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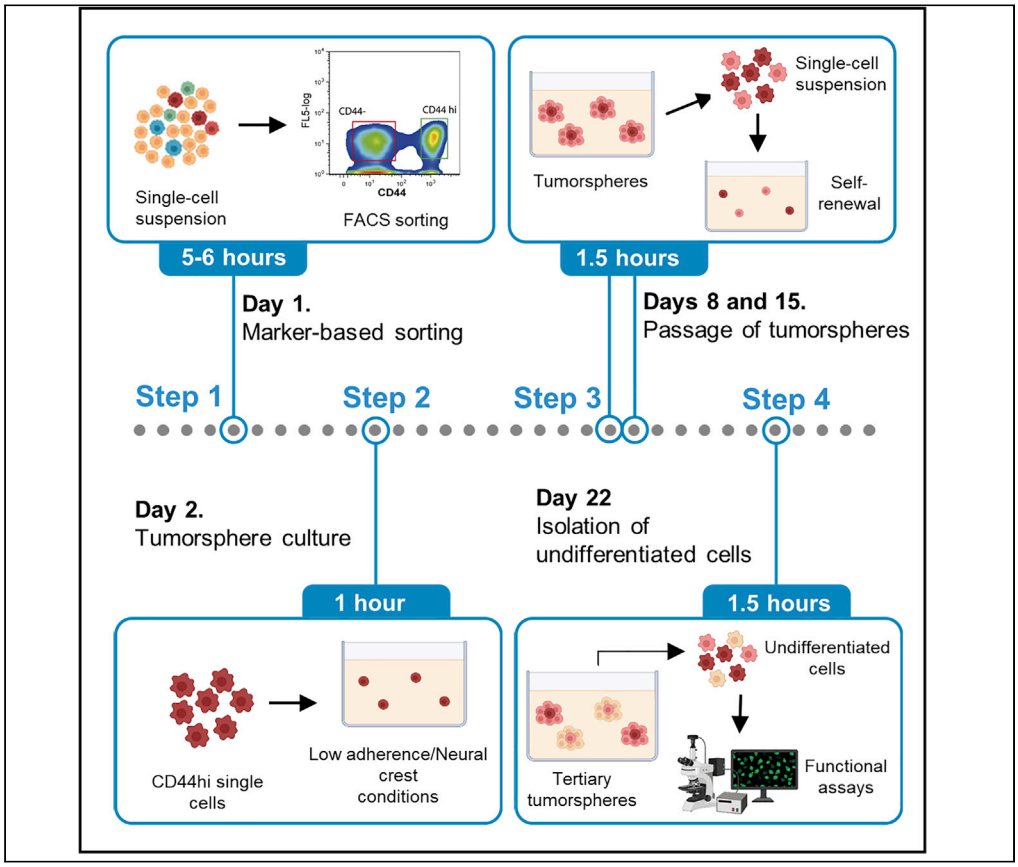
A protocol to enrich in undifferentiated cells from neuroblastoma tumor tissue samples and cell lines



The existence of a subpopulation of undifferentiated cells with stem-like properties has been suggested in neuroblastoma tumors, but a definitive biomarker for their successful isolation is missing. Here we describe an *in vitro* culture system for the enrichment in undifferentiated stem-like tumor cells for subsequent functional assays. We make use of clonal non-adherent cell culture conditions together with cell sorting with specific expression markers. This protocol allows for the differential study of heterogeneous cell population in neuroblastoma tumors.

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Highlights

Protocol to enrich neuroblastoma cell cultures in neuroblastoma undifferentiated cells

Obtaining individual undifferentiated cells suitable for cellular functional assays

Expansion, cryopreservation, and recovery to enable biobanking

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A protocol to enrich in undifferentiated cells from neuroblastoma tumor tissue samples and cell lines

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SUMMARY

The existence of a subpopulation of undifferentiated cells with stem-like properties has been suggested in neuroblastoma tumors, but a definitive biomarker for their successful isolation is missing. Here we describe an *in vitro* culture system for the enrichment in undifferentiated stem-like tumor cells for subsequent functional assays. We make use of clonal non-adherent cell culture conditions together with cell sorting with specific expression markers. This protocol allows for the differential study of heterogeneous cell population in neuroblastoma tumors.

For complete details on the use and execution of this protocol, please refer to Vega et al. (2019).

BEFORE YOU BEGIN

The experimental procedures included in this protocol describe the steps to enrich in undifferentiated neuroblastoma (NB) cells from the SK-N-SH cell line, a spontaneously immortalized heterogeneous human NB cell line. However, we have also used this protocol with other NB cell lines and patient-derived xenografts (PDX)-derived primary cells, with minimal modifications. The protocol has been successfully used with SK-N-AS, GI-MEN and GI-CAN cell lines, and can potentially be used to isolate undifferentiated cells from any heterogeneous cell line that present them in any proportion.

A tumorsphere is a solid, spherical formation developed from the proliferation of one cancer stem/progenitor cell, grown in non-adherent conditions with the suitable media. These tumorspheres are easily distinguishable from single or aggregated cells (Johnson et al., 2013). Tumorsphere cultivation, although with some limitations, is widely used to analyze the self-renewal capability of cancer stem cells (CSCs). In addition, the tumorsphere assay may be a promising in vitro strategy in the innovation towards future cancer therapeutics and may help in the screening of anti-cancer small-molecule chemicals (Lee et al., 2016).

The described protocol derives from the protocol used to isolate adult stem cells from peripheral nervous tissues like the carotid body (Pardal et al., 2007). Contrary to the protocols normally used with central nervous system and glioblastoma cells, in cells derived from peripheral nervous tissues or the neural crest, serum-rich (15–20%) media is used for the isolation of undifferentiated progenitor cells. Tumorspheres can also be formed using media without serum, but then the undifferentiated character and self-renewal of the cells obtained is compromised (Hong et al., 2012; Vega et al., 2019).

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In this protocol, we perform fluorescence-activated cell sorting (FACS) prior to tumorsphere culture to select for CD44 high expressing cells in SK-N-SH cells in order to eliminate adrenergic NB cells from the culture. CD44 is a putative stem cell marker in several solid tumors and it is expressed in a cell population with neural crest stem-like features in neuroblastoma tumors (Vega et al., 2019).

Institutional permissions

Ethical approvals and institutional permissions are required for the use of experimental mouse xenograft models and Patient-derived-xenografts (PDX). Tumor tissues from mouse xenografts referred in this protocol were obtained from the Instituto de Biomedicina de Sevilla (IBiS) animal facility with the approval from the local research ethics committee and the Andalusian Tissue Biobank. All experiments conform to the relevant regulatory standards.

Preparation of solutions

Timing: 1–2 h

Required solutions used in this protocol can either be prepared in advance and stored as indicated, or can be made fresh on the day of the experiment. Please refer to the materials and equipment for a complete list of solution recipes.

Culture cells

Timing: 3 days

1. Thawing and sub cultivation.
   a. Place cryovial of liquid nitrogen, frozen cells into a water bath at 37°C.
   b. Recover the cells from the vial by gently mixing with growing volume of fresh media to dilute DMSO concentration in the cell suspension.
   c. Centrifuge for 5 min at 300 × *g*.
   d. Resuspend cell pellet with 1 mL of fresh complete media and add to a 25 cm^2 cell culture flask with 4 mL of pre-warmed media.
   e. Incubate at 37°C in an incubator with 5% CO_2.
   f. Once at 80% confluence, sub cultivate and amplify cells by washing the cell monolayer with PBS without Ca^{2+} and Mg^{2+} twice, before trypsinising with the addition of 1 mL of 0.05% trypsin-EDTA solution to the culture.
   g. Incubate 5 min in the incubator, and add 4 mL complete media to inactivate trypsin and avoid cell damage.
   h. Make a uniform cell suspension by pipetting up and down, centrifuge for 5 min at 300 × *g* and resuspend pellet in 5 mL of fresh complete media.
   i. Quantify cell suspension and reseed the desired number of cells in a new flask.

   Note: we routinely sub cultivate cells by making a 1:20 surface dilution into a new flask. Alternatively, cell suspension can be seeded in bigger surface flasks for expansion. Ensure having a 70–80% confluent low passage (1–5) cell culture with enough cells to start procedure. We recommend starting with at least a 75 cm^2 flask.

Alternative: Dissociation of tissue sample into cells

Timing: 1.5 h

2. Primary tissue dissociation.
   a. Place the tumor tissue in a 100 mm dish. Mince it into small 2–4 mm pieces using a scalpel.

b. Place all pieces in a 15 mL plastic tube and add 3 mL of tumor dissociation media. Incubate for 10 min at 37°C in agitation.
c. Stop the enzymatic reaction by adding 5 mL of IMDM complete medium. Pipette up and down a few times until a homogeneous suspension is achieved.
d. Pass the result suspension through a 70 μm cell strainer.
e. Centrifuge 5 min at 300 × *g* and discard the supernatant, leaving behind dissociated cells pellet.
f. Add 5 mL of ACK buffer and resuspend carefully to eliminate red blood cells.
g. Incubate 5 min at room temperature (RT; 18°C–25°C). Centrifuge 5 min at 300 × *g* and discard the supernatant.
h. Repeat steps f and g.
i. Resuspend the cell pellet in IMDM complete media.
j. Count the number of cells in the suspension and calculate the viability by mixing a small aliquot with an equal volume of trypan blue. Use manual or automatic cell counting.

Note: Cells obtained can be seeded on a culture flask for primary culture at an approximate density of 1 × 10^5 cells in a 100 mm dish.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Purified Mouse Anti-Human CD44 (used at 1:500 dilution)	BD Biosciences	Cat#555476
Rabbit Anti-Nestin (used at 1:1,000 dilution)	Millipore	Cat#ABD69
Mouse Anti-Nestin (used at 1:1,000 dilution)	R&D Systems	Cat#MAB1259
Rabbit Anti-Dopa Decarboxylase (used at 1:500 dilution)	Cell signalling Technology	Cat#8786
Alexa488-donkey-α-mouse-IgG (used at 1:2,000 dilution)	Life Technologies	Cat#A32723
Alexa488-donkey-α-rabbit-IgG (used at 1:2,000 dilution)	Life Technologies	Cat#A32790
Alexa568-goat-α-rabbit-IgG (used at 1:2,000 dilution)	Life Technologies	Cat#A-11036
Chemicals, peptides, and recombinant proteins		
DMEM/F-12 medium	Gibco	Cat#10565018
DMEM medium	Gibco	Cat#61965059
IMDM medium	Gibco	Cat#12440-061
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140122
N-2 Supplement	Gibco	Cat#17502048
B-27 Supplement minus vitamin A	Gibco	Cat#12587010
Recombinant Human FGF	R&D Systems	Cat#233-FB
Recombinant Human EGF	R&D Systems	Cat#236-EG
Recombinant Human IGF-I	R&D Systems	Cat#291-G1
PBS	Gibco	Cat#10010023
Trypsin-EDTA (0.05%)	Gibco	Cat#25300062
Ammonium chloride (NH_4Cl)	Sigma-Aldrich	Cat#A0171
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	Cat#S8875
Ethylenediaminetetraacetic acid disodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$)	Sigma-Aldrich	Cat#E1644
HBSS buffer, no Ca^{2+}, no Mg^{2+}, no phenol red	Gibco	Cat#14175095
Collagenase from *Clostridium histolyticum*	Sigma-Aldrich	Cat#C6885
Elastase	Sigma-Aldrich	Cat#324682
Trypsin from bovine pancreas	Sigma-Aldrich	Cat#T8003
Deoxyribonuclease II from bovine spleen	Sigma-Aldrich	Cat#D8764
Fetal Bovine Serum (FBS)	TICO Europe	Cat#EU500
Leibovitz's L-15 Medium	Gibco	Cat#21083027
HEPES	Gibco	Cat#15630080
Bovine Serum Albumin	Sigma-Aldrich	Cat#A2153
Versene solution	Gibco	Cat#15040066

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
SK-N-SH	European Collection of Authenticated Cell Cultures (ECACC)	Cat#86012802
Biological samples		
Tumor tissue from human cell line derived- xenograft	SK-N-SH cells on C.B-17 SCID mice (Harland laboratories). Animal facility.	n/a
Tumor tissue from Patient-derived xenografts (PDX)	Own repository. Instituto de Biomedicina de Sevilla.	n/a
Oligonucleotides		
See Table 1	Sigma-Aldrich	n/a
Software and algorithms		
Fiji/ImageJ software	https://doi.org/10.1038/nmeth.2019	https://imagej.net/software/fiji/
Other		
Corning Costar Ultra-Low Attachment Microplates 6 well	Corning	Cat#3471
40 µm Cell Strainer	Corning	Cat#352340
50 µm Cell Strainer	BD Biosciences	Cat#340632
70 µm cell strainer	Falcon	Cat#352350
Plastic tubes	Corning	Cat#352063
Dimethyl sulfoxide	Sigma-Aldrich	Cat#D2438
Cell Scrapers	Biologix	Cat#70-1250
Thermo Scientific™ Mr. Frosty™ Freezing Container	Thermo Fisher Scientific	Cat#1535050
Olympus CKX41 inverted microscope	Olympus Life Sciences	n/a
Nikon DS-Fi2 camera	Nikon Instruments	n/a
BD FACS Aria cytometer	BD Biosciences	n/a

MATERIALS AND EQUIPMENT

Complete DMEM medium		
Reagent	Final concentration	Amount
DMEM medium	n/a	445 mL
FBS	10%	50 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	**n/a**	**500 mL**
Store at 4°C (maximum 1 month) and prewarm at 37°C before use.		

Note: DMEM medium is used for the growth of SK-N-SH cells. Use appropriate growing media for the used cell line or cell source.

Complete IMDM medium		
Reagent	Final concentration	Amount
IMDM medium	n/a	420 mL
FBS	15%	75 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	**n/a**	**500 mL**
Store at 4°C (maximum 1 month) and prewarm at 37°C before use.		

Neural Crest Medium (NCM)		
Reagent	Final concentration	Amount
DMEM/F12 medium	n/a	445 mL
IGF-I (100 μg/mL)	20 ng/mL	100 μL
EGF (500 μg/mL)	20 ng/mL	20 μL
FGF (10 μg/mL)	10 ng/mL	500 μL
FBS	15%	75 mL
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
B27 supplement (50×)	1×	10 mL
N2 supplement (100×)	1×	5 mL
Total	**n/a**	**500 mL**
Sterilize by filtration and store at 4°C until needed (maximum 2 weeks).		

Note: In order to preserve stability and activity of growth factors and supplements, let warm up at RT before use, and do not store neural crest medium for more than one month.

⚠ CRITICAL: Due to the short half-life of these growth factors, the neural crest medium is made fresh every two weeks in order to preserve stability and activity of compounds.

Tumor dissociation media		
Reagent	Final concentration	Amount
HBSS buffer	n/a	959.77 μL
Collagenase (60 mg/mL)	0.5 μg/mL	8.33 μL
Elastase (5 mg/mL)	0.05 μg/mL	10 μL
Trypsin (26.6 mg/mL)	0.25 μg/mL	9.4 μL
Deoxyribonuclease II 0.04% (32 mg/mL)	0.4 mg/mL	12.5 μL
Total	**n/a**	**1 mL**
Prepare fresh and keep at 4°C until use.		

Erythrocyte lysis buffer (ACK buffer)		
Reagent	Final concentration	Amount
Milli-Q water	n/a	100 mL
NH_4Cl	155 mM	829 mg
$NaHCO_3$	2,96 mM	24,867 mg
$EDTA \cdot 2H_2O$	3,72 mM	168,8 mg
Total	**n/a**	**100 mL**
Store at 4°C for 2 months maximum.		

Cryopreservation media		
Reagent	Final concentration	Amount
Neural Crest medium	n/a	45
DMSO	10%	5 mL
Total	**n/a**	**50 mL**
Store at 4°C for maximum 2 weeks.		

FACS media		
Reagent	Final concentration	Amount
Leibovitz's L-15 Medium	n/a	440 mL
BSA (2% (w/v)	0.2%	50 mL
HEPES (1 M)	10 mM	5 mL

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Continued		
Reagent	Final concentration	Amount
Penicillin-Streptomycin (10,000 U/mL)	100 U/mL	5 mL
Total	n/a	500 mL

Store at 4°C for 1 month maximum.

⚠ CRITICAL: The use of an inverted light microscope equipped with a 4× objective is essential to observe tumorspheres and to be able to recover them. In this protocol we use an Olympus CKX41 inverted microscope with a Nikon DS-Fi2 camera.

Alternatives: This protocol uses 6-well Ultralow binding plates. 24-well plates can also be used adapting volumes and number of cells seeded. Alternative low binding plates can be found in the market from different makers. However, we have found, particularly with primary cells, that some adhesion of tumorspheres to the plate surface can be observed with some products.

Alternatives: This protocol uses Counting Chamber/Hemocytometer or LUNA™ Automated Cell Counter with LUNA™ Cell Counting Slides from Logos Biosystems. Any other suitable cell counting method can be used, ensuring accuracy in cell number quantification and possibility to ensure single cell suspension.

Table 1. Primers

Oligonucleotide	Source	Identifier
Bmi-1: forward: TTGTTCGTTACCTGGAGACC; reverse: GGCAGCATCAGCAGAAGG	Sigma-Aldrich	n/a
Nestin: forward: GTGGCTCCAAGACTTCC; reverse: GCACAGGTGTCTCAAGG	Sigma-Aldrich	n/a
Oct3/4: forward: CTTGCTGCAGAAGTGGGTGGAGGAA; reverse: CTGCAGTGTGGGTTTCGGGCA	Sigma-Aldrich	n/a
TH: forward: GCGCAGGAAGCTGATTGC; reverse: CAATCTCCTCGGCGGTGTAC	Sigma-Aldrich	n/a
GATA3: forward: TCA TTA AGC CCA AGC GAA GG; reverse: GTCCCCATTGGCATTCCTC	Sigma-Aldrich	n/a
HAND1: forward: GAGAGCATTAACAGCGCATTCG; reverse: CGCAGAGTCTTGATCTTGGAGAG	Sigma-Aldrich	n/a
HAND2: forward: CGCCGACACCAAACTCTCC; reverse: TCGCCATTCTGGTCGTCCT	Sigma-Aldrich	n/a
PHOX2B: forward: AACCCGATAAGGACCACTTTTG; reverse: AGAGTTTGTAAGGAACTGCGG	Sigma-Aldrich	n/a
ISL1: forward: TACGGGATCAAATGCGCCAA; reverse: CACACAGCGGAAACACTCGAT	Sigma-Aldrich	n/a
KLF7: forward: AGACATGCCTTGAATTGGAACG; reverse: GGGGTCTAAGCGACGGAAG	Sigma-Aldrich	n/a
FOSL1: forward: CAGGCGGAGACTGACAAACTG; reverse: TCCTTCCGGGATTTTGCAGAT	Sigma-Aldrich	n/a
RUNX1: forward: CTGCCCATCGCTTTCAAGGT; reverse: GCCGAGTAGTTTTCATCATTGCC	Sigma-Aldrich	n/a
GLIS3: forward: GTTCAGCGACTGGGACTCATT; reverse: CCCTCTGTAAGCTAGGACTGAT	Sigma-Aldrich	n/a
ID1: forward: CTGCTCTACGACATGAACGG; reverse: GAAGGTCCCTGATGTAGTCGAT	Sigma-Aldrich	n/a
SOX9: forward: AGCGAACGCACATCAAGAC; reverse: CTGTAGGCGATCTGTTGGGG	Sigma-Aldrich	n/a
NOTCH2: forward: CAACCGCAATGGAGGCTATG; reverse: GCGAAGGCACAATCATCAATGTT	Sigma-Aldrich	n/a
SMAD3: forward: TGGACGCAGGTTCTCCAAAC; reverse: CCGGCTCGCAGTAGGTAAC	Sigma-Aldrich	n/a

STEP-BY-STEP METHOD DETAILS

CD44hi cell sorting – day 1

Timing: 5–6 h

CD44hi cells are sorted from neuroblastoma cell lines.

1. Obtain a single cell suspension for labelling.
   a. Aspirate media from a tissue culture flask with SK-N-SH cells and gently wash the cells twice with a volume of PBS without Ca^{2+} and Mg^{2+} enough to cover the cell layer (3 mL for a 25 cm^2 cell culture flask).
   b. Aspirate PBS and add a volume of Versene enough to cover the cell layer (500 μL for a 25 cm^2 cell culture flask). Incubate at 37°C in the cell incubator for 5 min.
   c. Stop the dissociation by adding 2 mL of FACS medium and resuspend cells by pipetting up-and-down until a homogeneous cell suspension is achieved.
   d. Transfer cell suspension to a sterile 15 mL plastic tube and centrifuge at 300 × *g* for 5 min.
   e. Discard the supernatant and resuspend the cell pellet with 1 mL of FACS medium gently pipetting up and down.
   f. Pass through a sterile cell strainer with 40 μm pore size into a sterile 50 mL tube to remove non-dissociated cell aggregates.
   g. Count the number of cells in the suspension and calculate the viability by mixing a small aliquot with an equal volume of trypan blue. Use manual or automatic cell counting.
   h. Prepare a suspension of at least 1 × 10^6 cells per 100–200 mL of FACS medium for labelling. Prepare also a suspension of at least 2 × 10^4 cells for unlabeled cells and 2 × 10^4 cells for cells to be labelled only with secondary antibody as controls.

   Note: Proper digestion and preparation of cells into a single cell suspension is crucial for the efficient collection of the fluorescently tagged population.

2. Labelling of cells for fluorescence-activated cell sorting (FACS).
   a. Incubate sample cell suspension with 1 μg of CD44 primary antibody for 40–60 min on ice.
   b. Wash cells 3 times with 1 mL cold FACS medium with gentle centrifugation (5 min at 300 g and 4°C) between washes.
   c. Incubate on ice for 30 min with Alexa488-donkey-α-mouse at 1:500 dilution in a volume of 200 μL. Keep protected from light from this step on.

   Note: pipet up and down gently in the middle of the incubation when possible.

   Alternatives: The use of a secondary antibody is not needed if a conjugated primary CD44 antibody is used.

   d. Wash cells 3 times with 1 mL cold FACS medium with gentle centrifugation (5 min at 300 g and 4°C) between washes.
   e. Place the cell suspension in the appropriate tube for sorting, passing the cell suspension through a 50 μm cell strainer before sorting.
3. Sorting of CD44hi cells.
   a. Use forward and side scatter density plots to exclude debris.
   b. Use secondary antibody control to set gates for positive fluorescence.
   c. Use CD44 expression distribution levels for gating strategy, defining the population with high CD44 expression (Figure 1).
4. Recovery of CD44hi cells.

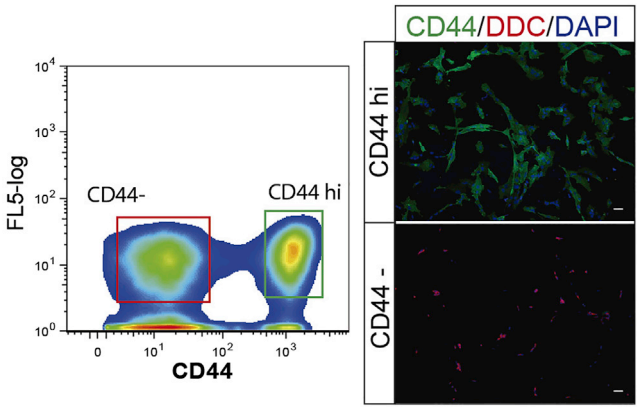

Figure 1. CD44 expression in SK-N-SH cells

Representative dot-plots showing CD44 expression and sorting strategy. Immunofluorescence show CD44 and DDC (adrenergic marker) expression in sorted populations. Scale bar: 25 μm. Published with author's permission.

a. Seed 1 × 10^5 of recovered sorted cells per well in a 6-well ultralow binding plate with 2 mL of pre-warmed NCM. Transfer the 6-well plate to a 37°C, 5% CO_2 incubator overnight (6–18 h).

Note: The flow cytometer to be used must have the proper lasers and filters to detect the emission spectrum of the fluorescent protein. We performed flow cytometry analyses and sorting in a BD FACS Aria cytometer, which allows for stringent gating to ensure the collection of a pure population. Users can adjust the gate depending on the stringency of their experiment. Also ensure that sorting is performed under sterile conditions as cells are going to be cultured long-term after the sorting.

Alternatives: The sorting can be carried out with dissociated cells from tissue samples, like PDXs, as the ones obtained from the alternative step 2 described above. In this case sorting is carried out right after dissociation with a similar strategy. CD24 is used as a negative selection marker in the case of xenographs to eliminate possible CD44+ host cells.

Alternatives: The following protocol can be performed using unsorted SK-N-SH cells as starting point. In this case, resulting tumorspheres will be enriched in any case in CD44hi cells which can be sorted later to eliminate possible differentiated cells present in the spheres. The main advantage of sorting early rather than sorting after obtaining the tumorspheres is that you can obtain more undifferentiated cells without the interference of the tumorspheres/aggregates formed by CD44- differentiated cells. We would only recommend sorting the resulting tumorspheres to save time at the beginning of the protocol or to remove possible differentiated cells in the tumorspheres.

Tumorsphere culture – day 2

Timing: 1 h

Cells are prepared and seeded for growing as tumorspheres.

Note: Use appropriate number of wells at start, as required, taking into account that the culture will be amplified in subsequent steps.

Note: Pre-warm complete DMEM medium and trypsin in a water bath at 37°C. Pre-warm NCM medium at room temperature.

Note: For unsorted SK-N-SH cells, start with a culture at around 70% confluency and follow steps 1.a–g. Use low passage cells as a starting point (below 5). For sorted SK-N-SH cells, continue from 5.a.

5. Obtain a homogeneous single cell suspension.
   a. Observe under the microscope and recover all previously sorted cells from the ultralow binding plate.
   b. Pass through a sterile cell strainer with 40 μm pore size to remove possible aggregates.

   Alternatives: alternatively dissociate aggregates resuspending in Versene after gentle centrifugation. Centrifuge again and recover cells in NCM media.

   c. Count the number of cells in the suspension and calculate the viability by mixing a small aliquot with an equal volume of trypan blue. Use manual or automatic cell counting.

   Note: We use an automatic cell counter with parameters adjusted to the cell line in use. Direct counting under a microscope with a hemocytometer is also possible. Either way, ensure you have an accurate quantification and, by imaging, that you have a single cell suspension.

   ⚠ CRITICAL: A single cell suspension is critical to ensure the clonality of the tumorspheres (see troubleshooting, problem 1).

6. Plating cells for primary tumorsphere.
   a. Add 2 mL of pre-warmed NCM per well in a 6-well ultralow binding plate.
   b. Mix the cell suspension a few times by pipetting before plating.
   c. For each well, pipette a volume of cell suspension so that the final concentration in the plate is 1–2.5 cells/μL (2,500–5,000 cells in 2 mL of media). Plate as many wells as necessary.
   d. Ensure even distribution of the cells in the well by slowly moving the plate drawing a cross and by gentle tapping.
   e. Carefully transfer the 6-well plate to a 37°C cell incubator for 7 days.

   ⚠ CRITICAL: Even distribution of the cells in the well is critical to ensure the clonality and growth of the tumorspheres. Ensure that the plate is maintained completely flat at all times in the incubator. Do not move or remove the plate form the incubator until the final day. Big groups or clumps can be formed otherwise (see troubleshooting, problem 1).

   Note: Plates can be incubated for 5–10 days, depending on the cells used and the desired outcome. We normally incubate for 7 days.

   Note: Tumorspheres can be also plated in 24-well plates, seeding 600 cells per well in 1 mL of NCM.

   Alternatives: The protocol can be easily adapted to a single cell tumorsphere assay to better asses clonality. In this case a single cell per well is seeded on a 96 well plate with 150 μL NCM. As only around 1% of cells will form tumorspheres, a high number of plates are needed.

Recovery and passage of primary tumorsphere – day 8

Timing: 1.5 h

Primary tumorspheres are imaged and collected. Once dissociated, cells can be plated to form secondary tumorspheres.

7. Imaging and collection of primary tumorspheres.
   a. Observe the plate under the microscope. Visible dispersed cell spheres should be observable.
   b. Gently rotate the plate in circles so that the tumorspheres concentrate in the middle of the well.

   Note: This motion will concentrate them by size, with the bigger spheres reaching the center quicker than the small ones or small aggregates. If the formed tumorspheres are too big or too small, see troubleshooting, problem 4. If tumorspheres are not obtained, see troubleshooting, problem 5.

   c. Take images of the resulting tumorspheres, ensuring that all spheres fall within the imaged field.

   Note: These images will serve to quantify the number and diameter of the resulting tumorspheres. Alternatively, spheres can be counted in situ under the microscope.

   d. While looking into the microscope, transfer all tumorspheres from the well with a 10 μL pipette, taking the minimum possible volume of medium, into a sterile plastic tube containing 500 μL 0.05% trypsin.

   Note: If different wells with the same condition are used, tumorspheres from these can be transferred into a single well, so that they can be recovered in the minimum volume possible.

   Note: Alternative to the use of 0.05% trypsin, tumorspheres can be transferred to a solution with a cocktail of enzymes for better dissociation. This can work better with sensitive spheres coming from primary tissue.

   Note: To avoid contamination, keep the plate open for the shortest time possible.

8. Dissociation and plating for secondary tumorspheres.
   a. Gently pipette the tumorspheres in the trypsin solution from time to time to help dissociation. Keep for approximately 5 min. Keep at room temperature and avoid long incubation times.

   Note: You can also collect the tumorspheres by transferring media with the cells directly into a 15 mL falcon tube. Then, allow the spheres to settle by gravity sedimentation for 5 min at room temperature. Aspirate the supernatant but leave approximately 200 μL in the falcon tube. Be careful not to aspirate the tumorsphere. Add 1 mlm of 0.05% trypsin to the tumorspheres and incubate for 5 min at room temperature. If your tumorspheres do not dissociate with trypsin or a gentler dispersion is needed, see troubleshooting, problem 2.

   b. Stop the trypsinization by adding 2 mL of NCM.
   c. Transfer cell suspension to a sterile 15 mL plastic tube and centrifuge at 300 × *g* for 5 min.
   d. Discard the supernatant and resuspend the cell pellet with 1 mL of NCM gently pipetting up and down.
   e. Pass through a sterile cell strainer with 40 μm pore size to remove non-dissociated cell aggregates.
   f. Count the number of cells in the suspension and calculate the viability by mixing a small aliquot with an equal volume of Trypan blue. Use manual or automatic cell counting.
   g. Reseed tumorspheres following step 6 to have secondary tumorspheres.

   Note: Cell aggregates are more common on the primary tumorspheres. Subsequent tumorsphere passages permit to obtain higher number of undifferentiated cells. Serial tumorsphere passage also permits to calculate the self-renewal potential.

Recovery and passage of secondary tumorspheres – day 15

Timing: 1.5 h

Secondary tumorspheres are imaged and collected following step 7. Once dissociated, cells can be plated to form tertiary tumorspheres following the procedure described in step 8.

Recovery of tertiary tumorspheres and isolation of undifferentiated cells – day 22

Timing: 1.5 h

9. Tertiary tumorspheres are imaged and collected following step 7 and dissociated following step 8.a to f. Once dissociated, cells can be used for characterization, functional assays, or single cell experiments.

   Optional: Complete tertiary tumorspheres without dissociation in single cells, can also be seeded on a tissue culture treated surface for them to adhere and open after a short incubation time. This allows the characterization of the cells in the spheres by immunofluorescence, video-microscopy or differentiation. Spheres can also be included fresh on optimal cutting temperature compound (OCT) and cryopreserved as frozen blocks that can be serially sliced using a cryotome to assess composition by immunofluorescence.

   Note: If the cells obtained from tertiary tumorspheres are not undifferentiated enough, see troubleshooting, problem 3.

Cryopreservation of undifferentiated cells

Timing: 15 min

Undifferentiated cells from tumorspheres can be preserved.

10. Freezing tumorspheres cells.
    a. Set a cryopreservation container filled with propan-2-ol aside, at 20°C–25°C.
    b. Dissociate tumorspheres into cell suspension as in step 8.a to f.
    c. Prepare enough cryopreservation media.
    d. After centrifugation, resuspend cell pellet in 1 mL of cryopreservation media, mixing gently.
    e. Transfer the cell suspension to a labelled 1.5–2 mL cryovial and place in the container.
    f. Transfer the cryopreservation container with the cryovial to a −80°C freezer.
    g. The day after, transfer cryovials into a liquid nitrogen tank for long-term storage.

Recovery of undifferentiated cells

Timing: 20 min

Note: The following steps describe the procedure to thaw a single cryovial.

11. Thawing of cryopreserved tumorspheres-derived cells.
    a. Pre-warm NCM at room temperature (RT), then place it under the tissue-culture hood.
    b. Place the cryovial containing frozen undifferentiated cells in a 37°C water bath for 1–2 min, or until fully thawed.
    c. Transfer all medium with cells from the cryovial into a 15 mL sterile tube containing 1 mL of NCM.

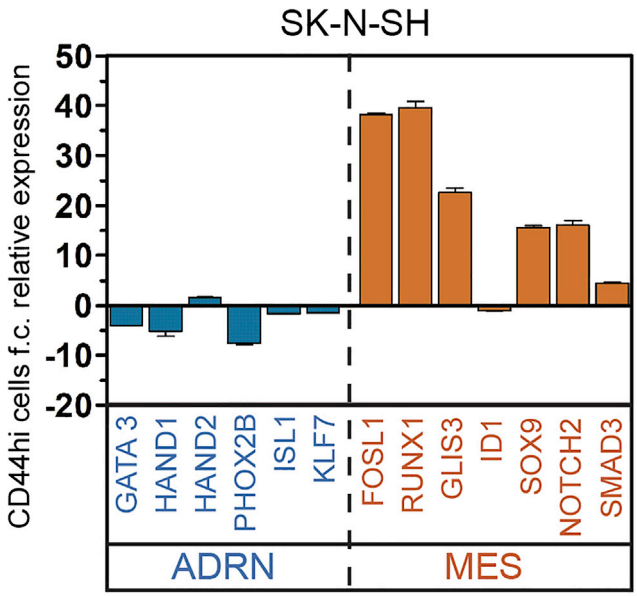

Figure 2. CD44hi cells present a MES gene expression signature
Data are represented as mean ± SEM. Published with author's permission.

d. Sequentially add NCM to double the current volume, gently agitating and incubating briefly after each addition to gradually dilute the DMSO. Repeat until you reach 10 mL of media.
e. Centrifuge at 300 × *g* for 5 min.
f. Aspirate supernatant.
g. Resuspend the cell pellet in 1 mL of NCM.
h. Proceed with the cells to form tumorspheres as described in steps 5 and 6.

Note: We normally plate cells for tumorsphere formation after thawing, to conserve undifferentiated character and let them recover.

EXPECTED OUTCOMES

Upon the completion of this protocol, we obtain multipotent, undifferentiated tumorspheres that are mainly composed by CD44hi/CD114+/Nestin+/DDC- cells. The tumorspheres should be solid, spheric structures, however their size varies, with an median diameter of around 80 μm. Gene expression analysis demonstrates that the cells obtained with this protocol present a mesenchymal (MES) gene expression signature, that can turn into an adrenergic (ADRN) type signature upon differentiation with retinoic acid (Vega et al., 2019) (Figure 2).

The undifferentiated cells obtained maintain their phenotypic characteristics for at least 10 days in culture when properly maintained. They can also be differentiated into neuronal, glial, or mesenchymal lineages using the appropriate differentiation protocols (Vega et al., 2019).

With an extended incubation time, tumorspheres will normally generate a visible crown of differentiated adrenergic DDC+ cells.

When sorting cells from fresh tissue samples, some cells from other tissues can express CD44. In our hands, high levels of CD44 in PDXs tumors are mainly seen on tumor cells. Nevertheless, is the tumorsphere serial passage culture condition the one finally selecting for undifferentiated neural crest like cell, not the initial CD44 sorting. Non desirable CD44+ cells initially sorted will not form tumorspheres and will disappear.

LIMITATIONS

Cells obtained with the use of this protocol in SK-N-SH cells present neural crest stem-like features but can not be considered bona-fide neural progenitor cells. Neural progenitor cells can be obtained with a similar protocol from different fresh tissues (Pardal et al., 2007; Platero-Luengo et al., 2014).

The number of cells obtained ranges between 0.5 and 2% of the starting cell number. Obtaining undifferentiated cells from cell lines or PDXs might not be a problem but starting sample material is a limitation when performed from patient samples. Furthermore, there is a great variability in the number of initial CD44hi sorted cells obtained from different samples and cell lines. A heterogeneous NB cell line like SK-N-SH is the optimal situation. Some mesenchymal cell lines might present uniform moderate levels of CD44. In this case it can be more difficult to sort the CD44 high expressing cells and it might be better to perform the tumorsphere assay directly without sorting.

The protocol does not work on cell lines that are very adrenergic and composed of only differentiated cells, such as IMR-32, from which we only obtained spheres with differentiated committed cells.

CD44 is not an exclusive marker of undifferentiated neuroblastoma cells, as it will also mark for example benign Schwann-like stromal cells. Also, only a small proportion of the CD44hi cells form undifferentiated tumorspheres in culture. This must be considered when sorting cell populations and interpreting the data obtained. As there are no definitive and specific cancer stem cell markers described for neuroblastoma tumors, functional experiments with the resulting cells are needed to address multipotency and other properties.

We have only used the protocol successfully with human cells. We have not been able to select for undifferentiated neural crest stem-like cells from spontaneous tumors in the TH-MYCN mouse model. This is probably due to the strong homogeneous adrenergic nature of these tumors (De Wyn et al., 2021). A different protocol to isolate sphere forming cells from TH-MYCN transgenic tumors has been described (Liu et al., 2016), but we have not been able to replicate it.

TROUBLESHOOTING

Problem 1

Big cell aggregates or clumps of tumorspheres are observed, especially on the primary tumorsphere culture (Figure 3; step 7).

Potential solution

Lower cell density on the original plate. Ensure starting with a single cell suspension. Ensure the minimum movement of the plate and an even flat surface during incubation. Alternatively, to ensure clonality, perform the single cell tumorsphere assay (described above).

Problem 2

Tumorspheres do not dissociate well into single cells in the passages (step 8).

Potential solution

Use a mechanical or enzymatic dispersion system that suits your cells.

Problem 3

Enough undifferentiated cells cannot be observed from the tumorspheres obtained (step 9).

Potential solution

Some differentiated cells will form also tumorspheres with this method, but with a limited self-renewal potential and no multipotency. Perform functional and expression analysis to ensure the undifferentiated character of the cells obtained with your starting sample/cell line.

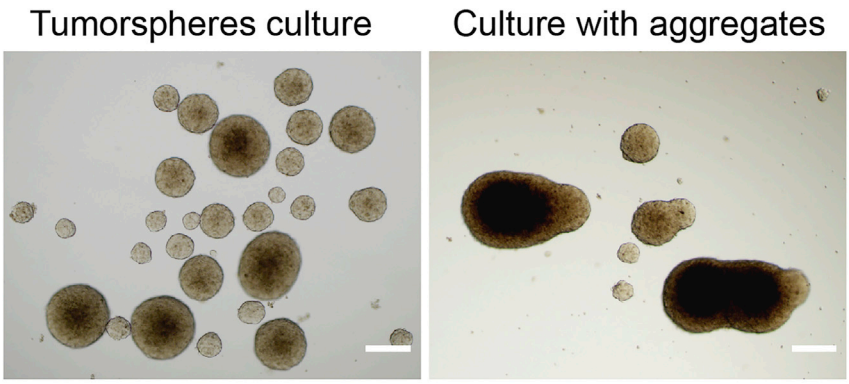

Figure 3. Examples of obtained tertiary tumorspheres and potential culture with aggregates
Scale bar: 200 μm.

Also, some differentiation will be observed in the tumorspheres. Reduce the incubation time on the tumorsphere assay to avoid differentiation and sort the resulting cells to eliminate undesired populations.

Problem 4
Too big or too small tumorspheres are obtained (step 7).

Potential solution
Adjust the incubation time for your sample/cell line so that you obtain tumorsphere big enough to be a source of undifferentiated cells, but not so big so that differentiation is occurring during tumorsphere formation.

Problem 5
Tumorspheres are not obtained (step 7).

Potential solution
Make sure to use fresh NCM medium and supplements. If you still do not obtain tumorspheres after 10–12 days ensure you start with a heterogeneous population as proper tumorspheres will not grow from already differentiated cells.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Francisco M. Vega (fmvega@us.es).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate/analyze new datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.A. and F.M.V.; Investigation, A.A., F.M.V., I.R., and M.A.G.; Writing – Original Draft, A.A. and F.M.V.; Writing – Review & Editing, A.A.A., F.M.V., R.P., and M.A.G.; Funding Acquisition, F.M.V. and R.P.; Supervision, F.M.V.

DECLARATION OF INTERESTS

The authors declare no competing interests

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