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## IMPORTANCE OF CALIBRATION IN FLOW CYTOMETRY ANALYSIS FOR HUMAN ADIPOSE TISSUE - DERIVED STEM CELL CHARACTERIZATION

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**ABSTRACT:** Human adipose tissue-derived stem cells (hADSCs) are promising for cellular therapy as they are multipotent, which consistently express high CD90, CD105, CD73 and low hematopoietic surface markers. Flow cytometry is a routine technology that is used in most laboratories and can be used to study hADSC characterization, but the instrument needs strict calibration to assure the quality of the results. However, for rarely used fluorophores, calibration is often neglected. This study aimed to compare calibrated and non-calibrated fluorescence (FL) in flow cytometry performances. hADSCs were isolated from lipoaspirate and cultured in human platelet lysate supplemented medium. Passaged cells were used for characterization. Cells stained with positive (CD90-FITC, CD105-PerCP Cy5.5, CD73-APC) and negative (CD34, CD45, CD11b, CD19, HLA-DR)-PE marker cocktails were subjected to flow cytometry analysis. In the instrument, FITC, PE, PerCP- Cy5.5, and APC were detected by FL1, FL2, FL3, and FL4 respectively. Routine calibration of FL1, FL2, and FL3 was done, except for FL4. Therefore, data from calibrated and non-calibrated FL4 were analyzed. hADSCs strongly and consistently expressed CD90, CD105, and negative surface markers at averages of 94.6%, 90.5%, and 2.8% respectively, hence in line with ISCT guidelines; in contrast to CD73 (FL4), whose average was 42%. Characterization of calibrated FL4 showed very good results on CD73 (above 94%), but not in non calibrated FL4, which showed inconsistent results, as values ranged between 0% - 82%. In conclusion, routine calibration of FL1, FL2, FL3, and FL4 is important to get a reliable result.

**INTRODUCTION:** Human adipose tissue-derived stem cells (hADSCs) are very promising in regenerative medicine and cellular therapy.<sup>1</sup>

They have multi-potential capacity to differentiate into osteogenic, chondrogenic, and adipogenic lineages, and consistently highly express positive stromal surface markers, *i.e.* CD90, CD105, CD73 ( $\geq 95\%$ ) and show low expression of hematopoietic surface markers ( $\leq 2\%$ ), which is in accordance with International Society and Cell Therapy (ISCT) guidelines.<sup>2</sup> However, according to data that was collected by Investigational New Drug Applications – (INDs) for Mesenchymal Stem Cell-Based product

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to FDA,<sup>3</sup> from 66 studies, the average values of positive surface marker expressions were  $88 \pm 7.54\%$ ,  $87 \pm 7.17\%$ , and  $86 \pm 7.24\%$  for CD105, CD90, and CD73 respectively. Moreover, from nine studies, the average value of negative surface markers was  $9 \pm 6.56\%$ .<sup>3</sup>

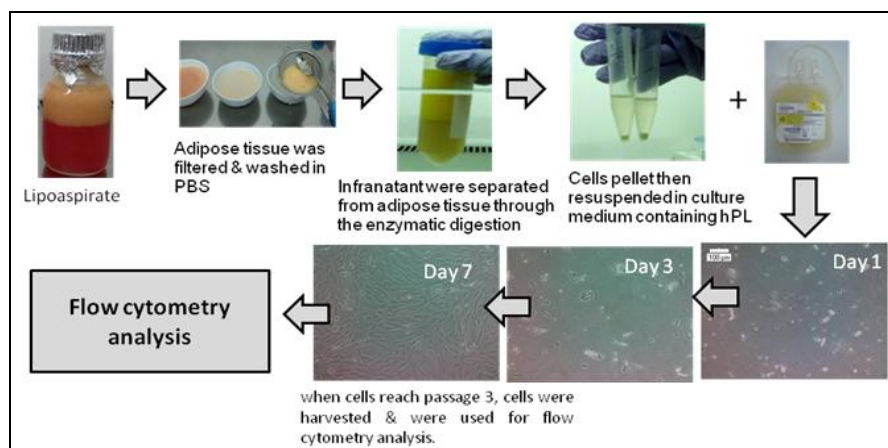
As cellular therapy products (CTPs) for regenerative medicine, the quality of the product should be controlled. Therefore, it is necessary to characterize the product including identification of phenotype markers.<sup>4</sup> Identification of surface markers can be done using immunocytochemistry or flow cytometry.<sup>5</sup>

Flow cytometry is one of the powerful tools to identify the surface marker phenotype of hADSCs. Our previous study used flow cytometry to identify surface markers of hADSCs.<sup>6</sup> However, operation of flow cytometry should follow quality control procedures to maintain high performance of the instrument. Calibration is one of the steps of

quality control procedures and strictly needs to be performed to assure the quality of the results.<sup>7</sup> However, for rarely used fluorophores, calibration was often neglected. Therefore, this study aimed to compare calibrated and non-calibrated flow cytometry performances.

**MATERIALS AND METHODS:** This is a descriptive study that compared calibrated and non-calibrated flow cytometry performances, that was done in July 2015 through July 2016 in the Department of Biology for isolation and culture of hADSCs, and in Integrated Laboratory, Faculty of Medicine Universitas Indonesia for flow cytometry analysis.

**Materials:** hADSCs were isolated from lipoaspirate of 3 donors (Ps-1, Ps-2, and Ps-3) as described previously,<sup>8</sup> and cultured in human platelet lysate supplemented medium (**Fig. 1**). Passaged cells were used for characterization.



**FIG. 1: hADSCs ISOLATION STEPS AND DAY-1, DAY-3, AND DAY-7 OF hADSCs CULTURES WITH 100X MAGNIFICATION, SCALE BAR: 100µM.**

**Processing of samples for flow cytometry:** When cells reach 80% confluent, cells were detached and cell pellets were re-suspended and washed in sterile PBS (Life technologies, USA) at a minimum concentration of 100.000 cells/tube. Appropriately labeled antibody against MSC markers were added according to the manufacturer's instructions (BD Stem flow human mesenchymal stem cell [hMSC] detection kit, Cat. 562245, Beckton Dickinson, USA). Monoclonal antibodies against CD73-APC, CD90-FITC, CD105-PECy5.5 as positive cocktail, and CD34-PE, CD-45PE, CD11b-PE, CD19-PE, HLA-DR PE as negative cocktail were used.

**Flow cytometry analysis:** Flow cytometry analyses were assessed using FACS Calibur Flow cytometer (Beckton Dickinson, USA). Each analysis was performed on 10.000 cells (10.000 gated events). Unstained cells were used to determine gating of cell population of interest and to eliminate debris. Positive and negative isotype controls were used to determine the base line. Ps-1 were analyzed at July 2015 (FL4 calibrated), while Ps-2 and Ps-3 were analyzed at November 2015 and April 2016 (non-calibrated FL4), respectively. FL1, FL2, and FL3 was frequently calibrated and was done using Calibrite beads 3-color kit that contained three different colors of BD calibrate

beads, *i.e.* FITC-labeled, PE-labeled, and Per CP-labeled beads, and unlabeled beads. APC-labeled beads, which was available separately was rarely used and calibration was often neglected.

Further, data from calibrated and non-calibrated FL4 were collected and tabulated. In other well cultures, prolonged hADSCs culture was also done until 21 days as was described in a previous method,<sup>6</sup> with the same medium and same condition with cell culture that was used for flow cytometry to show spontaneous chondrogenic differentiation capacity.

**RESULTS:** We found that hADSCs strongly and consistently expressed CD90 and CD105 at averages of 94.6% and 90.5% respectively. Moreover, hADSCs expressed hematopoietic surface marker only 2.8% (**Table 1**). These results

were in line with ISCT guidelines and INDS – FDA.<sup>2, 3</sup> However, the expression of CD73, which was conjugated with APC and detected by FL4 channel showed an average of only 42%. Characterization of hADSCs in calibrated FL4 showed very good results of CD73 that was above 94% (in July 2015), but not in non-calibrated FL4 (in November 2015 and April 2016), which showed inconsistent results, as values ranged in between 0% - 82% (SD= 42) (**Table 1**). When we do flow cytometry in November 2015, CD73 in all tubes were not detected (0%) and in April 2016 the CD73 expressions were inconsistent. We also found that hADSCs underwent spontaneous chondrogenic differentiation (**Fig. 2**), which was confirmed by alcian blue staining, which proved differentiation capacity of our cells.

**TABLE 1: hADSCs SURFACE MARKER EXPRESSION**

Sample	Calibrated/Non-Calibrated FL4	Group	Tube Number	CD90	CD73	CD105	Neg (CD34, CD45, CD11b, CD19, HLA-DR)	Dates
Ps-1	FL4 Calibrated	1	1	99.8	97.1	97.1	0.2	Jul-15
Ps-1	FL4 Calibrated	1	2	99.8	97.0	96.2	0.9	Jul-15
Ps-1	FL4 Calibrated	1	3	99.7	94.9	94.8	0.9	Jul-15
Ps-1	FL4 Calibrated	1	4	99.7	95.3	91.0	1.6	Jul-15
Ps-2	FL4 Non-Calibrated	2	5	96.5	0.34	96.8	4.8	Nov-15
Ps-2	FL4 Non-Calibrated	2	6	94.9	0.15	97.2	3.9	Nov-15
Ps-2	FL4 Non-Calibrated	2	7	96.6	0.08	97.8	3.0	Nov-15
Ps-2	FL4 Non-Calibrated	2	8	94.8	0.28	96.7	2.1	Nov-15
Ps-2	FL4 Non-Calibrated	2	9	95.2	0.33	96.6	2.5	Nov-15
Ps-2	FL4 Non-Calibrated	2	10	95.8	0.43	96.4	3.0	Nov-15
Ps-3	FL4 Non-Calibrated	2	11	79.8	82.2	81.7	1.1	Apr-16
Ps-3	FL4 Non-Calibrated	2	12	93.5	68.7	84.3	5.4	Apr-16
Ps-3	FL4 Non-Calibrated	2	13	95.56	46.02	84.38	4.03	Apr-16
Ps-3	FL4 Non-Calibrated	2	14	92.73	54.79	82.7	3.21	Apr-16
Ps-3	FL4 Non-Calibrated	2	15	93.42	34.1	80.72	4.41	Apr-16
Ps-3	FL4 Non-Calibrated	2	16	86.3	0.91	73.6	3.6	Apr-16
			Mean	94.6	42.0	90.5	2.8	
			STDEV	5.22	42.0	7.9	1.5	



**FIG. 2: hADSCs (Ps-1, Ps-2, Ps-3) UNDERGO SPONTANEOUS CHONDROGENIC DIFFERENTIATION, CONFIRMED BY ALCIAN BLUE STAINING. MAGNIFICATION 100X, SCALE BAR: 100µM**

**DISCUSSIONS:** Positive surface markers CD90, CD73, and CD105 are the most phenotype markers that are often used for hADSCs characterization.<sup>2-4</sup> CD90 or Thy-1 is a 25-30 kDa glycosylphosphatidylinositol (GPI)-linked membran glycoprotein that has been indicated to have a role in cell-cell and cell-matrix interactions as well as cell motility in various kind of cells including MSCs,<sup>9</sup> neural stem cells,<sup>10</sup> and is present in neural crest derived MSCs.<sup>11</sup> CD90 has been considered as a marker that is implicated in the hMSC inhibitory ability on lymphoproliferative response to phytohemagglutinin (PHA).<sup>12</sup> Moreover, CD90 is also found on LX2 cells that is similar to MSC as they are capable of adipogenic differentiation.<sup>13</sup> CD105 or endoglin has been found in MSCs and endothelial progenitor cells, and is involved in various biological processes including angiogenesis and neovascularization.<sup>14-17</sup>

CD105 has also been suggested in osteogenesis enhancement due to a reduction in TGF- $\beta$ 1/smad2 signaling.<sup>18</sup> CD73 has a regulatory function in controlling adipocyte, chondrogenic and osteogenic differentiation of MSCs.<sup>18, 19</sup> Moreover, CD 105 was regulated by cytokines, growth factors, as well as culture conditions *e.g.* hypoxia.<sup>18</sup> CD73 has an important role on mesenchymal stromal cell differentiation through adenosine receptor signaling.<sup>18</sup>

Although CD73 is known to be down-regulated during osteogenic, adipocyte, and chondrogenic differentiation,<sup>18</sup> until recently, there are no information whether CD73 acts as a transient or definite marker. Although CD90, CD105, and CD73 are not tissue specific markers and the expression are slightly reduced during differentiation,<sup>18, 19</sup> the combination is often used as defining criteria for MSCs.<sup>2-4</sup>

In our study, hADSCs underwent spontaneous chondrogenic differentiation for Ps-1, Ps-2, and Ps-3 (Figure 2). CD73 should be strongly expressed on early hADSCs culture (chondrogenic progenitor cell) and slightly declined during differentiation.<sup>18</sup> CD73 is also regulated by cytokines and growth factors such as TGF- $\beta$ . In our experiment, we used outdated human platelet lysate (outdated hPL) as FBS substitutes in culture medium, and outdated hPL was shown to be rich in growth factors.

Outdated hPL showed highest concentration of TGF- $\beta$ 1 compared to other growth factors,<sup>20</sup> and TGF- $\beta$ 1 level was not influences by freeze-thaw cycles.<sup>21</sup> This fact might influence the expression of CD73 on Ps-3, which had low expression (below 80%). However, according to Mendicino *et al.*,<sup>3</sup> from 16 studies, MSCs express CD73 at a minimum range of 88%. Moreover, Delorme *et al.*<sup>13</sup> stated that CD73 might be decreased on MSCs, but should not dropped severely or not expressed at all. We also found that spontaneous chondrogenic differentiation of hADSCs in culture (Ps-3) still expressed CD73 over 80% in calibrated and well maintained flow cytometer instrument (data not shown).

The BD Calibrite beads 3-color kit contains unlabeled beads, FITC-labeled, PE-labeled, and PerCP-labeled beads,<sup>22</sup> which are frequently used. However, APC-labeled beads, which are available separately, are rarely used. According to Adan and colleagues,<sup>23</sup> there are three basic procedures that affect the reliability and reproducibility of data. The first procedure includes examination of efficiency and performance of the laser tube, optical filters, log and linear amplifiers, and photomultipliers (PMT), which should be done once every 6 months. Daily calibration of the optical alignment is also included. Second procedure is frequent monitoring of instrument performance to identify both immediate and potential problems. Third procedure is calibrating the flow cytometer using a series of microbeads with at least four different predefined fluorescence (FL) intensities, plus non-fluorescent beads.

The third procedure can be performed at large intervals. Daily calibration practice is not a must, but the instrument must be quality controlled daily following calibration.<sup>24</sup> Moreover, over time calibration of FL channel must be performed since FL intensity is used to distinguish “positive” and “negative” cells in immunopheno-typing assays to assure the stability and reproducibility of FL intensities.<sup>7</sup> Moreover, if the instrument is changed or serviced, it is advisable to recalibrate.<sup>24, 25</sup>

**CONCLUSION:** Routine calibration of FL1, FL2, FL3, and FL4, or the use of labeled beads in 4-colors plus non-fluorescent beads is important to get a reliable result in flow cytometry analysis.

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**CONFLICT OF INTEREST:** None declared.

## REFERENCES:

- Pawitan JA: Future research in adipose stem cell engineering. In: Yves-Gerard Illouz, Aris Sterodimas, editors. Adipose stem cells and Regenerative Medicine. Heidelberg, Springer 2011: 257-272.
- Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8(4): 315-317.
- Mendicino M, Bailey AM, Wonnacott K, Puri RK, and Bauer SR: MSC-based product characterization for clinical trials: An FDA Perspective. *Cell Stem Cell* 2014; 14(2): 141-145.
- Bravery CA, Carmen J, Fong T, Oprea W, Hoogendoorn KH, Woda J, et al: Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy* 2013; 15(1): 9-19.
- Pawitan J: Role of visualization in stem cell characterization. *International Journal of Pharm Tech Research* 2016; 9(8): 214-220.
- Pawitan JA, Suryani D, Wulandari D, Damayanti L, Liem IK, and Purwoko RY: Prolonged culture in FBS and FBS-substitute containing media: Spontaneous chondrogenic differentiation of adipose tissue derived mesenchymal stem cells. *International Journal of Pharm Tech Research* 2014; 6(1):224-235.
- Kraan J, Gratama JW, Keeney M, and d'Hautcourt JL: Setting up and calibration of a flow cytometer for multicolor immunophenotyping. *J Biol Regul Homeost Agents* 2003; 17(3): 223-233.
- Pawitan JA, Liem IK, Suryani D, Bustami A, and Purwoko RY: Simple lipoaspirate washing using a coffee filter. *Asian Biomedicine* 2013; 7(3): 333-338.
- Jósvay K, Winter Z, Katona RL, Pecze L, Marton A, Buhala A, et al: Besides neuro-imaging, the Thy1-YFP mouse could serve for visualizing experimental tumours, inflammation and wound-healing. *Sci Rep.* 2014; 4:6776.
- Alic I, Kosi N, Kapuralin K, Gorup D, Gajovic S, Pochet R, et al: Neural stem cells from mouse strain Thy1 YFP-16 are a valuable tool to monitor and evaluate neuronal differentiation and morphology. *Neurosci Lett.* 2016; 634: 32-41.
- Yasui T, Mabuchi Y, Morikawa S, Onizawa K, Akazawa C, Nakagawa T, et al: Isolation of dental pulp stem cells with high osteogenic potential. *Inflammation and Regeneration* 2017; 37:8.
- Campioni D, Rizzo R, Stignani M, Melchiorri L, Ferrari L, Moretti S, et al: A Decreased Positivity for CD90 on Human Mesenchymal Stromal Cells (MSCs) Is Associated with a loss of immunosuppressive activity by MSCs. *Cytometry Part B* 2009; 76B: 225-230.
- Castilho-Fernandes A, de Almeida DC, Fontes AM, Melo FU, Picanço-Castro V, Freitas MC, et al. Human hepatic stellate cell line (LX-2) exhibits characteristics of bone marrow-derived mesenchymal stem cells. *Exp Mol Pathol.* 2011; 91(3):664-672.
- Duff SE, Li, Garland JM, and Kumar S: CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003; 17(9): 984-992.
- Levi B, Wan DC, Glotzbach JP, Hyun J, Januszyc M, Montoro D, et al: CD105 Protein depletion enhances human adipose-derived stromal cell osteogenesis through reduction of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) Signaling. *The Journal of Biological Chemistry* 2011; 286(45): 39497-39509.
- Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, et al: Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. *J Biol Chem* 2011; 286(34): 30034-30046.
- Shi X, Zhang W, Yin L, Chilian WM, Krieger J, and Zhang P: Vascular precursor cells in tissue injury repair. *Transl Res.* 2017; 184:77-100.
- Ode A, Schoon J, Kurtz A, Gaetjen M, Ode JE, Geissler S, et al: CD73/5'-ecto-nucleotidase acts as a regulatory factor in osteo-/chondrogenic differentiation of mechanically stimulated mesenchymal stromal cells. *Eur Cell Mater* 2013; 25: 37-47.
- Scarfì S: Purinergic receptors and nucleotide processing ectoenzymes: Their roles in regulating mesenchymal stem cell functions. *World J Stem Cells* 2014; 6(2): 153-162.
- Sari P, Luviah E, Nugraha Y, Pawitan JA, Lilianty J, and Purwoko RY: Various Growth Factor Yields In Various Platelet Rich Plasma Processing Methods. *Biotechnology: An Indian Journal* 2014; 9(11): 475-478.
- Nugraha Y, Sari P, Purwoko RY, Luviah E, and Pawitan JA: Effect of Lysed Platelet Count in Platelet Concentrates on Various Growth Factor Levels after Freeze Thaw Cycles. *Int J PharmTech Res* 2014; 6(7): 2036-2042.
- Dickinson B: Calibrate beads Instruction manual. USA: Beckton Dickinson: 2014. [Cited 2015 Oct 15]. Available from: <http://www.bdbiosciences.com>.
- Adan A, Alizada G, Kiraz Y, Baran Y, and Nalbant A: Flow cytometry: basic principles and applications. *Critical Reviews in Biotechnology* 2017; 37(2):163-176.
- Schmutz S, Valente M, Cumano A, and Novault S: Spectral Cytometry Has Unique Properties Allowing Multicolor Analysis of Cell Suspensions Isolated from Solid Tissues. *PLoS One.* 2016; 11(8): e0159961.
- NN: What is a flow cytometry laser and how flow cytometry optics function. *Excyte Expert Cytometry* 2016. [Cited 2017 June 9]. Available from: <https://expertcytometry.com/what-is-a-flow-cytometry-laser-how-flow-cytometry-optics-function/>

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