Factors Influencing Percentage Yield of Side Population Isolated in Ovarian Cancer Cell Line SK-OV-3

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Abstract: Isolation of side population (SP) cells has been recognized as a useful technique for the isolation and identification of hematopoietic stem cells or cancer stem cells (CSCs). Thus the yield and purity of isolated SP cells would have a profound influence on the research outcomes in these two important areas. Hoechst 33342 exclusion assay technique has been used for the identification of SP cells. However, diverse Hoechst staining protocols giving different SP yields even from the same tissue type or same cell line have been reported in different laboratories. In this study we systematically investigated the underlying factors influencing the SP yield using Hoechst dye staining and a robust platform of flow cytometric analysis of the human ovarian cancer cell line SK-OV-3. Our study revealed that SP yield was not only affected by the Hoechst 33342 concentration, staining cell density, staining cell viability, staining duration, staining medium, flow cytometric setting and SP gating strategy, but was also affected by the cell passage number in SK-OV-3. This is the first systematic study on the factors affecting SP yield in adherent cells that mimic many solid tumour tissues. Our results provide important technical guidelines to help ensure reproducible and comparable results in SP and CSCs study.

Keywords: Side population (SP), SK-OV-3, cancer stem cells (CSCs), flow cytometry (FCM), Hoechst 33342.

INTRODUCTION

Side population (SP) is a subpopulation of cells that is able to efflux the fluorescent dyes Hoechst 33342 and rhodamine 123. By making use of the fluorescence activated cell sorting (FACS) technique, SP cells can be isolated on the basis of their dye efflux characteristics. SP cells, originally identified in murine bone marrow by Goodell *et al.* contain a substantial proportion of cells with high repopulation potential, which are thought to be stem or progenitor cells [1]. This definition forms the basis of Hoechst staining protocol commonly used in many laboratories. Briefly, cells are incubated in 5 μg/ml Hoechst 33342 at 37^{o}C for 90 min in Dulbecco's modified Eagle's plus medium (DMEM+). The resulting flow-sorted 0.1% Hoechst-treated SP population in murine bone marrow was found to be phenotypically highly homogeneous to hematopoietic stem cell [1]. Since then SP staining has been employed as one of the most commonly used methods for the isolation and identification of stem cells or cancer stem cells (CSCs).

Evidence of SP acting as putative cancer stem cells that can initiate tumourigensis *in vivo* has been shown in several human cancers including AML [2], breast cancer [3-6], glioblastoma [7], lung cancer [8, 9], nasopharyngeal carcinoma [10], hepatocellular carcinoma [11, 12], gallbladder carcinoma [13, 14], colorectal cancer [15], epidermal squamous cancer [16], endometrial tumour [17], oral squamous cell carcinoma [18], and oesophageal carcinoma [19]. Thus SP detection is now one of the most commonly used methods for the isolation and identification of CSCs.

However, results from published SP studies have shown considerable variation in Hoechst staining protocols. For example, Hoechst staining concentration is 2.5 μg/ml for isolating SP cells from normal human endometrium [20], human umbilical cord blood [21], human tongue cancer cell line SCC25 [22], and C6 glioma cell line [23]; while 3 μg/ml is employed for isolating SP cells from zebrafish kidney hematopoietic tissue [24]; and 5 μg/ml is used for bone marrow [2, 25, 26], and breast cancer cell line MCF-7 [3, 5, 6]. Higher Hoechst staining concentrations have also been used for different cell lines. For example, 7.5 ug/ml for esophageal carcinoma cell line EC9706 [19] and 12.5 μg/ml for skeletal muscle [27]. Although it is likely that

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ISSN: 1929-2260 / E-ISSN: 1929-2279/14

different tissues or cell lines may require different Hoechst concentrations, diverse Hoechst staining concentrations are employed by different laboratories even on the same cell line. For example, to stain hepatocellular carcinoma cell HuH7, one laboratory used a Hoechst staining concentrations of 10 ug/ml [12], while other laboratories used a much higher concentration of 20 µg/ml [11, 28], for detecting SP in these same cells.

Another important point suggesting a strong need for systematic investigation of factors that may affect the percentage of SP, is the huge variation in the SP yield reported for the same tissue or cell line in different studies. The yields of a range of these studies are shown in Tables **1**, **2** and **3**.

To date, only few studies have addressed this important area of consistency of SP yield. Goodell *et al.* pointed out that optimal resolution of the SP profile is obtained by careful attention to the critical aspects of Hoechst concentration, cell concentration, staining time, and staining temperature [29]. As cell cohesiveness affects the Hoechest 33342 staining properties of epithelial cancer cells [30], it is advantageous to perform Hoechest 33342 labeling of epithelial cancer cells in subconfluent monolayer cultures [31]. Inter-individual variations in performing Hoechst 33342 labeling, the source of the cell lines used, and the cell culture conditions also strongly influence the results and may account for the interlaboratory variation in SP cell percentages of some common cell lines [30, 32]. In addition, it has been suggested that cancer SP cells preferentially prevail at low culture density [33], thus stressing the importance of factor of cell concentration. Also knowing that the efflux pumps are inhibited by ABC blockers such as verapamil [30, 34], and therefore information about the sensitivity of verapamil in the cells of interest acts a convenient control for inter-laboratory comparison in SP study.

Montanaro *et al.* demonstrated in studies of normal cell types of bone marrow, muscle and skin cells, that isolation parameters such as tissue dissociation, staining cell concentration, and Hoechst concentration affected the yield, viability, and homogeneity of SP cells [35]. We have also conducted a comprehensive investigation of factors that may affect SP staining using a non-adherent myeloma cell line RPMI-8226 [36]. The SP% was influenced by staining conditions including: viable cell proportion, dye concentration, staining cell density, incubation duration, staining volume, and mix interval. In addition, SP% was highest one day after passage, and dropped steadily over time. The relation between culture duration and SP% suggests that the incidence of SP cells may be related to cell proliferation and cell cycle phase [36]. However, as it is not clear the findings from non-adherent cells would be equally applicable to solid tumour SP cells,

Table 1: Prevalence of SP in Tissues

Tissue type	Species	SP percentage	Reference
Bone marrow	Murine	0.1%	[1]
		0.089%	[40]
		0.04%	[41]
		0.13%	[42]
	Human	0.02%	[42]
Mammary epithelial	Human	0.18%	[43]
	Mouse	0.45%	[43]
Limbal epithelium	Human	0.2%	[44]
	Rabbit	0.56%	[45]
	Rat	0.4%	[45]
Corneal epithelium	Rabbit	4.6%	[45]
Kidney	Rat	0.03 to 0.1%	[46]
	Mouse	0.18-0.23%	[47]
Periodontal ligaments	Human	3.9%	[48]
Ovary	Human	0.1-1.4%	[49]

Table 2: Prevalence of SP in Human Tumour Cell Lines

Tumour type	Cell line	SP percentage	Reference
Heptocellular carcinoma	Huh7	0.25%	[11]
	HuH7	0.38%	[28]
	HuH7	0.9%	[12]
	PLC/PRF/5	0.80%	[11]
	PLC5	0.51%	[28]
Lung cancer	H460	5.6%	[8]
		3.8%	[9]
Gallbladder carcinoma	SGC-996	1%	[13]
	GBC-SD	0.8%	[13]
		0.87%	[14]
Colorectal	SW1116	1.48%	[15]
	LoVo	0.479%	[15]
	HCT116	0.018%	[15]
	SW620	0.679%	[15]
Prostate tumour	LAPC9	0.07%	[32]
Breast cancer	MCF7	0.2%	[32]
		7.5%	[6]
		2-4%	[3]
		1.9%	[50]
		0.39%	[5]
Ovarian cancer	SKOV3	0.05%	[32]
	OVCAR3	0.9%	[51]
Glioma	D54	0.1%	[32]
	U87	0.05%	[32]
	U87-MG	1%	[52]
		1.9%	[53]
		0.7%	[7]
	U373	0.1%	[32]
	U373MG	1.5%	[53]
		0.4%	[7]
	KNS42	0.5%	[7]
	H4	2.2%	[53]
	C6	1%	[52]
	U251	0.04%	[32]
		0.1%	[7]
Neuroblastoma	JF, SK-N-SH, IMR32, LAN-1, LAN-5	4%-37%	[54]
Head and neck squamous carcinoma	UMSCC10B	0.69%	[55]
	HN12	0.91%	[55]
Endometrial tumor	AN3CA	0.02%	[17]
Tongue cancer	SCC25	0.23%	[22]

(Table 2). Continued.

Tumour type	Cell line	SP percentage	Reference
Esophageal carcinoma	EC9706	1.2%	[19]
	EC109	0.9%	[19]
Nasopharyngeal carcinoma	CNE-1	0.7%	[10]
	CNE-2	2.6%	[10]
	SUNE-1	6.8%	[10]
	NONE-1	1.8%	[10]
	C-666-1	0.1%	[10]

Table 3: Prevalence of SP in Human Tumour

Tumour type	Species	SP percentage	Reference
Neuroblastoma	Primary tumour cell sample	0.8%-51%	[54]
Glioblastoma	Primary glioblastoma cells	1.5%	[53]
Myeloma	Bone marrow samples	0%-4.9%	[56]
AML	Bone marrow (BM) specimens	0.07%	[26]
		0.00-16%	[2]
	Peripheral blood (PB) specimens	0.00-80%	[2]
	BM & PB specimens	8.1% (0.53-29.2%)	[25]
Ovarian cancer	Human ascetic fluid samples	0.3-9.7%	[49]
Breast cancer	Fine needle aspirate (FNA) clinical samples	0.4-1.5%	[50]
Colorectal cancer	Primary colorectal cancer samples	0.042%-10.6%	[15]
Oral squamous cell carcinoma	Primary tumour samples	2.27% (0.1-9.4%)	[18]

we attempted to investigate parameters affecting SP yield using the adherent human ovarian cancer cell line SK-OV-3 as the primary target.

This study aimed to unambiguously determine the factors that may affect SP identification and isolation leading to great inconsistencies of SP yield and correct identification. Since reduction of SP% could potentially be used as an indicator of efficacy of drugs specifically targeting the root problem of cancer – the SP cells, it is of paramount importance to understand and to control the factors that affect SP yield. Each laboratory needs to optimize its SP staining protocol when they commence SP isolation from a new cell line or tissue; and results from this study can provide useful information to assist in the development of the SP staining protocol in each laboratory from different cell sources.

In this study, we examined an array of Hoechst staining parameters in SK-OV-3 cells. Factors that may influence the SP yield and profile were Hoechst 33342 staining concentration, staining cell viability, staining cell density, staining duration, staining media, flow cytometric (FCM) settings and gating strategy. The effect of different cell passage number on SP yield in SK-OV-3 was also investigated.

MATERIALS & METHODS

Cell Line and Media for Cell Culture

The human ovarian epithelial carcinoma cell line SK-OV-3, purchased from the American Type Culture Collection (ATCC), was cultured at 37^{o}C in an incubator with a humidified atmosphere containing 5% carbon dioxide in McCoy's 5A complete growth medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 µg/ml of streptomycin. All reagents were obtained from Gibco, Invitrogen (Carlsbad, CA) except for FBS (Sigma-Aldrich, St Louis MO, USA).

Hoechst 33342 Dye Preparation

A stock solution of Hoechst 33342 was prepared by dissolving 100 mg of Hoechst 33342 (Invitrogen, Carlsbad, CA) in 100 ml of sterile water. This stock solution was aliquoted as 0.5-1 ml tubes and stored at -20^{o}C in the dark for long-term storage. It was warmed to 37^{o}C in an incubator before use.

Staining Media Preparation

In this study, four staining media, RPMI+, DMEM+, McCoy's 5A+, and HBSS+ were prepared as described below. RPMI+ is Advanced RPMI1640 supplemented with 10 mM HEPES, 2% FBS, 100 u/ml of penicillin and 100 µg/ml of streptomycin. DMEM+ is DMEM supplemented with 10 mM HEPES, 2% fetal bovine serum (FBS), 100 u/ml of penicillin and 100 µg/ml of streptomycin. McCoy's 5A+ is McCoy's 5A supplemented with 10 mM HEPES, 2% FBS, 100 u/ml of penicillin and 100 µg/ml of streptomycin. HBSS+ is filtered MQ water supplemented with 1X HBSS, 10 mM HEPES, 2% FBS, 100 u/ml of penicillin and 100 µg/ml of streptomycin. All reagents were obtained from Gibco, Invitrogen (Carlsbad, CA), unless otherwise specified.

Hoechst 33342 Staining Protocol

The Hoechst 33342 stock solution was first diluted to the desired concentration before the SK-OV-3 cells were detached from the tissue culture wells with 0.25% (w/v) Trypsin (Invitrogen, Carlsbad, CA) solution. The detached cells were washed with PBS and centrifuged at 400 x g for 5 min, and were then resuspended in the diluted Hoechst 33342 solution. Unless otherwise specified, the following conditions were used: final Hoechst 33342 staining concentration 3 µg/ml, staining cell density 10^6 cells/ml, staining medium McCoy's 5A+ , staining temperature 37°C, and staining duration 90 min.

For verapamil (Sigma-Aldrich, St Louis MO, USA) treatment, verapamil stock solution (5 mM) was added to the sample to give a final concentration of 200 µM prior to the addition of Hoechst 33342. For the non-staining aliquots of cells, neither Hoechst 33342 nor verapamil was added.

Cells were incubated in 500 µl of staining medium in a water bath at 37^{o}C for exactly 90 min and were gently mixed by tube inversion every 30 min during the 90-min incubation. After incubation, cells were centrifuged at 400 x g for 5 min at 4^{o}C, and the supernatant was discarded. Cells were resuspended in cold HBSS+ at 5 x 10^6 cells/ml and placed on ice to prevent dye efflux from the cells prior to FCM analysis.

The Hoechst 33342 staining protocol was modified for the study of the specific effects of a single factor that could impact SP yield. The parameter ranges employed for the tests were as follows: Hoechst 33342 concentrations from 1.25 to 90 µg/ml, staining cell densities from 0.1 to 2 x10^6 cells/ml, staining durations from 60 to 120 min. Four staining media, RPMI+, DMEM+, McCoy's 5A+, and HBSS+, were employed.

In order to study the specific effect of a single factor affecting SP%, for each experiment, only one parameter was varied while the others remained constant unless otherwise specified. For example, when the effect of Hoechst 33342 concentration on SP yield was tested, this concentration ranged from 2 to 90 µg/ml, while the staining cell density remained at 1x10^6 cells/ml, staining duration remained at 90 min and staining temperature remained at 37^{o}C.

As 90 min is the most frequently used staining duration, when analysing the effect of staining duration on SP yield, the SP yields at 60 min and 120 min were compared with the SP yield at 90 min duration, which was defined as a control group in the experiment.

Analysis of SP Cells by Flow Cytometry and Fluorescence Activated Cell Sorting (FACS)

All this work was performed at the Centenary Institute, University of Sydney. SP cells that had been stained with Hoechst 33342 dye were analyzed using a BD^{TM} LSR II flow cytometer (Becton Dickinson, San Joes CA, USA). Propidium iodide (PI; Sigma-Aldrich, St Louis, MO USA) was added to the samples at a concentration of 2 µg/ml to exclude dead cells before the analysis. The Hoechst dye was excited using a 350 nm laser, and its emitted fluorescence was measured at two wavelengths: Blue 440/30 nm and Red 670/40 nm. Both Hoechst blue and Hoechst red fluorescence were shown on a linear scale. PI was excited at 488 nm by argon laser and its fluorescence was detected using a 610/20 filter. The acquisition and FACS data were handled and organized using the BD FACSDiva^{TM} Flow Cytometry Acquisition & Analysis Software (BD Biosciences) which displays real-time statistical analysis.

The FACS (BD FACS VantageSE, BD Biosciences) was used for SP sorting. Hoechst 33342 was excited at 350 nm, and detected using 450/58 and 670 LP filters.

PI was excited at 488 nm and detected using 630/22 nm filters. For cell sorting, Hoechst 33342-stained cells were resuspended in HBSS+ at 10 x 10^6 cells/ml. Cells were passed through a 70-µm nylon strainer (BD Falcon, Bedford MA, USA) before being transferred to Falcon FAC tubes (Becton Dickinson, USA) to prevent subsequent potential blockage in the flow sorter.

Flow Cytometric Gating Strategy

From the FSC versus SSC scatter plot, all cells were firstly selected and debris was excluded. Single cells were then gated by selecting cells with a 1:1 ratio of FSC Height (FSC-H) versus FSC Area (FSC-A). Live cells were defined as PI negative. The gate for SP was set for cells which appeared as a tiny population on the lower left hand side of blue (440 nm) on the Y-axis against red (670 nm) on the X-axis plot of the flow cytometry diagram using linear scales on both axes. The SP gate of the flow analysis was established and defined using control cells stained with both Hoechst and verapamil. Non-SP (NSP) cells were gated at the centre of the main population (Hoechst 33342^{high}).

Cell Cycle Analysis by Flow Cytometry

The sorted cells were resuspended in 70% ethanol at 4°C for 24h. The cell pellet was then collected by centrifugation and the cells washed with PBS and centrifuged again. Finally, the samples were resuspended in PBS at 1x10^6 cells/ml, and incubated with RNase A (50 µg/ml) at 37°C for 30 min, before staining with PI (50 µg/ml) and incubating at RT for 5-10 minutes. Finally the samples were analysed on the LSRII flow cytometer.

Statistical Analysis

All data obtained of FCM and FACS analyses were exported as FCS files and were analyzed using FlowJo Flow Cytometry Analysis Software, version 8.5.3 (Tree Star, Inc. Ashland, USA).

GraphPad Prizm 5 (GraphPad Software, Inc.) was used for statistical analysis of the data. Pearson test, paired two-tailed student's t test, and one-way ANOVA were used in the statistical analyses where appropriate in this study.

RESULTS

The Effect of Hoechst 33342 Concentration on SP Yield and Profile

Hoechst 33342 concentration was shown to be the most critical factor influencing SP yield and profile among all the staining parameters. When the Hoechst 33342 concentration was lower than the optimal concentration, most cells were under-stained; and thus a higher proportion of cells remained in the SP gated region, without any clear cut-off between SP and NSP. At above optimal Hoechst 33342 concentration, cells were over-stained and the SP shifted to the NSP region. At optimal Hoechst concentration, SP appeared as a tiny "tail", NSP formed a tight, oval-shaped population and the boundary between SP and NSP was more clearly defined (Figure **1A**). It is important to note as Hoechst dye is cytotoxic above a threshold concentration, an optimal Hoechst 33342 staining protocol should aim to choose a non-cytotoxic concentration. In this study, the non-cytotoxic threshold was found to be 15 µg/ml in SK-OV-3 (Figure **1B**). Variation of other staining parameters resulted in the alteration of SP percentage through changing Hoechst concentration. For example, higher cell density meant more cells shared Hoechst 33342, which would reduce the free Hoechst 33342 concentration in the staining medium, resulting in more SP cells being harvested.

The Effect of Staining Cell Viability on SP Yield

To investigate the effect of the viable cell proportion on SP yield in SK-OV-3, a sample of "dead cells" was prepared by incubating cell culture aliquots in a water bath at 55°C for 15 min. A panel of samples was then prepared by mixing culture cells with various proportions of the above-mentioned heat-treated cells immediately before staining, while keeping the two factors of the cell density and volume consistent. Correlation analysis showed a positive correlation between staining cell viability and SP yield (Pearson r = 0.4988, P = 0.0415, r^2 = 0.2488). As the staining cell viability became lower, the SP percentage dropped in SK-OV-3 (Figures **2**, **3**).

While Hoechst 33342 can be used as a vital DNA binding dye, it also binds non-specifically with the DNA of dead cells. FCM analysis revealed that the blue fluorescence intensity of dead cells in SK-OV-3 was less than that in live cells (Figure **4A**). It has been reported that the ratio of the blue fluorescence to the red fluorescence is an accurate indicator of the intracellular dye concentration [37]. Our results showed that the ratios of the Hoechst 33342 blue fluorescence to the red fluorescence in dead cells were significantly lower than that of the live cells at a series of Hoechst 33342 concentrations enabling effective differentiation of the dead versus live cells (P<0.01, two tailed t test, n=7) (Figure **4B**).

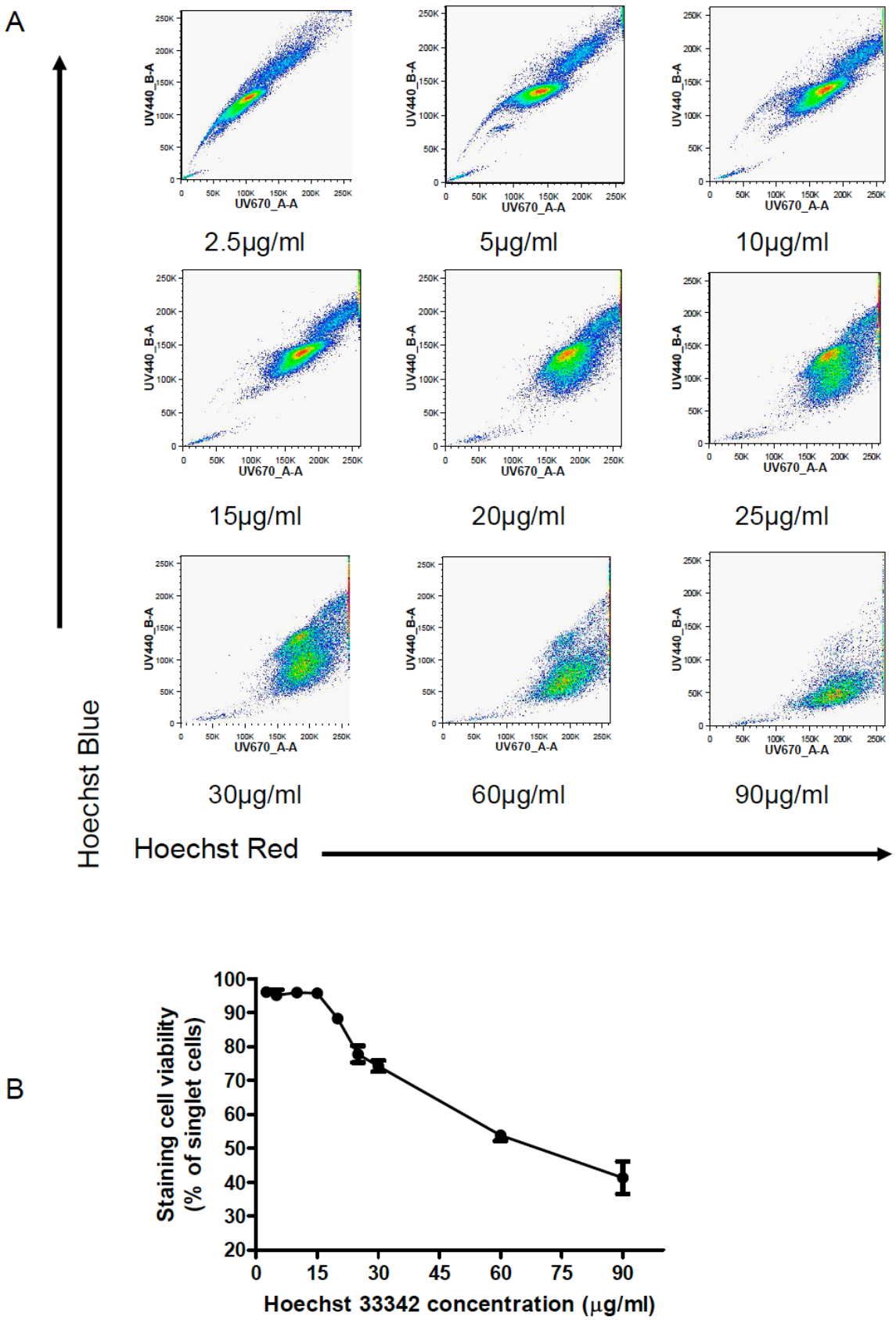

Figure 1: Effect of Hoechst 33342 concentration on SP yield.

A. Representative FCM profiles of SK-OV-3 cells stained in a range of Hoechst concentrations. **B**. Staining cell viability versus a range of Hoechst concentrations. Data are expressed as the mean ± SD (n=3) in an independent experiment.

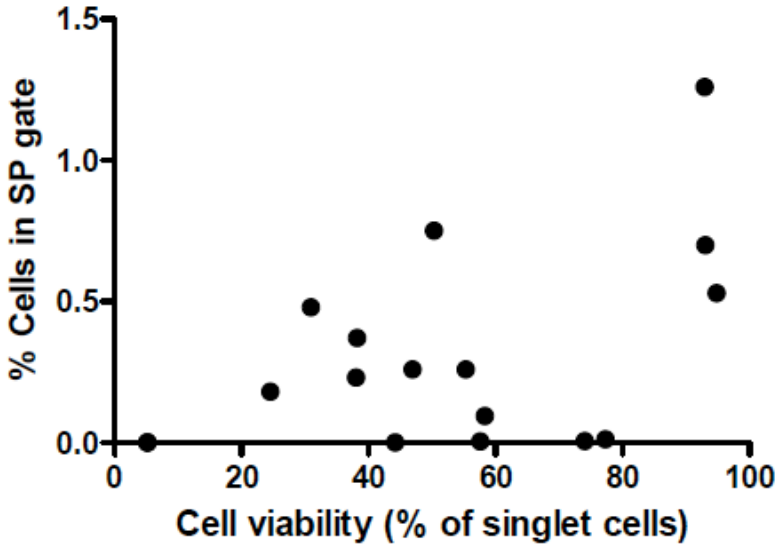

Figure 2: Effect of staining cell viability on the SP yield in SK-OV-3.
There was a positive correlation between staining cell viability and SP yield (Pearson r = 0.4988, P = 0.0415, r^2 = 0.2488). The cell viability was indicated by the data from the BD FACSDiva™ Flow Cytometry Acquisition & Analysis Software which displayed the real-time statistical analysis. Data were from three independent experiments (n=3).

The Effect of Staining Cell Density on SP Yield

As the Hoechst 33342 concentration was a critical factor affecting the SP yield and its flow profile, the staining cell density would be likely to affect the SP yield by changing the free Hoechst 33342 concentration in the staining medium. The results confirmed that SP yield was positively correlated with staining cell density (Figure **5A**). Furthermore, one-way ANOVA analysis showed there was no significant difference in the cell viability among different staining cell densities ranging from 0.5 to 2 x10^6 cells/ml (P>0.05) (Figure **5B**).

The Effect of Staining Duration on SP Yield

To investigate the effect of staining duration on SP yield, the range of 60 to 120 min of incubation at 37°C for the Hoechst 33342 staining was tested. The SP percentage at 60 and 120 min staining duration was compared with that of the 90 min staining duration group, and one-way ANOVA analysis showed that the SP% at 120 min was significantly lower than that at 90 min at both Hoechst concentrations of 3 µg/ml (P<0.05) and 5 µg/ml (P<0.01). The SP% at 60 min was significantly lower than that at 90 min only at a Hoechst concentration of 5 µg/ml (P<0.05) (Figure **6A**). There was no significant difference in the cell viability for different staining durations for either Hoechst concentration (Figure **6B**). To avoid the effect of cell passage number on the SP yield, the experiments were performed within six passages of SK-OV-3 cells.

The Effect of SK-OV-3 Cell Passage Number on SP Yield and Profile

When the SK-OV-3 cells were harvested from relatively low passage numbers (<5-10), it was difficult to obtain a yield of SP. At relatively higher passage numbers (>10-15), a yield of SP cells was more easily detected (Figure **7A**). An additional factor, the sub-G1 population, should be taken into consideration. Usually the sub-G1 cell percentage of the total population was higher in the lower passage than that in the higher passage. Longer culture would lead to lower sub-G1% in the total populations (Figure **7A**).

To further study the cell cycle phase of these two populations in low passage of SK-OV-3 cells, they were sorted for cell cycle analysis. Gate 1 cells were sorted out from the sub-G1 population, Gate 2 cells were sorted out from the main population or G1 population. Cell cycle analysis showed that the DNA content of sub-G1 population was basically half of the main population or G1 population (Figure **7B**).

In the SK-OV-3 cells within the higher passage and lower sub-G1%, SP was easily detected, and vice versa. Moreover, this characteristic was not influenced or reversed by the Hoechst concentration (Figure **8**).

The Effect of Staining Media on SP Profile

Four staining media, DMEM+, RPMI+, Mc Coy's 5A+ and HBSS+ were tested in this study under the following staining conditions: Cells were stained at

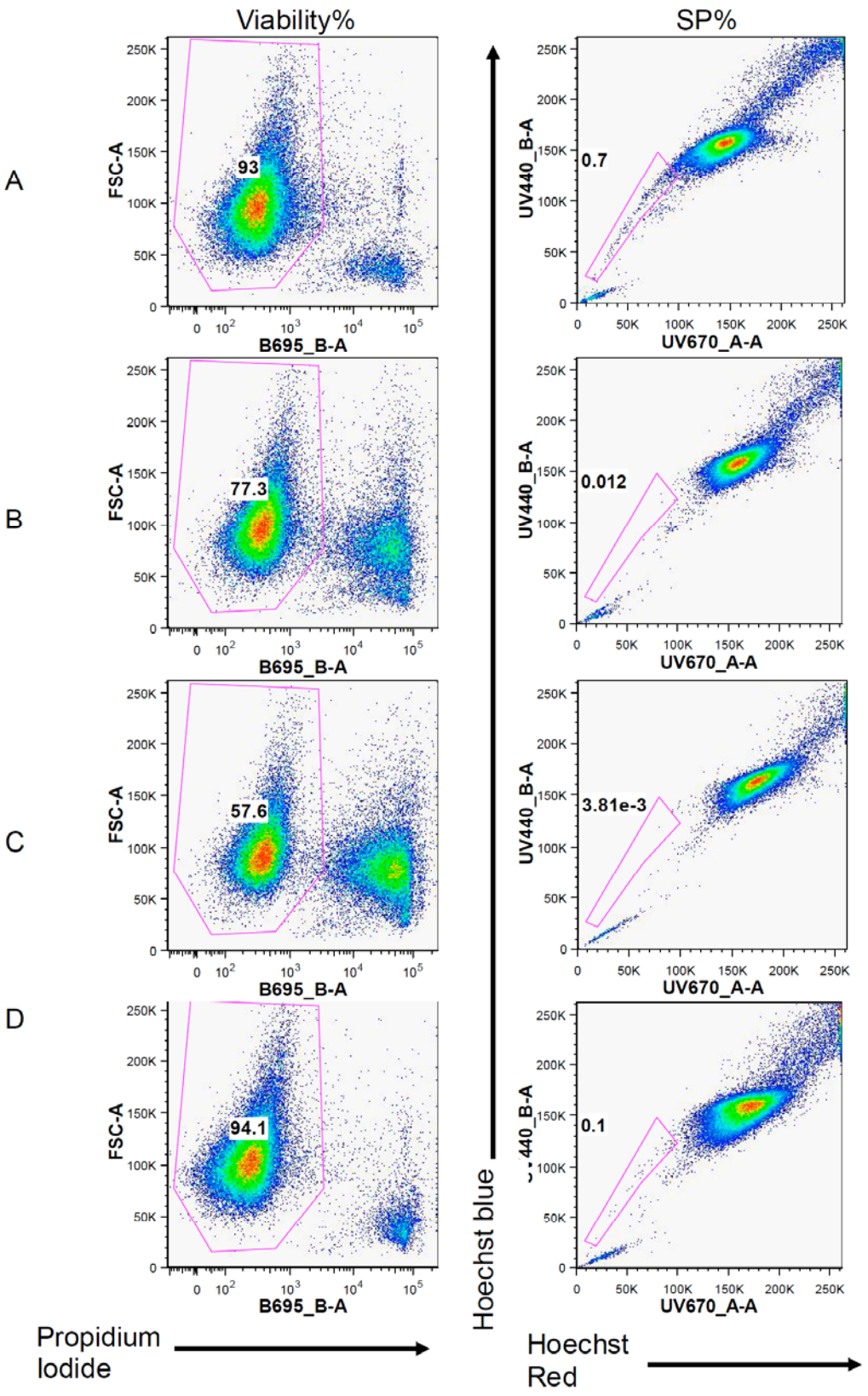

Figure 3: Cell viability and SP yield in SK-OV-3.

Representative FCM profiles of samples that contained various proportions of culture cells and heat-treated cells were analysed on LSRII. The samples were comprised of (**A**) 100% of culture cells; (**B**) 50% of culture cells and 50% heat-treated cells; (**C**) 100% of heat-treated cells or (**D**) Cells (100% of culture cells) that were stained with Hoechst with addition of verapamil were used as control cells for SP gating.

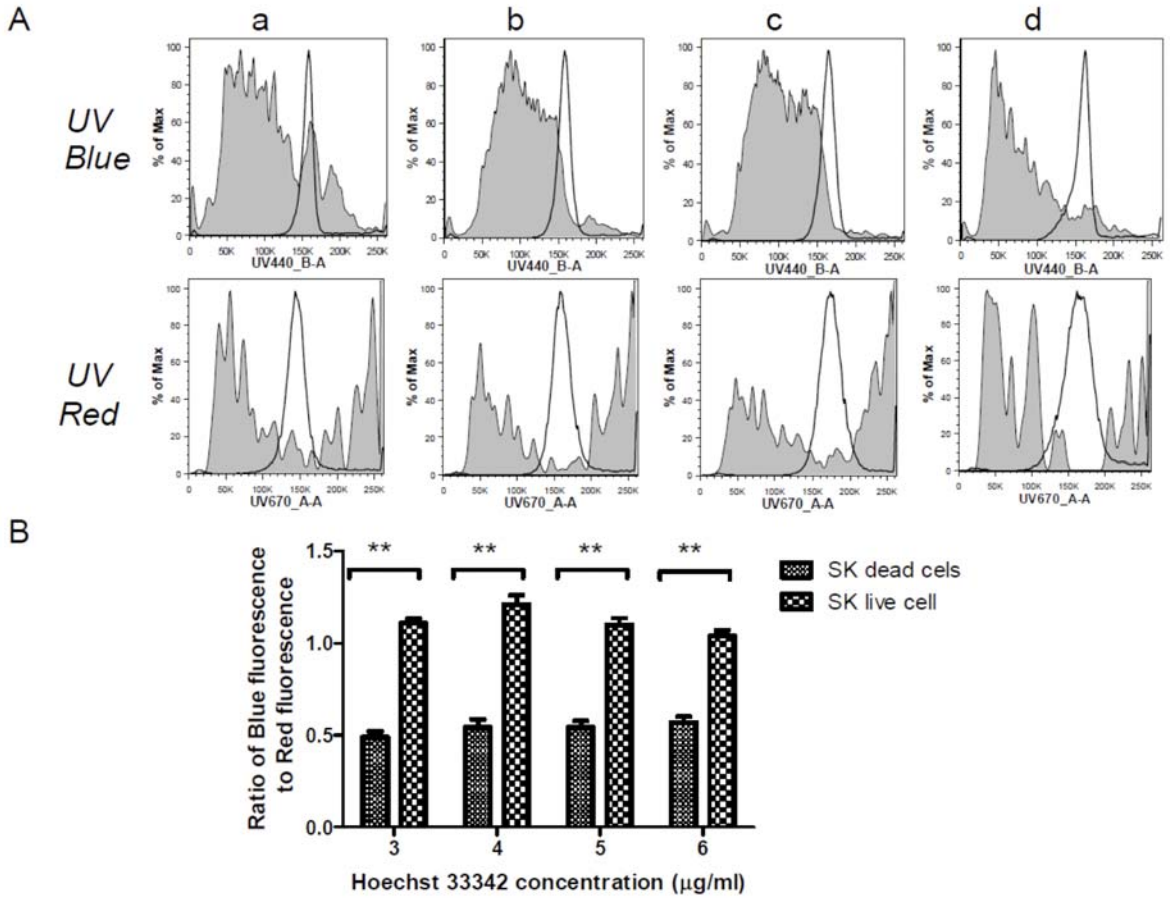

Figure 4: Hoechst 33342 fluorescence intensity in dead cells and live cells.

A. Hoechst 33342 blue and red fluorescence intensity in cells with different viabilities. Hoechst 33342 staining concentration was 3 µg/ml. Open histogram is the fluorescence of live cells, filled histogram is the fluorescence of dead cells. The samples comprised (a) 100% of culture cells, cell viability was 92.9%; (b) 50% of culture cells and 50% heat-treated cells, cell viability was 74.5%; (c) 100% of heat-treated cells, cell viability was 57.7% or (d) Cells (100% of culture cells, cell viability was 94.9%) that were stained with Hoechst with addition of verapamil were used as control cells for SP gating. **B**. The ratio of the Hoechst blue fluorescence to the red fluorescence in dead cells and live cells within SK-OV-3 at a series Hoechst 33342 staining concentration of 3 µg/ml, 4 µg/ml, 5 µg/ml and 6 µg/ml. **: P<0.01.

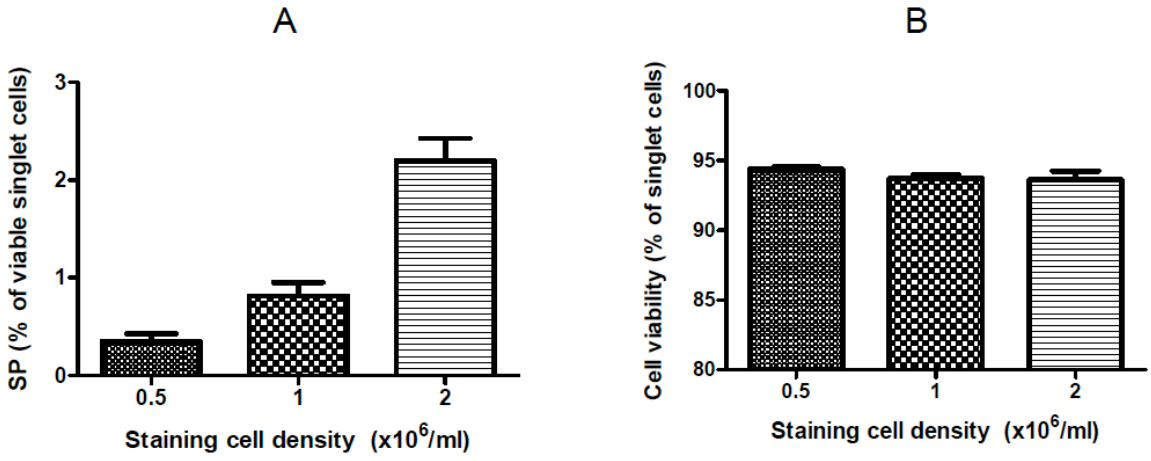

Figure 5: SP yield and cell viability over the staining cell density range.

A. SP yields over the staining cell density range. Data are expressed as the mean ± SEM of three independent studies (n=3). The correlation between SP yield and the staining cell density was significant (The Pearson r was 0.9961, P value was 0.0281, and r^2 was 0.9922). **B**. Cell viability over the staining cell density range. One-way ANOVA analysis showed there was no significant difference in the cell viability among different staining cell densities ranging from 0.5 to 2 x10^6 cells/ml (P>0.05).

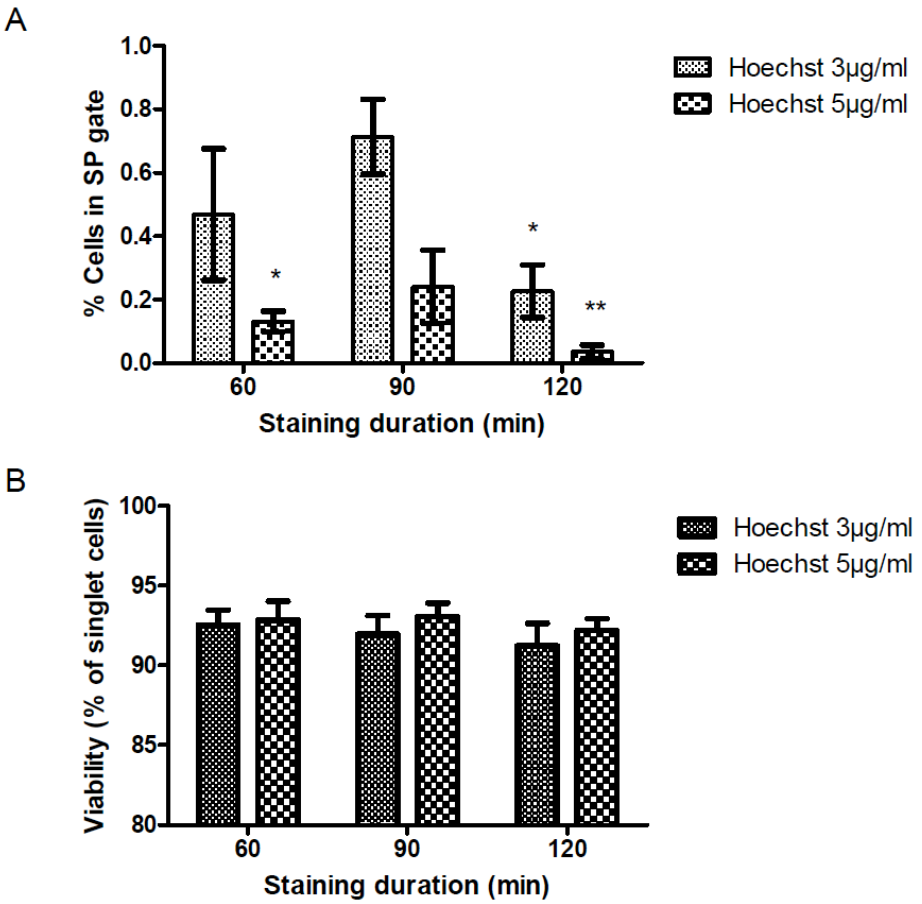

Figure 6: SP yield and cell viability within SK-OV-3 at different staining durations.

Data were from three independent experiments and expressed as mean ± SEM. **A**: SP yields for different staining durations of 60 min, 90 min and 120 min. *: P<0.05, **: P<0.01 versus 90 min of staining duration at the same Hoechst concentration. **B**: There was no significant difference in the cell viability of SK-OV-3 for different staining durations (P>0.05, One-way ANOVA) at Hoechst concentration 3 μg/ml and 5 μg/ml.

$1x10^6$ cells/ml, 37°C for 90 min, and Hoechst 33342 concentration of 5 μg/ml. When SK-OV-3 cells were stained in DMEM+, RPMI+, Mc Coy's 5A+, the sub-populations and SP cells could be distinguished clearly, suggesting that any of these could be used as suitable staining media. When HBSS+ was employed, the cells were under-stained with no clear cut-off between SP and NSP being seen. This suggests that HBSS+ was not a suitable staining medium (Figure **9A**).

FCM Analysis and Gating Strategy

In FCM, the optimal SP profile can be found by adjusting the Photomultiplier tube (PMT) voltage of UV blue and UV red channels. As the voltage is increased, the detector sensitivity increases, resulting in an increased signal [38].

The optimal voltage was selected based on the cell passage number, Hoechst staining concentration and flow cytometer setting in order to optimize the SP gating. A clear SP "tail" ensures not only a better display of SP, but also a greater accuracy of SP gating and SP cell counting. Alteration of UV blue and UV red voltages would change the SP profiles (Figure **9B**). Increasing UV blue and UV red voltages at the same time shifted the SP from the lower left hand of the profile to the upper right hand along the diagonal making the SP population easier to distinguish. The voltage of UV blue and UV red could not be changed on the FlowJo, the Flow Cytometry Analysis software used in this study. The optimized UV voltage was selected on the BD FACSDivaTM Flow Cytometry Acquisition & Analysis Software.

DISCUSSION

This study aimed to investigate the underlying factors that may significantly influence the yield and profile of SP cells. The SP profile would affect the SP gating and SP cell counting parameters. We observed different profiles from the same sample could lead to

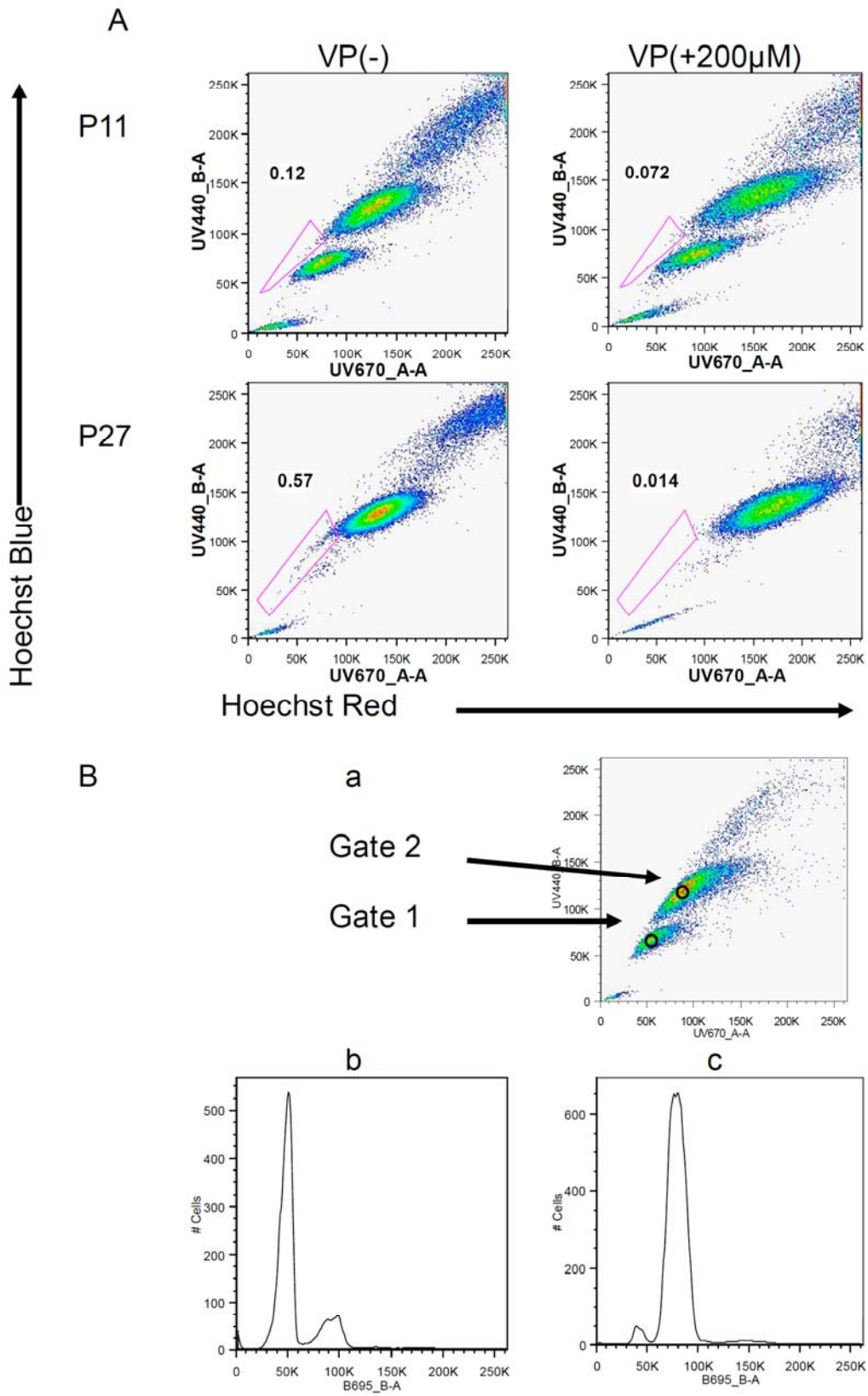

Figure 7: Effect of cell passage number on SP.

A: FCM profiles for SK-OV-3 of 2 passages in the same experiment: In the low passage (P11) SP cells were rare (SP%=0.12%). In the relative high passage (P27), SP was easily detected (SP%=0.57%). **B**: Cell cycle analysis of two populations in low passage SK-OV-3: (a) Cells in G1 and sub-G1 population in SK-OV-3 (passage 15) were sorted out for cell cycle analysis. (b) DNA content of sorted sub-G1 cells from Gate 1. (c) DNA content of sorted G1 cells from Gate 2.

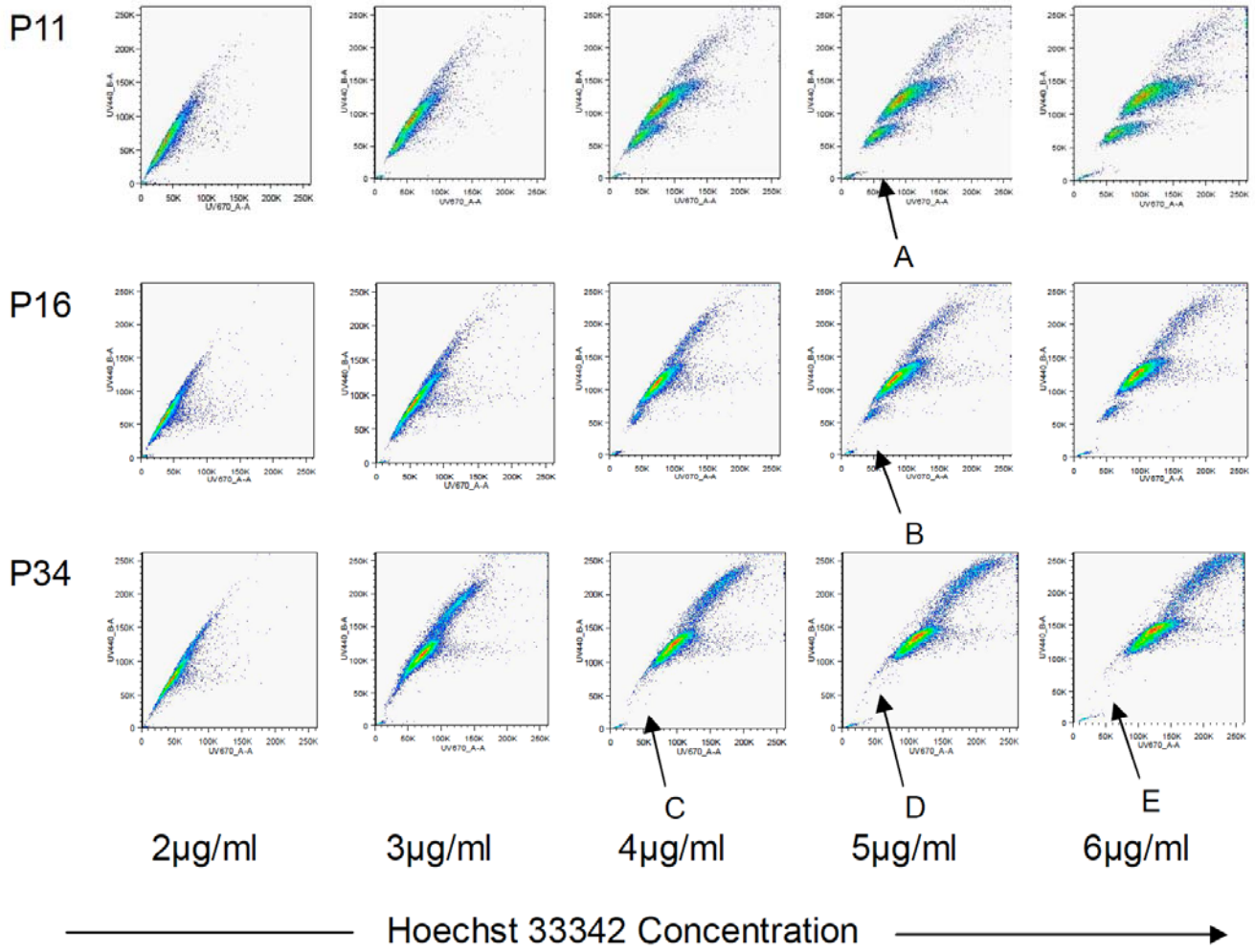

Figure 8: FCM profiles of SK-OV-3 of three passages versus Hoechst concentration range.
In the low passage (P11) there was a high percentage of sub-G1 population cells (Arrow A). As the cell passage number increased (P16), the sub-G1 population became smaller (Arrow B). In the SK-OV-3 cells in the higher passage (P34) with lower sub-G1 cells percentage, SP was easily detected (Arrow C, D, E). Data were derived from an independent experiment.

different SP%. Therefore FCM analysis and gating strategy should be included in the factors affecting SP yield accounting.

As expected, Hoechst concentration strongly influenced the SP profile pattern present as a useful distinct “tail” and greatly altered the proportion of cells present in the SP gate. It is therefore necessary to optimize the Hoechst concentration for different batches of dye. It is recommended that the same Hoechst 33342 batch be used for reproducible SP cell staining and subsequent isolation. Similarly, it is necessary to perform the Hoechst concentration optimization procedures when a new cell line is to be investigated.

Cell counts before Hoechst staining should be performed carefully to adjust the staining cell density precisely and accurately. Since Hoechst 33342 binds to all DNA-containing cells [35], the cell number in each of these categories of live, dying, and dead populations should be noted when considering the staining cell density. Our results showed that dead cells took up less Hoechst 33342 than live cells. It means that when the staining cell density is the same, if the viability of the cells is low, the effective Hoechst 33342 concentration (concentration of free dye in the staining medium) becomes relatively higher because the dead cells share less dye. This is the same effect as using a higher dye concentration resulting in a lower SP percentage. An important implication of this finding is that when quantifying SP yield in the screening of anti-CSC medicines, staining cell viability should be taken into consideration carefully because dead cells and live cells might share different amounts of the Hoechst dye, and only when their viabilities of the majority NSP components are similar, would the SP yields be comparable.

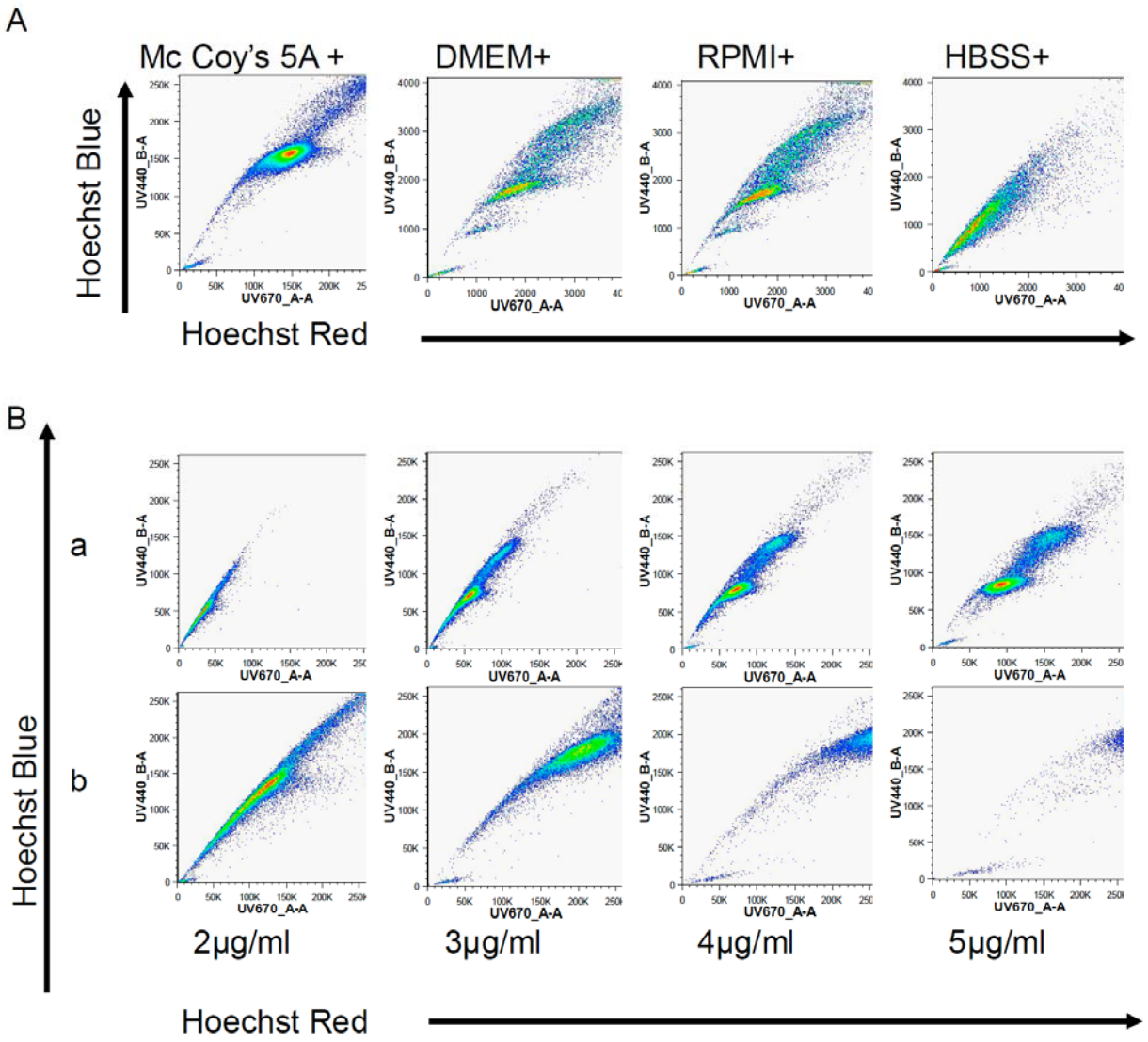

Figure 9: Effect of staining medium and FCM parameter setting on SP profiles.

A. Representative FCM profiles of cells stained in different media: SK-OV-3 cells were stained in Mc Coy's 5A DMEM+, RPMI+, and HBSS+, respectively. **B**. Representative FCM profiles of SK-OV-3 cells analyzed on LSR II with two UV blue and UV red voltage setting: The cells were stained at four Hoechst 33342 concentrations, the sample stained at the same concentration was analyzed on LSR II with two UV blue and UV red voltage settings. (a): UV 440_B-A: 250; UV670_A-A: 560. (b): UV 440_B-A: 280; UV670_A-A: 660. Data were from the same independent experiment.

Cells may have morphological or phenotype changes as a result of long term culture. Cell lines at high passage numbers experience alterations in cell morphology, response to stimuli, growth rates, protein expression, transfection, and signaling, compared to lower passage cells [39]. In our study cell line, higher and lower passage numbers exhibited different SP profiles and quantity. This highlights the importance of choosing cells of similar passage numbers to obtain accurate and comparable data.

Other factors examined in this study included staining duration and different types of culture medium as the SP staining media. It was found that 90 min was optimal for the cell line SK-OV-3 which is similar to the time reported for RPMI-8226 [36]. Our study also showed that staining medium from a range of commonly used sources such as DMEM+, HBSS+, PBS/BSA and RPMI+ should also be carefully tested for optimal Hoechst staining protocol when new tissues or cell lines are being studied.

In conclusion, this study examined a range of important factors that affected both the yield and profile of SP cells. These factors included the passage number of the cell line, Hoechst 33342 staining concentration, staining cell density, staining cell viability, staining duration, staining media, FCM setting

and gating strategy. Results from this study provide a useful evaluation system for laboratories to establish and optimize their SP staining protocol in order to generate reproducible and comparable results in SP and CSCs study.

ACKNOWLEDGEMENTS

This work was supported by grants from Cancer Institute NSW (D.M.Y.S). We also like to acknowledge the support of the National Institute of Complementary Medicine Centre Grant of Collaborative Centre of Traditional Chinese Medicine. We want to express our great thanks to Dr Adrian Smith, Christopher Brownlee and Robert Salomon -- the flow cytometry experts at the Centenary Institute, Sydney for their technical expertise. Last but not least, we like to thank Kei Fan, Shihong Yang, Jia Li in the Faculty of Pharmacy, University of Sydney for their help and critical discussion.

AUTHORSHIP

Contribution: Y.C. designed experiments, performed research, analyzed / interpreted data, made figures and wrote the paper; S.L.M. designed experiments, performed research, analyzed / interpreted data; F.W.S.W designed experiments, analyzed / interpreted data, G.Q.L. analyzed / interpreted data and wrote the paper; Y.S.L. performed research; B.D.R. helped design experiments, M.V.B. edited the paper, D.M-Y.S. designed experiments and wrote the paper; all coauthors reviewed and discussed the manuscript.

CONFLICT-OF-INTEREST DISCLOSURE

The authors declare no competing financial interests.

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Received on 06-12-2013 Accepted on 22-01-2014 Published on 13-02-2014

DOI: http://dx.doi.org/10.6000/1929-2279.2014.03.01.5