

Flow Cytometric Sorting of Cells from Solid Tissue – Reagent Development and Application

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1. Introduction

The isolation and study of cells from solid tissues is one of the great current challenges for flow cytometry. The vast majority of mammalian cells reside within solid organs, but the application of flow cytometric techniques to such cells remains limited. The study of hematopoietic biology has been revolutionized by the development of standardized monoclonal antibodies recognizing defined antigens that permit flow cytometric isolation, definition, and characterization of blood cell types (Bendall et al., 2011; Fleming et al., 1993; Hoffman et al., 1980; Nakeff et al., 1979). Antibodies have been used to better identify and understand the role different blood cells play in the immune system (Springer et al., 1979). They have also been used to help zero in on the stem cell populations and to learn more about the processes of hematopoietic differentiation (Ikuta et al., 1992; Spangrude et al., 1988). Their use has impacted virtually every facet of hematopoietic study.

This same approach is likely to bring similar benefits to the study of solid tissue. Unfortunately, the reagents and techniques needed for studying cells from solid tissues are woefully limited. Most available surface-reactive antibodies have been defined by their activity against hematopoietic antigens. The lack of understanding about the surfaces of tissue-resident cells is one of the reasons why the isolation of adult stem cells and culture of primary cells from solid tissue remains problematic. In order to advance the study and characterization of these cells, new reagents and techniques must be developed. The development of monoclonal antibodies against solid-tissue specific antigens will help researchers sort and study subsets of cells from other organs. It will allow for comparison between subsets of cells from the same tissue and similar subsets from different tissues. It is likely that, as with the hematopoietic field, new subsets and classes of cells will be discovered. Hopefully, the development of more monoclonal antibodies will not only move research forward but will also aid in the development of therapeutic technologies.

This chapter will include information about the history of monoclonal antibodies with a focus on how they impacted the field of flow cytometry. The development of novel monoclonal antibodies and examples of their successful application to the field of solid

tissue biology will be described, and suggestions will be offered for the advancement of flow cytometric research beyond the hematopoietic field.

2. A brief overview of the history of hematopoietic antibody production

In order to better understand how antibodies will benefit the study of solid tissues, it is helpful to examine how they have benefited the study of hematopoietic tissue. The study of hematopoietic tissue has been immensely successful and there is much benefit to be gained by understanding and learning from what has been done before.

2.1 Hybridomas

The advent of monoclonal antibody-producing hybridomas revolutionized the field of cytometry. Kohler and Milstein originally described the creation of immortal cells that produced monoclonal antibodies against a predefined target (Kohler and Milstein, 1975). They did this by fusing cells from a plasmacytoma line with splenocytes from a mouse that had been immunized with sheep erythrocytes. The resulting clonal cell line possessed the useful attributes of each parent; they could be cultured indefinitely and also continued to produce the desired immunoglobulin.

Prior to the development of hybridomas, polyclonal serum was used for cell or protein recognition. While there are applications where the use of polyclonal serum is advantageous, it has the disadvantages of being a finite resource and that the antigens are not fully characterized.

The innovation of monoclonal antibodies revolutionized cell analysis by allowing precise and accurate reproducibility both between experiments and between researchers. In the beginning of the prolific development of monoclonal antibodies, individual labs would make their own antibody clones (Lemke et al., 1978; Solter and Knowles, 1978). Very quickly, it became clear that a more standardized approach would be useful; it was not always clear, for example, whether antibodies that appeared to label the same cells were binding to the same antigens. During the first HLDA (Human Leucocyte Differentiation Antigens) workshop in 1992, 15 antibodies with known targets were standardized as CD (cluster of differentiation) markers (Zola and Swart, 2005). As was reflected in the name, the HLDA was concerned primarily with blood cells, which were readily accessible and a logical place to start.

2.1.1 Uses

Flow cytometry is not the only use for monoclonal antibodies; their application in biomedical and basic research is extensive. Although immunoassays were in use prior to the development of monoclonal antibodies, the increased availability and consistency of this new reagent dramatically increased the number of potential applications. Radioimmunotherapy would not be possible without the superb specificity of monoclonal antibodies. Pathogen classification used to require hours, or even days; there are now many cases where it can be done in a matter of minutes. Microscopy has taken advantage of monoclonal antibodies to produce exquisite images and reveal the internal structure of cells in ways not previously imagined. Many other examples exist, but a comprehensive list of monoclonal antibody applications is beyond the scope of this chapter.

2.2 Use of antibodies for flow cytometry

Even before Kohler and Milstein devised monoclonal-producing hybridomas, antibodies were being used for flow cytometry. The Herzenberg group, in 1971, became the first to employ antibody labeling as a cell identification tool in flow cytometry (Herzenberg, 1971). Polyclonal anti-sera were employed in subsequent studies, such as Epstein's work on lymphocyte biology (Epstein et al., 1974). In 1977, the first paper using fluorescently-labeled monoclonal antibodies was published, and the field has never looked back (Williams et al., 1977). Since then, thousands of publications have included the use of monoclonal antibodies in flow cytometry. They have become an indispensable tool for biomedical research.

2.3 Advances in flow cytometry

Advancements in the technical aspects of flow cytometry were driven largely by the rapid generation and characterization of new monoclonal antibodies. The demand for more fluorophores, more detectors, and more lasers in order to assess multiple properties on the same cell was the impetus for significant advancements in the field. Multicolored flow cytometry allowed for the discovery of multiple subsets of cells. Three color detection using a dual laser system was developed by 1983 (Parks et al., 1984). In 1985, BD utilized microcomputing in the new FACScan, increasing cell throughput and simplifying all aspects of the cell isolation process. By the turn of the millennium, eight simultaneous 'colors' (fluorochromes) were used in flow cytometry experiments (Roederer et al., 1997). Any time a new instrument becomes available, researchers immediately begin pushing the limits of the new technology. Advances in photomultiplier tube technology and the development of better dichroic filters also helped in the development of bigger and better flow cytometers.

The rise in technology necessitated new standardized ways of analyzing the data. The FCS (Flow Cytometry Standard) was proposed in 1984 by Murphy and Chused and was summarily adopted by the scientific community at large (Murphy and Chused, 1984). This facilitated data sharing between labs.

3. Antibody production targeting cells from solid tissue

The rapid development of antibodies and cell sorting technology to meet the needs of the hematopoietic research community has laid much of the ground work required for studies of other cell types. There is an ever-growing need to be able to identify subpopulations of cells from solid tissues. Diseases such as diabetes, liver disease, and a variety of cancers have benefited, and will continue to benefit from, an expanding pool of monoclonal antibodies that can be used to further investigate them (Jarpe et al., 1990; Larsen et al., 1986; Muraro et al., 1989). Using the same approach pioneered by Milstein and Kohler, research into all systems of the body has begun to follow in the footsteps of the hematopoietic field. While there are other ways of making hybridomas, the immunization of a rat or mouse with whole cells continues to be valuable because antigens are presented in their native configuration. Thus, the resulting antibodies are likely to recognize the epitopes available in a live, intact cell. In order to use these antibodies to identify and isolate cells for growth and/or functional studies, this is an essential property.

3.1 Immunization

Modern monoclonal antibody production uses a procedure that has changed little from the methods of Köhler and Milstein (Kohler and Milstein, 1975). In brief, the mouse or rat is immunized on multiple occasions using cells obtained from the tissue of interest. Four days after the final immunization the animal is sacrificed and the spleen harvested. Splenocytes are fused with myeloma cells using polyethylene glycol and cultured on semi-solid HAT (hypoxanthine/aminopterin/thymidine) media to prevent the growth of un-fused myeloma cells. Hybridoma clones are isolated and grown individually to produce antibody for testing, and interesting clones are expanded to larger cultures and cryopreserved for further study. Of these steps, the selection of the immunogen and the treatments used to isolate it from solid tissue prior to immunization are the most critical to the success of the procedure. One can immunize with partially dissociated tissue, fully dispersed cells in suspension or a FACS-purified cell subpopulation. Each of these has advantages and disadvantages.

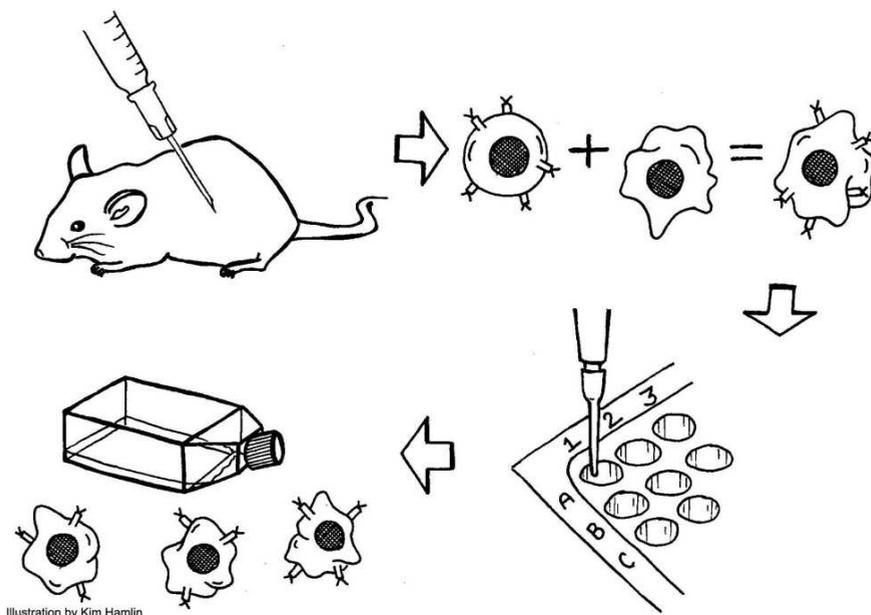


Fig. 1. Making Hybridomas. A mouse is injected with an immunogen, antibody producing splenocytes are fused with myeloma cells to produce hybridoma lines, clones are grown in 96-well plates using drug selection to exclude un-fused myeloma cells and selected clones are scaled up

Immunization using minimally-dispersed fragments of tissue is simple, quick and requires limited (if any) proteolytic enzyme treatment. Thus, this approach is most likely to preserve sensitive antigens. Because it does not exclude indigestible material (which can include important tissue substructures), this method also offers the most complete representation of all antigens in the tissue. The disadvantage is that this method includes antigenic material that gives rise to immune responses against unwanted antigens that are present in debris.

If tissue is instead enzymatically dissociated to a single-cell suspension, much of the intercellular debris can be eliminated and the cell-surface antigens are more available. Although native epitopes might be altered by proteolysis, it is important to consider that this same process will be required to recover cells for future analysis; raising antibodies against a proteolysis-sensitive antigen is of limited utility. Unfortunately, it is not always clear what the optimal digestion protocol will turn out to be for a given tissue type. If it is possible to perfuse the organ with a collagenase, this can be a very productive approach (Klaunig et al., 1981). Regardless of whether perfusion is performed, dispersal to a single-cell state usually requires three elements: Mechanical dissociation (by mincing and/or passage through a narrow aperture such as a needle), collagenase treatment (to digest extracellular matrix) and a broad-specificity protease (e.g. trypsin) to disperse tightly-associated cells. Specific protocols successfully used for tissues such as mouse liver (Dorrell et al., 2008b), and human pancreas (Dorrell et al., 2008a) are described in the next section.

Immunization with a subset of a single-cell suspension allows for the generation of antibodies targeted against a subset of cells. At a minimum, it should be possible to exclude defined “contaminating” populations such as blood and endothelium. The cell subset may be isolated using flow sorting, immunomagnetic separation, or physical properties such as density or size. This approach has the same downside as tissue digestion because the researcher must know how to digest the tissue in order to liberate the cells of interest without damaging them. In addition, the number of recovered cells tends to be smaller, meaning that the immunization might be less efficient.

A useful method for increasing the likelihood of obtaining antibodies targeted to a particular cell type is subtractive immunization (Williams et al., 1992). In this method, the first immunization uses tissue, cells and/or debris containing antigens which are irrelevant or undesirable. Subsequent treatment with cyclophosphamide kills the lymphocytes which have responded to any of these antigens, so that after subsequent immunizations with the tissue/cells of interest, only the new and relevant antigens cause an immune response. This approach was used successfully to raise antibodies against human pancreatic islet cells after pre-immunization with trypsin and calf serum, which might otherwise have been confounding antigens required for islet dispersal and cell storage (Dorrell et al., 2008a). Subtractive immunization is compatible with any of the immunogen sources described above.

3.1.1 Proven immunization strategies for mouse liver and human islets

This chapter will focus on two different tissue preparations from two dissimilar tissues. Understanding why they would be treated differently will help the researcher determine the best course of action for future tissue digestion.

Preparing single-cell solution from a mouse liver requires several stages. The first is a two-step portal vein perfusion with calcium and magnesium-free Hanks salt solution followed by a solution containing collagenase. After about twenty minutes, when the liver is visibly degraded it is removed, placed in a dish with media, and teased apart with forceps to release as many cells as possible. The resulting slurry should be allowed to flow through a 40 μm strainer. The filtered cells can be spun for two minutes at 50g in order to pellet the hepatocytes; other cell types will remain in suspension after this treatment. Tissue that does

not pass through the filter should be subjected to an *ex vivo* digest of about 20 minutes in 2.5 mg/mL collagenase D at 37°C before collection through a 40 µm strainer. Any remaining solid tissue is then digested with 0.05% trypsin and mechanically dissociated with a pipetter. Following all of these treatments little or no undigested material should remain. At each stage, it is important to collect dissociated cells by passing them through a 40µm strainer and to store them on ice without further exposure to enzyme (Dorrell et al., 2008b; Klaunig et al., 1981).

We receive intact human islets from the Islet Cell Resource Center network. They are essentially small aggregates of cells obtained as the end product of a whole-organ perfusion protocol, and therefore require only a simple enzymatic digest to reduce to a single cell suspension. Islets are washed with calcium- and magnesium-free DPBS (Dulbecco's Phosphate-Buffered Saline) and then incubated in 0.05% trypsin at 37°C. Gentle pipetting is done every three minutes in order to aid the dissociation until the islets are visibly dispersed into a single-cell state.

3.2 Hybridoma screening

Regardless of the immunization strategy and target cell population, most of the resulting hybridomas will not produce desirable antibodies. It is therefore essential to have a strategy for evaluating many hybridomas to obtain as many useful ones as possible. Labeling of tissue sections with hybridoma supernatants followed by microscopic evaluation can be labor-intensive, but it provides invaluable information regarding cell type-specificity. A detailed anatomical knowledge of the target tissue is not generally required by the screener; the important requirement at this stage is the identification of clones that selectively label populations of cells found in common structures (e.g. ducts, blood vessels, islets, etc.).

For the screening of large numbers of hybridomas for reactivity, cryosections of OCT (Optimal Cutting Temperature) cryomatrix-embedded tissue is recommended. To save time, hybridoma supernatants can be pooled and evaluated in batches. Batches exhibiting a promising tissue labeling pattern are then reevaluated, clone-by-clone. Figure 2 illustrates immunofluorescent labeling using an antibody with an interesting labeling pattern that emerged after an immunization series using human islet cells; this one made the cut. "HIC1-8G12" labels both the ducts (as shown by co-labeling with KRT19) and a subset of cells within the islet.

Further examination revealed that these islet cells were alpha cells (as shown by co-labeling with transthyretin [TTR], which is strongly expressed in this population (Dorrell et al., 2011b)). Using "interesting labeling pattern" as a criterion for antibody selection is subjective, of course, but the study of markers like HIC1-8G12 can lead to the identification of previously unexpected relationships between cell types. Flow cytometric screening of this antibody (described in the next section) showed that this particular antibody recognizes a cell surface antigen and can be used for live cell flow cytometry studies.

4. Flow cytometry

Protocols describing analysis and sorting by flow cytometry have been developed with primary blood cells and/or transformed cell lines in mind. For solid-tissue derived cells, some modifications and special considerations are needed.

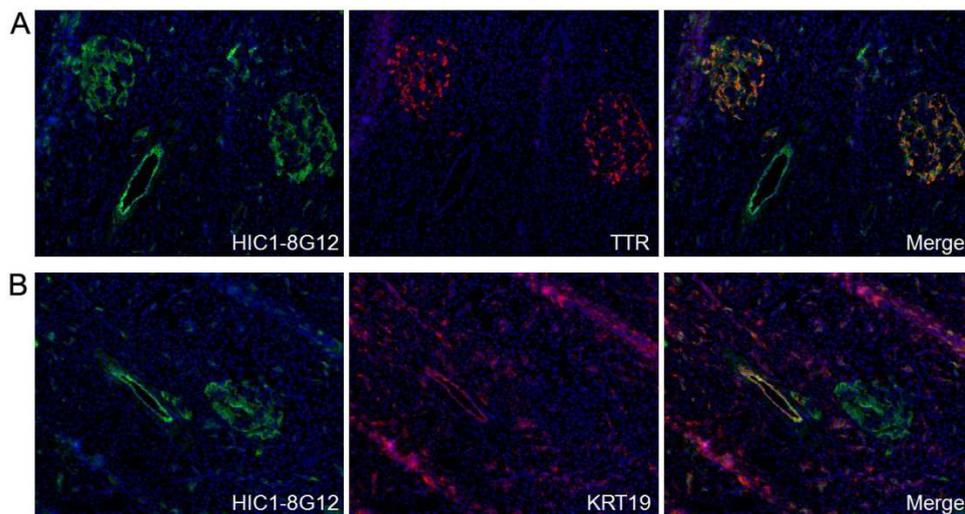


Fig. 2. Human pancreatic tissue labeling by HIC1-8G12. Co-labeling with TTR (A) or KRT19 (B) shows that this antibody binds an antigen that is present on both ducts and alpha cells

4.1 Selecting antibodies of interest by flow cytometric screening

After the initial screening on slides, it is important to determine which antibodies have cell surface reactivity. Flow cytometry is ideal for this purpose. Because the goal is to create antibodies against surface molecules, if an antibody with an interesting tissue labeling pattern does not label live cells with sufficient intensity and specificity to be distinguished by flow cytometry, it is not worth pursuing.

As discussed previously, there are many ways of getting cells into a single-cell suspension. It is recommended that if an enzymatic dispersal was used for immunization then the same method should be used to prepare cells for flow cytometry. If no enzymatic dispersal was used, it is worth taking the time to do the screening using a variety of digestion methods.

Many people worry that the dispersal method will influence the preservation of epitopes. They are right to worry. Some methods will alter or remove antigens of interest rendering them unrecognizable. We have found, for example, that the antigen recognized by one of our in-house favorite antibodies (MIC1-1C3) is unusually sensitive to digestion with Pronase, a mixture of strong proteases (Dorrell et al., 2011a). It is very important to try to optimize your digestion protocol but bear in mind that, at the end of the day, if you can't get the cells into single-cell suspension then they can't be sorted and studied in isolation.

With precious human samples, it can be much more difficult to do the experiments and learn which digestion method will work best but it is worth optimizing in the beginning for higher reproducibility later on.

As with screening on tissue sections, hybridoma supernatants can be pooled for screening by flow cytometry. This may not be necessary if the tissue section screen reduced the candidate list to a reasonable number, but will be necessary if tissue section screening was

not performed or if few cells are available. During screening by flow cytometry, the antibody isotype can also be conveniently determined. Cells incubated in the supernatant from a single clone are detected using a combination of isotype-specific secondary antibodies selective for mouse or rat IgG or IgM. Figure 3 is a dot plot showing cells labeled with a mixture of primary antibodies, some IgG and some IgM. Because these were detected with both PE-conjugated anti-mouse IgM and APC-conjugated anti-mouse IgG, primary antibodies of these two isotypes can be used together.

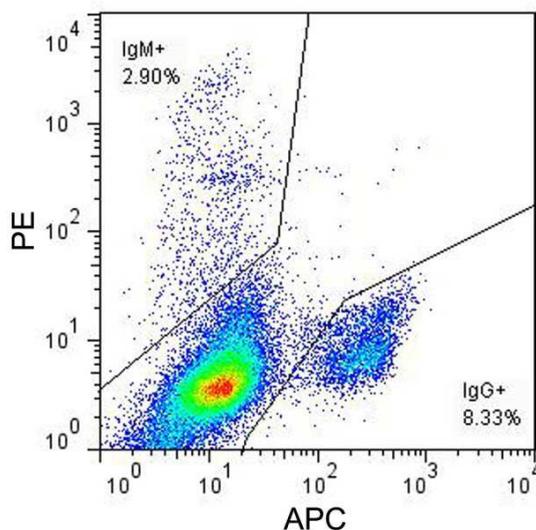


Fig. 3. Isotype specific secondary antibodies permit simultaneous detection of IgG and IgM-labeled cells

4.2 Multi-step antibody labeling to isolate populations of interest

Using unconjugated monoclonal antibodies has advantages and disadvantages. On one hand, it takes longer to label the cells because a secondary antibody is required for detection, necessitating additional steps. On the other hand, the researcher is not limited by pre-conjugated fluorochromes and can change “colors” to suit the experiment or instrument. Although pre-conjugated monoclonal antibodies marking known lineages can be an important tool in the study of solid tissue cells, such reagents are limited and most experiments will require the use of novel, unconjugated antibodies as well. Thus, despite its added complexity, a multi-step labeling protocol incorporating both fluorochrome-conjugated and unconjugated antibodies is ideal.

Generally speaking, monoclonal antibodies made against mouse antigens are made in rats and monoclonal antibodies targeting human antigens are made in mice. It is possible to do combination labeling using both conjugated and unconjugated antibodies raised in the same host species, but special care is required; a detailed protocol is provided later in this section. Polyclonal antibodies are produced by immunization of a variety of species, and are often easy to combine with monoclonal antibodies for co-detection. It is vital to know the host

species of each antibody, its isotype and whether or not each secondary antibody is adsorbed to prevent cross-reactivity against immunoglobulins from another species.

Once an antibody has been found with reactivity against a cell population of interest, it is important to do a simple titration in order to ensure that the optimal concentration is being utilized. This helps to optimize the intensity of labeling by minimizing non-specific “background” labeling and also avoids waste. A proper titration involves labeling a fixed number of cells with antibody a range of different concentrations. When the antibody concentration is unknown (which is often the case for hybridoma supernatants), it is convenient to test a series of dilutions (e.g. undiluted, 1:10, 1:100, 1:500, 1:1000). We prefer to use the highest concentration that does not result in a detectable elevation in non-specific labeling of “negative” cells. It is important to have an excess of antibody to ensure that all target antigens are saturated. The staining will be much easier to reproduce if the researcher takes the time to do one titration experiment at the beginning.

Once the sample is dissociated into single cells, it can be resuspended in holding buffer at a concentration of between 1×10^6 and 1×10^7 per ml. This has proven to be a good working concentration for the labeling of solid-tissue derived cells. A recommended general-purpose holding buffer is DMEM with 1% FBS and 0.1 mg/ml DNase I; this mixture provides nutrients, “blocks” cells against non-specific antibody binding by exposure to bovine immunoglobulins and reduces cell aggregation by digesting nuclear material released by dead cells.

It can be advantageous to use a combination of newly developed and commercially available antibodies. This allows for the exclusion (by electronic gating) of unwanted cell types. If multiple antibody-defined cell types are undesirable, the experiment can be simplified by using a common fluorochrome to label them for combined exclusion. This is sometimes referred to as a “dump channel”. For example, if both blood and endothelium are unwanted, anti-CD45-FITC and anti-CD31-FITC can be employed together.

We have found it useful to employ a labeling technique incorporating two different unconjugated primary antibodies and three conjugated antibodies in a five color sort. This allowed us to learn whether the new antibodies were reactive against blood or endothelium in addition to epithelial subpopulations.

A sample labeling protocol follows:

- Step 1.** Using 5ml round-bottom tubes (polystyrene or polypropylene, of the specific type required by the instrument), incubate the appropriate samples with unconjugated primary antibodies on ice for 20 minutes. Primary antibodies that can be distinguished by isotype or host species can be combined at this stage. If the cells are prone to settling, resuspend them periodically by vortexing.
- Step 2.** Wash by increasing the volume and centrifuging at the speed appropriate for your cells. Aspirate all but about 50 μ l of supernatant.
- Step 3.** Resuspend the pellet in the small volume that remains by gently flicking the tube until there is no longer a visible pellet. Do this before adding appropriate volume of holding buffer. (Failure to do this may result in clumping)
- Step 4.** Add all secondary antibodies and incubate for 20 minutes on ice.
- Step 5.** Wash as described above and resuspend in blocking buffer. This solution is holding buffer supplemented with serum/sera of the species in which the conjugated

antibodies to be used in Step 6 were raised. The immunoglobulins present in such sera will block unoccupied sites on the divalent secondary antibodies added in Step 4. Allow 20 minutes for blocking to proceed, on ice.

Step 6. Add conjugated primary antibodies and incubate for 20 minutes on ice.

Step 7. Wash and resuspend in holding buffer containing a dead cell-marking agent such as propidium iodide. The optimal concentration for sorting tends to be between 1×10^6 and 1×10^7 depending on the amount of debris present after cell isolation. At this point, it is important to visually inspect each tube for the presence of cell clumps. If these cannot be dispersed by vortexing they must be excluded by cell straining to avoid clogging the sorter's nozzle. BD makes tubes with strainer caps that allow convenient filtration of samples immediately prior to sorting.

4.3 Sorting

There are several instrument-related considerations for sorting tissue-derived cells. The selection of optimal nozzle diameter, fluidic pressure, forward and side scatter voltage settings are all vital and will be different depending on the organ and tissue type. Furthermore, because tissue-derived cells are being stored in an unnatural environment - a single cell suspension - care must be taken to avoid cell aggregation and to minimize ongoing cell death. These are issues that seldom arise in the world of hematopoietic sorting.

Cells from solid tissues tend to be larger than hematopoietic cells so it is often necessary to use a larger nozzle in order to avoid damaging the cells or clogging the nozzle. Cells from solid tissues are also often more fragile and are potentially more likely to be damaged by the shear forces when they pass through a smaller nozzle. The correct nozzle diameter must be determined empirically, but when in doubt one should err towards using a larger nozzle and accepting reduced cell throughput.

For sorting cells from human pancreatic islets or nonparenchymal cells isolated from a mouse liver, we use a 100 μm nozzle at a pressure of 15 PSI. Under these conditions, cells were sorted at between 2,000 and 4,000 events per second with over 98% purity (Dorrell et al., 2008b). When sorting mouse hepatocytes, which are large and fragile, the optimal nozzle for highest post-sort viability is the 150 μm nozzle at 4.5 PSI (Duncan et al., 2010).

There are a few tricks to keeping cells in a single cell suspension. If a visible pellet of settled cells is observed in the source tube, the sorter's tube vortexer or manual flicking/vortexing should be employed. As discussed previously, minimizing the amount of calcium can reduce cell aggregation; a low $[\text{Ca}^{2+}]$ holding buffer may be helpful. Keeping the sample cold using a chilling apparatus will also inhibit clumping.

Size-standard beads (e.g. 10 μm diameter) should be used to establish consistent forward and side scatter voltages. This facilitates the direct comparison of the size and granularity of cell samples collected on different days and even on different instruments. Sample-to-sample variability of solid tissue derived cells can be much greater than that of hematopoietic cells, so this sort of calibration is very important.

It is common practice when using flow cytometry with hematopoietic cells to set the voltages for the fluorescent detectors so that negative population falls below 10^1 . This approach is not recommended for the more heterogeneous populations found in isolated

tissue-derived cells, however. It is important to set the voltage on the PMTs so that very few cells are against the axis; if this means that some of the “negative” cells exhibit a significant degree of non-specific labeling, so be it. Note that this will be less of an issue when using software that permits visualization using a logical scale. Figure 5 illustrates how it becomes very difficult to identify distinct populations when detector voltages are set so that not all cells are on scale.

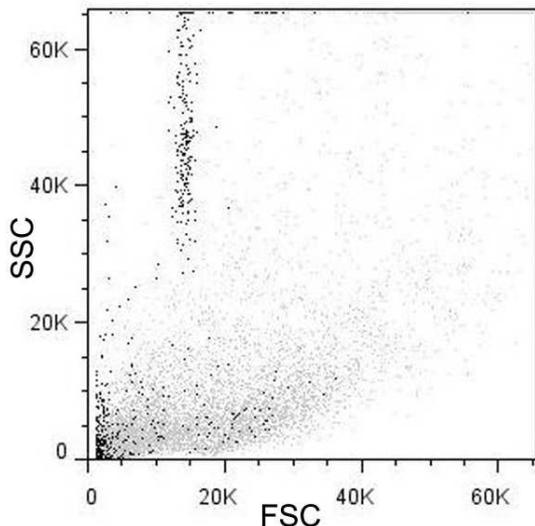


Fig. 4. 10 micron diameter beads and cells both on scale

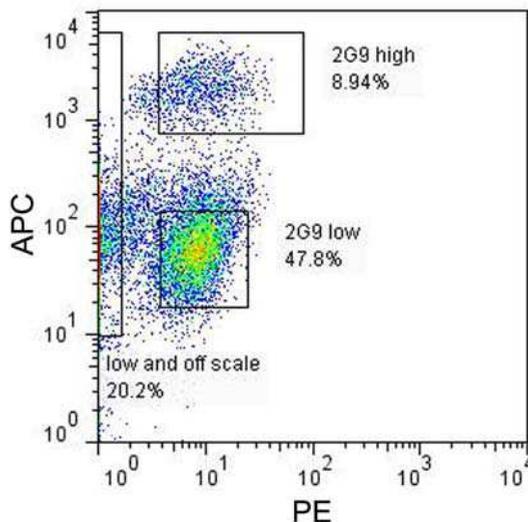


Fig. 5. Some cells are “off-scale low”

The use of “back-scattering” to evaluate the size/granularity of antibody-defined cell subpopulations requires the ability to gate on distinct populations. Since these properties are hard to predict on solid tissue-derived cells, back-scattering can reveal where the cells appear on a FSC/SSC plot and thus insure that both the optimal voltages and gating are employed.

5. Post-sort analysis

While flow cytometry data is valuable, it is the ability to isolate cell populations and do follow-up experiments that it such an overwhelmingly powerful tool. Cell populations can be analyzed for RNA content, they can be cultured, reprogrammed, transplanted, or even simply cytopspun and stained for other markers.

5.1 Microarray and RT-PCR

Microarray and QRT-PCR are arguably two of the most powerful tools for more comprehensive understanding of each cell type. When post-sort RNA analysis is the goal, there are a few things to keep in mind in order to acquire the highest quality RNA.

A very high purity sorted sample will result in less contamination of RNA from other cell types. A typical sort will result in a purity of 95% or higher. Unless the available cell numbers are very limited, a higher purity level is probably desirable. Re-sorting can be advantageous because it can allow you to make the initial sort faster, not aborting any events.

There are, of course, advantages and disadvantages of sorting and then resorting. The obvious advantage is that the resorted sample is very close to 100% pure; there are very few contaminating cells that might negatively affect results. A potential disadvantage is that the cells are being put through the sorter again. This increases the likelihood of them being damaged. The question of whether the RNA expression might change as the cells are subject to shear forces multiple times is a valid but hypothetical concern.

5.2 Cell culture

Culturing cells that have never been isolated before poses exciting challenges. How does one optimize culture conditions for cells that have not been thoroughly characterized? It requires trial and experimentation but at least if the cells can be analyzed first to see what genes they are expressing, the researcher has information that will aid with making educated guesses.

5.2.1 Stem cell assays

If the goal of sorting is to isolate stem/progenitor cells, there are a number of things that can be done in order to determine whether the cells of interest can be accurately described as stem cells.

One good example of this is the work done by Sato and colleagues, where LGR5 positive cells were isolated from the mouse intestine and used to initiate three dimensional cultures in Matrigel (Sato et al., 2009). In this case, previously gained knowledge, about what the

stem cells were expressing and what the requirements were to maintain them, were used in order to cultivate the intestinal stem cell and maintain them indefinitely.

The most rigorous stem cell assay is transplantation. If the unique population is sorted and found to be able to engraft a recipient animal and give rise to multiple types of daughter cells it is a strong indicator that the population contains a stem cell. It is fortunate that many human stem cells are transplantable into immune-deficient mice and give rise to cells that can replace the equivalent mouse cell (Grompe, 2001; Kamel-Reid and Dick, 1988; Wang et al., 2001).

5.2.2 Cell reprogramming

There are several ways that new antibodies might contribute to the success of cell reprogramming. First, if tissue-resident progenitors can be identified and isolated, their inherent plasticity would make them obvious targets for reprogramming. Additionally, if antibodies are created against cells in various stages of development, it will aid in the understanding of cell expression and signaling during development, and the replication of these events in adult cells. Knowing what cells express at various stages of development also allows for quick assays to determine whether the researcher is on the right path.

5.2.3 Culture for research and for transplant

Currently, it is very difficult to get cells for experimentation from cadaveric human donors. Researchers need sources of tissue and unfortunately the cell lines that are available are almost always tumor-derived. This limits their utility with regard to learning about the normal biology of certain cell types. Great strides have already been made in the field of solid tissue culture with the goal of future transplantation. Tissue culture has been shown to be remarkably successful at producing skin (Ueda, 2011). There has also been some success culturing hepatocytes with the hope of creating an external liver support device (Niu et al., 2009). As our understanding becomes more comprehensive, so too will our ability to maintain these cells in culture. Eventually, tissue banks might augment or even supplant the need for cadaveric organ donation.

6. Conclusion

The more tools we have to work with, the more quickly and thoroughly we can address issues of disease and expand our understanding of healthy tissue. Being able to physically isolate different cell subtypes is crucial to that understanding if we want to utilize powerful tools, such as microarray and RNA sequencing, which require large numbers of highly purified populations of cells. Experiments using up to 17 fluorochromes and 31 metal-conjugated antibodies are already being performed on blood and bone marrow cells (Bendall et al., 2011; Perfetto et al., 2004). In our lab, we have produced a number of antibodies against solid tissue and used these to isolate and analyze defined subpopulations that were not previously accessible by flow cytometry (Dorrell et al., 2011b; Dorrell et al., 2011c). As the need for these antibodies continues, it is hoped that more researchers will contribute to the array of tools that can be used in solid-tissue research.

The HLDA has expanded their mission to include characterization of surface epitopes found on all tissues relevant to the immune system. This expansion was reinforced with a name

change; they are now the HCDM (Human Cell Differentiation Molecules) (Zola et al., 2007) Unfortunately there is not the same sort of all-inclusive drive toward a comprehensive characterization and evaluation of antibodies against tissue-bound cells. We feel that a comparable initiative directed towards solid organs, using the strategies and techniques described in this document, would yield many additional tools and reagents for the study of normal and pathological tissues.

7. Acknowledgements

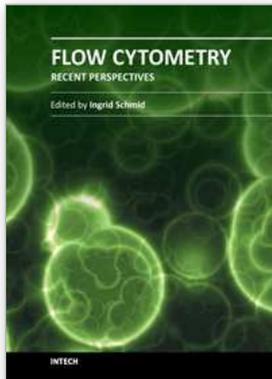
The authors would like to thank Kim Hamlin for illustrating figure 1.

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"Flow Cytometry - Recent Perspectives" is a compendium of comprehensive reviews and original scientific papers. The contents illustrate the constantly evolving application of flow cytometry to a multitude of scientific fields and technologies as well as its broad use as demonstrated by the international composition of the contributing author group. The book focuses on the utilization of the technology in basic sciences and covers such diverse areas as marine and plant biology, microbiology, immunology, and biotechnology. It is hoped that it will give novices a valuable introduction to the field, but will also provide experienced flow cytometrists with novel insights and a better understanding of the subject.

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