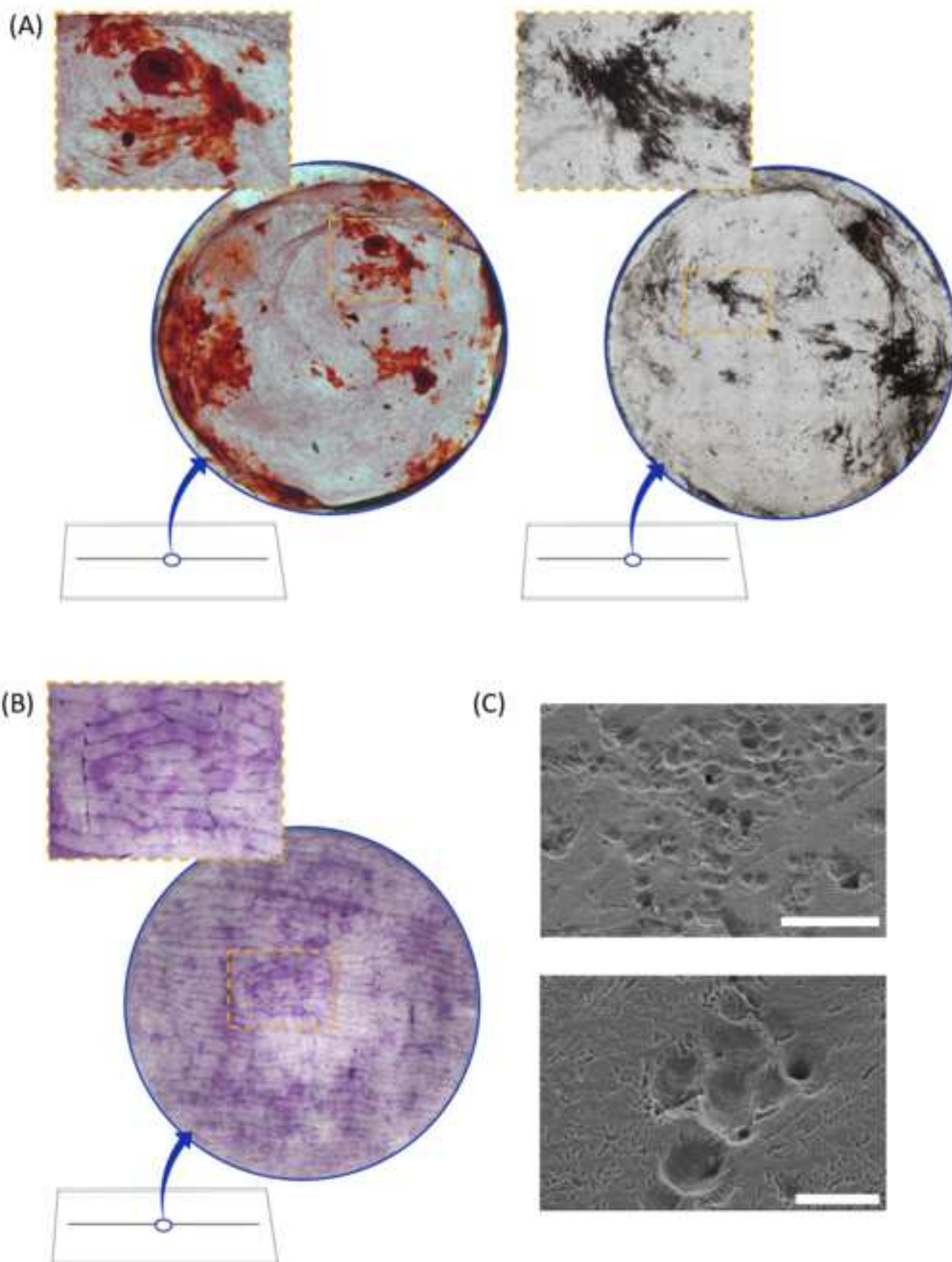
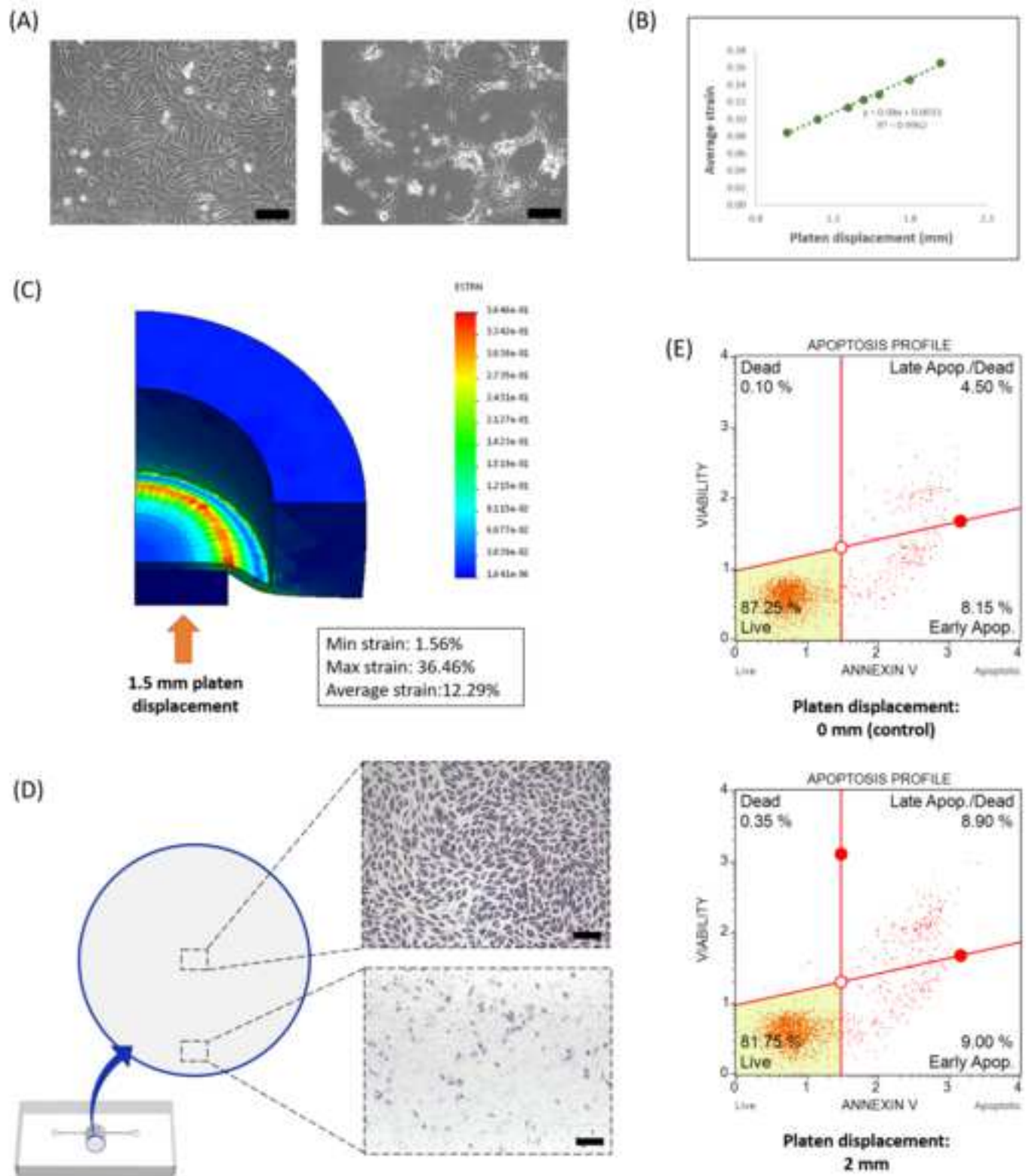


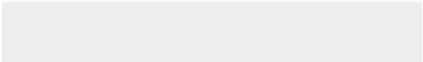
Figure 4







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Name	Company
Acrylic sheet	Optix
Angled dispensing tips	Jensen Global
Biopsy punch	Robbins Instruments
Bone wafers	Boneslices.com
Bovine calf serum	Hyclone
Calipers	Global Industrial
Cell spatula	TPP
Chip mask	ProtoLabs
Cork borer	Fisher Scientific
Cotton tipped applicator	Puritan
Culture dish (100 mm)	Corning
Culture dish (150 mm)	Corning
Double sided tape	3M Company
Fetal bovine serum	Hyclone
Forceps	Fisher Scientific
Leveling box	Custom-made
Masking tape	3M Company
MC3T3-E1 preosteoblasts	ATCC
Mechanical loading device	Custom-made
Minimum essential alpha medium	Gibco
MLO-Y4 osteocytes	--
Packaging tape	Duck Brand
Paraffin film	Bemis Parafilm
Penicillin/streptomycin	Invitrogen
Plasma cleaner	Harrick Plasma
Polydimethylsiloxane kit	Dow Corning
Polystyrene coverslips	Nunc Thermanox
Oven	Qunicy Lab
RAW264.7 preosteoclasts	ATCC
Scalpel	BD Medical
Silicone tubing	Saint-Gobain Tygon
SolidWorks software	Dassault Systèmes
Spray adhesive	Loctite
Syringe (5 ml)	BD Medical
Syringe pump	Harvard Apparatus
Tapered laboratory spatula	Fisher Scientific
Two-part epoxy	Loctite
Type I collagen	Corning
Vacuum desiccator	Bel-Art
Waterproof sealant	Gorilla

Catalog Number	Comments
--	3.175 mm thick
JG18-0.5X-90	Remove plastic connector prior to use
RBP-10	1 mm diameter
0.4 mm thick	Bovine cortical bone
SH30072	
T9F534164	
99010	
Custom-designed	Print material: Accura SL 5530
07-865-10B	
806-WCL	
430591	Sterile, Non-tissue culture treated
430597	Sterile, Non-tissue culture treated
Scotch 237	
SH30910	
22-327379	
--	3D printed
Scoth 2600	
CRL-2593	Subclone 4
--	3D printed
12571-063	
--	Gift from Dr. Lynda Bonewald
--	Standard packaging tape
PM999	
p4333	
PDC-001	Expanded plasma cleaner
Sylgard 184	
174942	Sterile, tissue culture treated
12-180	
TIB-71	
372611	
ABW00001	ID: 1/32" (0.79 mm), OD: 3/32" (2.38 mm)
--	Used to generate 3D printed models and perform FEA
2323879	Multi-purpose adhesive
309646	Sterile
70-2213	Pump 11 Pico Plus
21-401-10	
1395391	5 minute quick set
354236	Rat tail collagen
F42010-0000	
8090001	100% silicone sealant

January 13, 2020

Vineeta Bajaj, Ph.D.
Review Editor
Journal of Visualized Experiments

Dear Dr. Vineeta Bajaj,

We are pleased to resubmit to the Journal of Visualized Experiments our manuscript, "A lab-on-a-chip platform for stimulating osteocyte mechanotransduction and analyzing functional outcomes of bone remodeling" (JoVE1076). We are grateful for the constructive and thoughtful comments provided by the reviewers and for the opportunity to revise our manuscript. We believe that by addressing these comments, our manuscript has been improved. We responded to the comments in bold, italicized text below. Changes made within the manuscript have been tracked. We look forward to correspondence with you and hope our edits will be satisfactory to make our manuscript fitting for publication in your respectable journal.

Respectively,



Sharon Truesdell

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Akron, OH USA 44325-0302

Email: slt79@zips.uakron.edu

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please ensure that the Summary is between 10-50 words limit.
3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.
4. Please ensure you answer the "how" question, i.e., how is the step performed?
5. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
6. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
7. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The authors thank the editor and confirm that these changes have been made.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this protocol, the authors design chips combined with a 3D printed loading device that can be used to induce mechanotransduction. Then, this system can be used to study the effects of bone remodeling and mechanotransduction. This system has been used in several other works previously published by the authors. The authors describe in detail all the steps needed for the assembly of this system with clear explanations and informative figures. This is a well-written protocol that I'm certain will be of interest to other researchers in the field. I have one major concern regarding the anticipated results provided in this protocol.

Major Concerns:

The authors describe and present a loading device capable of inducing mechanotransduction, however no functional results of this system are presented throughout the protocol (as opposed to what the title implies). In figure 5, it is shown that the strain largely affects the outer edge of the chip, leading to increased apoptosis. Are there any functional changes that occur to the remaining osteocytes? This is a crucial point that should be presented, as the main point of this protocol is the ability to induce mechanotransduction and analyze its outcomes. This should

also provide the readers with a clear quantification assay that could help them study such effects.

By functional outcomes of bone remodeling, the authors were referring to bone formation and bone resorption. We apologize for this confusion and thank the reviewer for pointing this out. We have attempted to clarify this distinction within the article summary. Further, to address the reviewers concern of a lack of functional results, we have added data describing the amount of bone formation and resorption that was observed from culturing osteoblasts and osteoclasts within the device (lines 365-371).

For osteocyte cultures, we have focused our analyses on the average response of all cells within the device as we feel this is a better representation of the signaling response in vivo. Within the bone matrix osteocytes are subjected to a range of strain values and the osteocyte network allows for a high degree of intracellular signaling. We are interested in studying how this overall response affects the activity of osteoclasts and osteoblasts. As an example, we cited a study in which we used this system to mechanically load drug-induced osteocytes and found a significant change in the cytokine expression of conditioned medium when compared to non-loaded cells (lines 506-509).

Reviewer #2:

Manuscript Summary:

This manuscript describes the development of a lab-on-a-chip (LOC) platform and related protocols for analyzing a bone remodeling process. The developed LOC can be used to actually evaluate the functional outcomes of bone remodeling (such as formation and resorption of bone) for a long period of culture time (up to seven weeks) with or without mechanical loading by associating with 3D printed mechanical loading device. Further, the developed LOC platform addressed sterility and cytotoxicity concerns. All required protocols were described succinctly and very efficiently. I would like to suggest the acceptance of this protocol article with very minor revisions:

Major Concerns:

None

Minor Concerns:

1. Line 65: microfluidic devices are have been > microfluidic devices have been

This correction has been made.

2. In Discussion explaining the strain magnitude (around line 374), it will be great if some description of the available frequency of the mechanical loading is included. Is the frequency of mechanical loading adjustable?

The authors agree with the reviewer that load frequency is an important discussion point, and we would like to thank the reviewer for pointing out that this information is missing from the manuscript. The mechanical loading device described here applies a static load to the cells. This was clarified in the introduction (line 78) and in the results section (line 365). Conceivably, the loading device could be modified to apply a dynamic load by

controlling rotation of the dial with a small stepper motor. In this case the motor could be easily programmed to apply load over a wide range of frequencies.

Reviewer #3:

The technology addresses behaviour of bone-forming or bone remodeling cells when cultivated on an elastic surfaces with respect to terminal analyses. It will not allow on-line monitoring. It is a single well design thus direct comparisons of analyses under controlled conditions are restricted by the fact that a battery of such devices need to be built.

Comments:

In this manuscript, the authors compile a wealth of technical details to describe an interesting way of testing cells involved in bone formation and remodeling. The device has already, at least in part, been described in other publications. Indeed, citations are given and regarding many questions arising during the description references are made to specific publications. Hence in order to generate a complete protocol, important information from many other sources have to be gathered and brought into line. Examples are mask production, and details related to the cultivation of cells on special substrates and making of chip holder and loading device.

Regarding the description of representative results: no details and/or specification regarding assay conditions and reagents are given. Here references to publications applying comparable methods would be greatly appreciated

The text is very well written, highly instructive and informative for the researcher who wants to build and apply the described technology. Together with the previously published experimental results and analysis, it is well conceivable that this description is highly supportive in establishing this type of method and analysis in the lab. The above mentioned consideration that quite often more than one device is needed when intending to perform experiments in parallel and not in series should be mentioned in the discussion. Have you experienced drawbacks regarding differences in performance and handling when working with different devices and using hand-made chips for the cell tests. Are analytical results highly reproducible? Please discuss!

The authors would like to thank the reviewer for the thoughtful comments. We agree with the reviewer that in some steps additional sources are required to obtain a complete protocol. The authors feel that presenting the material in this way is required for the sake of brevity. However, we have added detail to some steps in this manuscript, such as mask production, in an attempt to clarify the information presented here. Also, in the results section of the manuscript we have added additional references that detail assay conditions and reagents.

We have included some analytical results with standard errors of the means (lines 364-371) to demonstrate the current degree of reproducibility. We are currently in the process of developing an automated feeding system that we believe will minimize any errors introduced during the feeding procedure and increase the reproducibility of the results. Further, an automated feeding system will allow for chips with numerous wells to be used as the feeding can be performed while the chips remain within the incubator. Currently, chips are fed one at a time, and as such, a single well chip allows us to minimize the time

in which it is removed from the incubator. Also, we are using the current chips as a foundation towards a design that incorporates all three cell types into a single chip. This will greatly decrease the overall number of chips that need to be fabricated.

Minor concerns:

It is not entirely clear to this reviewer, why parts of the manuscript are marked in yellow?

As per the instructions from the journal, the parts of the manuscript marked in yellow are the portions of the procedure that we would like to include in the video protocol.

line 25 functional out comes from osteoclasts and osteoblasts: ???

Functional outcomes from osteoclasts and osteoblasts is referring to bone resorption and formation. We apologize for this confusion. To clarify, we have added this information to the summary.

line 65: delete additional verb

The additional verb has been deleted.

1. Chip mask production: most procedures are described in full detail, yet not the mask production. It is not clear why, albeit described elsewhere. This is greatly destructing for the reader; please add a short outline what type of mask and how the mask is actually applied in during layer 2 production. As of now, this is confusing.

The authors apologize for this confusion and thank the reviewer for bringing this to our attention. We designed the masks in-house using CAD software and then have them commercially fabricated using high resolution stereolithography. This information has been added to the protocol (lines 96-97). Additionally, we have added to Figure 1 to incorporate images of the mask design and final product. Here we used the deep-well mask design as an example. Additionally, we have provided an image of the leveling box that the mask is set into in order to cast the polymer used for layer 2.

3. Functional activity substrate: please briefly indicate and introduce the two option of cell culture plastic or bone as a substrate to cultivate and test adherent cells. Consider mentioning the technical constraint also in the introduction

We have updated the introduction section of the manuscript to incorporate this information (lines 74-76).

also briefly mention specifications of the bone wafer, there is at least 2 thicknesses offered by Boneslices Inc.

The authors thank the reviewer for pointing out that this information is not stated within the manuscript. The thickness of the bone wafers used was specified within the materials list. However, to further clarify this we have also added the bone wafer specifications to the protocol (line 223).

line 296: note ref 12: is under review - just in case essential information is buried in there.

The essential information can be found in reference 11, which is also cited on this line. However, reference 12 is now in the second round of review and we anticipate its publication.

6. Experimentation

6.1.5: how is feeding performed: flushing media through the chip, please indicate flowrate, is it done every 72 hrs, when performing a long-term experiment? Is it conceivable to perform continuous or discontinuous flow by using syringe pumps. If not, please explain.

For the shallow-well design, chips were fed every 72 h by flushing medium through the chip with a syringe pump set to a flow rate of 4 ml/h. For the deep-well design, the extra depth allowed chips to be fed by manual administering medium with a syringe without disrupting the cell monolayer. The spent medium is first removed from the chip and then replaced with 100% fresh medium. When maintaining MLO-Y4 osteocytes within the chip beyond 72 h, feedings should be performed every 72 h. The protocol (lines 314-316 and line 344) has been updated to include this information.

We are currently in the process of developing an automated feeding system that will allow for intermittent feedings using a significantly lower flow rate. This system will allow us to maintain a large number of chips with a single syringe pump. Also, the increased accuracy of this system should allow us to reproducibly feed cells using less than 100% medium replacement. We believe this is an important step due to the high amount of paracrine signaling present among these cells.



Part Name	Description
Leveling Mounts	1" Long 3/8"-16 Threaded Stud
Socket Head Screws	18-8 Stainless Steel, M6 x 1 mm thread, 18 mm long
Pan Head Phillips Screw	18-8 Stainless Steel, M6 x 1 mm thread, 12mm long
Washers	18-8 Stainless Steel, for M6 screw size

Vendor	ID#	Quantity Needed
McMaster Carr	23015T64	3
McMaster Carr	91292A136	3
McMaster Carr	92000A424	3
McMaster Carr	93475A250	6



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