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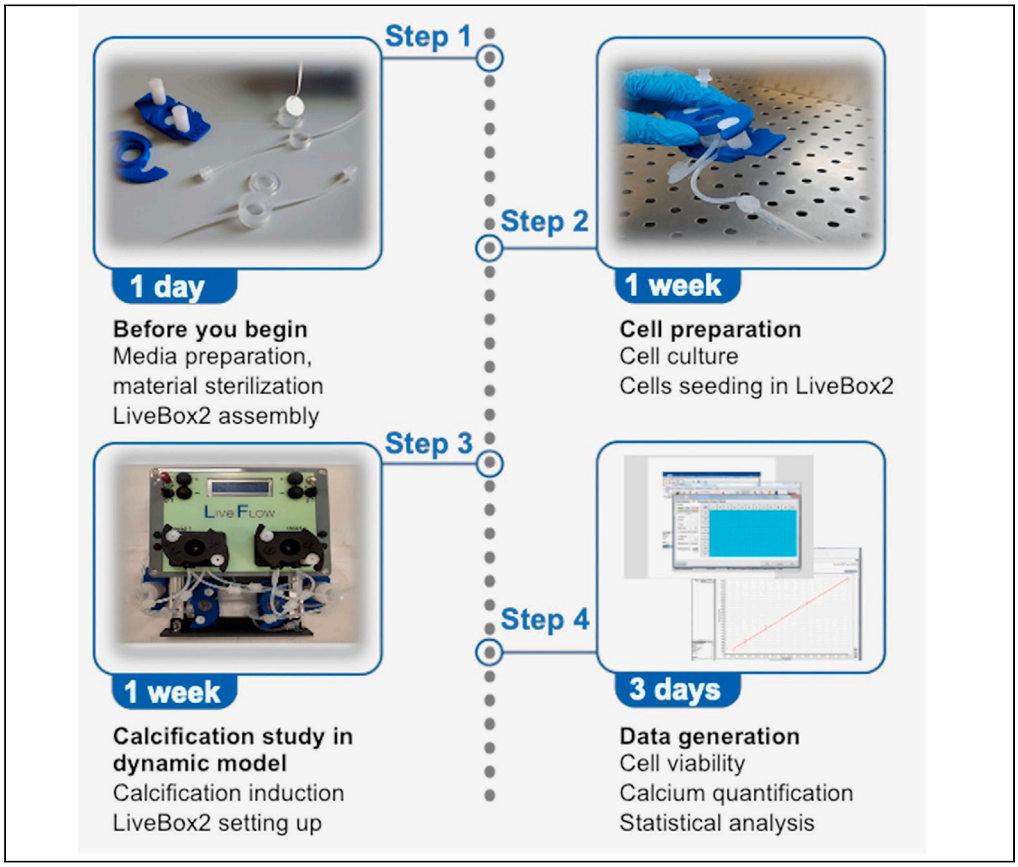
Protocol to generate an *in vitro* model to study vascular calcification using human endothelial and smooth muscle cells



Vascular calcification is a systemic disease characterized by calcium salt deposition within vascular walls. Here, we present a protocol for establishing an advanced dynamic *in vitro* co-culture system using endothelial and smooth muscle cells to replicate vascular tissue complexity. We describe steps for cell culture and seeding in a double-flow bioreactor that recreates the action of blood in humans. We then detail the induction of calcification, setting up of the bioreactor, followed by cell viability assessment and calcium quantification.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

An *in vitro* system to mimic cardiovascular hemodynamic and cell crosstalk environment

Assembly of a double-flow bioreactor to recreate dynamic conditions in human circulation

Steps to induce calcification, assess cell viability, and quantify intracellular calcium

The protocol versatility allows for its use in other pathologies

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Protocol to generate an *in vitro* model to study vascular calcification using human endothelial and smooth muscle cells

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SUMMARY

Vascular calcification is a systemic disease characterized by calcium salt deposition within vascular walls. Here, we present a protocol for establishing an advanced dynamic *in vitro* co-culture system using endothelial and smooth muscle cells to replicate vascular tissue complexity. We describe steps for cell culture and seeding in a double-flow bioreactor that recreates the action of blood in humans. We then detail the induction of calcification, setting up of the bioreactor, followed by cell viability assessment and calcium quantification.

BEFORE YOU BEGIN

Experimental setup and preparation

Timing: 1 day

1. Membrane assembly (Methods video S1, Membrane assembly).
   a. Under the laminar flow hood, place the polyethylene terephthalate (PET) membrane (0.45 μm pore size, Figure 1A) in a Petri dish and soak it in 70% ethanol for 15 min.
   b. Transfer the membranes to a 12-multiwell using sterile tweezers and allow them to dry for approximately 2 h.
   c. Sterilize the membranes by activating the hood's UV for 15 min.
   d. Seal the plate with parafilm before taking it out from the hood.
   e. Wear sterile gloves in the laminar flow hood to perform the following operations.
      i. Place the core (Figure 1B) in a Petri dish.
      ii. Using sterile tweezers, wet the membrane (Figure 1A) with PBS, remove the excess liquid and place it onto the surface of the core.
      iii. Place the shield (Figure 1C) over the core and press downwards until touching the assembling surface to firm the membrane properly.
   f. Store the membranes in a Petri dish, sealing them with parafilm before removing them from the hood.
2. Gelatin solution preparation for glass disc coating (optional step). Dissolve the gelatin powder in PBS to achieve a 1% w/v final working solution. Store the solution in a glass bottle at 4°C (problem 1). Sterilize the gelatin solution in 5 mL aliquots in an autoclave and store at +4°C.

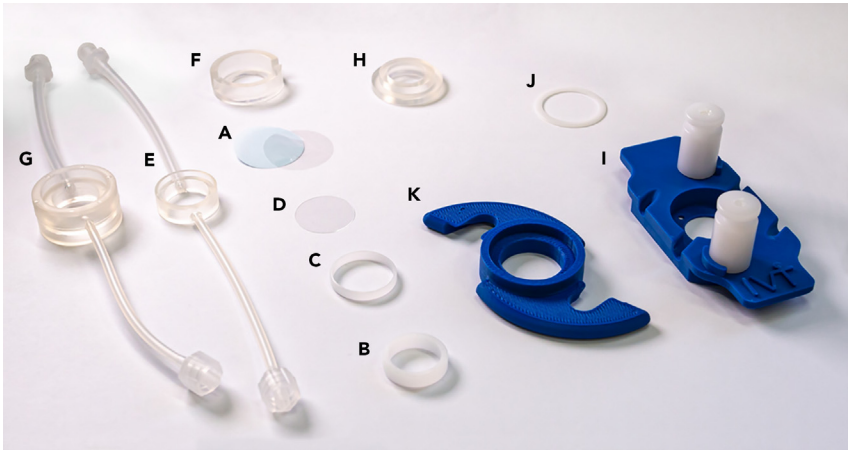

Figure 1. LiveBox2 components, essential for the chamber's assembly

(A) PET membrane.
(B) Core.
(C) Shield.
(D)Glass disc.
(E) Inner cylinder.
(F) Bottom housing.
(G) Upper cylinder.
(H) Top.
(I) Base.
(J) Low friction ring.
(K) S-shape element.

3. Chambers assembly (Methods video S2, chambers assembly). Perform this step on a stable surface.
   a. Insert a glass disc (Figure 1D) and then the inner cylinder (Figure 1E) into the slot of the bottom housing (Figure 1F), leaving the side with the slot oriented upwards. This represents the bottom chamber.
   b. Insert a glass disc (Figure 1D) into the upper cylinder (Figure 1G) with the word "UP" facing up (Figure 2) and then seal with the top (Figure 1H). These components constitute the upper chamber.
4. Component sterilization.
   a. Using gas plasma sterilization (preferably, otherwise, autoclave sterilization might be possible), sterilize the bottom and upper chambers separately, leaving the tubes open.
   b. Individually sterilize the core (Figure 1B), the shield (Figure 1C), and the base (Figure 1I) of each LiveBox2.

Figure 2. Upper cylinder with the word "UP" marked on a side

This helps with both the chamber and the LiveBox2 assembly.

c. Sterilize tweezers.

5. LiveBox2 assembly (Methods video S3). In the safety cabinet, wear sterile gloves to perform the following operations.
   a. Insert the bottom chamber into the slot of the base (Figure 1I), fitting the lower tubes with the holes designed.
   b. Place the membrane (already mounted with core and shield as previously described) onto the LiveBox2 bottom chamber and gently press it down to join them with their male-female joint.

   Optional: coat the membrane with 300 µL of sterile gelatin solution 1% w/v. Wait 15 min and remove the excess solution.

   c. Place the upper chamber over the bottom chamber and gently press downwards to join them through their male-female joint.
   d. Place the low friction ring (Figure 1J) onto the top surface of the upper cylinder and then the S-shape element (Figure 1K).
   e. Rotate it, avoiding forcing it vertically and fitting the s-shape with the slot of the columns. An alternative way to assemble and sterilize can be found on the official IVTech LiveBox2 page.

Preparation of incubation media

Timing: 1 h

6. Prepare a bottle of complete Human Coronary Artery Smooth Muscle Cells (HCASMC) medium.
   a. Mix 500 mL of Medium 231 with Smooth Muscle Growth Supplement (SMGS) and Penicillin/Streptomycin for a final concentration of 100 IU/mL and 100 µg/mL, respectively.
   b. Store at +4°C, protected from light if not used immediately. Supplemented medium is stable for up to a month.
7. Prepare a bottle of complete Human Coronary Artery Endothelial Cells (HCAEC) medium.
   a. Mix 500 mL of Endothelial Cell Growth with Supplements and Penicillin/Streptomycin for a final concentration of 100 IU/mL and 100 µg/mL, respectively.
   b. After adding the supplements, the shelf life of the complete medium is 6 weeks at +4°C in the dark.
8. Prepare a bottle of Reference medium, otherwise named culture medium, prepared as follows. It must be stored at +4°C for up to five weeks. Note that FBS should be heat-inactivated, filter-sterilized (0.2 µm filter), aliquoted and stored at –20°C.

Reference medium

Reagent	Final concentration	Amount (mL)
DMEM High glucose	/	500
FBS	10%	50
L-Glutamine	1%	5
Penicillin/Streptomycin	1%	5

⚠ CRITICAL: Freezing of complete media is not recommended. Always warm each medium up to +37°C before adding it to the cells.

9. Prepare stock and working solution of Calcifying medium as follows.[1] It must be freshly prepared before each use.
   a. Prepare 100 mM stock solutions of Na_2HPO_4 and NaH_2PO_4 monthly, filter sterilized (0.2 µm filter), aliquot and store at –20°C. Mix Na_2HPO_4 and NaH_2PO_4 solutions in 1:1 proportion.

Note: The final phosphate concentration is 100 mM.

b. Add the stock solution to the reference medium for a final **phosphate** concentration of 1.9 mM[1].

⚠ CRITICAL: Preparing reference medium (i.e., DMEM and FBS) before adding phosphates is crucial to avoid precipitation.

10. Prepare hydrogen chloride 0.6 M solution. It is suggested to make a 100 mL final volume and to operate under a fume hood.

Note: Special caution should be taken when handling HCl. Personal Protective Equipment (PPE) is required. This solution must be stored in a glass bottle and is stable for several months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Medium 231	Thermo Fisher Scientific	M231500
Smooth Muscle Growth Supplement (SMGS)	Thermo Fisher Scientific	S00725
CellTiter-Blue® Cell Viability Assay	Promega	G8081
Calcium Colorimetric Assay kit	Merck	MAK022
Endothelial Cell Growth Medium MV2	PromoCell	C-22221
Endothelial Cell Growth Medium MV 2 Supplement Pack	PromoCell	C-39221
Dulbecco's Modified Eagle's Medium - high glucose	Merck	D6546
Fetal bovine serum	Merck	F2442
L-Glutamine	Merck	G7513
Penicillin/streptomycin	Merck	A5955
Gelatin powder	Merck	G2500
Hydrogen chloride	Merck	H3162
Disodium hydrogen phosphate (Na_2HPO_4)	Merck	71643
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck	S5011
PBS	Merck	D8537
Trypsin/EDTA	Merck	T4174
Calcium Colorimetric Assay Kit	Merck	MAK022
Experimental models: Cell lines		
Primary Human Coronary Artery Endothelial Cells	PromoCell	C-12221
Primary Human Coronary Artery Smooth Muscle Cells	Thermo Fisher Scientific	C0175C
Other		
Polyethylene terephthalate (PET) membrane	it4ip	2000M12/620N453/25
Glass coverslip	Marienfeld	0111600
T25 and T75 flasks	Merck	C6481/ C7231
12-multiwell plate	VWR	734–1598
LiveBox2	IVTech	https://www.ivtech.it/Products/
LiveFlow (peristaltic pump)	IVTech	https://www.ivtech.it/Products/

STEP-BY-STEP METHOD DETAILS

It was necessary to use endothelial cells in co-culture with muscle cells to recreate the *in vivo* vascular environment and to perform calcification experiments on a dynamic model in LiveBox2. In our *in vitro* cell model, we used HCASMCs and HCAECs, respectively. The cells were purchased in

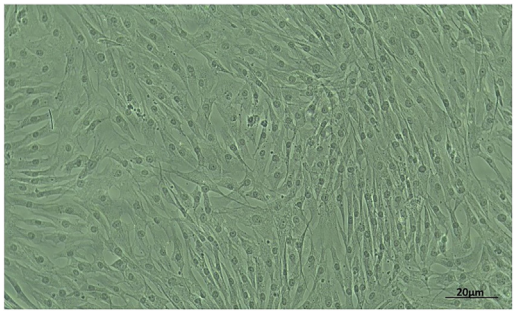

Figure 3. HCASMCs in the culture at a confluence of 90%
Scale bar represents 20 μm.

cryopreserved form, and the starting culture was carried out following the manufacturer's instructions. The starter culture should be set up on their first use.

Step 1: HCASMCs culture for a dynamic vascular model

Timing: 1 week

This section describes the steps for HCASMCs culture initiation and maintenance.

Note: Typically, HCASMCs of passages 3 to 8 were used for vascular calcification studies.

1. HCASMCs culture initiation from cryopreserved batch.
   a. Remove the vial of cells from liquid nitrogen and rapidly thaw by placing it at 37°C in a water bath with gentle agitation until only a tiny piece of ice remains visible.
   b. When the materials of the vial have thawed, wipe the outside with disinfecting solution and move it to a safety cabinet.

   Optional: determine the number of viable cells per ml (mixing cell solution with trypan blue) using a hemocytometer.

   c. Dilute the vial's contents (1 mL) with supplemented Medium 231 (10 mL for a T75 flask, 5 mL for a T25 flask). Gently swirl the flask.
   d. Incubate in a 37°C, 5% CO_2 humidified incubator, and do not disturb for at least 24 h after the culture has been initiated. Change the medium the following day.
2. HCASMCs culture maintenance.
   a. Following the first medium change, replace with fresh medium every other day until the culture is approximately 90% confluent (Figure 3), and then they are ready to be split. Most of the cells should have a spindle-shaped morphology at this stage.
   b. To achieve the highest cell densities, change the culture medium every day until reaching confluence. Such prepared cells are ready to be seeded in LiveBox2.

Step 2: HCAECs culture for a dynamic vascular model

Timing: 1 week

This section describes steps for HCAECs culture initiation and maintenance.

Note: Typically, HCAECs of passages 4 to 7 were used for vascular calcification studies.

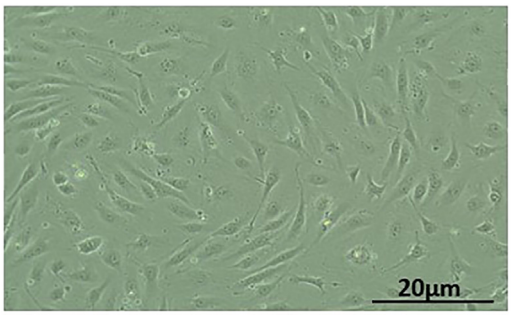

Figure 4. HCAECs in culture
When cells reach a confluence of 80%, they are ready to be split. Scale bar represents 20 µm.

3. Defrosting of cryopreserved HCAECs by passing briefly in a thermostatic bath at 37°C.

   Note: Follow the same steps as in the HCASMCs section using supplemented endothelial growth medium MV2.

4. HCAECs subculture and proliferation.
   a. After the first medium change 24 h after thawing, replace the medium completely every 48 h.
   b. Upon reaching a cell density of about 80% (Figure 4), pass the cells into flasks for maintenance and/or seed in plates or LiveBox2 for calcification experiments.

Step 3: Day 0 - Cells seeding in the LiveBox2

Timing: 6.5 h

This section describes steps for HCASMCs and HCAECs seeding in the bioreactor.

Note: The allometric scaling described in the supplementary information describes the ratio between the HCASMCs and the HCAECs that have to be seeded in a LiveBox2 chamber.

Note: The HCAECs and HCASMCs were kept at 37°C and 5% CO_2 and maintained in HCAEC medium and Medium 231, respectively, replacing medium every 2 days. When 80% confluent, HCAECs and HCASMCs need to be split. All the following procedures must be performed in sterile conditions under a laminar flow safety cabinet.

5. Pre-warm Endothelial and 231 complete medium, trypsin/EDTA solution and PBS to room temperature (20°C–22°C).
6. Aspirate medium in HCASMCs flasks and wash twice with PBS each. Add 1 mL trypsin/EDTA solution to a T25 flask and incubate for 3–5 min.
7. Add 4 mL of Medium 231 and dispense the medium over the cell layer until they are detached from the flask.
8. Transfer the cell suspension into a 15 mL tube and proceed with the centrifugation step at 900 × *g* for 5 min.
9. Perform a cell count using a hemocytometer.
10. Remove the supernatant and resuspend the cells in Medium 231 to have 30,000 HCASMCs in 200 µL.
11. Using a 1,000 µL pipette, insert 200 µL of HCASMCs suspension into the lower chamber of LiveBox2 using the inlet tube (Figure 5A). The cells will grow adhering to the glass coverslip.
12. Add 600 µL Medium 231 to allow all cells to enter the chamber. Proceed slowly to avoid the formation of air bubbles (problem 2).

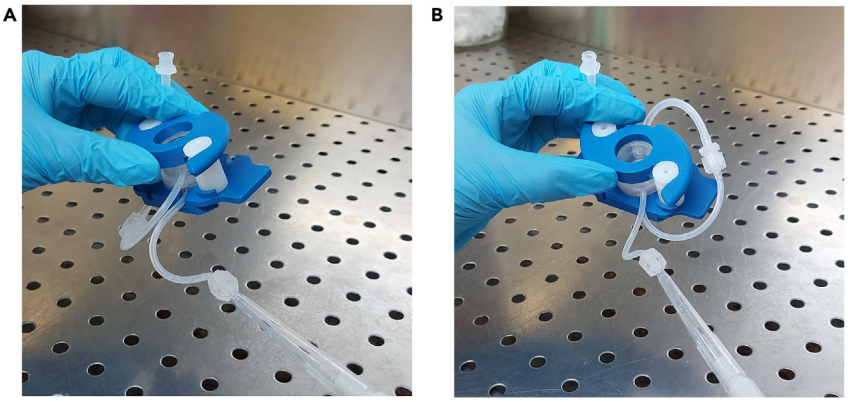

Figure 5. Cell seeding in LiveBox2

(A) HCASMCs are seeded in the lower chamber through the inlet tube using a 1,000 μL pipette.

(B) HCAECs are inserted into the upper chamber using the inlet tube.

13. Incubate the LiveBox2 in a 37°C, 5% CO_2 humidified cell culture incubator. Wait for the attachment on the glass coverslip before proceeding with the seeding of the HCAECs (about 4 h).
14. Afterwards, aspirate the medium in HCASMCs flasks and wash twice with PBS. Add 1 mL trypsin/EDTA solution to a T25 flask and incubate for 3–5 min.
15. Add 4 mL of Endothelial medium and dispense it over the cells until HCAECs are detached.
16. Transfer the cell suspension into a 15 mL tube and proceed with the centrifugation step at 900 × *g* for 5 min.
17. Remove the supernatant and resuspend the cells in the HCAEC medium to have 45,000 HCAECs in 200 μL.
18. Using a 1,000 μL pipette, insert 200 μL of HCAECs suspension into the upper chamber of LiveBox2 using the inlet tube (Figure 5B). The cells will grow, adhering to the membrane surface.
19. Add 600 μL HCAEC medium to move all cells into the chamber. Proceed slowly to avoid the formation of air bubbles.
20. Incubate the LiveBox2 in a 37°C, 5% CO_2 humidified cell culture incubator for 24 h.

Step 4: Day 1 - Start dynamic experimental condition

Timing: 30 min

This section describes steps for bioreactor connection to LiveFlow and how to start the dynamic flow.

Note: All the following procedures must be performed in sterile conditions under a laminar flow hood.

21. Using a 1,000 μL pipette, aspirate the Medium 231 in the lower chamber of LiveBox2 using the inlet tube, and replace it with 1,000 μL of fresh medium (either reference medium or calcifying medium).
22. Using a 1,000 μL pipette, aspirate the HCAEC medium in the upper chamber of LiveBox2 using the inlet tube, and replace it with 1,000 μL of fresh medium.
23. Insert 15 mL of HCAEC medium into the reservoir bottles, one for each LiveBox2.
24. Connect the inlet tube of the bottle to the peristaltic pump and the outlet tube of the bottle to the upper chamber of LiveBox2. Then, connect the inlet tube of the upper chamber to the peristaltic pump (Methods video S4).
25. Turn on the pump from the main switch and set the flow at 250 μL/min with counterclockwise rotation (CCW, Methods video S5 and Figure 6). Wait for the chamber to be completely filled with medium.

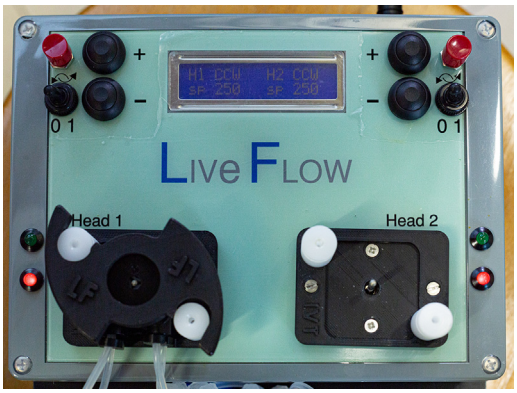

Figure 6. Peristaltic pump in action
The screen displays the flow speed and the direction of the flux.

Note: Make sure there are no air bubbles.

26. Incubate the LiveBox2s in a 37°C, 5% CO_2 humidified cell culture incubator until day 4.

Step 5: Day 4 - Refresh medium

Timing: 30 min

This section describes the steps for refreshing the culture chamber medium.

Note: All the following procedures must be performed in sterile conditions under a laminar flow hood.

27. Stop the pump flow from the main switch.
28. Using a 1,000 µL pipette, aspirate the reference or calcifying media in the lower chamber from the inlet tube, and refresh with fresh, warmed-up medium. The removed media could be stored at –80°C for further analysis.
29. Repeat the same for the Endothelial medium in the upper chamber of LiveBox2 using the inlet tube. Replace the HCAECs medium bottle with one containing 15 mL of Endothelial cells growth fresh medium and connect with the upper chamber.
30. Turn on the pump and set the CCW flow at 250 µL/min. Wait for the upper chamber to be completely filled. Make sure there are no air bubbles.
31. Incubate the LiveBox2 in a 37°C, 5% CO_2 humidified cell culture incubator until day 7.

Step 6: Day 7 - Stop dynamic experimental condition

Timing: 30 min

This section describes the steps for stopping the dynamic flow.

Note: All the following procedures must be performed in sterile conditions under a laminar flow hood.

32. Stop the pump flow and disconnect the LiveBox2.
33. Using a 1,000 µL pipette, aspirate the media from the upper and bottom chambers of LiveBox2 using the inlet tube. These media can be stored at –80°C for further analyses.

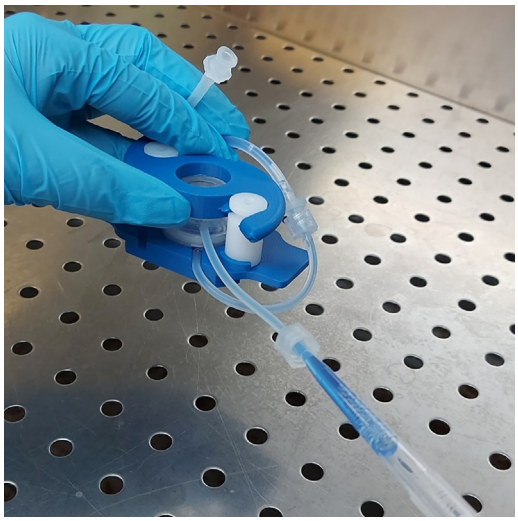

Figure 7. Cell viability assay
CellTiter-Blue® reagent is mixed with either HCASMC or HCAEC medium and added to the bottom or upper chamber respectively, using the inlet tube.

Step 7: Post-treatment cell viability

Timing: 30 min plus incubation

This section describes the steps for cell viability assessment.

Note: The metabolic capacity of HCASMCs and HCAECs as a mirror of the cell viability can be assessed through the CellTiter-Blue® Cell Viability Assay kit. The rationale behind the kit is that viable, healthy cells convert resazurin into resorufin, a fluorescent product that can be quantitatively measured through spectrometer detection.

34. Using the outlet tube, aspirate the media from the bottom chamber (media in contact with HCASMCs) and transfer it to a 1.5 mL tube. Repeat the procedure for the upper chamber (media in contact with HCAECs).
35. For each chamber, add 1 mL of CellTiter-Blue® solution at 10% v/v by mixing Reference medium or Endothelial Cell Growth complete medium for HCASMCs or HCAECs respectively, with CellTiter-Blue® reagent (Figure 7). In separate tubes, prepare blank controls for HCASMCs or HCAECs.
36. Incubate for 2 $^{1}/_{2}$ hours.
37. Transfer 100 µL of the such-prepared mix to a 96-multiwell plate; loading each sample in triplicate is recommended. Shake the plate for 10 s and record fluorescence at 560/590 nm. Subtract fluorescence generated after recording to the appropriate blank (either HCASMCs or HCAECs medium).

Step 8: LiveBox2 disassembly for subsequent analyses

Timing: 15 min

This section describes the steps for LiveBox2 disassembling and cells recovering for subsequent analyses.

Note: The following operations do not require a sterile hood.

38. Disassemble the LiveBox2, rotating the S-shape lock and remove the upper cylinder.
39. Collect the membrane using tweezers. Wash twice with PBS and store inside a 1.5 mL tube at –80°C for further analyses.
40. Gently remove the bottom cylinder from the base to retrieve the glass disc (Methods video S6).

Note: Pay close attention. The glass disc could break at this stage.

Step 9: HCASMCs lysis for calcium quantification

Timing: 2 h plus overnight incubation (18–24 h)

This section describes the steps for HCASMCs lysis to release the intracellular calcium deposits.

Note: Calcium deposition occurs when cells are exposed to a pro-calcifying environment. After the incubation period with the appropriate medium, typically not less than 7 days, HCASMCs can be processed for calcium quantification measurement. To release intracellular deposits, HCASMCs must be lysed first.

41. After LiveBox2 disassembly, move each coverslip into a 12 multiwell plate. Remember to label each sample.
42. Gently wash HCASMCs twice with PBS (previously equilibrated to room temperature (20°C–22°C)).
43. Lyse cells by adding 200 µL per well (for a 12 multiwell format) of HCl 0.6 M.
44. Incubate for 1 h at 4°C.
45. Following this period, dislodge cells vigorously by pipetting and scraping with a pipette tip.
46. Move the plate to a –20°C freezer overnight (18–24 h) to further promote the cell lysis.
47. The following day, scrape each well and check with a microscope whether cell lysis occurred. Collect cell lysates in 0.5 mL tubes and proceed with calcium quantification (problem 3).

Note: Special caution should be taken when handling HCl. Personal Protective Equipment (PPE) is required. This also includes sample handling when stored in HCl.

Pause point: Samples can be stored at −20°C for longer if not processed after overnight incubation (18–24 h).

Step 10: Intracellular calcium quantification

Timing: 30 min

This section describes the steps for HCASMCs intracellular calcium quantification.

Note: The calcium ion concentration is determined by detecting a chromogenic complex (calcium ions and o-cresolphthalein). The reaction is very specific, and the signal intensity is directly proportional to the quantity of calcium ions in the sample. This analysis is performed using Calcium Colorimetric Assay Kit.[2]

48. Prepare six standard dilutions as per manufacturer's suggestion. These need to be freshly prepared before each use and in a final volume of 50 µL. Follow the scheme reported below. Prepare enough volume to perform duplicates for each dilution.

Calcium µg/well	Standard solution (µL)	H_2O (µL)
Blank	\	50
0.4	2	48
0.8	4	46
1.2	6	44
1.6	8	42
2	10	40

49. Transfer 50 μL of each sample to a 96-multiwell transparent plate. Within the same plate, also transfer the diluted standard.
50. Using a multichannel pipette, add 90 μL per well of Chromogenic reagent and gently mix with the pipette. Avoid vigorous pipetting as it may cause the formation of foam.
51. Add 60 μL per well of Calcium assay buffer with a multichannel pipette and gently mix.
52. Incubate for 10 min at room temperature (20°C–22°C), protected from light.
53. Record absorbance at 575 nm.

EXPECTED OUTCOMES

The *in vitro* cell model described here represents a valuable approach to studying the pathophysiology of VC. The dynamic environment allows for better mimicking of the cellular and mechanical characteristics of VC *in vivo*.

This model is going to complement the organ-on-chip system. These two methods share the common purpose of replacing animal testing and avoiding ethical and financial issues. Thanks to the recreation of a microenvironment and the use of a microfluidic component, both systems mimic human settings. Additionally, it is possible to control cell-to-cell interactions spatiotemporally when co-culturing different cells in a microfluidic device. Despite many technical and analytical advantages, they both present disadvantages linked to the difficulties in controlling the fluid flow (laminar or turbulent flow). In our specific case, we preferred using the LiveBox2 (IVTech system) for the following reasons: easier interaction between user and cells, higher amount of sample can be processed, direct access to the cell-seeding site and protocol transferability from the standard static multiwell format to the LiveBox2.

Based on the experiments run in our laboratory, we noticed that calcifying treatment substantially decreased HCASMCs viability compared with reference media (Figure 8). Considering that 7 days of exposure to calcifying media were enough to allow intracellular calcium deposits to form (Figure 9), we considered one week as the best compromise between cell viability and calcification.

Dynamic conditions can modulate cell viability, compared with static (Figure 10). Assessing cell viability in both HCASMCs and HCAECs is noticeable a decrease when dynamic experimental conditions are used. This is a normal consequence of the mechanical stress that cells are exposed to and is also due to the natural turnover of the cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Normalization of intracellular calcium quantity can be performed through different methods, however, in our protocol we applied the cell number normalization.

This type of normalization requires the construction of a calibration curve, as described below. A defined number of HCASMCs serially diluted was seeded in a 24 multiwell plate. We recommend plating a number of HCASMCs per well ranging from 4,000 to 200,000 to cover the most likely degree of confluence. Cells were allowed to settle for 24 h, and cell viability was measured as previously described.

Creating a standard curve links the fluorescent value (cell viability) with the relative number of cells. The template generated could be applied to obtain the cell number from each cell viability experiment. Following calcium quantification, normalize the raw data with the corresponding number of cells to obtain the calcium quantity in each cell (Figure 9).

LIMITATIONS

Bacterial and fungal contaminations represent a threat in all *in vitro* protocols that require sterility. This is valid for the present protocol, especially considering the peculiarity of the assembly and

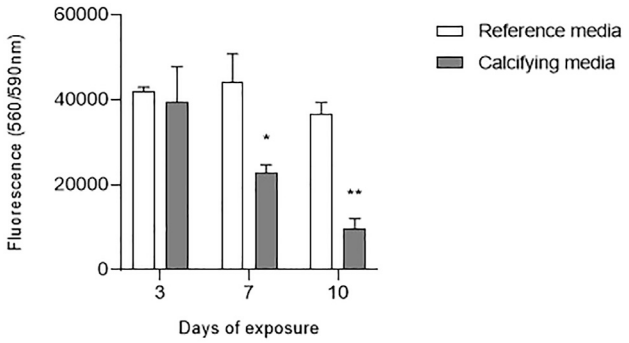

Figure 8. HCASMCs viability assay results after 3, 7 and 10 days of exposure to calcifying media

Statistical analysis with 2-way ANOVA and Sidak's post hoc test, the bars represent the mean value ± SD. *p ≤ 0.05; **p ≤ 0.01. Representative results of three independent experiments.

disassembly of the system. In this context, it is advisable to pay close attention to preserving the *in vitro* cultures described by our protocol. Since the procedure in its integrity requires many handling steps, it is also prone to contamination. Therefore, it is crucial to sterilize each LiveBox2 component and to work under laminar flow hoods whenever possible to minimize this problem. Furthermore, it is highly advisable to sterilize each component in contact with LiveBox2 (e.g., tweezers) and to use sterile gloves when assembling the system.

As for many other experiments involving cells, a limiting factor is the amount of serum in the HCASMC growth and calcifying medium. We previously conducted preliminary experiments aiming to optimize FBS concentration. Evaluating the balance between cell viability and the calcification process, we observed that a concentration of 10% in all media adopted here was optimal. Nevertheless, variations in FBS amount are likely to better perform in alternative cell types and/or experimental settings, therefore, it is recommended to test different concentrations when using LiveBox2 in calcification studies.

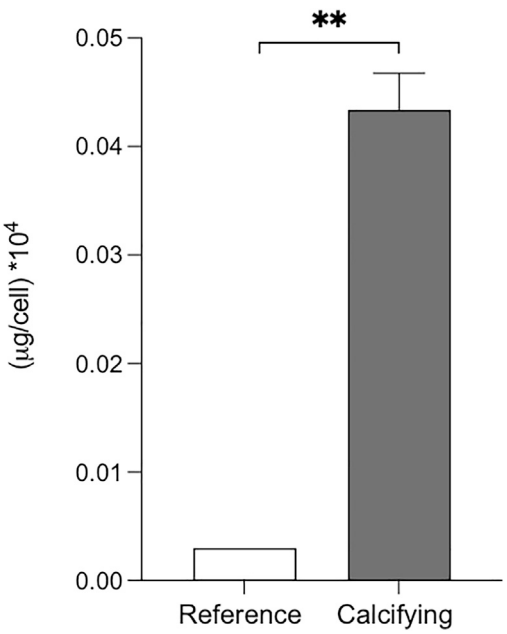

Figure 9. Quantity of intracellular calcium content normalized versus the number of viable cells

HCASMCs were treated with 1.9 mM phosphate and left for 7 days. Cells were lysed as described, and intracellular calcium content was analyzed with Calcium Colorimetric Assay Kit (Merck). Statistical analysis was performed using t-test, the bars represent the mean value ± SD. **p ≤ 0.01. Representative results of three independent experiments.

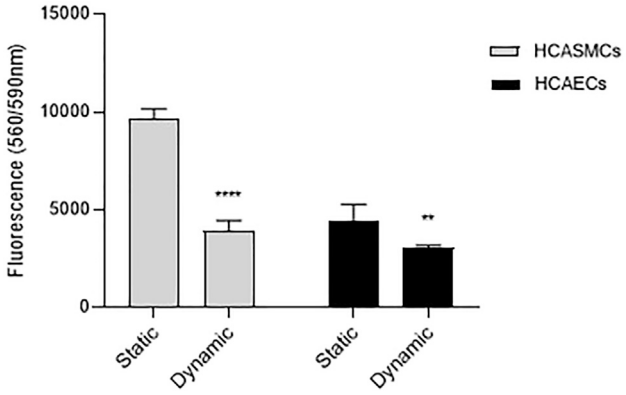

Figure 10. HCASMCs and HCAECs viability under static versus dynamic conditions

Statistical analysis with 2-way ANOVA and Sidak's post hoc test, the bars represent the mean value ± SD. **p ≤ 0.01; ****p ≤ 0.0001. Representative results of three independent experiments.

The culture media used here are commercially available and ready-to-use products. However, we cultured HCASMCs in Medium 231 completed as, in our experience, it allows rapid expansion of cells. Whenever intracellular calcification needed to be improved, we applied DMEM high glucose added with 10% FBS, together with phosphate mix. This was selected for mere congruence and direct comparison with previously reported in the literature. Notably, the induction of calcification dissolving phosphate in Medium 231 is a possibility that merit to be explored.

In our laboratory, we performed many experiments involving the cell types reported here, and various batches were used. We found minimal differences in cell survival, growth, and calcification. Since we purchased primary cells systematically from one provider, discrepancies between manufacturers are possible.

TROUBLESHOOTING

Problem 1

The cells do not attach to the membrane and/or to the glass coverslip (See experimental setup and preparation).

Potential solution

Try coating the membrane and/or the grass coverslip with the gelatin solution 1% w/v to promote cell adhesion. If the problem persists, replace it with brand-new chambers since damage might affect cell attachment.

Problem 2

During sample loading, you observe bubbles inside LiveBox2 chambers (See step 3: day 0 - cells seeding in the LiveBox2).

Potential solution

Load the sample slowly and turn the LiveBox2 with the outlet tube opened and oriented upwards to allow air bubbles to move out of the chamber.

Problem 3

Cells are still attached to the well or intact after lysis with HCl (See step 9: HCASMCs lysis for calcium quantification)

Potential solution

It may happen that after the incubation time with HCl, cells are still intact and visible under the microscope. In this case, scrape with a cell scraper, pipette vigorously, and leave cells at –20°C for 1–2 h. After this period, repeat scraping and pipetting and check again under a microscope.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vozzi Federico (vozzi@ifc.cnr.it).

Materials availability

This study did not generate new unique materials.

Data and code availability

The data presented in this work are available from the lead contact upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102328.

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AUTHOR CONTRIBUTIONS

E.P., E.C., and I.G. performed the experiments and analyzed the data; E.P. and E.C. wrote the manuscript; A.C. and F.V. designed the research.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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