Chapter 1

Physical versus Immunological Purification of Mesenchymal Stem Cells

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Abstract

A prerequisite before dealing with any cell type is to identify it and isolate it from the heterogeneous cell population that it belongs to. Mesenchymal stem cells (MSC) can be found in nearly all tissues and are mostly located in perivascular niches.

MSC isolated from Bone marrow, adipose tissue, peripheral blood and different organs had shown promising potential for proliferation and differentiation into different cell types. They exhibit plastic-adherence under standard culture conditions, and this physical method of isolation is widely used as it is the most economic method and yet reveals relatively purified populations of cells after 3 or 4 passages. The complete purification still needs a specific call to different MSCs subsets. This could be achieved by immunological sorting, which depends on identifying cell marker(s) of such cells. Selecting these cells using antibodies against their specific markers then sorting the cells either by Magnetic or florescence based techniques named Magnetic Activated Cell Sorting (MACS) or Florescence Activated Cell Sorting (FACS) respectively is the principle of such purification techniques.

The aim of this chapter is to thoroughly define MSCs and compare between the different available methods for their purification

Keywords: MSC surface markers, MSC isolation, purification techniques

1. Definition of MSC

In 1970, Friedenstein [1] discovered in the bone marrow a rare stromal cell population forming around 0.0001 to 0.01% of nucleated cells. These cells are having the ability to proliferate in culture, and now commonly called mesenchymal stem or stromal cells (MSCs).

No unique cell surface marker clearly distinguishes MSCs, which makes a uniform definition difficult. The International Society for Cell Therapy proposed criteriathat comprise (1) adherence to plastic in standard culture conditions; (2) expression of the surface molecules CD73 (ecto-5′-nucleotidase marker), CD90 (Thy1 marker), and CD105 (endoglin marker) in the absence of CD34 (hematopoietic stem cell marker), CD45 (leukocyte marker), HLA-DR (human leukocyte antigen class II), CD14 or CD11b (monocyte and macrophage markers), CD79 or CD19 (B cell marker), and (3) a capacity for differentiation to osteoblasts, adipocytes, and chondroblasts *in vitro* [2].

These criteria were established to standardize human MSC isolation but may not apply uniformly to other species. The expression of these markers may decline over sub-passaging yet with the preservation of its proliferative, self-renewal and multilinage differentiation capability. Although the latter criteria are more consistent in defining MSCs, the above mentioned definition is discussed thoroughly as follows:

1.1. Adherence to plastic in standard culture conditions

MSCs grow as adherent monolayers, and unless they have transformed and become anchorage independent, after tissue disaggregation or subculture they will need to attach and spread out on the substrate before they will start to proliferate, thus giving rise to the criterion of adherence to plastic in standard culture conditions.

Cell adhesion is a complex event that refers to binding of cells to a surface. This surface may be another cell, the surrounding extracellular matrix (ECM) or a substrate. Mammalian cells coexist *in vivo* in intimate contact with each other and the surrounding ECM. Adhesion between these surfaces is directed at the molecular level by two different types of interactions. One is the "cell-cell adhesion" which is regulated by membrane expression of specialized integral membrane proteins called "cell adhesion molecules" (CAMs) that are generally clustered together at specialized points of cell contact with the cytoplasm of neighboring cells and thus can regulate signal transduction. A large number of CAMs exist and fall into four major families: the cadherins, immunoglobulin (Ig) superfamily, integrins, and selectins [3–5].

While the other is the "cell- matrix adhesion" through which the cells adhere indirectly by binding of a membrane adhesion receptor to specified components of ECM. The ECM is an organized network of proteins and polysaccharides secreted by cells that play a key regulatory role in determining the development, organization, and biological behavior of cells. In mammalian systems, three types of molecules are abundant in the ECM of all tissues: collagens, multi-adhesive matrix proteins, and proteoglycans. While collagen fibers and proteoglycans provide mechanical support, they are primarily the adhesive matrix proteins that bind to cell-surface adhesion receptors and other ECM components.

By way of these two types of interactions, cells can communicate bidirectionally with each other and respond to changes in the extracellular environment [6].

The process of adhesion regulates cell shape and biomechanics and is required for a variety of other cellular processes including proliferation, differentiation, migration, and invasion [7].

Originally, it was found that MSCs would attach to, and spread on, glass that had a slight net negative charge. They would also attach to some plastics, such as polystyrene, if the plastic was appropriately treated with strong acid, a plasma discharge, or high-energy ionizing radiation.

As cell adhesion is mediated by specific cell surface receptors for molecules in the extracellular matrix, so it seems likely that spreading may be preceded by the cells' secretion of extracellular matrix proteins and proteoglycans. The matrix adheres to the charged substrate (glass or treated plastic), and the cells then bind to the matrix via specific receptors. Thus, glass or conditioned plastic in which previous cells were grown upon can often provide a better surface for attachment, and substrates pretreated with matrix constituents, such as fibronectin or collagen, or derivatives such as gelatin, will help more demanding cells' attachment and proliferation.

1.2. Expression of the surface molecules (cell markers)

As previously mentioned, the definition of MSCs included the expression of certain cell markers together with the other criteria of their adherence and differentiation capacity.The selection of such criteria was to obtain easier comparisons between different studies and to adapt standards for the characterization of MSC. Nevertheless, these markers represent differentiation potential of MSC. Furthermore, these criteria apply to human MSCs, but do not necessarily extend to other species [8], also following culture, these markers may be lost or new markers may arise. So, some results fail to meet these criteria, making the comparison difficult. Thus, it was more convincing to agree on referring to human MSCs as stem cells when they prove self-renewal capability and showing their capacity for multilinage differentiation [9].

The expression of surface molecules (**Table 1**) and thus the phenotyping of human MSCs have been illustrated by many researchers based on the characterization of cultured cells.

MSCs have immunomodulatory properties as they express moderate levels of human leukocyte antigen (HLA), major histocompatibility complex class I, lack major histocompatibility complex class II expression, and do not express costimulatory molecules B7 and CD40 ligand [11–13]. The allogeneic transplantation of MSCs is well tolerated due to this unique immunophenotype together with the powerful immunosuppressive activity via cell-cell contact with target immune cells and secretion of soluble factors, such as nitric oxide, indoleamine 2,3-dioxygnease, and hemeoxygenase-1 [14–17]. MSCs produce an immunomodulatory effect by interacting with both innate and adaptive immune cells.

The innate immune cells (neutrophils, dendritic cells, natural killer cells, eosinophils, mast cells, and macrophages) are responsible for a nonspecific defense to infection, and MSCs have been shown to suppress most of these inflammatory cells. The adaptive immune system, composed of T and B lymphocytes, is capable of generating specific immune responses to pathogens with the production of memory cells. MSCs have been shown to suppress T cell proliferation in a mixed lymphocyte culture [18, 19].

Common name	CD locus	Detection
Adhesion molecules*		
ALCAM	CD166	Positive
ICAM-1	CD54	Positive
ICAM-2	CD102	Positive
ICAM-3	CD50	Positive
E-selectin	CD62E	Negative
L-selectin	CD62L	Positive
P-selectin	CD62P	Negative
LFA-3	CD58	Positive
Cadherin 5	CD144	Negative
PECAM-1	CD31	Negative
NCAM	CD56	Positive
HCAM	CD44	Positive
VCAM	CD106	Positive
Hyaluronate receptor	CD44	Positive
Growth factors and cytokine receptors*		
IL-1R (α and β)	CD121a, b	Positive
IL-2R	CD25	Negative
IL-3R	CD123	Positive
IL-4R	CD124	Positive
IL-6R	CD126	Positive
IL-7R	CD127	Positive
Interferon γ R	CDw119	Positive
TNF-α-1R	CD120a	Positive
TNF-α-2R	CD120b	Positive
FGFR		Positive
PDGFR	CD140a	Positive
Transferrin receptor	CD71	Positive
Integrins*		
VLA-α1	CD49a	Positive

Common name	CD locus	Detection
VLA-α2	CD49b	Positive
VLA-α3	CD49c	Positive
VLA-α4	CD49d	Negative
VLA-α5	CD49e	Positive
VLA-α6	CD49f	Positive
VLA-β chain	CD29	Positive
β4 integrin	CD104	Positive
LFA-1 α chain	CD11a	Negative
LFA-1 β chain	CD18	Negative
Vitronectin R α chain	CD51	Negative
Vitronectin R β chain	CD61	Positive
CR4 α chain	CD11c	Negative
Mac1	CD11b	Negative
Additional markers*		
T6	CD1a	Negative
CD3 complex	CD3	Negative
T4, T8	CD4, CD8	Negative
Tetraspan	CD9	Positive
LPS receptor	CD14	Negative
LewisX	CD15	Negative
—	CD34	Negative
Leukocyte common antigen	CD45	Negative
5′terminal nucleotidase	CD73	Positive
B7-1	CD80	Negative
HB-15	CD83	Negative
B7-2	CD86	Negative
Thy-1	CD90	Positive
Endoglin	CD105	Positive
MUC18	CD146	Positive
BST-1	CD157	Positive

*Data are from Pittenger et al. [9] and Azizi et al. [10], or are previously unreported communication.

Table 1. Phenotyping of MSCs.

2. Sources, isolation, and types of MSCs

Firstly, MSCs were found to be isolated from BM [9], adipose tissue [20], synovial tissue [21], lung tissue [22], umbilical cord blood [23], and peripheral blood [24] are heterogeneous, with variable growth potential, but all have similar surface markers and mesodermal differentiation potential [25]. Later, MSCs have also been isolated from nearly every tissue type of adult mice, suggesting the existence of such cells in almost postnatal organs [26].

The bone marrow (BM) is the major source of hematopoietic stem cells (HSCs), the precursors of red blood cells, platelets, monocytes, and granulocytes. MSCs found in the BM act as a support to the microenvironment termed the "hematopoietic niche" through which HSCs are housed. This microenvironment is necessary for development and differentiation of HSCs [27, 28]. Physiologically, MSCs do not migrate easily in the peripheral blood, and available protocols are not very successful in inducing the translocation of this cell pool from the BM to the periphery. Therefore, isolation and culture expansion of MSCs is usually necessary for therapeutic purposes.

3. Methods for isolation and purification of MSCs

3.1. Plastic adherence of MSCs

Plastic adherence of MSCs, as discussed before, is now the most adapted method of their isolation from more heterogonous cell population sample as bone marrow or mononuclear cell layer known as the buffy coat. The advantage of this isolation technique lies in its feasibility. The only limitation is the inability of selecting, thus culturing a named subpopulation of MSCs, and also it needs several passaging to purify more and more MSCs from non-MSCs in the cell culture. This procedure resulted in a heterogeneous population, which contains both single stem cell-like cells as well as progenitor cells with different linage commitment.

3.2. Magnetic-activated cell sorting

Knowing that cells could be selected by their markers, different mechanisms by which these cells can be sorted without affecting their viability, morphology, or function are developed. One of these mechanisms is the use of magnetic power for attracting these cells when labeled with antibodies conjugated to magnetic beads.

Magnetic beads are microscopic, synthetic beads provided with a core of magnetite or other magnetic material, and coated with a thin polymer-shell, are subjected to chemical modification, facilitating covalent protein attachment.

The magnetic particles used for labeling of the cells, are divided into micro and nanobeads. Microbeads range from 0.5 to 5 nm in diameter, while nanobeads range from 100 to 500 nm. Such beads are provided commercially, for example, as Dynal (microbeads 1–3 nm;

Invitrogen, Carlsbad, USA), MACS (nanobeads 20–100 nm; Miltenyi Biotec, Bergisch Gladbach, Germany), IMAG nanobeads 100–500 nm; BD Biosciences, San Jose, USA), EasySep (nanobeads about 150 nm; Stem Cell Technologies, Vancouver, Canada), or MagCellect beads (nanobeads about 150 nm; R&D Systems (Techne), McKinley Place NE, USA) [29–32].

In a classic practice, magnetic beads are added to the media in which the cells are cultured. They are then incubated for variable duration according to the given protocol. The magnetic beads then attach to cells mostly via antibodies but sometimes through other substances. The selection of the right biomarker for a given cell population guarantees the labeling of only desired cells. When these labeled cells are placed with the entire mixed-cell population into a biomagnetic separation system, the targeted cells are attracted by magnetic force to the tube wall or paramagnetic column, separating them from other cells in the culture.

3.2.1. Methods of cell labeling

Labeling of cells can be either direct or indirect. Direct labeling is when cells are labeled with antibodies that are readily conjugated to the magnetic beads. It is the fastest way of magnetic labeling as only one incubation step is required. Direct magnetic labeling requires a minimal number of washing steps and therefore minimizes cell loss.

While, indirect labeling is done in two-step procedure. Firstly, cells are labeled with a primary antibody directed against a cell surface marker. Secondly, the cells are magnetically labeled with magnetic beads, which either bind to the primary antibody or to a molecule that is conjugated to the primary antibody.

The primary antibody can either be unconjugated, biotinylated, or fluorochrome-conjugated. These antibodies will be further labeled with the magnetic beads that will be antiimmunoglobulin, antibiotin, or antifluorochrome beads, respectively.

3.2.2. Positive versus negative selection for cell separation

The selection can be positive by labeling the cells targeted for analysis or culture and thus the unlabeled cells are discarded. Alternatively, negative selection labels unwanted cells that are left in biomagnetic separation system and the unlabeled cells are extracted without them; it's also called cell depletion method. Comparison between the two methods is shown in **Table 2**.

In the context of magnetic cell separation technologies, two main methods are provided: the tube-based method and column-based separation method (**Figure 1**).

3.2.3. Methods of separation technology

3.2.3.1. Tubular cell separation method

Tubular cell separation is fully implemented in a single vessel. Magnetic beads are added to a cell-sample, which is incubated. Targeted cells are pulled into the tube wall toward the magnet when its power is applied, effectively separating cells with attached beads.

	Positive selection	Negative selection, cell depletion
Pros	Only one antibody is required that binds to the targeted cell marker (easy, cheap, fast)	No bound antibodies to the cells of interest
	High purity of sorted cells	Purification of cell population with unknown specific marker
		Combination with subsequent positive selection is possible
Cons	Potential interference with biological function of antibody-bound marker	Relatively impure
	Antigen expression must be unique to the cells of interest	Many antibodies necessary

Table 2. Comparison between the positive and the negative selection for magnetic cell sorting.

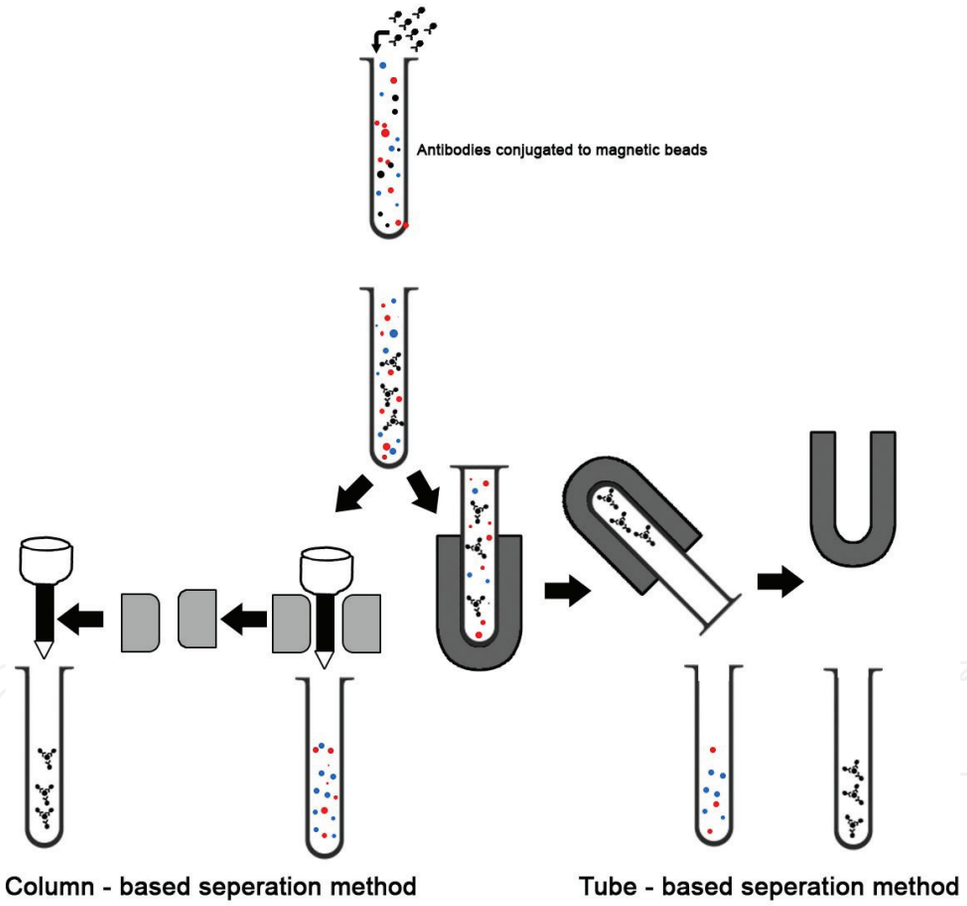

Figure 1. Tube-based magnetic separation method and column-based magnetic separation method.

3.2.3.2. Column-based separation method

Column is a vessel that contains an optimized matrix to generate a strong magnetic field when placed in a permanent magnet. Magnetic beads are added to a cell-sample, which is incubated.

Targeted cells are pulled into the surfaces of the magnetic spheres forming the matrix when the column is placed in the magnetic field.

The advantages and disadvantages of the two methods are shown in **Table 3**.

3.3. Fluorescence-activated cell sorting (FACS)

A significant improvement has been made since the initial commercialization of flow cytometry (FC) and fluorescence-activated cell sorting (FACS) in 1968. However, numerous points of weakness still exist, starting with the high cost and ending with the acceptance of the technology by many laboratories.

Flow cytometry is a widely used method for characterizing and defining different cell types in a heterogeneous cell population. It analyzes the expression of cell surface and intracellular molecules as well as the size and the shape of the cell. It also assesses the purity of isolated subpopulations.

In conventional laser flow cytometry, cells after passing through the flow cell will be treated as a waste. In fluorescence-activated cell sorting (FACS), the characteristics of the cells determined in the flow cell is the tool by which these cells will be further sorted into different paths in the equipment. Thus based on fluorescent labeling, FACS will separate a population of cells into subpopulations.

Sorting involves more complex mechanisms in the flow cytometer than a nonsorting analysis. Cells stained using fluorophore-conjugated antibodies can be separated from one another depending on which fluorophore they have been stained with.

Fluorescent dyes, or fluorochromes, are dyes that absorb light energy of a certain wavelength and reemit it at a longer wavelength. The main types of these dyes are; small dyes (e.g., fluorescein isothiocyanate/FITC and alexa dyes), protein dyes (phycoerythrin [PE] allophycocyanin

	Tubular cell separation method	**Column-based cell separation method**
Advantages	Eliminates undue cell stress that can be generated by column-based separation methods or from exposure to iron spheres forming the column matrix	Minimal cell labeling with nanosized beads is sufficient to isolate cells effectively due to the high surface area and the generated strong magnetic field
	Diminishing the risk of experimental procedures negatively impacting cell function and phenotype	Gain the benefits of minimal labeling; no nonspecific labeling and no cell activation
Disadvantages	Low gradient of magnetic force that is only applied to the tube wall	Exposure of the cells to undue stress due to the exposure to iron particles
	Massive labeling required that may lead to nonspecific labeling and/or cell activation	The high cost and waste of disposable columns that must be periodically changed after a limited number of cell separation runs

Table 3. Comparison between the tubular and column-based cell separation methods.

[APC] GFP), tandem dyes, where a protein dye collects laser light, transfers it to a small dye, and the tandem emits at the wavelength of the smaller dye (e.g., perCP, APC-Cy7), quantum dots, and polymer dyes (brilliant violet). All have advantages and disadvantages, but the protein and small molecule dyes have been the mostly used in flow cytometry.

The choice of fluorochromes to use in an experiment is based on the lasers and filters available on your flow cytometer or FACS, the relative richness of the targets—brighter fluorochromes should be used on less abundant molecules—and if any of the targets are intracellular. Intracellular targets need brighter dyes than that used for the cell surface. PE is typically the brightest, followed by APC, so they should be conjugated to antibodies to intracellular or low abundance targets.

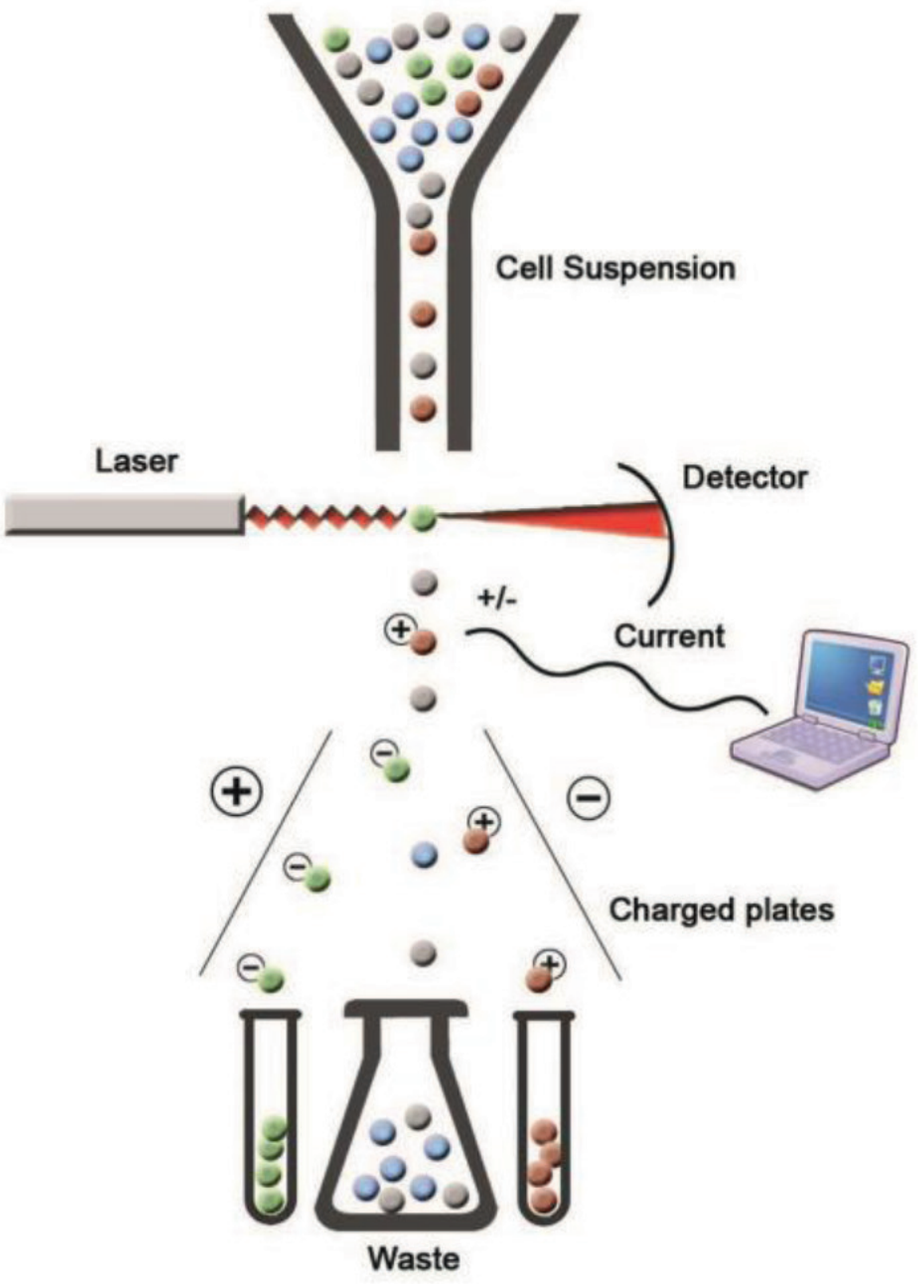

Figure 2. Principle of fluorescence-activated cell sorting (FACS).

Cells stained using fluorophore-conjugated antibodies are then taken into a column of pressurized sheath fluid, and as they emerge from the nozzle, they pass through one or more laser beams. At this point, the moment of analysis, the cytometer collects information about the fluorescence characteristics of the cell. After passing through the stream for the break-off distance, the stream is charged when the cell breaks off into a drop (moment of charging). Charged drops then pass through two high-voltage deflection plates and are deflected into collection vessels or aspirated to waste (**Figure 2**).

3.3.1. Points of weakness for FACS

Technical weakness could be like, the difficulty in detecting low abundance molecules in intracellular compartments, the great variability in cell permeabilizing chemistries, confounding effects from cell autofluorescence, overlap of emission spectra between used fluorochromes, and sometimes the unavailability of reagents for targeting molecules of interest.

Specifically for cell sorters, cell survival after pressure stress during droplet formation and collection, dilution of the sorted cells prior to reanalysis or culture, and the long duration it takes to obtain sufficient number of viable cells are considered to be some of the major problems. Lastly, data analysis is complicated, especially when dealing with low abundance targets.

4. Comparison between MACs and FACS cell sorting techniques

Although both methods are efficient, knowing their relative strengths and weaknesses can help make an informed choice on the technique used.

Each technique has "what it's best for" that gives it a privilege over the other. FACS is best in the following conditions: (1) when you want your sorted cell population to have a higher purity and recovery; (2) when sorting is based on an intracellular molecule (to which magnetic beads would not have access); FACS can sort cells labeled with fluorescent probes for intracellular targets; (3) when an information is needed about cell surface molecules, such as membrane protein receptors especially if these are of low density. It also can sort cells according to presence, absence, and density of the receptors.

On the other hand, sorting cells using magnetic beads is suitable for the separation of cells according to one separation criterion or characteristic, rather than several. It is also best as a method that classifies and sorts simultaneously and not sequentially as FACS separation. Magnetic beads separation is often used as a preparatory step prior to FACS.

MACs is a must use method when cells exhibit a high level of intrinsic cell fluorescence (auto fluorescence), which would disrupt the ability of a FACS instrument to detect signals.

Some aspects of comparison between the two techniques are shown in **Table 4**.

Fluorescence-activated cell sorting (FACS) can be combined with **magnetic-activated cell sorting** (MACS) if fluorescent magnetic microspheres are bound to the cells of interest. This added specificity can be useful in complicated sorts. Magnetic-activated cell sorting relies on

	MACs	FACS
Technology complexity	Low	High
Purity	Intermediate (90–98%)	High (98%)
Specificity	High	High
Negative selection	Possible (low purity)	Possible
Positive selection	Possible	Possible
Multimarker selection	Very limited	Possible
Risk for bacterial contamination	Low	Intermediate
Sorting for distinct expression levels	Not possible	Possible
Sorting of cells with intracellular fluorescence (e.g., eGFP)	Not possible	Possible
Simultaneous sorting of different populations	Very limited and not simultaneous	Possible

Table 4. Comparison between the MACs and FACS techniques for cell sorting.

the introduction of an external magnetic field to control the movement of magnetic-particle-bound cells in a cell lysate. Typically, the magnetic field traps the cells of interest on the sides or bottom of a tube while the unwanted solution and contaminants are washed away. While MACS alone is less expensive than FACS, it is unable to provide information about individual cells and cannot isolate one cell at a time. Therefore, **it is beneficial to use a combination of MACS and FACS**. Magnetic-activated cell sorting is used to obtain the purest sample possible before sending it through the FACS machine. This means that the sample must be incubated with magnetic particles and fluorophores. To save time, one can purchase fluorescent magnetic particles. These particles allow MACS and FACS to be performed sequentially with only one incubation period.

5. Sorting specific MSC subsets before culture

As per the definition of MSCs mentioned in the beginning of this chapter, all the criteria mentioned perfectly define MSC only in culture; however, how to identify these cells *in vivo* is still unrecognized. This, mainly due to their minimal existence among other cell populations *in vivo*, forms only 0.001–0.01% of cells in the BM as described by Pittenger et al. [9]. Also, undifferentiated cells with no specific phenotype make them rather more complicated. Many investigators directed their efforts to find markers for the identification of these cells, which help their purification through specific selection, rather than the adherence based purification method.

CD271 (LNGFR) has been described as one of the most specific markers for the purification of human BM-MSCs [33, 34]. CD271, also known as low-affinity nerve growth factor receptor (LNGFR), nerve growth factor receptor (NGFR), or p75NTR (neurotrophin receptor), belongs to the tumor necrosis factor superfamily [35], yet it would not be considered as a universal marker to identify MSC before culture, as it is not adequate in the isolation of MSC from some tissues such as umbilical cord or umbilical cord blood.

6. Conclusion

MSCs express several cell markers that differ according to the cells source, also, these markers could be lost or changed with further culturing of these cells. Thus, the immunologic isolation of all MSC subsets may be a difficult thing to do. Accordingly, the adherence to plastic in standard culture conditions is still the gold standard method for MSC isolation and purification. Yet their characterization before use is a must, either by using these cell markers or more expedient by proving their multilinage differentiation capability.

Abbreviations

MSCs	Mesenchymal stem cells
MACS	Magnetic-activated cell sorting
FACS	Fluorescence-activated cell sorting
CD	Cluster of differentiation
ECM	Extracellular matrix

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