EXPERIMENTAL PROTOCOL: Tumor organoid-T-cell co-culture system

Author/s: Mantrana A., Guil-Luna S., Aranda-Aguilar E., Rodríguez Ariza A.

Contact information: ana.mantrana@imibic.org;v22gulus@uco.es

Introduction

This protocol is a procedure for establishment and culture of tumor organoids from human colorectal cancer tissues.

Reagents

- 1X RBC Lysis Buffer (eBioscience, 00-4333-57)
- Colorectal Cancer Organoid Media (see below)
- Dispase type II (Sigma-Aldrich, cat. no. D4693)
- Human recombinant interferon gamma (Peprotech, cat. no. 300-02)
- Human serum, from human male AB plasma (Sigma-Aldrich, cat. no. H3667)
- Lymphoprep (Stem Cell, 07851)
- Matrigel (Corning #354230)
- Mouse anti-human CD28 (eBioscience,CD28.2, cat. no. 16-0289-81, RRID:AB_468926)
- Organoid Culture Media (see below)
- Recombinant Human Interleukin-2 (Bio-Rad, PHP042)
- T-cell culture medium (see below)
- T-cell thawing medium (see below)
- TrypLE Express (Gibco, cat. no. 12604-013)
- Ultraglutamine type I (Lonza, cat. no. BE17-605E)

Instruments and tools

- Falcon tubes, 15 mL (Sarstedt, cat. no. 62.554.502)
- Falcon tubes, 50 mL (Sarstedt, cat. no. 62.547.254)
- Plates, 24 well (Greiner, cat. no. 662160)
- Plates, 6 well (Greiner, cat. no. 657165)
- Plates, 96 well, U-bottom (Greiner, cat. no. 650180)

Before start

Preparation of Reagents:

Organoid Culture Medium (OCM)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
Advanced DMEM/F12	Sigma #D6421	500 mL	486.9 mL	1x
HEPES	Media Kitchen (Life Technologies #15630080)	1 M	5 mL	10 mM
Glutamax	Media Kitchen (Life Technologies #35050-061)	100x (200 mM)	5 mL	1x (2 mM)
Zell Shield	Minerva Biolabs GmbH, Berlin, Germany	100x	5 mL	1x

Colorectal Cancer Organoid Media (CRC Medium)

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
OC Medium	See above	-	47.75 mL	1x
A 83-01	TOCRIS #2939-10mg	5 mM	5 µL	0.5 µM
B27	ThermoFisher Sc. #12587010	50x	2000 µL	2x
EGF	Sigma Aldrich #SRP3027-500UG	500 ug/mL	25 µL	0.25µg/ml
Gastrin	Sigma Aldrich #G9145-0.5MG	1 mg/mL (480.7461 uM)	50 µL	1 ug/mL (480.7461n M)
N acetyl Cyst	Sigma Aldrich #A9165-5G	5g/50mL (612.78mM)	100 µL	1.23mM
SB202190	Sigma Aldrich #S7067-5MG	3.333 mg/mL = 10.0592 mM	25 µL	5. 0296 µM
Y27632	Sigma Aldrich #Y0503-5mg	10mM	50 µL	10 µM

T-cell thawing medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
RPMI 1640	Gibco, cat. no. 11875093	500 mL	44 mL	1x
ZellShield	Minerva Biolabs GmbH, Berlin, Germany	100x	0.5 mL	1x
FBS	Biowest, S1810	500 mL	5 mL	10%
Ultraglutamine I	Lonza, cat. no. BE17-605E		0,5 mL	1%

T-cell culture medium

Component	Company/Cat#	Stock Conc.	Volume	Final Conc.
RPMI 1640	Gibco, cat. no. 11875093	500 mL	44 mL	1x
ZellShield	Minerva Biolabs GmbH, Berlin, Germany	100x	0.5 mL	1x
Human Serum	Sigma-Aldrich, cat. no. H3667	50 mL	5 mL	10%
Ultraglutamine I	Lonza, cat. no. BE17-605E	100 mL	0,5 mL	1%

PROCEDURE

Priorities:

1) PBMCs activation towards tumour samples.
2) Expansion of activated PBMCs.
3) Performance of downstream assays such as cytotoxicity assays or cell death assays.

Organoid isolation for co-culture (day -2)

1. Aspirate medium from 24-well plate containing organoids and resuspend in 500 µL of pre-heated (37°C) dispase II (2mg/mL PBS). This allows organoids isolation from matrigel.
2. Resuspend organoids with a p1000 pipette and incubate at 37°C for 15 minutes.
3. Transfer organoid suspension to a 15 mL Falcon tube.
4. Add 100 µL EDTA (0.5 M) for every 1 mL of dispase used and fill up tube up till 10 mL with PBS
5. Pellet organoids (300g, 5', RT) and aspirate supernatant.
6. Resuspend organoids in complete organoid culture medium and plate 2-4 mL/ well of a tissue culture-treated 6-well plate. Use 2 mL for every well used in step 1.
7. Culture organoids for 24h at 37°C.

Human PBMCs isolation for co-culture and co-culture preparation (day -1)

Human PBMCS isolation:

1. Collect blood samples and mix an equal volume of blood and PBS up to a final volume of 50 mL per Falcon tube.
2. Prepare 50 mL Falcon tubes containing 15mL Lymphoprep previously warmed to RT. Slowly, transfer 35 mL of blood-PBS mixed samples to each lymphoprep Falcon.
3. Centrifuge for 20 minutes at 2500 rpm, RT, adjusting acceleration to 1 and no break.
4. There will be a bottom layer of Red Blood Cells, followed by a thin layer of PBMC and then a layer of plasma. Remove plasma to reach the PBMC. Collect PBMC in a separate tube by carefully pipetting up the cells from the layer.
5. Wash the collected PBMCs with PBS (up to 50 mL) at 1500 rpm, 10 minutes, RT at maximum break and acceleration. Discard supernatant.

6. If necessary, remove erythrocytes by resuspending the pellet in 3 mL of RBC lysis buffer and incubate for 10 minutes RT.
7. Wash with 3mL PBS and remove supernatant. (1500rpm, 10 minutes, RT)
8. Resuspend and count PBMCs using a hemocytometer or automated cell counter.
9. Culture PBMCs at 2 x10^6 cells/mL in T-cell culture medium with no interleukin 2 (resting) for 24 hours at 37°C.

Co-culture preparation (day -1)

1. Add 200 ng/mL IFNγ to organoids to enhance antigen presentation
2. Coat tissue culture-treated 96-well U-bottom plate with 5 µg/mL anti-CD28 in PBS (50 µL/well).
3. Wrap plate in parafilm and incubate for 24 h at 4 °C.

Co-culture (day 0)

1. Collect medium containing organoids in suspension that have been incubated with IFNγ overnight and spin down (300g, 5', RT). Remove supernatant.
2. Resuspend pellet in 1 mL/well TrypLE and combine with remaining cells that have adhered to the culture plate. 63) Incubate for 5-15' at 37 °C.
3. Resuspend every few minutes and check using a microscope if organoids or big clusters of cells are still present. Stop when they have dissociated into single cells. Some small cell clusters are aceptable.
4. Transfer dissociated organoids to 15 mL Falcon tube and fill up with PBS.
5. Pellet dissociated organoids (300g, 5', RT).
6. Remove supernatant and resuspend pellet in 1 mL T cell culture medium. Count cells using a hemocytometer.
7. Resuspend dissociated organoids at 5 x 10^4 cells/mL in T cell culture medium.
8. Resuspend PBMC and count using hemocytometer or automated cell counter.
9. Wash PBMC in PBS (500g, 5', RT).
10. Resuspend PBMC at 1x10^6 cells/mL in T cell culture medium, supplemented with 2ng/mL IL-2 (2x concentrated).
11. Mix equal volume of dissociated organoids and PBMC for a PBMC: tumor cell ratio of 20:1.
12. Wash anti-CD28-coated plate 2x with PBS. Do not leave plate dry.
13. Plate 200 µL dissociated organoid / PBMC suspension per well and incubate at 37°C during 24-48h.
14. Proceed with downstream assays.