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Isolation and expansion of muscle precursor cells from human skeletal muscle biopsies

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Running head: Isolation of human muscle precursor cells

ABSTRACT

One of the major issues concerning human skeletal muscle progenitor cells is represented by the efficient isolation and *in vitro* expansion of cells retaining the ability to proliferate, migrate and differentiate once transplanted. Here we describe a method i) effective in obtaining human muscle precursor cells both from fresh and frozen biopsies coming from different muscles, ii) selective to yield cells uniformly positive for CD56 and negative for CD34 without FACS sorting, iii) reliable in maintaining proliferative and *in vitro* differentiative capacity up to passage 10.

Key words: human muscle precursor cells, skeletal muscle biopsy, CD56, enzymatic digestion, human muscle cell primary culture.

1. INTRODUCTION

Two main approaches are currently employed for the obtainment of myogenic precursor cells, namely single fiber isolation, firstly described by Rosenblatt and colleagues [1] and applied also for human samples [2], and whole muscle enzymatic digestion with different adaptations [3,4]. The first method allows to get a virtually pure population of activate satellite cells but is a quite complex procedure, starting from the muscle collection which has to provide myofibers preserved in their length, whereas the second one is more simple and fast but yields a mixed cell population and usually need further passages such as preplating or fluorescence-activated cell sorting [5,6]. In terms of surface antigen expression, different proteins have been investigate to better define the precursor cells in human muscle, however so far the literature is quite unanimous in identifying the cells restricted to a myogenic fate in the $CD56^{+}CD34^{-}$ population [7,8].

Here we described a protocol to efficiently isolate and expand in culture human muscle precursor cells from different skeletal muscles through a whole muscle digestion method without the need of further cell processing. This is possible following the combination of (i) careful biopsy collection and cleaning, (ii) double enzymatic digestion, (iii) defined medium composition and appropriate

plastic ware. This method allows obtaining homogeneous $CD56^+CD34^-$ cell population that maintain proliferative and *in vitro* differentiative capacity up to passage 10. Moreover, although other improvements mainly regarding culture conditions are needed in the perspective of a future possible clinical application, we defined a freezing procedure that does not affect cell isolation and characteristics.

2. MATERIALS

2.1 Culture media and plastic ware

1. Prepare the Collagenase I 0.2% (w/v) solution: weigh 200 mg of lyophilized Collagenase from Clostridium histolyticum for general use, Type I (Sigma Aldrich) and resuspend with 2 mL of DMEM 1X 1 g/L glucose (+) L-Glutamine (+) Pyruvate (GIBCO, Thermo Fisher Scientific), then add 98 mL of DMEM. Filter the solution through a 0.22 µm filter and stock single-use aliquots at -20°C.
2. Trypsin 0.05 % with EDTA and Phenol Red (GIBCO, Thermo Fisher Scientific).
3. Red blood cell lysis buffer: Auto Lyse Plus (Biosource).
4. Washing medium: DMEM 1X 1 g/L glucose (+) L-Glutamine (+) Pyruvate containing 20% fetal bovine serum (FBS) (GIBCO, Thermo Fisher Scientific) and 1% Penicillin Streptomicin (pen/strep) (GIBCO, Thermo Fisher Scientific).
5. Proliferation medium: DMEM 1X 1 g/L glucose (+) L-Glutamine (+) Pyruvate containing 20% FBS, 10^{-6} M dexamethasone (Sigma Aldrich), 10 ng/mL recombinant mouse FGF basic protein (R&D Systems), 10 µg/mL insulin (Insuman Rapid 100UI/mL, Sanofi Aventis) and 1% pen/strep.
6. Differentiation medium: MEM Alpha (1X), (+) L-Glutamine, (+) Ribonucleosides, (+) Deoxyribonucleosides, (-) Ascorbic Acid (GIBCO, Thermo Fisher Scientific) containing 2% horse serum (GIBCO, Thermo Fisher Scientific), 10 µg/mL insulin and 1% pen/strep.

7. Freezing medium: 70% FBS, 10% DMEM 1X 4.5 g/L glucose (+) L-Glutamine (+) Pyruvate (GIBCO, Thermo Fisher Scientific), 20% dimethyl sulfoxide.
8. Non-Tissue Culture Treated (NTC) Plate, polystyrene, flat bottom: 96-well, 24-well and 6-well plates (Falcon, Corning). Use these plates for cell expansion and proliferating cell characterization.
9. Non-Tissue Culture (NTC) polystyrene Petri dishes, 100 x 20 mm and 150 x 25 mm (Falcon, Corning). Use these plates for cell expansion.
10. Tissue Culture Treated (TC) Plate, polystyrene, flat bottom: 24-well plates (Falcon, Corning). Use these plates for myogenic differentiation.

2.2 Flow cytometry analysis

1. Tubes or other supports suitable for your cytometer.
2. Antibodies anti-human antigens: CD34-FITC clone 581, CD56-PE clone B159, 7-aminoactinomycin D (7AAD) (all from BD Pharmigen). Isotype controls: mouse IgG1-FITC (IOTest Immunotech), PE mouse anti-human Ig k (BD Pharmigen).

2.3 Immunofluorescence analysis

1. Routine reagents: 4% paraformaldehyde (PFA) for fixation, 0.5% Triton-X100 in phosphate-buffered saline (PBS) for permeabilization, 10% horse serum in PBS for saturation, 1% bovine serum albumin (BSA) in PBS for antibodies dilution, 4',6-diamidino-2-phenylindole (DAPI) fluorescence mounting medium for nuclei counterstaining.
2. Proliferating cell characterization. Primary antibodies: monoclonal rabbit IgG anti-human Ki-67 clone SP6 (Novusbio), monoclonal mouse IgG_1 anti-human PAX7 clone PAX7 (R&D systems), polyclonal rabbit IgG anti-human MYOD clone M318 (Santa Cruz Biotechnology), polyclonal rabbit IgG anti-human MYF5 clone C20 (Santa Cruz Biotechnology). Secondary antibodies: chicken anti-rabbit IgG (H+L) Alexa Fluor® 594 conjugate (Thermo Fisher Scientific), goat anti-mouse IgG (H+L) Alexa Fluor® 594 conjugate (Thermo Fisher Scientific).

3. Differentiated cell characterization. Primary antibody: monoclonal mouse IgG_{2B} anti-human Myosin Heavy Chain clone MF20 (R&D systems). Secondary Antibody: goat anti-mouse IgG (H+L) Alexa Fluor® 594 conjugate (Thermo Fisher Scientific).

3. METHODS

3.1 Cell isolation

1. Carry the muscle biopsy (*see* **Note 1**, **Fig. 1** and **Fig. 2**) in sterile saline from operating theatre to the laboratory cell processing room (*see* **Note 2**).
2. Transfer the sample on a Petri dish using disposable sterile plastic pincers and quickly rinse it with Betadine® or similar products and PBS.
3. Transfer the sample in a clean Petri dish and carefully remove visible vasculature, connective tissue and potential adipose tissue (*see* **Note 3**).
4. Divide the muscle in pieces weighting up to 500 mg (*see* **Note 4**) and treat every piece separately.
5. Mince the sample in very small pieces (1-2 mm^3) using two disposable sterile scalpels.
6. Cover minced sample with 3 mL of 0.2% (w/v) Collagenase I in a well of 6-well plate and incubate for 90 min at 37°C.
7. Transfer the digestion product in a 15 mL conical centrifuge tube, add 10 mL of washing medium and homogenate with serological pipette, then centrifuge at 300 x *g* for 10 min.
8. Take out the supernatant and resuspend the pellet with 2 mL of 0.05 % Trypsin-EDTA, transfer in a well of 6-well plate and incubate for 60 min at 37°C.
9. Transfer the digestion product in a 15 mL conical centrifuge tube, add 10 mL of washing medium and homogenate with serological pipette, then filter twice the cell suspension first through 70 µm and then 40 µm cell strainers. Centrifuge at 300 x *g* for 10 min.
10. Resuspend the pellet in 3 mL red blood cell lysis buffer for 5 min, add 10 mL of washing medium and centrifuge at 300 x *g* for 10 min.

11. Resuspend the pellet in 2 mL of proliferation medium and seed cells in a well of 6-well NTC plate.
12. Refresh proliferation medium after 48-72 hours (*see* **Note 5**). Usually attached cells appear after 5-7 days from enzymatic digestion (*see* **Note 6**).

3.2 Sample freezing

1. Transfer minced sample obtained at **step 5 of section 3.1** in a 2 mL cryovial and add 1 mL of freezing medium.
2. Proceed with slow cooling protocol as for cell samples and store in liquid nitrogen (*see* **Note 7**).

3.3 Sample thawing

1. Take out sample from liquid nitrogen and thaw in water bath at 37°C.
2. Transfer sample in a 15 mL conical centrifuge tube, add 10 mL of washing medium and centrifuge at 300 x *g* for 10 min.
3. Proceed with **step 6 of section 3.1**.

3.4 Cell expansion

1. Take out the medium from the well or dish and wash twice with PBS. Add the appropriate volume of 0.05 % Trypsin-EDTA (300 µL for wells of 6-well plate, 1 mL for 100 x 20 mm dishes, 2 mL for 150 x 25 mm dishes) and incubate for 5-10 min at 37°C.
2. Add washing medium, collect detached cells and centrifuge at 300 x *g* for 10 min. Remove the supernatant, resuspend cell pellet in proliferation medium and seed cells on a suitable NTC well or dish. Usually around 1 x 10^5 cells in a 100 x 20 mm dish and 2.5 x 10^5 cells in a 150 x 25 mm dish are seeded. We recommend never exceeding the 60-70% of confluence. In our experience cells maintain unaltered characteristics up to passage 10.

3.5 Cell characterization

1. Doubling time analysis (Fig. 3A): seed 1 x 10^4 cells for each well of 24-well NTC plate in proliferation medium, after 48 h detach cells by trypsin treatment and proceed with

counting. Consider at least three wells separately. Calculate the doubling time (g) using the following equation $g = 48\ h \times [\log 2 / \log (N_{48h}/N_0)]$ in which g is the generation (or doubling) time during the logarithmic phase of the growth curve, N_{48h} is the cell number at 48 h after seeding and N_0 is the cell number at time 0 [9].

2. Flow cytometry analysis (Fig. 3B): detach cells by trypsin treatment and proceed with incubation using antibodies described in the **Materials 2.2 section**. Usually we observe more than 90% of CD56 positive cells and no cells positive for CD34.
3. Immunofluorescence analysis of proliferating cells (Fig. 3C-D): seed 5×10^3 cells for each well of 96-well NTC plate in proliferation medium, after 48 h take out the culture medium, wash twice with PBS and fix cells by incubation with 4% PFA at 4°C for 10 min. Proceed with the immunostaining protocol using the antibodies described in the **Materials 2.3 section paragraph 2**.
4. Myogenic differentiation and myogenic index evaluation (Fig. 3E): seed 1.5×10^4 cells for each well of 24-well TC plate in proliferation medium and let cells proliferate for 48 h, then take out the medium and add differentiation medium. After 48 h refresh differentiation medium and after other 48 h take out the medium, wash twice with PBS and fix cells by incubation with 4% PFA at 4°C for 10 min. Proceed with the immunostaining protocol using the antibodies described in the **Materials 2.3 section paragraph 3**. Calculate the myogenic index defined as the number of nuclei residing in cells containing three or more nuclei divided by the total number of nuclei [10]. Consider at least 5 fields at 20X magnification for each sample.

4. NOTES

1. We experienced efficient isolation and expansion of muscle precursor cells starting from biopsies obtained from different adult skeletal muscle listed in Fig. 1. We also applied our

method to pediatric abdominal muscle biopsies and derived muscle precursor cells with similar features to those observed in cells of adult origin (described in Fig. 3).

2. If it is not possible to process the biopsy immediately after surgery it can be maintained in sterile saline at 4°C not longer than 24 hours.
3. We considered biopsies from adult healthy subjects aged from 25 to 60 years undergoing scheduled orthopedic operations or surgery after trauma, with muscle being virtually free of ectopic adipose tissue deposition that is instead present during aging or in some pathological condition [11].
4. For samples weighting less than 200 mg adjust reagent volumes and plastic ware as following:

   - 1.5 mL of 0.2% Collagenase I in a well of 24-well plate (step 6 of section 3.1)
   - 1 mL of 0.05% Trypsin-EDTA in a well of 24-well plate (step 8 of section 3.1)
   - avoid incubation with red blood cells lysis buffer (step 10 of section 3.1)
   - seed cells in 1 mL of proliferation medium in a well of 24-well NTC plate (step 11 of section 3.1)

   In case of small samples attached cells could appear slightly later (7-10 days).
5. Despite of washing and filtering procedures it is possible to still observe cell and fiber debris at this stage, we thus recommend taking out medium and washing cells with PBS or washing medium before adding fresh proliferation medium.
6. If attached cells appear before 5 days, it is possible that fibroblasts overcame the proliferation of myogenic cells. This may occur despite the careful removal of connective tissue from biopsies and an accurate muscle sample collection during surgery can help in avoiding this inconvenience. Although the large and granular appearance of fibroblasts is easily distinguishable from the elongated and thin shape of myogenic cells, we recommend performing flow cytometry analysis at earlier passage as possible to evaluate the percentage of CD56 positive cells.

7. In our experience there are not differences between freshly processed and frozen/thawed biopsies, apart a possible delay in the appearance of attached cells.
8. Both adult and pediatric biopsies were taken after approval by local ethical committee and informed consent acceptance by patients (Prot N. 2682P and 3030P, Azienda Ospedaliera di Padova).

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FIGURE LEGENDS

Fig. 1 Muscle sources. Biopsies were obtained from different skeletal muscles, as highlighted in pink in the illustration; the numbers of samples analyzed for each muscle are listed in the table.

Fig. 2 Method outline. After washing and removal of non-muscle tissue (1) samples are directly subject to enzymatic digestion (2.1) or frozen for later use (2.2). Isolated cells are cultured for several passages (3) and characterized in terms of surface antigen expression, proliferation, myogenic marker expression and myogenic index (4).

Fig. 3 Cell characterization. A) Representative image of cell appearance in culture at phase contrast microscope (scale bar = 100μm) and bar graph of doubling time calculated at different passages. **B)** Representative dot-plot of expanded cells (passage 5) analyzed for the expression of CD56 and CD34 and bar graph of CD56 expression at different passages. **C)** Representative pictures of Ki67 and myogenic markers expression (passage 5) (scale bar = 100μm). **D)** Percentage of cells positive for Ki67 and myogenic markers at different passages (at least 5 fields for each independent experiment). **E)** Representative picture of myosin heavy chain staining (passage 5)

(scale bar = 100μm) and myogenic index calculation at different passages (at least 5 fields for each independent experiment).

REFERENCES

1. Rosenblatt JD, Lunt AI, Parry DJ, Partridge TA (1995) Culturing satellite cells from living single muscle fiber explants. In Vitro Cell Dev Biol Anim 31 (10):773-779. doi:10.1007/BF02634119

2. De Coppi P, Milan G, Scarda A, Boldrin L, Centobene C, Piccoli M, Pozzobon M, Pilon C, Pagano C, Gamba P, Vettor R (2006) Rosiglitazone modifies the adipogenic potential of human muscle satellite cells. Diabetologia 49 (8):1962-1973. doi:10.1007/s00125-006-0304-6

3. Agley CC, Rowlerson AM, Velloso CP, Lazarus NR, Harridge SD (2013) Human skeletal muscle fibroblasts, but not myogenic cells, readily undergo adipogenic differentiation. J Cell Sci 126 (Pt 24):5610-5625. doi:jcs.132563 [pii]

10.1242/jcs.132563

4. Gaster M, Kristensen SR, Beck-Nielsen H, Schroder HD (2001) A cellular model system of differentiated human myotubes. APMIS 109 (11):735-744

5. Jankowski RJ, Haluszczak C, Trucco M, Huard J (2001) Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. Hum Gene Ther 12 (6):619-628. doi:10.1089/104303401300057306

6. Xu X, Wilschut KJ, Kouklis G, Tian H, Hesse R, Garland C, Sbitany H, Hansen S, Seth R, Knott PD, Hoffman WY, Pomerantz JH (2015) Human Satellite Cell Transplantation and Regeneration from Diverse Skeletal Muscles. Stem Cell Reports 5 (3):419-434. doi:S2213-6711(15)00222-2 [pii]

10.1016/j.stemcr.2015.07.016

7. Bareja A, Holt JA, Luo G, Chang C, Lin J, Hinken AC, Freudenberg JM, Kraus WE, Evans WJ, Billin AN (2014) Human and mouse skeletal muscle stem cells: convergent and divergent mechanisms of myogenesis. PLoS One 9 (2):e90398. doi:10.1371/journal.pone.0090398

PONE-D-13-42782 [pii]

8. Pisani DF, Dechesne CA, Sacconi S, Delplace S, Belmonte N, Cochet O, Clement N, Wdziekonski B, Villageois AP, Butori C, Bagnis C, Di Santo JP, Kurzenne JY, Desnuelle C, Dani C (2010) Isolation of a highly myogenic CD34-negative subset of human skeletal muscle cells free of adipogenic potential. Stem Cells 28 (4):753-764. doi:10.1002/stem.317

9. Scarda A, Franzin C, Milan G, Sanna M, Dal Pra C, Pagano C, Boldrin L, Piccoli M, Trevellin E, Granzotto M, Gamba P, Federspil G, De Coppi P, Vettor R (2010) Increased adipogenic conversion of muscle satellite cells in obese Zucker rats. Int J Obes (Lond) 34 (8):1319-1327. doi:ijo201047 [pii]

10.1038/ijo.2010.47

10. van der Velden JL, Schols AM, Willems J, Kelders MC, Langen RC (2008) Glycogen synthase kinase 3 suppresses myogenic differentiation through negative regulation of NFATc3. J Biol Chem 283 (1):358-366. doi:M707812200 [pii]

10.1074/jbc.M707812200

11. Vettor R, Milan G, Franzin C, Sanna M, De Coppi P, Rizzuto R, Federspil G (2009) The origin of intermuscular adipose tissue and its pathophysiological implications. Am J Physiol Endocrinol Metab 297 (5):E987-998. doi:00229.2009 [pii]

10.1152/ajpendo.00229.2009

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Muscle type	Biopsies Number
Biceps	2
Deltoid	3
Extensor forearm	2
Flexor carpi	2
Flexor longus pollicis	1
Flexor hallucis longus	3
Peroneus	14
Pronator teres	2
Quadriceps rectus femoralis	3
Sovraspinatus muscle	3
Tibialis	4
Triceps	1
Vastus lateralis	2

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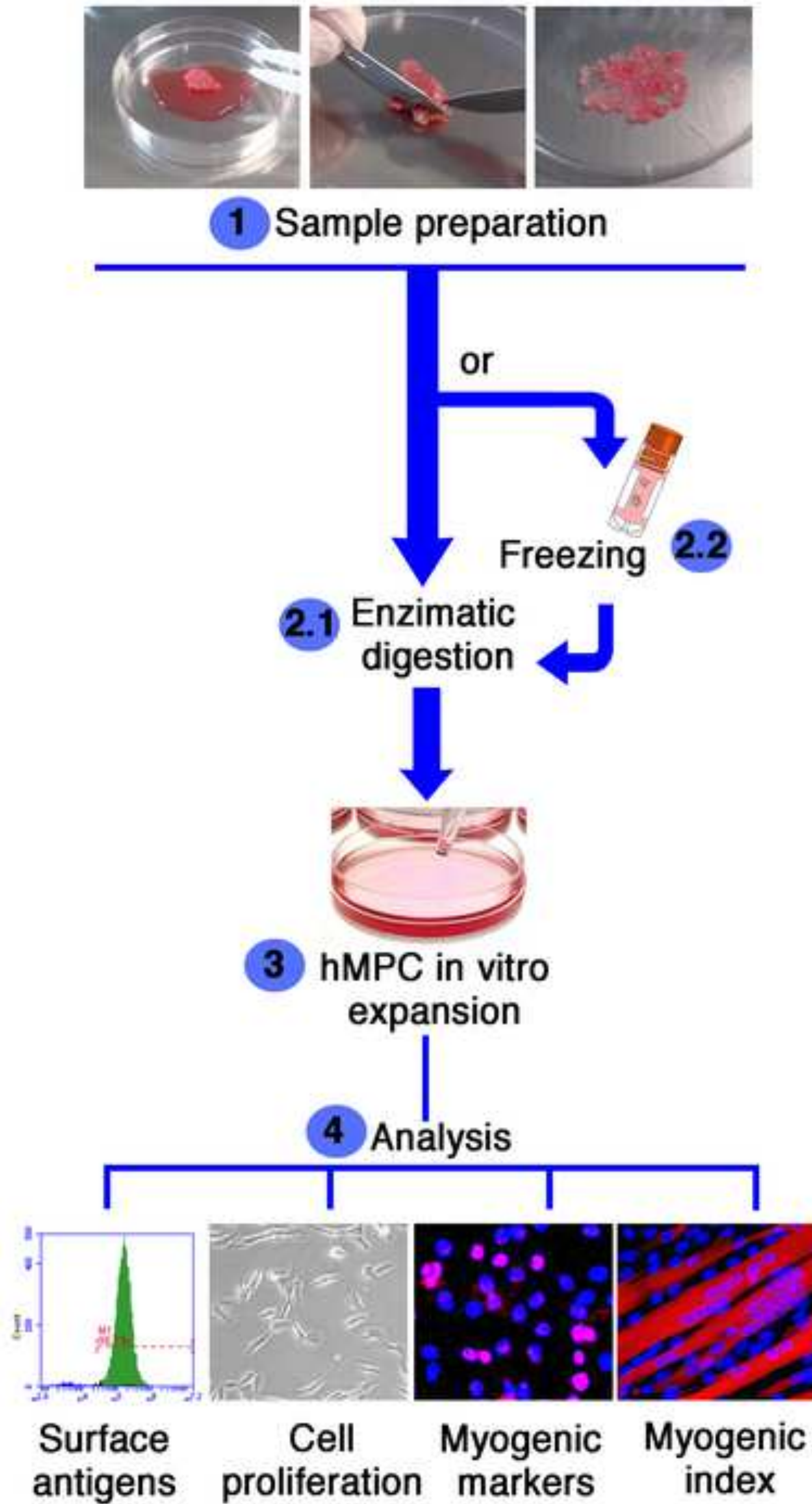

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