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SOP –ADSI-3.0

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DRUG-SCREENING OF TUMOR AND WT ORGANOIDS IN CO-CULTURE WITH CAF CONDITIONED MEDIA

Purpose

The SOP-ADSI-3.0 was issued to describe how to perform drug screenings of tumor or WT organoids cultured with CAF conditioned media.

Scope

SOP-ADSI-3.0 is intended to cover all resources, personnel and equipment needed to perform drug screenings of organoids with CAF conditioned media. This SOP is preceded by SOP-ADSI-1.0 for organoid generation and SOP-ADSI-2.0 for generation of CAF conditioned medium.

Introduction

Cancer cells are embedded in a complex microenvironment of stromal cellular components. Components of this sort are fibroblasts. In context of tumors these fibroblasts are called cancer-associated fibroblasts (CAFs). Fibroblasts are producers of many growth factors and hormones, with involvement in tumorigenesis by remodeling of the extracellular matrix. In addition, they can promote the malignancy of tumors. Frequently used cell culture platforms represent only one cell type, lacking a complex tumor microenvironment. Tumor organoids in combination with CAFs derived media constitutes an improved tool to identify patient-specific therapy options by drug-screens. Therefore, generated tumor organoids are cultivated with CAFs conditioned medium. The organoids are treated with a library of drugs for 24 and 72 hrs, with subsequent assessment of organoid viability by bioluminescence. In advance to the drug screens tumor or wt organoids need to be established from patient biopsies (SOP-ADSI-1.0) with parallel generation of CAFs conditioned medium (SOP-ADSI-2.0). The aim of this SOP is to provide protocols for the analysis of tumor or WT organoids viability in response to anti-cancer drugs. *Table .1* proposes a time schedule for drug testing on a week-day basis.

Weekday	Day	Media	RTG timepoint	Imaging
Friday	0	Seed in respective cultivation medium	-	-
Saturday	1	-	-	-
Sunday	2	-	-	-
Monday	3	CAFs conditioned medium + drug / vehicle + RTG	1 h, 2 h	+
Tuesday	4	-	24 h	+
Wednesday	5	-	48 h	+
Thursday	6	-	72 h	+

Table 1. Time schedule for drug testing

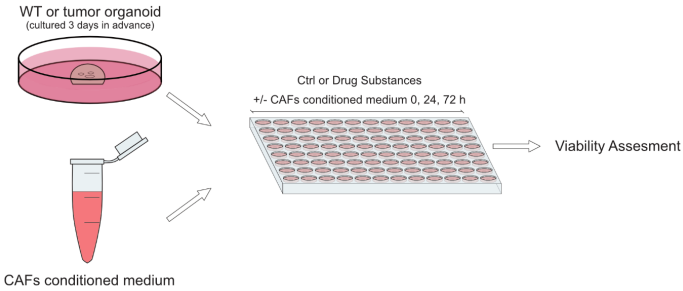


Figure 1. Schematic procedure – Drug-screening of tumor or WT Organoids in co-culture with CAF conditioned media

1. Reagents, solutions, and cell culture media

1.1 Reagents

- Real Time – GloTMMT Cell Viability Assay (#G9711 Promega)
- Matrigel (#356231 Corning)
- DPBS, 1 x no calcium, no magnesium (Gibco#14190-094)
- Pen/Strep (100X) (#15070063 GIBCO)

- Primocin – 500mg (Cat.Code: ant-pm-1 InvivoGen)
- Advanced DMEM/F-12(#12634028 Gibco-Thermo Fisher)
- Glutamax 100 x (#35050038 GIBCO)
- HEPES (1 M) (#15630080 GIBCO)
- Fetal Bovine Serum (#S1810-500 Biowest)
- WCM – Wnt3a-conditioned media (2016 Broutier)*
- RCM – R-Spondin-conditioned media (2016 Broutier)**
- NCM – Noggin-conditioned media (2016 Broutier)***
- R-Spondin (#120-38 PEPROTECH) optional instead of RCM (100 ng/mL)
- Noggin (#250-38 PEPROTECH) optional instead of NCM (1000 ng/mL)
- Wnt3a – if required 100 ng/mL (PEPROTECH)
- Nicotinamide (#N0636-100G Sigma-Aldrich)
- N-Acetylcyteine (#A9165-25G Sigma-Aldrich)
- B-27 (#17504044 Thermo Fisher)
- m-EGF (#315-09 PEPROTECH)
- SB 202190 (S7067- Sigma-Aldrich 5MG)
- A83-01 (#2939 R&D Systems Europe)
- Gastrin (#3006/1 R&D Systems Europe)
- Prostaglandin E2 (#P0409 Sigma-Aldrich)
- Y-27632 (#120129 Abcam)

*Wnt3a conditioned medium was obtained from L-Wnt3a cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CTUtrecht, The Netherlands

**R-spondin1-conditioned medium was produced from 293T-HA-Rspol-Fc producer cell line; MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CTUtrecht, The Netherlands

***Noggin-conditioned medium from HEK293-mNoggin-Fc cell line. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

1.2 Compositions

- **GF-**

500 mL Advanced DMEM/F12

5 mL P/S (100X)

5 mL of HEPES (1M = 100X)

5 mL of Glutamax (100X)

Store at 4 °C

- **RCM**

Store at -20 °C

Thaw on ice

(4 mL for the generation of 40 mL media)

- **NCM**

Store at -20 °C

Thaw on ice

(2 mL for the generation of 40 mL media)

- **WCM (50%)**

Freshly prepared (every 2 weeks)

Store at 4°C

- **Nicotinamide (1 M = 100X)**

1,464 g

Fill up to 12 mL with 1 x PBS

Dissolve by shaking and leave at RT

Filter sterilize 0,22 µm

Make aliquots 500 µl and store at -20°C

(5000 µl for 500 mL culture medium = 10 mM)

- **N-Acetyl cysteine solution**

3 mL sterile water

245 mg N-Acetyl cysteine

Dissolve by shaking and leave at RT

Filter sterilize 0,22 µm

Make 500 µl aliquots in Eppendorf tubes and store at -20 °C

(1250 µl for 500 mL culture medium)

- **B27 (50X)**

Thaw on ice

Prepare 2 mL aliquots

Store at -20°C

(1000 µl for 500 mL culture medium)

- **m-EGF (500 µg/mL = 10000x)**

Dissolve 500 µg m-EGF in 1000 µl PBS/0.1 % BSA

Make 50 µl aliquots, store at -20 °C

(4 µl in 40 mL culture medium or 50 µl in 500 mL culture medium = 50 ng/mL)

- **Y-27632 (10 mM = 1000x)**

10 mg

Dissolve in 3122 µl sterile water

Make 400 µl aliquots (10 mM)

(40µl in 40 mL culture medium = 10 µM)

Store at -20°C

- **A83-01 (major stock = 50 mM and 1,5 mM = 3000x)**

Stock:

Dissolve 10 mg A83-01 in 474 µl DMSO

Make 18 µl aliquots (50 mM)

Store at -20°C

Pre-dilution stock:

Quickly thaw an 18 µl major stock aliquot

Dilute with 582 µl DMSO

Make 13,3 µl aliquots (1,5 mM = 3000 x)

(13,3 µl for 40mL culture medium)

Store at -20°C

- **SB202190 (30 mM = 3000x)**

Dissolve 5 mg SB202190 in 503 µl DMSO

Make 13,3 µl aliquots

(13,3 µl for 40 mL culture medium)

Store at -20°C

- **Gastrin (100 µM = 10000x)**

Dissolve 1 mg gastrin

in 4,77 mL PBS (without Calcium and Magnesium)

Make 50 µl aliquots or smaller

(4 µl for 40 mL culture medium = 10 nM)

Store at -20°C

- **PGE2 (100 µM = 10000x)**

Dissolve 1 mg PGE2 in 4,77 mL PBS (without Calcium and Magnesium)

Make 50 µl aliquots or less

(4 µl for 40 mL culture medium = 10 nM)

Store at -20°C

- **Primocin (500x)**

(80 µl for 40 mL culture medium = 2x)

Store at -20°C

2. Equipment

- Incubator Binder APT. lineTM (150CE2)
- Laminar Air-Flow Labculture Plus ESCO, Class II BSC
- Micro Plate Reader (Mithras LB940)
- Centrifuge (4°C) Hettich Zentrifugen ROTIXA A50 RS
- Brightfield microscope Motic AE31 and camera ProgRes CF cool JENOPTIKA
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)
- Waterbath
- Polystyrene box
- Box with crashed ice
- Pipette Aid
- Set of pipettes 10 µL, 200 µL, 1000 µL and pipette tips

3. Plastics

- 24-well-plates (Greiner bio-one #662160)
- 96-well-plates (Corning #3603)
- 50 mL conical tubes (#ET5050B Euroclone)
- 15 mL conical tubes (#ET5015B Euroclone)
- Sterile 0,5 mL, 1,5 mL, 2mL and 5 mL tubes
- Pipettes 10 mL, 25 mL, 50 mL

4. Preparation of tumor organoids and CAFs conditioned media

Before drug screening WT or tumor organoids are generated and cultured in accordance to SOP-ADSI-1.0 in 24 well plates. In parallel, CAFs conditioned media are generated in accordance to SOP-ADSI-2.0.

5. Seeding of organoids into 96-well-plates

5.1 Preparation of material and reagents

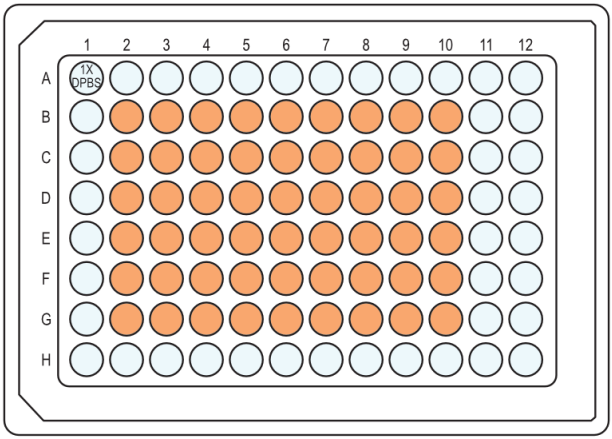
Harvest organoids after expansion from 24-well-plates according to SOP-ADSI-1.0 (Proceed here directly after step 8.4.6.). One well of organoids from a 24-well-plate is required per drug testing condition.

- Matrigel: thaw 1 mL aliquot on ice (keep on ice until usage)
- Box with crashed ice
- Put GF- on ice
- Pre-warm 96-well-plates (Corning #3603) at 37 °C in incubator
- Prepare and pre-warm required culture media (complete ready to use media for WT or tumor organoids (see SOP-ADSI-1.0 1.3. & 1.4.)
- 15 mL conical tube
- Pipette tips and pipettes (10µl, 200µl, 1000µl)

5.2 Procedure of seeding

- Resuspend the cells in GF- at 1.5 x 106 cells/mL in the required amount GF- (example: 3.3 µl GF- and 6.7 µl Matrigel per well in 96-well-plate) resulting in 5000 cells/ well.
- Seed 10 µl droplets per well. Pipette fast to avoid cooling of the pre-warmed plate (seeding scheme see Figure 2)
- Incubate for 10-15 min at 37 °C, 5 % CO2 and 95 % r.H.
- Carefully add 150 µL pre-warmed organoid cultivation medium per well.
- Incubate at 37 °C, 5 % CO2 and 95 % r.H. for 3 days (see *Figure 2*).
- After 3 days, organoids are ready for the drug screening (RTG viability assay).

Figure 2. Seeding scheme of organoids into 96-well-plate. Orange coloured wells indicate wells with organoids.



6. Drug treatment of organoids cultured with CAFs conditioned medium

This SOP shows a simple example with 2 different drug substances + 1 control (vehicle) tested on one batch of organoid culture. The experimental setup allows the comparison of the anti-cancer activity of a drug in context of CAFs conditioned media from the same patient.

6.1 Preparation of material and reagents

- Put Real-Time-Glo (RTG) Kit at RT, vortex and centrifuge
- Pre-warm CAFs conditioned medium (24 h and 72 h medium) at 37 °C
- Prepare and pre-warm organoid cultivation medium at 37 °C (protocol see *SOP-ADSI-1.0* or *Table 2.*)
- 5x stock solution of the RTG enzyme and substrate is prepared in GF-. This is essential to guarantee equal amounts of Real-Time-Glo throughout the entire assay.
- The 5x RTG in GF-/CAFs conditioned medium is then used to prepare the 5x drugs/vehicle stock solutions (The 5x solution mixed with the 1,25x medium results in a 1x medium in the well). The amount of stock solution required depends on how many wells per 96 well plate is to be tested.

Media	Expansion media			M#2 +Y	M#3 +Y	M#4 + Y	M#5 + Y	M#6+Y = WT +Y
Compound	**Stock conc.**	**Final conc.**	**Dilution**	**µL**	**µL**	**µL**	**µL**	**µL**
Final volume				5500.00	5500.00	5500.00	5500.00	5500.00
GF- / CAF conditioned medium				3379.20	3376.82	3377.37	3374.98	624.43
WCM	1x	50 %	2x	-	-	-	-	2750.00
RCM	10x	10 %	10x	550.00	550.00	550.00	550.00	550.00
NCM	20 x	5 %	20x	275.00	275.00	275.00	275.00	275.00
mEGF	10000x	50 ng/mL	10000x	-	0.55	-	0.55	0.55
Nicotinamide	1 M	10 mM	100x	55.00	55.00	55.00	55.00	55.00
N-acetyl*	500 mM	1.25 mM	400x	13.75	13.75	13.75	13.75	13.75
B27	50 x	1 x	50x	110.00	110.00	110.00	110.00	110.00
Y-27632 (ROCK-inh)	10 mM	10 µM	1000x	5.50	5.50	5.50	5.50	5.50
A83-01 (TGFß-inh)	1.5 mM	500 nM	3000x	-	-	1.83	1.83	1.83
SB202190 (P38 inh)	30 mM	10 µM	3000x	-	1.83	-	1.83	1.83
Primocin	50 mg/mL	100 µg/ mL	500x	11.00	11.00	11.00	11.00	11.00
Gastrin	100 µM	10 nM	10000x	0.55	0.55	0.55	0.55	0.55
PGE2	100 µM	10 nM	10000x					0.55
Volume without GF- or CAF conditioned medium				1020.80	1023.18	1022.63	1025.02	3775.57
1.25 stock medium (total)				4400.00	4400.00	4400.00	4400.00	4400.00

Table 2. Media composition

*N-acetyl needs to be added last because of its low pH!

6.2 Drug Treatment

- Remove medium from wells of 96-well-plate (do not touch Matrigel drop. Use P1000+P10 tip).
- Carefully add 80 µL of pre-warmed 1.25x media
- Add 20 µL of pre-warmed 5x RTG in GF- or CAF conditioned medium containing drugs or vehicle, as indicated by *Figure 3*
- Incubate at 37 °C, 5 % CO2 and 95 % r.H.

- Perform luminescence measurement at time points: 1 h, 4 h, 24 h, 48 h, and 72 h.
- Take pictures of every well with organoids at time points: 1 h,24 h, 48 h, 72 h, (optionally also 96 h, 120 h, and 144 h).
- If you plan to image longer than 72 h, then refresh medium after 72 hours to medium without enzyme and substrate, but with drug substance.

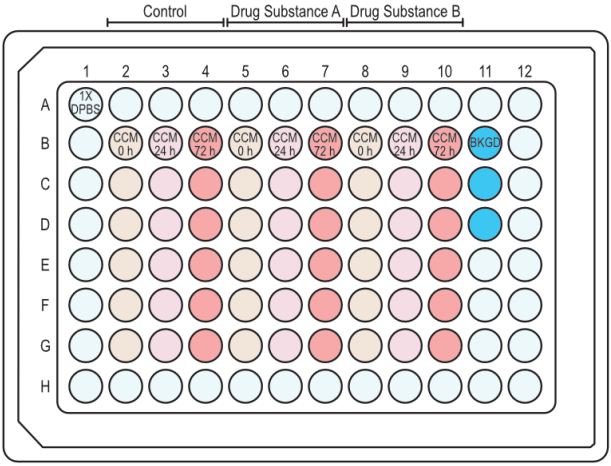


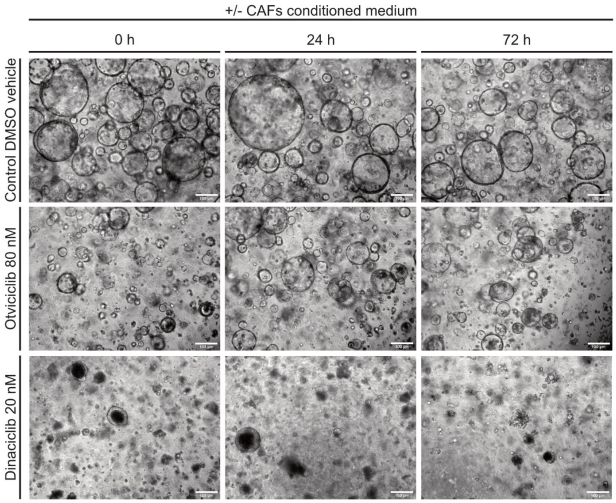
Figure 3. Drug-screening pipetting scheme. CCM = CAFs conditioned media, BKGD = Background. CCM 0 h = organoid cultivation medium (no CAF conditioned medium), CCM 24 h and 72 h = organoid cultivation medium with CAF conditioned medium (24 h or 72 h respectively) instead of GF-.

6.3 Measurement

- Switch on the luminometer (Mitras) at least 1 h before measurement.
- Turn on the PC and open the program MikroWin 2000.
- Open program to measure luminescence for 1 and 0.5 s according to your instrument setting. Make sure that the luminometer is at 37 °C
- Use a filter-set allowing luminescence measurement.
- The measurement is very temperature sensitive. Keep plates in a styrofoam box for transfer from the incubator to luminometer. Try to be as fast as possible to prevent the plate from cooling down.
- Export the data as EXCEL file for further calculations

Figure 4 gives an example of organoids cultured with CAFs conditioned media and treatment with CDK-inhibitors by images.

Figure 4. Example of brightfield images from drug-screening of tumor or WT Organoids in co-culture with CAFs conditioned media. Size bar = 100 µm.



7. Applicable to ADSI SOPs

SOP-ADSI-1.0

SOP-ADSI-2.0

8. References

Broutier L, Andersson-Rolf A, Hindley C J, Boj S F, Clevers H,Koo B K, Huch M. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat Protoc. 2016 Sep;11(9):1724-43.doi: 10.1038/nprot.2016.097. Epub 2016 Aug 25.

Lebret SC, Newgreen DF, Thompson EW, Ackland ML. Induction of epithelial to mesenchymal transition in PMC42-LA human breast carcinoma cells by carcinoma-associated fibroblast secreted factors. Breast Cancer Res. 2007;9(1):R19–R19.

Lo Y-H, Karlsson K, Kuo CJ. Applications of Organoids for Cancer Biology and Precision Medicine. Nat Cancer. 2020;1(8):761–73.

NOTES