

An Integrated Three-Dimensional Osteocyte Culture, Simulated Microgravity, and Fluid Shear Platform with Real-Time Calcium Imaging

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Abstract

Osteocytes are the primary mechanosensors of bone, continuously translating mechanical loading into biochemical signals that regulate skeletal homeostasis. *In vivo*, osteocytes transition seamlessly between loading and disuse, and their responses to reloading are shaped by this preceding disuse history. *In vitro* methods have been previously developed that facilitate 3D culture and exposure of immortalized osteocyte-like cell lines to loading or disuse conditions to model *in vivo* experiences of osteocytes, but few studies have provided methodology that allows all three conditions to be studied sequentially without requiring manipulation of the cells, such as trypsinization and replating, which disrupts cytoskeletal architecture and confounds interpretation of disuse-specific responses. The goal of this protocol is to provide an integrated *in vitro* platform that couples simulated microgravity-induced disuse with subsequent fluid shear reloading within a single three-dimensional (3D) osteocyte culture. Osteocyte-like cells (Ocy454) are seeded onto porous polystyrene scaffolds, differentiated into a mature dendritic phenotype, ~~exposed as 3 mm discs to a rotating wall vessel bioreactor to simulate disuse, and~~ then transferred intact into a parallel-plate flow chamber, where real-time calcium signaling dynamics are captured by live fluorescence imaging during reloading. This approach is expected to preserve cytoskeletal and dendritic architecture throughout the transition, yielding a method that is reproducible across biological replicates, compatible with downstream gene expression and immunostaining analyses, and adaptable to studying how altered gravitational or loading environments shape osteocyte mechanosensation.

Introduction

Osteocytes serve as the primary mechanosensors of bone, translating fluid shear stress within the lacunar-canalicular network into biochemical signals that regulate skeletal homeostasis^{1,2}. Among these signals, transient intracellular calcium (Ca²⁺) waves represent one of the earliest and most rapid indicators of mechanoactivation, occurring within seconds of fluid shear exposure^{3,4,5}. Disuse, whether from spaceflight, prolonged bedrest, or hindlimb unloading, remodels the osteocyte cytoskeleton, alters the cell's differentiation state, and influences

mechanosensitive responses upon reloading⁶⁻⁹. This is of particular relevance to spaceflight, where skeletal deterioration scales with mission duration and is projected to affect the majority of crew on transit-length Mars missions^{10,11,12,13}; laboratory platforms that reproduce the disuse-to-reloading sequence are therefore of practical value. The overall goal of the method described here is to provide an accessible and reproducible *in vitro* platform that integrates 3D osteocyte culture, simulated microgravity, and application of fluid shear with real-time calcium

imaging, enabling direct investigation of how prior disuse alters osteocyte mechanosensation upon reloading.

Several established systems address parts of this question, but each carries an important limitation that the present method is designed to overcome. Two-dimensional (2D) monolayer studies using parallel-plate flow chambers have generated foundational insights into osteocyte calcium signaling^{3,14}, yet they lack the dendritic 3D architecture that characterizes osteocytes *in situ*. Rotating wall vessel bioreactors provide a robust ground-based simulation of microgravity and reliably elevate disuse-associated genes such as *Sost*, *Tnfrsf11 (Rankl)*, and *Dkk1* in osteocyte-laden 3D scaffolds^{15,16,17}, but in prior reports, the scaffolds were not subjected to subsequent mechanical loading. A recent clinostat-based study coupled simulated microgravity to fluid shear, but required cells to be trypsinized and replated onto glass slides between disuse and reloading¹⁸, an intermediate handling step that has been reported to disrupt the cytoskeleton, alter cell stiffness, and promote detachment-related signaling; all of these represent confounding variables in the interpretation of disuse-induced changes in mechanotransduction^{19,20,21}. More recent 3D culture platforms, including hydrogel-confined osteocyte networks²², have been reported to enable real-time calcium imaging during fluid shear, but have not been combined with simulated microgravity. Furthermore, mineralized bone is a structurally rigid tissue, whereas softer hydrogels may not provide sufficient mechanical integrity to maintain a stable osteocyte microenvironment and may not be compatible with subsequent assembly into a parallel-plate flow chamber for fluid-shear application. *In vivo* intravital calcium imaging using genetically encoded calcium indicators provides physiologically appropriate readouts^{3,23,24}, but requires specialized transgenic animals and complex surgical preparation, while offering limited control over the precise mechanical environment of individual osteocytes.

The protocol described here addresses these gaps by directly transferring cell-seeded disc-shaped scaffold constructs from a microgravity-simulating bioreactor into a parallel-plate flow chamber for real-time fluorescence calcium imaging, without trypsinization, replating, or recovery culture required between disuse and reloading interventions¹⁵. This seamless transition is expected to preserve the cytoskeletal state, dendritic architecture, and substrate attachment of the disuse-conditioned osteocytes²⁵. The method is appropriate for investigators studying how altered gravitational or loading environments shape osteocyte mechanotransduction, and the modular scaffold-based design can be adapted to other osteocyte-like cell lines, primary osteocytes, alternative shear regimens, the addition of pharmacological agents, or additional imaging-based readouts. Several practical considerations determine the suitability of this method for a given application. The full protocol spans approximately 16–18 days from initial seeding to imaging (**Figure 1A**) and requires access to a

rotating wall vessel bioreactor, a parallel-plate flow chamber, and a fluorescence or confocal imaging system, equipment that may not be available in all laboratories. Because light scattering through the porous scaffold limits reliable signal acquisition to approximately the upper 50 μm of the 200 μm construct, the method is best suited to investigators studying superficial osteocyte populations rather than those requiring uniform sampling throughout the full scaffold depth. Finally, as with any parallel-plate flow system, the applied shear stress represents a bulk chamber-level value rather than the precise local stress experienced by individual cells within the porous architecture, a consideration relevant to investigators requiring single-cell-resolved mechanical quantification.

Protocol

Cell line authentication, mycoplasma testing, and any institutional biosafety approvals required for the use of immortalized osteocyte-like cell lines should be obtained before initiating this protocol. All cell culture work is performed in a Class II biosafety cabinet using sterile techniques. Reagents and equipment are listed in the **Table of Materials**.

1. Maintenance of osteocyte-like cells

1. Maintain Ocy454 cells in 10 cm tissue culture dishes pre-coated with 0.3 mg/mL type I collagen solution. Culture cells in complete growth medium consisting of α -minimum essential medium (α -MEM) supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution¹⁵; this complete growth medium formulation is used throughout the protocol unless otherwise specified.
2. Culture cells in a humidified incubator at 33 °C with 5% CO₂, which is the proliferative permissive temperature for the temperature-sensitive T-antigen used to immortalize this line¹⁵.
3. Passage cells when they reach approximately 70% confluency using 0.05% trypsin-Ethylenediaminetetraacetic acid (EDTA). Avoid allowing cultures to exceed 80% confluency to prevent premature differentiation.

2. Three-dimensional scaffold preparation

1. Transfer 22 mm diameter sterile porous polystyrene scaffolds (200 μm thickness, 36–40 μm pore size) into individual wells of a sterile 6-well plate using sterile forceps.
2. Add 4 mL of 70% ethanol to each well and incubate for 5 min at room temperature to render the hydrophobic scaffolds hydrophilic.
3. Aspirate the ethanol and rinse each scaffold twice with 4 mL of sterile phosphate-buffered saline (PBS) to remove residual ethanol.

4. Prepare a chilled 0.3 mg/mL type I collagen solution in cell culture-grade water. Add 500 μ L of the collagen solution to the center of each scaffold, replace the 6-well plate lid, and incubate at room temperature for 1 h to coat the scaffold surfaces.
5. Aspirate the collagen solution and allow the scaffolds to air-dry for 10 min under sterile conditions before cell seeding.

3. Cell seeding and osteocytic differentiation on scaffolds

1. Detach Ocy454 cells from the culture dish at ~70% confluency using 0.05% trypsin-EDTA. Neutralize trypsin with growth medium, centrifuge cells at $200 \times g$ for 5 min, and resuspend in growth medium.
2. Count the cells using a hemocytometer or automated cell counter. Adjust the cell density so that 1×10^6 cells are delivered per scaffold in a volume of 200 μ L.
3. Pipette the cell suspension dropwise onto the center of each collagen-coated scaffold. Place the plate in a 33 °C, 5% CO₂ incubator for 90 min to allow cell attachment.
4. After 90 min, gently add 7 mL of pre-warmed growth medium to each well, ensuring that medium is added along the wall of the well to avoid dislodging cells from the scaffold.
5. Continue to culture the cell-seeded scaffolds at 33 °C for 3 days to promote proliferation.
6. After 3 days at 33 °C, transfer the cell-seeded scaffolds to a 37 °C, 5% CO₂ humidified incubator and culture for an additional 10–12 days to induce osteocytic differentiation. Refresh growth medium every 3 days.

4. Exposure of scaffolds to simulated microgravity (disuse)¹⁵ (Figure 1B)

1. Use a sterile 3 mm diameter biopsy punch to cut disc-shaped constructs from each differentiated scaffold. Each 22 mm scaffold typically yields approximately 10 discs of 3 mm diameter.

NOTE: To avoid punch-induced edge deformation, place the full scaffold flat against a rigid sterile surface (e.g., the inverted lid of a 10 cm dish) and apply firm, vertical pressure with the biopsy punch. Handle cell-seeded discs by grasping the lateral edge with fine sterile forceps. All subsequent imaging and quantification are restricted to the non-edge region of each disc, which is unaffected by the cutting process.

2. Transfer 3 mm discs designated as static controls into 10 cm tissue culture dishes containing 12 mL of pre-warmed growth medium (Figure 1C) and place at 37 °C, 5% CO₂.

3. Transfer 3 mm discs designated for simulated microgravity into the chamber of a rotating wall vessel bioreactor (Slow Turning Lateral Vessel, 120 mL working volume; see **Table of Materials**) pre-filled with growth medium.

NOTE: In this protocol, 10 discs are loaded per vessel per culture condition; the vessel can accommodate up to 20 discs without disc–disc collisions during rotation.

1. Completely fill the chamber with growth medium and seal the large port with its solid stopper. Insert one-way stopcocks (luer-lock, swivel-male) into the two remaining smaller ports.
4. Connect two 5 mL syringes to both of the one-way stopcocks. Tilt the bioreactor laterally back and forth while alternately drawing on and releasing the syringe plunger to dislodge and aspirate trapped air bubbles. Repeat until no visible air remains within the chamber, then close the stopcocks.
5. Mount the bioreactor onto its rotation base. Set the rotation speed to 18.6 rpm for the first 24 h, then increase to 20.9 rpm for an additional 48 h, for a total of 72 h of simulated microgravity exposure.

NOTE: These speeds were selected to maintain solid-body rotation and minimize scaffold–wall collisions, as previously described^{15,16}. Solid-body rotation is confirmed visually: at the appropriate speed, the scaffold discs remain gently suspended and orbit synchronously with the medium without settling to the bottom, colliding with the vessel wall, or tumbling. The rotation speed is increased partway through the culture (18.6#20.9 rpm) because the discs become denser as cells proliferate and settle faster, requiring a higher speed to maintain suspension. The operator monitors the discs during this ramp and adjusts the speed as needed to keep them suspended and collision-free.

6. At the end of the 72 h disuse period, halt rotation and orient the bioreactor vertically to allow scaffold discs to settle to the bottom of the chamber. Connect a sterile glass Pasteur pipette attached to a vacuum aspirator and remove approximately two-thirds of the medium from the upper portion of the chamber.
7. Tilt the bioreactor chamber so that all scaffold discs settle to the bottom by gravity. Insert a sterile glass Pasteur pipette connected to a vacuum aspirator through the port, and aspirate the residual medium from the uppermost surface of the liquid, keeping the pipette tip near the meniscus and well away from the settled discs.
 1. As the liquid level drops, gradually increase the tilt angle toward horizontal while continuing to aspirate from the top of the remaining liquid, ensuring the pipette tip never approaches the scaffolds at the bottom. This

top-down aspiration removes the medium completely without disturbing the scaffold discs.

- Open the chamber and gently retrieve each scaffold disc with sterile forceps, transferring the discs into a 10 cm dish containing 10 mL of sterile PBS to hold them until they are processed in subsequent experimental steps.

5. Loading of scaffold cultures with the calcium indicator dye

NOTE: Cell-permeant acetoxymethyl (AM) ester calcium indicators are susceptible to hydrolysis in solution and are highly light-sensitive. Reconstitute dye stocks in anhydrous dimethyl sulfoxide (DMSO) immediately before use and protect all dye-containing solutions from light at every step.

- Prepare a 2 mM stock solution of the cell-permeant fluorogenic calcium indicator (50 µg per aliquot, see **Table of Materials**) by adding 23 µL of anhydrous DMSO directly to the lyophilized aliquot. Mix gently by pipetting and protect from light.

CAUTION: DMSO is a potent solvent and skin-penetrating agent. Handle in a fume hood with appropriate personal protective equipment. Dispose of all DMSO-containing dye solutions and other hazardous chemical waste generated during this protocol (e.g., residual ethanol, spent culture medium containing biological waste) in accordance with institutional Environmental Health and Safety (EHS) guidelines, using designated hazardous waste containers; do not dispose of DMSO-containing solutions down the sink.

- Prepare 1× Hanks' balanced salt solution (HBSS) by diluting 1 mL of 10× HBSS stock in 9 mL of ultrapure water. Add 4.8 mg of HEPES per mL of 1× HBSS to generate Hanks and HEPES buffer solution (HHBS).
- Prepare HHBS containing 0.04% poloxamer 188 (HHBS+P188) by adding 40 mg of poloxamer 188 per 100 mL of HHBS. This non-ionic surfactant facilitates uniform dye loading.
- Prepare a 20 µM working dye solution by performing a 1:100 dilution of the 2 mM cell-permeant fluorogenic calcium indicator stock into HHBS+P188 (10 µL stock per 1 mL HHBS+P188).
- Transfer 3–5 scaffold discs into an individual 1.5 mL tube containing 200 µL of the 20 µM working dye solution diluted 1:1 with complete growth medium (yielding a final serum concentration of ~5%), for a final loading concentration of 10 µM cell-permeant fluorogenic calcium indicator. Wrap the tubes in aluminum foil to protect the dye from light.

NOTE: Growth medium is included to maintain cell viability over the ~90–120 min loading period. Loading in serum-free HHBS+P188 alone is also acceptable and may further reduce extracellular AM-ester hydrolysis.

- Incubate the scaffolds at 37 °C for 60–90 min, followed by an additional 30 min at room temperature to allow complete intracellular hydrolysis of the AM ester and stable cytosolic dye retention.
- During the final 30 min of room-temperature incubation, add the cell-permeant blue-fluorescent nuclear stain directly to the dye solution at a final concentration of 5 µg/mL to counterstain cell nuclei.
- Aspirate the dye-loading solution and rinse the scaffolds twice with PBS supplemented with 1.8 mM CaCl₂ to remove unincorporated dye. Proceed immediately to fluid shear application (Step 6).

6. Application of laminar fluid shear stress with real-time calcium imaging

- Assemble a parallel-plate flow chamber on a flat stage (**Figure 2**). Place a gasket (0.25 mm thickness, 0.25 cm flow path width) onto the chamber base to define the flow channel above the scaffold.

NOTE: The manufacturer-supplied flow chamber is designed to be sealed against a small dish using a vacuum line connected to its peripheral vacuum port. To simplify on-stage assembly and to remove the vacuum tubing and vibration that can interfere with imaging, the chamber was modified for routine use as follows: the chamber-slide assembly was sealed mechanically by compressing the chamber housing against the glass slide using two adjustable zinc alloy clamps (originally designed as adjustable glass-shelf brackets) applied to opposing sides. This clamping configuration provides a watertight seal across the flow path without requiring a vacuum (**Figure 2**).

- Position one dye-loaded scaffold disc in the center of the flow path on the gasket. Place a clean glass slide on the bottom of the chamber and apply two adjustable zinc alloy glass clamps on opposing sides to compress the slide against the chamber housing and seal the flow path.
- Connect the inlet port of the flow chamber to a programmable syringe pump pre-loaded with sterile flow medium consisting of PBS supplemented with 1.8 mM CaCl₂. Connect the outlet to a waste reservoir.

NOTE: A colorless PBS-based flow medium is used to avoid the autofluorescence and light absorption of phenol red present in standard growth medium, which would otherwise interfere with the calcium indicator signal. It is supplemented

add "freshly"

in standard growth medium. It is supplemented with the shear-evoked calcium re

with 1.8 mM CaCl_2 to provide the extracellular calcium required for the shear-evoked calcium response; glucose, bicarbonate, and Mg^{2+} are not needed over the brief 5-min flow window, and omitting bicarbonate avoids pH drift in the open, non- CO_2 -equilibrated flow path during imaging.

- Mount the assembled flow chamber on the stage of an inverted confocal microscope and image through the glass slide from below using a 10 $\times$ /0.40 NA air (dry) objective (working distance $\sim$ 2.2 mm). All imaging is performed at room temperature. Configure the acquisition software to record time-lapse fluorescence images at 1 frame every 1.73 s using settings appropriate for the calcium indicator (excitation/emission 490/525 nm for the cell-permeant fluorogenic calcium indicator; 350/461 nm for the cell-permeant blue-fluorescent nuclear stain).
 - Begin time-lapse acquisition approximately 10 s before the onset of flow to capture baseline fluorescence.
 - Initiate fluid flow at a constant volumetric flow rate of 4.83 mL/min (290 mL/h) to deliver a bulk laminar shear stress of 30 dynes/cm² (3.0 Pa) across the flow path, using the parallel-plate flow chamber equation $\tau = 6\mu Q/(bh^2)$, where τ is the wall shear stress, μ is the fluid viscosity (taken as 0.01 poise for the aqueous PBS-based flow medium at room temperature), Q is the volumetric flow rate, b is the channel width (0.25 cm), and h is the gasket thickness (0.25 mm). Maintain flow continuously for 5 min (300 s). 0.254 mm
- NOTE:** Computational modeling indicates that the porous architecture of the scaffold modifies the local shear stress experienced by individual cells, so the bulk shear stress of 30 dynes/cm² represents a bulk reference rather than the precise shear stress at every cell²².
- After 5 min of flow, stop the syringe pump and end image acquisition. Save the time-lapse data using a non-compressed format for downstream analysis.

7. Image analysis and quantification of calcium signaling dynamics

NOTE: In this protocol, time-lapse calcium imaging was acquired and analyzed using the analysis software supplied with the confocal microscope, which permits user-defined regions of interest (ROIs) to be drawn directly on the time-lapse stack and exports fluorescence intensity versus time data as tabular files. Equivalent functionality is available in ImageJ/Fiji and other image analysis suites.

- Open each time-lapse stack in the image analysis software. In the image analysis software, select the item in the **Open projects** panel, click the **Quantify** tab, and select the **Tools** tab. From the "Tool" dropdown, select **Intensity # Stack Profile**, set

Dimension to **t**, and select only the calcium channel (Channel 2, cell-permeant fluorogenic calcium indicator/FITC).

- Using the cell-permeant blue-fluorescent nuclear dye-stained nuclei (Channel 1) as reference points, scrub along the time axis and draw a minimum of 30 randomly selected osteocyte ROIs per scaffold within the non-edge region of the disc using the circle annotation tool. Select **Sort ROIs** to report each intensity–time trace separately.
- For each ROI, extract the calcium-channel fluorescence intensity over time by clicking on **Report** to export the intensity–time data to a spreadsheet file. Because subtle scaffold motion may occur during flow onset, define the baseline fluorescence (F_0) not as the pre-flow value but as the mean fluorescence intensity over the stable plateau interval immediately preceding the highest calcium peak within the 300 s observation window.
- Plot the fluorescence-versus-time data exported from each ROI and calculate the maximum fold change in calcium fluorescence over baseline (F/F_0) for the highest peak in each ROI. Exclude ROIs that fail to demonstrate at least a 2-fold change over baseline as non-responsive cells.
- For each responsive ROI, also quantify (i) the duration (in seconds) of the highest calcium wave above baseline and (ii) the total number of distinct calcium waves (defined as transient F/F_0 excursions $\geq$ 2-fold) over the 300 s window.
- Average the per-ROI metrics across all responsive ROIs in each scaffold to generate one biological replicate value per scaffold for: (a) average maximum F/F_0 , (b) average duration of the highest calcium wave, and (c) average number of calcium waves.

NOTE: Each independently processed and imaged scaffold constitutes one biological replicate; reproducibility is assessed by comparing these per-scaffold values across replicates ($n = 6$ per condition in the companion study²⁵). Exclude any ROI showing a sustained, non-decaying fluorescence signal that does not return to baseline, as this suggests irreversible loss of membrane integrity rather than physiological calcium signaling.

Results

Successful execution of this protocol yields a series of representative outcomes that span the full workflow, from confirmation of a mature osteocyte phenotype through real-time acquisition and quantitative analysis of calcium signaling during reloading.

Dendritic morphology and molecular validation of the disuse intervention

After 10–12 days of differentiation at 37 °C, scaffold-cultured Ocy454 cells were fixed and stained with phalloidin (F-actin) and cell-permeant blue-fluorescent nuclear stain (nuclei) as previously described²⁵; the resulting confocal images reveal a robust dendritic network that fills the porous architecture of the scaffold (**Figure 3**). This mature dendritic phenotype provides a morphological benchmark for confirming successful differentiation prior to the disuse and reloading interventions. To confirm that the rotating wall vessel bioreactor step produces a genuine disuse phenotype rather than merely altering the mechanical environment, the expression of established disuse-responsive osteocyte genes can be assessed following the 72 h simulated microgravity period. Using this platform, scaffolds cultured under simulated microgravity showed elevated mRNA expression of *Sost*, *Tnfrsf11 (Rankl)*, and *Dkk1* relative to static controls²⁵, consistent with the transition to a disuse state and confirming that the bioreactor intervention functions as intended before reloading.

Representative output of the image-analysis pipeline

Applying the Step 7 workflow to a representative time-lapse dataset from a single static control (normal-gravity) scaffold illustrates how raw fluorescence imaging is converted into quantified calcium signaling metrics (**Figure 4**). For each ROI drawn over an individual osteocyte using the cell-permeant blue-fluorescent nuclear stain channel as reference (**Figure 4A**), calcium-channel fluorescence intensity is extracted over time as a fluorescence-versus-time trace (**Figure 4B**). The baseline (F_0) is defined as the mean intensity over the stable pre-peak plateau interval immediately preceding the highest calcium wave, the peak (F) as the maximum intensity of that wave, and their ratio yields the maximum fold change over baseline (F/F_0). The duration of the

highest wave (interval above baseline) and the number of distinct calcium waves (excursions ≥ 2 -fold over baseline across the 300 s window) are quantified from the same trace.

The same pipeline is also used to distinguish analyzable from non-analyzable ROIs (**Figure 4C**). A responsive osteocyte shows one or more transients rising ≥ 2 -fold above a stable baseline and returning toward baseline after each wave. Two patterns are excluded: an ROI whose fluorescence never exceeds the 2-fold threshold is classified as non-responsive (**Figure 4C**, blue), and an ROI in which fluorescence rises but fails to return to baseline—suggesting irreversible loss of Ca^{2+} extrusion capacity (i.e., loss of membrane integrity) rather than physiological signaling—is likewise excluded (**Figure 4C**, red). The full quantitative efficacy dataset, including aggregate metrics and the disuse-versus-control statistical comparison across $n = 6$ biological replicate scaffolds per condition, is reported in the companion study using this same integrated platform²⁵.

Real-time calcium signaling responses to laminar fluid shear

A representative video (**Supplementary Video 1**) demonstrates the real-time calcium signaling responses of Ocy454 osteocytes cultured within the 3D scaffold during the induction of laminar fluid shear stress. Following the cell-permeant fluorogenic calcium indicator loading and assembly of the scaffold into the flow chamber, the construct is mounted on the confocal stage and imaged under continuous laminar perfusion at a bulk shear stress of 30 dynes/cm². Over the 5 min imaging window, time-lapse acquisition captures stochastic, asynchronous Ca^{2+} transients across the dendritic network, visualized as dynamic increases in the cell-permeant fluorogenic calcium indicator fluorescence intensity within individual osteocyte ROIs.

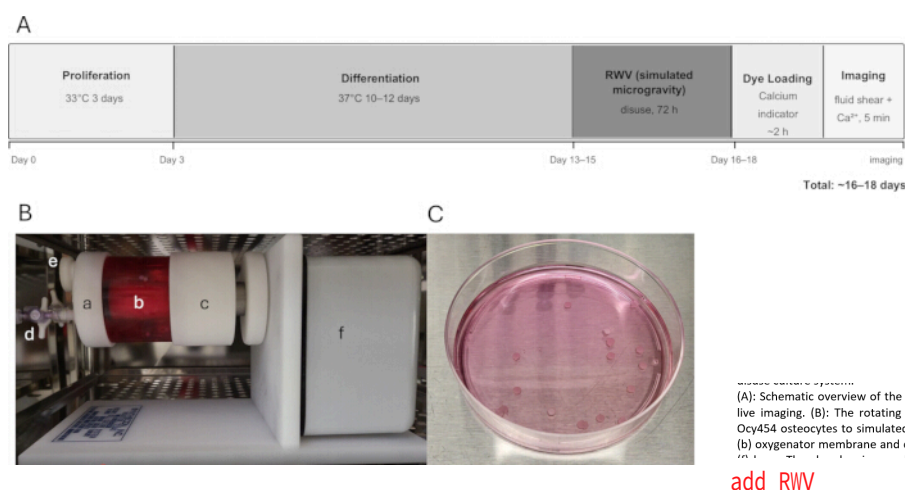


Figure 1: Schematic overview of the complete workflow and macroscopic configuration of the disuse culture system. (A) Schematic overview of the complete workflow, from cell seeding through dye loading and live imaging. (B) The rotating wall vessel (RWV) bioreactor used to expose scaffold-cultured Ocy454 osteocytes to simulated microgravity (disuse). Labeled components: (a) front end cap; (b) oxygenator membrane and core; (c) rear end cap; (d) syringe ports; (e) 1/2-inch fill port plug; (f) base. The chamber is completely filled with growth medium and sealed, with trapped air aspirated through

the syringe ports prior to rotation. **(C)** Disc-shaped constructs (3 mm diameter) cut from differentiated scaffolds using a sterile biopsy punch, shown in growth medium prior to loading into the bioreactor chamber. [Please click here to view a larger version of this figure.](#)

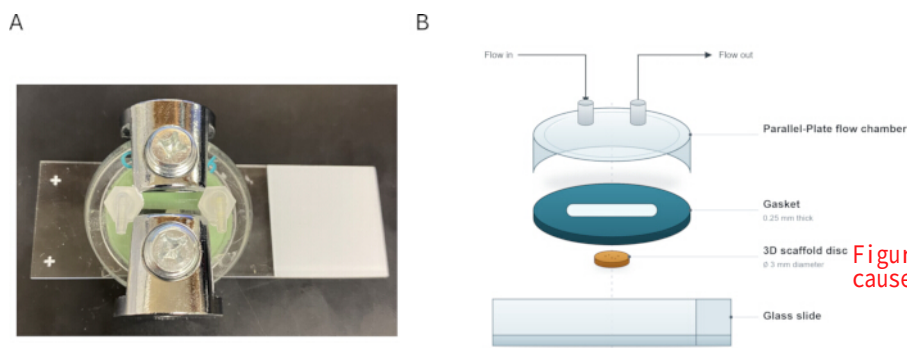


Figure 2: Modified parallel-plate flow chamber assembly. **(A)** Photograph of the assembled flow chamber mounted on a glass slide. The chamber housing is compressed against the slide using two adjustable zinc alloy clamps applied to opposing sides, providing a watertight seal across the flow path without the vacuum line specified by the manufacturer; this modification simplifies on-stage assembly and removes the tubing and vibration that interfere with imaging. **(B)** Exploded schematic of the assembly layers, from top to bottom: the Parallel-Plate flow chamber (with flow inlet and outlet ports), a gasket (0.25 mm thickness) that defines the flow channel, a single 3 mm diameter 3D scaffold disc positioned in the center of the flow path, and the underlying glass slide. [Please click here to view a larger version of this figure.](#)

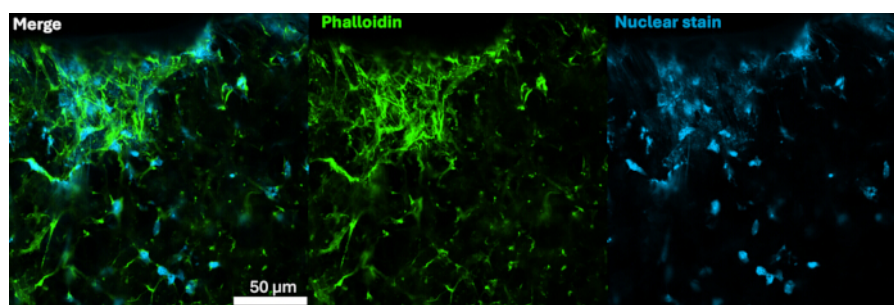


Figure 3: Dendritic morphology of scaffold-cultured Ocy454 osteocytes. Ocy454 cells were cultured within the 3D porous scaffold and differentiated for 10–12 days at 37 °C, then fixed and stained with phalloidin to label the F-actin cytoskeleton (green) and the cell-permeant blue-fluorescent nuclear stain to label nuclei (cyan). Confocal imaging reveals a robust dendritic network that extends throughout and fills the porous architecture of the scaffold, consistent with prior reports²⁵ of this culture system and comparable to *in vivo* osteocyte morphology. Left: merged channels. Middle: phalloidin (F-actin) alone. Right: the cell-permeant blue-fluorescent nuclear stain (nuclei) alone. Scale bar: 50 μm. [Please click here to view a larger version of this figure.](#)

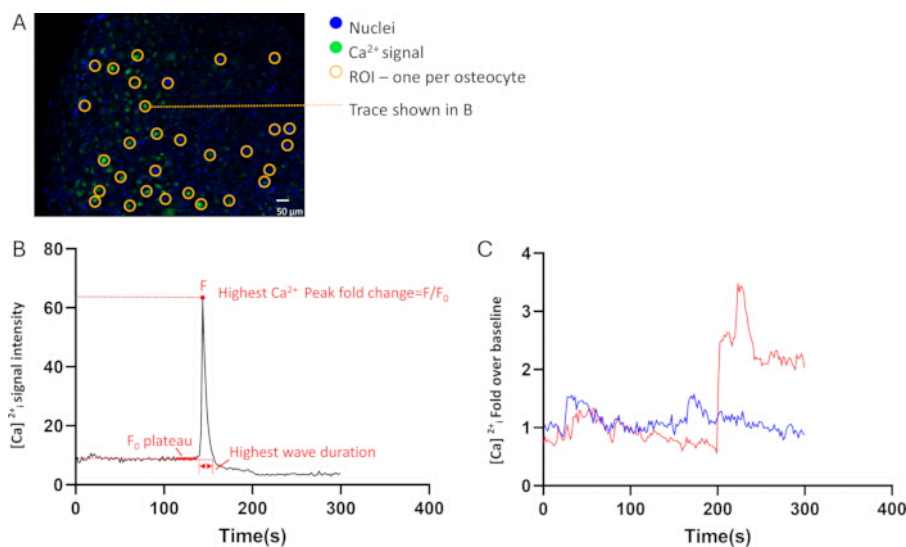


Figure 4: Representative output of the image-analysis pipeline for quantifying osteocyte calcium signaling. (A) Representative fluorescence micrograph of Ocy454 osteocytes within a 3D scaffold following cell-permeant fluorogenic calcium indicator loading and laminar fluid shear, showing the cell-permeant blue-fluorescent nuclear stain-labeled nuclei (blue) and the cell-permeant fluorogenic calcium indicator signal (green). Circular regions of interest (ROIs; orange) are drawn over individual osteocytes using the nuclear channel as a positional reference (≥ 30 ROIs per scaffold); the highlighted ROI corresponds to the trace in (B); Scale bar, 50 μm . (B) Representative annotated fluorescence-versus-time trace from a single ROI over the 300 s window. Because subtle scaffold motion can occur at flow onset, the baseline (F_0) is defined not as the pre-flow value but as the mean intensity over the stable plateau interval immediately preceding the highest calcium wave (red segment). The peak (F) is the maximum intensity of that wave, and its ratio gives the maximum fold change over baseline (F/F_0). The duration of the highest wave (interval above baseline) and the number of distinct calcium waves (excursions ≥ 2 -fold over F_0) are quantified from the same trace. (C) Representative excluded traces. The blue trace never exceeds the 2-fold threshold and is classified as non-responsive; the red trace rises but fails to return to baseline, suggesting loss of Ca²⁺ extrusion capacity (i.e., loss of membrane integrity/cell death) rather than physiological signaling. Both are excluded. Fluorescence in (C) is expressed as fold change over baseline. [Please click here to view a larger version of this figure.](#)

Supplementary Video 1: Real-time calcium signaling dynamics during laminar fluid shear.

Time-lapse confocal video of Ocy454 osteocytes cultured within the 3D scaffold and loaded with the cell-permeant fluorogenic calcium indicator (green) and the cell-permeant blue-fluorescent nuclear stain. The construct was assembled into the modified parallel-plate flow chamber and imaged under continuous laminar perfusion delivering a bulk shear stress of 30 dynes/cm² over 5 min (300 s), with frames acquired every 1.73 s using a 10 $\times$ objective. Dynamic increases in the cell-permeant fluorogenic calcium indicator fluorescence within individual osteocyte regions of interest reflect stochastic, asynchronous intracellular Ca²⁺ transients evoked by fluid shear. Scale bar: 100 μm . [Please click here to download this file.](#)

Discussion

The principal methodological advance of this protocol is the integration of post-disuse fluid shear reloading with real-time intracellular calcium imaging in the same 3D osteocyte culture, enabling the immediate mechanotransduction response to reloading to be visualized as it unfolds in disuse-conditioned cells. While rotating wall vessel bioreactor cultures of 3D scaffold-seeded osteocytes have been used to model disuse, those scaffolds were not subsequently subjected to mechanical

reloading^{15,16,17}, and while Liu et al. recently combined *in vivo* hindlimb unloading with *ex vivo* tibial loading and real-time osteocyte calcium imaging, and additionally exposed monolayer-cultured osteocytes to simulated microgravity followed by fluid shear after trypsinization and replating, an analogous *in vitro* framework that preserves the disuse-conditioned cellular architecture throughout the disuse-to-reloading transition has not, to our knowledge, been described^{3,18,23,24}. The present platform closes this gap by applying defined laminar fluid shear directly to the disuse-conditioned scaffold while live calcium dynamics are recorded by time-lapse fluorescence microscopy, providing a tractable *in vitro* window into the earliest mechanosensation events of reloading. A second beneficial feature of the method is the direct transfer of scaffold discs from the bioreactor into the parallel-plate flow chamber, which is expected to preserve the cytoskeletal state, dendritic architecture, and substrate attachment of the disuse-conditioned osteocytes throughout the disuse-to-reloading transition by avoiding the enzymatic dissociation required in trypsinization-and-replating approaches. In contrast, another prior approach that coupled simulated microgravity with fluid shear-evoked calcium imaging required cells to be trypsinized and replated onto glass slides after the disuse period¹⁸. Beyond the profound cytoskeletal rearrangements and reductions in cell stiffness known to

accompany enzymatic detachment^{19,20,21}, cell detachment itself has been shown to activate Hippo-pathway signaling, driving phosphorylation of its mechanosensitive effector YAP within minutes via cytoskeletal reorganization²⁶. Because YAP/TAZ signaling is itself an established mechanotransduction pathway in osteocytes²⁷, inserting a trypsinization-and-replating step between disuse and reloading could plausibly superimpose a detachment-induced mechanotransduction event on the disuse-specific responses under study, confounding their interpretation.

Several practical points are critical for reproducible results. Ocy454 cells proliferate rapidly and undergo contact inhibition–induced premature differentiation even at the 33 °C permissive maintenance temperature¹⁵; maintenance cultures must therefore be passaged before they exceed ~80% confluence, otherwise the proliferative pool is prematurely depleted, and the controlled 3-day proliferation / 10–12-day differentiation timeline on the scaffold cannot be reliably established. Accurate cell counting at the time of seeding (1×10^6 cells per scaffold) is equally critical, since over- or under-seeding shifts the density of the dendritic network that subsequently develops at 37 °C and confounds comparisons across biological replicates.

During scaffold culture, it is advisable to verify successful colonization at the mid-culture stage rather than waiting until the end of the 10–12-day differentiation period. A single representative scaffold can be removed, stained with the cell-permeant blue-fluorescent nuclear stain, and examined under a standard fluorescence microscope: nuclei distributed throughout the scaffold indicate successful seeding and colonization, and the remaining scaffolds can be left to mature. If few or no nuclei are visible across the scaffold, colonization has likely failed, and the culture should be restarted immediately, avoiding the loss of time incurred by discovering an uncolonized scaffold only at the differentiation endpoint. During calcium imaging in the reloading experiments, an absence of detectable cell-permeant fluorogenic calcium indicator signal most commonly reflects inadequate dye penetration. This typically occurs when multiple scaffolds are stacked or clumped together in the same tube during the cell-permeant fluorogenic calcium indicator incubation, preventing the acetoxymethyl ester dye from reaching cells within the scaffold interior. To ensure uniform penetration, scaffolds should be physically separated with forceps during dye incubation or incubated individually with only one scaffold per tube.

The method has limitations that should be acknowledged when planning experiments. First, although the scaffolds are 200 μm thick, fluorescence imaging through the porous polystyrene matrix is constrained by light scattering at the polymer–aqueous interfaces, restricting reliable calcium signal acquisition to approximately the upper 50 μm of the scaffold; osteocytes embedded in deeper regions are therefore not represented in the analyses described here. Second, the bulk shear stress applied

across the chamber is not necessarily equivalent to the local shear experienced by individual cells within the porous scaffold architecture; computational fluid-structure interaction modeling indicates that local shear can deviate substantially in either direction from bulk values²², so the 30 dynes/cm² value utilized here should be interpreted as a chamber-level reference rather than the precise stress at any individual cell, which may be locally amplified or blunted depending on pore geometry. As an initial implementation, this platform has limitations that warrant further refinement, including its reliance on simplifying *in vitro* assumptions and its focus on cell-level readouts rather than a multiscale or subcellular-level analysis; these represent valuable directions for future development.

Within these limits, the platform may have utility in mechanistic studies of osteocyte responses relevant to astronaut bone health^{10,11,12,13}, clinical immobilization, prolonged bed rest, and spinal cord injury, and it is designed to accommodate simultaneous testing of pharmacological agents (e.g., mechanotransduction modulators or anti-resorptives) under controlled mechanical conditions. The modular design can be adapted to other osteocyte-like cell lines or primary osteocytes from genetically engineered mice, and the underlying workflow, 3D differentiation on a porous scaffold, controlled mechanical conditioning, direct transfer to a parallel-plate flow chamber, and real-time calcium imaging, is conceptually transferable to other mechanosensitive cell types in which fluid shear-evoked calcium dynamics are biologically relevant. Under conditions that change to hypophosphatemic, osteocyte-like cell lines or primary osteocytes can be used in the workflow-3D differentiation and transfer to a parallel-plate flow chamber.

Disclosures

The authors declare no conflicts of interest. The first author confirms that AI-based large language model tools were used solely to assist with language editing and polishing (grammar, clarity, and conciseness) of author-written text. No AI tool was used to generate, analyze, or interpret experimental data, or to produce scientific conclusions. All AI-assisted text was reviewed, verified, and edited by the authors, who take full responsibility for the accuracy and integrity of the entire manuscript.

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