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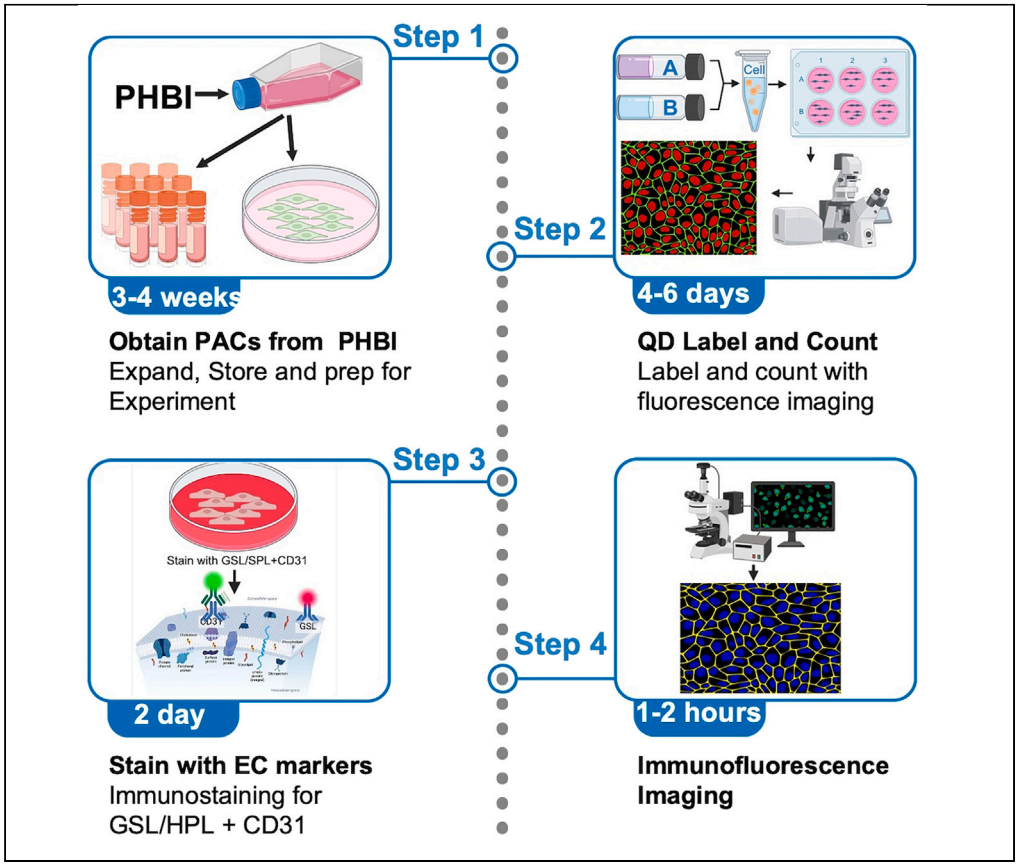
Protocol

# Protocol for quantum dot-based cell counting and immunostaining of pulmonary arterial cells from patients with pulmonary arterial hypertension




Currently, there is no protocol for growing and culturing primary pulmonary arterial cells (PACs) available from the Pulmonary Hypertension Breakthrough Initiative (PHBI). Here, we present a protocol for cultivating and maintaining three major PACs collected from patients with pulmonary arterial hypertension (PAH): endothelial (PAH-ECs), smooth muscle (PAH-SMCs), and adventitial cells (PAH-ADCs). We describe steps for obtaining PACs from PHBI, evaluating the growth of cells labeled with quantum dots (QDs), and staining endothelial cell (EC) markers for immunofluorescence imaging.

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

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## Highlights

Protocol for culturing PAH-affected pulmonary arterial cells

Guidance for the labeling of cells with quantum dots and their growth analysis

Instructions for coating of cell culture plates with gelatin

Steps for immunofluorescence imaging of pulmonary arterial endothelial cells

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# Protocol for quantum dot-based cell counting and immunostaining of pulmonary arterial cells from patients with pulmonary arterial hypertension

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## SUMMARY

**Currently, there is no protocol for growing and culturing primary pulmonary arterial cells (PACs) available from the Pulmonary Hypertension Breakthrough Initiative (PHBI). Here, we present a protocol for cultivating and maintaining three major PACs collected from patients with pulmonary arterial hypertension (PAH): endothelial (PAH-ECs), smooth muscle (PAH-SMCs), and adventitial cells (PAH-ADCs). We describe steps for obtaining PACs from PHBI, evaluating the growth of cells labeled with quantum dots (QDs), and staining endothelial cell (EC) markers for immunofluorescence imaging.**

**For complete details on the use and execution of this protocol, please refer to Al-Hilal et al.[1]**

## BEFORE YOU BEGIN

We used this protocol to label PAH-ECs, PAH-ADCs, and PAH-SMCs in traditional dishes with QD Tracker and to generate growth curves for the QD-labeled cells. Further, we characterized PAH-ECs via immunofluorescence imaging. This protocol extends previous work that generated growth assays for QD-labeled smooth muscle cells and utilized immunofluorescence for cell characterization in a microfluidic device.[1]

1. Plan your experiments ahead of receiving the cells from PHBI, as cells typically do not grow well after the 7th or 8th passage.
2. Apply to the PHBI at http://phbi.org/ to collect cells (PAH-ECs, PAH-ADCs, and PAH-SMCs) from pulmonary artery origins.
3. Coordinate the delivery of cells with PHBI only after ensuring you have all necessary culture media on hand and are prepared to immediately care for the cells upon arrival.
4. Prepare separate culture media supplemented with the respective cell culture kits for both endothelial cells and smooth muscle cells.
5. Obtain the necessary QD trackers, primary and secondary antibodies to characterize cells of pulmonary origin.
6. Prepare gelatin coated T-25 flasks, T-75 flasks, 6-well and 24-well plates as described below.

### Institutional permissions

Ensure necessary institutional ethics or protocol approval for cultivating, storing and maintaining primary pulmonary arterial cells (PACs) collected from human subjects. This includes obtaining permission from the Pulmonary Hypertension Breakthrough Initiative (PHBI) for the use of their cell specimen banks.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| CD31 monoclonal antibody (MEM-05), Alexa Fluor 488 (1:400) | Thermo Fisher Scientific | Cat# MA5-18135 |
| Chemicals, peptides, and recombinant proteins | | |
| EBM-2 (endothelial cell basal medium-2) | Lonza | Cat# CC-3156 |
| EGM 2 MV SingleQuots | Lonza | Cat# CC-4147 |
| SmGM-2 (smooth muscle basal medium-2) | Lonza | Cat# CC-3181 |
| SmGM 2 SingleQuots | Lonza | Cat# CC-4149 |
| PBS (1X), calcium, magnesium | Thermo Fisher Scientific | Cat# 14040133 |
| Trypsin-EDTA (0.05%), phenol red | Thermo Scientific | Cat# 25300062 |
| Trypsin-EDTA (0.25%), phenol red | Thermo Scientific | Cat# 25200072 |
| Gelatin from bovine skin | Sigma-Aldrich | Cat# G9391 |
| 16% Formaldehyde (w/v), methanol-free | Thermo Fisher Scientific | Cat# 28906 |
| Triton X-100 | Thermo Fisher Scientific | Cat# A16046.AE |
| Lectin from *Helix promatia* | Sigma-Aldrich | Cat# L6512-1MG |
| Lectin from *Bandeiraea Simplicifolia* | Sigma-Aldrich | Cat# L2140 -2MG |
| Streptavidin, Texas Red conjugate | Thermo Fisher Scientific | Cat# S872 |
| Hoechst 33342 solution (20 mM) | Thermo Fisher Scientific | Cat# 62249 |
| Normal goat serum | Fisher Scientific | Cat# 10-000-C |
| Trypan blue solution, 0.4% (w/v) in PBS | Corning | Cat# 25-900-CI |
| CellTracker Blue CMHC dye | Thermo Fisher Scientific | Cat# C2111 |
| Qtracker 525 Cell Labeling Kit | Thermo Fisher Scientific | Cat# Q25041MP |
| Qtracker 605 Cell Labeling Kit | Thermo Fisher Scientific | Cat# Q25001MP |
| Qtracker 705 Cell Labeling Kit | Thermo Fisher Scientific | Cat# Q25061MP |
| Cell Banker 2 (100 mL) cryopreservation medium | Amsbio | Cat# 11914 |
| Gibco Antibiotic-Antimycotic (100X) | Thermo Fisher Scientific | Cat# 15240062 |
| Gibco Penicillin-Streptomycin (10,000 U/mL) | Thermo Fisher Scientific | Cat# 15140122 |
| Experimental models: Cell lines | | |
| Idiopathic pulmonary arterial endothelial cells (PAH-ECs) | PHBI | N/A |
| Idiopathic pulmonary arterial smooth muscle cells (PAH-SMCs) | PHBI | N/A |
| Idiopathic pulmonary arterial adventitial cells (PAH-ADCs) | PHBI | N/A |
| Software and algorithms | | |
| GraphPad Prism (version 10.2.3) | Dotmatics, MA, USA | N/A |
| Leica Application Suite X (LAS X) LAS-X | Leica, USA | N/A |
| Other | | |
| Falcon tissue culture-treated flasks (T-25) | Fisher Scientific | Cat# 10-126-10 |
| Falcon tissue culture-treated flasks (T-75) | Fisher Scientific | Cat# 13-680-65 |
| 6-well clear TC-treated multiple well plates | Fisher Scientific | Cat# 07-200-83 |
| 24-well clear TC-treated multiple well plates | Fisher Scientific | Cat# 09-761-146 |
| Serological pipettes (5 mL) | Thermo Scientific | Cat# 12-567-602 |
| Serological pipettes (10 mL) | Thermo Scientific | Cat# 12-567-603 |
| Polypropylene conical centrifuge tubes (50 mL) | Fisher Scientific | Cat# 14-955-239 |
| Polypropylene conical centrifuge tubes (15 mL) | Fisher Scientific | Cat# 14-959-70C |
| Cell freezing vial containers | Corning | Cat# 07-210-003 |
| Freezing container | Thermo Scientific | Cat# 5100-0001 |
| 250 mL filter system, 0.22 μm PES filter, 75 mm diameter, sterile | CELLTREAT Scientific | Cat# 50-202-044 |
| P10 Barrier pipette tips | Santa Cruz Biotechnology | Cat# sc-201721 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Exel International TB SYR 1CC LL LOW-WST CAP C/PK | Fisher Scientific | Cat# 22-841-001 |
| Benchmark SureTherm180 $CO_2$ incubator | Benchmark | H3565-180 |
| Class II biological safety cabinet | Labconco | Cat# 302519100 |
| Pump aspirator | Welch | Cat# 2534B |
| Water bath (Precision 183) | Precision Scientific | Cat# 66551-27 |
| Disposable glass pipets, 9 inch | Fisher Scientific | Cat# 50-136-7739 |
| Biological microscope | Proway Optics & Electronics Co. | XSZ-PW107 |
| LEICA DMi8 manual microscope | Leica Microsystems | DMi8 |
| Benchtop centrifuge | Eppendorf AG | 5805F |
| Bright-Line hemacytometer | MilliporeSigma | Cat# Z359629 |

## MATERIALS AND EQUIPMENT

**0.2% Gelatin Solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| Gelatin | 0.2% | 0.2 gm |
| $ddH_2O$ | N/A | 100 mL |
| **Total** | **N/A** | **100 mL** |

Store at 2°C–4°C for up to 2 weeks.

**Blocking Buffer**

| Reagent | Final concentration | Amount |
|---|---|---|
| Normal goat serum | 5% | 2.5 mL |
| Triton X-100 | 0.05% | 25 μL |
| PBS | 1X | 47.5 mL |
| **Total** | **N/A** | **50 mL** |

Store at 2°C–4°C for up to 1 week.

**Cell Permeabilization Solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| Triton X-100 | 0.1% | 20 μL |
| PBS | 1X | 20 mL |
| **Total** | **N/A** | **20 mL** |

Store at 2°C–4°C for up to 2 months.

**Helix pomatia agglutinin (HPA)/ Griffonia simplicifolia lectin (GSL) Solution**

| Reagent | Final concentration | Amount |
|---|---|---|
| HPA/GSL | 1 mg/mL | 1 mg |
| PBS | 1X | 1 mL |
| **Total** | **N/A** | **1 mL** |

Store at 2°C–4°C for up to 1 week.

**Pulmonary Endothelial Cell (ECs) Medium**

| Reagent | Final concentration | Amount |
|---|---|---|
| EBM-2 | N/A | 470 mL |
| EGM 2 MV SingleQuots (contains bellow components) | | |
| FBS | 5.0% | 25.0 mL |
| Hydrocortisone | 0.04% | 0.2 mL |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| hFGF-B | 0.4% | 2.0 mL |
| VEGF | 0.1% | 0.5 mL |
| R3-IGF-1 | 0.1% | 0.5 mL |
| Ascorbic Acid | 0.1% | 0.5 mL |
| hEGF | 0.1% | 0.5 mL |
| GA-1000 | 0.1% | 0.5 mL |
| **Total** | **N/A** | **500 mL** |
| Store at 2°C–4°C up to 2 months. | | |

| **Pulmonary Smooth Muscle/Adventitial Cell (SMCs/ADCs) Medium** | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| SmBM | N/A | 472.5 mL |
| SmGM 2 SingleQuots (contains bellow components) | | |
| FBS | 5.0% | 25.0 mL |
| Insulin | 0.1% | 0.5 mL |
| hFGF-B | 0.2% | 1.0 mL |
| GA-1000 | 0.1% | 0.5 mL |
| hEGF | 0.1% | 0.5 mL |
| **Total** | **N/A** | **500 mL** |
| Store at 2°C–4°C up to 2 months. | | |

## STEP-BY-STEP METHOD DETAILS

### Obtaining pulmonary arterial cells from the pulmonary hypertension breakthrough initiative (PHBI)

Timing: 2–3 weeks

Researchers from academic and non-profit organizations in the USA can apply to the Pulmonary Hypertension Breakthrough Initiative (PHBI) for access to cells obtained from patients with PAH.

1. Create an account on the PHBI electronic Tissue Utilization Committee (eTUC) application system at http://phbi.org/index.do.
2. Pay the associated fee for the sample (~$400/each).
3. Receive the replicating cells from PHBI in a T-25 flask.
4. Replace the old media with 5 mL of fresh, warm (37°C) respective cell culture media.
5. Incubate the T-25 flask in an incubator at 37°C with 5% $CO_2$ until the cells reach 80%–90% confluency.

### Gelatin (0.2%) coating of cell culture plates

Timing: 7–8 h

Gelatin, derived from collagen found in bovine skin, is typically a colorless substance and serves as an excellent substrate for cell culture attachment.[2,3]

*Note:* We exclusively used gelatin-coated plates for culturing pulmonary endothelial cells, while regular tissue culture treated plates were employed for all other cell types.

6. Preparation of 0.2% Gelatin Solution.

a. Weigh out 0.2 g of gelatin powder.
b. Transfer the powder to a volumetric flask and add enough warm sterile water to reach a total volume of 100 mL.
c. Stir vigorously until the solution becomes clear.

7. Autoclave the 0.2% gelatin solution at 121°C and 15 psi for 35 min to ensure sterility.
8. Store the sterilized gelatin solution at 2°C–8°C for future use, if necessary.
9. Coating plates with gelatin solution.
   a. Add 500 μL of the 0.2% gelatin solution to each well of a 6-well and a 24-well plate, 1.5 mL to a T-25 flask, and 5 mL to each T-75 flask.
   b. Ensure the solution covers the whole working area of the flask/well.
   c. Close the lids securely and incubate the flasks for 2 h in 22°C–25°C.
   d. Aspirate the gelatin solution from the flasks.
   e. Incubate them to air dry in a cell culture hood for at least 4 h.
   f. Keep the flasks slightly ajar during drying to facilitate airflow.
   g. Ensure the flasks are completely dry and free from any residual liquid before use.

*Note:* Store the gelatin-coated flasks or plates at 2°C–8°C for up to 4 weeks, ensuring they are properly sealed to prevent contamination. Do not use the gelatin-coated flasks or plates if there is a color change or if cracks are observed on the surface.

### Expansion, storage, and maintenance of pulmonary arterial cells received from PHBI

Timing: 1–3 weeks

PACs from PHBI generally maintain their regular phenotypic characteristics up to passages 6/7. Therefore, plan accordingly to save enough vials in liquid nitrogen within passages 3–4.

10. Check the cells daily until they reach 80%–90% confluency.
11. Pre-warm the culture media, PBS, and trypsin-EDTA to 37°C before use.
12. Before aspirating the medium, observe and document cell morphology under a microscope to ensure the cells are healthy and free of contamination.
13. Aspirate the culture medium from the T-25 flask.
14. Rinse the T-25 flask gently with 5 mL of 1X PBS solution.
15. Add 1.5 mL of trypsin-EDTA (0.25%) solution to the T-25 flask.
16. Place the flask with the trypsin-EDTA solution into a cell culture incubator at 37°C for 3–5 min.

*Note:* Monitor the trypsinization process; cells should detach from the flask surface and float in the solution, taking on a round or spherical shape.

17. After incubation, quickly transfer the flask to the cell culture hood and add an equal or greater volume of culture medium to neutralize the trypsin.
18. Dislodge all rounded cells from the surface by vigorous tapping and pipetting.
19. Transfer the cell suspension from the flask to a 15 mL sterile Falcon tube.
20. Centrifuge the Falcon tube at 4°C, 413 *g* for 5 min.
21. Carefully aspirate the supernatant from the Falcon tube, without disturbing the cell pellet.
22. Add 2–3 mL of cell culture growth medium to the pellet and resuspend it.
23. Count the cells using a hemocytometer or cell counter.
24. Seed approximately $1 \times 10^6$ cells in a T-75 flask containing 12 mL cell culture media and incubate in a humidified 37°C incubator with 5% $CO_2$ and 95% air for further expansion.
25. Cryopreserve $5 \times 10^5$ cells per vial in 1.5 mL of Cell Banker 2 media for future use, ideally from early passages.
26. Monitor the cell growth progress of PAH-ECs (Figure 1), PAH-SMCs (Figure 2), and PAH-ADCs (Figure 3) until the next passage.

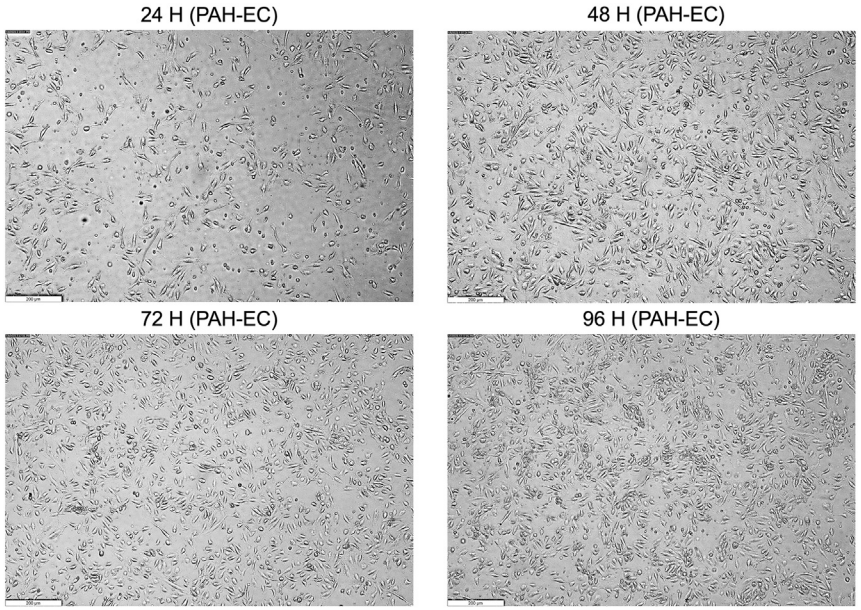


**Figure 1. The growth of endothelial cells from PAH-affected pulmonary arteries (PAH-ECs) in T-75 flasks was monitored over 96 h**

Bright-field microscopy at 2.5X magnification captured cellular expansion and distribution at 24-h intervals of PAH-ECs. Representative image showing PAH-EC with a 200 μm scale bar for reference.

## Label PACs with QD tracker and seed into gelatin-coated 6-well plates

Timing: 2 h

QD Tracker Nanocrystals[4,5] and QD Tracker Carrier will be mixed in a 1:1 volume/volume (v/v) ratio according to the manufacturer's instructions to create a labeling solution. Primary arterial cells (PACs), including endothelial cells (ECs), smooth muscle cells (SMCs), and adventitial cells (ADCs), will then be labeled with the freshly prepared QD tracker solution. The labeled cells will be incubated and subsequently counted using fluorescence microscopy.

*Note:* We used three different QD trackers to label PAH-ECs, PAH-SMCs, and PAH-ADCs separately. To identify or track each cell type in a mixed culture, you need to label them separately. If you intend to study each cell type individually, you can use the same QD tracker for all cells. Specifically, we used QD Tracker 525 for SMCs, QD Tracker 605 for ADCs, and QD Tracker 705 for ECs.

27. Prepare PACs for labeling with QD tracker.
    a. Transfer 6 × $10^5$ cells into a 2 mL Eppendorf tube.
    b. Centrifuge at 413 *g* for 5 min at 4°C.
28. Prepare fresh QD tracker (10 nM) solution for each cell line.
    a. Add 1 μL of Qtracker Nanocrystals to 1 μL of carrier in a separate 2 mL Eppendorf tube.
    b. Mix via vortexing or pipetting and incubate for 5 min at 22°C–25°C.
    c. Add 1 mL of fresh media to the tube.
29. Mix and Incubate QD tracker with the PACs.
    a. Remove the supernatant from the cells (Step 27) and add 1 mL of freshly prepared QD tracker solution (Step 28).
    b. Incubate the cells in a humidified incubator with 5% CO2 at 37°C and 95% air for 45–60 min.
30. Wash QD tracker from cell suspension.
    a. Centrifuge at 413 *g* for 5 min at 22°C–25°C and remove the supernatant.

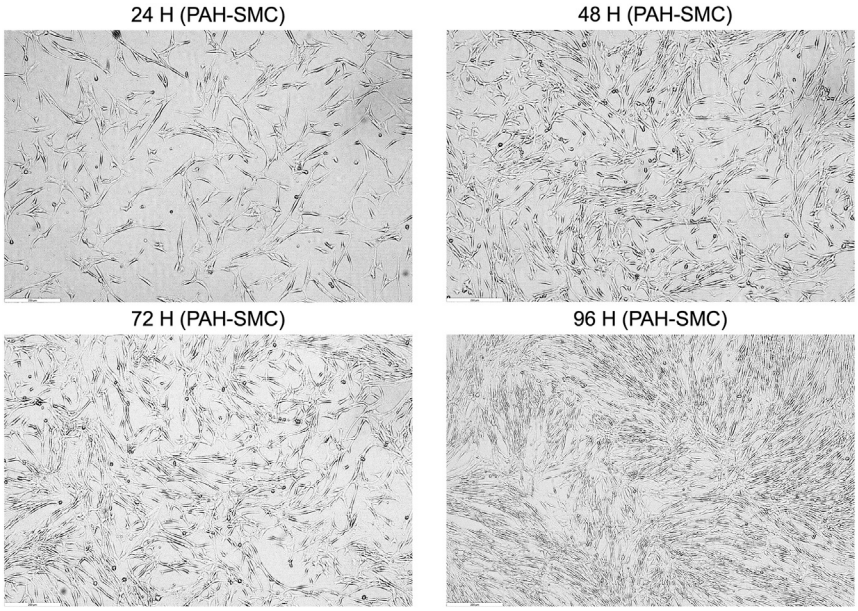


**Figure 2. Smooth muscle cells (PAH-SMCs) from PAH-afflicted human pulmonary arteries were cultured in T-75 flasks and observed over 96 h**

Each panel, captured via Bright-field microscopy at 2.5X magnification at 24-h intervals, shows the increasing density and morphological changes as the cells proliferate. Representative image showing PAH-SMC with a 200 μm scale bar for reference.

b. Resuspend the cells in 1 mL of 1X PBS, centrifuge again, and remove the supernatant to eliminate the QD tracker.
c. Resuspend the cells in 1 mL of cell culture media.

31. Prepare working cell concentration ($6 \times 10^4$ cells/mL) in a 15 mL Tube.
    a. Add 9 mL of cell culture media to a 15 mL Falcon tube.
    b. Transfer and mix the cells from Step 30 into the 15 mL tube.
32. Seed QD labeled cells in collagen coated 6-Well Plate.
    a. Add 1 mL of cell suspension to each well of the collagen-coated 6-well plate.
    b. Incubate in a humidified incubator with 5% CO2 at 37°C and 95% air.

### Quantification of PACs using QD tracker fluorescence microscopy

Timing: 2–6 h

The cells were imaged using a LEICA DMi8 fluorescence microscope at 24-, 48-, 72-, and 96-h post-incubation, and the total number of QD tracer signal containing cells were counted manually.

33. Incubate PACs (PAH-ECs, PAH-SMCs, and PAH-ADCs) seeded in a 6-well plate (Step 32) for 24 h, 48 h, 72 h, and 96 h.
34. Capture bright field and corresponding QD tracker fluorescence images of each well daily using a 10X objective on a LEICA DMi8 fluorescence microscope.
35. Manually count PAH-ECs (Figure 4), PAH-SMCs (Figure 5), and PAH-ADCs (Figure 6) in multiple imaged fields at each time interval.
36. Consider the total number of cells counted after 24 h as 100%.
37. Plot the percentage data using GraphPad Prism or any alternative software (Figure 7).

### Characterize pulmonary arterial cells using immunofluorescence imaging in a 24-well plate

Timing: 48 h

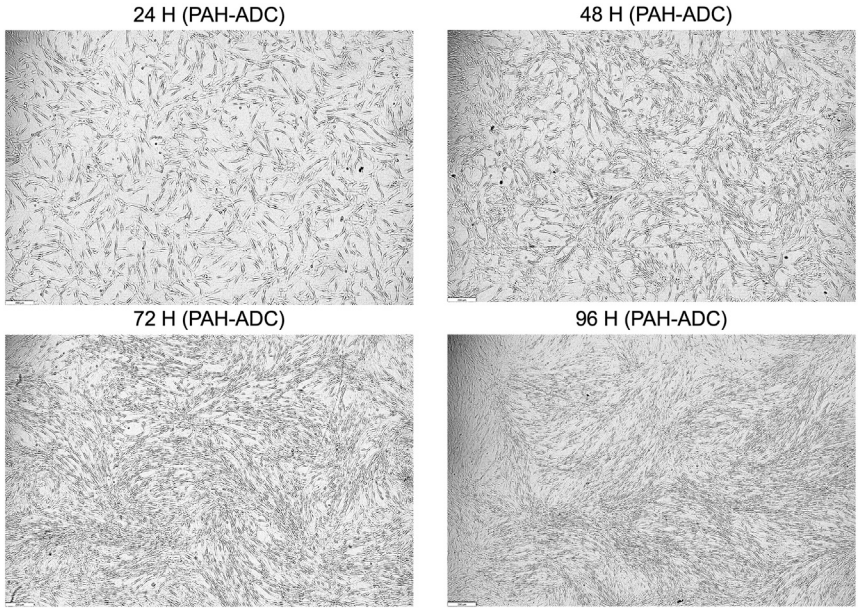


**Figure 3. Adventitial cells (PAH-ADCs) from PAH-afflicted human pulmonary arteries were cultured in T-75 flasks and observed over 96 h**

Images at 24, 48, 72, and 96 h show morphological changes and growth density. Representative image showing PAH-ADC with a 200 μm scale bar for reference.

PAH-ECs obtained from PHBI, originating from the pulmonary artery, were subjected to labeling with Helix pomatia lectin[6] (HPL)/Griffonia simplicifolia lectin[7] (GSL) followed by counterstaining CD31 a membrane protein of PAH-ECs to confirm their identity as endothelial cells (ECs).

38. Coat the 24 well plate using 0.2% gelatin as described in the steps 6–9.
39. Seed 2 × $10^4$ endothelial cells (ECs) onto each well containing 500 μL of a 24-well plate.
40. Incubate the plate in a humidified incubator with 5% CO2 at 37°C and 95% air for 24 h to allow cell attachment and growth.
41. After incubation, wash the cells twice with 500 μL of 1x PBS to remove non-adherent cells and debris.
42. Fix the cells by adding 500 μL of 4% formaldehyde solution to each well and incubate at 4°C for 15 min.
43. Remove the formaldehyde solution and permeabilize the cells by adding 500 μL of permeabilization solution.
44. Wash the cells three times with 500 μL of 1x PBS to remove residual fixative and permeabilization solution.
45. Block non-specific binding sites by adding 500 μL of blocking buffer to each well and incubate for 30 min at 22°C–25°C.
46. Wash the cells three times with 500 μL of 1X PBS to remove excess blocking buffer.
47. Prepare a solution of *Helix pomatia* agglutinin (HPA) or *Griffonia simplicifolia* lectin (GSL) according to manufacturer's instructions and add 500 μL to each well. Incubate for 15 min at 22°C–25°C. Wash the cells twice with 500 μL of 1X PBS to remove unbound lectin.
48. Stain the cells by adding streptavidin Texas Red at a 1:500 dilution and Alexa Fluor 488-conjugated anti-CD31 antibody at a 1:400 dilution in 500 μL of staining buffer. Incubate for 1 h at 22°C–25°C, protected from light.
49. Wash the cells twice with 500 μL of 1X PBS.
50. Stain the nuclei by adding Hoechst 33342 solution at a 1:2000 dilution in 500 μL of staining buffer and incubate for 10 min at 22°C–25°C, protected from light.

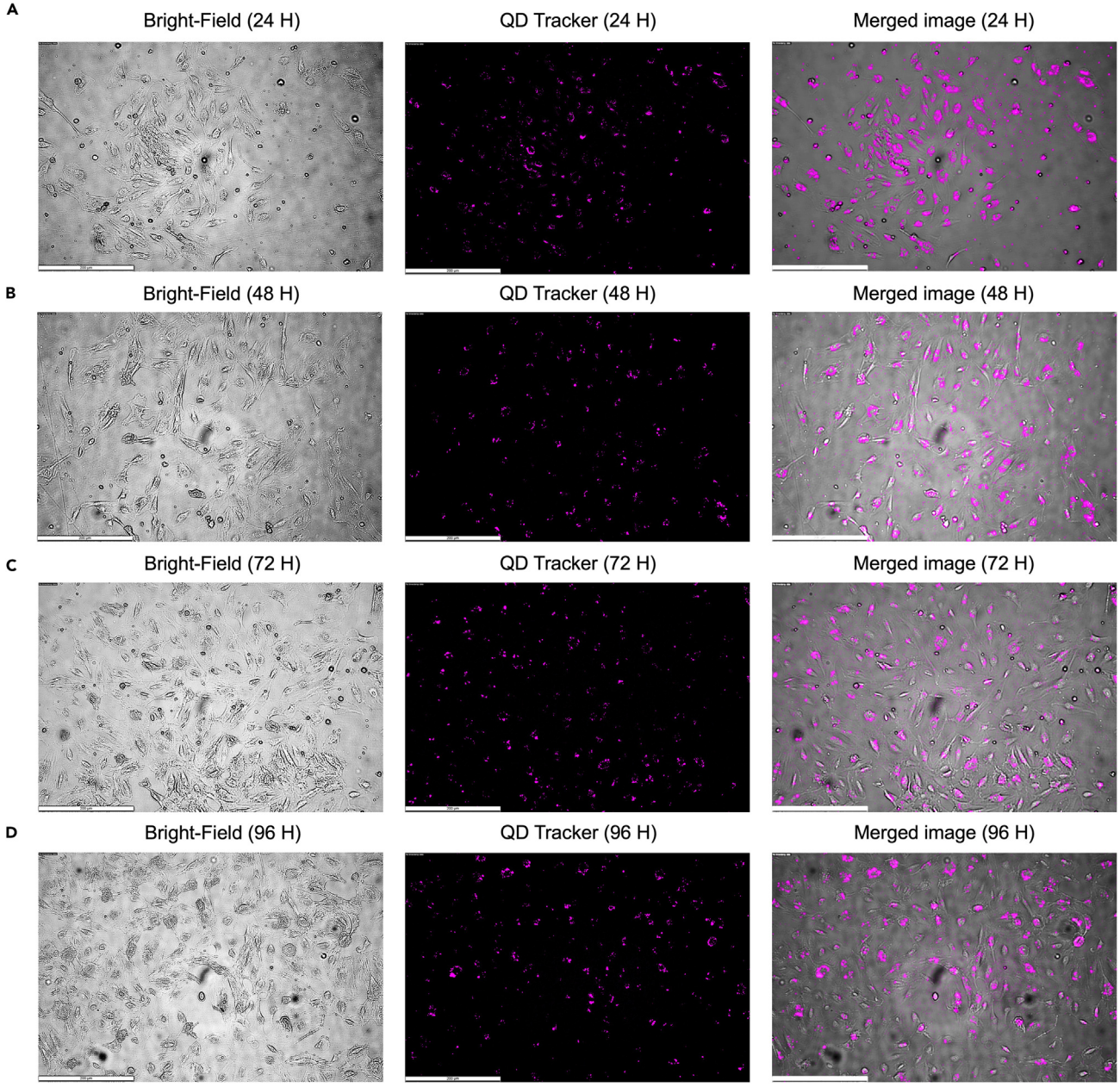


**Figure 4. Pulmonary arterial hypertension endothelial cells (PAH-ECs) were labeled with magenta quantum dot (QD 705) trackers and monitored over a 24 to 96-h period**

(A–D) Imaging at 10X magnification was performed using a Leica fluorescence microscope, capturing bright-field, fluorescence, and merged images. Representative image showing PAH-EC with a 200 μm scale bar for reference.

51. Wash the cells twice with 500 μL of 1x PBS.
52. Finally, image the stained cells using a DMi8 fluorescence microscope at 20X magnification (Figure 8).

## EXPECTED OUTCOMES

This protocol outlines methodologies for obtaining, culturing and preparing cells for counting and immunostaining pulmonary arterial cells from PAH human subjects. PAH-SMCs, PAH-ADCs, and PAH-ECs reach confluency in four days in early passages (Figures 1, 2, and 3). Growth patterns

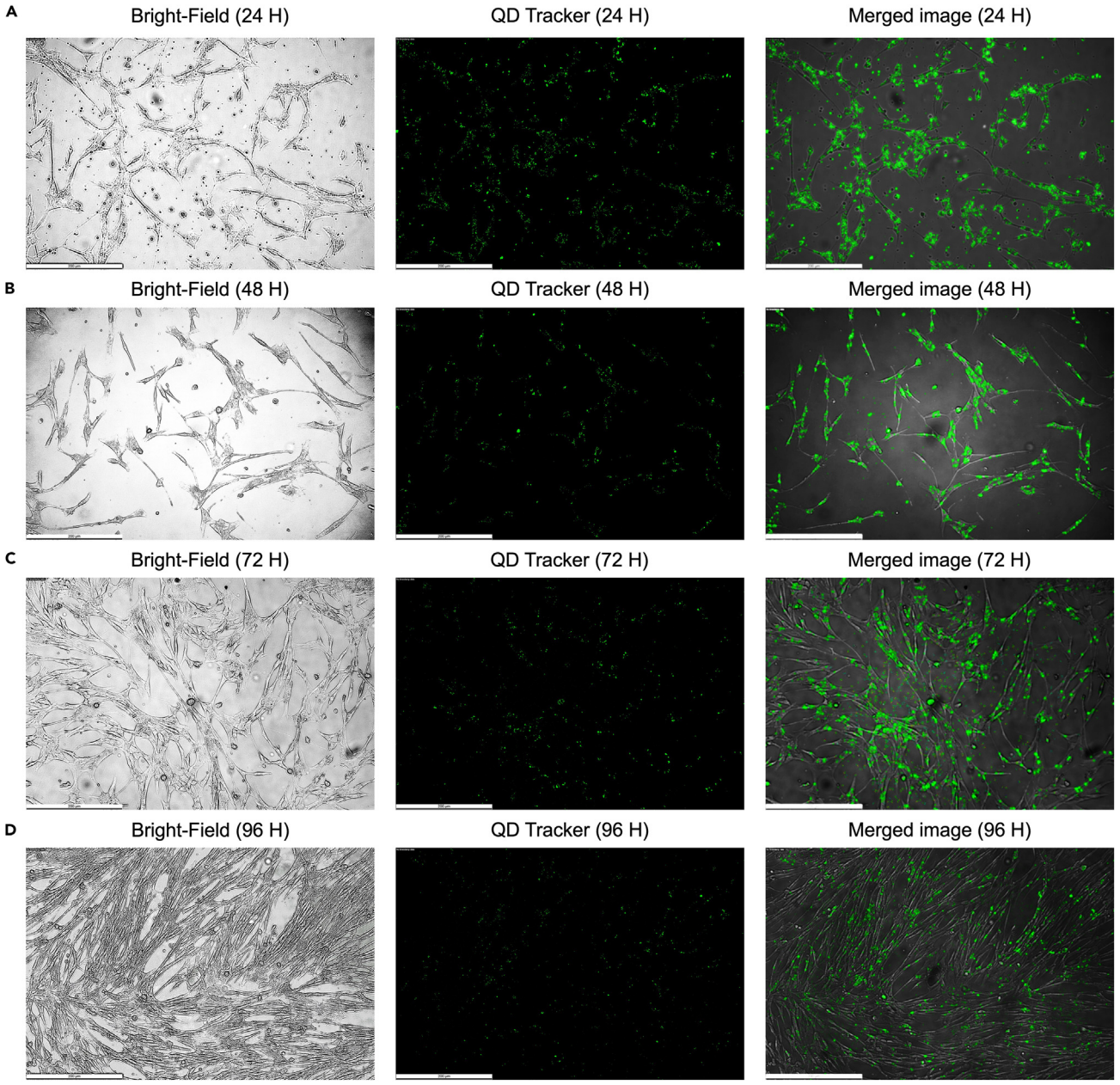


**Figure 5. Pulmonary arterial hypertension smooth muscle cells (PAH-SMCs) labeled with green, fluorescent quantum dot (QD 525) trackers, captured at 24, 48, 72, and 96 h**

(A–D) The 10x magnification images include bright-field, fluorescence, and merged views. Representative image showing PAH-SMC with a 200 μm scale bar for reference.

can be monitored using single and multiple quantum dot (QD) trackers (Figures 4, 5, 6, and 7). Immunostaining confirms the pulmonary arterial origin of ECs (Figure 8). This protocol assists PAH researchers by providing a detailed guide for cell culture, facilitating studies in cell morphology, drug screening, and therapy evaluation. For detailed outcomes, please refer to our study.[1]

## QUANTIFICATION AND STATISTICAL ANALYSIS

We performed t-tests/one-way/two-way ANOVA using GraphPad Prism (version 10.1.2, GraphPad Software LLC, Boston, MA). The sample size (n) indicated in the results represents the number of replicates.

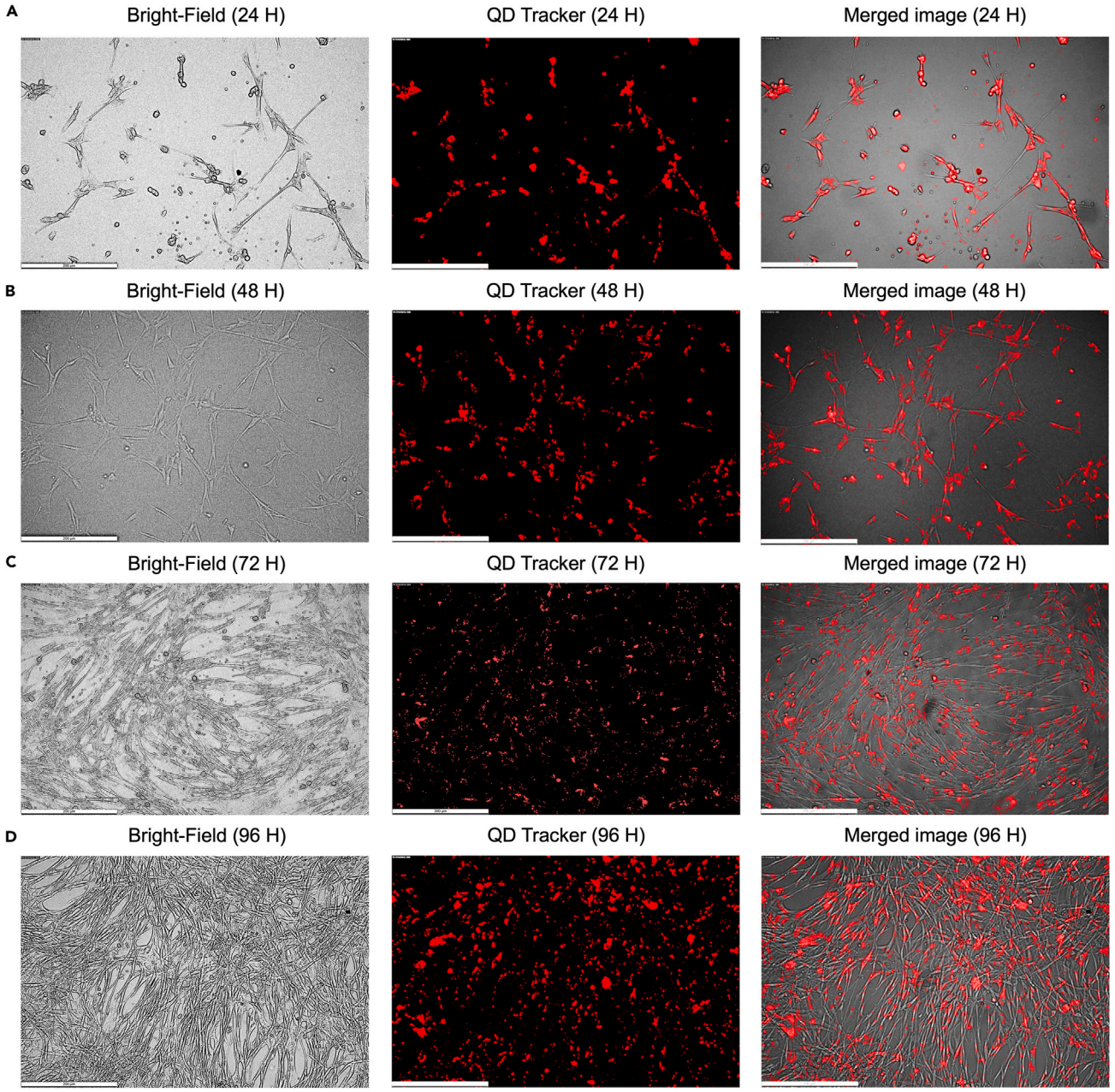


**Figure 6. Pulmonary arterial hypertension adventitial cells (PAH-ADCs) labeled with red-emitting quantum dot (QD 605) trackers, observed at 24, 48, 72, and 96 h**

(A–D) Images at 10X magnification include bright-field, fluorescence, and merged views. Representative image showing PAH-ADC with a 200 μm scale bar for reference.

## LIMITATIONS

A key limitation of this protocol is that the Pulmonary Hypertension Breakthrough Initiative (PHBI) is the sole source for collecting pulmonary arterial hypertension (PAH) cells. This reliance on a single source may affect the generalizability of the results, as variability in cell quality and characteristics can occur due to differences in donor backgrounds, handling, and processing techniques. Additionally, availability of PAH cells from PHBI may be limited, potentially impacting the reproducibility and scalability of the study.

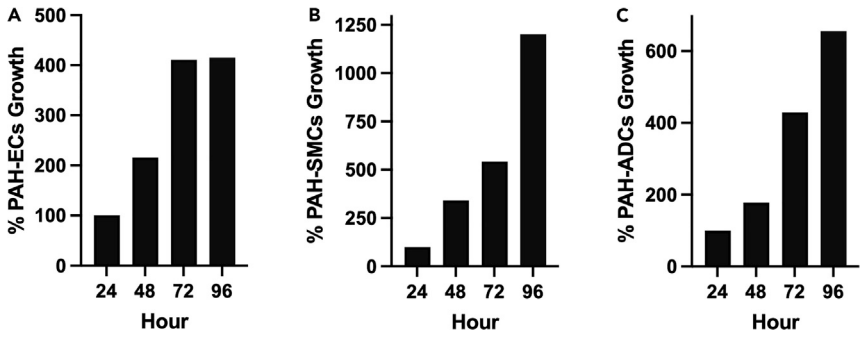


**Figure 7. Growth curves of pulmonary arterial hypertension (PAH) cell types with quantum dot (QD) trackers**
(A) IPAH endothelial cells (PAH-ECs) with QD 705, (B) IPAH smooth muscle cells (PAH-SMCs) with QD 525, and (C) IPAH adventitial cells (PAH-ADCs) with QD 605. The graphs depict the percentage growth over time for each cell type.

## TROUBLESHOOTING

### Problem 1

Fungal/mold contamination during shipping (Step 3) or in the lab (Figure 9).

### Potential solution

Thoroughly decontaminate the flask with 70% ethanol upon receiving it from PHBI. Check the cells daily for any signs of white mold colonies floating on the media. If colonies are observed, remove the media immediately and treat the cells with Gibco antifungal/antibiotic (Anti/Anti) agents for at least 2 to 3 passages.

### Problem 2

Bacterial contamination in cells.

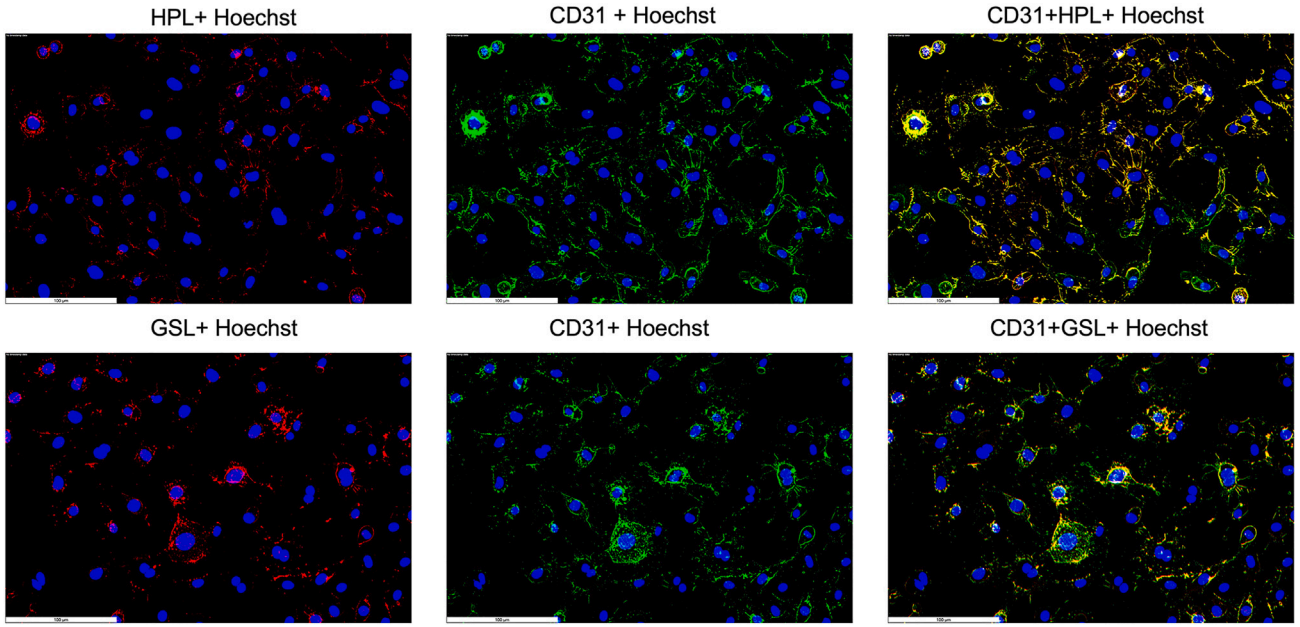


**Figure 8. Endothelial cells (ECs) stained with *Helix pomatia* lectin (HPL, red) and *Griffonia simplicifolia* lectin (GSL, red), alongside anti-CD31 antibody (green) to identify pulmonary arterial origin**
Top row: HPL, anti-CD31, and their colocalization. Bottom row: GSL, anti-CD31, and their colocalization. Nuclei are stained blue with Hoechst dye. Representative image showing ECs with a 100 μm scale bar for reference.


**Figure 9. T25 flask containing PAH-ADCs received from PHBI exhibited media leakage during shipping**
Fungal colony growth was observed on the surface of the media after 3 days.

### Potential solution
If bacterial contamination occurs, the media will become turbid. To eliminate the bacteria, treat the cells with Gibco Penicillin-Streptomycin (Pen/Strep) for at least 2 to 3 passages.

### Problem 3
Slower growth after passage 6/7.

### Potential solution
Avoid planning new experiments with cells beyond passage 6/7, as cellular senescence and reduced proliferation rates may affect experimental outcomes.

### Problem 4
Weak fluorescence signaling of QD tracker.

### Potential solution
Increase the concentration of the QD tracker to 15 nM and extend the cell incubation time with the QD tracker up to 90 min to enhance fluorescence signaling.

### Problem 5
Poor cell attachment in gelatin-coated flasks/6-well/24-well plates.

### Potential solution
Increase the gelatin concentration up to 0.5% during the coating process to improve cell attachment.

## RESOURCE AVAILABILITY

### Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fakhrul Ahsan (fakhrul.ahsan@cnsu.edu).

### Technical contact
Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Sakib Moinuddin (sakib.moinuddin@cnsu.edu).

### Materials availability
This study did not generate new unique reagents.

### Data and code availability
Added data are available from the corresponding author on request. There is no code generated during this study.

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## AUTHOR CONTRIBUTIONS

Conceptualization, S.M.M., M.I., and F.A.; methodology, S.M.M., T.S., and F.A.; investigation, S.M.M.; writing – original draft, M.I. and S.M.M.; writing – review and editing, F.A. and M.S.H.; validation, M.I.; visualization, M.I.; funding acquisition, F.A.; resources, T.S. and M.R.; supervision, F.A.; project administration, F.A.

## DECLARATION OF INTERESTS

F.A. discloses partial ownership of Medluidics LLC, located in Elk Grove, California. M.I. discloses partial ownership of Oncovask Therapeutics LLC in Sacramento, California.

## DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT (GPT-4) to proofread and increase readability and clarity. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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