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Multiparameter flow cytometry for the characterization of human embryonic stem cells (hESC)

Kathryn Brosnan, Andrew Want, Karen Coopman and Christopher J Hewitt*

Centre for Biological Engineering, Department of Chemical Engineering, Loughborough University, Leicestershire, LE11 3TU, UK

**Author for correspondence. (Phone: +44-1509-222506; Fax: +44-1509-223923; E-mail: c.j.hewitt@lboro.ac.uk)*

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Abstract

Using multiparameter staining methods and flow cytomeyry to investigate the pluripotency of HUES7 human embryonic stem cell cultures it was found that the multidimensional approach of marker co-expression allowed the different cell populations to be easily identified and demonstrated cross reactivity between the SSEA 4 and SSEA 1 antibodies, resulting in a substantial false positive SSEA 1 population. It is the accepted norm to apply control gates at a 95% confidence level of the isotype control, however this study found that adjusting the control gate to a 99% confidence level significantly reduced the effect of this cross reactivity. Though conversely, this gating shift also decreased the positive marker expression of SSEA 4 and Tra-1-60, indicating that there is a need for strongly expressing markers coupled with increased optimization of fluorophore/antibody combinations before a gating strategy of 99% can be implemented on a more routine basis.

Introduction

Flow cytometry is a well-established, analytical technology employed across a wide range of medical and research areas to study the functional and structural properties of a cell. This is often accomplished by targeting protein markers with the application of fluorescently labeled monoclonal antibodies, both within and at the surface of the cytoplasmic membrane. Immunophenotyping is used extensively within hematology, and allows identification of multiple phenotypic and functional markers enabling the comprehensive interrogation of cells on a single cell level (Czechowska et al. 2008; Shapiro 1883). The advantage of flow cytometry is the combination of multiplexed assays; measuring several features of a single cell simultaneously, whilst sampling a statistically significant number of cells (~5000).

The understanding of the biological and chemical processes that regulate a stem cells' pluripotent and multipotent states would not have developed at the rate, and extent to which, it has, without flow cytometric analysis. Flow cytometry allows cells with any biologically interesting characteristics to be qualified and coupled with the application of fluorescence activated cell sorting (FACS), isolated. This at-line quality control testing and product purification while indispensable at laboratory scale will be critical in bringing clinical therapies to the market (Want *et al.* 2012).

A number of human embryonic stem cell (hESC) pluripotency associated markers have been documented, both intracellular (e.g. transcription factors NANOG, SOX2 and OCT4 (Chambers *et al.* 2003; Medvedev *et al.* 2008; Nichols *et al.* 1998; Adachi *et al*. 2010; Fong *et al.* 2008)) and surface (e.g. glycolipids SSEA 3/4, keratan sulfate antigens Tra-1-60 and Tra-

1-81 (Draper *et al.* 2002; Wright and Andrews 2009; International Stem Cell Initiative 2007; Henderson *et al.* 2002)), however unlike their multipotent counterparts, mesenchymal stem cells (Dominici *et al.* 2006), no definitive phenotypic marker panel has been agreed upon (International Stem Cell Initiative 2007; Laslett *et al.* 2003; Carpenter *et al.* 2004). This is predominantly due to the fact that of the numerous embryonic cell lines that have been established (167 lines approved for use in UK alone (MRC UK Stem Cell Line Registry), for the limited number of these that have been compared at least, the expression levels for the reported pluripotency markers have been shown to be similar but not identical (International Stem Cell Initiative 2007; Carpenter *et al.* 2004). The contribution of divergent culture techniques in different laboratories to this diversity is debatable (Carpenter *et al.* 2004; Allegrucci *et al.* 2005; Allegrucci and Young 2007). Additionally, the lack of knowledge about documented target markers has also hindered the selection of a universal marker panel. This is particularly true for surface markers where for most, their physiological function remains unclear (Henderson *et al.* 2002; Allegrucci *et al.* 2005; Allegrucci and Young 2007) especially considering some are not exclusive to hESCs and are expressed on various other cell types (Allegrucci and Young 2007; Barraud *et al.* 2007; Linju-Yen *et al.* 2005; Gang *et al.* 2007). Still, given the potential of non-invasive FACS, surface markers are the ideal standard marker panel candidate. Nevertheless, until such a time that a universal phenotypic marker panel is agreed upon, the current generally accepted minimum criteria for defining pluripotency of a hESC population is by demonstrating the expression of a combination of these markers (Hoffman and Carpenter 2005; Cai *et al.* 2006), usually with the addition of at least one known differentiation marker as a negative control.

Current hESC literature is awash with flow cytometric data, but the vast majority employ laborious secondary (indirect) antibody methods, where a single fluorophore-conjugated

antibody is employed to detect all of the markers individually, or single color primary (direct) antibody staining where a fluorophore-conjugated antibody specific for the antigen of interest is applied. These conventional methods while effective in obtaining reliable and reproducible data are somewhat cumbersome and, more critically, information-poor in comparison to the capabilities of modern multiparameter flow cytometry. Multiparameter (a.k.a. multiplex or polychromatic) staining methods, apply a cocktail of fluorophore conjugated antibodies allowing multiple targets to be interrogated simultaneously, each indicated by a different fluorescent color. Pioneered in hematology laboratories in the 1980s (De Rossa *et al.* 2003), integration into stem cell laboratories has been relatively slow compared to other biological disciplines. Multiparameter flow cytometry is cost efficient, time saving and more robust, while requiring less sample volume than conventional staining methods (Prowse *et al.* 2009). Most crucially, it allows for the capture of multidimensional data (Jansen *et al.* 2008) which may otherwise have been left undisclosed. Multiparameter flow cytometry does, however, require a greater degree of understanding of the properties of fluorescent molecules and a greater variety of controls to ensure proper interpretation of data. This, in combination with limited availability of fluorophore-conjugated antibodies and the larger amount of post-acquisition analysis has retarded the progression of multiparameter flow cytometry into stem cell research. These challenges are diminished however, by the ability of multiparameter flow cytometry to produce more information-rich datasets with the possibility of observing interactions between markers within a single cell. Currently, only a handful of stem cell focused publications (International Stem Cell Initiative 2007; Carpenter *et al*. 2004; Henderson *et al*. 2002; Gang *et al.* 2007; De Rossa *et al.* 2003; Prowse *et al.* 2009; Pruszak *et al.* 2007; Ramirez *et al.* 2011) have demonstrated the benefits of this technique but given the strength of the data these have presented, it is predicted that this number will continue to rise.

Here relatively simple three color multiparameter flow cytometry was employed to investigate the pluripotency of HUES7 hESC cultures. Three extracellular markers were chosen, due to their widespread use within hESC research SSEA 4, Tra-1-60 and SSEA 1. SSEA 1 which has been shown previously to be down regulated on pluripotent hESC and up regulated upon differentiation (Andews *et al.* 1996; Thomson *et al.* 1998) was included as a negative control. The main objective of this study was to illustrate the strength of multiparameter flow cytometry methods within stem cell research and how the acquired data can be exploited beyond the scope of the more conventional single color staining methods.

Materials and Methods

HUES7 cells were cultured feeder free on Matrigel^{TM} basement matrix ((BD Biosciences) Thomas *et al.* 2009). Monolayer cultures were maintained in 5% CO_2 37°C incubators between passages 24 and 36. Cells were passaged using 0.05% Trypsin/EDTA (Sigma Aldrich) for a 1 minute 37°C incubation followed by centrifugation for 5 minutes at 100g.

All assay reagents and antibody conjugates were supplied as part of a surface antigen kit, FlowCellect Human ESC (Tra-1-60) Surface Marker Characterization Kit (Merck Millipore, Darmstadt, Germany). Briefly, single cell suspensions were prepared in wash buffer at a cell concentration of $1x10^6$ cell/ml. 250µl cell suspension was transferred to each test sample tube before centrifugation at 600g for 3 minutes. Cells were resuspended in 500µl assay buffer and the centrifugation cycle repeated. After the supernatant was aspirated to waste, cells were resuspended in 100µl of assay buffer. Antibody (Tra-1-60-FITC, SSEA 4 -PE and SSEA 1-PE/CY5) and negative control conjugates (IgM-FITC, IgG3-PE and IgM-PE/CY5) were applied (5µl per test) before being placed in a light proof container at 2-8°C for 1 hour. After incubation 400µl of assay buffer was added and test samples were centrifuged as before. Cells

were resuspended in a final volume of 500µl assay buffer and analyzed. Preliminary studies indicated that fixation of the cells had no impact on the assay (data not shown) so this step was omitted.

All data were collected using a Guava easyCyte 8HT (equipped with 488 and 640 nm lasers); post-acquisition analysis was performed using FlowJo (v.7.6.5). Compensation adjustments were carried out at the time of analysis using Incyte^{TM} acquisition software (v.2.2.2) on the analyzer. During post-acquisition analysis all compensation parameters were verified using the FlowJo^{TM} compensation tool. A minimum of 5000 gated events per sample was collected.

Throughout the study, cells were periodically karyotyped using standard G-banding of 30 metaphase spreads at the Centre of Medical Genetics, Nottingham, UK. The samples were prepared according to the method described by Thomas *et al.* (2009).

Results

The multiparameter assay was conducted on 4 different HUES7 cultures, each at a different passage number; only two assays were subsequent passages of the same culture (Experiment 1 at p34 and Experiment 2 at p36 culture). Cells were found to be karyotypically normal with a minimum of 28/30 nuclei analyzed having 46XY with no increase in abnormalities reported. Also, morphology of the cultures was consistent with pluripotent cells grown in monolayer. The expression levels of the extracellular markers (Figure 1) were indicative of a predominantly pluripotent culture, with each test sample strongly expressing SSEA 4 (≥96%) and, while Tra-1-60 was not as strongly expressed, the proportions shown here (≥ 60%) were similar to those reported elsewhere (Draper *et al.* 2002; Henderson *et al.* 2002). Figure 1(*i*)

clearly demonstrates the power of the multiparameter approach allowing for the quantification of the cell subpopulations that coexpress both positive target markers (SSEA 4 and Tra-1-60). Notably, almost all Tra-1-60 positive cells were SSEA-4 positive. Figure 1(*ii*) shows similar data expressing SSEA 4 against the negative marker expression SSEA 1, again the use of quadrants allowing the heterogeneous population to be clearly deciphered. The representative dot plot in Figure 1(*ii*) shows that approximately 15% of the cell population was expressing SSEA 1 however it was the positioning of this sub population on the SSEA 4 axis that was interesting, because it seemed that the SSEA 1 positive population (median 13.3) was also emitting the strongest SSEA 4 intensity (median 421). On further analysis it was discovered that this was not an isolated event and was consistent throughout each test sample. This was particularly unexpected as it has previously been shown that upon differentiation, SSEA 4 is one of the first markers to be down regulated (International Stem Cell Initiative 2009; Liang *et al.* 2010).

To explore this observation, the positive SSEA 1 population was backgated on to the SSEA 4 Tra-1-60 dot plot (seen previously Figure 1(*i*)) to investigate where and how this sub population was distributed among the positive marker population. It was revealed that the SSEA 1 positive (SSEA 1^{+ve}) population was preferentially distributed high in the double positive quadrant (Figure 2A). The scale of this preferential distribution cluster can be seen for each experiment in Figure 2B where the SSEA 1^{+ve} population was isolated within SSEA 4 Tra-1-60 quadrant gates to attain the percentage distribution.

Figure 2B indicates that the SSEA 1^{+ve} population is positioned at the upper limit SSEA 4^{+ve} intensity. This combined with the low SSEA 1^{+ve} fluorescence intensity, led to the hypothesis that this was the result of cross reactivity between the two glycolipid epitopes and not a true

SSEA 1^{+ve} population. To test this the isotype control gate was expanded from 95% to 99%. (Figure 3A). The 4% adjustment had a significant effect, almost totally eradicating the SSEA 1^{+ve} population, which can be seen when comparing Q6 from Figure 1(*iv*) with Q6 in Figure 3A(*iv*). This comparison can be seen even more clearly in Figure 3B which illustrates an example of the before and after backgated dot plot, where the SSEA 1^{+ve} population was reduced from 15.4% to 2.9%.

Conversely, while expanding the gates reduced the SSEA 1^{+ve} population, the gating shift also reduced the positive population of the other two markers (SSEA 4^{+ve} and Tra-1-60^{+ve}; Table 1). The decrease in the SSEA 4^{+ve} was negligible with the majority of test samples retaining expression >95%, however Tra-1-60 expression, which in comparison was already relatively low, was considerably reduced with the shift. With expression levels lower than <45% the 99% gate shift has inadvertently disqualified Tra-1-60 as a suitable hESC pluripotency marker for this cell line. To make such a conclusive assumption is beyond the scope of this work and will require a much more in depth study. However it is noteworthy that, when running successive passages (Exp. 1 and Exp. 2), a marked decrease in Tra-1-60 expression was observed over the 6 day culture period, shown in Table 1. Admittedly, SSEA 1 expression was also significantly decreased during this culture period although the high SSEA 1^{+ve} population seen in Exp. 1 could be attributed to the aforementioned cross reactivity as on average it was reduced from 36% to <3% when the isotype gates were shifted to 99%. SSEA 4 expression was not affected however, and remained constant >95% during this period, leading to uncertainties with regard Tra-1-60 expression stability and by extension, further question the suitability of this marker in the panel.

Discussion

Multiparameter flow cytometry offers an enormous opportunity to greatly improve the quality and quantity of available data acquired from stem cell analysis. In this present study hESC cultures were investigated for expression of known pluripotency surface markers using a simple 3 color assay. The benefits compared to more conventional secondary or single color primary staining protocols were evident almost immediately. It was calculated that per multiparameter experiment where 8 tubes (1 isotype control, 3 compensation controls and 4 multicolor test samples) were prepared, at least 15 tubes (3 isotype controls and 12 one color test samples) would be required to obtain the same level of repeatability if using conventional methods. This method instantly reduced preparation times, sample size requirements and costs. The inclusion of compensation controls in these initial assay development experiments meant significant savings of antibody volumes were not achieved. However, it was found that the compensation parameters for these experiments was very consistent with only very slight inter-assay adjustment being required (+/- 0.06 < 3%; data not shown) suggesting perhaps that once a stable protocol has been established, a single run of compensation controls could be used for future experiments. This is particularly true considering any inter assay variation could be corrected using post-acquisition analysis software. Removing these controls from some experiments at least, would decrease operator workload by 75% per experiment over conventional methods not to mention the increased cost efficiency.

Furthermore, the capacity of additional data output when using multiparameter flow cytometry was enormously beneficial. The multidimensional aspect of marker co-expression was most useful in deciphering the different cell populations and demonstrated that there may be some cross reactivity between the SSEA 4 and SSEA 1 epitopes. This was an unexpected discovery and one that would have been impossible to deduce from conventional staining methods. After a comprehensive review of the recent publications that have reported

multiparameter flow cytometry methods, surprisingly few have employed SSEA 1 as a negative marker, despite its frequent use in publications that applied conventional staining techniques. As a consequence, similar evidence of this potential cross reactivity does not seem to have been reported, though Strain *et al.* (2009) did report finding staining in markers with ≤10% of cells above the isotype threshold was most often due to nonspecific binding, and additionally recommended increasing the isotype gates to ensure such events were gated out. The populations measured by flow cytometry can be approximately modeled by a normal distribution: this means that confidence intervals can be utilized that encompass a given proportion of the population. Gating the negative isotype control at a 95% confidence level literally applies a vertical delimiter defining any population to the left of this point as negative with 95% probability. Similarly, to the right is deemed a positive population. Current literature is replete with articles where the data has been gated at 95% of the negative control however this study shows sufficient evidence that this is inadvisable, especially when the implication of such false positives in cells destined for the therapeutic market is considered, some of the samples investigated in this study presented with SSEA 1^{+ve} population of up to 35%, potentially leading to massive product and therefore potential financial losses. On the other hand, if the isotype gates were to be shifted to 99%, then to ensure the utmost confidence in the data, there is a need for strongly expressing markers coupled with increased optimization of fluorophore antibody combinations. Otherwise, there is the risk that target populations will be gated out, again potentially leading to marked financial losses. The consequence of this can be seen in the Tra-1-60 marker expression which was effectively gated out when the isotype gate was increased to 99%. The average level of positive expression seen in this study (>60%) when gated at 95% of the isotype control are in line with values reported elsewhere in the literature (Draper *et al.* 2002; Henderson *et al.* 2002). However more interestingly, it has been shown that Tra-1-60 expression is particularly

sensitive to the cell culture conditions (Schinzel *et al.* 2011) and demonstrated how, by using different culture platforms, the Tra-1-60 expression could be increased or decreased accordingly. This in mind, it is difficult not to consider the possibility that under different culture conditions the recorded loss of expression between passages may have been greatly reduced if not totally eradicated and similarly the gate shift to 99% may not have been so detrimental to the marker expression in general. This paper lends weight to the opinions of Allegrucci and Young (2007) and the International Stem Cell Initiative (2007), that the regulation and standardization of human embryonic culture conditions among research laboratories could be the first steps required to limit expression marker variation in embryonic cell lines. Perhaps also by extension the first steps towards defining a universal phenotypic marker panel for human embryonic stem cells.

Defining and fully understanding the characterization of pluripotent stem cells remains a major challenge in stem cell biology. Nevertheless, the considerable progress that has been documented to date strongly suggests that this is not an insurmountable task and with time this goal will be achieved. However for this to materialize, it will be imperative that researchers fully exploit current technologies and laboratory protocols to maximize the depth of all available data. Multiparameter flow cytometry is a powerful cell characterization technique that maximizes the capture of significantly more data than conventional flow cytometry methods. This technique has a multitude of benefits to offer current and future stem cell research with available data increasing geometrically with the addition of each parameter (Preffer and Dombkowski 2009). It promises practically limitless potential, with considerable scope remaining for the introduction of new more advanced antibody conjugates to address the fundamental questions that are being raised as the regulatory establishment that controls pluripotent cell states is further elucidated. The application of multiparameter flow cytometry

in stem cell research is still in its infancy but recent publications (International Stem Cell Initiative 2007; Carpenter *et al.* 2004; Gang *et al.* 2007; De Rosa *et al.* 2003; Prowse *et al.* 2009; Pruszak *et al.* 2007; Ramirez *et al*. 2011) suggest this technique is actively being incorporated into stem cell laboratories worldwide, representing hopefully the first important steps towards ensuring high resolution data-rich experiments become the accepted norm.

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Figure and Table Legends

Figure 1 – Cell Distribution at 95% Isotype Control Gate (*i*) and (*ii*) representative dot plots for the three markers, (*i*) SSEA 4/PE against Tra-1-60/FITC and (*ii*) SSEA 4/PE against SSEA 1/ PE-CY5. Each dot plot is flanked by two histograms highlighting the positioning of the quadrant boundaries corresponding to 95% of the isotype control. (*iii*) and (*iv*) bar charts are used to represent a specific quadrant on the density plot, with each bar showing the mean of the samples within a single experiment (error bars are calculated from the standard error). $n \geq 3$ in all cases.

Figure 2 - SSEA 1^{+ve} Population Distribution (A) The isolated positive SSEA 1 population, shown here in the single color histogram was backgated onto the SSEA 4 Tra-1-60 dot plot. Backgating illustrated SSEA 1^{+ve} population preferentially distributed high in the double positive quadrant Q2. (red black dot plot, where the red is the SSEA 1 positive population). Note slight increase in positive percentage from 15.2% on figure 1(*ii*) to 18.9% on histogram due to fact quadrant dot plot is gated at 95% of two colors and not just single color as in the case of the histogram. (B) The positive SSEA 1 population was isolated within the SSEA 4 Tra-1-60 quadrant gates to identify the percentage distribution of this daughter population for each sample. The percentage distribution is displayed on a bar chart next to the parent SSEA 4 Tra-1-60 population distribution.

Figure 3 - Cell Distribution at 99% Isotype Control Gate *(*A) (*i*) and (*ii*) show representative dot plots for the three markers, (*i*) SSEA 4/PE against Tra-1-60/FITC and (*ii*) SSEA 4/PE against SSEA 1/ PE-CY5. Each dot plot is flanked by two histograms highlighting the positioning of the quadrant boundaries corresponding to 99% of the isotype control. (*iii*) and (*iv*) bar charts are used to represent a specific quadrant on the density plot, with each bar showing the mean of

the samples within a single experiment (error bars are calculated from the standard error). $n \geq 3$ in all cases. (B) Illustrates a representative backgated dot plot before and after the gate shift. (*i*) shows the distribution of the SSEA 1^{+ve} population at 95% of the isotype control on the left (shown previously in Figure 2) and (*ii*) on the right shows same population after the isotype control gate has been increased to 99%.

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Table 1. Expression (%) of surface markers recorded at 95% and 99% gating. The column immediately following the marker title column (2^{nd} from left) highlights the single color expression levels for each marker. A loss of Tra-1-60^{+ve} expression was observed between subsequent cultures (76.5 – 55.0%). Cells were in culture 6 days between analyses. Multiparameter marker co-expression can be read by cross referencing the marker title (column 1) against the markers titled within the multiparameter column. Values given are the mean of the samples within a single experiment (± are calculated from the standard error) for each run, $n \geq 3$ in all cases.

Table 1

% Expression											
		Single Parameter Data		Multiparameter Data							
				SSEA 4				Tra-1-60			
		95%	99%	95%	99%	95%	99%	95%	99%	95%	99%
				+ve		-ve		+ve		-ve	
Exp 1 (n=3)	SSEA 4 **+ve**	97.5±1.5	81.9±10.7								
	SSEA 4 **-ve**	3.2±1.2	18.1±10.7								
	Tra-1-60 **+ve**	75.6±4.0	42.1±5.2	73.1±3.3	34.4±0.6	2.5±0.1	7.8±5.7				
	Tra-1-60 **-ve**	25.3±4.4	2.6±1.2	24.6±4.8	47.5±10.2	0.7±0.4	10.3±5.1				
	SSEA 1 **+ve**	36.1±9.0	2.6±1.2	35.9±9.1	2.5±1.3	0.2±0.1	0.1±0.0	31.8±4.9	4.8±1.9	10.8±4.4	1.8±1.4
	SSEA 1 **-ve**	63.8±9.0	97.4±1.2	61.6±8.5	80.2±9.6	2.28±1.6	17.2±10.3	33.0±6.9	43.2±6.2	24.3±4.4	50.4±4.6
Exp 2 (n=4)	SSEA 4 **+ve**	98.5±0.1	96.3±0.3								
	SSEA 4 **-ve**	1.5±0.1	3.7±0.3								
	Tra-1-60 **+ve**	55.0±1.8	25.9±1.4	54.7±1.8	25.6±1.4	0.2±0.1	0.3±0.1				
	Tra-1-60 **-ve**	45.0±1.8	74.1±1.4	43.8±1.9	70.7±1.6	1.2±0.1	3.4±0.2				
	SSEA 1 **+ve**	6.9±0.2	1.3±0.1	6.7±0.24	1.2±0.1	0.2±0.0	0.1±0.0	5.8±0.3	0.9±0.1	0.64±0.0	0.3±0.0
	SSEA 1 **-ve**	93.0±0.2	98.7±0.1	92.1±0.2	95.8±0.2	0.9±0.1	2.9±0.3	46.5±1.9	22.9±1.4	47.0±1.8	75.9±1.4
Exp 3 (n=4)	SSEA 4 **+ve**	99.8±0.1	98.2±0.2								
	SSEA 4 **-ve**	0.2±0.0	1.7±0.2								
	Tra-1-60 **+ve**	79.2±0.9	42.3±1.5	79.2±0.9	42.2±1.5	0.1±0.0	0.1±0.0				
	Tra-1-60 **-ve**	20.8±0.9	57.7±1.5	20.7±0.9	56.1±1.3	0.2±0.0	1.6±0.2				
	SSEA 1 **+ve**	14.6±0.6	2.7±0.1	14.6±0.6	1.2±0.1	0.0±0.0	0.0±0.0	8.91±0.2	2.1±0.1	0.74±0.1	0.8±0.1
	SSEA 1 **-ve**	85.4±0.6	97.3±0.1	85.2±0.6	95.8±0.2	0.2±0.0	1.6±0.2	69.1±1.1	33.9±1.4	21.1±0.9	63.3±1.3
Exp 4 (n=4)	SSEA 4 **+ve**	98.9±0.1	89.5±0.5								
	SSEA 4 **-ve**	1.1±0.1	10.5±0.5								
	Tra-1-60 **+ve**	57.6±1.4	29.8±1.5	57.5±1.4	28.8±1.4	0.1±0.0	1.0±0.1				
	Tra-1-60 **-ve**	42.3±1.4	70.2±1.5	41.3±1.4	60.7±1.4	1.0±0.1	9.5±0.5				
	SSEA 1 **+ve**	17.4±1.9	1.8±0.4	17.1±1.8	1.7±0.4	0.3±0.1	0.1±0.0	11.5±0.9	1.2±0.2	7.7±1.2	0.7±0.2
	SSEA 1 **-ve**	82.6±1.9	98.2±0.4	81.5±1.8	87.8±0.5	1.1±0.1	10.4±0.5	39.2±0.6	23.5±1.5	41.6±2.8	74.5±1.3

Figures

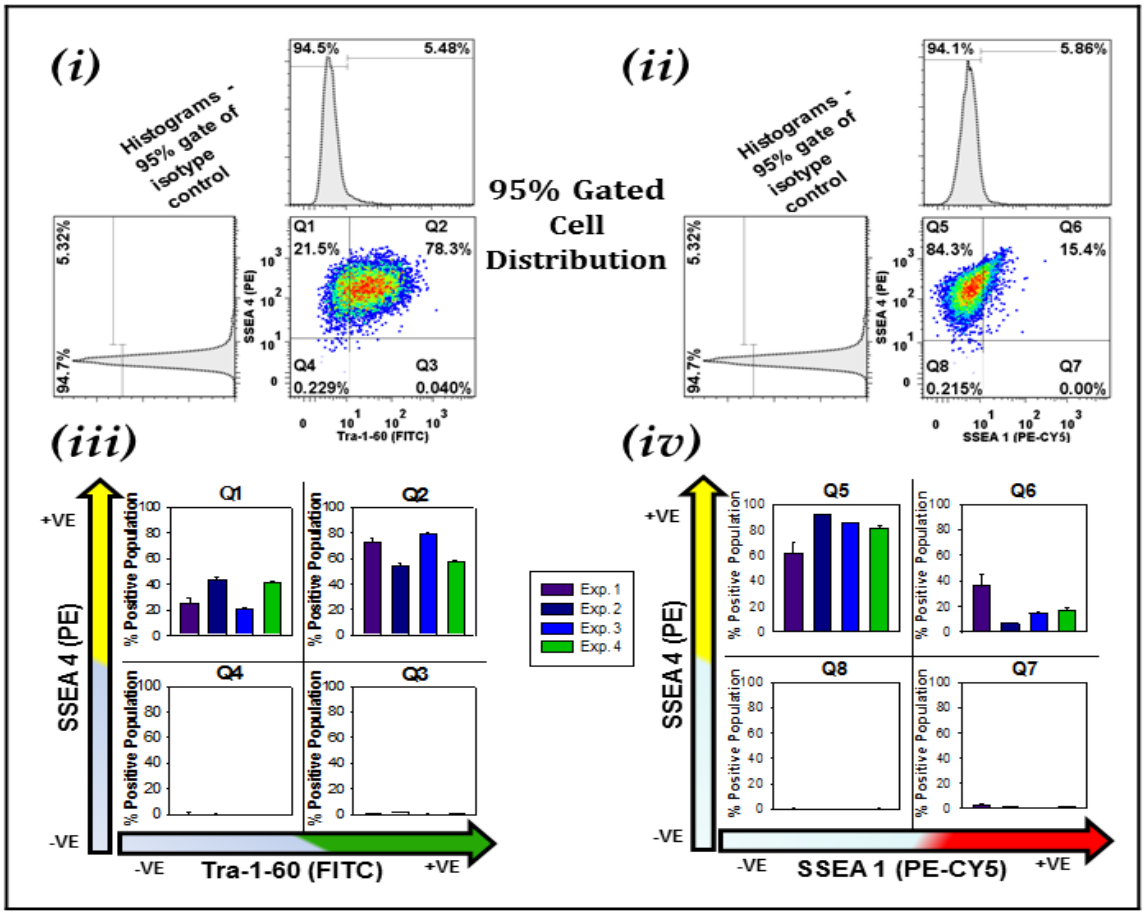


Figure 1

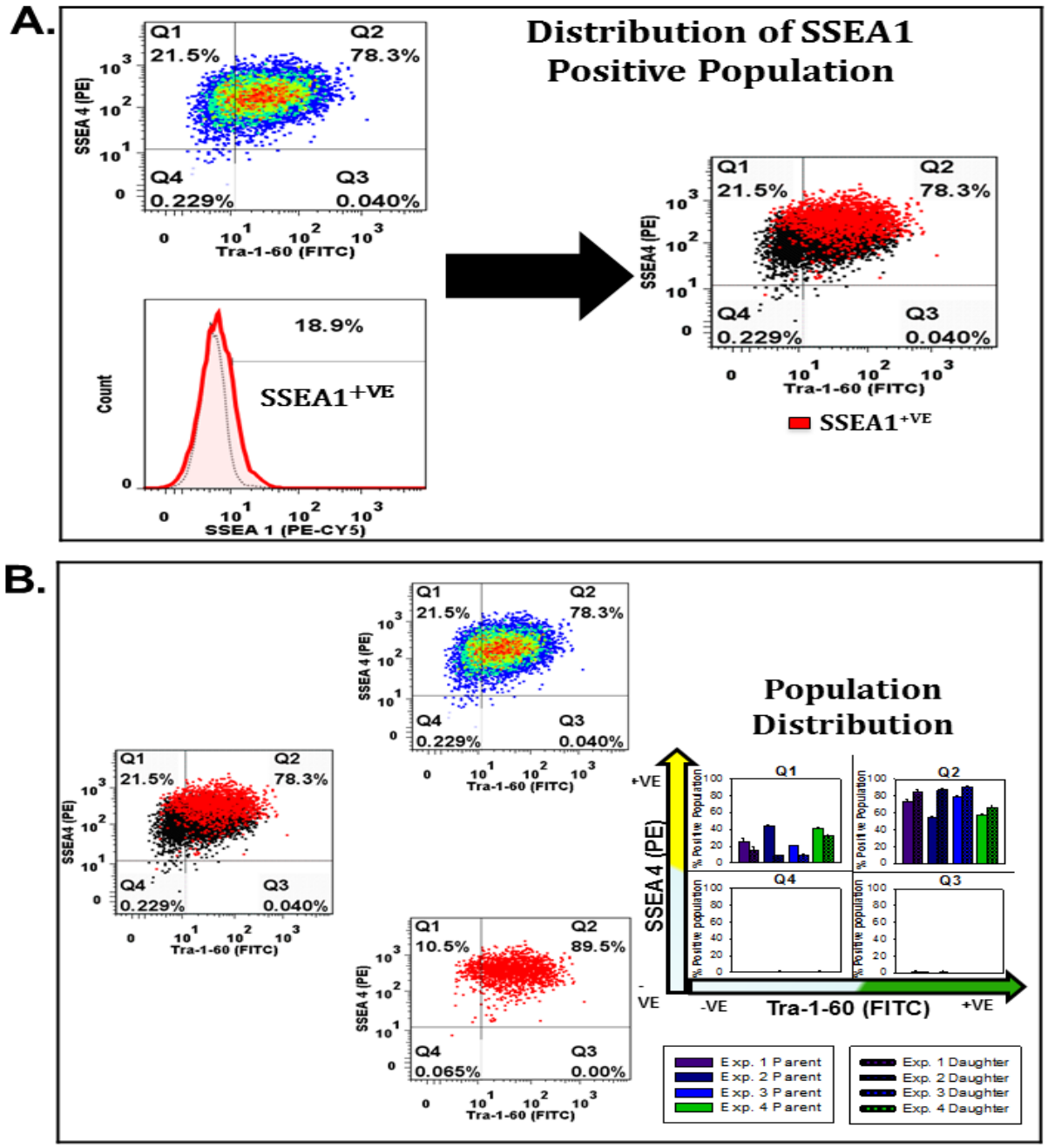

A.
Distribution of SSEA1 Positive Population
Q1 21.5%
Q2 78.3%
Q4 0.229%
Q3 0.040%
SSEA 4 (PE)
Tra-1-60 (FITC)
18.9%
SSEA1+VE
Count
SSEA 1 (PE-CY5)
SSEA4 (PE)
SSEA1+VE
B.
Q1 10.5%
Q2 89.5%
Q4 0.065%
Q3 0.00%
Population Distribution
+VE
-VE
% Positive Population
Exp. 1 Parent
Exp. 2 Parent
Exp. 3 Parent
Exp. 4 Parent
Exp. 1 Daughter
Exp. 2 Daughter
Exp. 3 Daughter
Exp. 4 Daughter

Figure 2

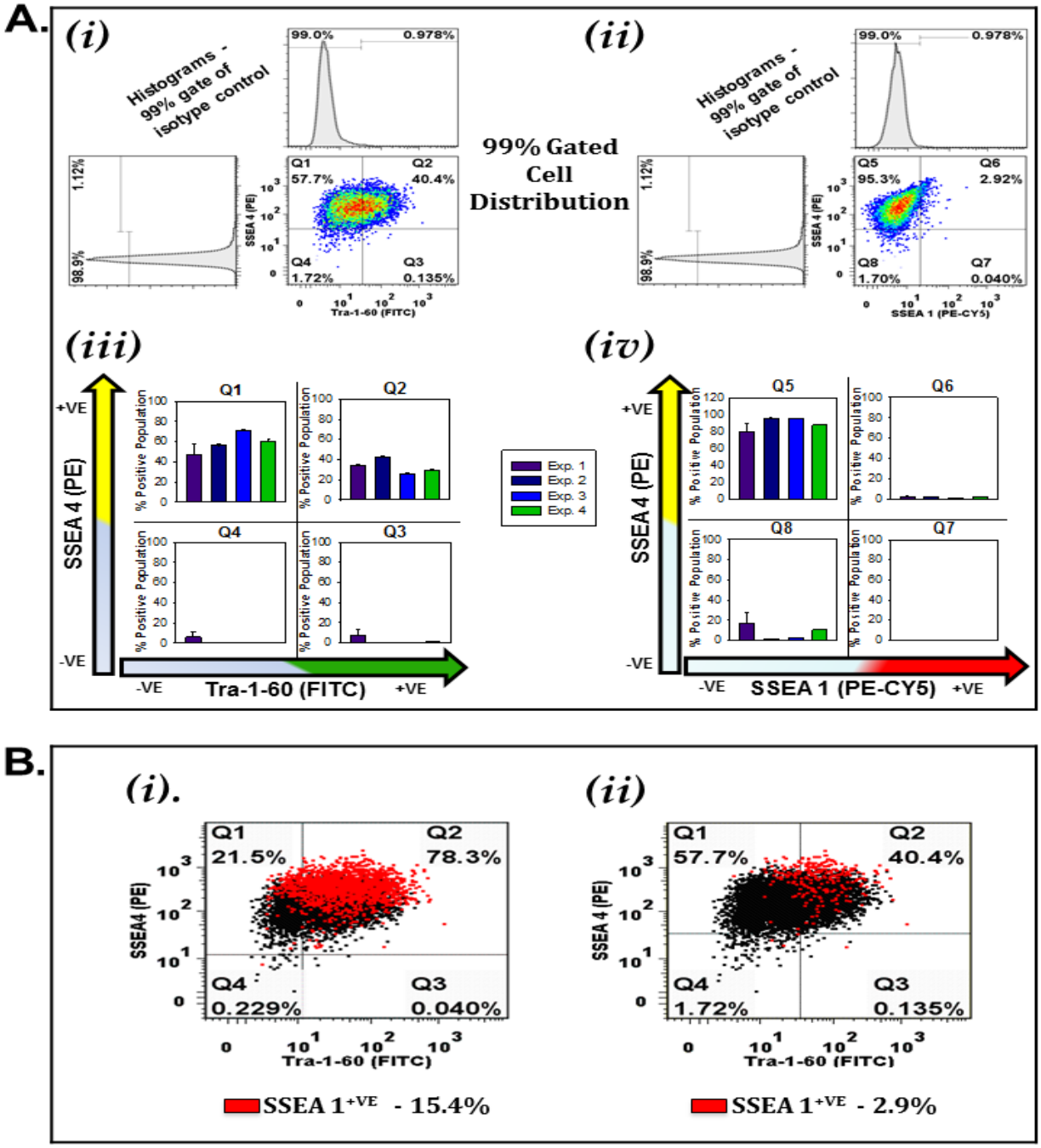
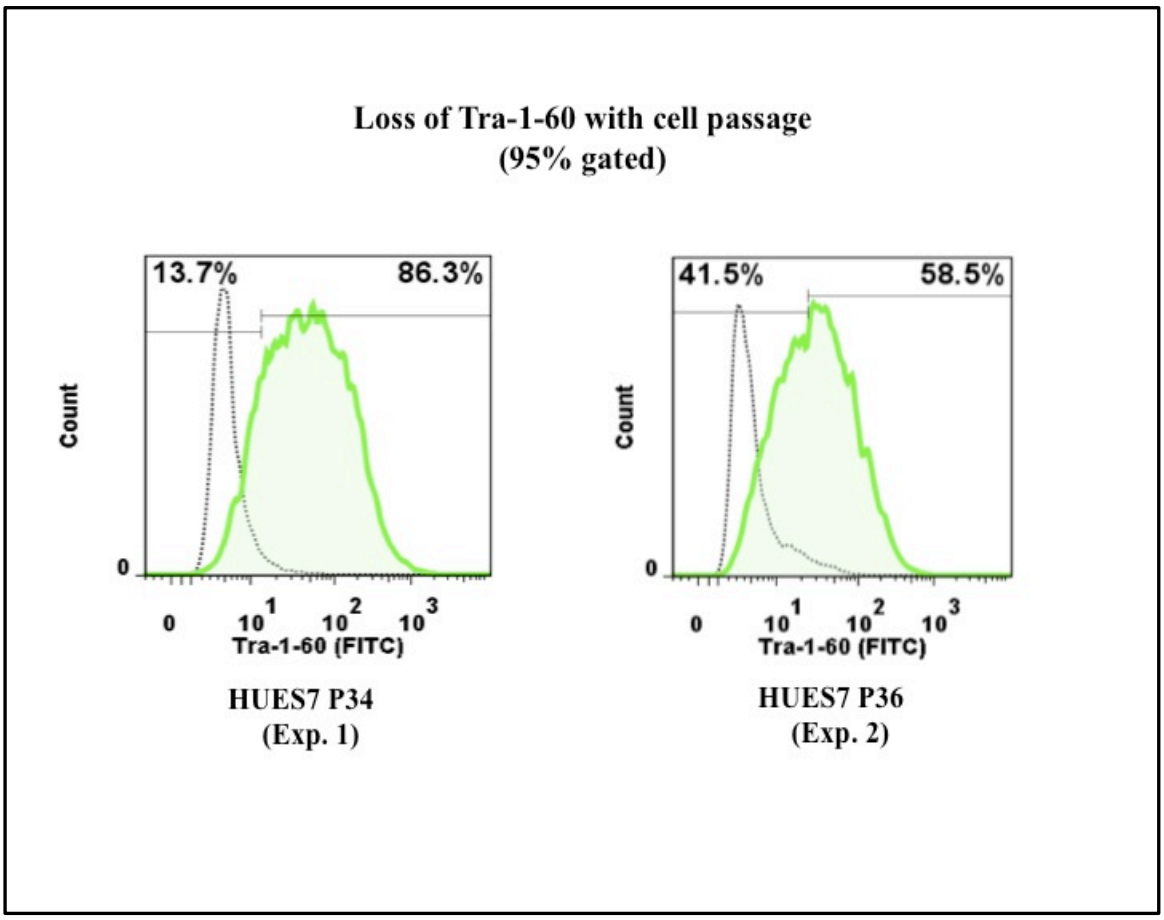

A.
(i)
Histograms - 99% gate of isotype control
99.0%
0.978%
1.12%
98.9%
Q1
57.7%
Q2
40.4%
Q4
1.72%
Q3
0.135%
SSEA 4 (PE)
Tra-1-60 (FITC)
99% Gated Cell Distribution
(ii)
Histograms - 99% gate of isotype control
99.0%
0.978%
1.12%
98.9%
Q5
95.3%
Q6
2.92%
Q8
1.70%
Q7
0.040%
SSEA 4 (PE)
SSEA 1 (PE-CY5)
(iii)
+VE
SSEA 4 (PE)
-VE
Q1
Q2
Q4
Q3
% Positive Population
Exp. 1
Exp. 2
Exp. 3
Exp. 4
-VE
Tra-1-60 (FITC)
+VE
(iv)
+VE
SSEA 4 (PE)
-VE
Q5
Q6
Q8
Q7
% Positive Population
-VE
SSEA 1 (PE-CY5)
+VE
B.
(i).
Q1
21.5%
Q2
78.3%
Q4
0.229%
Q3
0.040%
SSEA4 (PE)
Tra-1-60 (FITC)
SSEA 1+VE - 15.4%
(ii)
Q1
57.7%
Q2
40.4%
Q4
1.72%
Q3
0.135%
SSEA 4 (PE)
Tra-1-60 (FITC)
SSEA 1+VE - 2.9%

Figure 3

Supplementary Figures



Supplementary Figure 1. Representative single color histograms demonstrating the Tra-1-60^{+ve} expression of two successive passages, p34 (Exp. 1) and p36 (Exp. 2) where there was a significant reduction in positive expression. Cells were in culture 6 days between analyses.