SOP40103: Passaging and Sub-culture of Patient-Derived Organoid (PDOrg) Cultures

Effective Date: 3/5/2020

Please check for revision status of the SOP at

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PDMR NCI Patient-Derived Models Repository
An NCI Precision Oncology Initiative℠ Resource

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VERSION INFORMATION

1. Change History

Revision	Description
8/25/2017, v003	Internal SOP used by PDMR In Vitro Laboratory
2/13/2018	Standardize SOP for posting to PDMR internal site for use by designated NCI intramural laboratories
2/20/2019	Updated reference SOPs. Appendix 1 added with representative PDOrg culture images. Updated vendor information for BME2. Additional details about passaging low density PDOrg cultures added.
3/5/2020	Updated Cultrex BME, Type2 item description

2. Related SOPs

SOP30101: Recipes for Complete Media for Patient-Derived In Vitro and Organoid Cultures
SOP40102: Thawing and Initial Culture of Patient-Derived Organoid (PDOrg) Cultures
SOP40104: Cryopreservation of Patient-Derived Organoid (PDOrg) Cultures

1.0 PURPOSE/SCOPE

This Standing Operating Procedure (SOP) describes the procedures for passaging of Patient-Derived Organoid (PDOrg) cultures under BSL-2 safety criteria. Optimal tissue culture media for use with specific PDOrg lines will be provided with the individual **Certificate of Analysis**.

This SOP is used/performed by the Biological Testing Branch (BTB) at NCI-Frederick, Frederick National Laboratory for Cancer Research.

2.0 SAFETY

BTB treats all patient-derived material under Biosafety Level 2 (BSL2) conditions even when PCR-based screening has not detected the presence of a known set of human pathogens. All work is conducted in a biological safety cabinet (BSC) using personal protective equipment and avoiding the use of sharps where possible. All materials potentially exposed to human-derived material are disinfected by exposure to a 10% bleach solution for a minimum of 10 minutes, double bagging for autoclaving or incineration. Consult with your facility safety professionals regarding the safe handling of BSL2 studies.

3.0 CLEAN-UP

3.1 All materials in contact with patient tissue, as well as the mice carrying patient tumor samples and cultures derived from patient tumor samples, are treated as a potential health threat (BSL-2 precautions) since the human tissues could retain human pathogenic agents even if they do not replicate in mouse cells (e.g., EBV, HPV, etc).

3.2 Flush/soak any items (e.g., tubes, syringes, petri dishes, lab mats, etc) that were in contact with human tissue with disinfectant (e.g., 10% bleach, commercial hydrogen peroxide disinfectant, 2% Virkon®) for a minimum of 10 minutes before disposal in biohazard waste or sharps containers (follow institutional guidelines and manufacturer's recommendations).

3.3 For items that can't be rinsed (e.g., micropipettors), wipe down thoroughly with bleach-soaked gauze or other appropriate disinfectants.

4.0 EQUIPMENT

4.1 **Certificate of Analysis** for PDOrg culture to be grown.

4.2 Media

4.2.1 Complete Media:

4.2.1.1 Prepare the recommended Complete Media per the PDOrg-specific Certificate of Analysis (COA).

4.2.1.2 Prepare fresh weekly and warm to room temperature 2-3 hours before use.

4.2.2 Wash Media:

Item	Catalog	Volume
Basic Media	*see SOP30101	100 mL
FBS	HyClone Laboratories, Cat#: SH30071.03HI	2.5 mL

4.2.2.1 Sterile filter with 0.22 µm filter. Prepare fresh weekly and warm to room temperature 2-3 hours before use.

4.3 Reagents, Material & Equipment

4.3.1 Accutase, in DPBS without Ca++ or Mg++ (Innovative Cell Technologies, Inc., Cat#: AT-104)

4.3.2 Dispase II (ThermoFisher Scientific, Cat#: 17105-041)

4.3.3 Cultrex™ PathClear Reduced Growth Factor BME, Type 2, (R&D Systems, Cat#: 3533-005-02; 8-12 mg/mL [high protein content])

4.3.4 25-mL, 10-mL, 5-mL pipettes, sterile

4.3.5 50-mL polypropylene tubes, sterile

4.3.6 Pipetman and sterile tips

4.3.7 24-well tissue culture plate, flat bottomed, sterile (e.g., Costar, Cat#: 3524)

4.3.8 Aspirator pipettes (e.g., Fisher Scientific, Cat#: 357501)

4.3.9 Sterile screw cap tubes (e.g., Sarstedt, Cat#: 72692.005)

4.3.10 Ice bucket with ice

4.3.11 Benchtop Centrifuge equipped with sealed buckets

4.3.12 37°C Incubator (5% CO_2, humidified)

4.3.13 Biological Safety Cabinet (BSC) meeting biosafety level 2 (BSL2) standards

4.3.14 Micro-centrifuge (e.g., Corning Model 6765)

5.0 PROCEDURE

5.1 Guidance on Passaging

PDOrg cultures have a range of phenotypes, sizes, and growth densities; see Appendix 1 for examples.

5.1.1 Organoids are ready to be passaged based on 2 criteria: **density and size**.

5.1.1.1 **High-density Organoid Cultures**: Passage into new wells at 1:1.5 or 1:2 ratio. For new cultures, we recommend starting at 1:1.5 and then adjusting as the researcher becomes familiar with the growth properties of the culture.

- Figure 1 **and** Figure 2: Left panels: Example of phase contrast images of PDOrg cultures at high density when passaging should occur.

5.1.1.2 **Low-density Cultures with Large Organoids**: Passage into new wells at 1:1 or 1:0.5 ratio.

- Figure 1 **and** Figure 2: Right panels: Example of PDOrg cultures at low density when feeding (SOP Step 5.6) is all that is required.
- Figure 3: Examples of PDOrg cultures where individual organoids have grown large while the rest of the culture are smaller. If individual organoids within a culture reach this size they need to be passaged as they will "pop" or disintegrate. The Figure shows several organoids at different stages of disintegration.
- Timing and size of this phenomena is different from model to model, but it should be noted that this can happen overnight, where one day the organoid is intact and healthy and the next day it has "popped".
- If the model being grown is low-density but has large organoids, the culture should be passaged and the large organoids broken up by mechanical breakage or the addition of Accutase preheated to 37°C followed by mechanical breakage.
- If the density of organoids is light instead of splitting 1:1, condense the number of wells (e.g., split 1:0.5) to increase the density.

5.1.2 PDOrg cultures should be passaged if any of the following criteria are met:

- PDOrg cultures have reached high-density per the examples demonstrated in Figure 1 and Figure 2.
- A low-density culture has large organoids within in it that are likely to "pop" or disintegrate per the examples demonstrated in Figure 3.
- If the BME2 dome becomes "wobbly." This can happen during initial culture development as well as at later passages if the BME2 is diluted with media or if a low-protein content lot of BME2 is received.

5.2 Prepare Reagents

5.2.1 Before starting, pre-warm the Complete Media and Wash Media to room temperature for 2-3 hours. Addition of cold or cool media to the BME2 dome will cause the BME dome to dissipate.

5.2.2 Thaw BME2 on ice before use (approx. 5 hrs) or in a 4°C refrigerator overnight.

5.2.3 Prepare Splitting Media: 1.5 mg/mL Dispase II in Wash Media, sterile filter with a 0.22 µm filter, and store on ice. Can be prepared fresh each week, stored at 4°C.

5.2.4 Perform all procedures under sterile conditions in a Biosafety Level 2 certified biosafety cabinet.

5.3 Passaging Organoid Cultures

Note: When passaging organoids, you should never split PDOrg culture more than 1:2. Follow passaging recommendations in SOP Section 5.1.

5.3.1 Add a volume of Splitting Media equal to the volume of Complete Media present in each well to be passaged. This provides a final concentration of Dispase II at 0.75 mg/mL.

5.3.1.1 For a single well in a 24-well plate, this would be 750 µL Splitting Media per well to the already present 750 µL Complete Media.

5.3.2 Place the plate with the Splitting Media in a 37°C tissue culture incubator and incubate for 1.5 to 2 hours.

5.3.3 Using a P1000 Pipetman, gently pipette the Splitting Media up and down in each well to dissociate any residual BME dome.

5.3.4 Transfer the dissociated BME2 domes, organoids, and Splitting Media into a sterile 50-mL conical tube and add an equal volume Wash Media to dilute and neutralize the Dispase II.

5.3.5 Centrifuge the organoid solution at 200xg for 5 min.

5.3.6 With a 10-mL pipette, carefully remove as much Splitting/Wash Media as possible.

5.3.7 Add 10-mL Wash Media, cap and invert the tube several times to wash the organoids. Then centrifuge the organoid solution at 200xg for 5 min.

5.3.8 With a 10-mL pipette, carefully remove all but 1.0-1.5 mL of Wash Media.

5.3.9 With a P1000 Pipetman, triturate the organoids to mechanically break them apart. Transfer the remaining 1.0-1.5 mL Wash Media/organoid suspension into a 1.5-mL sterile screw cap tube.

5.3.10 Using a mini-centrifuge, short-pulse (~1 sec/pulse) spin the tube 3 times. Be careful as prolonged mini-centrifugation can damage the cells.

5.4 Prepare new BME2 Domes

5.4.1 With a P1000 Pipetman, carefully remove as much Wash Media as possible from the pellet.

5.4.2 Repeat short-pulse (~1 sec/pulse) spins and Wash Media removal using a P200 and then a P20 Pipetman so that ALL excess media is removed (see Figure 4 for appearance of final pellet) as any excess media will dilute the BME2 causing a "wobbly" dome. Try to limit spinning so the cells are not damaged.

5.4.3 Place the tube on ice and add the appropriate amount of BME2; **avoid** creating bubbles. Volume to add is dependent on the density of the original culture and organoid size as outlined in SOP Section 5.1. Use tables below as references for calculations. Each BME2 dome will be 35.

- High-density Organoid Cultures (Definitions: SOP Section 5.1.1.1)

Starting Well#	Splitting Ratio	Volume BME2 to Add	Final #Wells in a 24-Well Plate
4	1:1.5	210 µL	6
	1:2	280 µL	8
6	1:1.5	315 µL	9
	1:2	420 µL	12

- Low-density cultures w/ Large Organoids (Definitions: SOP Section 5.1.1.2)

Starting Well#	Splitting Ratio	Volume BME2 to Add	Final #Wells in a 24-Well Plate
4	1:0.5	70 µL	2
	1:1	140 µL	4
6	1:0.5	105 µL	3
	1:2	210 µL	6

5.4.4 Keeping the PDOrg/BME2 solution on ice. Using a P200 Pipetman, carefully pipette 35 µL PDOrg/BME2 droplets into the center of the wells of a 24-well plate (see Figure 5A). Again, **avoid** creating bubbles.

- **Note**: Organoids settle quickly to the bottom of the tube, thus it will be necessary to resuspend the organoid solution each time an aliquot is removed from the tube by gently drawing the solution up and down in the pipet tip.
- **Hint**: Turning the plate diagonally with a corner pointing toward you while plating the domes will increase the visibility in the plate and make placing the dome in the center of the well a bit easier.

5.4.5 Quickly and smoothly, turn plate upside-down and gently place into a 37°C incubator for 20 min so the BME 2 polymerizes (see Figure 5B).

Important: The In Vivo/In Vitro Development and Evaluation Laboratory supporting the PDMR has noticed there can be lot-to-lot variations in the surface coating of the 24-well plate that can result in flattened/spreading domes that is not related to the protein content of the BME2. A new lot of plates should be used.

5.5 Sub-culture PDOrg/BME2 Domes

5.5.1 Remove plate from incubator, return to the hood, place plate right-side up, and add 750 µL Complete Media per well.

5.5.2 Place plate right-side up in a 37°C humidified, 5% CO_2 tissue culture incubator to culture the organoids.

5.5.3 Check PDOrg cultures 1-2 times/week and follow the recommendations for timing of passaging the PDOrg cultures at the beginning of this SOP.

5.6 Feeding PDOrg Cultures

5.6.1 Each week, completely remove the media from the wells and add 750 µL of fresh Complete Media to each well.

6.0 RECOMMENDED QUALITY CONTROL

6.1 Maintain a record of reagents used to prepare media.

6.2 Document vendors and lot numbers of all media components.

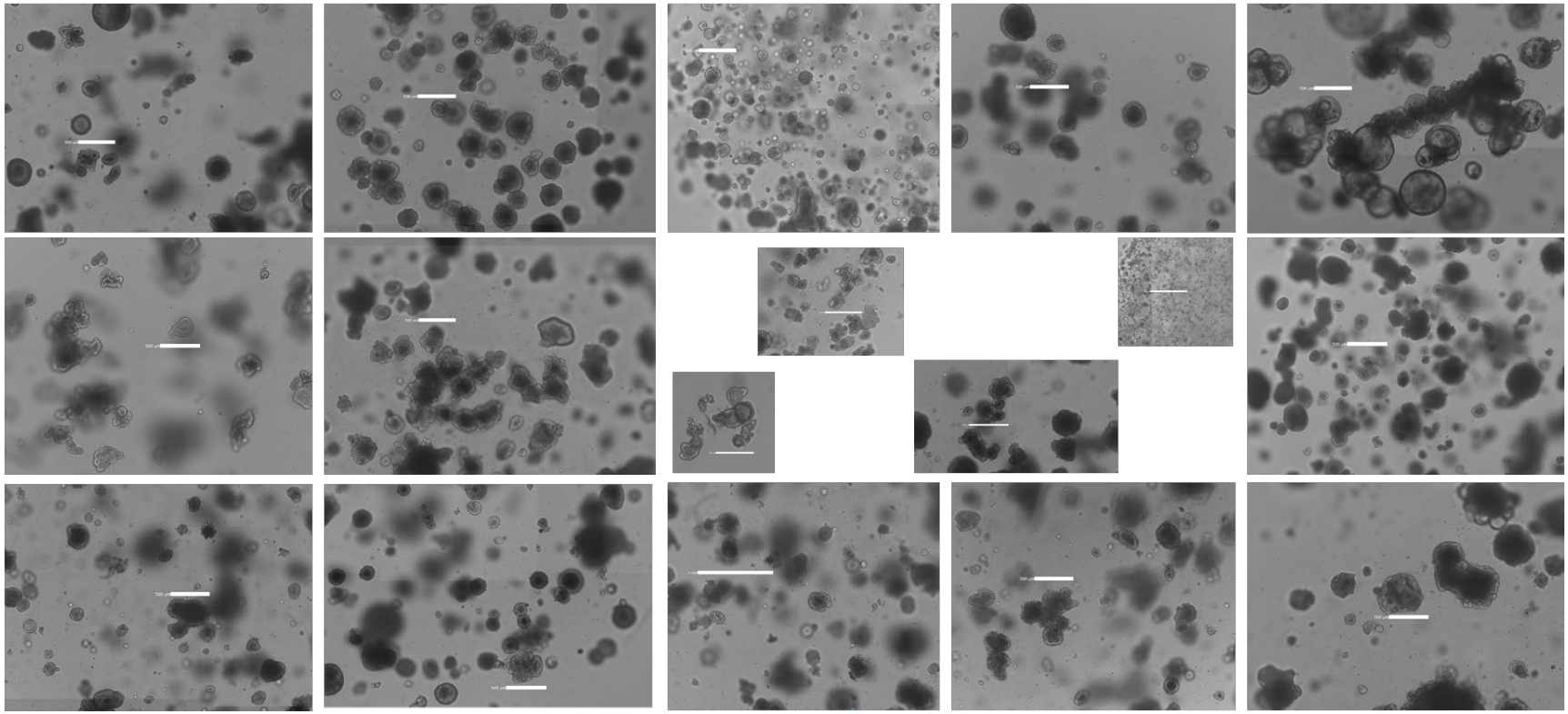
6.3 At change-over, parallel new reagents with existing lots prior to placing a new lot into service.

7.0 REFERENCES

- Sato, T., et al., *Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium.* Gastroenterology, 2011. **141**(5): p. 1762-72. https://www.ncbi.nlm.nih.gov/pubmed/21889923
- Tuveson Laboratory Protocols, Cold Spring Harbor Laboratory. *Murine and Human Organoid Protocols* (Version: 4/27/2016). Link to protocol: http://tuvesonlab.labsites.cshl.edu/wp-content/uploads/sites/49/2017/01/20160427-TuvesonOrganoidProtocols.pdf

APPENDIX 1: PHASE CONTRAST IMAGES OF PDORG CULTURES

Representative images PDOrg cultures from multiple histologies to demonstrate the range of organoid phenotypes, sizes, and growth densities that can be expected. Size of all images adjusted so that they are of similar magnification; scale bars range from 0.5-1 mm.



APPENDIX 2: FIGURES

Figure 1: Example #1 - High density (**A,C**) and low density **(B,D**) PDOrg cultures. (**C, D**) 500 µm scale bar.

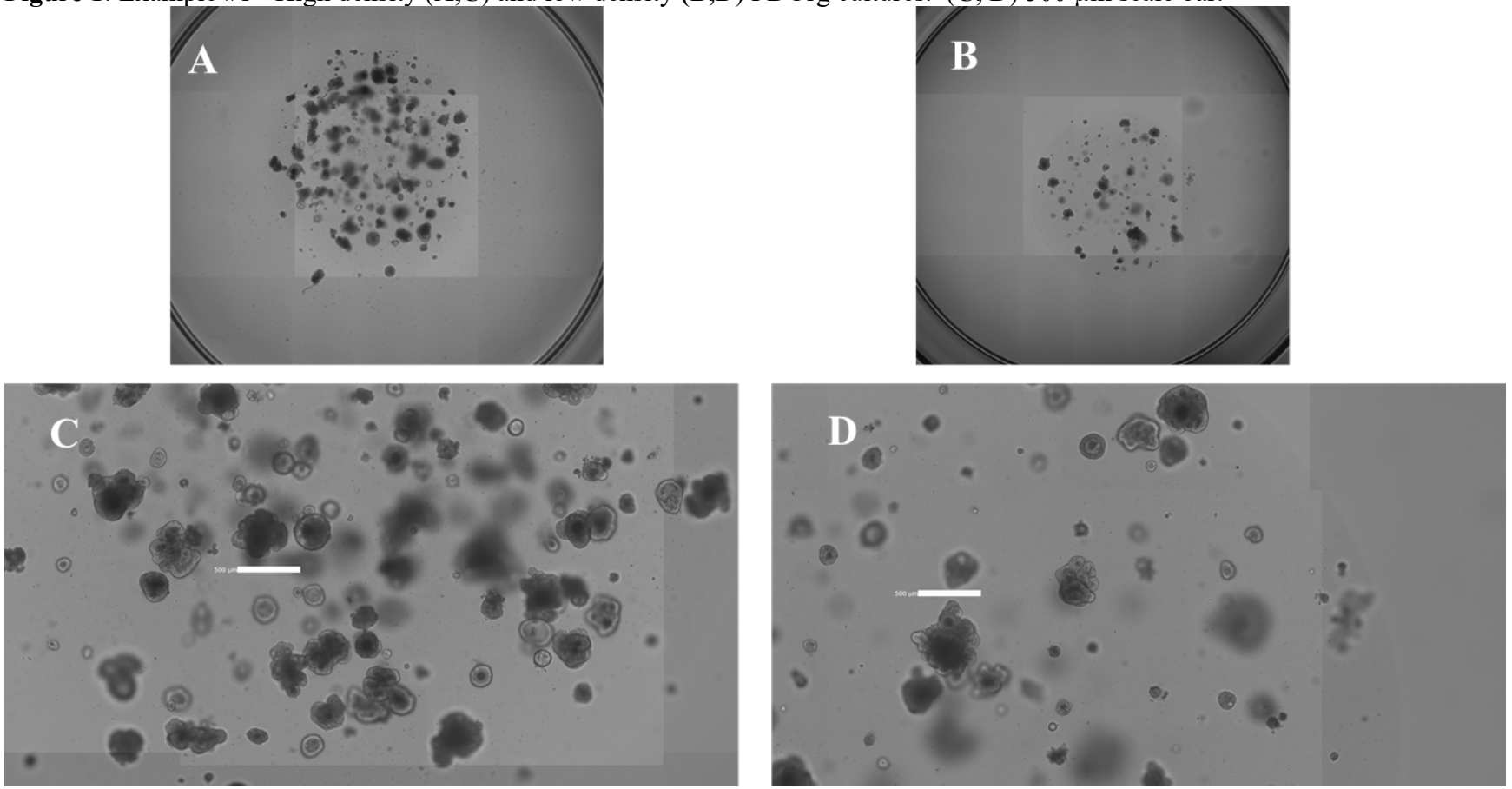


Figure 2: Example #2 - High density (**A,C)** and low density **(B,D**) PDOrg cultures. (**B,C**) 1 mm scale bar, (**D**) 500 µm scale bar.

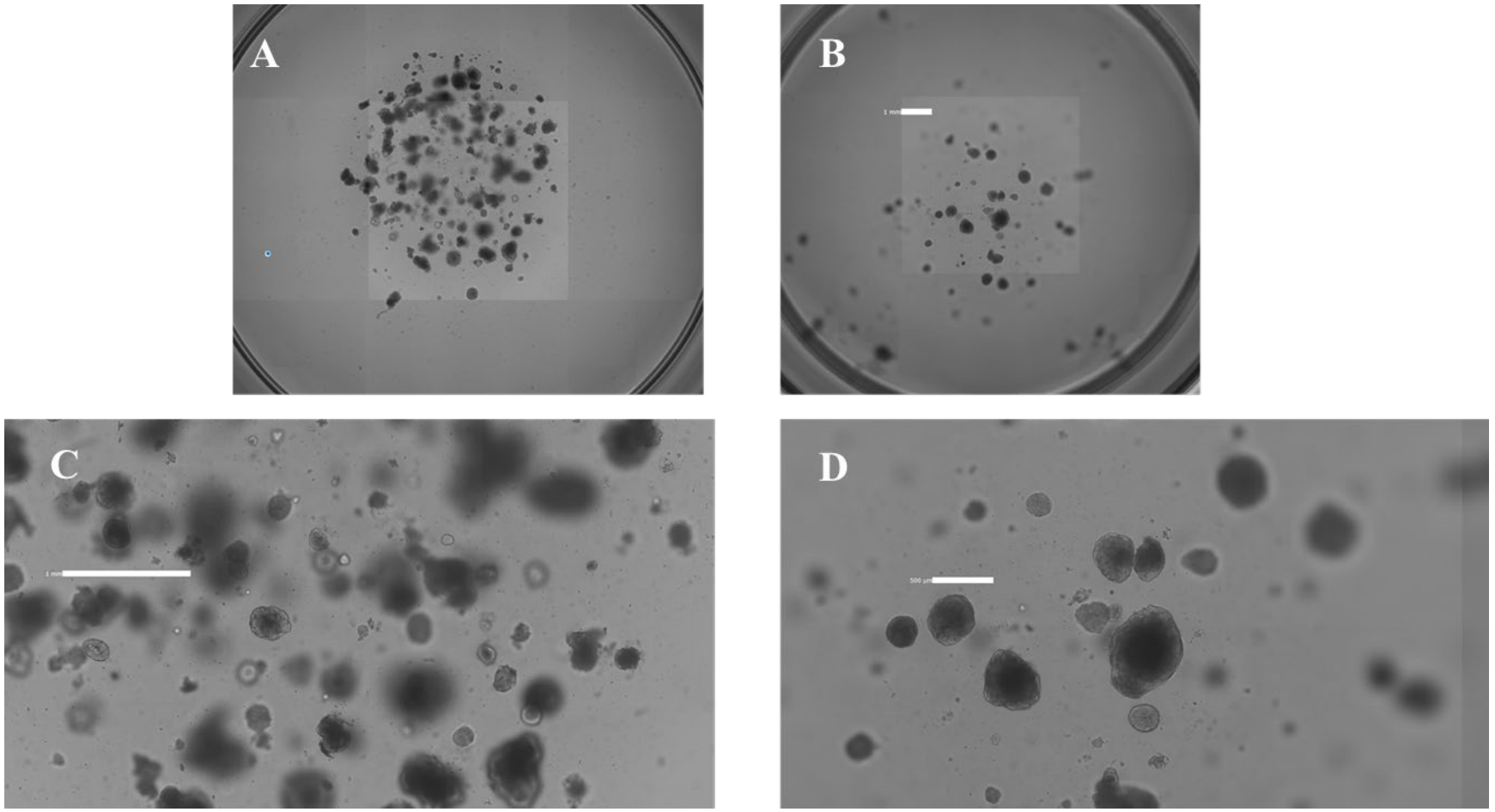


Figure 3: Low and high magnification of a PDOrg culture with large organoids that have "popped" and are at various stages of disintegration (red arrows).

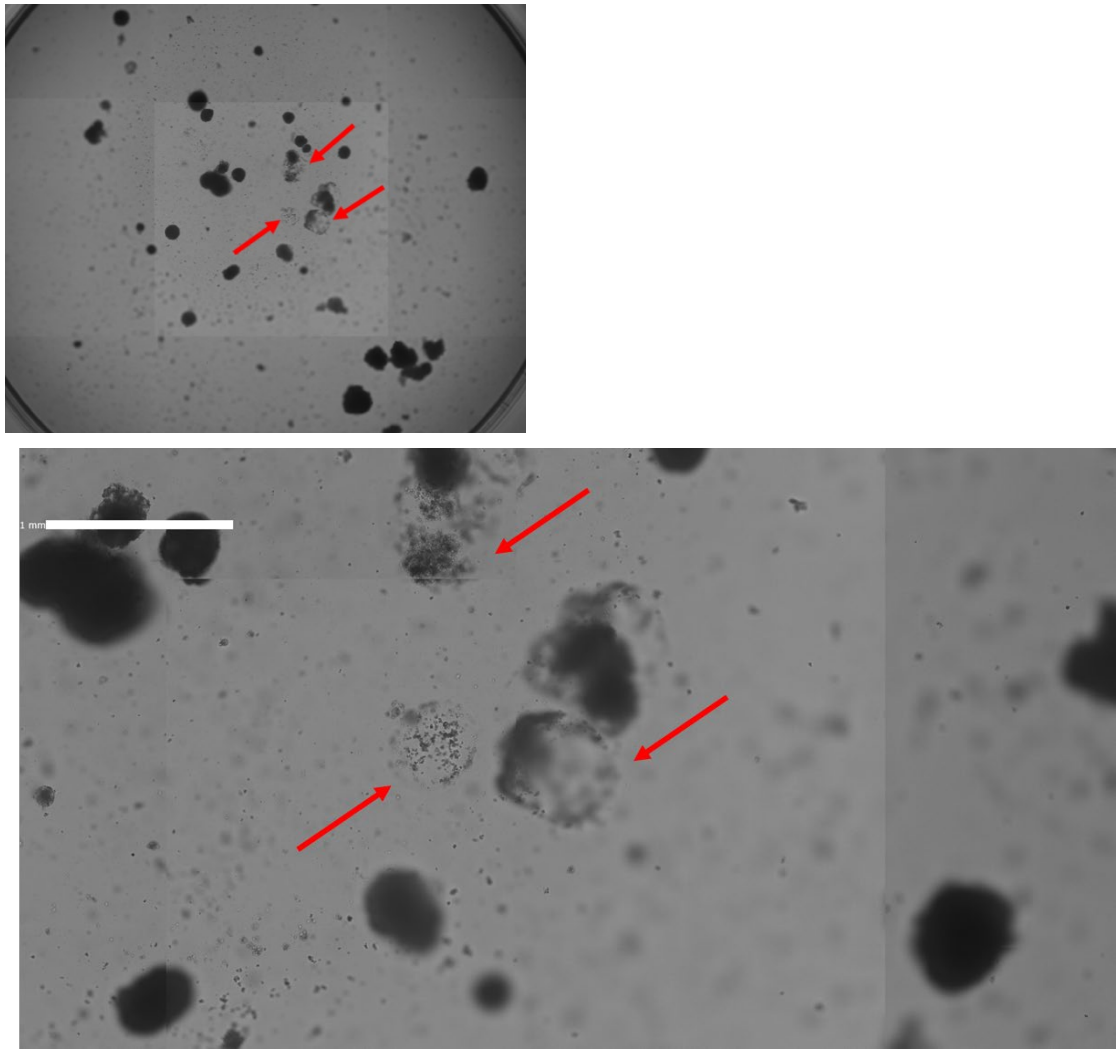


Figure 4: Appearance of pelleted PDOrgs just prior to BME addition.

Figure 5: (A) Appearance of non-polymerized BME2 droplets, freshly pipetted onto bottom of a sterile, 24-well plate. **(B)** Appearance of polymerized BME2 domes, ready for addition of Complete Media