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Ashleigh S. Boyd *Editor*

Immunological Tolerance

Methods and Protocols

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Edited by

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Preface

"Immunological Tolerance," the state in which the body is unresponsive to antigen, ensures the development of a "non-self" reactive T cell repertoire, as well as development of tolerogenic regulatory immune populations that protect us from disease. Continuing to understand how tolerance develops and, in the context of diseases such as autoimmunity and cancer, falters and breaks down will offer invaluable insight into how to manipulate these mechanisms to improve treatment of these diseases. This knowledge may also be applied to induce immunological tolerance in the setting of allogeneic transplantation as first described by Peter Medawar and Frank Macfarlane Burnet, who were awarded a Nobel Prize in 1960 for their seminal work in transplantation tolerance.

Since then the concept of immunological tolerance has blossomed into an entire, separate discipline in immunology, where advances have been regularly observed in small and large animal model systems but as of yet have largely failed to be replicated in the clinical setting. Yet, with recent advances in immunotherapy and techniques such as genome editing in concert with the isolation and characterization of novel subsets of tolerogenic cells, the prospect for harnessing the power of the immune system clinically to control unwanted responses to both self and foreign antigen now appears to be edging closer to reality than ever before.

With this in mind, I have developed this edition with a strong emphasis on techniques that can be used to understand or manipulate tolerance in human cells and to assess human disease in this setting directly. The chapters have been organized thematically into five parts. In Part 1, we focus on the isolation of tolerogenic cell types from stem cells, which are a self-renewing cell type responsible for producing and maintaining tissues in the body. While their biology in this respect is well established, different classes of stem cells and their progeny are now emerging as potential agents of immunomodulation, and we explore this capacity from both ex vivo isolated stem cells and laboratory-engineered stem cells (so-called induced pluripotent stem cells). Part 2 extends the premise of isolating tolerogenic cell populations for study and therapeutic utility by covering the ex vivo isolation of tolerogenic lymphocytic cells, including recently characterized B regulatory populations. In Part 3, we explore multiple methods to study the mechanisms underpinning tolerance, as well methods to induce tolerance through thymus progenitors which may be utilized in the future to reconstruct the thymus, the key site for central tolerance induction. How such methods of tolerance induction are invoked practically, as first envisioned by Medawar and Burnet, in context of transplantation, is considered in Part 4. Finally, Part 5 includes methods to assess the breakdown of immune tolerance in specific pathological conditions.

When I was asked to compile this special edition of *Methods in Molecular Biology* on "Immunological Tolerance" I was both honored and somewhat daunted by the task of covering the massive scope of the field. I have attempted to focus on what I consider to be important contemporaneous issues and methods, which I sincerely hope you will enjoy and find useful in your quest to further advance the field.

Finally, it would be remiss of me not to acknowledge my husband, Neil, for his steadfast support of my academic career (and in life in general), and to our daughter, Olivia, the light of our lives, who makes us realize what really matters in the end.

London, UK ***Ashleigh S. Boyd***

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Part I

Isolation of Stem Cells and Their Progeny for Immunological Tolerance Applications

Chapter 1

Isolation and Characterisation of Human Adipose-Derived Stem Cells

Anna Wilson, Melisse Chee, Peter Butler, and Ashleigh S. Boyd

Abstract

Recently, adipose-derived stem cells (ASCs), obtained from fresh human lipoaspirate, have shown promise as immunomodulatory agents having demonstrated immunosuppressive functionality both in vitro and in vivo. A number of researchers have described the isolation of ASCs through the enzymatic digestion of fat samples, followed by a number of purification steps, involving centrifugation and filtration. Here, we utilize a standard isolation technique, with the added purification of putative ASCs by fluorescence activated cell sorting (FACS). ASCs are an extremely valuable resource in clinical applications, including reconstruction, regeneration, and investigations into immune activity. This method could be used to isolate and purify ASCs for such downstream applications.

Key words Adipose-derived stem cells, Adipose stem cells, ASCs, Isolation, Characterization, Immunosuppression, Immunomodulation, Stromal vascular fraction, Collagenase, Lipotransfer, Lipoaspirate

1 Introduction

Similar to bone marrow-derived mesenchymal stem cells (MSCs), adipose-derived stem cells (ASCs) are multipotent adult stem cells which, as their name suggests, can be isolated from adipose (fat) tissue [1]. First identified in 2001, obtained via liposuction, ASCs were initially named processed lipoaspirate (PLA) cells [1]. Morphologically similar to fibroblasts, upon differentiation, ASCs can give rise to adipocytes, osteocytes, and chondrocytes. This plasticity may offer potential for their use in regenerative medicine as large numbers of these daughter cells can be generated from an easily isolated stem cell source. Furthermore, since aspirated fat is in plentiful supply from many plastic surgery procedures, such as liposuction and liposculpture, and the precursor cells can be purified by a variety of processing and enzymatic techniques to obtain the ASC-rich stromal vascular fraction (SVF) it is relatively straightforward to obtain ASCs [2–4].

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A further similarity between MSCs and ASCs is their promising immunomodulatory capacity [5–11], which has been observed to be either cell-contact dependent [6] or mediated by cytokines and trophic factors such as TNF-α, IFN-γ, IDO, PGE-2, and IL-17 [12–15]. In vivo, ASCs have been used for this purpose in spinal cord injury and neurodegenerative diseases [16, 17], allergic [10] and autoimmune diseases (for example, rheumatoid arthritis and inflammatory bowel disease) [18, 19], and in reducing Graft Versus Host Disease (GVHD) [20]. Clinical trials are bringing the use of injectable and implantable ASCs closer to becoming a reality for patients [21–24].

However, adipose-derived stem cells have proven problematic to identify in culture, and studies have been carried out to point to particular cellular markers, which may make them easier to recognize. Previous work has characterized ASCs based on their morphology [25–27], cell surface marker expression [28], and/or by assessing their ability to differentiate into specific lineages [29]. Once isolated, adipose stem cells have an even, round phenotype, in contrast to the irregular shape of endothelial cells. When cultured, they adhere to plastic and assume a fibroblast-like morphology within several days [30].

Rigotti's groundbreaking work showed positive characterization by flow cytometry of ASCs with respect to antibodies against CD105, CD73, CD29, CD44, and CD90. ASCs correlated negatively with CD31, CD45, CD14, and CD34 expression [28]. This characterization was echoed in another study which reported ASC positive expression of CD13, CD44, CD73, CD90, and CD105, but which did not express CD45, CD69, CD117, or HLA-DR [26]. These findings were consolidated by the International Society for Cellular Therapy (ISCT), who recently published guidelines for the identification of pluripotent and multipotent stem cells [27]. Herein, we isolated ASCs from human fat tissue using a combined approach of washing, centrifugation, and filtration followed by cell sorting based on their expression of the markers CD90, CD73, CD105, and CD44 and lack of expression for the markers CD45 and CD31. This method could be used to isolate and purify ASCs for downstream applications such as for regenerative medicine and reconstruction or for immune suppression studies.

2 Materials

All solutions should be prepared using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature, unless stated otherwise. Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 Isolation of ADSCs

1. Washing solution: Phosphate-buffered saline (PBS) (Sigma-Aldrich, Poole, UK), 1% penicillin/streptomycin liquid (P/S, Gibco, Life Technologies, Thermo Fisher Scientific, Paisley, UK).
2. HBSS solution: Hanks Balanced Salt Solution (HBSS, Gibco), 1% P/S (Gibco).
3. Tissue culture medium: Alpha-MEM (Gibco), 10% fetal bovine serum (FBS, cat#10091-148, Gibco), 1% P/S (Gibco).
4. Flow cytometry buffer: 50 mL PBS (Sigma)/0.25 mL FBS (0.5%, as before).
5. Collagenase type 1 (#17100017, Life Technologies, Thermo Fisher Scientific).
6. Beckton Dickinson 40 and 100 mm cell strainers, 15 and 50 mL Corning centrifuge tubes, 25 cm^2 tissue culture flasks, 0.2 mm syringe filters, 10 cc syringes, and weighing boats (all from VWR International, Lutterworth, UK).
7. Trypan Blue solution 0.4% (Thermo Fisher Scientific).

2.2 Culture and Expansion of ASCs

1. Washing solution: PBS (Sigma), 1% P/S (Gibco).
2. Tissue culture media: Alpha-MEM (Gibco), 10% FBS (Gibco), 1% P/S (Gibco).

2.3 Flow Cytometry

1. Flow cytometry buffer: 50 mL PBS/0.25 mL FBS (0.5%).
2. 5 mL polypropylene flow cytometry tubes (#352063, BD Biosciences).
3. 0.25% Trypsin-EDTA (1×), Phenol red (all Thermo Fisher Scientific).
4. Alpha-MEM, 10% FBS, 1% P/S (all Thermo Fisher Scientific).
5. Anti HLA-DR PerCP (#ab91333, Abcam, Cambridge, UK).
6. MSC Phenotyping Kit (Miltenyi Biotec, Oxford, UK).
7. 7-aminoactinomycin D (7AAD ThermoFisher Scientific).
8. Propidium iodide (PI, Sigma).
9. OneComp eBeads (#01-1111-42, eBioscience, Aachen, Germany).

2.4 Differentiation

1. Stempro adipogenic differentiation kit (Thermo Fisher Scientific).
2. Stempro chondrogenic differentiation kit (Thermo Fisher Scientific).
3. Stempro osteogenic differentiation kit (Thermo Fisher Scientific).
4. 12-well plates (VWR).

5. Cover slips (VWR).
6. ASC basic media (Alpha-MEM, 10% FBS, 1% pen/strep).
7. Paraformaldehyde 4% (Sigma Aldrich).
8. Isopropanol 60% (Sigma Aldrich).
9. Oil-Red-O (#ab150678, Abcam).
10. Alizarin Red (#ab142980, Abcam).
11. Alcian Blue 8GX (#ab145250, Abcam).

2.5 Fluorescence Activated Cell Sorting (FACS)

1. BD Biosciences flow cytometry tubes (as before).
2. 0.25% Trypsin-EDTA (1×), Phenol red.
3. Alpha-MEM, 10% FBS, 1% penicillin/streptomycin (all Thermo Fisher Scientific).
4. Flow cytometry buffer: 50 mL PBS/0.25 mL FBS (0.5%) (suppliers as before).
5. MSC Phenotyping Kit (Miltenyi Biotec).
6. Isotype control cocktail (Miltenyi Biotec).
7. Anti HLA-DR PerCP (Abcam).

3 Methods

All procedures should be carried out in a laminar flow hood, at room temperature, unless otherwise specified. We recommend processing human lipoaspirate within 24 h of collection, having stored it in sterile laboratory conditions at room temperature [29].

3.1 Adipose Tissue Harvest

Surplus fatty tissue can be collected following Coleman fat transfer procedures from adult human patients undergoing routine plastic surgery procedures. It is essential that local ethics approval is in place and all guidelines are followed. Patients are to be consented for the donation of surplus fatty tissue and its use in research, and provided with a patient information sheet. The procedures can be performed with or without the use of local anesthetic solution infiltration preoperatively. Fat can be harvested from the abdomen in six distinct areas, typically through a 2 mm-diameter cannula, into a 10 cc syringe. The fat filled syringes should then be centrifuged at 400 × *g* for 3 min to separate the oil, supernatant, and blood (Fig. 1), which can be poured off manually. Surplus fat is then collected.

3.2 Isolation of ASCs

From each patient, up to two samples of 5 g can be acquired. The process of ASC isolation is summarized in Fig. 1. Dispense each sample into a sterile specimen tube and wash three times with 20 mL PBS solution. Add 30 mg of collagenase type I (6 mg per

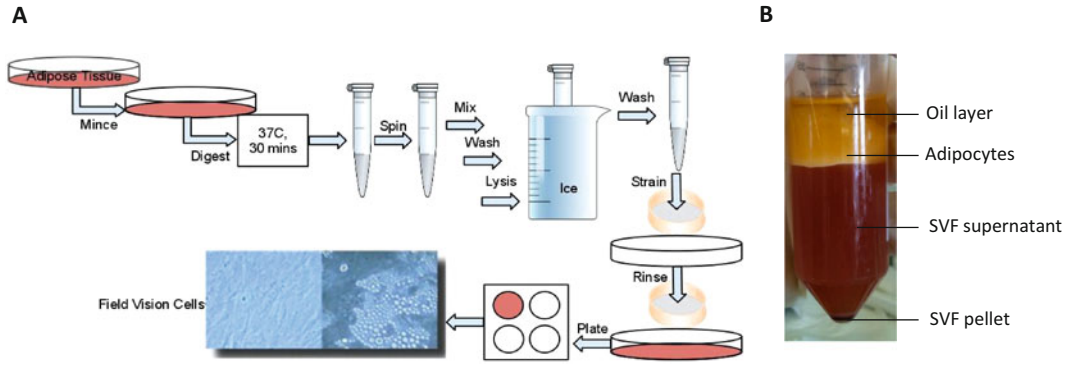

Fig. 1 Diagrammatic summary of the process of ASC isolation from adipose tissue and the appearance of the stromal vascular fraction (SVF). (**a**) Depiction of the steps involved in ASC isolation from lipoaspirate. (**b**) The stromal vascular fraction is first apparent following centrifugation of the aspirated fat

1 g adipose tissue, made up in 15 mL HBSS) to each sample. Shake the suspension and incubate for 2 h at 37 °C, 5% CO_2, 90% humidity. Immediately after this, centrifuge the sample at 300 × *g* for 5 min, and aspirate the supernatant. Resuspend the resulting pellet in complete culture media (Alpha-MEM, 10% FBS, 1% p/s), and combine both the samples into one tube, filtering sequentially through 100 μm and then 40 μm cell strainers. Centrifuge the samples again at 300 × *g* for a further 5 min, then aspirate the supernatant and resuspend the final pellet in 1 mL culture medium (*see* Fig. 1b to ascertain the appearance of the final cell pellet and SVF supernatant). Determine the cell number using the Trypan Blue assay. Incubate at 37 °C, 5% CO_2, 90% humidity in a 25 cm^2 culture flask.

3.3 Trypan Blue Cell Counting Protocol

With the cell pellet still in 1 mL of complete media, mix cells by pipetting up and down or vortexing. In a 96-well plate, pipette 90 μL of Trypan Blue solution into two adjacent wells. Pipette 10 μL of cells in media, into the first well and mix well. Then pipette 10 μL of cells from the first well into the second well, and mix well. Pipette 10 μL from the second well into the haemocytometer, and count cells. The resulting cell number represents a 1 in 100 dilution; therefore, the average number obtained should be multiplied by 10^6 in order to calculate the number of cells per 1 mL of media, i.e., per 10 g of fat.

3.4 Expansion of ADSCs In Vitro

Following the placement of ASCs into culture flasks in culture media (Alpha-MEM, 10% FBS, 1% p/s), allow them to adhere to culture plastic for 72 h. After this, wash cells twice with PBS, and replace culture media. Repeat after a further 48 h. At 7 days from isolation, the cells can be detached from the plate using trypsin, and counted using the Trypan Blue assay [31, 32].

3.5 Phenotypic Analysis of Putative ADSCs by Flow Cytometry

Following isolation, in culture putative ADSCs retain markers in common with other mesenchymal stromal/stem cells (MSCs), including CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31. Using these markers ADSCs can be immunephenotyped by flow cytometry. Control samples required include unstained controls, single stain controls, and isotype stain controls using the same cells as to be analyzed for test antibodies. Since as most cells emit low level auto-fluorescence, an unstained control is used to set up the photomultiplier (PMT) voltages on the flow cytometer to a level wherein the unstained cells should appear in the first quartile of a four decade logarithmic scale for each fluorochrome measured. Isotype controls are also required. They are used as a specificity control to distinguish specific from nonspecific binding. Together, these controls allow for the appropriate gating of the desired cell population. The following sections describe the process for antibody labeling and analysis of samples on a flow cytometer [33].

3.5.1 Flow Cytometry Cell Staining

1. Make up two aliquots of one million putative ADSCs each, in culture media.
2. Centrifuge tubes at 300 × *g* for 10 min. Then aspirate the supernatant.
3. Resuspend cells in 90 μL buffer.
4. Add 10 μL of MSC Phenotyping cocktail and 10 μL of Anti-HLA-DR PerCP to tube 1, and 20 μL of isotype control cocktail to tube 2.
5. Vortex to mix and incubate tubes for 10 min in the refrigerator.
6. Add 3 mL of flow cytometry buffer to each tube, then centrifuge at 300 × *g* for 10 min. Aspirate the supernatant.
7. To stain for cell viability, resuspend each cell pellet in 100 μL buffer containing 7AAD or PI (1:250 dilution). Vortex to mix and incubate tubes in the refrigerator for 10 min.
8. Add 3 mL of flow cytometry buffer to each tube, then centrifuge at 300 × *g* for 10 min. Aspirate the supernatant.
9. Resuspend each cell pellet in 400 μL flow buffer in preparation for acquisition.
10. Place all tubes on ice prior to acquisition on a flow cytometer.

3.5.2 Compensation for Flow Cytometry

Compensation is the process of subtracting the spectral spillover from one fluorochrome into the detector of another. Single stain controls can be used to set the instrument to compensate for this fluorescence spill over between channels. However, fluorescently conjugated beads (such as OneComp eBeads™, eBioscience) provide an alternative to cells in achieving this, and allow for a reliable and reproducible benchmark with the added advantage that they replace precious samples, enabling every cell to be retained for analysis.

1. Aliquot 500,000 cells each (or one drop of OneComp eBeads; except for in the "no stain" tube, for which cells should always be used) into five separate tubes. Label as "PerCP," "PE," "APC," "FITC," and "no stain." These tubes will be used as single stain controls and an unstained control.
2. Add 100 μL of flow cytometry buffer to each tube. Then add the following to the appropriate tube:
   (a) PerCP tube—add 10 μL CD73 Biotin antibody.
   (b) PE tube—add 10 μL CD105 PE antibody.
   (c) APC tube—add 10 μL CD73 APC antibody.
   (d) FITC tube—add 10 μL CD90 FITC antibody.
   (e) No stain tube—500 μL buffer only, no antibody.
3. Vortex to mix and incubate tubes for 10 min in the refrigerator.
4. Add 2 mL of flow cytometry buffer to each tube to dilute antibody, centrifuge at 300 × *g* for 10 min. Aspirate the supernatant.
5. Add 10 μL Anti-Biotin PerCP to the PerCP aliquot, mix well, and incubate in the refrigerator for 10 min. Then wash with 1 mL flow cytometry buffer, centrifuge at 300 × *g* for 10 min, and aspirate the supernatant.
6. Resuspend each pellet in 400–500 μL buffer, ready for flow cytometry, and store on ice. Process as soon as possible.

3.5.3 Acquisition and Analysis on a Flow Cytometer

The samples and controls should be acquired on a flow cytometer. Our samples were acquired using a MACSQuant 10 (Miltenyi Biotec, Germany) and MACSQuant Analysis software (Miltenyi Biotec) to identify progression of surface marker characteristics of isolated ASCs.

3.6 Differentiation

In order to show that isolated ASCs have stem cell potential, in common with MSC differentiation, ASCs can be induced to differentiate into three different cellular lineages namely adipocytes, osteocytes, and chondrocytes which together are referred to as the tri-lineages. This section describes the process for inducing differentiation toward each lineage.

3.6.1 Directed Differentiation

1. Determine cell number.
2. Make up differentiation media as per StemPro instructions for adipogenic, chondrogenic and osteogenic differentiation (Gibco, UK).
3. Sterilize cover slips by dipping them into 70% ethanol, then place into wells in 12-well plate.
4. Wash each well with PBS (1% penicillin/streptomycin).

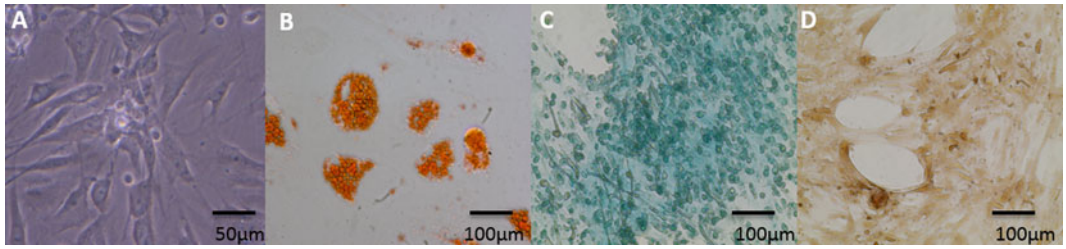

Fig. 2 Appearance of ASC in culture and following targeted tri-lineage differentiation. (**a**) Appearance of adipose derived stem cells (ASCs) in culture at day 7. Following isolation, ASCs were driven to undergo targeted differentiation to the tri-lineages (**b**–**d**). (**b**) Oil red O staining of lipid droplets generated via adipocyte differentiation of ASCs. (**c**) Alcian blue staining of chondrocyte nuclei following ASC chondrocytic differentiation. (**d**) Alcian red staining of osteoblasts following osteogenic differentiation of ASCs. Images captured on a EVOS XF light microscope and are representative of $n = 3$ independent experiments

5. Seed 30,000 cells per well and add 1.5 mL culture media. Incubate at 37 °C, 90% humidity, 5% CO_2.
6. For chondrogenesis, allow the cells to adhere for 2–3 h prior to changing the medium to chondrogenic differentiation medium. For adipogenesis, allow the cells to adhere for 24 h prior to changing the medium to adipogenic and osteogenic differentiation media, respectively.
7. Wash wells and replace differentiation media once per week, for 21–28 days, while monitoring cell growth and differentiation.
8. Staining for adipose cells can be performed at 21 days, and for chondrocytes and osteocytes at 28 days (as below; Fig. 2).

3.6.2 Phenotyping of Differentiated ASC–Derived Cells

In order to show that isolated ASCs have stem cell potential, in common with MSC differentiation, ASCs can be induced to differentiate into three different cellular lineages namely adipocytes, osteocytes, and chondrocytes. This section describes the process of staining the differentiated cells to show that they have become committed to the MSC/ASC tri-lineages (*see* Fig. 2).

Staining of ASC-Derived Adipocytes

1. Fix with 4% PFA for 1 h.
2. Mix three parts ORO with two parts distilled water.
3. Remove PFA, rinse with DI water.
4. Add 2 mL 60% isopropanol to each well and leave for 5 min.
5. Remove and add 2 mL ORO working solution, leave for 5 min.
6. Remove ORO, rinse with DI water.

Staining of ASC-Derived Osteocytes

1. Fix with 4% PFA for 1 h.
2. Aspirate and rinse twice with DI water.
3. Cover wells with Alizarin red solution, leave for 30 min.
4. Remove Alizarin red and wash 4× with DI water.

Staining of ASC-Derived Chondrocytes

1. Fix with 4% PFA for 1 h.
2. Aspirate and rinse twice with DI water.
3. Stain with 1% Alcian Blue solution for 30 min.
4. Rinse with DI water.

3.7 Fluorescence Activated Cell Sorting (FACS)

To isolate putative ASCs from lipo-aspirate, rather than simply to analyze surface marker expression, the cells should be stained in a similar manner as for flow cytometry analysis. The benefit of using FACS is that the cells can be separated based on their expression of ADSC markers and retained for downstream experiments.

Current ASC enrichment techniques include monolayer expansion and surface marker-based sorting. Cultured ASCs grown in monolayers are defined by their ability to adhere to plastic surfaces and fast proliferation rates; this method is yield-effective because non-adherent cell types, for example, erythrocytes and leukocytes, are washed away enabling more efficient proliferation of ASCs. The ideal solution would be to immediately isolate and characterize the ASCs, based on cell surface marker expression, such that a precise fraction of specific cells could then be re-injected into the operative site [34].

1. Determine cell number. If possible, use >ten million cells for cell sorting.
2. Perform staining as per flow cytometry protocol, amending the amount of antibody used; for example, use 25 μL of MSC phenotyping cocktail for every ten million cells stained.
3. Stain further samples to set compensation controls.
4. Place all samples on ice and acquire on flow cytometer as soon as possible following staining.

4 Notes

1. 5ccs of lipoaspirate provides sufficient volume of fat for isolation of ASCs, with relative ease in processing.
2. Washing is an essential first step in isolation of ASCs from lipoaspirate; the more thoroughly this is done, the better the adhesion of ASCs to tissue culture plastic will be.
3. During filtration of the SVF through cell strainers, it may prove helpful to aspirate some air through the cell strainer while pouring the digested fat to be filtered. This needs to be done slowly to prevent clogging of the cell strainers with large debris.
4. Exclude dead cells from analysis by staining with propidium iodide (PI) or 7-aminoactinomycin D (7AAD) in conjunction with their forward and side light scattering properties.
5. When ASCs are processed clumps of dead fat cells can arise which may cause line blockages during flow cytometry. We