# Porcine Collagen Micro Granule Matrix, Petakas, and Osteocyte Culture for Mitigating Spaceflight Osteopenia

**PI:** Michael E. Kjelland, M.S., Ph.D.

**Undergraduate Student researchers:** Hayle Boechler, Taylor Painter, Erin Walcker, Julia Kohls, Madisen Knudsvig

Mayville State University, Mayville, North Dakota, USA
Email: michael.kjelland@mayvillestate.edu

# Introduction

As NASA progresses in space exploration, astronauts are continuing to be exposed to microgravity environments for long periods of time. This is resulting in increased bone absorption leading to decreased bone density and loss of bone mass (Ohshima, Hiroshi. 2012).

Our research falls under TA 6, with specific focus on 6.3, 6.3.2, and 6.3.2.2.

Novel Hypothesis & Human Health, Clinical relevance: Novel tissue-specific substrate for bone tissue regeneration can overcome done lose due to microgravity environments. This was effectively demonstrated in hypoxia using novel gas exchange. The advanced porcine bone tissue serves as a transplantable substrate for bone cells and tissues when bone grafts are used. The ACRO porcine bone granules and power can be pipetted into the Petaka port and form the substrate for bone cell development.

# Objectives:

1) Grow human mesenchymal stem cells and differentiate them into osteocytes using both traditional and non-traditional cell culture flasks and ACRO bone plug 3D scaffolding in 5% $CO_2$ conditions.
2) Reproduce traditional culture conditions in Petaka cell culture chambers and compare ability to grow and differentiate MSCs.
3) Pipette ACRO bone powder and granules into Petaka cell culture chambers and test possibilities for use in microgravity conditions.

# Goals:

1) Provide a basis for “an automated cell culture and animal model system that can be used with nanosatellite platforms to carry out space environment effects on cells, microbes, plants and microcellular organisms.”
2) Merge advanced cell culture matrix technology (porcine collagen powder and micro granules) with an innovative bioreactor (Petaka).
3) Elucidate Petaka cell culture readiness and practicality for space flight and determine if there are any modifications needed.

# Materials & Methods

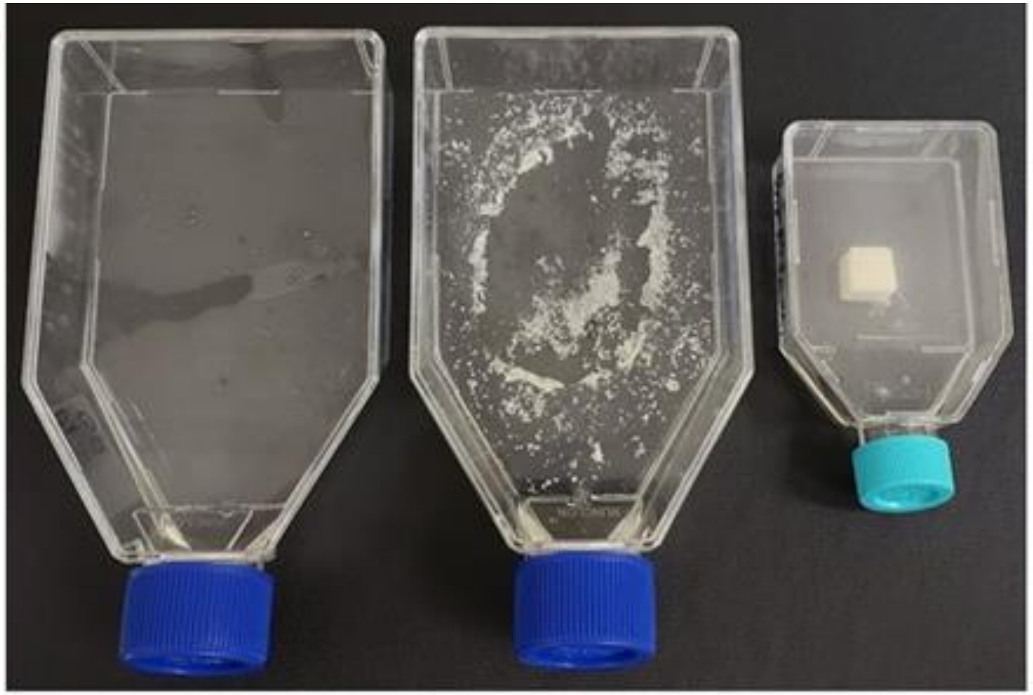
During this research the PI and undergraduate students performed the following:

- human mesenchymal stem cell culture and osteocyte differentiation (i.e., RoosterBio protocol),
- fluorescence and brightfield microscope techniques, as well as the imaging software for capturing microphotographs for presenations and publication,
- determining cell concentrations using a Neubauer Hemocytometer,
- cell centrifugation,
- live/dead staining of cells,
- processing cells for cell sorting using a flow cytometer,
- cell reactive oxygen species determination using the DCFDA - Cellular ROS Assay Kit / Reactive Oxygen Species Assay Kit (Abcam),
- use of both Petaka and traditional cell culture flasks (i.e., T-25/T-75/T-200) in cell culture,
- use of ABCcolla® porcine collagen matrix, and
- other cell culture and aseptic techniques involved with the culturing of cells *in vitro*.

Statistical Analyses: Two-way ANOVA and Dunnett's multiple comparisons test ($\alpha = 0.05$), GraphPad Prism v. 9.1.0.221 for Windows, GraphPad Software, San Diego, California USA.

a)


b)



c)

Petaka LOT Average DO = 1 mg/L (0.5 to 1.1 mg/L depending on cell type), equivalent to average 22.7 mmHg (Torr) in tissues or equivalent to average 3.11 %O2 in hypoxia chambers.

a) ABCcolla® porcine collagen matrix (ACRO Biomedical Co., Ltd.) in granule and powder form, b) T-75 flask without porcine collagen matrix (left, control), versus porcine collagen matrix powder (center, treatment), and porcine collagen matrix cube (i.e., bone plug) tested in T-25 and T-75 flasks only (right, treatment), and c) Petaka with porcine collagen matrix powder.

**Materials:**

**RoosterVial-hBM-10M** Part: MSC-001 Lot: **00175** (RoosterBio, Inc., USA), RoosterVial-hBM-10M Part: MSC-003 Lot: **310267** (RoosterBio, Inc., USA), RoosterVial-hBM-10M Part: MSC-003 Lot: **310263** (RoosterBio, Inc., USA)
**Petaka G3™ LOT** - The Cell Culture Shuttle (Celartia Ltd, USA)
T25 Flask, **T75 Flask**, T225 Flask,
Pipette tips: 20-200 µL, 1 mL, & 10 mL,
Sterile 20 mL disposable syringe (Lot: 2017001 American Health Service, USA) or 60 mL Syringe with needles
15 & 50 mL Test Tubes,
70% isopropyl alcohol,
Neubauer Hemocytometer,
**Gibco Cell Therapy Systems - TrypLE™ Select CTS™ (trypsin)**, Lot: 2204774, 2279511, 2175157 (Life Technologies Corporation, USA),
Gibco StemPro® – 1g/L D-Glucose, 110 mg/L Sodium Pyruvate, GlutaMAX™-I, Lot 2185924 (Life Technologies Corporation, USA),
Gibco StemPro - Osteocyte/Chondrocyte Differentiation Basal Medium,
**Gibco STEMPRO® Osteogenesis Supplement**, Lot: 2121137 (Life Technologies Corporation, USA),
Gibco Cell Therapy Systems - CTS™ DPBS (1X) (without Ca, Mg) - Dulbecco's Phosphate Buffered Saline, Lot: 2152927 (Life Technologies Corporation, USA),
**RoosterBio® - RoosterBasal-MSC-CC** - Lot: 19002, 20005 (RoosterBio, Inc., USA), RoosterBio® - RoosterBooster™-MSC - Lot: 00242, 310274 (RoosterBio, Inc., USA),
ABCcolla® Bone Graft, TFDA: Class II Medical Device; USFDA: 510(k) Clearance (ACRO Biomedical Co., Ltd., Taiwan), and
**ABCcolla® Collagen Matrix, Tissue Engineering Collagen Scaffold**, Cancellous Bone Scaffold powder (ACRO Biomedical Co., Ltd., Taiwan).

## Materials (cont.) - Cell Staining:

**Propidium Iodide**, Solution in Water, 1 mg/mL Lot: 19PO628, Mwt: 668.4 (Biotium, Inc., USA)

**NucBlue® Live Cell Stain** ReadyProbes™ reagent (Hoechst 33342 Special Formulation, Lot: 1583115) (Molecular probes® by Life Technologies Corporation, USA)

**Vybrant™ DyeCycle™ Violet Stain**, 5 mM solution in deionized water, Lot# 1499378 (Molecular Probes®, Invitrogen™, V35003, Life Technologies Corporation, USA)

**Vectashield Mounting Medium with Propidium Iodide**, H-1300, Lot: T0226, (Vector Laboratories, Inc., USA)

**Ghost Dye Red 780**, 1 uL/test in DMSO (Tonbo Biosciences, USA)

**SYBR® Green I**, nucleic acid gel stain, 10,000X concentrate in DMSO, Lot No. 24117W (Molecular Probes, Invitrogen™, Thermo Fisher Scientific, USA)

**Alizarin Red S**, Lot No. SR01904, Mwt: 342.25 (MP Biomedicals, LLC, USA)

**DCFDA/H2DCFDA** - Cellular ROS Assay Kit / Reactive Oxygen Species Assay Kit (ab113851, Lot: GR3313162-5 Abcam)

# Mesenchymal Stem Cell Experiment

| | Donor 1 (Male, 25 years) | Donor 2 (Male, 19 years) | Donor 3 (Female, 20 years) |
|---|---|---|---|
| **With 3D Collagen Matrix** | Petaka (n=3)<br>T-75 Flask (n=3) | Petaka (n=3)<br>T-75 Flask (n=3) | Petaka (n=3)<br>T-75 Flask (n=3) |
| **Without 3D Collagen Matrix** | Petaka (n=3)<br>T-75 Flask (n=3) | Petaka (n=3)<br>T-75 Flask (n=3) | Petaka (n=3)<br>T-75 Flask (n=3) |

Metrics:
1) Time to Confluency
2) Cell Concentration
3) % Live/Dead
4) Cellular Reactive Oxygen Species (DCFDA/H2DCFDA)
5) Alizarin Red S (Calcium Content)

# Mesenchymal Stem Cell Experiment – 5% $CO_2$ versus Ambient $CO_2$

| | **Donor 1 (Male, 25 years)** | [redacted] | **Donor 3 (Female, 20 years)** |
|---|---|---|---|
| **With 3D Collagen Matrix 5% $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |
| **With 3D Collagen Matrix Ambient $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |
| **Without 3D Collagen Matrix 5% $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |
| **Without 3D Collagen Matrix Ambient $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |

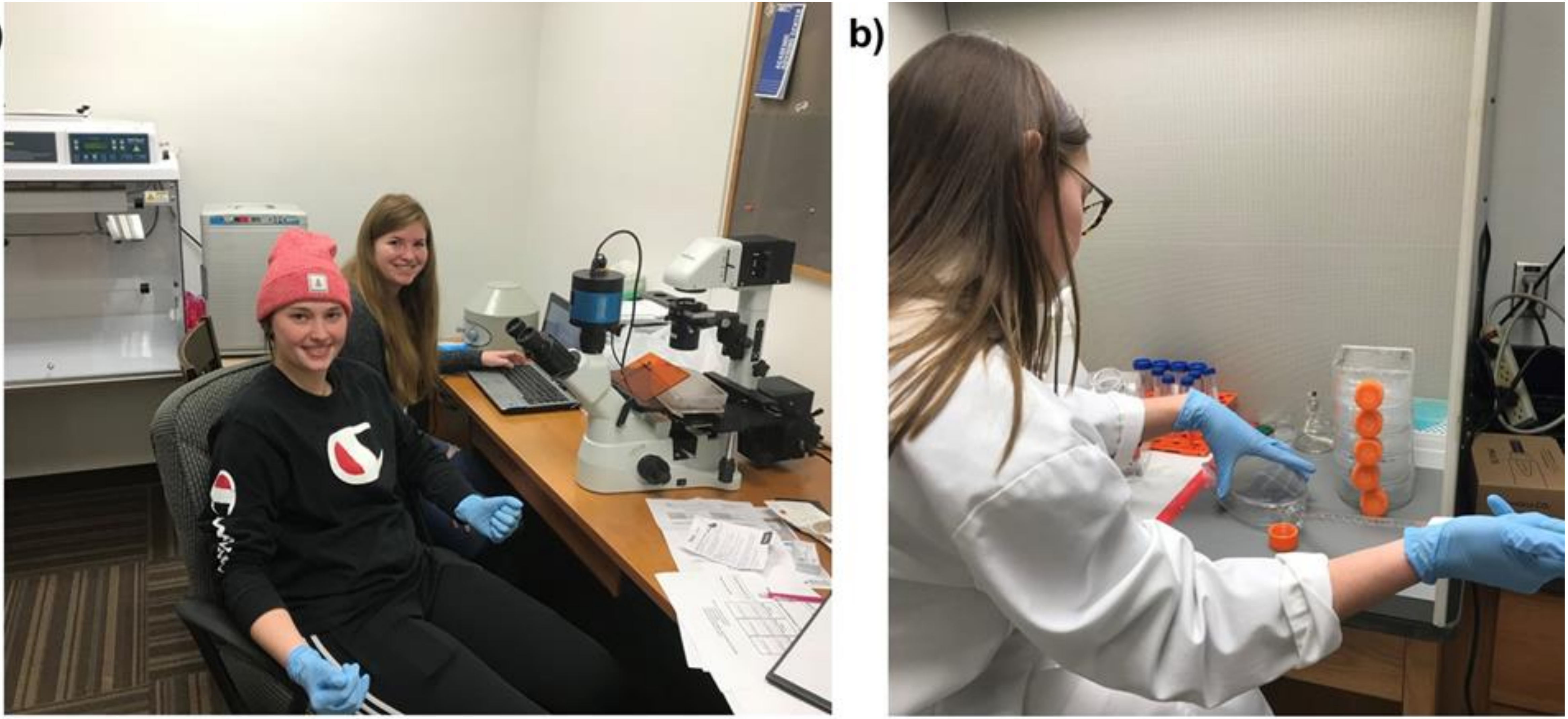
Metrics:
1) Time to Confluency
2) Cell Concentration
3) % Live/Dead
4) Cellular Reactive Oxygen Species (DCFDA/H2DCFDA)
5) Alizarin Red S (Calcium Content)

# Mesenchymal Stem Cell Experiment – Normal Duration versus Extended Duration

| | Donor 1 (Male, 25 years) | [redacted] | Donor 3 (Female, 20 years) |
|---|---|---|---|
| [redacted] | [redacted] | [redacted] | [redacted] |
| **With 3D Collagen Matrix Ambient $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |
| [redacted] | [redacted] | [redacted] | [redacted] |
| **Without 3D Collagen Matrix Ambient $CO_2$** | Petaka (n=3)<br>T-75 Flask (n=3) | [redacted] | Petaka (n=3)<br>T-75 Flask (n=3) |

a)

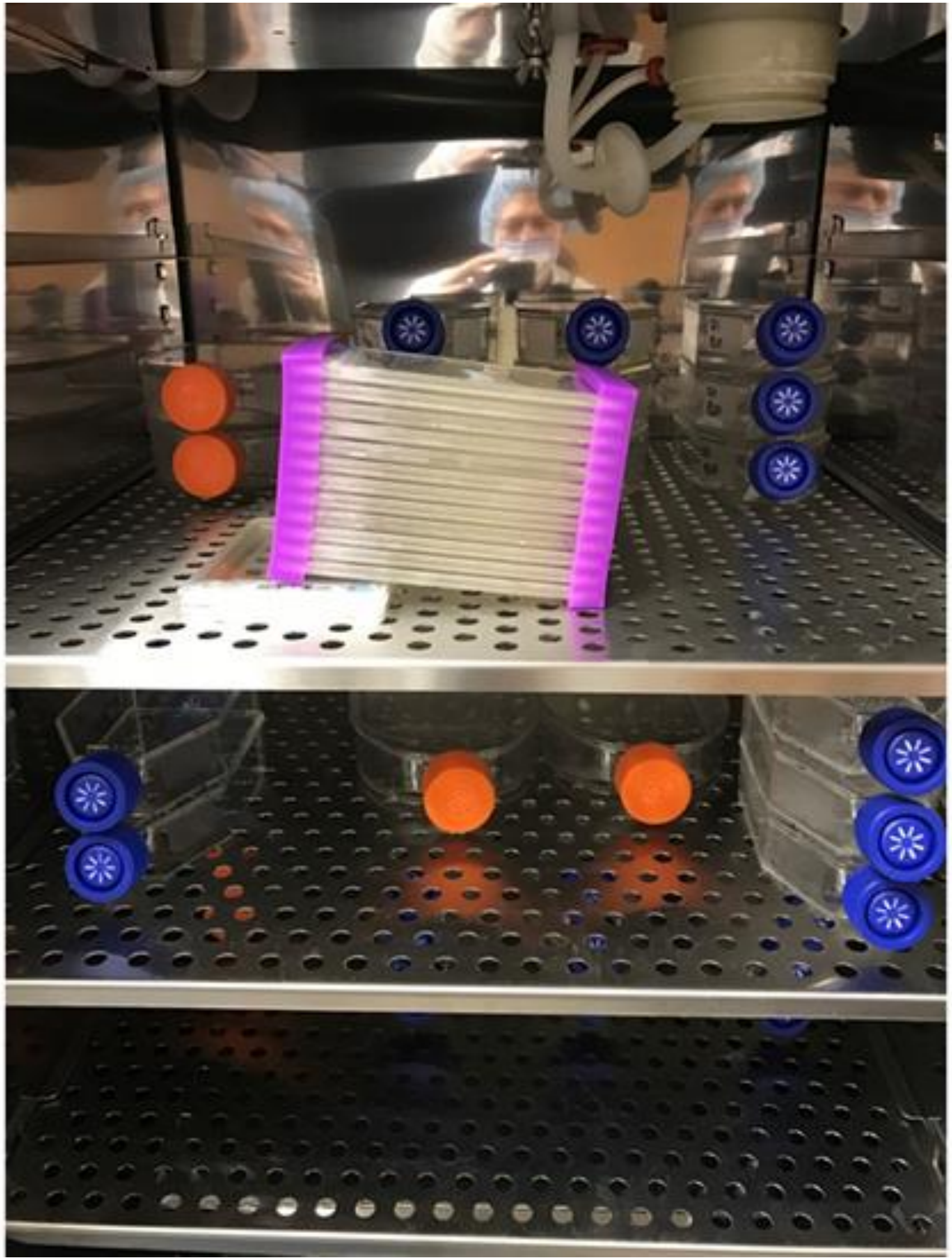
b)

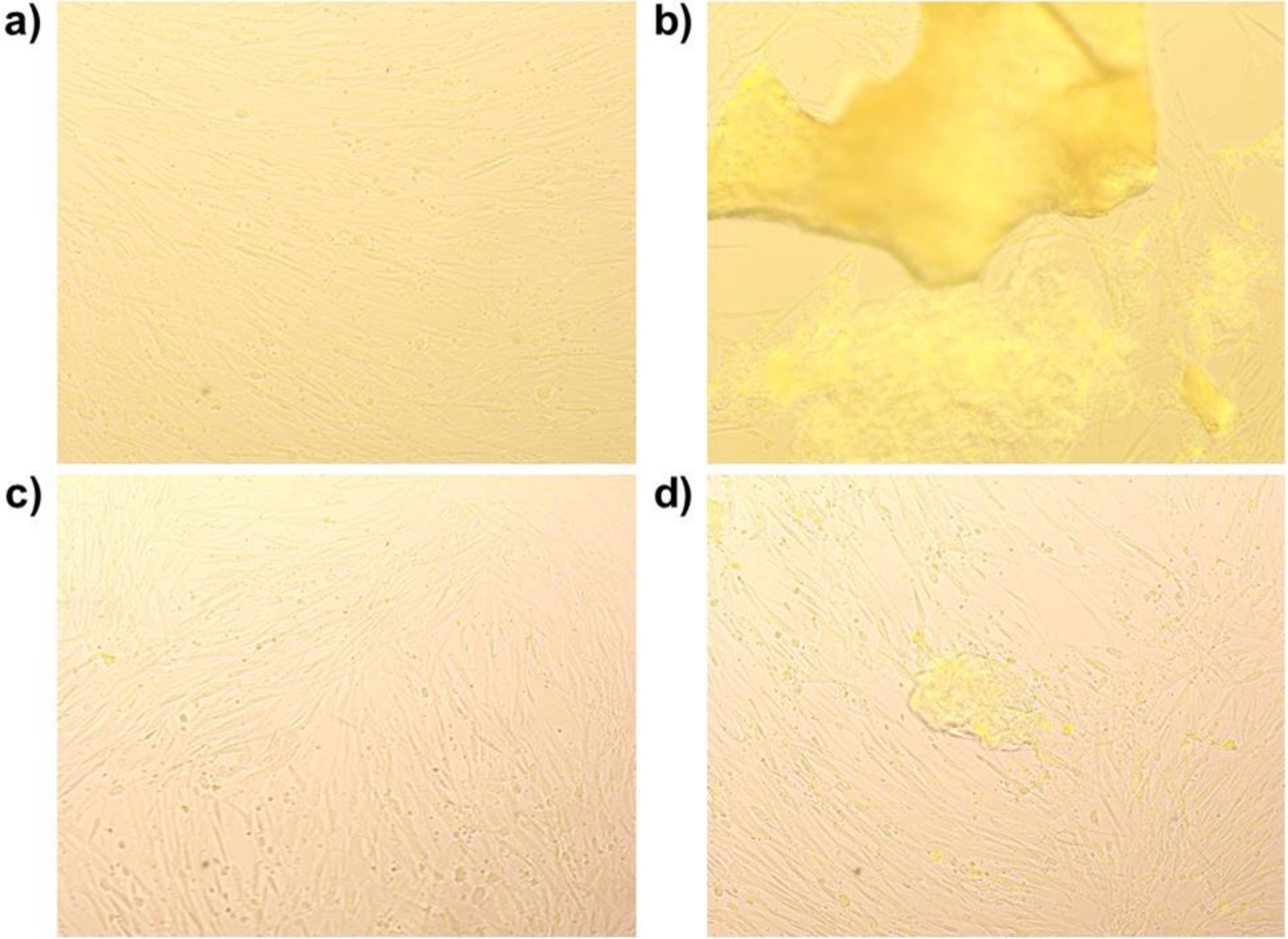


a) Undergraduate student researchers in the Integrated Tissue Culture and Bio-imaging Laboratory at Mayville State University, and b) Hayle Boechler changing spent cell culture medium with fresh cell culture medium for the human mesenchymal stem cells (note: typically, a mask would be worn during this procedure but the masks that were ordered never arrived due to the mask shortage caused by the pandemic, which was an early indicator for us just how bad things were getting at that point in time).

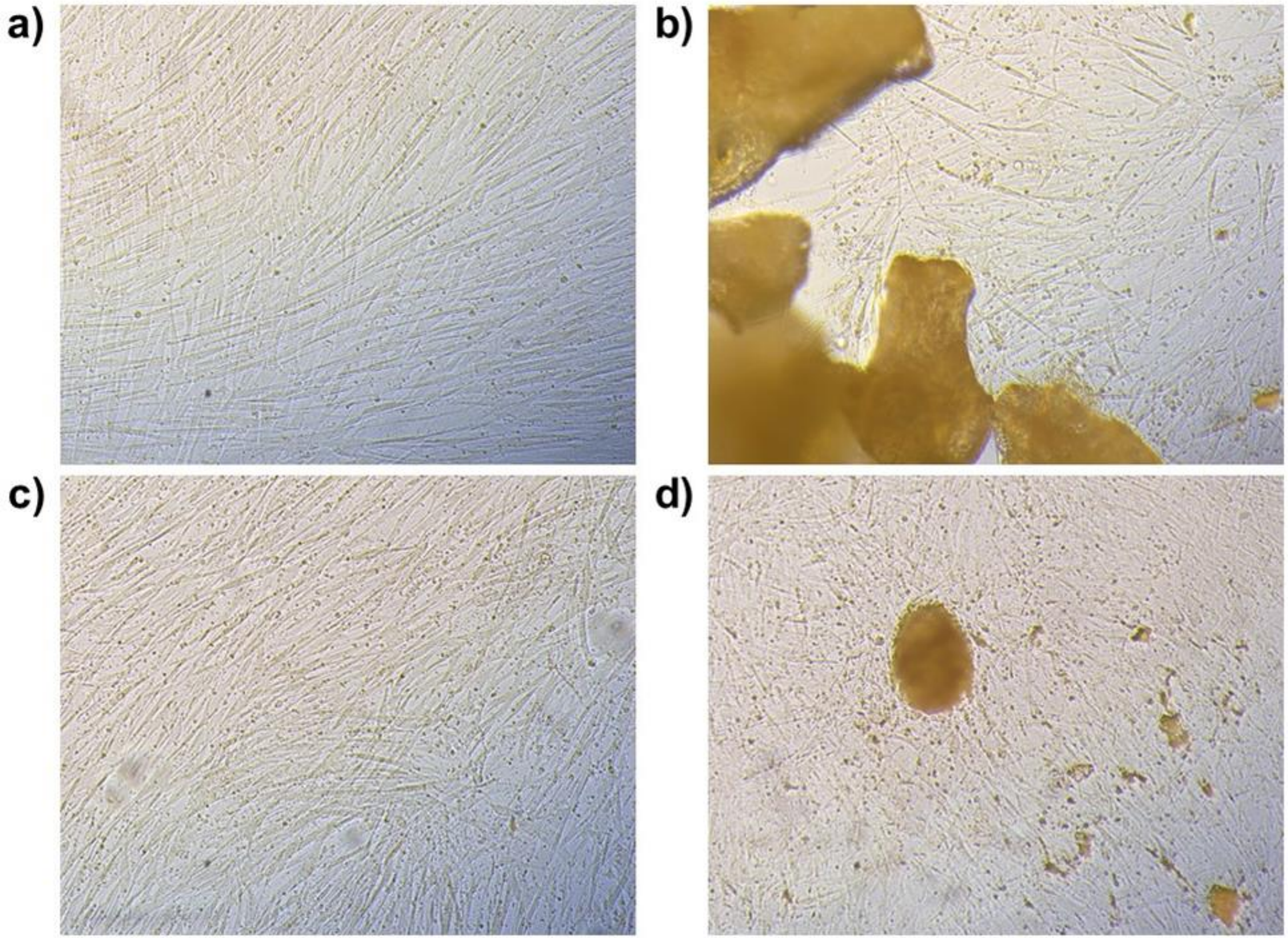
# Results

Petakas and traditional T-75 and T-225 cell culture flasks containing human mesenchymal stem cells and within the cell culture incubator demonstrating the space saving benefit of the stacked Petakas.

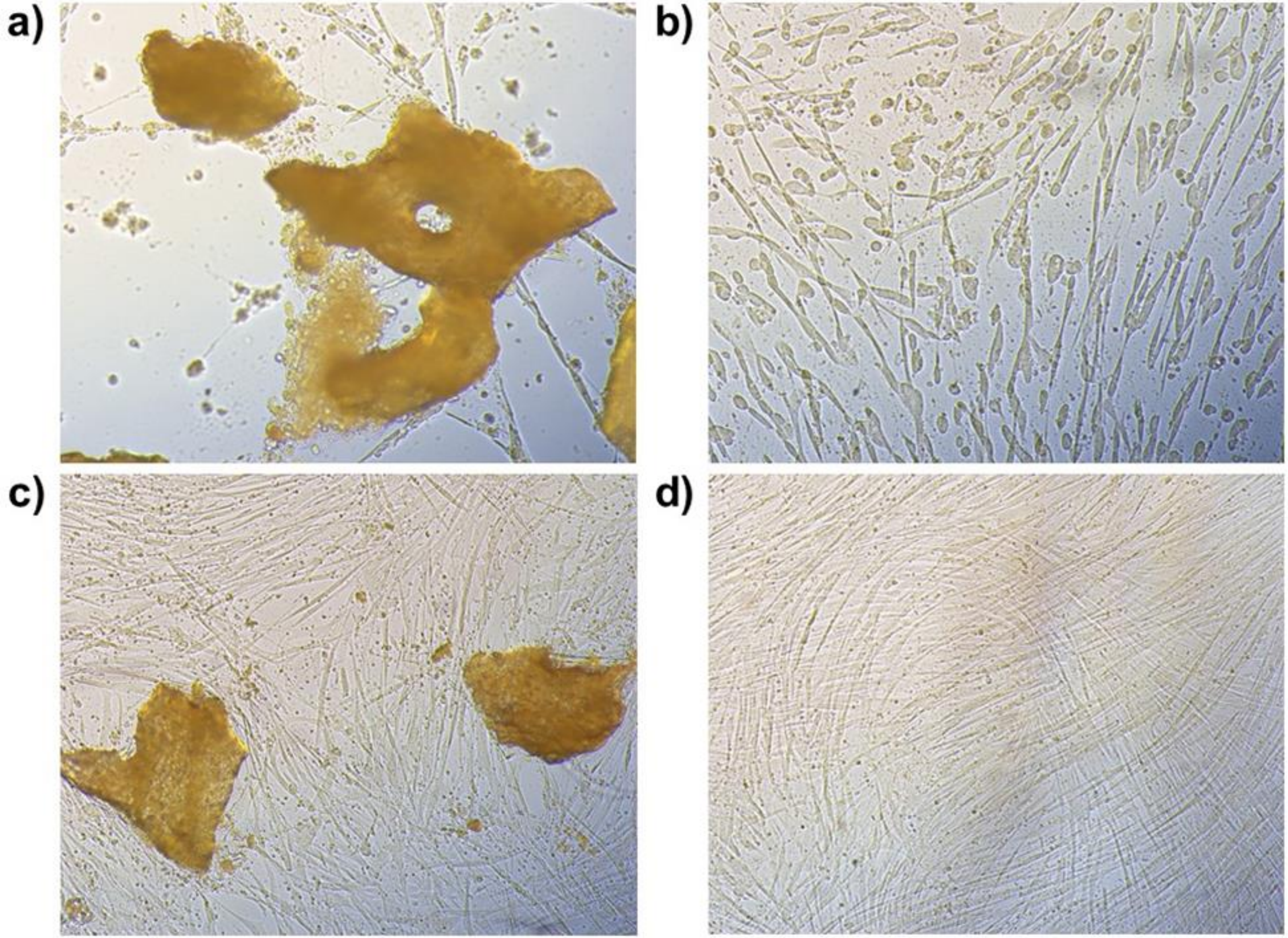




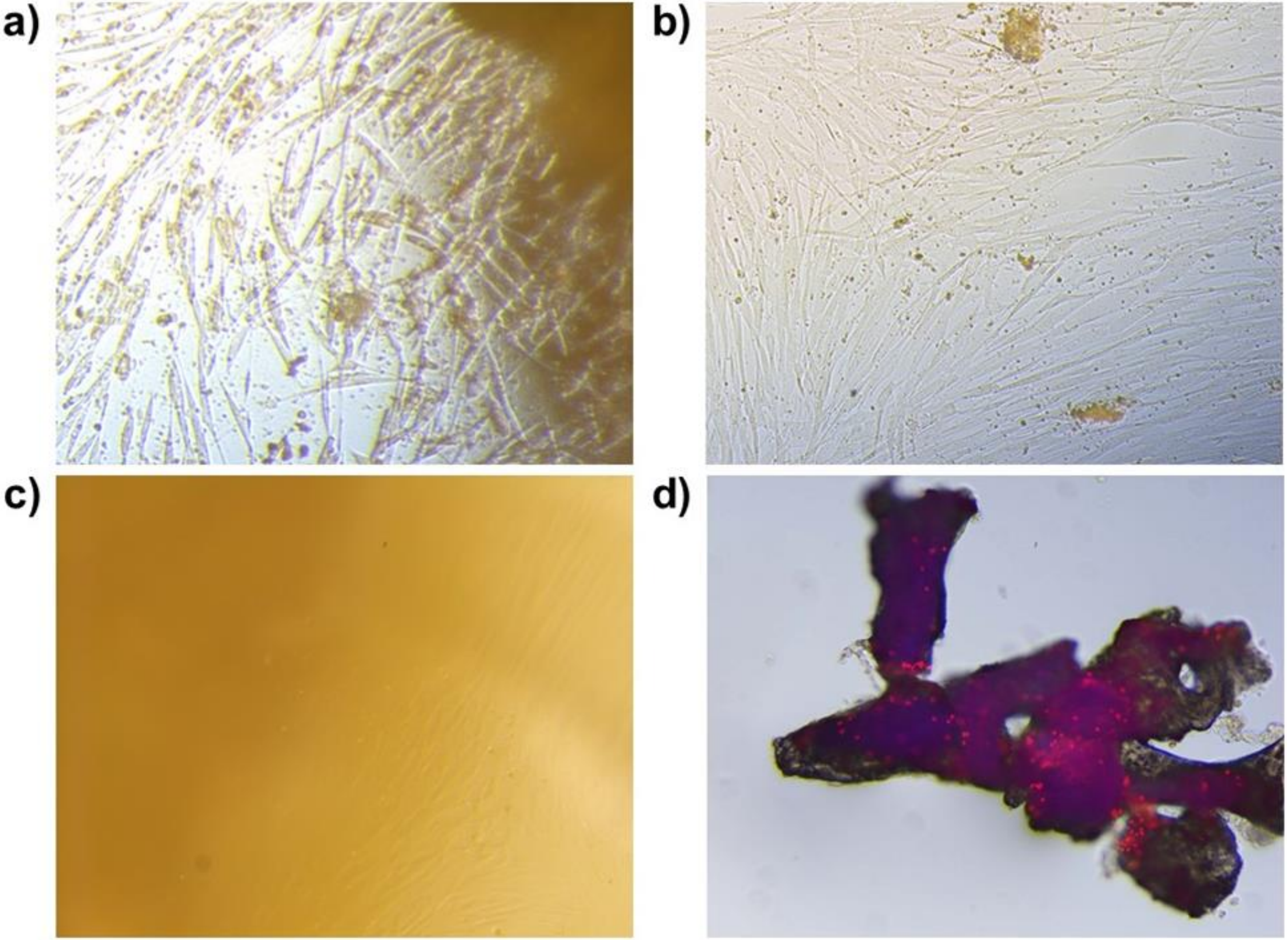

a) Without Collagen & 5% $CO_2$ – T-75 Flask (D3, 37.5X), b) With Collagen & 5% $CO_2$ – T-75 Flask (D3, 37.5X), c) Without Collagen & 5% $CO_2$ – Petaka (D3, 37.5X), and d) With Collagen & 5% $CO_2$ – Petaka (D3, 37.5X).



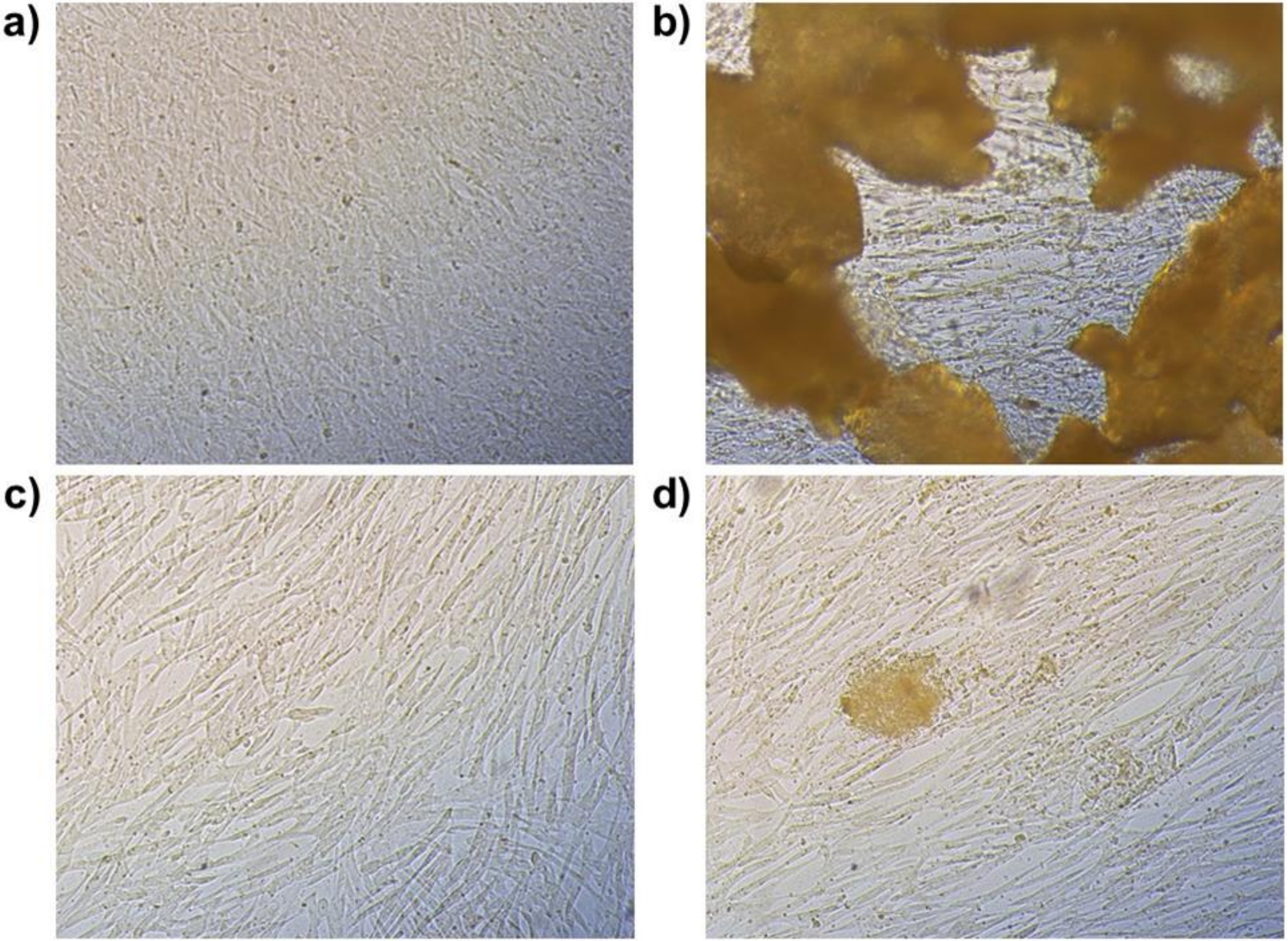

a) Without Collagen & 5% $CO_2$ – T-75 Flask (D3, 37.5X), b) With Collagen & 5% $CO_2$ – T-75 Flask (D3, 37.5X), c) Without Collagen & 5% $CO_2$ – Petaka (D3, 37.5X), and d) With Collagen & 5% $CO_2$ – Petaka (D3, 37.5X).



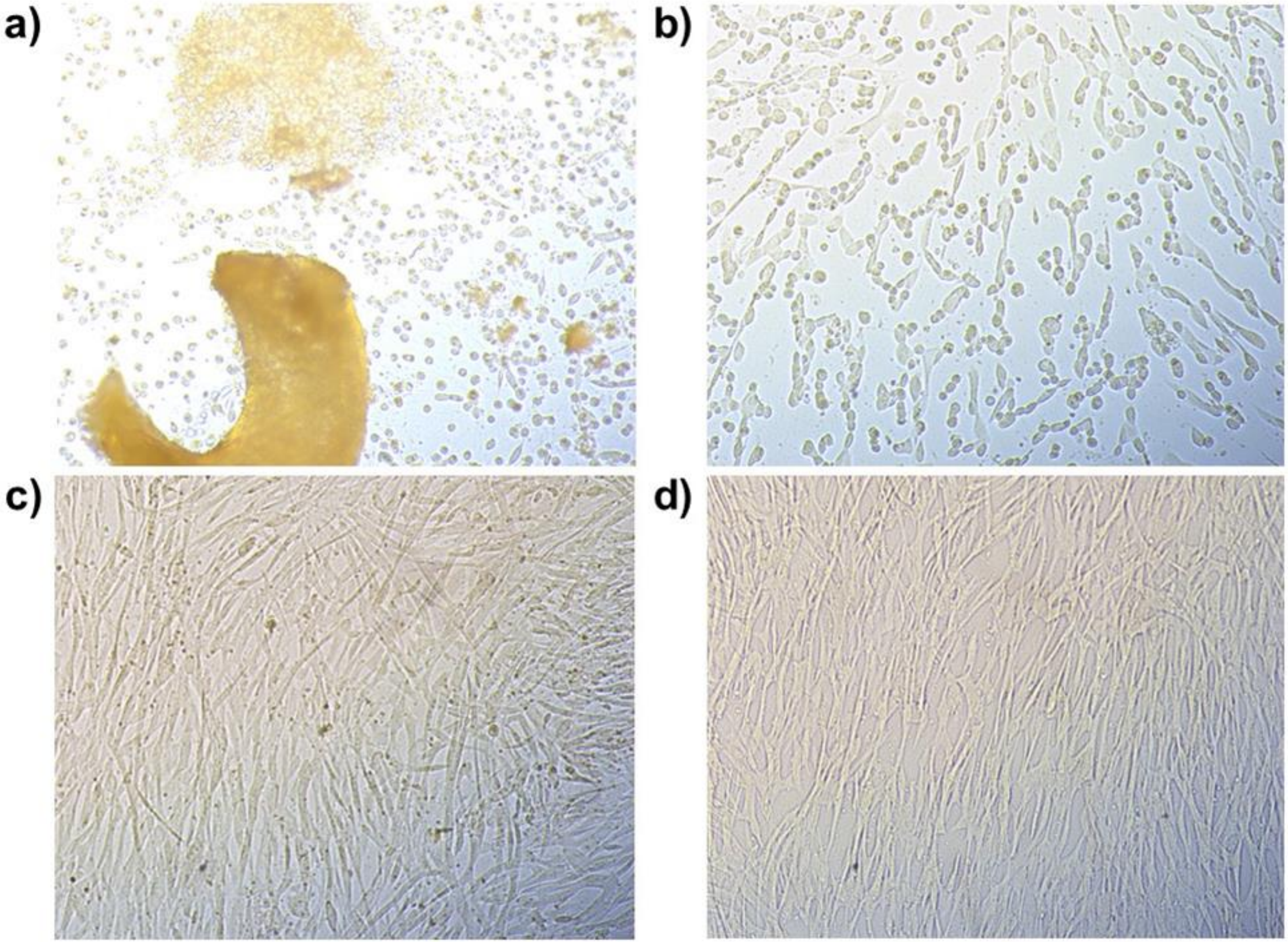

a) With Collagen & Ambient $CO_2$ – T-75 Flask (D3, 150X), b) Without Collagen & Ambient $CO_2$ – T-75 Flask (D3, 150X), c) With Collagen & Ambient $CO_2$ – Petaka (D3, 37.5X), and d) Without Collagen & Ambient $CO_2$ – Petaka (D3, 37.5X).



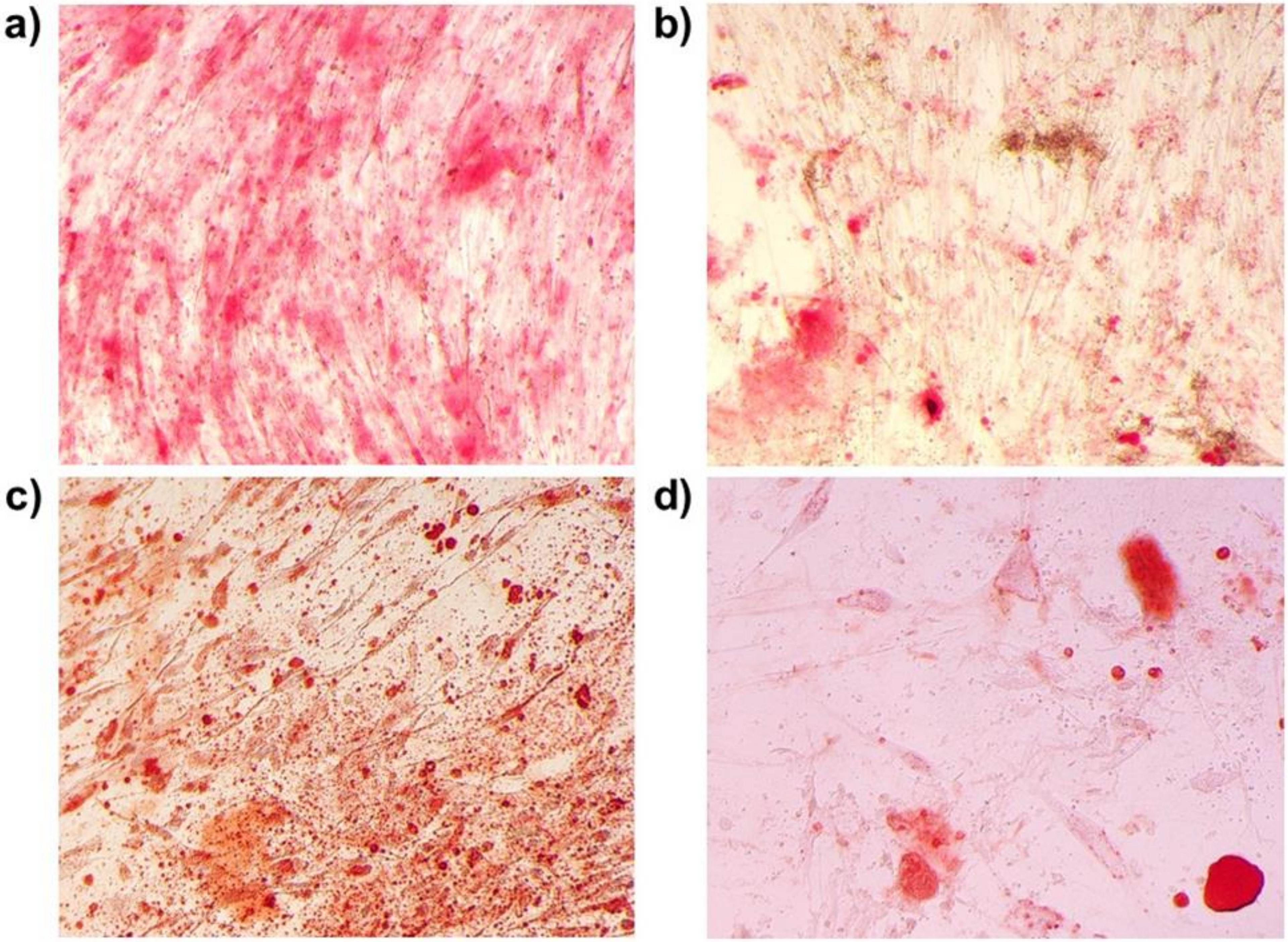

a) With Bone Plug Collagen & Ambient $CO_2$ – T-75 Flask (D3, 150X), b) With Bone Plug Collagen & 5% $CO_2$ – T-75 Flask (D3, 37.5X), c) With Bone Plug Collagen & 5% $CO_2$ – T-75 Flask (D1, 37.5X), and d) D1 with collagen $CO_2$ (75X VV, PI + BF).



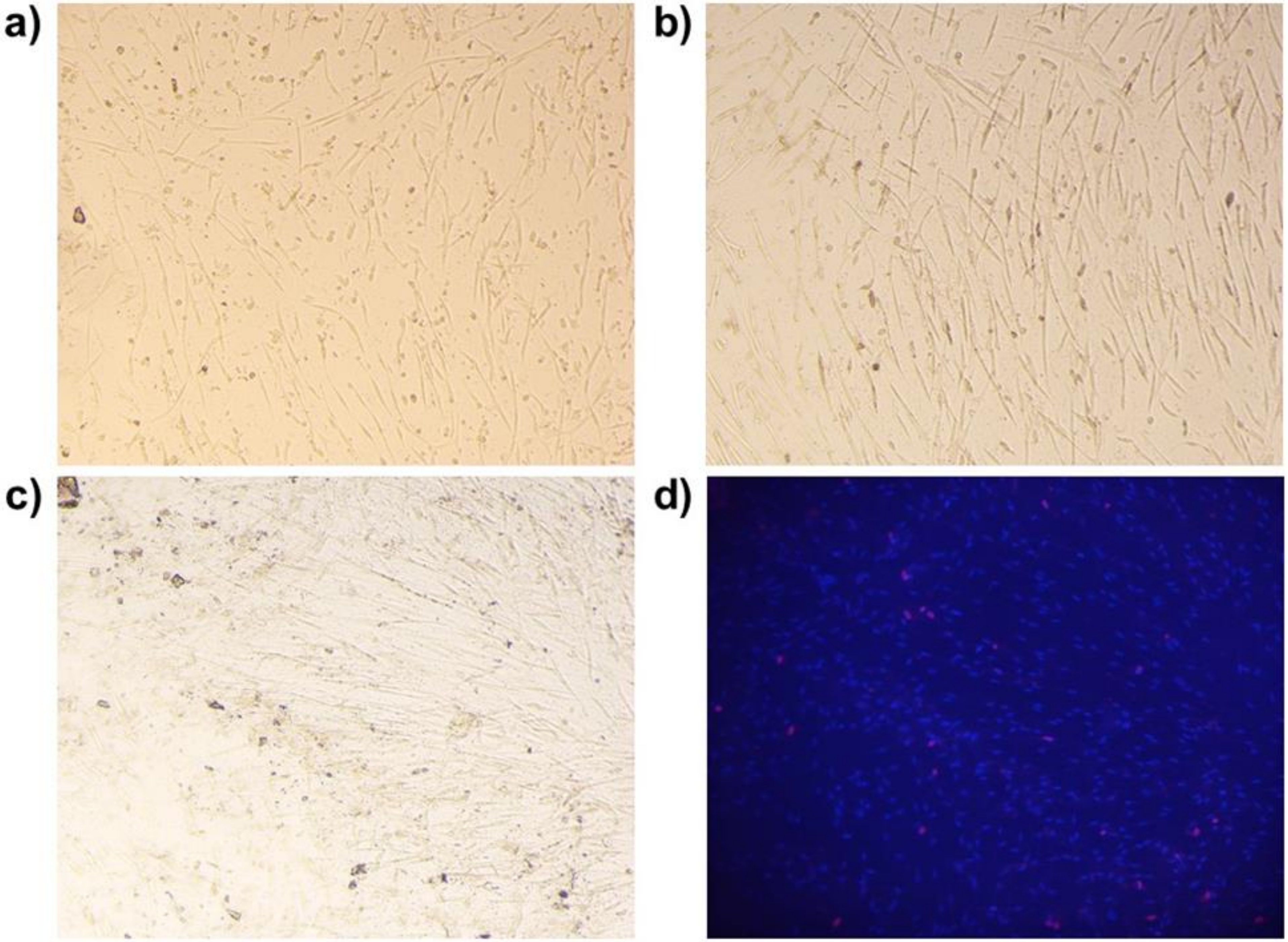
a) Without Collagen & 5% $CO_2$ – T-75 Flask (D1, 37.5X), b) With Collagen & 5% $CO_2$ – T-75 Flask (D1, 37.5X), c) Without Collagen & 5% $CO_2$ – Petaka (D1, 150X), and d) With Collagen & 5% $CO_2$ – Petaka (D1, 150X).




a) With Collagen & Ambient $CO_2$ – T-75 Flask (D1, 37.5X), b) Without Collagen & Ambient $CO_2$ – T-75 Flask (D1, 150X), c) With Collagen & Ambient $CO_2$ – Petaka (D1, 150X), and d) Without Collagen & Ambient $CO_2$ – Petaka (D1, 150X).




a) MSC D3 control T-75 differentiation 37.5X, b) MSC D3 T-75 with collagen differentiation 37.5X, c) MSC D3 control differentiate Petaka 75X, and d) D3 with collagen 300X Petaka Alizarin Red S.

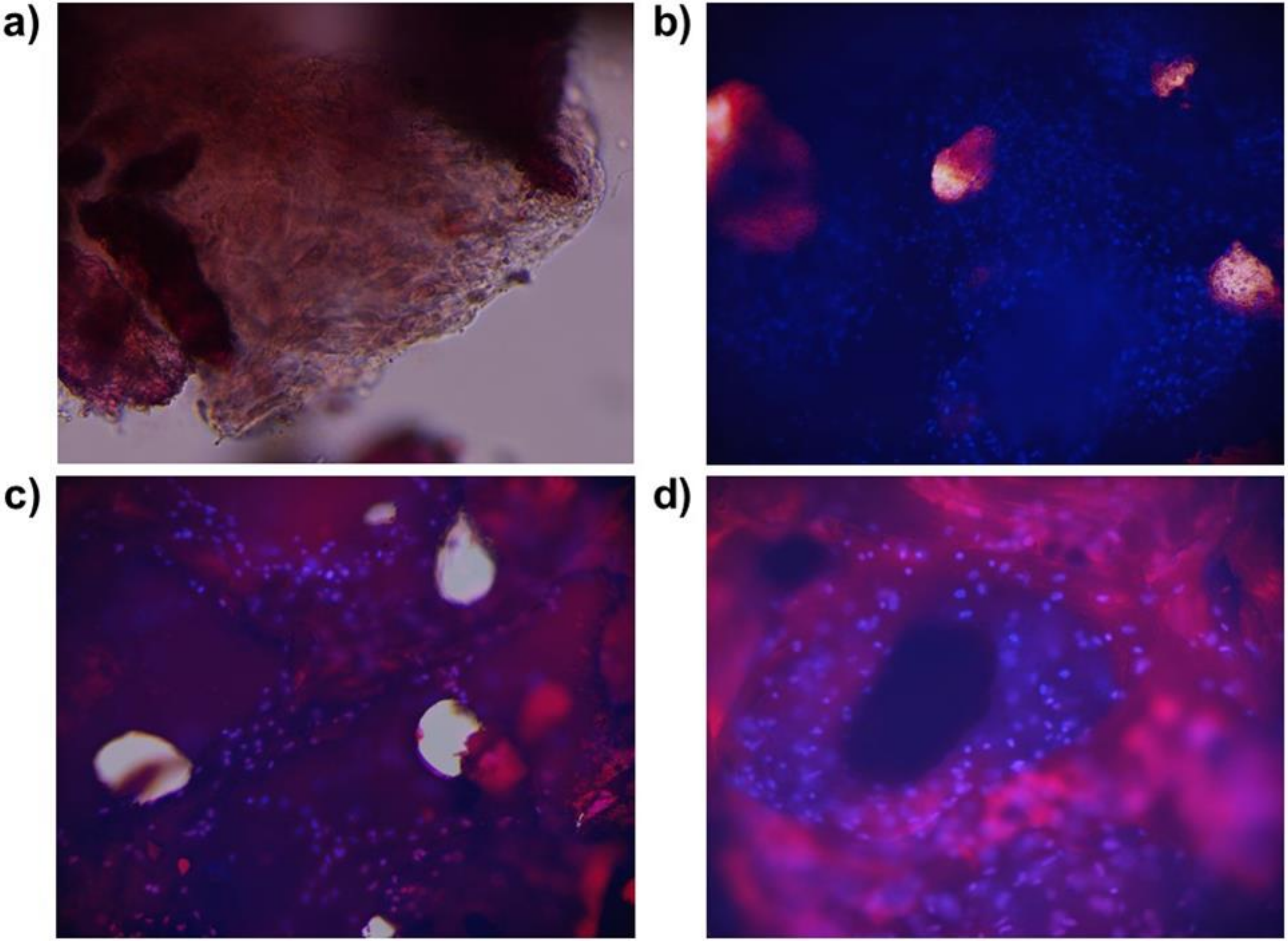



a) Without Collagen & Ambient $CO_2$ – Petaka (D1, 37.5X), b) Without Collagen & Ambient $CO_2$ – Petaka (D3, 37.5X), c) With Collagen & Ambient $CO_2$ – Petaka (D3, 37.5X), and d) With Collagen & Ambient $CO_2$ – Petaka (D3, 37.5X), PI and Vybrant Violet.

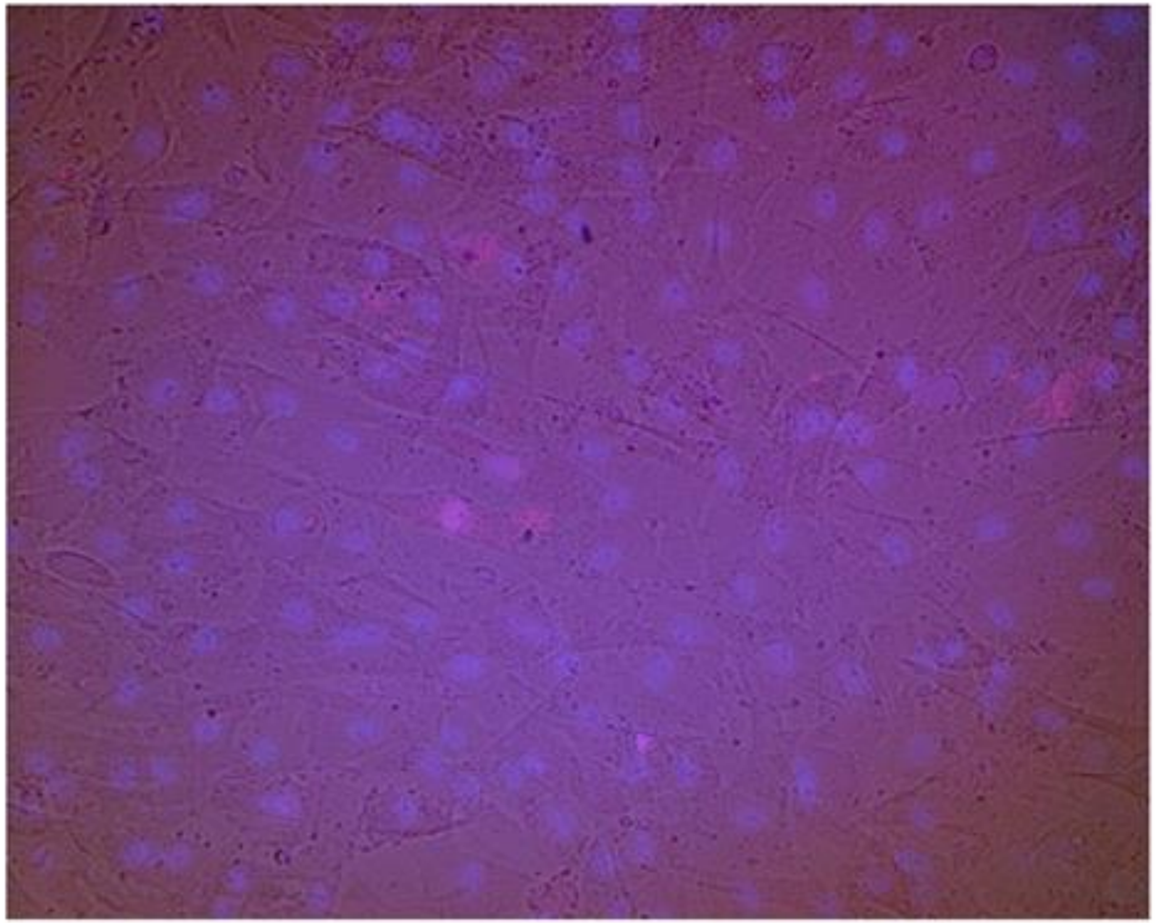
29 days no cell culture medium refresh

**T-75 cells dead but**

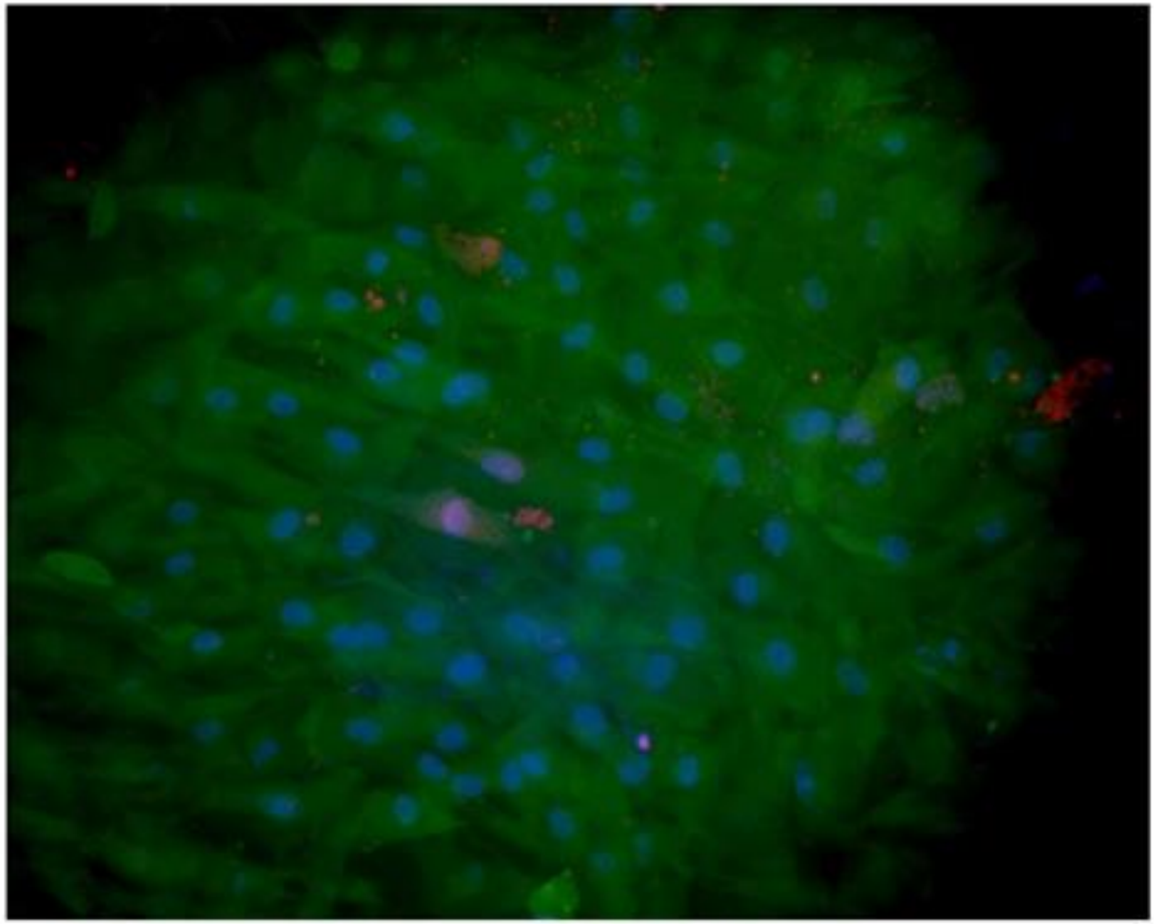
**Petaka cells alive.**




a) Small bone plug sectioned (D1 HO & PI + BF, 150X), b) small bone plug sectioned (D1, 37.5X), c) small bone plug sectioned (D1, 75X), and d) small bone plug sectioned (D1, 75X).

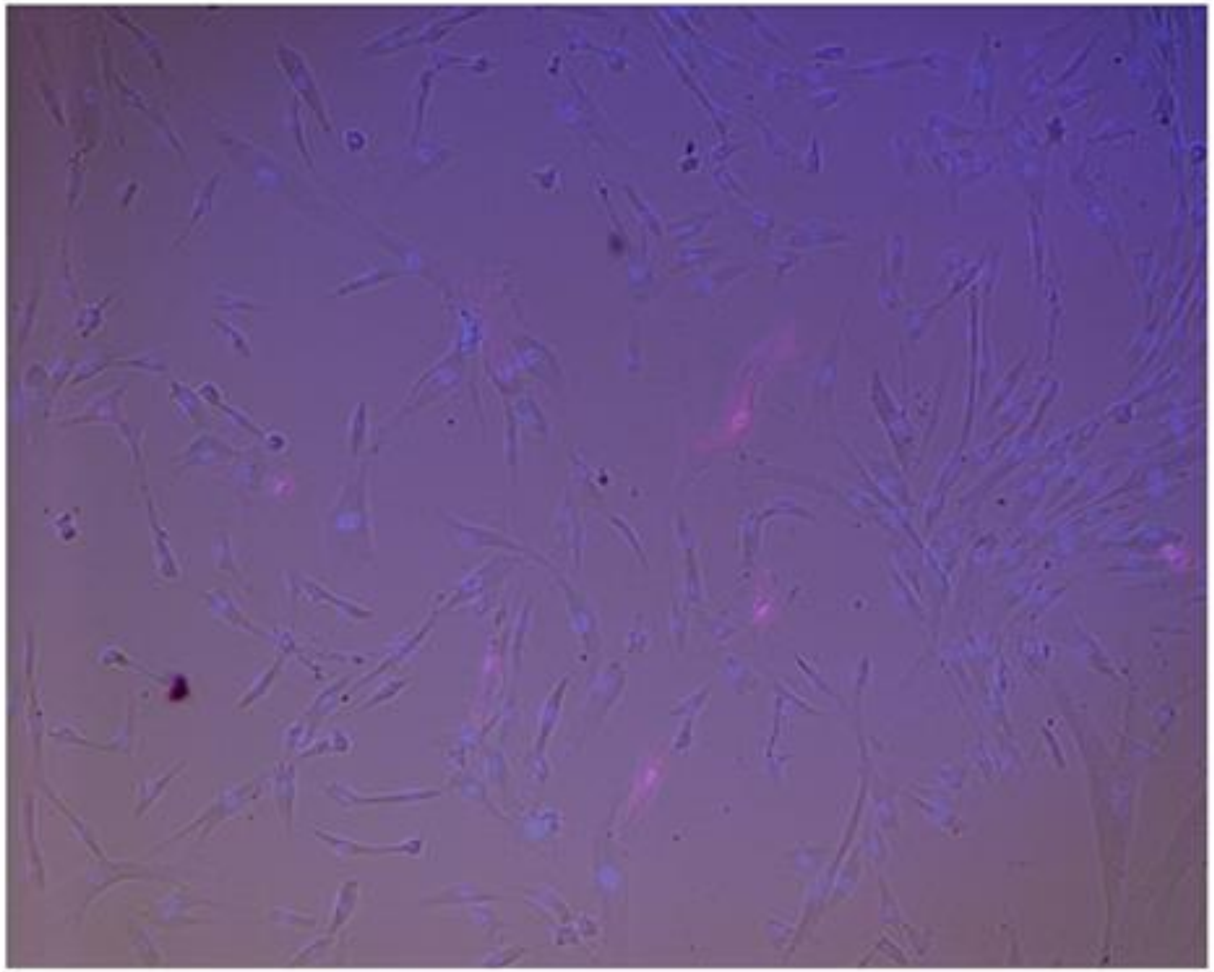


Vybrant Violet + Propidium Iodide (300X)

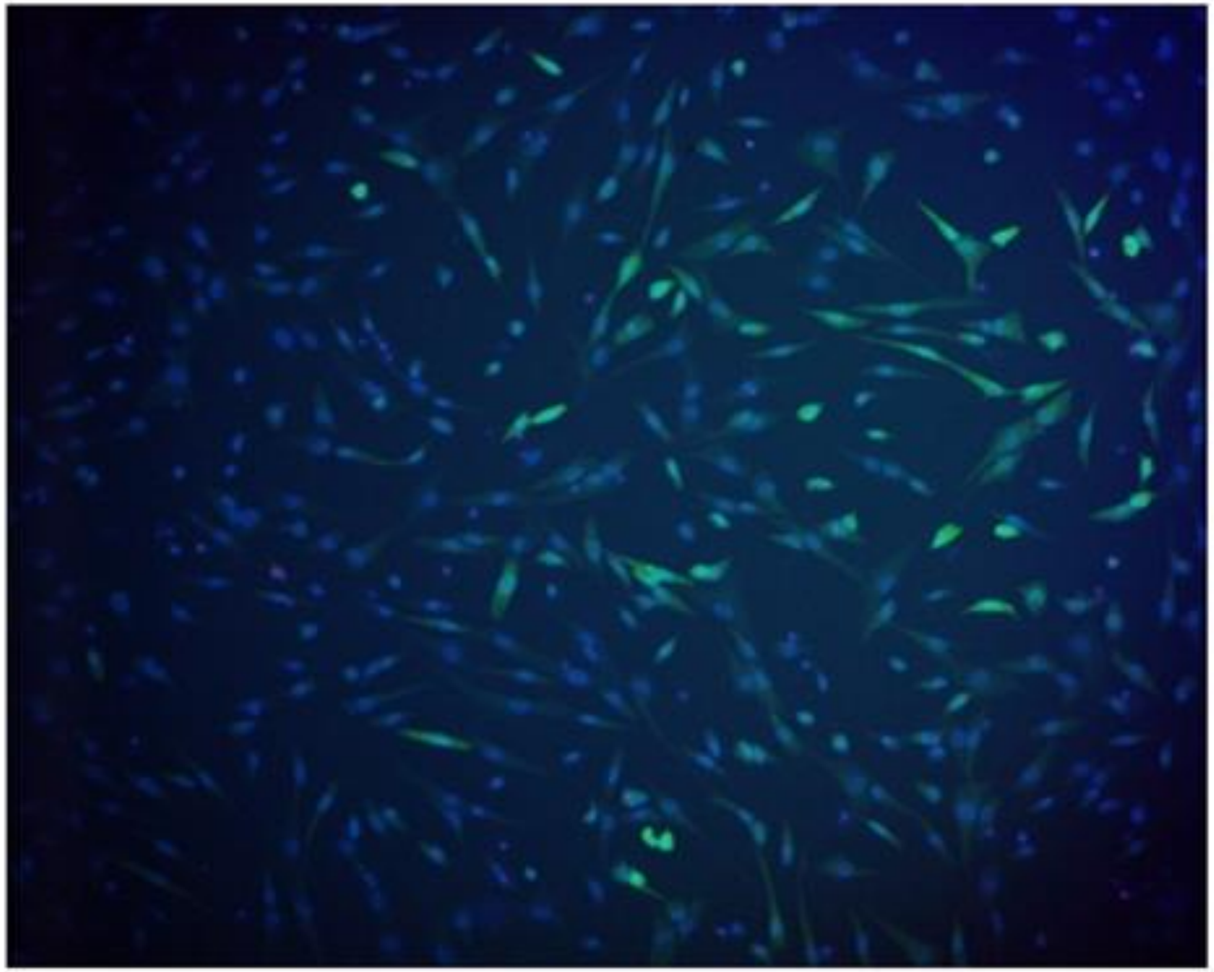


DCFDA (ROS) + Vybrant Violet + Propidium Iodide (300X)

**Human Mesenchymal Stem Cells**

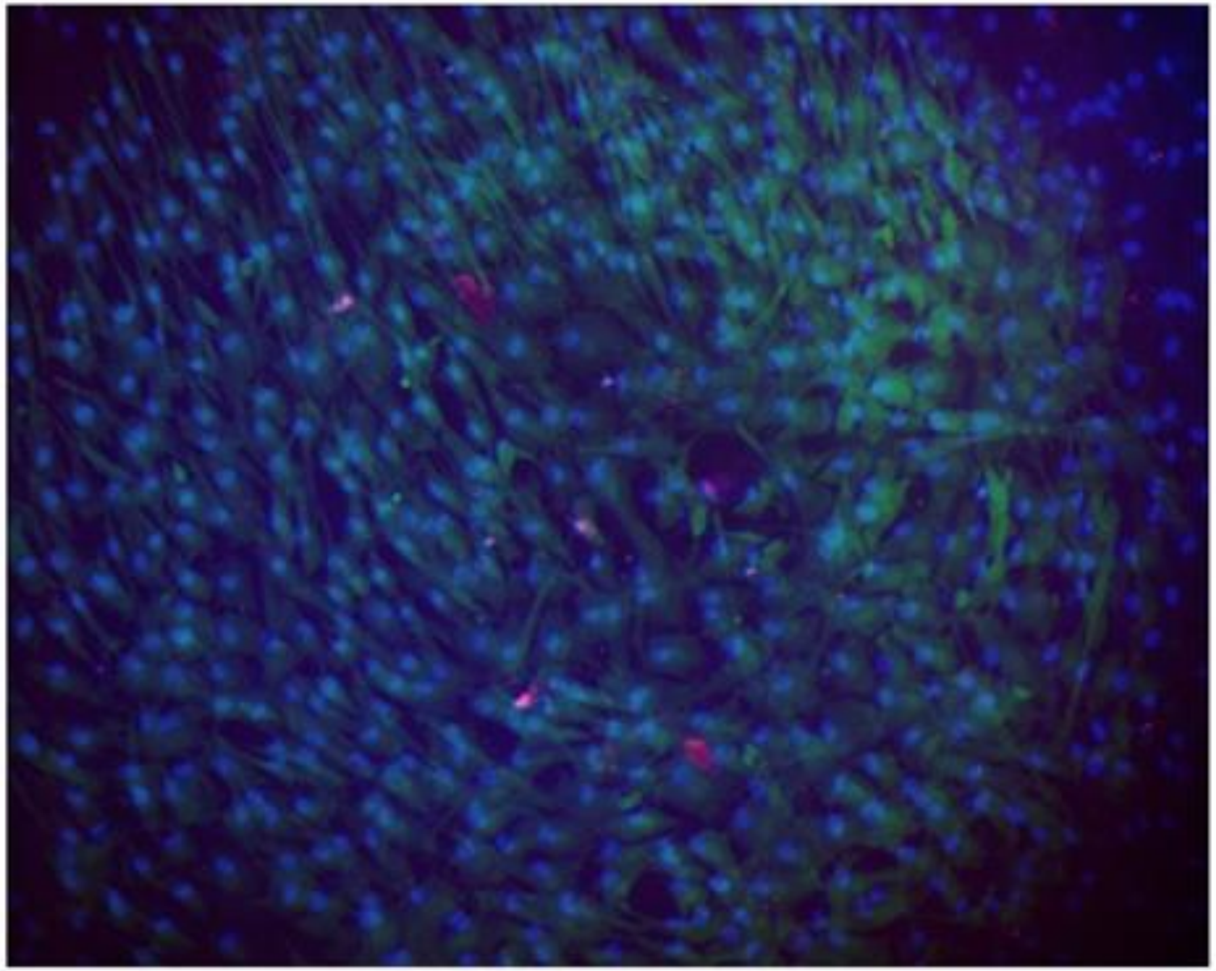


Vybrant Violet + Propidium Iodide (150X)

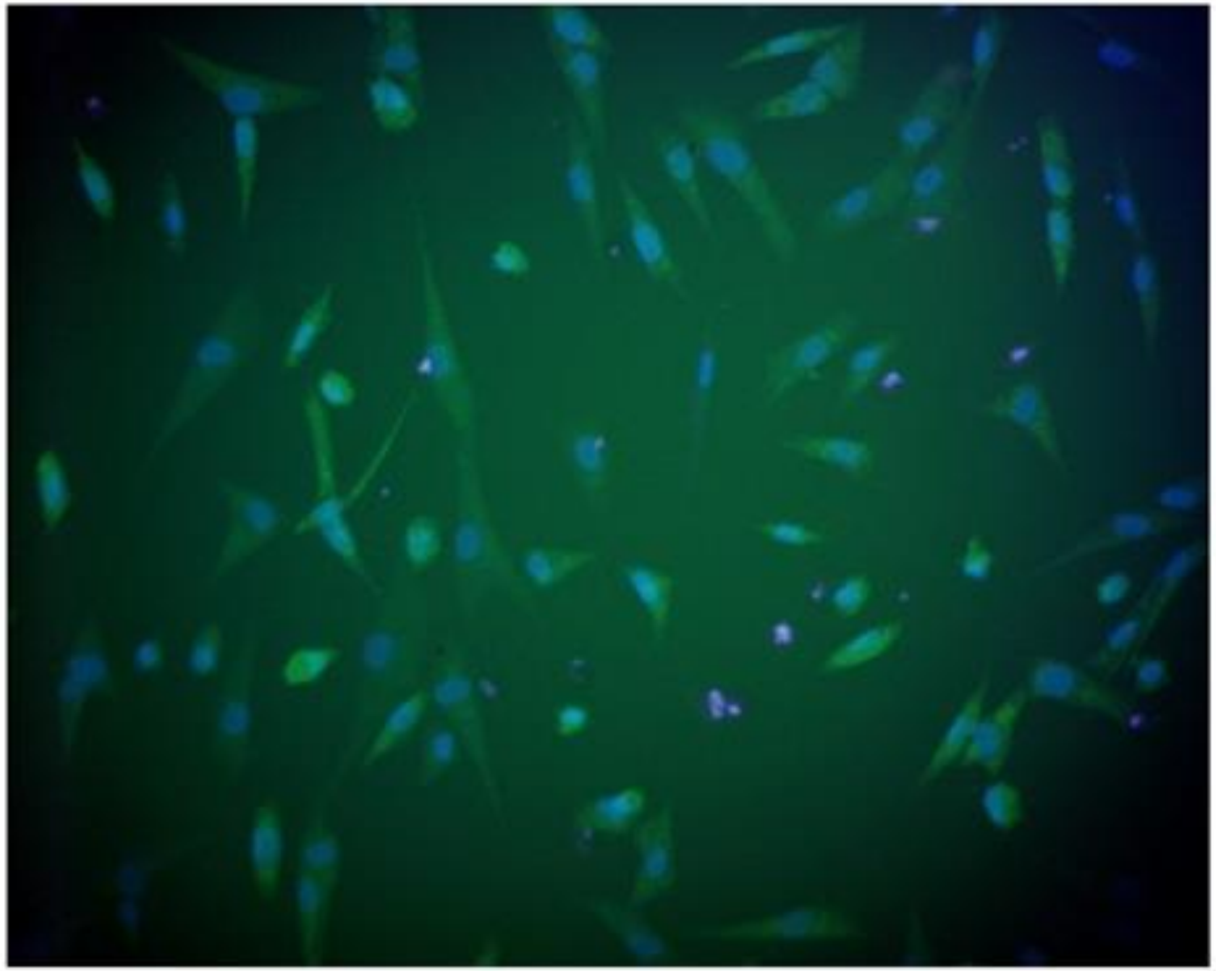


DCFDA (ROS) + Vybrant Violet (150X)

**Human Mesenchymal Stem Cells**

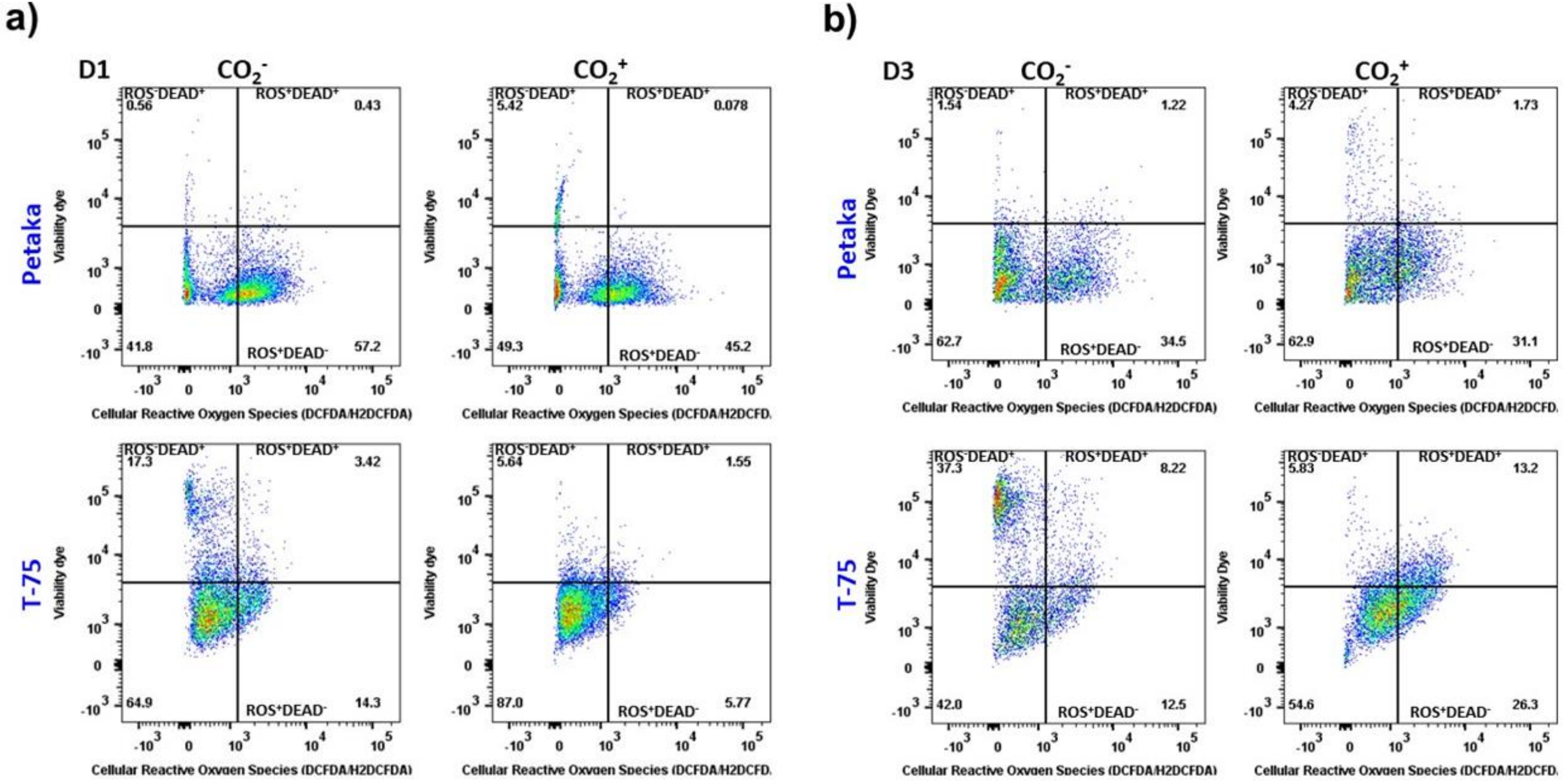


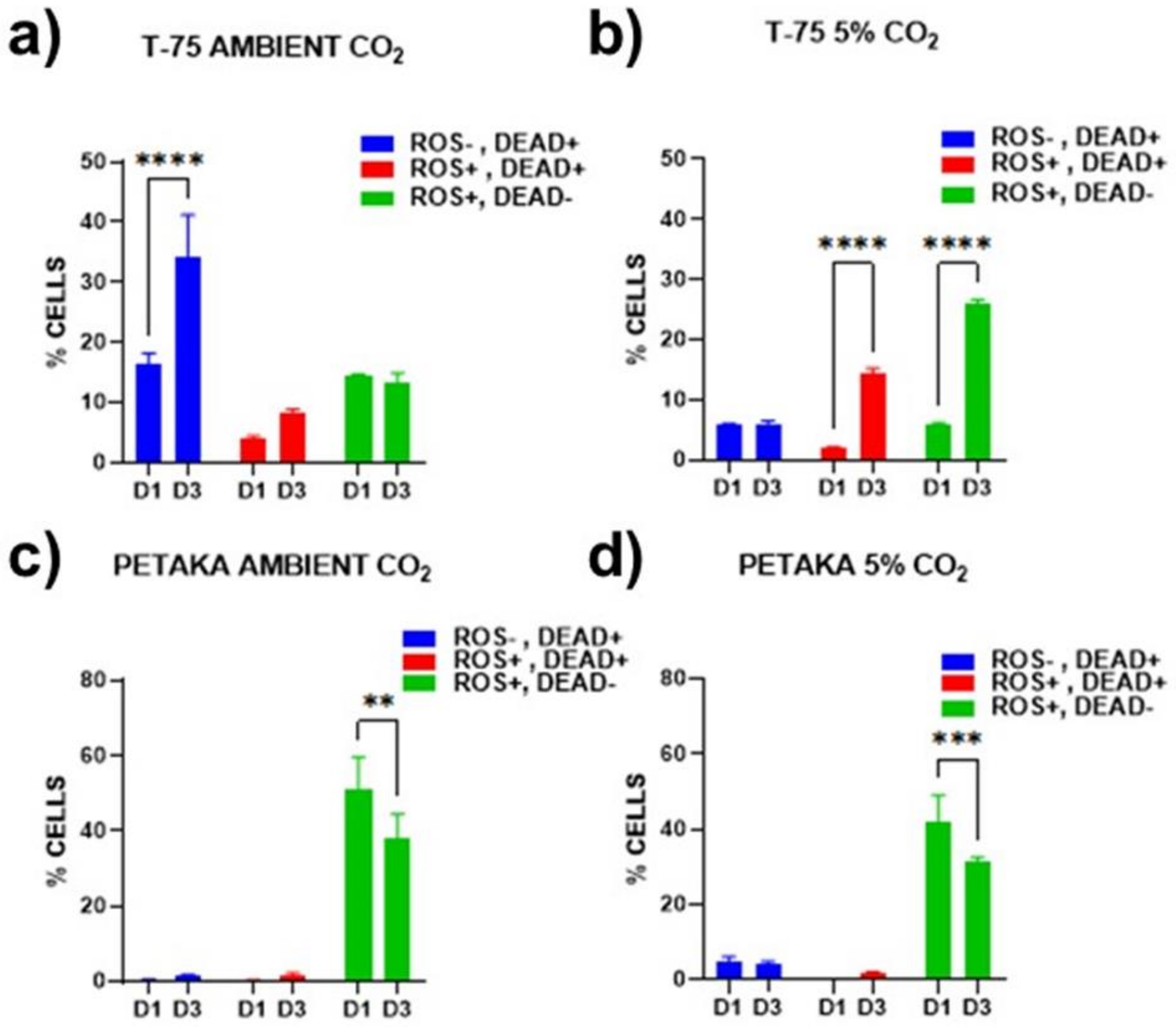
DCFDA (ROS) + Vybrant Violet + Propidium Iodide (150X)



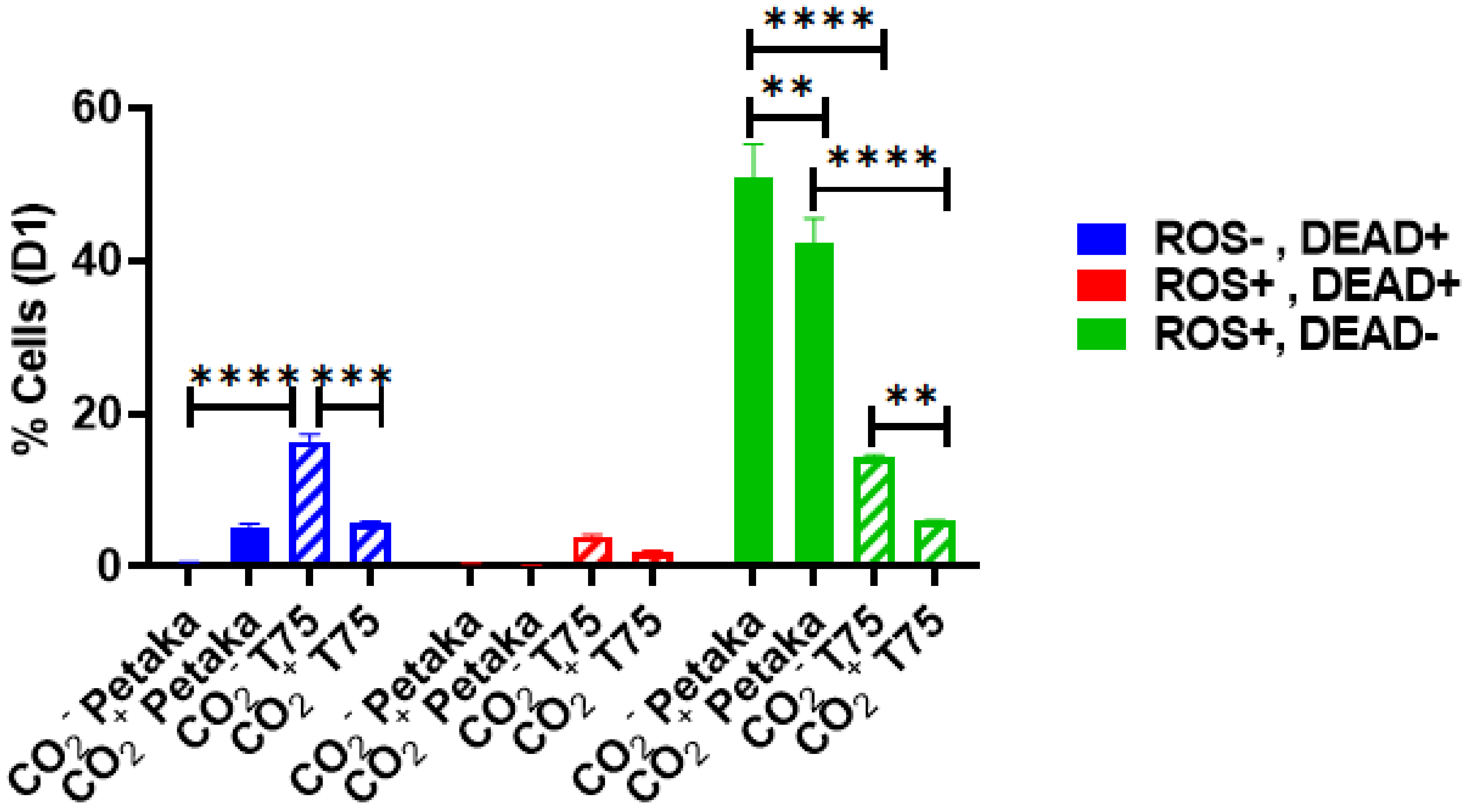
DCFDA (ROS) + Vybrant Violet + Propidium Iodide (300X)

**Human Mesenchymal Stem Cells**


a)
b)
D1
D3
$CO_2^-$
$CO_2^+$
Petaka
T-75
ROS⁻DEAD⁺
ROS⁺DEAD⁺
ROS⁺DEAD⁻
Viability dye
Viability Dye
Cellular Reactive Oxygen Species (DCFDA/H2DCFDA)
0.56
0.43
41.8
57.2
5.42
0.078
49.3
45.2
17.3
3.42
64.9
14.3
5.64
1.55
87.0
5.77
1.54
1.22
62.7
34.5
4.27
1.73
62.9
31.1
37.3
8.22
42.0
12.5
5.83
13.2
54.6
26.3



a)
T-75 AMBIENT $CO_2$
b)
T-75 5% $CO_2$
c)
PETAKA AMBIENT $CO_2$
d)
PETAKA 5% $CO_2$
ROS- , DEAD+
ROS+ , DEAD+
ROS+, DEAD-
% CELLS
D1 D3 D1 D3 D1 D3
****
**
***



% Cells (D1)
60
40
20
0
****
**
****
**
****
****
CO2- Petaka
CO2x Petaka
CO2- T75
CO2x T75
CO2- Petaka
CO2x Petaka
CO2- T75
CO2x T75
CO2- Petaka
CO2x Petaka
CO2- T75
CO2x T75
ROS- , DEAD+
ROS+ , DEAD+
ROS+, DEAD-

- Confluency typically occurred on day 7, presence of porcine collagen matrix did not seem to impact confluency speed
  - Cell count and confluency was confirmed by DNA staining.
  - Size, shape, and other morphological features were not quantified, but appeared similar.
    - More understanding on osteoporosis model and the osteoblast/osteoclast relationship needed.
- Petakas outperformed T-75 flasks for the following:
  - More expansion in MSCs from days 7-14.
  - Quicker to form tissue layers, especially when porcine collage matrix present.
  - Viable cells = 90%+ after 14 days in low $CO_2$ conditions.
  - Maintained better cell adhesion and viability under stress.
- In extended duration experiment (29 days), with not medium refresh and low $CO_2$ , Petakas maintained MSC viability whereas the T-75 flasks did not.
- RoosterBio donor cell lines 1 and 3 outperformed donor cell line 2 after post-thaw cell culture (i.e., lower viability).
  - On day 29, no medium refresh:
    - Cell line 1 ~50%
    - Cell line 3 > 90%
      - Cell line 3 can be expanded up to 50 million ready-to-print (3D bioprinter) MSCs to limit number of required passages and medium exchange.

# Discussion & Conclusions

ACRO bone powder and granules can be pipetted into Petakas, although granules cause occasional cell and culture medium port blockages.

ABCcolla® porcine collagen matrix granule and powder had the tendency to not completely adhere to cell culture flask bottom if too much medium was used.

Petakas greatly outweigh T-75 in terms of space saving, which is important to consider when study is conducted in the confines of limited launches and the International Space Station.

Statistical difference found between Donor cell lines (e.g., ROS and live/dead).

Statistical difference found between Petakas and T-75 variable (e.g., ROS and live/dead).

Issue: Human MSC medium very expensive, had to adjust experimental design from 3 to 2 donor cell lines.

Future Research: Present study's results leveraged for ND EPSCoR research funding and equipment grant (i.e., 3D Bioprinter) & CASIS pre-proposal approved (ISS), full proposal stage now. (https://www.issnationallab.org/iss360/nlra-2021-3-technology-advancement/)

# Acknowledgements:


ND Space Grant Consortium

Special thanks to Suba Nookala, Peter Knopick, and Steven Adkins at the North Dakota Flow Cytometry Cell Sorting Core (UND), supported by Institutional Development Awards (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103442 & P20GM113123.

ND EPSCoR STEM grants program, Equipment Grant 2019


<u>Collaborators</u>:

ACRO Biomedical Co., Ltd.,
Blue Kick, LLC,
Rhodium Scientific, LLC, and
SpacePharma SPAd

## References


NASA Technology Roadmaps, TA6: Human Health, Life Support, and Habitation Systems. (July 2015) National Aeronautics and Space Administration. NASA Headquarters, Washington, DC.

NASA Strategic Technology Investment Plan 2017. (2017) National Aeronautics and Space Administration. NASA Headquarters, Washington, DC.

Ohshima, Hiroshi. 2012. Preventing Bone Loss in Space Flight with Prophylactic Use of Bisphosphonate: Health Promotion of the Elderly by Space Medicine Technologies. Space Biomedical Research Office, JAXA.
https://www.nasa.gov/mission_pages/station/research/benefits/bone_loss.html (Date accessed:5/15/2019)