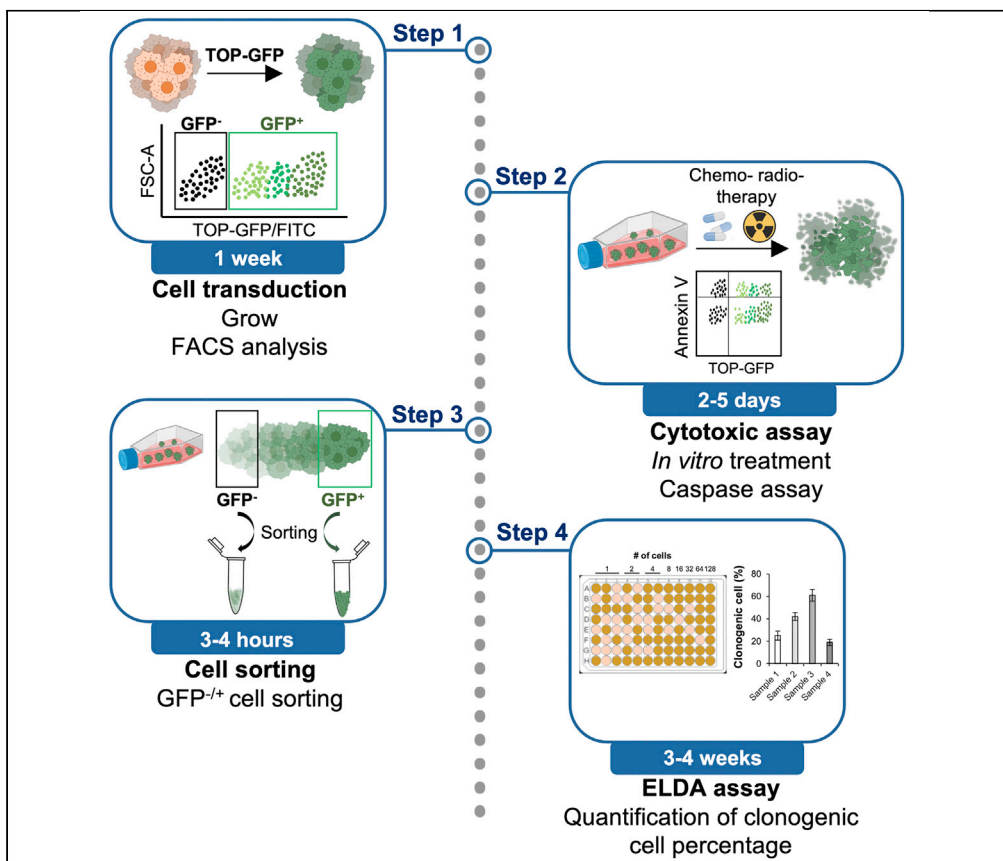


Protocol

FACS-based protocol to assess cytotoxicity and clonogenic potential of colorectal cancer stem cells using a Wnt/ β -catenin signaling pathway reporter



Cancer stem cells (CSCs) play a key role in tumor initiation and progression. A real-time tool to evaluate the activation of CSC-specific signaling pathways is crucial for the study of this cancer cell subset. Here, we present a protocol to monitor, *in vitro*, the activation of Wnt/ β -catenin signaling pathway, which is considered a functional biomarker for colorectal CSCs (CR-CSCs). This flow-cytometry-based protocol allows it to isolate CR-CSCs and to evaluate their cytotoxicity upon anti-tumor treatments.

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Highlights

Protocol to investigate the CSC cytotoxicity based on the use of Wnt reporter

Stable lentiviral transduction of colorectal cancer cells with Wnt reporter

Flow cytometry analysis of apoptosis induction in Wnt^{+/-} colorectal cancer cells

Extreme limiting dilution analysis (ELDA) on colorectal cancer cells

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Protocol

FACS-based protocol to assess cytotoxicity and clonogenic potential of colorectal cancer stem cells using a Wnt/ β -catenin signaling pathway reporter

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SUMMARY

Cancer stem cells (CSCs) play a key role in tumor initiation and progression. A real-time tool to evaluate the activation of CSC-specific signaling pathways is crucial for the study of this cancer cell subset. Here, we present a protocol to monitor, *in vitro*, the activation of Wnt/ β -catenin signaling pathway, which is considered a functional biomarker for colorectal CSCs (CR-CSCs). This flow-cytometry-based protocol allows it to isolate CR-CSCs and to evaluate their cytotoxicity upon anti-tumor treatments.

For complete details on the use and execution of this protocol, please refer to Di Franco et al. (2021).

BEFORE YOU BEGIN

Lentiviral particles preparation

⌚ Timing: 5 days

This section describes the transfection protocol of early passage HEK293 cells (15–40 passages) to produce lentiviral particles, using 2nd generation helper plasmids.

1. HEK293 cells preparation for transfection.
 - a. Twenty-four hours before the transfection (i.e., Monday, at 16:00), seed $0.8\text{--}1 \times 10^6$ HEK293 cells (or 293T cells) in a 25 cm² Corning® cell culture flask (canted neck, vented cap) with 7 mL of Eagle's Minimum Essential Medium (EMEM), supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics/antimycotics (complete EMEM).

Note: FBS should be heat-inactivated before use by heating it for 30 minutes at 56°C with mixing, to inactivate complement proteins.

- b. Gently swirl the flask to avoid the formation of cell clumps in the middle of the flask.
- c. Incubate at 37°C, 5% CO₂ for 18–24 h.



Note: Be aware that after 40 passages, HEK293 cells start changing their morphology, thus randomly affecting the transfection efficiency.

2. HEK293 cells transfection for lentiviral particles production.
 - a. At the time of transfection (i.e., Tuesday, at 16:00), after 18–24 h of incubation, the HEK293 cells should have reached 70%–80% of confluence.

△ CRITICAL: cell confluence >80% should be avoided since it could affect the transfection efficiency.
 - b. Add 500 μ L of Opti-MEM Medium, 12 μ L of X-tremeGENE HP DNA Transfection Reagent, 2.6 μ g of psPAX2 (#12260, Addgene), 1.4 μ g of pMD2.G (#12259, Addgene) (second generation helper plasmids) and 4 μ g of TOP-GFP.mC lentiviral vector plasmid (#35491, Addgene), or FOP-GFP.mC (#35492, Addgene) as control (ratio X-tremeGENE HP:DNA = 3 μ L : 2 μ g), into a 2 mL polypropylene tube and gently swirl the tube. The mix needs to be incubated for at least 30 min at room temperature (23°C–25°C) before proceeding to next steps.
 - c. Before transfection, replace the cell culture medium of HEK293 cells with 7 mL of EMEM (10% FBS) without antibiotics/antimycotics, which could interfere with the transfection efficiency.
 - d. Add the transfection mix to HEK293 cells in a drop wise manner and incubate at 37°C, 5% CO₂.
 - e. After 16–24 h of transfection (i.e., Wednesday, at 9:00), gently remove the cell culture medium from the flask and replace it with 7 mL of complete EMEM.
3. Lentiviral particles collection and storage.
 - a. After 48 h from transfection (i.e., Thursday, at 16:00), gently collect the cell culture medium from transfected HEK293 cells (first batch, to be stored at 4°C for 24 h) and replace it with fresh complete EMEM.

Note: At this time, it is highly recommended to check the expression of RFP in HEK293 cells transfected with both TOP-GFP.mC and FOP-GFP.mC lentiviral plasmids, since this is a useful indicator of transfection efficiency. Indeed, both the plasmids constitutively express the mCherry RFP, but only cells transfected with TOP-GFP.mC will express GFP according to Wnt pathway activity (due to a mutation in the TCF/LEF response elements of FOP-GFP.mC plasmid, used as control) If RFP expression is not observed after 48 hours, be aware that the transfection protocol may have not worked properly.

- b. After 72 h from transfection (i.e., Friday, at 16:00), gently collect the second batch of cell culture medium from transfected HEK293 cells and mix it with the first batch collected the day before.
- c. Spin the collected medium at 1500 g to remove most of the cell components from the supernatant.
- d. Filter the medium with 0.45 μ m filters to avoid having any residual HEK293 cell and debris in the medium.
- e. The lentiviruses are concentrated using the Lenti-X Concentrator (for detailed instructions visit <https://www.takarabio.com/documents/User%20Manual/PT4421/PT4421-2.pdf>). The obtained pellet is resuspended in 1/10 to 1/100th of the original volume, using Opti-MEM Medium, and stored at –80°C. One fresh lentivirus aliquot should be used for titration to determine the multiplicity of infection (MOI), following a standard titration protocol for lentiviral preparations carrying a fluorescent protein marker (<https://www.addgene.org/protocols/fluorescence-titering-assay/>). As an alternative, high-titer lentiviral preparations can be obtained by performing ultracentrifugation in presence of sucrose (Brown et al., 2020).

▣▣ Pause point: Proceed with the following steps of the protocol. Alternatively, lentiviral particles can be stored at –80°C for long storage (up to 1 year).

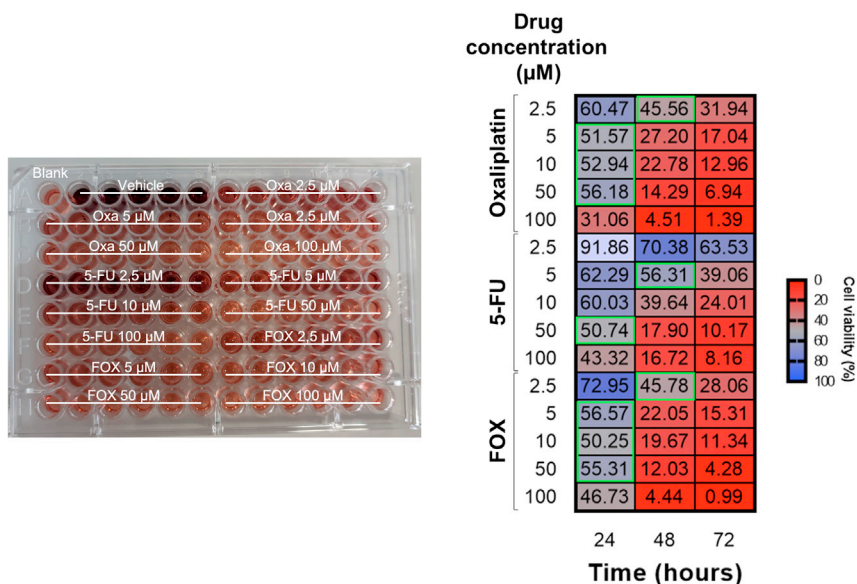


Figure 1. IC₅₀ drug screening

Plate layout for MTS proliferation assay to evaluate the IC₅₀ of anti-tumor drugs, or combinatorial treatments, at 48 h (left panel). Heatmap of cell viability calculated from drug dose-response analysis of HCT-116 cell line treated with Oxaliplatin, 5-FU or drug combination (FOX). Green boxes indicate IC₅₀ values for each time point. Cells treated with vehicle have been used as control for cell viability, calculated as [(Absorbance of cells treated with drug / Absorbance of cells treated with vehicle) * 100].

Note: When working with lentiviral plasmid in absence of constitutive fluorescent protein marker expression, as TOP-GFP (cat. #35489, Addgene), or FOP-GFP as control (#35490, Addgene), lentiviral particles titration can be performed by isolating lentiviral RNA using the NucleoSpin RNA Virus kit (cat. #740956, Macherey-Nagel) and quantifying it using the Quant-X One-Step qRT-PCR TB Green kit (cat. #638317, Takara).

Chemotherapeutics preparation and cell treatment

⌚ Timing: 1 h

4. Preparation of 5-FU and Oxaliplatin solution.
 - a. Resuspend the powders in DMSO under sterile conditions to obtain a 10 mM solution. These reconstituted drugs are stable for at least 2 years if stored at -80°C .
5. *In vitro* treatment of colorectal cancer (CRC) cells (<50 passages).
 - a. Before proceeding with treatment of CRC line, perform a dose-response curve to determine the IC₅₀ of both single and combinatorial treatment, at different time points (Figure 1).
 - b. Cells are treated with Oxaliplatin 2.5 µM, and after 3 h with 5-FU 5 µM, by mimicking the clinically used schedule and doses for FOX (de Gramont et al., 2000; Sorbye et al., 2004).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's Phosphate Buffered Saline	Euroclone	Cat# ECB4004L
Bovine Serum Albumin	Sigma-Aldrich	Cat# A9418

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Trypsin-EDTA	Euroclone	Cat# ECB3052D
StemPro Accutase™ Cell Dissociation Reagent	Thermo Fisher Scientific	Cat# A1110501
5-FU	Selleckchem	Cat# S1209
Oxaliplatin	Sigma-Aldrich	Cat# O9512
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega	Cat# G3582
X-tremeGENE™ HP DNA Transfection Reagent	Sigma-Aldrich	Cat# 06366236001
Polybrene	Sigma-Aldrich	Cat# H9268
CS&T beads (FacsLyric)	BD Biosciences	Cat# 656505
CS&T beads (FacsMelody)	BD Biosciences	Cat# 661414
Accudrop beads (FacsMelody)	BD Biosciences	Cat# 661612
Fetal Bovine Serum (FBS)	Corning	Cat# 35-079-CV
EDTA	Merck	Cat# E6758
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	Cat# 15250061
Dimethyl sulfoxide (DMSO)	Merck	Cat# D5879
Eagle's Minimum Essential Medium (EMEM)	ATCC	Cat# 30-2003
DMEM, high glucose	Thermo Fisher Scientific	Cat# 11965084
RPMI-1640 Medium	ATCC	Cat# 30-2001
McCoy's 5a Medium Modified	ATCC	Cat# 30-2007
Advanced DMEM/F-12	Thermo Fisher Scientific	Cat# 12634010
HEPES (1 M)	Thermo Fisher Scientific	Cat# 15630106
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat# 25030024
N-Acetyl-L-cysteine	Sigma-Aldrich	Cat# A7250
N-2 Supplement (100X)	Thermo Fisher Scientific	Cat# 17502048
Penicillin-Streptomycin 100X (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140122
Animal-Free Recombinant Human EGF	Peprotech	Cat# AF-100-15
Animal-Free Recombinant Human FGF-basic (154 a.a.)	Peprotech	Cat# AF-100-18B
B-27 Supplement (50X), serum free	Thermo Fisher Scientific	Cat# 17504044
Nicotinamide	Sigma-Aldrich	Cat# N3376
Gastrin I human	Sigma-Aldrich	Cat# G9020
OPTI-MEM	Thermo Fisher Scientific	Cat# 31985070
Antibiotic-Antimycotic 100X	Euroclone	Cat# ECM0010D
Lenti-X Concentrator	TaKaRa	Cat# 631232
Critical commercial assays		
Brilliant Violet 421 Annexin V	BioLegend	Cat# 640924
Cell Staining Buffer	BioLegend	Cat# 420201
Annexin V Binding Buffer	BioLegend	Cat# 422201
CaspGLO Red Active Caspase Staining Kit	BioVision	Cat# #K190
CaspGLOW™ Red Active Caspase-3 Staining Kit	BioVision	Cat# #K193
NucleoSpin RNA Virus kit	Macherey-Nagel	Cat. #740956
Quant-X One-Step qRT-PCR TB Green kit	Takara	Cat. #638317
Recombinant DNA		
psPAX2	Addgene	Cat# 12260
pMD2.G	Addgene	Cat# 12259
TOP-GFP.mC (with constitutive RFP)	Addgene	Cat# 35491
FOP-GFP.mC (with constitutive RFP)	Addgene	Cat# 35492
TOP-GFP (only Wnt-driven GFP)	Addgene	Cat# 35489
FOP-GFP (only Wnt-driven GFP)	Addgene	Cat# 35490
Experimental models: Cell lines		
HEK293	ATCC	Cat# CRL-1573
293T	ATCC	Cat# CRL-3216
DLD-1	ATCC	Cat# CCL-221
HT-29	ATCC	Cat# HTB-38
HCT-116	ATCC	Cat# CCL-247

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
ELDA: Extreme Limiting Dilution Analysis Software	ELDA software	http://bioinf.wehi.edu.au/software/elda/
Graphpad Prism 8	GraphPad Software	http://www.graphpad.com/scientificsoftware/prism/
FlowJo_v10.7.1	BD	https://www.flowjo.com/solutions/flowjo
Other		
25 cm ² Rectangular Canted Neck Cell Culture Flask with Vent Cap	Corning	Cat# 430639
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	Thermo Fisher Scientific	Cat# 339650
Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes	Thermo Fisher Scientific	Cat# 339652
Vortex mixer VORTEXCLONE	Euroclone	Cat# EMXS
Medical Millex-GP Syringe Filter Unit, 0.22 µm, polyethersulfone, 33 mm, sterilized by gamma irradiation	Merck	Cat# SLGPM33RS
Millex-HP Syringe Filter Unit, 0.45 µm, polyethersulfone, 33 mm, gamma sterilized	Merck	Cat# SLHP033R
1.7 mL microcentrifuge tubes	Costar	Cat# 3620
Costar® 6-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates, Individually Wrapped, Sterile	Corning	Cat# 3471
Nunclon Delta Surface 6-well plate	Thermo Fisher Scientific	Cat# 140675
Costar® 24-well Clear Flat Bottom Ultra-Low Attachment Multiple Well Plates, Individually Wrapped, Sterile	Corning	Cat# 3473
Costar 24-well Clear TC-treated Multiple Well Plates, Individually Wrapped, Sterile	Corning	Cat# 3526
70 µM cell strainer	Merck	Cat# CLS431751
40 µM cell strainer	Merck	Cat# CLS431750
FACSMelody cell sorter	BD	Cat# 653885
Falcon 5 mL Round Bottom High Clarity PP Test Tube, with Snap Cap, Sterile	Corning	Cat# 352063

MATERIALS AND EQUIPMENT

HEK293 cell culture medium

Reagent	Final concentration	Amount
Eagle's Minimum Essential Medium (EMEM)		445 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Antibiotic-Antimycotic 100X	1X	5 mL
Store at 2°C–8°C until expiration date		

Alternatives: DMEM high glucose can be used instead of EMEM.

DLD-1 cell culture medium

Reagent	Final concentration	Amount
RPMI-1640		445 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Antibiotic-Antimycotic 100X	1X	5 mL
Store at 2°C–8°C until expiration date		

HT-29 and HCT-116 cell culture medium

Reagent	Final concentration	Amount
McCoy's 5a Medium Modified		445 mL
Fetal Bovine Serum (FBS)	10%	50 mL
Antibiotic-Antimycotic 100X	1X	5 mL
Store at 2°C–8°C until expiration date		

Primary CRC organoids cell culture medium

Reagent	Final concentration	Amount
Advanced DMEM/F-12		457.5 mL
Hepes (1 M)	10 mM	5 mL
L-Glutamine (200 mM)	2 mM	5 mL
N-Acetyl-L-cysteine (stock solution 500 mM)	1 mM	1 mL
N-2 Supplement (100X)	1X	5 mL
Penicillin-Streptomycin 100X (10,000 U/mL)	1X	5 mL
Animal-Free Recombinant Human EGF (stock solution 50 µg/mL)	50 ng/mL	500 µL
Animal-Free Recombinant Human FGF-basic (154 a.a.) (stock solution 100 µg/mL)	100 ng/mL	500 µL
B-27 Supplement (50X), serum free	1X	10 mL
Nicotinamide (stock solution 500 mM)	10 mM	10 mL
Gastrin I human (stock solution 10 µM)	10 nM	500 µL

Store at 4°C for 2 weeks.

As alternative strategy, EGF and basic-FGF can be freshly added only to the daily used aliquot of medium, as to do so, the cell culture medium can be stored at 4°C for 2 months.

N-Acetyl-L-cysteine stock solution

Reagent	Final concentration	Amount
N-Acetyl-L-cysteine	500 mM	4.079 g
ddH ₂ O	n/a	50 mL

Dissolve 4.079 g of N-Acetyl-L-cysteine in 50 mL of bi-distilled sterile water in a 50 mL tube and mix by inverting the tube (or using vortex mixer) until complete dissolution is reached. Sterilize the solution by filtering it with 0.22 µm filters and aliquot in sterile (autoclavable) 1.7 mL microcentrifuge tubes (1 mL per aliquot). Store at –20°C until use. Upon reconstitution, the aliquot should be used within six months.

Animal-free recombinant human EGF stock solution

Reagent	Final concentration	Amount
Animal-Free Recombinant Human EGF	50 µg/mL	1 mg
ddH ₂ O	n/a	20 mL

Dissolve 1 mg of Animal-Free Recombinant Human EGF in 20 mL of bi-distilled sterile water in a 50 mL tube and mix by inverting the tube (or using vortex mixer) until complete dissolution is reached. Sterilize the solution by filtering it with 0.22 µm filters and aliquot in sterile (autoclavable) 1.7 mL microcentrifuge tubes (500 µL per aliquot). Store at –20°C until use. Upon reconstitution, the aliquot should be used within six months.

Animal-free recombinant human FGF-basic (154 a.a.) stock solution

Reagent	Final concentration	Amount
Animal-Free Recombinant Human FGF-basic	100 µg/mL	1 mg
PBS 0.1% BSA	n/a	10 mL

Dissolve 1 mg of Animal-Free Recombinant Human FGF-basic in 10 mL of sterile PBS 0.1% BSA in a 15 mL tube and mix by inverting the tube (or using vortex mixer) until complete dissolution is

reached. Sterilize the solution by filtering it with 0.22 μm filters and aliquot in sterile (autoclavable) 1.7 mL microcentrifuge tubes (500 μL per aliquot). Store at -20°C until use. Upon reconstitution, the aliquot should be used within six months.

Nicotinamide stock solution		
Reagent	Final concentration	Amount
Nicotinamide	500 mM	1.22 g
ddH ₂ O	n/a	20 mL

Dissolve 1.22 g of Nicotinamide in 20 mL of bi-distilled sterile water in a 50 mL tube and mix by inverting the tube (or using vortex mixer) until complete dissolution is reached. Sterilize the solution by filtering it with 0.22 μm filters and aliquot in 15 mL tubes (10 mL per aliquot). Store at -20°C until use. Upon reconstitution, the aliquot should be used within six months.

Gastrin I human stock solution		
Reagent	Final concentration	Amount
Gastrin I human	10 μM	0.5 mg
PBS 0.1% BSA	n/a	23.8 mL

Dissolve 0.5 mg of Gastrin I human in 23.8 mL of sterile PBS 0.1% BSA in a 50 mL tube and mix by inverting the tube (or using vortex mixer) until complete dissolution is reached. Sterilize the solution by filtering it with 0.22 μm filters and aliquot in sterile (autoclavable) 1.7 mL microcentrifuge tubes (500 μL per aliquot). Store at -20°C until use. Upon reconstitution, the aliquot should be used within six months.

STEP-BY-STEP METHOD DETAILS

Lentiviral transduction of cancer cells

⌚ Timing: 1 week

This protocol is aimed at the lentiviral transduction of CRC cells for the stable expression of Wnt-reporter gene.

1. Cell plating.
 - a. Collect cells at single-cell level by trypsinization (or by exposing them to Accutase).

Note: This protocol can be applied to both commercial CRC cell lines (grown in adhesion) and primary isolated CRC cells (grown in suspension as spheroids, in ultra-low adhesion plastic) (see [Figure 2](#)). Commonly commercially available CRC cell lines that have already been efficiently transduced with TOP-GFP reporter plasmids include DLD-1, HT-29 and HCT-116 ([Vermeulen et al., 2010](#)).

- b. Count viable cells by using Trypan blue and plate 500,000 cells in a well of a 6 well plate (adhesion or ultra-low adhesion, depending on the cell type) with 2 mL of cell culture medium supplemented with 8 $\mu\text{g}/\text{mL}$ of Polybrene (to increase the lentiviral transduction efficiency).

⚠ CRITICAL: a prolonged exposure to polybrene could affect cell viability. Before proceeding with lentiviral transduction of cells it would be useful to assess the sensitivity of the cells to polybrene in terms of concentration and exposure time to set the best transduction condition.

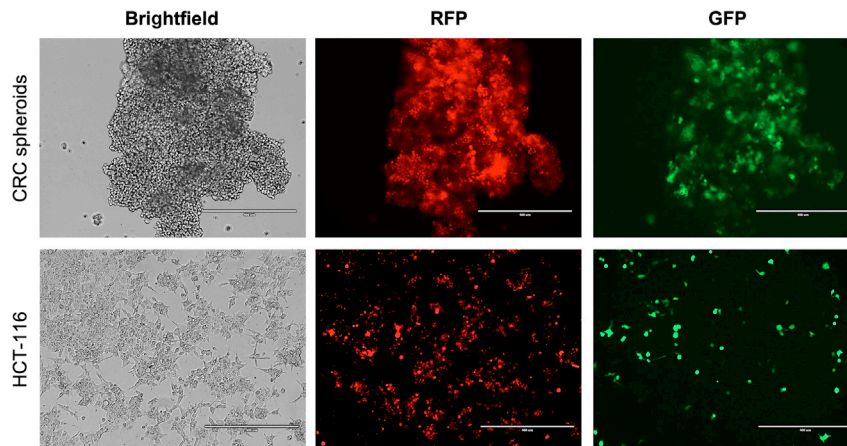


Figure 2. Transduction of CRC cells with Wnt reporter construct

Fluorescence analysis of lentiviral transduction efficiency in CRC spheroids (grown in suspension) and HCT-116 CRC cells (grown in adhesion). Scale bar, 400 μm .

2. Lentiviral transduction.

- a. Add lentiviral particles, diluted or concentrated (MOI of 10-100), to the cells and mix gently.

Note: The optimal multiplicity of infection (MOI) for each cell line to be stable transduced should be previously assessed to obtain the best balance between transduction efficiency and cell viability (<https://www.genecopoeia.com/resource/lentivirus-thats-my-moi-and-im-sticking-to-it-lentivirus-moi-cell-lines/>).

- b. Incubate the cells at 37°C, 5% CO₂ for 24 h.

- c. After 24 h from lentiviral transduction, put the cells back in culture in fresh cell culture medium.

3. Evaluation of lentiviral transduction.

- a. After 72 h from lentiviral transduction the cells should start expressing the constitutive RFP to be observed under a fluorescent microscope. On the other hand, the expression of GFP should be heterogeneous, with GFP^{-/+} cells, according to their Wnt pathway activity (Figure 2).

- b. After expansion of the transduced cells, it would be useful to evaluate the transduction efficiency by FACS, and eventually, enrich the cells for the RFP⁺ cell population (Figure 3) (see step #7 for Brilliant Violet 421 Annexin V staining, to discriminate alive cells within TOP-GFP cells).

Pause point: Proceed with the following steps of the protocol. Alternatively, sorted RFP⁺ cells can be stored in liquid nitrogen for long storage (>1 year).

Note: Typical transduction efficiency obtained using this protocol is around 75%–85%.

Cytotoxicity assay of cancer cells

⌚ Timing: 2–5 days

This section describes a protocol for determining the cytotoxicity of certain treatment on cancer cell subpopulation with differential Wnt/ β -catenin signaling based on TOP-GFP intensity. It is conventionally believed that CSC population, usually displaying high Wnt/ β -catenin activity, is more resistant to therapies (Di Franco et al., 2014; Lenos et al., 2018; Vermeulen et al., 2010). With the Wnt/ β -catenin

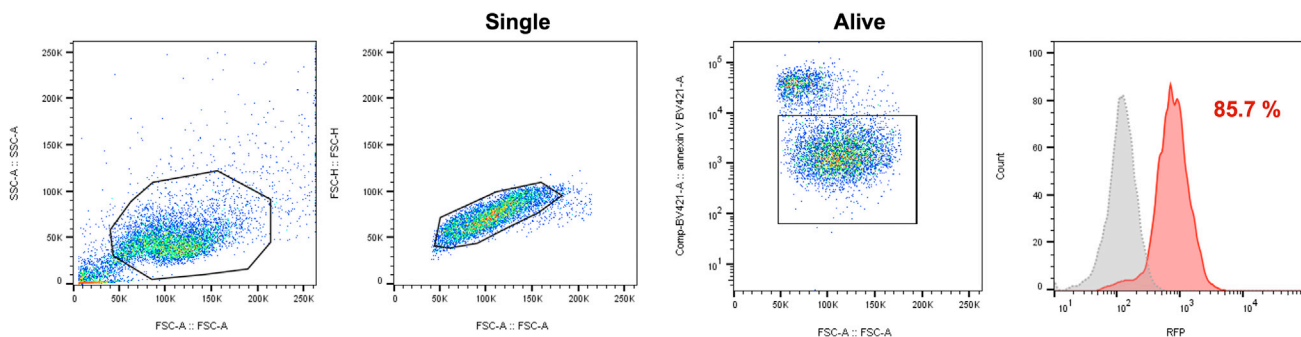


Figure 3. Evaluation and sorting of transduced CRC cells
Gating strategy for the sorting of TOP-GFP RFP⁺ transduced CRC spheroids.

signaling reporter TOP-GFP, CSC population that exhibit high TOP-GFP signal could be specifically screened in cytotoxicity assessment. Consequently, this protocol allows researchers to distinguishably assess the efficiency of the treatment on cancer stem cell or more differentiated population, by FACS, analyzing the induction of cell death (using a dye compatible with GFP and mCherry-RFP) in GFP^{high} (Wnt^{high}-CSCs) versus GFP^{low-neg} (Wnt^{low-neg}-progenitor/differentiated) CRC cells.

4. Plate Cells.

- a. Harvest RFP⁺-sorted TOP-GFP transduced cells by trypsinization. Mechanically open the cell clumps by pipetting up and down with harsh pressure if cells grow in sphere or island.

△ CRITICAL: When pelleting the treated cells growing in adhesion cell culture conditions, for Brilliant Violet 421 Annexin V staining, make sure to collect the supernatant and pool it together with the cell suspension after the trypsinization. The less viable cell population would detach and float up after treatment. To avoid losing this population and bringing in artificial bias in calculation of apoptotic percentage, floating cells should be collected as well as attached ones. Additionally, high speed (no more than 500 g) should be used to spin and pellet in order to ensure that the less viable cells are properly pelleted.

- b. Count the living cells with Trypan Blue exclusion and seed 50,000 cells per well, in a 24 well plate. Treatment conditions could be set up in duplicate or triplicate according to experiment design.
- c. Culture cells at 37°C, 5% CO₂ overnight (8–16 h) before treatment. Proceed to next step if cells grow in suspension.

5. Treatment.

- a. Dilute drugs and prepare solvent control.
- b. Add the diluted drugs or solvent control in a dropwise manner.
- c. Culture the cells for different time, according to time point setting.

Note: Before to perform a flow cytometry analysis of cell viability in Wnt⁺ versus Wnt⁻ cells, it would be useful to perform a dose-response curve of cancer cells to 5-FU+Oxaliplatin chemotherapy (or any other treatment), to select the best option in terms of drug concentration and time point to perform the analysis (or the following sorting of cells spared by the treatment) (see Figure 1).

6. Collect and pellet the treated cells.

- a. Collect the supernatant in 15 mL polypropylene centrifuge tube. Label the tubes properly.
- b. Trypsinize and collect the cells in the corresponding tubes. Dissociate the clumps by pipetting up and down with harsh pressure if cells grow in sphere or island, in order to obtain a single-cell suspension.
- c. Spin at 750 g for 4 min and pellet the cells.

Note: Untreated cells, and cells treated with high concentration of drugs (previously determined by the IC₅₀ study), could be used as Annexin V staining negative and positive controls, respectively.

7. Label the cells with Brilliant Violet 421 Annexin V.
 - a. Wash cells twice with the Staining Buffer (cat. #420201) and resuspend cells in Annexin V Binding Buffer (cat. # 422201) at a concentration of 1×10^6 cells/mL (minimum volume 100 μ L)
 - b. Transfer 100 μ L of cell suspension in a Falcon 5 mL Round Bottom High Clarity PP tube and add 5 μ L of Brilliant Violet 421 Annexin V.

Note: When working with the fluorescent substrate, keep it always in the dark as it is light sensitive.

- c. Gently vortex the cells, and incubate them for 15 min at R.T., in the dark.
 - d. Add 400 μ L of Annexin V Binding Buffer (cat. # 422201) to each tube. Analyze by flow cytometry.
8. Filter the cells through a 70 μ m cell strainer, to avoid the formation of cell clumps.

Note: The use of a 40 μ m cell strainer is highly recommended to avoid collection of cell doublets, or when working with sticky cells, to further prevent the formation of cell clumps.

9. Measure the fluorescence of Brilliant Violet 421 Annexin V by Flow Cytometry.
 - a. Start up BD FACSMelody cell sorter (for detailed instruction visit: <https://www.bdbiosciences.com/content/dam/bdb/marketing-documents/BD-FACSMelody-FACSCorus-Quick-Reference-Guide.pdf>).
 - b. Run daily fluidics startup.

Optional: perform the flow cell clean.

- c. Install the sort nozzle.
 - d. Run Cytometer Setup and Drop Delay
 - i. Prepare a tube of B&D CS&T RUO beads by mixing 500 μ L of PBS + 2 drops of beads.
 - ii. Vortex the mix of beads for at least 20 s before loading the tube and performing the Optical configuration.
 - iii. Prepare a tube of Accudrop beads by mixing 500 μ L of PBS + 1 drop of beads.
 - iv. Vortex the mix of beads for at least 20 s before loading the tube and performing the drop delay.
 - e. Create protocol by setting forward scatter (FSC), side scatter (SSC), FITC, PE and Brilliant Violet421 Channels for measurement, for an appropriate gating strategy (Figure 4).

Note: Given the heterogeneous activation of Wnt pathway in CRC cells, it is always required the analysis of untransduced cells, as Wnt negative control (see small inset in the third panel of Figure 3). This will lead to a correct discrimination of Wnt^{neg} versus Wnt^{pos} (high and low, defined as ~10% highest versus ~10% lowest positivity) CRC cells.

- f. Run and record the experiment (Figure 5).
 - g. Export FCS files for data plotting and analysis.
10. Data analysis on FlowJo software.

Cell sorting + extreme limiting dilution analysis (ELDA)

⌚ Timing: 3–4 hours (cell sorting) + 3–4 weeks (ELDA)

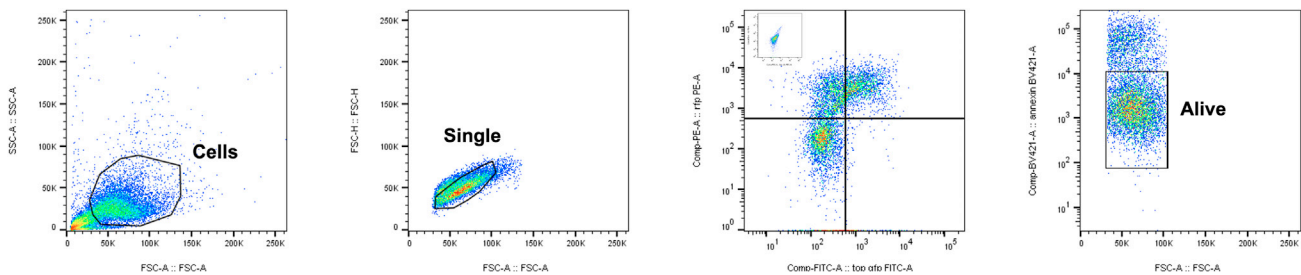


Figure 4. Cytotoxicity assay

Gating strategy for the analysis of cytotoxicity in TOP-GFP transduced CRC spheroids. Small inset represents the untransduced CRC cells, used as GFP/RFP negative control.

This section describes how to perform the isolation of specific CRC cell population (CSCs vs progenitor/differentiated cells) for downstream *in vitro* extreme limiting dilution assay (ELDA), in naive cells, or following a specific anti-tumor treatment.

11. Harvest cells

- a. Trypsinize and collect the cells in the corresponding tubes. Disassociate the clumps by pipetting up and down with harsh pressure if cells grow in spheres or islands.
- b. Label the cells with Brilliant Violet 421 Annexin V.
- c. Filter the cells through a 70 μm cell strainer, to avoid the formation of cell clumps.

△ CRITICAL: To obtain reproducible results in terms of clonogenic potential of cancer cells it is crucial to not introduce any variation from cell collection to cell sorting. Be aware that the cell culture conditions must be maintained the same for all the replicates (starting cell viability of the cells, number of plated cells to be treated, volume of medium and time of treatment), as well as the sample storage conditions between cell collection and cell sorting. In this case, it is preferable to keep the samples at room temperature in a mix 1:1 of cell culture medium: PBS, 2% FBS, 1 mM EDTA. FBS should be heat-inactivated before use by heating it for 30 minutes at 56°C with mixing, to inactivate complement proteins. It is crucial not to keep the sample for too long in the tubes, while waiting for the sorting. For this reason, it is suggested to prepare each sample in slightly advance compared to the scheduled time of the sorting.

12. Start up BD FACSMelody cell sorter (see point #9 for detailed information).
13. Load a previously saved experiment layout or create a new one.
14. Load the tube of cells that need to be analyzed/sorted and create the appropriate gating strategy to analyze/sort the viable cells (SSC-A versus FSC-A: cells; FSC-H versus FSC-A: single cells; BV421 versus FSC-A: alive cells) (see [Figure 3](#)).
15. In the "Set up" sort tab, select the format (plate), the volume and the sort mode (single cells).
16. Limited dilution sorting and cell plating.
 - a. Prepare 96 well plates. Add 200 μL of complete culture medium in each well.
 - b. Change the recipient stand to 96 well plate holder.
 - c. Plan the 96 well plate as shown in [Figure 6](#). Number in each well of the table indicates the cell number to be seeded.

Note: When working with a highly clonogenic cell population, the setup of the 96 well plate can be changed by plating only single cells in all the wells of the plate, to better define their clonogenic potential. In this scenario, given the variable efficiency of the cell sorter ($\geq 60\%$ in the best sorting conditions), it is preferable to use the U-Shaped-Bottom 96-well plate, as to do so you will be able, just few minutes after the sorting, to check every single well, in order to identify the well containing a single cell. Indeed, using the U-Shaped-Bottom microplate, the

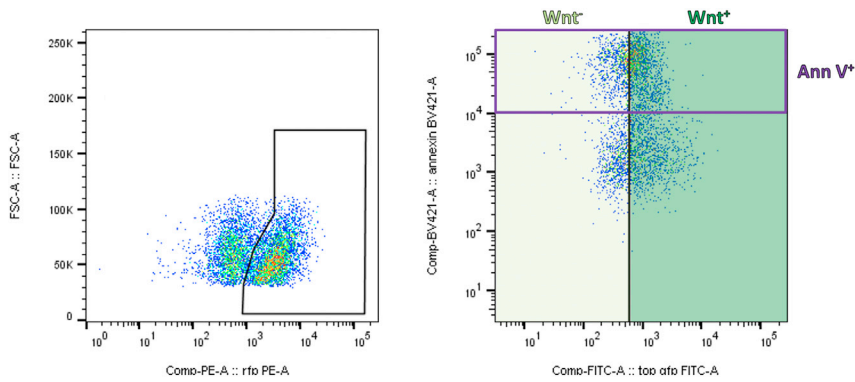


Figure 5. Evaluation of cell death induction in $Wnt^{+/-}$ CRC cells

Analysis of cytotoxic effects in CSCs vs differentiated cancer cells in TOP-GFP transduced CRC spheroids.

sorted cells will fast seed into the center of the wells, making it easy for their microscope observation.

17. Start the sorting and record the events.

△ CRITICAL: During the sorting protocol, pay attention to the stability of the cell cloud to be sorted (sometimes it can be affected by oscillations in laser intensity or fluidics change), which could lead to the isolation of an unwanted cell population. Be aware that even small variations in the room temperature where the instrument is located could affect the stability of the selected parameters.

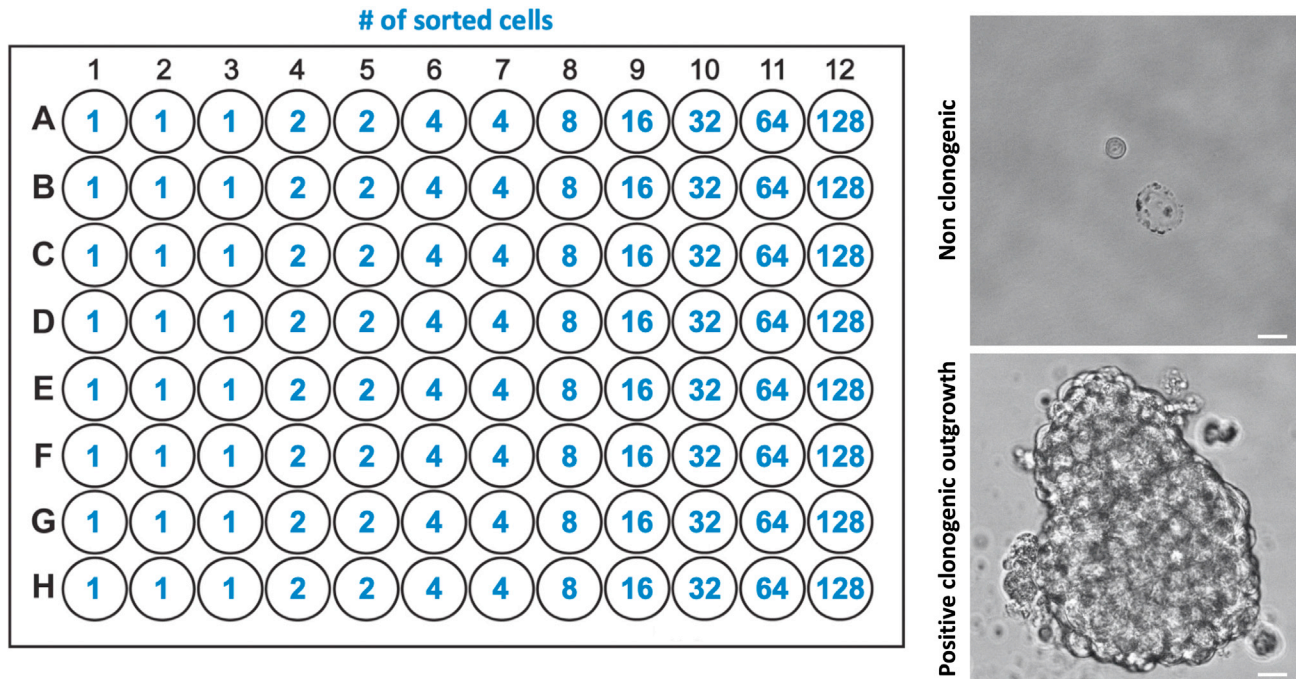


Figure 6. Setup of FACS-based limiting dilution assay

96 well plate ELDA layout for the sorting of CRC cells to test their clonogenic potential. (Right panel) Phase contrast analysis of non clonogenic cell versus positive clonogenic outgrowth of CRC cells growing in suspension, 7 days after cell sorting. Scale bar, 20 μ m.

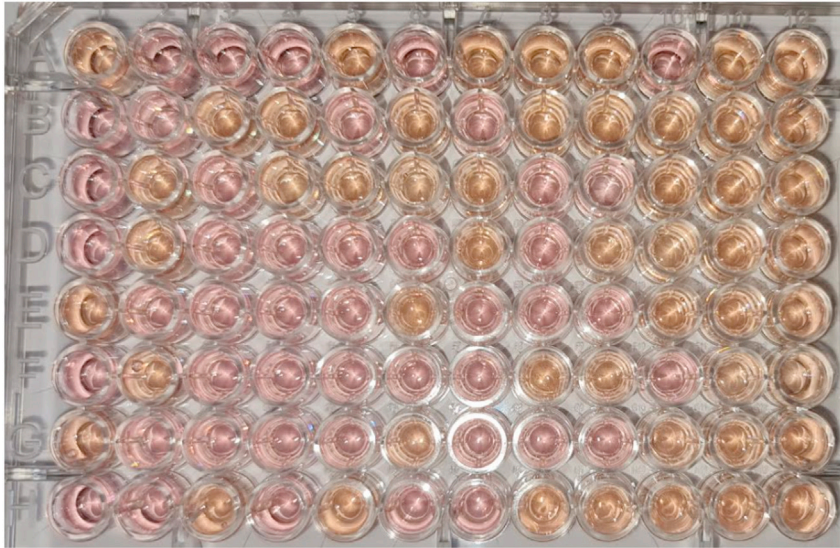


Figure 7. Evaluation of CRC cells colony forming potential

Yellow wells represent those considered positive for the clonogenic outgrowth (acidification of the medium due to cell metabolic activity), while pink-colored wells represent the negative clonogenic potential ones (no growth of colonies, unaltered pH of the cell culture medium).

18. Place the 96 well plates back to incubator and culture it at 37°C for 2 weeks, or longer, according to the proliferation rate of each cell line.
19. Count the number of wells in which tumor sphere(s) grows out (Figure 7).

Note: When determining the outgrowth rate of the clonogenic cells, only count the well in which tumor clones form instead of counting the colonies in the wells.

20. Calculate the clonogenic potential by ELDA online tool (<http://bioinf.wehi.edu.au/software/elda/>) (Figure 8).

EXPECTED OUTCOMES

Lentiviral transduction of CRC cells will allow to monitor the Wnt pathway activity in real-time by simply analyzing the Wnt-driven GFP expression by FACS. This protocol is mainly useful to discriminate CRC stem cells, endowed with high Wnt pathway activity, and progenitors/differentiated cells that are characterized by low activity of Wnt pathway. The evaluation of GFP expression by flow cytometry gives the possibility to investigate the biological properties of these specific cell subsets (i.e., the clonogenic potential) (Figures 6, 7, and 8), as well as to study the cytotoxic activity of selected anti-tumor compounds (Figures 4, 5, and 9).

QUANTIFICATION AND STATISTICAL ANALYSIS

To quantify the clonogenic potential following the ELDA online tool (<http://bioinf.wehi.edu.au/software/elda/>), four parameters need to be entered as input to calculate the confidence intervals defined as $1/(\text{stem cell frequency})$.

1. Dose: number of cells in each culture, herein specifically 1, 2, 4, 8, 16, 32, 64, and 128 for each dilution.
2. Tested: number of cultures tested, herein specifically 24 tested wells for 1; 16 for 2 and 4; 8 for 8, 16, 32, 64, and 128 dilutions.
3. Response: number of positive cultures, herein, specifically refer to the number of wells in which it has been observed a positive outgrowth of the colony.

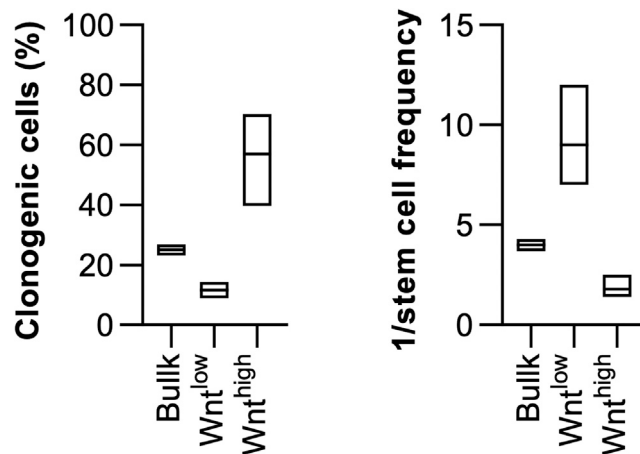


Figure 8. CRC cells clonogenic potential calculation

Comparison of clonogenicity of bulk versus TOP-GFP^{low/high} CRC cells. The confidence intervals for 1/ (stem cell frequency) are calculated by ELDA online tool (*left panel*) and the percentage of clonogenic cells are plotted (*right panel*).

- Group (optional): label for the population group to which cells belong (i.e., untreated versus treated) (see [Figure 9](#)).

LIMITATIONS

Although most tumor cells possess sufficient levels of Wnt pathway activations to visualize GFP expression (in particular in cancer cells bearing *APC*, *BRAF* or *KRAS* mutations), in some cases this may not occur, making it impossible to isolate and study specific tumor populations (CSCs, progenitors and differentiated cells).

TROUBLESHOOTING

Problem 1

Absence of GFP expression following TOP-GFP transduction of CRC cells (step 3a).

Potential solution

Even if it is likely a rare event, it is possible to notice the absence of GFP expression in CRC cells upon transduction with TOP-GFP lentiviral vector ([Figure 10](#)). This can be a serious issue if the lentiviral plasmid without the constitutive expression of mCherry RFP is used (cat. #35489 and #35490, Addgene), because the absence of GFP could be read as a failure in cell transduction, thus leading to a serious waste of time. In this case, the use of mCherry RFP bearing lentiviral plasmid is highly suggested (cat. #35491 and #35492, Addgene). Moreover, it would be useful to first transiently transfect the CRC cells to evaluate if their Wnt pathway activity is enough to drive the expression of detectable levels of GFP, before proceeding with CRC stable lentiviral transduction.

Problem 2

Absence of violet laser/filter in the flow cytometer/sorter (step 9).

Potential solution

In this case it is highly recommended the use of a Wnt-reporter plasmid without the constitutive expression of RFP (Addgene, cat. #35489; #35490), thus making it possible the use of a kit of apoptosis with a dye in the red fluorescent channel (i.e., CaspGLO Red Active Caspase Staining Kit, BioVision, cat. #K190 or #K193). Be aware that in this scenario, as mentioned before, the negative expression of GFP after cancer cell transduction, could be due to a non-optimal transduction, or to the low activity of the Wnt pathway, thus making difficult the interpretation of the results.

Data Entry

Data should be input as a data table of three or four columns, separated by any combination of commas, spaces or tabs. The data can be typed directly into the text field below, or can be cut and pasted from any spreadsheet application. Each row of data gives results for a particular cell dose. The columns are:

1. Dose: number of cells in each culture
2. Tested: number of cultures tested
3. Response: number of positive cultures
4. Group (optional): label for the population group to which cells belong

Enter your data in this box:

```
1 24 8 Vehicle
2 16 5 Vehicle
4 16 7 Vehicle
8 8 4 Vehicle
16 8 5 Vehicle
32 8 6 Vehicle
64 8 8 Vehicle
128 8 8 Vehicle
1 24 3 Drug
2 16 3 Drug
4 16 4 Drug
8 8 2 Drug
16 8 4 Drug
32 8 5 Drug
64 8 5 Drug
128 8 6 Drug
```

Limiting dilution data entered.

Counter	Dose	Tested	Response	Group
1	1	24	8	Vehicle
2	2	16	5	Vehicle
3	4	16	7	Vehicle
4	8	8	4	Vehicle
5	16	8	5	Vehicle
6	32	8	6	Vehicle
7	64	8	8	Vehicle
8	128	8	8	Vehicle
9	1	24	3	Drug
10	2	16	3	Drug
11	4	16	4	Drug
12	8	8	2	Drug
13	16	8	4	Drug
14	32	8	5	Drug
15	64	8	5	Drug
16	128	8	6	Drug

The number of lines of data entered = 16

Confidence intervals for
1/(stem cell frequency)

Group	Lower	Estimate	Upper
Drug	57.2	36.9	23.85
Vehicle	14.2	9.5	6.36

Figure 9. Online ELDA tool set-up

Analysis of ELDA using the online tool (<http://bioinf.wehi.edu.au/software/elda/>) in CRC cells treated with Drug, or Vehicle as control.

Problem 3

Evaluation of cytotoxic effects of compounds affecting Wnt pathway activity (steps 9 and 10).

Potential solution

If the compound(s) used for evaluation of cytotoxicity in Wnt^{high} versus Wnt^{low} CRC cells is known to affect the Wnt pathway activity (or when no information has been previously reported), it would be crucial to perform a cell sorting to isolate the two cell subsets before to perform the cytotoxic assay. This experimental setting will allow to evaluate at the same time the induction of cell death and the possible reprogramming in terms of Wnt pathway activity (Wnt^{high} to Wnt^{low} , and vice versa).

Problem 4

No evidence of cytotoxicity of selected anti-tumor drugs (steps 9 and 10).

Potential solution

To note, following cell sorting, it is common to observe the induction of cell cycle arrest (G0-G1 phase) in CRC cells for 12–24 h, due to the stress of cell sorting procedure. In this scenario, that need to be evaluated in advance, it is crucial to wait 24 h following cell sorting before starting the cytotoxic assay (in particular when using cytotoxic drugs that specifically target proliferating cells).

Problem 5

Excess of cell culture medium evaporation during clonogenic outgrowth (step 18).

Potential solution

Given the low amount of medium in each well of the 96 well plate used for clonogenic assay (about 100–120 μ L), and the long-term incubation of the plated cells (15–21 days to observe a clonogenic outgrowth), it is commonly observed a decrease in cell culture medium due to evaporation, which could lead to cell sufferance and compromise the clonogenic outgrowth of cancer cells. If the sorted cells are grown in basal medium, it is possible to refresh the medium in each well over time (adding 20–50 μ L once a week). Otherwise, if the cells are plated in cell culture medium containing a specific

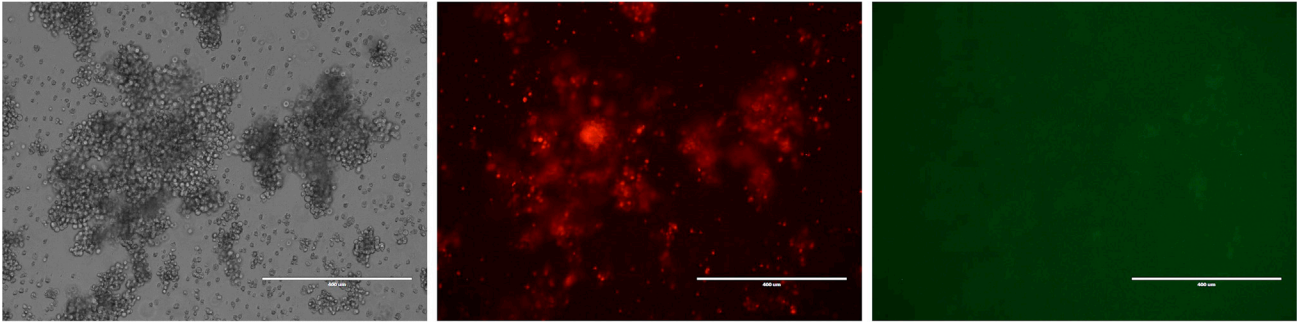


Figure 10. Monitoring the transduction of CRC cells with Wnt reporter construct

CRC spheroids efficiently transduced with Wnt reporter gene (with constitutive RFP expression), lacking the expression of Wnt-driven GFP. Scale bar, 400 μm .

anti-tumor drug, or cells are grown in suspension, to avoid manipulating the wells (this will introduce a variability in the assay), it is highly recommended to wrap the plate with a single thin layer of parafilm.

Problem 6

No difference in clonogenic potential between Wnt^{high} and Wnt^{low} cell subsets (steps 19 and 20).

Potential solution

We have already experienced the possibility to not find any difference in the clonogenic potential of Wnt^{high} and Wnt^{low} CRC cells. In this scenario, researchers should be aware that the mutational background, in particular the presence of activating mutations occurring in Wnt pathway components (i.e., β -catenin mutation in HCT-116 cell line), could affect CRC cells' clonogenic potential. Thus, this biological condition leads to a boost in basal Wnt pathway activity, thus making it impossible to notice any difference between Wnt^{high} and Wnt^{low} CRC cells in terms of clonogenic potential (Vermeulen et al., 2010).

Problem 7

Sorted cells show no clonogenic potential (steps 19 and 20).

Potential solution

It is important to highlight that some CRC cells strongly suffer from the cell sorting procedure, thus making it difficult to perform the clonogenic assay due to the high induction of cell death. To avoid facing this issue after CRC cell transduction, it would be crucial to select the right cell lines to work with, by performing a preliminary screening of cell lines not suffering from cell sorting protocol.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Giorgio Stassi (giorgio.stassi@unipa.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze data sets/code.

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AUTHOR CONTRIBUTIONS

Conceptualization and visualization, S.D.F., J.P.M., and G.S.; methodology, S.D.F., L.Z., M.G., and M.L.I.; writing – original draft, S.D.F. and L.Z.; writing – review & editing, S.D.F., L.Z., M.G., M.L.I., J.P.M., and G.S.; supervision and funding acquisition, J.P.M. and G.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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