Video Article

Harnessing the DNA Dye-triggered Side Population Phenotype to Detect and Purify Cancer Stem Cells from Biological Samples

Maximilian Boesch*[1,2,3], Elisabeth Hoflehner[2,3], Dominik Wolf[2,3,4], Guenther Gastl[2], Sieghart Sopper*[2,3]

[1]Institute of Immunobiology, Kantonsspital St. Gallen

[2]Internal Medicine V, Medical University of Innsbruck

[3]Tyrolean Cancer Research Institute (TKFI)

[4]Medical Clinic III, Oncology, Hematology, Immunoncology and Rheumatology, University Clinic Bonn (UKB)

*These authors contributed equally

Correspondence to: Maximilian Boesch at Maximilian.Boesch@kssg.ch

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Abstract

Cancer is a stem cell-driven disease and eradication of these cells has become a major therapeutic goal. Deciphering vulnerabilities of Cancer Stem Cells (CSCs) and identifying suitable molecular targets relies on methods that allow their specific discrimination in heterogeneous samples such as cell lines and *ex vivo* tumor tissue. Flow cytometry/FACS is a powerful technology to multi-parametrically dissect biological samples at the single cell level and is to date the method of choice to recover live cells for downstream analyses. Surface markers such as CD44 and CD133 as well as detection of aldehyde dehydrogenase enzymatic activity have often been used to define and sort out CSCs from tumor samples by FACS. A complementary approach, depicted here in methodological detail, makes use of functional dye extrusion by ABC drug transporters, which identifies a distinct population of fluorescence-dim cells commonly referred to as side population (SP). SP cancer cells exhibit canonical stem cell characteristics and can be abrogated and functionally confirmed using agents that inhibit the dye-extruding drug transporter (most frequently ABCB1/P-glycoprotein/MDR1/CD243 and ABCG2/Bcrp1/CD338). Moreover, the SP assay is compatible with other flow cytometric evaluations such as staining of surface antigens, aldehyde dehydrogenase detection and dead cell discrimination (*e.g.*, with 7-AAD or propidium iodide (PI)). Thus, we describe a valuable and broadly applicable method for CSC identification, isolation and sub-characterization mechanistically based on a functional, rather than a phenotypic parameter. Although originally performed with Hoechst 33342 as triggering dye, we here focus on the more recent Violet dye-based SP phenotype that is resolvable on any flow cytometer equipped with a violet laser source.

Video Link

The video component of this article can be found at https://www.jove.com/video/55634/

Introduction

The efficacy of primary cancer therapies has improved substantially over the last decade due to compelling advances in the genetic and molecular understanding of the disease and the advent of targeted drugs such as therapeutic antibodies and small molecule inhibitors. In contrast, metastatic and recurrent disease is still typically incurable, and morbidity and mortality remain high in these clinical settings. CSCs represent a distinct subpopulation within tumors and are endowed with canonical stem cell properties such as clonogenicity/tumorigenicity, multi-drug resistance and asymmetric cell division[1,2]. Thus, CSCs not only drive metastatic progression and tumor heterogeneity, but also persist during treatment to predispose the patient to relapse. Therapeutic CSC elimination is therefore an important medical need to prevent disease recurrence and allow long-term cure of cancer[3].

Identification of vulnerabilities and elucidation of strategies to eradicate CSCs heavily depends on methods that allow their purification from biological samples for subsequent expression profiling and/or functional investigation. In turn, such methods rely on surface, intracellular or functional markers that are specific for these cells. CSC-specific surface markers include, but are not limited to, CD44, CD133, CD24 and CD90, and have been used to identify CSC populations in a variety of tumor entities including breast cancer and colon cancer[4]. Another marker, aldehyde dehydrogenase (ALDH), shows intracellular localization and can be functionally detected providing a respective substrate whose enzymatic conversion produces light. Using this test, CSC populations have been identified in diverse tumor entities as well[5]. A complementary method, commonly referred to as SP analysis and portrayed here in methodological detail, harnesses active dye efflux by ABC drug transporters to identify small populations of fluorescence-dim stem-like cells[6,7,8]. To achieve this, a given sample is incubated in the presence of a lipophilic DNA-binding fluorophore which enters all cells through passive diffusion and targets nuclear and mitochondrial DNA for binding. Non-CSCs devoid of ABC drug transporter expression accumulate the dye resulting in bright fluorescence, whereas CSCs actively extrude the dye which reduces fluorescence. Pharmacological inhibition of drug transporter activity abrogates and functionally confirms the SP phenotype and should

be used for control purpose. CSC populations exhibiting SP characteristics have been disclosed, amongst others, in ovarian cancer[9,10], prostate cancer[11,12], breast cancer[13], lung cancer[14], endometrial cancer[15], glioma[16,17] and bone sarcoma[18]. Importantly, the SP assay is compatible with both cancer cell lines and primary tumor tissue, even though primary material poses additional challenges such as the requirement for a specific tumor cell discrimination strategy (certain host cell populations can exhibit SP characteristics as well)[19,20].

The two most established SP-conferring drug transporters are ABCB1/P-glycoprotein/MDR1/CD243 and ABCG2/Bcrp1/CD338[8,9,21]; however, other drug transporters can be a molecular determinant of the SP phenotype too (*e.g.*, ABCB5)[22]. ABCB1 can be efficiently blocked with verapamil whereas the activity of ABCG2 can be specifically abrogated with fumitremorgin C (FTC)[6,19]. A particular strength of SP analysis is that it can be combined with other stainings (*e.g.*, surface markers and ALDH) and that it allows live cell recovery thus being compatible with downstream functional investigations. Moreover, SP detection is broadly applicable owing to the high conservation of ABC drug transporters among CSC populations[9,23].

Originally, SP detection has been performed using Hoechst 33342 as a triggering dye[24]. This dye achieves excellent resolution but requires ultraviolet laser excitation; hence its applicability is naturally limited to high-end flow cytometric instruments. The advent of DyeCycle Violet (DCV)[25] has opened new avenues for SP analysis and extended the applicability of this method to standard flow cytometric instruments lacking an ultraviolet laser source (a violet laser source suffices to resolve DCV-SP cells). Importantly, Hoechst 33342 and DCV share common pump specificities, indicating that either dye should identify the same cell populations.

Here, we provide a detailed experimental protocol of DCV-based SP analysis for quick and easy reproduction in independent labs. We thus perceive our article as a resource for CSC researchers that should contribute to the optimization and standardization of this useful cell-biological method.

Protocol

This protocol is in full compliance with standard Institutional Ethical Review Board guidelines. If human or animal tissue is investigated using the protocol depicted here, researchers are obliged to have approval from the relevant review board of their institution or country.

Caution: Standard precautions for the safe handling of biological samples apply to this protocol. This includes wearing gloves and a lab coat, and performing the work under a biosafety cabinet whenever possible.

1. Preparation of Cells

1. **Cancer cell lines**
    1. Culture a respective human or murine cancer cell line at 37 °C in 10-30 mL of appropriate medium (*e.g.*, RPMI 1640 supplemented with 10% (*v/v*) FBS, 2 mM L-glutamine and 1x penicillin/streptomycin) and harvest the cells at sub-confluency (*i.e.* 70-90%) using digestion with 0.05% trypsin-EDTA or some other de-attachment procedure. Determine the cell count and check for viability using a counting chamber and trypan blue staining. The fraction of viable cells should exceed 85%.
    NOTE: Examples of human cancer cell lines harboring SP compartments include MCF7/HTB-22 and SKBR3/HTB-30 (breast cancer), A2780 and SKOV-3/HTB-77 (ovarian cancer), and A549 (lung cancer)[26].

2. ***Ex vivo* tumor tissue**
    1. Take a fresh surgical tumor specimen or alternatively, harvest tumor tissue from a transplantable or genetically-engineered mouse tumor model. Take 200-1,000 mg of tissue and generate a single cell suspension by mechanical disaggregation (*e.g.*, using scissors or a scalpel) and enzymatic digestion (*e.g.*, using a collagenase/dispase/DNase cocktail).
    2. Filter the sample through a 70 µm cut-off strainer. Determine the cell count and check for viability using a counting chamber and trypan blue staining.
    NOTE: Digestion cocktails frequently contain 200 µg/mL collagenase P, 0.8 U/mL dispase and 25 µg/mL DNase I, and incubation times typically range from 20 - 50 min. Ideally, tissue disaggregation is performed according to a protocol optimized for the tissue under investigation [27,28,29].

2. Test Samples

1. Adjust cell concentration to 1 x 10^6 cells/mL in fresh culture medium (a nutrient-containing vehicle is important as dye extrusion is an active energy-consuming process). Finally, transfer 1 mL of sample to an ordinary flow cytometry/FACS round-bottom tube (labelled 'test').
NOTE: For optimal results, it can be necessary that the cell concentration is titrated (for instance 5 x 10^5-1 x 10^6-2.5 x 10^6 cells/mL), but a lower cell concentration generally yields a higher resolution[19]. Only for sorting applications where a maximum number of SP cells shall be recovered, it is recommended to increase the cell concentration to up to 1 x 10^7 cells/mL.
2. Add 10 µM of DCV to the sample and mix well, either by gentle vortexing or resuspending for 3-5x. Proceed to inhibition controls.
NOTE: Optimal results might depend on titration of the triggering dye (for instance 2.5; 5; 10; 20 µM), but a higher dye concentration generally yields a higher resolution[19].

3. Inhibition Controls

1. Transfer 250 µL of the dye-containing cell suspension to a new flow cytometry/FACS round-bottom tube (labelled 'control'). Add 50 µM of verapamil or 20 µM of FTC and mix well by pipetting.

NOTE: Verapamil and FTC are the most well-established inhibitors for SP analysis but have different efflux pump specificity: Verapamil inhibits several ABC drug transporters including ABCB1 and ABCB5, whereas FTC is specific for ABCG2[6,19]. If the SP status of an individual sample is unknown, it is thus recommended to use both verapamil and FTC in separate tubes.

4. Staining

1. Cap both the 'test' and 'control' tubes and incubate them at 37 °C in the dark for 90 min, with gentle agitation every 15 min. Most of the dye extrusion in SP cells will occur within the first 45 min, but dye accumulation in non-SP cells (contributing to resolution) is only maximal after 75-90 min[19].
2. After staining is complete, wash the cells in 3-4 mL ice-cold PBS and resuspend the pellet in a volume of 100 µL (PBS as well). Keep the cells in the dark and chilled on ice, and proceed to the staining of additional markers. Alternatively, directly perform flow cytometric analysis.

5. Costaining for Other Markers

1. Add a desired panel of fluorochrome-conjugated antibodies to the DCV-stained cells, mix well and incubate the cells at 4 °C in the dark for 20-30 min. Wash the cells in 3 - 4 mL ice-cold PBS and proceed to dead cell discrimination.
   NOTE: Antibody formats that are easily compatible with DCV and virtually do not require compensation include PerCP or PerCP-Cy5.5, PE-Cy7, APC, Alexa Fluor 647, APC-Cy7, APC-H7, BV711, Alexa Fluor 750 and BV786. Formats that are compatible with DCV but may require compensation include FITC, Alexa Fluor 488, PE and BV650. Formats that are virtually not compatible with DCV include BV421, V450 and V500.
2. Add 7-AAD (or PI) to the DCV-stained cells at the concentration recommended by the particular manufacturer and incubate the samples for 5 - 10 min in the dark. Filter the samples through 70 µm cut-off strainers and proceed to flow cytometric analysis.

6. Flow Cytometric Analysis

NOTE: It is recommended to switch on the respective flow cytometric instrument during the staining process already so that everything is set as soon as the samples are ready. This includes also standard maintenance procedures such as system performance tracking and tank refilling.

1. **Acquire the cells on the flow cytometer and gate them on a bivariate FSC/SSC dot plot (Figure 1B). Exclude doublets and aggregates by comparing the different signals of FSC (*i.e.* height, width, area) (Figure 1C).**
   1. If a dead cell marker has been included, gate on the viable cell fraction (*e.g.*, 7-AAD-negative) (**Figure 1D**). Visualize the viable singlet cells on a bivariate dot plot for 'blue' and 'red' DCV emission. To this end, display the 'blue' fluorescence channel on the y-axis (*e.g.*, 450/50) and the 'red' fluorescence channel on the x-axis (*e.g.*, 525/50).
   2. Switch both channels to linear mode and adjust the detector voltages correspondingly so that the SP locates to the *side* of the plot in the lower left quadrant. Conversely, the non-SP locates to the upper right quadrant of the plot reflecting the cell cycle distribution (G1 and G2). Gate the SP and stop the acquisition (**Figure 1E**).
      NOTE: DCV-defined SP cells can be detected using flow cytometric instruments equipped with a violet laser excitation source (*i.e.* a 405 nm laser line). It is one specific of SP analysis that the emitted fluorescence is measured in linear mode and in two separate channels displayed on a bivariate dot plot. The 'blue' emission of DCV is measured at approximately 450 nm (*e.g.*, with a 450/40 standard filter) and the 'red' emission of DCV is measured at approximately 510 - 585 nm (*e.g.*, with a 510/50, a 525/50 or a 585/15 filter)[19]. Due to the particular chemistry of the assay, the separation between SP and non-SP cells is generally higher in the 'red' fluorescence channel[19].
2. Load the inhibition control(s) and acquire the data. The SP cells have now disappeared or are reduced substantially (**Figure 1F**). If necessary, refine the SP gate based on these data.
3. To investigate surface marker expression by SP cells, leave the 'red' fluorescence of DCV on the x-axis and display a respective fluorescence channel on the y-axis in logarithmic mode (*e.g.*, APC). Adjust the detector voltages correspondingly and gate the fraction of SP cells staining positive for the investigated marker(s) (**Figure 1G**). Co-expression of a particular marker by SP cells yields a respective signal in the upper left quadrant of the plot[30].
4. Record the data and/or sort the SP cells.

Representative Results

Provided is a representative SP analysis of A2780 cells, a human ovarian cancer cell line previously shown to harbor a SP accounting for less than 1% of cells[9]. Cells were cultured at 37 °C in RPMI 1640 supplemented with 10% (*v/v*) FBS, 2 mM L-glutamine and 1x penicillin/streptomycin and harvested at 85% confluency using treatment with 0.05% trypsin-EDTA. Cells were washed in PBS and filtered through 70 µm cut-off strainers. The cell count was determined using a counting chamber and trypan blue staining and 1 x 10^6 cells/mL (fresh medium) were stained with 10 µM DCV for 90 min at 37 °C in the dark. In parallel, an aliquot thereof was incubated in the presence of 20 µM FTC under exactly the same conditions. Cells were washed in 4 mL PBS and an APC-conjugated antibody against ABCG2 was added to the 'test' tube at a pre-titrated concentration. After incubation for 25 min at 4 °C, cells were washed in PBS, and 7-AAD was added to label dead cells. Samples were chilled on ice and analyzed on a FACS instrument. The depicted gating strategy is more or less universally applicable to other samples even though highly heterogeneous material such as samples from tissue specimens may require pre-definition of the target cell fraction using specific surface markers (*e.g.*, EpCAM, CD45, CD31). The results shown are intended to serve as reference for DCV-based SP detection in other labs. Hence, the relative localizations of the investigated cell populations should be roughly comparable, but surely not identical, to those obtained by other researchers with independent biological samples.

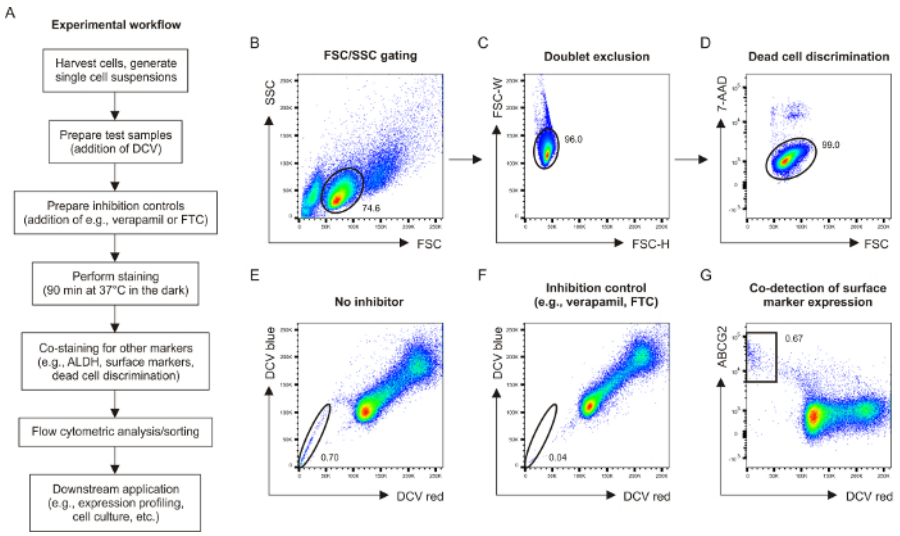

Figure 1: Workflow and Representative Results. (A) Principal flow chart for SP detection using DCV staining. Costaining for additional markers is optional, but dead cell discrimination is highly recommended. (**B-G**) Representative SP analysis of A2780 human ovarian cancer cells performed on a FACS instrument. Cells are gated according to FSC/SSC characteristics (B) and doublets and aggregates are excluded by comparing the different parameters of FSC (*e.g.*, height and width) (**C**). Thereafter, dead cells are discriminated by gating on the 7-AAD-negative (or PI-negative) cell fraction (**D**). Live single cells are then displayed on a linear bivariate dot plot for blue and red DCV emission and the SP (and the corresponding non-SP) is gated (**E**). The same analysis is now done with a respective inhibition control (in this case FTC was used), and this should lead to almost-complete disappearance of cells within the SP gate (**F**). Finally, the identified SP can be more closely characterized, *e.g.*, by determining the ABC drug transporter(s) that might be responsible for dye extrusion hence conferring the SP phenotype (in this case ABCG2, in line with FTC sensitivity). (**G**). FTC, fumitremorgin C; ALDH, aldehyde dehydrogenase. Please click here to view a larger version of this figure.

Discussion

Progress in the clinical management of cancer also depends on the development of treatment modalities that target CSCs. Methods allowing the reliable isolation of CSCs from biological samples will accelerate the identification of suitable targets and are therefore of critical importance in this endeavor. SP analysis is an established and valuable technique to identify CSC populations that express ABC drug transporters and the implementation of DCV has extended its applicability to standard flow cytometric instruments. Mechanistically, SP discrimination harnesses active dye efflux by ABC drug transporters to detect CSCs based on low fluorescence entailing a characteristic localization at the *side* of the plot bottom left to the corresponding non-SP. Importantly, DCV-based SP detection is a robust and straightforward method, with the most critical steps being (i) the preparation of cells (ii) the staining process (iii) the selection of an appropriate inhibitor and (iv) the flow cytometric analysis. Because dye extrusion is an active energy-consuming process, it is particularly relevant to start off with a good cell viability; only viable (drug transporter-expressing) cells can produce a SP. Moreover, it often makes sense to optimize the ratio between dye concentration and cell density and thereby enhance the separation of SP cells (in turn, this requires titration of both DCV and cells). A general rule is that both a higher dye concentration and a lower cell concentration result in improved separation[19]. Key to a successful DCV experiment is the functional validation of the SP using pharmacological inhibitors of ABC drug transporter activity. As stated earlier, verapamil and FTC are among the most established inhibitors for SP analysis and have different pump specificities (FTC is specific for ABCG2, whereas verapamil inhibits several pumps). For samples with unknown SP status, it is thus recommended to use both inhibitors in separate tubes. Another option is the use of the pan-ABC drug transporter inhibitor reserpine[4]. However, researchers should be aware that autofluorescence of this compound can potentially interfere with the DCV signal[19]. Once the cells are analyzed for blue and red DCV emission, the detector voltages should be adjusted so that both populations (*i.e.* SP and non-SP) are visible with maximum separation. If the picture looks strange, logarithmic scaling might be the reason why (in this case, switch both channels back to linear mode).

It is also important to note that the DCV-based SP assay is neither specific for cancer nor has it been originally developed for application in oncology. Instead, it is specific for ABC drug transporters and thus also facilitates the detection and isolation of physiological (tissue) stem cells, such as lineage$^-$ Sca-1$^+$ c-kit$^+$ hematopoietic stem cells[25]. In addition, differentiated cell types establishing dedicated barriers at crucial body sites can also exhibit SP characteristics[20]. Applying DCV-based SP detection to primary tissues hence requires specific discrimination of the target cell population using suitable markers.

In view of the high conservation of drug efflux pumps among CSC populations[9,23], DCV-based SP detection is broadly applicable, a fact that is also exemplified by its successful use across tumor entities including entities of different germ layer origin[9,17,18]. Other advantages include that (i) cells with direct implications in drug resistance are identified (particularly important for the tackling of clinically-relevant cancer cell subsets mediating disease recurrence) and that (ii) the detection of a functional, rather than a static, parameter might confer more assay specificity and improve the resolution[19,31,32]. From the conceptual/technological side, DCV-based SP detection is clearly distinct from antibody-mediated surface stainings: (i) The target structure shows intracellular localization and represents nucleic acid, not protein. (ii) The assay detects protein *activity*, not the mere presence or density. (iii) The target cells are defined by virtue of low fluorescence, not by a positive signal. (iv) As a DNA-binding dye, DCV is sensitive to even slight variations in the cell or dye concentration during staining[19]. (v) DCV fluorescence is measured in linear mode, not logarithmically. (vi) DCV fluorescence (*i.e.* the fluorescence generated by a single fluorophore) is measured in two separate channels, not in a single wavelength range. Despite these specifics, DCV-based SP analysis is easy to perform and evidently extends the flow cytometric toolbox for CSC research.

DCV-based SP analysis also offers many possibilities for costaining for other markers, being compatible with both conventional antibody-mediated surface stainings (numerous formats possible) and the commercial assay detecting ALDH enzymatic activity. However, we here focused on surface staining and the reader is referred to published literature[4] to see methodological details on codetection of ALDH activity. In any case, DCV-triggered SP analysis should be combined with dead cell discrimination and obvious reagents for this purpose are 7-AAD and PI.

Of note, DCV-defined SP cells (and non-SP control cells) can be live-purified using the sorting application of corresponding flow cytometric instruments. Because the SP assay detects a functional parameter that only viable cells can produce, the viability of the recovered SP cells can generally be expected to be high (sometimes approximate 100%). If there should be problems with post-sort viability nonetheless, different technical parameters can be checked such as the size of the sorter nozzle, the nature of the buffering agent, and the temperature during sorting (using a 70 µm nozzle we never had problems with viability, but a larger nozzle might still help for very delicate cells). The recovered cells can be subjected to downstream analyses such as qRT-PCR, Western blotting and global expression profiling (*e.g.*, using gene array or the more recent RNASeq technology). Furthermore, isolated SP cells can be investigated for functional stem cell characteristics (*e.g.*, single cell clonogenicity and serial transplantation), or sub-cultured to establish cell lines of enriched or even pure SP cells that might be stable for months[9]. In our study, we used normal medium and conventional 2D plastic flasks to sub-culture DCV-defined CSCs[9], but stem cell-selective modalities such as anchorage-independent/3D culture conditions and specialized media will also be feasible. We propose that the most favorable conditions for *in vitro* expansion of a given SP compartment will need to be checked on an individual basis.

In summary, we here depict the detailed experimental workflow of CSC identification/isolation by means of DCV-based SP analysis. Our paper shall assist CSC researchers to implement and establish this useful method in their own labs, which should further promote the elucidation of therapeutic anti-CSC strategies for improved clinical management of cancer.

Disclosures

The authors have no conflicts of interest to declare. There is also no non-author involvement in the preparation of the manuscript.

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References

1. Pattabiraman, D.R. and R.A. Weinberg, Tackling the cancer stem cells - what challenges do they pose? *Nat Rev Drug Discov.* **13** (7), 497-512 (2014).
2. Boesch, M., *et al.*, Heterogeneity of Cancer Stem Cells: Rationale for Targeting the Stem Cell Niche. *Biochim Biophys Acta.* **1866** (2), 276-289 (2016).
3. Li, Y., *et al.*, Suppression of cancer relapse and metastasis by inhibiting cancer stemness. *Proc Natl Acad Sci U S A.* **112** (6), 1839-44 (2015).
4. Greve, B., R. Kelsch, K. Spaniol, H.T. Eich, and M. Götte, Flow cytometry in cancer stem cell analysis and separation. *Cytometry A.* **81** (4), 284-93 (2012).
5. Marcato, P., Dean, C.A., Giacomantonio, C.A., and P.W. Lee, Aldehyde dehydrogenase: its role as a cancer stem cell marker comes down to the specific isoform. *Cell Cycle.* **10** (9), 1378-84 (2011).
6. Golebiewska, A., N.H. Brons, R. Bjerkvig, and S.P. Niclou, Critical appraisal of the side population assay in stem cell and cancer stem cell research. *Cell Stem Cell.* **8** (2), 136-47 (2011).
7. Hirschmann-Jax, C., *et al.*, A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci U S A.* **101** (39), 14228-33 (2004).
8. Zhou, S., *et al.*, The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.* **7** (9), 1028-34 (2001).
9. Boesch, M., *et al.*, The side population of ovarian cancer cells defines a heterogeneous compartment exhibiting stem cell characteristics. *Oncotarget.* **5** (16), 7027-39 (2014).
10. Szotek, P.P., *et al.*, Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian Inhibiting Substance responsiveness. *Proc Natl Acad Sci U S A.* **103** (30), 11154-9 (2006).
11. Brown, M.D., *et al.*, Characterization of benign and malignant prostate epithelial Hoechst 33342 side populations. *Prostate.* **67** (13), 1384-96 (2007).
12. Mathew, G., *et al.*, ABCG2-mediated DyeCycle Violet efflux defined side population in benign and malignant prostate. *Cell Cycle.* **8** (7), 1053-61 (2009).
13. Engelmann, K., H. Shen, and O.J. Finn, MCF7 side population cells with characteristics of cancer stem/progenitor cells express the tumor antigen MUC1. *Cancer Res.* **68** (7), 2419-26 (2008).
14. Ho, M.M., A.V. Ng, S. Lam, and J.Y. Hung, Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res.* **67** (10), 4827-33 (2007).
15. Kato, K., *et al.*, Endometrial cancer side-population cells show prominent migration and have a potential to differentiate into the mesenchymal cell lineage. *Am J Pathol.* **176** (1), 381-92 (2010).
16. Bleau, A.M., *et al.*, PTEN/PI3K/Akt pathway regulates the side population phenotype and ABCG2 activity in glioma tumor stem-like cells. *Cell Stem Cell.* **4** (3), 226-35 (2009).
17. Harris, M.A., *et al.*, Cancer stem cells are enriched in the side population cells in a mouse model of glioma. *Cancer Res.* **68** (24), 10051-9 (2008).
18. Murase, M., *et al.*, Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. *Br J Cancer.* **101** (8), 1425-32 (2009).

19. Boesch, M., D. Wolf, and S. Sopper, Optimized Stem Cell Detection Using the DyeCycle-Triggered Side Population Phenotype. *Stem Cells Int.* **2016**, 1652389 (2016).
20. Golebiewska, A.*, et al.*, Side population in human glioblastoma is non-tumorigenic and characterizes brain endothelial cells. *Brain.* **136** (Pt 5), 1462-75 (2013).
21. Boesch, M.*, et al.*, Drug transporter-mediated protection of cancer stem cells from ionophore antibiotics. *Stem Cells Transl Med.* **4**(9), 1028-32 (2015).
22. Fukunaga-Kalabis, M.*, et al.*, Tenascin-C promotes melanoma progression by maintaining the ABCB5-positive side population. *Oncogene.* **29** (46), 6115-24 (2010).
23. Dean, M., T. Fojo, and S. Bates, Tumour stem cells and drug resistance. *Nat Rev Cancer.* **5** (4), 275-84 (2005).
24. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med.* **183** (4), 1797-806 (1996).
25. Telford, W.G., Bradford, J., Godfrey, W., Robey, R.W., and Bates, S.E. Side population analysis using a violet-excited cell-permeable DNA binding dye. *Stem Cells.* **25** (4), 1029-36 (2007).
26. Boesch, M.*, et al.*, High prevalence of side population in human cancer cell lines. *Oncoscience.* **3** (3-4), 85-7 (2016).
27. Ali, M.Y., Anand, S.V., Tangella, K., Ramkumar, D., and Saif, T.A.- Isolation of Primary Human Colon Tumor Cells from Surgical Tissues and Culturing Them Directly on Soft Elastic Substrates for Traction Cytometry. *J Vis Exp.* (100), e52532 (2015).
28. Azari, H.*, et al.*, Isolation and expansion of human glioblastoma multiforme tumor cells using the neurosphere assay. *J Vis Exp.* (56), e3633 (2011).
29. Hasselbach, L.A.*, et al.*, Optimization of high grade glioma cell culture from surgical specimens for use in clinically relevant animal models and 3D immunochemistry. *J Vis Exp.* (83), e51088 (2014).
30. Boesch, M.*, et al.*, DyeCycle Violet used for side population detection is a substrate of P-glycoprotein. *Cytometry A.* **81** (6), 517-22 (2012).
31. Patrawala, L.*, et al.*, Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. *Cancer Res.* **65** (14), 6207-19 (2005).
32. Guha, P.*, et al.*, Nicotine promotes apoptosis resistance of breast cancer cells and enrichment of side population cells with cancer stem cell-like properties via a signaling cascade involving galectin-3, alpha9 nicotinic acetylcholine receptor and STAT3. *Breast Cancer Res Treat.* **145** (1), 5-22 (2014).

