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## Separating Stem Cells By Flow Cytometry: Reducing Variability for Solid Tissues

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### Summary

Until there are valid identifiers that visualize stem cells *in vivo*, we rely upon flow cytometry to enrich for subpopulations with stem cell function. However, data reporting styles for flow cytometric analyses are typically inconsistent, creating challenges in comparing results across publications. In our view, clear reporting guidelines could improve reproducibility of stem cell analyses in solid tissues.

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Presently, the field of cancer stem cell biology (and somatic stem cell biology in general) is awash with reports of two-parameter dot plots that do not resemble one another, despite originating from similar tissues immuno-phenotyped with the same antibodies. Regardless of the lack of resemblance to original publications, authors will often attribute specific properties to cells that exhibit particular phenotypic traits by flow analysis without confirming their identity or function. Overall, this practice leads to the accumulation of unvalidated conclusions and misinformation on the behavior of stem cells or other cell types (such as differentiated epithelial or non-epithelial cell types).

We encourage the community to make use of the following proposed recommendations for the presentation of stem cell data obtained by flow cytometry. These criteria are not new: Broadly stated, standards have been set out by MIBBI (Minimum Information for Biological and Biomedical Investigations), and are outlined by Lee et al (Lee et al., 2008), as a consensus of opinion from cytometry professionals, and have even been implemented as the minimum accompanying information for flow cytometric results by some journals. Detailed

criteria and techniques have been discussed by Roederer and Herzenberg, especially with respect to setting standards for hematopoietic cell analysis (Herzenberg et al., 2006; Moore and Roederer, 2009; Perfetto et al., 2006; Perfetto et al., 2004; Roederer, 2002a, 2008). Within the broader stem cell community, however, there has been little standardization applied to the separation of cell fractions from solid tissues, to date. This oversight is unfortunate, since the enzymatic dissociation procedures that are used to generate cell fractions make this analysis even more variable than the analysis of non-adherent cell types. It seems timely to highlight specific practices that may offer the stem cell field improved consistency in reporting across published accounts, given the wildfire adoption of cytometric procedures by laboratories not previously specialized in multi-chromatic analyses of cell populations.

We propose that a detailed list of experimental details and specific examples be included in submissions that utilize flow cytometric methods (Table 1). We offer our insight as to how the provision of such details will improve consistency across related reports, and outline potential pitfalls that might be avoided by following this pattern of experimental reporting.

There are many reasons why flow histograms of the same tissue type may not look similar. Some of these variables are hard to control for, and include differences between cytometers (even the same model), or areas of the world in which the experiments are conducted. These issues can only be truly solved by repeating all the functional characterization in each independent laboratory. Others sources of discrepancy might be attributed to variability between human tumors, or to substantial differences between profiles from inbred mouse strains, or to different regimens for proteolytic dissociation of tissues. Specifically, the inclusion of the following information should enable valid cross-comparisons and ensure improved reproducibility, as described below.

### **Antibody binding conditions used to label cell populations**

Flow cytometry is a quantitative technique when antibody binding is saturating. Individual laboratories should take pains to test “new” antibodies for saturation binding (see Kantor and Roederer (1997) as a source of information on basic experimental guidelines). To describe a staining reaction in enough detail that it can be reproduced, the cell and antibody concentrations utilized should be specified (within the limits of the manufacturer’s description; see Supplementary Figures 1 and 3). Indeed, the antibody clone and specific fluorochrome used often influence the binding reactions in ways that are difficult to rationalize, making the provision of more detailed methodological information important.

### **Make and settings of the flow cytometer**

To be able to reproduce functional data from live cell sorts, machine factors that can affect sample recovery, viability, and function, such as nozzle tip diameter, sheath pressure and fluid composition should be reported (see examples in Supplementary Figures). The laser power could be included, if this figure is known to be an important determinant of success. Note that these settings differentially affect various cell types; for example, for cells from the mammary gland, high pressure and low nozzle tip diameter can lead to fewer differentiated cells or basal cells. Specification of the make of the flow cytometer and the name of the software package(s) used during the sort and for any subsequent analysis provides most of the important machine-based parameters needed for background information. There are a great diversity of options for laser wavelengths and emission filters and an expanding repertoire of new fluorochromes (Chattopadhyay et al., 2008); thus the wavelength of the laser (and possibly the emission filter, if that is not predictable) should be stated, since these parameters can determine the relative efficiency of fluorochrome signals (see Supplementary figures 1 and 3).

## Compensation Procedures

To preserve the quantitative aspect of flow cytometry, fluorescent signals that bleed from one channel to another should be subtracted. For example, if an immunophenotyping reaction includes a bright fluorochrome with an emission spectrum that closely aligns with that of other fluorochromes used in the same sample, a correction factor will be necessary to prevent cell populations from being shifted inappropriately to different quadrants of the dot plot. In other words, cells that are labeled with one bright fluorochrome may read as false positive expressors for a fluorochrome with a closely overlapping emission spectrum. The correction factors applied to prevent false positive signals are termed compensation procedures (Roederer, 2002a), and a description of compensation procedures (single stains for each dye, use of CompBeads (Becton Dickinson), or lot numbers for tandem dyes with variable spectral properties, and whether the correction is applied by the operator as either a machine-based correction, or calculated by software after flow cytometry (Herzenberg et al., 2006; Tung et al., 2004)) should be included in the Methods section (see Supplemental figures 1, 3 and 5 for examples, or <http://www.drmr.com/> for more detailed technical advice). Overall, reproducible patterns and quantitation of polychromatic flow histograms require a consistent set-up procedure. This paradigm has been elegantly described by Perfetto et al., and can be presented as a stepwise dissection including system optimization, calibration and continuous monitoring of the fluorescent signals with respect to sensitivity, accuracy and precision (Perfetto et al., 2006).

## Display of Manual Gates Applied

Accurate quantitation of cell subpopulations depends as much on accounting for the cells that are left out as the cells that are included on the final histogram. A description of the gating procedure should include a list and display of the sequential gates applied to exclude debris, to select single cells, to assay only live cells, and to exclude irrelevant cells. This pattern is summarized in a gating tree, also known as a population hierarchy (see Supplementary figures 1-5). For example, PI-positive cells (marking dead cells with permeable membranes) and debris can be “sticky” and non-specifically bind antibody and/or fluorochromes and/or also emit autofluorescent signals that contribute spurious, false-positive signals (Supplementary Figure 2). Cell doublets will bind proportionally more antibody than single cells, and can often appear in stem cell fractions, given that stem cell-enriched fractions are often defined as “high” expressors of various cell surface antigens, and so should be gated out. The typical 2D histogram relies on the exclusion of cells not directly relevant to the analysis, since they will often express ligands that bind the analytical antibodies, as well. They can cloud the view of rare target cells, and decrease the purity of a target population. The most common gate applied to epithelial cell populations when attempting to enrich for a rare stem or progenitor pool is described as a Lineage<sup>+</sup> gate (Lin<sup>+</sup>, or “dump channel”, based historically on the application of a similar gate during hematopoietic separations), and includes a panel of antibodies labeled with the same fluorochrome that bind endothelial and hematopoietic cells (such as CD31 and CD45). However, since these irrelevant populations are not completely excluded by Lin<sup>+</sup> antibodies (either because cells are not homogeneously positive, or the antigens are clipped off cell surfaces during cell preparation), and their number can be high, marking the location of these cells on the final epithelial cell histogram can be important to subsequent interpretation (if for example, endothelial cells overlie a putative stem cell-enriched fraction; see Supplementary Figure 2). Knowing where any spurious populations from non-subject lineages lie on the final histogram may convince the reader that any changes in the fractions of interest are, indeed, specific.

In addition to indicating how unwanted events are eliminated, it is equally important to clarify how a positive signal is defined. Thus, for many antigens, an unselected population exists as a continuum of negative-, low-, and high-staining cells, rather than as a collection of obviously discrete populations. Subtraction of background binding can be based on (in order of rigor): 1) a truly negative population, for example genetically null cells (see Supplementary Figure 4), 2) a non-expressing population, known to be negative by prior understanding (see Supplementary Figure 3), or 3) relevant singly-stained fluorescently-labeled isotype-matched antibody controls and/or fluorescence-minus-one strategies (Roederer, 2002b; Tung et al., 2004)(see Supplementary Figure 3). Applying one or more of these strategies is particularly important for rare or specialized antigens, often visualized by adding a labeled secondary antibody that binds to the antigen-specific primary antibody, and which are particularly prone to high background binding (Supplemental figure 4). Furthermore, if it is clear that unstained cell populations have significant autofluorescence that is detected in (all) analytical channels, the strategy used to exclude this contribution should be described. Many times, staining is described by the subjective terms “high” and “low”. Instead, any cutoff used to distinguish relative levels of staining should be delimited quantitatively (for example, “CD49<sup>fl</sup> cell fractions were gated as the lower 50% of the population”, or “The median fluorescence of the CD49<sup>fl</sup> was 10-fold less than that of the CD49<sup>thi</sup> cells”; see Supplemental Figure 3).

After having completed the gating hierarchy, both to negatively and positively select for the population of interest, it should be clear what proportion of cells is shown in the final analytical window. For some cell preparations, the total cell population represented on the final flow histogram may be a relatively minor fraction of the starting cell preparation. Thus, if one cell type is more susceptible to damage than others (especially with respect to the stem/differentiated cell fractions), this trait could lead to large discrepancies between laboratories (for example, if one laboratory displays data that represent 50% of the starting population, whereas another presents 5%). That is, without knowing which cells were lost during the course of the gating procedure, the final reported frequency of the population of interest is impossible to compare across laboratories.

Variations in outcome due to mechanical susceptibilities of isolated cells might be minimized by establishing a reproducible pattern of cell release from solid tissues, which is aided by providing detailed information on mechanical disaggregation, cell dissociation media and agitation patterns. Perhaps counter-intuitively, tumor cells are often more fragile than their normal counterparts, and easy to destroy during preparation. Necrotic tumors containing aneuploid cells may be particularly hard to handle.

Devising a method for quality control for the enzymatic release procedure is useful. For example, enzymatic digestion can strip epithelial cell surface antigens, causing the epithelial cells to appear at high frequency in other non-epithelial populations. This effect can be detected by analyzing non-epithelial cell fractions for their expression of epithelial keratins (such as keratin-5 or -8).

An estimate of the total number of events retrieved from a flow cytometer, compared to the cell number added to the analysis tube, will reveal serious discrepancies that relate to the extensive particles and debris that can be produced during live tissue processing. The number of events is often assumed to equal the number of cells, but this relationship may not be accurate if/when epithelial or tumor cells are disrupted into numerous debris particles. The presence of scraps of extracellular matrix can also contribute to machine detected ‘events’ that are actually debris. Thus, in cases where significant debris is present, the % recovery of live cells may be significantly higher than implied by the values reported in the gating tree.

## Display of the Raw Data

Flow cytometry follows the same rules of reporting as other scientific assays; it requires a sufficient n number (independent sample determinations) to show statistical significance. If the number of independent assays is sufficient, the absolute number of events required can be low (Roederer, 2008). If one histogram is shown to illustrate the properties and frequency of an enriched population, this point should be indicated  $\pm$  standard deviations based on multiple independent determinations. Note that axis ticks should always be visible and clear (particularly to distinguish between logarithmic and linear scales), and contour or other density plots are often more visually quantitative than dot plots. Finally, it should be made clear whether methods such as BiExponential Data Transformation are used to visualize compensated data points that fall below the axis on a logarithmic scale (Herzenberg et al., 2006; Roederer et al., 2004).

## Validation of results: Evaluating the purity of sub-populations

Cell fractions of interest should be characterized after sort-mediated purification (using relevant assays, such as cyto-spin-immunohistochemistry (see Supplementary Figure 1) or genetic tests), with the aim of confirming their identity. Note that claims of purity are also supported by re-analysis of sorted cell fractions, but these methods can harbor false negatives if antibodies or antigens and/or epitopes are lost during mechanical separation, due to temperature changes following the sort, or to photo-bleaching of particular fluorochromes, and thus could result in an under-estimate of the sort efficiency. When various cell populations are tested to determine their functional activity, their relative viability should be specified (using a live cell reporter such as trypan blue). Ideally, enriched fractions should be compared to stained, unseparated populations that have also passed through the sorting apparatus, to control for loss of function due to mechanical shear or other stress such as temperature or nutrient shock (Supplementary Figure 6).

Among the purest stem cell populations reported to date are the hematopoietic stem cells ( $\geq 1$  in 3) purified by Morrison and colleagues (Kiel et al., 2005). Isolated fractions that harbor relatively more cells with stem cell activity should be labeled stem cell-enriched (SCE), rather than “stem cells” (see Supplemental Figure 5), since this label can be misleading for readers, and is often incorrectly summarized in media sound-bites. For example, the MRU mammary stem cell fraction, though it contains all the stem cell activity isolated from this tissue, may still be only 5% pure (Shackleton et al., 2006; Stingl et al., 2006; Supplementary Figure 6).

Experiments that claim to enrich for stem cell activity must be validated via experimental means so as to demonstrate that functional enrichment has, indeed, been accomplished. Thus, the non-purified cell population should be compared with purified cell fractions, recording the % recovery of activity and fold-enrichment (per cell), to illustrate how much of the functional activity has been accounted for (Supplementary Figure 6). It is important to perform functional analyses on all cell subsets, including those claimed to be stem cell-deficient. The process of flow sorting itself can compromise stem cell activity, by separating the test population from non-stem cell types usually required to support stem cell activity, or due to mechanical damage, to blocking functional epitopes with cell surface-binding antibodies, or to antigenicity of fluorochromes (together, often responsible for 90% loss of activity) (Britt et al., 2009). Importantly, in order to make specific claims about stemness, there should be a functional evaluation of stem cell activity.

## Concluding thoughts

To summarize, we encourage authors and reviewers to keep the following questions in mind when assessing whether submitted cytometric data is sufficient to support the claims made in a given study. Are the methods described in sufficient detail that the experiment can be reproduced, and include procedures for mechanical and enzymatic dissociation, antibody sources and binding reactions, make and settings for the flow cytometer, and any relevant software? For any major findings, are the gating procedures presented, and the rationale for gate placement clearly defined? Are the relevant controls present that confirm specificity of staining? Is the overall percent cell recovery presented? Is the reproducibility of fractionation indicated? What is the basis for correlating a specific population with an activity or a phenotype? Is the percent purity presented for any given phenotype in a cell fraction? If functional activities are presented, what is the percent activity recovered in purified cell fractions compared to the starting population? Are the cell fractions given accurate names? That is, if a subpopulation is described as stem cell-enriched, what is the estimated percentage of purity? What efforts are made to determine whether surrogate stem cell markers are accurate, and truly specific to the stem cell-containing fraction?

It is typically expensive and time-consuming to set up flow cytometric analyses of animal or human tissues, especially when searching for rare populations. We hope that widespread adoption of reporting guidelines, such as those outlined previously (Lee et al., 2008), and that we have proposed to specifically target challenges faced during the analysis of cells isolated from solid tissues, will enable the comparison of data generated across laboratories worldwide, to yield more accurate conclusions, and reduce the frustration of new investigators in this area.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

To report in submitted publications	Purpose	Specific details to include	Templated example
Antibody binding conditions used to label cell populations	Establish consistency of high / low antibody binding immunophenotypes	<ul style="list-style-type: none"> <li>Antibody clone &amp; fluorochrome used.</li> <li>Antibody concentrations and time, temperatures used for labeling.</li> </ul>	Figure S1
Make and settings of the flow cytometer	Clarify any discrepancy in reporting due to machine factors such as physical pressures exerted, laser calibration, wavelength and filters used	<ul style="list-style-type: none"> <li>Make / model of cytometer used.</li> <li>Nozzle tip diameter, sheath pressure and fluid composition.</li> <li>Laser power and wavelength used.</li> <li>Software package(s) used for sorting and analysis.</li> </ul>	Figures S1 and S3
Compensation procedures	Achieve consistent elimination of artifacts associated with spectral overlap between fluorochromes	<ul style="list-style-type: none"> <li>Report use of single stains, or fluorescence-minus-one stains</li> <li>Indicate whether compensation was performed prior to separation and/or during post-sort analysis</li> <li>Calculated by operator or with a software-based algorithm</li> </ul>	Figures S1, S3, and S5
Display of manual gates applied	Improve equivalence of quantitation across independent experiments	<ul style="list-style-type: none"> <li>Display the gating hierarchy used.</li> <li>Indicate the number and percent of events excluded at each step in the "tree".</li> <li>Gates used to eliminate dead cells, cell doublets, debris, and irrelevant live cells.</li> </ul>	Figures S1-5
Display of the raw data	Establish transparency of number of events examined and reveal degree of separation between populations	<ul style="list-style-type: none"> <li>raw data plots demonstrating a sufficient event count to establish an adequate 'n'.</li> <li>axis ticks that distinguish between log and linear scales should be visible.</li> <li>summary percentages should include a degree of error determined in independent replicates.</li> </ul>	Figures S1-5
Validation of results	Evaluation of extent of purification of isolated cells and verify their functional status and degree of enrichment	<ul style="list-style-type: none"> <li>re-analysis of fluorescent profile post-sort can establish degree of sort purity</li> <li>examination of morphology and/or genetic traits can verify the identity and purity of sorted cells</li> <li>functional assays comparing to similarly handled, unselected cells are needed to determine fold-enrichment and degree of activity recovered.</li> </ul>	Figures S1, S5, and S6