Interreg Italia-Österreich
precanmed

ADSI –CCO-SOP-5.0

Author:
P. Filipek, I. Krainer
Approved:
R. Gstir

Issued:
26/03/2019
Revised:

IMMUNOFLUORESCENCE OF ORGANOID CULTURES -WHOLE MOUNT STAINING

Purpose

The ADSI – CCO-SOP-5.0 was issued to describe the procedure how to perform immuno-stainings with organoids as well as tumor organoid samples in order to do organoid characterization.

Scope

ADSI – CCO -SOP-5.0 is intended to cover resources, personnel and equipment needed to perform whole mount immuno stainings with organoids and how to prepare organoid cultures for paraffin embedding.

Introduction

Molecular characterization of organoids can be performed using different techniques. Here ww describe procedures for immunofluorescence analysis of human organoids using antibodies for specific marker proteins.

1. Reagents, equipment and materials

1.1 Equipment

- Laminar Air-Flow Labculture Plus ESCO, Class II BSC
- Centrifuge (4°C) Hettich Zentrifugen ROTIXA A50 RS
- Thermomixer
- Microwave
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)

1.2 Materials

- Sterile forceps
- Sterile scissors

- Sterile scalpel + blades
- Sterile Erlenmeyer flask (200 ml)
- Sterile 15ml tubes
- Sterile 50ml tubes
- Sterile 0,5ml, 1,5ml, 2ml and 5ml tubes
- Set of pipettes 10µl, 20 µl, 200µl, 1000µl and pipet tips
- Pipette Aid and pipettes (10ml, 25ml, 50ml)
- Microscope slides (A.Hartenstien OTMM)
- Coverslips (A.Hartenstien DKR1)
- 96 well plates (Corning CLS3603)
- Box with ice

1.3 Chemicals

- Advanced DMEM/F-12 (Gibco-Thermo Fisher #12634028)
- Glutamax 100x (GIBCO #35050038)
- HEPES (1M) (GIBCO #15630080)
- Pen/Strep 100x (GIBCO #15070063)
- DPBS, 1x no calcium, no magnesium (Gibco #14190-094)
- Sterile water
- Fetal Bovine Serum (Biowest #S1810-500)
- Goat Serum (SIGMA G6767)
- Triton X-100 (SIGMA #11332481001)
- BSA – Albumin Fraktion V (Roth #8076.4)
- PFA 4% (Homemade)
- TO-PRO-3 (ThermoFischer T3605)
- Hoechst 33342 (ThermoFisher H3570)
- Moviol (Homemade)
- IMM (ibidi #50001)
- Ultra PureTM Agarose (Invitrogen #16500-100)
- 70% Ethanol
- Antibodies of interest

1.4 Buffer and Solutions

- **Pen/Strep (100x)**
  (long term storage -20°C)
  Store at 4°C
- **HEPES (1M=100x)**
  Store at 4°C
- **Glutamax (100x)**
  (long term storage at -20°C)
  Store at 4°C
- **GF- (Growth Factors – Medium = Washing Medium)**
  Advanced DMEM/F12 - 500ml
  Pen/strep (100x) - 5ml
  HEPES (1M=100x) - 5ml
  Glutamax (100x) - 5ml

  All buffers and solutions listed above are prepared under sterile conditions in the Laminar Air-Flow
- **Blocking Buffer**
  5% BSA, 10% Goat Serum, 0,2% Triton X-100 in 1xPBS
- **PFA 4%**
  Homemade
- **Permeabilization buffer**
  1% Triton X-100 in 1xPBS
- **Washing buffer**
  1xPBS + 2% FBS

2. Procedures

2.1 Fixation of Organoids / Tumoroids

2.1.1 Preparatory work

- Pre-cool GF-, 1 x PBS and 15 ml falcons on ice
- Cut tips P1000
- 1,5ml tubes (Pre-coated with 100% FBS for >10 min at RT)
- 4% PFA

2.1.2 Harvest

- Remove medium
- Add 0.5 ml of ice cold GF- to each well
- Detach matrigel with P1000 tip

- Pipette gently up and down 5 – 10 x with P1000 (CUT TIP) and transfer to precooled 15 ml falcon
- Repeat for all required wells (not more than 12 wells, 6 wells are optimal)
- Wash wells with 1 ml GF- (optional)
- Add GF- to the final volume of 8 – 10 ml
- Settle the organoids under gravity (10 min) on ice

2.1.3 Wash

- Remove supernatant
- Add 10ml of ice cold GF-
- Invert the falcon several times to gently re-suspend the organoids
- Settle the organoids under gravity (10 min) on ice
- Repeat 3 - 5 times
- Remove supernatant leaving 1ml
- Transfer organoids to pre-coated 1,5ml tube using pre-coated P1000 cut tip
- Wash once more using 1ml ice cold 1xPBS. (optional)
- Settle the organoids under gravity (5-10 min) on ice (optional: centrifuge 3 s small table top centrifuge 2000g)
- Remove supernatant as much as possible, add 500µl Corning Cell Recovery Medium and gently agitate the tube to re-suspend the organoids (optional)
- Incubate on ice for 1 h

2.2.2 Fixation

- Remove supernatant as much as possible
- Add 500 µl of 4 % PFA, gently agitate the tube to re-suspend the organoids
- Incubate for 1h at RT
- Remove supernatant as much as possible
- Add 1 - 1,5 ml 1 x PBS, gently agitate the tube to re-suspend the organoids
- Store the sample at 4˚C

2.2 Immunofluorescent whole-mount staining after PFA fixation in suspension.

2.2.1 Preparatory work

- Permeabilization buffer: 1% Triton X-100 in 1xPBS
- Blocking Solution: 5 % BSA, 10 % Goat Serum, 0.2 % Triton X-100 in 1 x PBS
- Washing buffer: 1 x PBS + 0.5 % FBS

2.2.2 Procedure

Entire protocol is performed in tubes pre-coated with 100 % FBS for more than 10min at RT.

- Remove the 1 x PBS in which organoids were stored
- Permeabilize in 300 µl of 1% TritonX-100, gently agitate the tube to re-suspend the organoids
- Incubate for 30 min at RT
- Settle the organoids under gravity
- Carefully aspirate the solution as far as possible
- Incubate in 300 µl Blocking Buffer (5 % BSA, 10 % Goat Serum, 0.2 % Triton X-100 in 1 x PBS) 1-4h at RT.
- Settle the organoids under gravity
- Carefully aspirate the solution as much as possible
- Incubate with primary antibodies (100 µl / sample in Blocking Buffer) overnight to 24h at 4°C with gentle agitation
- Remove the Primary antibody solution as far as possible
- Wash 5 x 5min:
    - Add 300-500µl 1 x PBS + 0.5 % FBS, gently agitate the vials several times to re-suspend the organoids
    - Incubate 5 – 10 min at RT (allow organoids to settle under gravity)
    - Carefully aspirate the solution
    - Repeat 4 x
- Prepare the secondary antibodies in Blocking Buffer
- Centrifuge the secondary antibodies in Blocking Buffer at 17000g for 5 min before use
- Incubate in secondary antibody (in blocking buffer) for 1-2h at RT
- Wash 2 x 5min: as described above

- Incubate 5min in 250µl of 1xPBS + 0.5%FBS with TOPRO-3 (1µM) or Hoechst (2µg/ml) (optional)
- Wash 2 x 5min: as described above
- Carefully remove the solution leaving 50-80 µl

Transfer organoids in small volume of 1 x PBS + 0.5% FBS (40-80µl) on the coverslip or into the well of 96 well plate using cut P200 tips (resuspend well to get rid of clumps) and leave 20-30 min to sediment, carefully remove the buffer and add 20µl of Moviol or 50µl IBIDI Mounting medium (IMM).

Leave the microscopic glass to dry at RT at least overnight. The 96 well plate is ready for imaging.

4. Reference

ADSI – CCO -SOP-1.0, ADSI – CCO -SOP-2.0, ADSI – CCO -SOP-3.0