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ADSI –CCO-SOP-3.0

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COLON ORGANOID CULTIVATION AND CRYO-CONSERVATION

Purpose

The SOP-ADSI-3.0 was issued to describe how to maintain and cryopreserve organoids from colon cancer samples and the respective healthy tissue.

Scope

SOP 3.0 is intended to cover all resources, personnel and equipment needed to maintain and cryopreserve organoids from fresh tissue of colon cancer samples as well as healthy tissue.

Introduction

Organoid cultures need to be passaged/splitted in regular intervals to prevent overgrowth causing uncontrolled differentiation and death of cultures. WT organoids can be splitted as single cell suspensions. Tumor organoid cultures should be preferentially splitted as small cell aggregates, which are still visible under the microscope. After segregation in smaller cell-aggregates, organoids can be re-seeded or cryo-conserved. The provided protocol describes technical procedures for organoid splitting and cryo-conservation in liquid nitrogen as well as procedures for the thawing of organoids.

1. Splitting WT organoids / tumor organoids

Reagents, Media and Instrumentation

For reference see SOP-ADSI-1.0

- Thaw required amount of matrigel on ice, always keep on ice

- 0.25% Trypsin / EDTA, 4°C
- Put GF- on ice
- Pre-warmed 24 well Culture Plates
- Prepare required culture media
- 15 ml tube
- Pipette tips and Pipettes (10µl, 200µl, 1000µl)
- Pipette aid and pipettes (10 ml)
- Pre-cool the centrifuge to 4°C

2. Procedure

2.1 Harvest

- Remove medium from the wells (check if the Matrigel is stable otherwise you might loose the cultures!)
- Add 500 µl ice cold GF- to every well
- Detach matrigel drop with 1000 µl tip
- Transfer to 15 ml tube (kept on ice)
- Wash wells with 500 µl cold GF- to collect all cells
- Add GF- up to 10-14 ml
- Centrifuge for 3 min 800 rpm at 4°C
- Remove supernatant (matrigel pellet with the organoids is visible by eye).
- If the matrigel pellet is very big, remove the supernatant and resuspend the pellet with 3ml ice cold GF-. Pipette up and down. Add 7ml of ice cold GF- and centrifuge again for 3 minutes at 1200 rpm at 4°C.

2.2 Trypsinization

- Add 200 to 500 µl 0.25% Trypsin-EDTA (depending on the size of the pellet)
- Pipette up and down
- Incubate 7 min at 37°C (water bath)
- Pipette up and down with a 100 µl pipette tip (if cells will not

get loose either extend the incubation time or attach a 200µl pipette tip to the 1000µl pipette tip)

- Check repeatedly under the microscope (in the tube).
- Repeat incubation cycles until the majority of cells are single cells
- If single-cells still do not appear after 3 times of checking under the microscope, add additional 200 µl 0.25% Trypsin-EDTA and extend incubation cycles at 37°C

2.3 Seeding

- When single cells suspension is obtained, add 10 ml GF-
- Centrifuge for 5 min, 1500 rpm at 4°C
- Optional: cell counting
- Remove supernatant
- Resuspend in 200 µl of GF-
- Count cells (in duplicate)
- Calculate how many cells you need
- Transfer the required amount to a 15 ml tube
- Add 10ml GF-
- Centrifuge for 3 min at 1500 rpm at 4°C
- Remove the supernatant and resuspend in the required amount GF- (example: 10 µl GF-, 20 µl matrigel per well in 24 well plate) and add matrigel according to the dilution.
- Seed 30 µl droplets per well. Pipette fast to avoid cooling of the pre-warmed plate.
- Incubate for 10-15 min at 37°C (5% CO2)
- Carefully add 500 µl pre-warmed medium per well.
- Incubate at 37°C (5% CO2)
- Replace medium every 2 to 3 days.

3. Cryopreservation of Organoids / Tumoroids

Organoids as well as tumor organoids can be stored at -80°C (temporary – days/ a few weeks) and in liquid nitrogen (long term). The best way to freeze them is in the Recovery™ Cell Culture Freezing Medium. It is a ready-to-use complete cryopreservation medium for mammalian cell cultures. In order to achieve the ideal cooling rate of -1°C/ min use a Mr. Frosty freezing container. Freeze 6 wells of a 24 well plate in 500 µl freezing medium. Perform all procedures on ice unless otherwise indicated.

3.1 Reagents, Media and Instrumentation

For reference see SOP-ADSI-1.0

- Put Recovery™ Cell Culture Freezing Medium of GIBCO on ice
- Label cryotubes (passage number, date, organoid ID (e.g. TPIO-19), amount of wells frozen, medium, operator)
- Pre-cool cryotubes on ice
- Mr. Frosty freezing container should be filled with isopropanol according to the manufacturer's protocol and be kept at RT

3.2 Procedure

3.2.1 Harvest organoids

- Remove medium from the wells (check if the matrigel is stable not to lose the cultures!)
- Add 500 µl ice cold GF- to every well
- Detach matrigel drop with 1000 µl tip
- Transfer to 15 ml tube (kept on ice)
- Wash wells with 500 µl cold GF- to collect all cells
- Add GF- up to 10-14 ml
- Centrifuge for 3 min 800 rpm at 4°C
- Remove supernatant (matrigel pellet with the organoids is visible by eye).
- If the matrigel pellet is very big, remove the supernatant and resuspend the pellet with 3ml ice cold GF-. Pipette up and down. Add 7ml of ice cold GF- and centrifuge again for 3 minutes at 1200 rpm at 4°C

3.2.1 .Trypsinisation

- Add 200 to 500 µl 0.25% Trypsin-EDTA (depending on the size of the pellet)
- Pipette up and down
- Incubate 7 min at 37°C (water bath)
- Pipette up and down with a 100 µl pipette tip (if cells will not get loose either extend the incubation time or attach a 200µl pipette tip to the 1000µl pipette tip)
- Check repeatedly under the microscope (in the tube).
- Repeat incubation cycles until the majority of cells are single cells
- If single-cells still do not appear after 3 times of checking under the microscope, add additional 200 µl 0.25% Trypsin-EDTA and extend incubation cycles at 37°C

3.2.1 Freezing – Cyro-Conservation

- Remove supernatant as far as possible
- Resuspend pellet carefully in Recovery™ Cell Culture Freezing Medium
- (500µl / vial) using a 1000µl pipette tip (work fast)
- Transfer the cryovials to the Mr. Frosty
- Put Mr. Frosty at -80°C for at least 2 days
- Put tubes in liquid nitrogen (-196°C) for long term storage
- Tubes can be stored for up to 4 weeks at -80°C
- Put samples to liquid nitrogen after at least 2-4 days at -80°C

4. Cryopreservation of Organoids / Tumoroids

Start organoid culture from frozen vials in the ratio 1:1 or 1:2 from the amount of wells you have frozen. Omit adding the apoptosis inhibitor for at least 4 days after thawing organoids. Perform all procedures on ice unless otherwise indicated.

For reference see SOP-ADSI-1.0:

4.1 Reagents, Media and Instrumentation

- Thaw aliquot matrigel on ice
- Pre warmed 24 well cell culture plates in the incubator
- Always use an aliquot GF-, keep on ice
- Pre-warm water bath

- Put 15 ml tubes on ice
- Pipette tips and Pipettes (10µl, 200µl, 1000µl)
- Pipette aid and pipettes (10 ml)
- Prepare culture medium according the media preparation protocol. Add Y-27632 (concentration according the protocol). Pre-warm at 37°C just before use

4.2 Procedure

4.2.1 Thawing of cells

- Pick up cryovial from liquid nitrogen (Check Biobank – sign vial out from the database)
- Keep vial in dry ice or liquid nitrogen until shortly before thawing
- Thaw in your palm until only a small block of ice is left
- Add 10 ml ice cold GF- to a 15 ml tube
- Pipette the cell suspension carefully from the cryovial into the prepared 15 ml tube with the 10 ml GF-
- Rinse the cryovial with 500 µl GF- to collect all cells and add them to the 15 ml tube
- Centrifuge for 5 min at 1500 rpm at 4°C
- Remove the supernatant

4.2.2 Seeding of cells:

- Resuspend the pellet in GF- (10 µl per well) and matrigel (20 µl per well)
- Use a pre-warmed 24 well plate
- See 30 µl droplet per well (24 well plate)
- Incubate 15 min at 37°C (5% CO2)
- Carefully add 500µl prewarmed desired culture medium (M#2, M#3, M#4, M#5, M#6) with 10 µM Y-27632 per well
- Incubate at 37°C (5% CO2)
- Record passage number: p (form vial) +1
- Refresh medium after 2-4 days with culture medium.

5. Applicable references

ADSI-CCO-SOP-1, ADSI-CCO-SOP-2