Methods for isolation and evaluation of Muse cells

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Be sure to read the following comments because these are important issues influencing the Muse cell yield.

1) Materials:
1-1) Commercial culture cells as a source for Muse cells
1-2) Reagents, instruments and equipment

2) Cell culture:
2-1) Culture medium
2-2) Serum lot check
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2-5) Cryopreservation of the cells for making stocks

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3-5) Staining and washing with secondary antibodies

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6) Bulk production of Muse cell-clusters using methylcellulose gel
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9) Evaluation of pluripotency of Muse cell-clusters: Gelatin culture of clusters
[Flowchart of Muse cell collection]

1. Preparation of cell suspensions

   ![Flowchart of Muse cell collection](image)

   - Adhesion culture
   - Cell suspension

2. Muse cell staining with anti-SSEA-3 antibodies (primary antibodies)

   - Anti-SSEA-3 (Rat, IgM)
   - SSEA-3 → Anti-SSEA3
   - Staining of cell suspensions
   - Figure: Staining image of SSEA-3 antigens

3. Staining with FITC-labeled anti Rat IgM (secondary antibodies)

   - FITC-anti-Rat IgM
   - SSEA-3 + Anti-SSEA3
   - FITC-anti-Rat IgM
   - Staining of cell suspensions
   - Figure: Staining image of secondary antibodies

4. Analysis and isolation of Muse cells by FACS

   - Laser
   - Polarizer
   - Isolated Muse cells

   - Mixture of cells and buffers
   - Laser irradiation, droplet formation and electric charge
   - Collection of targeted cells

   - Analysis of Muse cells with anti-SSEA-3 antibodies + labeled secondary antibodies
1) Materials

1-1) Commercial culture cells as a source for Muse cells (cells used in our laboratory)

- Human Bone Marrow Mesenchymal Stem Cells; BM-MSCs (Cat.#PT-2501, Lonza)
- Normal Human Dermal Fibroblasts-adult skin; NHDF (Cat.#CC-2511, Lonza)
- Human Dermal Fibroblasts-adult; HDFa (Cat.#2320, Lonza)
- Human Adipose Derived Stem Cells; ADSCs (Cat.#PT-5006, Lonza)

1-2) Reagents, instruments and equipment

- [Important] Human-FGF-2, premium grade (used for culture of BM-MSCs, Cat.#130-093-840, Miltenyi)
- [Important] Rat anti-SSEA-3 antibody (Cat.#330302, BioLegend or Cat#MA1-020, Thermo)
- Rat IgM Isotype control (Cat.#400801, BioLegend)
- Goat anti-Rat IgM antibody (FITC-labeled) (Cat.#112095-075, Jackson ImmunoResearch)
- HyClone-FBS (Fetal bovine serum) (use for cell culture, Cat.#SH30910.03, GE Healthcare)

  Be sure to read "Lot check for FBS" in the next section.

- FBS (Fetal bovine serum) (use to inactivate trypsin, no manufacturer specified)
- [Important] Low-glucose DMEM+GlutaMAX (Cat.#10567, Thermo) → Use to culture BM-MSCs, NHDF and HDFa
- [Important] High-glucose DMEM+GlutaMAX (Cat.#11965, Thermo) → Use to culture ADSCs
- Kanamycin (100 X) (Use at 1X in media, Cat.#15160-054, Thermo)
- PBS (10 X) (Cat.#27575-31, Nacalai tesque)
- Sterile water (1X, use for PBS preparation) (Cat.#06442-95, Nacalai tesque)
- Trypsin (0.25 %)/EDTA (Cat.#25200-072, Thermo)
- FluoroBrite DMEM (Cat.#A18967-01, Thermo)
- BSA (Bovine serum albumin) (Cat.#01860-65, Nacalai tesque)
- EDTA (Cat.#15111-45, Nacalai tesque)
- Gelatin (Cat.#G-1890, Sigma)
- Poly-HEMA [poly(2-hydroxyethyl methacrylate)] (Cat.#P3932, Sigma)
- MethoCult H4100 (Cat.#04100, StemCell Technologies)
- Cellbanker 1 plus, cryopreservation solution for culture cells (Cat.#CB021, ZENOAQ)
- CryoTube vials (Cat.#377267, Thermo)
- BiCell, CryoTube container for deep freezing (see picture)
- [Recommended] 10-cm dish (Cat#150464, Thermo)
- 1.5-mL tube (Cat#BM-15, BMBio)
- 15-mL tube (Cat#352096, Corning)
- 50-mL tube (Cat#352070, Corning)
- Cell strainer (40 μm) (Cat#352346, Corning)
- 0.22 μm filter (Cat#SLGV033RS, Merck Millipore)
- Centrifuge (15 mL, 50 mL) (swing rotor, no manufacturer specified)
- Centrifuge (1.5 mL) (swing rotor with cooler, no manufacturer specified)
- Cell sorter (BD FACS Aria II) (used in our laboratory)
- FACS analysis software (BD FACSDiva) (used in our laboratory)
2) Cell culture

2-1) Culture medium

- BM-MSCs: Low-glucose DMEM, 10% FBS, 1 ng/ml FGF-2, 0.1 mg/ml Kanamycin
- NHDF, HDFa: Low-glucose DMEM, 10% FBS, 0.1 mg/ml Kanamycin
- ADSCs: High-glucose DMEM, 15% FBS, 0.1 mg/ml Kanamycin

**ATTENTION!** Use low-glucose DMEM or high-glucose DMEM depending on the cell type. For cultures of BM-MSCs, NHDF and HDFa, always use 10% FBS (HyClone) / low-glucose DMEM (Gibco), and not high-glucose DMEM. Use of high-glucose DMEM induces hypoproliferative capacity and decreases Muse cell yield. On the other hand, for culture of ADSCs, use 15% FBS (HyClone) / high-glucose DMEM (Gibco).

**ATTENTION!** Use FGF-2 (bFGF) for culture of bone marrow-derived mesenchymal stem cells. For culture of BM-MSCs, add FGF-2 (concentration: 1 ng/mL, Cat#130-093-840, Miltenyi). Be aware that Muse cell yields change markedly using products from several manufacturers (refer to the following figure). For the latest information, contact Prof. Dezawa (mdezawa-med.tohoku.ac.jp) because the SSEA-3-positive rate depends on the lots from some manufacturers. Please convert “*” into “@”.

Figure. Differences in SSEA-3-positive cell rates with FGF-2 from different manufacturers (as of 2017)

**Differences in SSEA-3 positive cell rates with FGF-2 by manufacturer (BM-MSCs)**

- Rat anti-SSEA-3 monoclonal antibody (MC-631) (BioLegend, Cat#330302, 0.5 μg/100 μl)
- Isotype control: Purified Rat IgM, κ Isotype Control (BioLegend, Cat#400801, 0.5 μg/100 μl)
- Human bone marrow-derived mesenchymal stem cell (Passage 7)

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>FGF-2 Concentration</th>
<th>SSEA-3 Positive Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miltenyi</td>
<td>1 ng/mL</td>
<td>3.3%</td>
</tr>
<tr>
<td>A</td>
<td>1 ng/mL</td>
<td>2.5%</td>
</tr>
<tr>
<td>B</td>
<td>1 ng/mL</td>
<td>1.3%</td>
</tr>
</tbody>
</table>

FITC
**ATTENTION!** These are examples of cell sources that can be used to reproduce our data. Other mesenchymal cells may also work as a source for Muse cells, but the outcome cannot be guaranteed unless the above cell types are used.

**ATTENTION!** When purchasing cells, culture media specialized for the cells should also be bought from the same company to maintain the cells according to the manufacturer’s instructions.

**ATTENTION!** NHDF, HDFa, BM-MSCs, ADSCs have a number of different lots. Because the cell growth rate and Muse cell ratio may differ among lots, we recommend purchasing a couple of lots and then selecting the best lot for the experiment.

2-2) Serum lot check
- Mesenchymal cells are used for the lot check. Expanded mesenchymal cells are collected by trypsin incubation.
- Count the number of cells and suspend cells at a concentration of $1\times10^4$ cells/500 μl in 10% FBS (for lot check) in DMEM (+Kanamycin) and plate them individually in a 24-well plate.
- Incubate cells at 37°C, 5% CO$_2$.
- Exchange medium the next day. Culture medium is exchanged every 2 to 3 days.
- When cells reach 90% confluence, they should be subcultured. Cells should be expanded to 1:2. Never exceed 1:3.
- Incubate cells at 37°C, 5% CO$_2$.
- Cells should be subcultured at least 2 to 3 times in the same lot of FBS before evaluation. Do not use the first culture to check the FBS lot because the effect of the serum from the past medium may not be eliminated.
- The quality of the FBS lot will be determined based on cell growth, morphology, and spontaneous formation of specific cell clusters (i.e., Muse cell clusters).
- If possible, several FBS concentrations should be evaluated (from 5% to 20%) and cell growth and morphology should be compared among lots and concentrations to confirm the best FBS.
- For the lot check, it is desirable to obtain several lots of FBS from several companies (a total of ~20 lots).
- **The FBS does NOT need to be inactivated.**
2-3) Thawing frozen cells

**ATTENTION!** Carefully read the manufacturer’s instructions prior to thawing purchased cells. Purchased cells usually arrive frozen in a vial. Transfer the vial into liquid N₂ as soon as it arrives, and keep it there until thawing the cells.

- Wash hands, and clean hands using 70% EtOH. Use sterilized gloves if necessary.
- Set a water bath to 37°C.
- Clean the clean bench using 70% EtOH.
- Remove the frozen vial from the liquid N₂, and quickly transfer to the 37°C water bath to thaw the cells. Take care not to touch the vial cap to the water, otherwise the cells will be easily contaminated by bacteria.

**ATTENTION!** To avoid cell death, remove the vial from the water bath before it is completely thawed. The best time to remove the vial from the water bath is the point at which the solution still contains a piece of ice.

- Clean the vial using 70% EtOH, and then bring it to the clean bench.
- Carefully open the cap, and melt the piece of ice with gentle pipetting.
- Transfer cells to a 15-ml Falcon tube.
- Slowly add culture medium to the tube to a final volume of 10 ml.
- Centrifuge the 15-ml tube at 300 g for 5 min.
- Remove the supernatant, loosen the cell pellet with gentle pipetting, and add 10 ml culture medium.

**ATTENTION!** The cells should be plated uniformly.

- Incubate the cells overnight at 37°C, 5% CO₂.
- Remove the supernatant, loosen the cell pellet with gentle pipetting, and add 10 ml culture medium the next day. If several floating dead cells are visible, wash a couple of times with culture media to remove the dead cells.

**ATTENTION!** In the case of cells purchased from a company, thawing the frozen cells and culturing for the first time is counted as passage 1 (P=1) in this protocol.

**ATTENTION!** The basic principle is to start culturing the thawed cells at the same scale as just before cryopreservation of the cells. For example, if one 10 cm-dish that was 90% confluent was cryopreserved into one tube, the cells after thawing should be plated in one 10 cm-dish using 10 ml culture medium and maintained.
**ATTENTION!** If the cells were cryopreserved at P=4 and then thawed, the cells are counted as P=4 at this point and passage numbering is continued (*i.e.*, P=5, P=6, ...

☐ When the cells reach 90% confluence, subculture them as described in 2-4.
2-4) Subculture of mesenchymal cells (example of a 10-cm dish scale culture)

**ATTENTION!** When the cells reach 90% confluence, subculture the cells to allow for expansion. NHDFs, HDFa, BM-MSCs, and ADSCs are not immortalized cells but are derived from primary culture. In such cases, cell growth will be suppressed by contact inhibition when they reach 100% confluence.

Best timing for subculture (~90% confluent; some spaces are remaining)

Bad timing for subculture (over confluent; cells are already piling up)

NHDF
HDFa

BM-MSCs
Each time the cells reach 90% confluence, begin the next subculture. Remove the medium, wash the cells by adding and removing 10 ml serum-free DMEM.

Add 2 ml trypsin per 10-cm dish. Rotate the dish to distribute the trypsin uniformly, and then incubate at 37°C in 5% CO₂ for 5 min.

After 5 min incubation, check under a phase contrast microscope to determine whether the cells have detached from the dish.

**ATTENTION!** If all the cells are not detached, incubate for 5 more minutes at 37°C in 5% CO₂ or add 1 ml trypsin and incubate for a couple of minutes. If these treatments do not work, the trypsin itself might be inactivated. In this case, fresh trypsin should be used.

If cell detachment is confirmed, add 1 ml serum to inactivate the trypsin reaction.

Gently pipette the cells and solution to dissociate the cells using a P-1000 with a blue tip.

**ATTENTION!** The P-200 yellow tip is **NOT** appropriate because the opening is not wide enough and the cells will be damaged.

Prepare a new 50-ml tube, and transfer the cells and reagents. Add 7 ml serum-free DMEM to the dish to collect the remaining cells, and transfer the cells and reagents to the 50-ml tube.

Centrifuge the 50-ml tube at 300 g for 5 min.

Discard the supernatant, and loosen the cell pellet by gentle pipetting.

Add 20 ml culture medium to the cells and plate into two 10-cm dishes.

**ATTENTION!** The culture medium is the specified medium provided by the company for P=2, and is 10% FBS in DMEM (+Kanamycin) beginning with P=3.

**ATTENTION!** Always subculture the mesenchymal cells at a ratio at 2:1. Never expand cells to 3:1 or more. Otherwise, cell growth or Muse cell ratio will decrease. In case of a 10-cm dish, for example, expand one 10-cm dish to two 10-cm dishes.

Incubate the cells overnight at 37°C, 5% CO₂. Exchange the medium the next day.

Cells are maintained by exchanging medium every 2 to 3 days.

**ATTENTION!** Cells can be kept expanded and then used directly for Muse cell collection. We usually use P=4-P=10 for collecting Muse cells and analysis, while cells at P=11 or greater are not used for experiments. When cultured cells are not to be used in the near-term, we strongly recommend that the cells be stocked in liquid N₂ rather than kept in subculture for a long time.
2-5) Cryopreservation of the cells for making stocks

At P=3 or P=4, we usually dispense the cells and make a stock by cryopreservation

- When the cells reach 90% confluence, begin the next subculture. Remove the medium, and wash the cells by adding and removing 10 ml serum-free DMEM.
- Add 2 ml trypsin per 10-cm dish. Rotate the dish to uniformly distribute the trypsin, and then incubate at 37°C, 5% CO₂ for 5 min.
- After 5 min incubation, check under a phase contrast microscope to examine whether the cells have detached from the dish.
- Once cell detachment is confirmed, add 1 ml serum to inactivate the trypsin reaction.
- Gently pipette the cells and solution to disassociate cells using a P-1000 with a blue tip.
- Prepare a new 15-ml tube, and transfer the cells and reagents. Add 7 ml serum free-DMEM to the dish to collect the remaining cells, and transfer the cells and reagents to the 15-ml tube.
- Centrifuge the 15-ml tube at 300 g for 5 min.
- Discard the supernatant, and loosen the cell pellet by gentle pipetting.
- Add Cellbanker 1plus (1mL) to the cells, and gently mix by pipetting.

**ATTENTION!** The correct volume of Cellbanker 1plus is 1 ml per one 10-cm dish when the cells are 90% confluent.

- Transfer the 1 ml Cellbanker 1plus + cells to a cryotube and screw the lid to form a seal.
- Place the tube into the BiCell and gradually freeze at -80°C for 24 h.
- After freezing at -80°C for 24 h, transfer the tube into liquid N₂ and store the cells.

**ATTENTION!** The cells can be stored at -80°C for a short time without losing activity, but for a longer storage period, the cells must be stored in liquid N₂.
3) Procedures for immunostaining of Muse cells

**ATTENTION!** After collecting Muse cells, prepare poly-HEMA-coated plates in advance of clustering. For details, refer to section [Poly-HEMA coating of wells and dishes].

3-1) Preparation of secondary antibodies
- Dissolve commercial FITC-labeled anti-rat IgM antibodies at 1.0 mg/mL in sterile water, dispense, and store at −30°C before use.

3-2) Preparation of FACS buffer
- Prepare FACS buffer just before use and cool on ice constantly after preparation. Do not keep remainder.

**FACS buffer**

- 5% BSA: 5 mL
- 100 mM EDTA: 1 mL
- PBS or FluoroBrite DMEM: 44 mL
- Total: 50 mL/50-mL tube

**ATTENTION!** 5% BSA solution: dissolve in PBS or FluoroBrite DMEM, sterilize with 0.22 µm filter and store at 4°C. 100 mM EDTA solution: dissolve in PBS or FluoroBrite DMEM, sterilize with 0.22 µm filter and store at 4°C. If cell viability is decreased by PBS, it is recommended to use FluoroBrite DMEM.

3-3) Preparation of cell suspensions

**ATTENTION!** In contrast to the subculture methods described above, the cells must reach 100% confluence before subjecting them FACS analysis to stably obtain Muse cells.

Best timing for FACS (100 % confluent; locus almost starting to be piling up is seen)

![NHDF](image.png)

**ATTENTION!** For FACS analysis, cells must be completely dissociated. Because mesenchymal cells are sticky cells and the cells are at 100% confluent before trypsin incubation, incubation with trypsin should be longer than the usual subculture to obtain completely dissociated cells.
**ATTENTION!** When obtaining Muse cells from frozen mesenchymal cells, the cells must be subcultured at least once before being subjected to FACS analysis. Cells after thawing and plating are still weak and unstable and have a lower ratio of SSEA-3+ Muse cells. After thawing, the cells should be cultured to reach 90% confluence, then subcultured once until they reach 100% confluence, and then subjected to FACS analysis.

**ATTENTION!** When primary cultured cells are to be analyzed by FACS, the cells should be P=4 to P=10. If the cells are earlier than P=3, the Muse cells will be weak, and if later than P=10, the Muse cells will be present in a lower ratio or have lower differentiation activity.

- Use cells cultured in a 10-cm dish (the following procedures are for a 10-cm dish × 1).
- Remove culture supernatant in a dish and wash and remove the cell surface with media not containing PBS or serum.
- Add trypsin (0.25%)/EDTA (2 mL) and heat the culture in an incubator at 37°C until cells are detached (5-10 min).
- Stop trypsin reaction by adding FBS (1 mL) and transfer cell suspensions into a 15-mL tube.
- Collect remaining cells in the dish with serum free-DMEM (7 mL) and transfer them into the same 15-mL tube (total: 10 mL).
- Centrifuge (400 g, 5 min, room temperature)

**ATTENTION!** Use centrifuge with swing rotor.
- Remove supernatant and resuspend cell pellets in PBS or FluoroBrite DMEM (total: 10 mL).
- Centrifuge (400 g, 5 min, room temperature)
- Remove supernatant, resuspend cell pellets with FACS buffer (1 mL), and sample a part of the cells for counting.
- Add FACS buffer (9 mL) (total: 10 mL).
- Centrifuge (400 g, 5 min, room temperature) Count the number of cells during the above processes.
- Remove supernatant and resuspend cell pellets in FACS buffer to establish cell density of 1×10^6 cells/100 µL.

**ATTENTION!** Be sure that cell density does not exceed 1×10^6 cells/100 µL, since this results in cell aggregation. The upper limit of a cell suspension stainable in a 1.5-mL tube is 1×10^7 cells/1000 µL. If the number of cells exceeds the upper limit, use more tubes.

### Table 3-1. Dispensing and cell number examples

<table>
<thead>
<tr>
<th>#</th>
<th>Sample</th>
<th>Dispensing volume</th>
<th>Number of cells</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Without staining</td>
<td>~20 µL</td>
<td>~2×10^5 cells</td>
<td>Up to 100 µL with FACS buffer</td>
</tr>
<tr>
<td>2</td>
<td>Secondary antibodies only (anti-Rat IgM, FITC)</td>
<td>~20 µL</td>
<td>~2×10^5 cells</td>
<td>Up to 100 µL with FACS buffer</td>
</tr>
<tr>
<td>3</td>
<td>Rat IgM Isotype Control + Secondary antibodies only</td>
<td>100 µL</td>
<td>1×10^6 cells</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Rat anti-SSEA-3 + Secondary antibodies only</td>
<td>100 µL</td>
<td>1×10^6 cells</td>
<td>–</td>
</tr>
</tbody>
</table>

3-4) Staining and washing with primary antibodies
ATTENTION! Be aware that anti-SSEA-3 antibodies produced by several manufacturers do not work for collecting Muse cells. Recommended manufacturers of anti-SSEA-3 (anti-stage-specific embryonic antigen-3, clone MC-631, Rat IgM) antibodies are BioLegend (Cat#330302) or Thermo (Cat#MA1-020). Anti-SSEA-3 antibodies of clone MC-631 are available from several other manufacturers; however, be sure they can be used because Muse cells may not be stained (refer to the following figure). For the latest information, contact Prof. Dezawa (mdezawa@med.tohoku.ac.jp) because the SSEA-3-positive rate markedly depends on the lots from some manufacturers. Please convert “*” into “@”.

Figure. Differences in anti-SSEA-3 antibody staining by manufacturer (as of 2017)

Rat anti-SSEA-3 monoclonal antibody (MC-631) (0.5 µg/100 µl)
Isotype control: Purified Rat IgM, κ Isotype Control (BioLegend, Cat#400801, 0.5 µg/100 µl)
Human bone marrow-derived mesenchymal stem cell (Passage 7)

Antibodies from D company had a high positive rate, but had increased background in negative populations, and are not recommended.

ATTENTION! When using cells that are not used in our laboratory, perform blocking of FcR. When using cells except BM-MSCs, ADSCs, NHDFs and HDFs, perform blocking with 10% normal human serum at 4°C for 20 min before staining with primary antibodies.

ATTENTION! Prepare samples of Isotype control for accurate estimation of the rate of SSEA-3-positive cells in FACS. Our laboratory uses Rat IgM Isotype Control (Cat#400801, BioLegend) and evaluates the SSEA-3-positive cell rate based on the gating of Isotype Control in FACS analysis.

- Transfer Samples #1 to 4 on ice (samples are left on ice until immediately before analysis).
- Add rat IgM isotype control at a concentration of 0.5 µg/100 µL in Sample #3, stir slowly with a pipette, and incubate on ice for 1 h (stir slowly every 10 min with a pipette).
- Add manufacturer-recommended rat anti-SSEA-3 antibodies at a concentration of 0.5 µg/100 µL in Sample, stir slowly with a pipette, and incubate on ice for 1 h (stir slowly every 10 min with a pipette).
**ATTENTION!** After adding antibodies, stir slowly with a pipette to ensure a thorough reaction. For the video on pipetting, refer to → [http://www.stemcells.med.tohoku.ac.jp/protocol/movie/suspension_good.mp4](http://www.stemcells.med.tohoku.ac.jp/protocol/movie/suspension_good.mp4)

When adding antibodies (both primary and secondary) to cell suspensions for reaction, stir slowly with a pipette. Specifically, stir until cells settling at the bottom refloat. For many suspensions (700-1000 µL/tube), stirring in a slow rolling manner is effective. However, do not agitate or use strong vortexing because this may damage cells and result in cell death.

### Table 3-2 Primary antibody list

<table>
<thead>
<tr>
<th>#</th>
<th>Sample</th>
<th>Rat IgM Isotype Control</th>
<th>Rat anti-SSEA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Without staining</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Secondary antibodies only</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Rat IgM Isotype Control + Secondary antibodies</td>
<td>+ (0.5 µg/100 µL)</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Rat anti-SSEA-3 + Secondary antibodies</td>
<td>–</td>
<td>+ (0.5 µg/100 µL)</td>
</tr>
</tbody>
</table>

- Start washing 1 h after. Add FACS buffer (1 mL in total) to a 1.5-mL tube.
- Centrifuge (400 g, 5 min, 4°C)

**ATTENTION!** For 1.5-mL tubes, use a centrifuge with a swing rotor.

- Remove supernatant until the 0.1-mL line of the 1.5-mL tube (FACS buffer of 0.1 mL remains).
- After resuspending pellet with slow stirring with a pipette, add FACS buffer (0.9 mL).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube.
- After resuspending pellet with slow stirring with a pipette, add FACS buffer (0.9 mL).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube.
- After resuspending pellet with slow stirring with a pipette, add FACS buffer (0.9 mL).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube.
- Resuspend the pellet with slow stirring using a pipette.

### 3-5) Staining and washing with secondary antibodies

**ATTENTION!** Nonspecific staining may occur due to impurities in antibody solutions. To prevent such conditions, centrifuge secondary antibodies immediately before use (10,000 g, 3 min, 4°C) to precipitate impurities, and use supernatants only to eliminate impurities. Store remaining secondary antibodies at 4°C. Expiration date is within 2 weeks. Our laboratory uses anti-rat IgM antibody, FITC conjugates (Cat#112-095-075, Jackson Immunoresearch); however, anti-rat IgM antibody, APC conjugates (Cat#112-136-075) are also usable and available from this manufacturer.

- Thaw on ice prepared secondary antibodies (FITC-labeled anti-rat IgM antibodies) that were stored at −30°C.
- Homogenize thawed secondary antibody solutions with a pipette, centrifuge (10,000 g, 3 min, 4°C), and use supernatants for cell staining. Store remaining secondary antibodies at 4°C. Expiration date is within 2 weeks
- Add secondary antibodies at a concentration of 1 µg/100 µL in Samples #2, 3 and 4, stir slowly with a pipette and incubate on ice for 1 h (stir slowly every 10 min with a pipette)
### Table 3-3 Secondary antibody list

<table>
<thead>
<tr>
<th>#</th>
<th>Sample</th>
<th>FITC-labeled anti-rat IgM antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Without staining</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Secondary antibodies only</td>
<td>+ (1 µg/100 µL)</td>
</tr>
<tr>
<td>3</td>
<td>Rat IgM Isotype Control + Secondary antibodies</td>
<td>+ (1 µg/100 µL)</td>
</tr>
<tr>
<td>4</td>
<td>Rat anti-SSEA-3 + Secondary antibodies</td>
<td>+ (1 µg/100 µL)</td>
</tr>
</tbody>
</table>

- Start washing 1 h after. Add FACS buffer (1 mL in total) to a 1.5-mL tube.
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube (FACS buffer of 0.1 mL remains).
- After resuspending the pellet with slow stirring with a pipette, add FACS buffer (0.9 mL).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube (FACS buffer of 0.1 mL remains).
- After resuspending the pellet with slow stirring with a pipette, add FACS buffer (0.9 mL).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube.
- Resuspend the pellet in FACS buffer and prepare for analyses.

**Option:** If the number of cells is large (≥1×10^7 cells) and aggregates are detected in suspensions before FACS
- Resuspend the pellet in an appropriate amount of FACS buffer and filter with a cell strainer (40 µm).
- Centrifuge (400 g, 5 min, 4°C)
- Remove supernatant until the 0.1-mL line of the 1.5-mL tube.
- Resuspend the pellet in FACS buffer and prepare for analyses.
4) Analysis of SSEA-3-positive cells with FACS and procedures for collecting Muse cells

**ATTENTION!** A sample containing many dead cells is inappropriate for analysis and collection. Nonspecific staining is likely to occur for dead cells, resulting in an inaccurate measurement of the Muse cell rate. Even if sampling is forced, cell survival is frequently decreased. In the FSC-A vs. SSC-A plot in FACS analysis, dead cells are evaluable (refer to the following figure).

- Normal samples
  (Successful example)
  - One large population

- Samples with many dead cells
  (Inappropriate for analysis and collection)
  - Two populations

4-1) Gating setting and data acquisition (The following data were collected from a BD FACS Aria II)

- Load unstained sample #1.
- Adjust sensitivity of SSC (side scattering light) and FSC (frontal scatting light) and perform Area Scaling. Refer to the following figure.

  - Area scaling (before setting)
  - Area scaling (after setting)

  * Place dots on a diagonal line to upper right.

- Develop a plot of SSC-A vs. FSC-A. Refer to the following figure.

  - Draw a Gate P1.
  - Set SSC and GSC sensitivities so that the P1 %population is ≥95%
  - Set [Show Populations] of the plot to All Events.
Develop a histogram of Count vs. FITC-A. Refer to the following figure.

- Adjust FITC voltage so that the whole histogram is covered.
- Set [Show Populations] of the plot to All Events.
- Draw a central bar using an appropriate tool.

(The bar is not always necessary to find the center of the histogram)

Develop a plot of SSC-A vs. FITC (screen to determine SSEA-3-positive rate). Refer to the following figure.

- Make gating so that dots in Gate P1 are analyzed in Gate P2.
- Shift Gate P2 to the left to the area not including dots.
- Set [Show Populations] of the plot to P1.

After setting all conditions, obtain data for an unstained sample loaded without change (#1).

Load a stained sample (#2) containing secondary antibodies only and obtain data.

- Adjust the position of Gate P2 not to include dots.
  - Gate P2: 0% (%SSEA-3)

Load a stained sample (#3) containing Rat IgM Isotype Control + secondary antibodies and obtain data.

- Adjust the position of Gate P2 not to include dots.
  - Gate P2: 0% (%SSEA-3)

Load a stained sample (#4) containing Rat anti-SSEA-3 + secondary antibodies and obtain data.

- Do not change the position of Gate P2 established for Isotype control and obtain data.
  - Gate P2: 5.2% (e.g. rate of BM-MSC-derived SSEA-3 positive cells)
4-2) Muse cell sorting

- To eliminate doublets, develop a plot FSC-W vs. FSC-H and SSC-W vs. SSC. Refer to the following figure.

  - Make gating to analyze SSEA-3-positive populations (Gate P2 of sample #4) in Gate P3.
  - Set [Show Populations] of the plot to P2.
  - Eliminate deviated dots from gates.

  [Diagram of FSC-W vs. FSC-H and SSC-W vs. SSC plots]

- Make gating so that dots in Gate P3 are analyzed in Gate P4.
- Set [Show Populations] of the plot to P2.

- Set a tube containing 10% FBS/DMEM (choose low-glucose or high-glucose type appropriate to cells) at a sorting site to receive cells.

- Start sorting of Muse cells (collect populations in Gate P4)
5) Poly-HEMA coating of wells and dishes

5-1) Add 1.2 g of poly-HEMA (poly 2-hydroxyethyl methacrylate, SIGMA, P3932) to 40 ml of 95% EtOH (95% EtOH = 38 ml 99.5% EtOH + 2 ml MilliQ)

**ATTENTION!** Poly-HEMA is very difficult to dissolve (it will not dissolve in 100% EtOH). First fill the tube with 95% EtOH solution and then add the poly-HEMA to the 95% EtOH.

5-2) Shake the solution for several hours at 37°C to dissolve the poly-HEMA. Do not tilt the tube, or the poly-HEMA at the bottom of the tube will not dissolve.

3. Add the dissolved poly-HEMA to the dish/well, and rotate the dish to homogenously cover the bottom of the dish/well.

<table>
<thead>
<tr>
<th>Dish</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 cm</td>
<td>3.2 ml</td>
</tr>
<tr>
<td>6 cm</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>3.5 cm</td>
<td>500 μl</td>
</tr>
<tr>
<td>12 well</td>
<td>200 μl</td>
</tr>
<tr>
<td>24 well</td>
<td>100 μl</td>
</tr>
<tr>
<td>48 well</td>
<td>70 μl</td>
</tr>
<tr>
<td>96 well</td>
<td>25 μl</td>
</tr>
</tbody>
</table>

4. Leave the cover off the dish, and allow the dish to completely dry out in a clean cabinet overnight. The door of the cabinet should be left open 10 to 20 cm. Do not turn on a UV light.

**ATTENTION!** When evaporated EtOH is saturated in a clean cabinet, the dishes will not dry completely, even when allowed to sit overnight. Do not to make too many poly-HEMA coated dishes at one time.

**ATTENTION!** Dried dishes can be stored in the dark, at room temperature for a couple of months.

4. Wash the poly-HEMA-coated dish at least 3 times by PBS before use.
6) **Bulk production of Muse cell-clusters using methylcellulose gel**

**ATTENTION!** Overnight preparation of **poly-HEMA coated dishes** is required before performing the FACS isolation (see Poly-HEMA coating section).

**ATTENTION!** Methylcellulose (MC, MethoCult H4100, StemCell Technologies, 04100) must be purchased prior to initiating the experiments.

1. Stain FACS-sorted cells using trypan-blue, and count the number of live cells.

2. According to the following table, place cells, fetal bovine serum (FBS), and methylcellulose (see above for purchasing information) into each well. Methylcellulose has a very high viscosity. Therefore, you must first cut off the end of a blue or yellow pipette tip to facilitate sucking up the methylcellulose medium.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Cell number</th>
<th>Cell + DMEM (μl)</th>
<th>FBS (μl)</th>
<th>2.6% MC (μl)</th>
<th>Total (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>25,000</td>
<td>1700</td>
<td>300</td>
<td>1000</td>
<td>3000</td>
</tr>
<tr>
<td>12 well</td>
<td>10,000</td>
<td>705</td>
<td>125</td>
<td>420</td>
<td>1250</td>
</tr>
<tr>
<td>24 well</td>
<td>5000</td>
<td>400</td>
<td>70</td>
<td>230</td>
<td>700</td>
</tr>
<tr>
<td>48 well</td>
<td>3000</td>
<td>230</td>
<td>40</td>
<td>130</td>
<td>400</td>
</tr>
<tr>
<td>96 well</td>
<td>1000</td>
<td>77</td>
<td>13</td>
<td>40</td>
<td>130</td>
</tr>
</tbody>
</table>

※The final solution will contain 10% FBS and 0.9% MC in DMEM

※Only poly-HEMA coated dishes should be used.

3. Slowly and gently stir the cells, FBS, and methylcellulose medium using a cell scraper. Pay careful attention not to scratch the poly-HEMA coating. Use a phase microscope to confirm the homogeneous distribution of the cells.

4. After plating, the cells should be maintained by the addition of 10% FBS in Low-glucose DMEM (according to the volume indicated in the table below) every 3 days. Cells should be cultured for 7 to 10 days and clusters picked up for analysis.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 well</td>
<td>1300</td>
</tr>
<tr>
<td>12 well</td>
<td>530</td>
</tr>
<tr>
<td>24 well</td>
<td>300</td>
</tr>
<tr>
<td>48 well</td>
<td>170</td>
</tr>
<tr>
<td>96 well</td>
<td>60</td>
</tr>
</tbody>
</table>

Muse cell-cluster in methylcellulose culture on day 7
7) Making Muse cell-clusters in single cell-suspension culture

**ATTENTION!** 96 well poly-HEMA coated dishes need to be prepared before doing FACS isolation. It takes over night for preparation. (see Poly-HEMA coating of wells and dishes)

**ATTENTION!** Use 10% FBS in low-glucose DMEM to culture BM-MSCs, NHDFs and HDFs, and 15% FBS in high-glucose DMEM to culture ADSCs. Furthermore, be sure to add FGF-2 (bFGF) to culture BM-MSCs.

1. Stain FACS sorted cells by trypan-blue, and count the live cell number.

2. Calculate cell number and prepare the cell solution in medium so as to make 1 cell in each well, namely, limiting dilution.

   For example: each well needs 100 μl medium. For 96 well-one plate, 96 cells are suspended in 9600 μl medium. However, we routinely adjust the medium to make 1.5~2 cells per well. This will make 1 cell in each well. Logical calculation is usually too strict to make single cell in each well.

3. Next day, observe each well under phase microscope and check vacant well and well with multiple number of cells from counting. Those wells should be eliminated from counting the cluster formation ratio.

4. Add 30μl of medium for each well every 3 days. Culture for 7~10 days, and pick up Muse cell-derived clusters for analysis.

Clusters formed in single cell-suspension culture at day 7.
8) Evaluation of pluripotency of Muse cell-clusters: Alkaline phosphatase reaction

**ATTENTION!** Purchase Leukocyte Alkaline Phosphatase Kit (Cat#86R-1KT, Sigma).

**ATTENTION!** Do not use PBS for alkaline phosphatase (ALP) reaction. PBS is generally used for stopping the reaction.

1. Prepare ALP solution according to the manufacture's protocol. In brief, 
   Mix 10 μl of Sodium Nitrite Solution and 10 μl of FRV-Alkaline Solution. Both solutions are provided in the kit. Leave for 2 min in room temperature, and then add 450 μl saline.
   
   ↓
   Add 10μl Naphthol AS-BI Alkaline Solution to the above solution (this is also provided in the kit).

2. Collect Muse cell-clusters in 1.5 ml tube, add 1ml of saline and suspend the clusters. Do not use PBS!!

3. Centrifuge at 400 g in room temperature for 5 min. Remove the supernatant.

4. Add 1ml saline and suspend the clusters.

5. Centrifuge at 400 g in room temperature for 5 min. Remove the supernatant.

6. Add 1ml saline and suspend the clusters.

7. Centrifuge at 400 g in room temperature for 5 min. Remove the supernatant as much as possible.

8. Fix the clusters by 4% paraformaldehyde. -> This is optional. Reaction would be stronger without fixation. We usually skip this procedure.

9. Add 200 μl ALP solution to the clusters. Incubate in 37°C incubator for 15min. -> The manufacture protocol instructs us to incubate in room temperature, but in our experience, 37°C gives better reaction.

10. Add 800 μl PBS to stop the ALP reaction.

11. Centrifuge at 400 g in room temperature for 5 min. Remove the supernatant.

12. Add 1ml PBS and suspend the clusters.

13. Centrifuge at 400 g in room temperature for 5 min. Remove the supernatant.

14. Transfer clusters to slide class and observe under light microscope.
9) Evaluation of pluripotency of Muse cell-clusters: Gelatin culture of clusters

**ATTENTION!** Prepare gelatin coated dish or cover slip before doing following experiment.

1. Preparing Gelatin coated dish or cover slip.
   Gelatin : SIGMA Cat#G-1890 → Stock solution is 0.1% gelatin in PBS. Sterilize by autoclaving and use.
   For coating, load plentiful amount of 0.1% gelatin solution in plastic wells or wells placed cover slips (we usually use 18 mm diameter round cover slip for 24 well plate) in the bottom, incubate at 37 °C at least for 30 min.
   For use, aspirate gelatin solution and directly use for experiment without washing.
   After coating, pay attention NOT TO DRY the coated wells or cover slips.

2. Pick up formed clusters. Use glass capillary or pipetman (P20 scale) for picking up.
   Attention) In the case of clusters formed in methylcellulose, wash clusters by Low-glucose DMEM for a couple of times because cellulose cling to cluster disturb its adherence to dish or cover slip. In brief, supply 200 μl Low-glucose DMEM into each well of 4 well plate, transfer clusters into Low-glucose DMEM and wash several times by pipetting.

3. Remove gelatin solution after incubation in 37 °C, and quickly supply 10% FBS in Low-glucose DMEM into each well. Pay attention not to dry the dish or cover slip.
   Initially, the volume of medium should be a bit lesser than usual. Lesser volume makes transferred clusters easier to adhere to the bottom of dish or to the set cover slip.
   For example, 250 μl for 24 well scale, ~800 μl for 12 well.

4. Transfer clusters into above well.

5. After a couple of hours, add the 10% FBS in Low-glucose DMEM for volume-up. For 24 well, 300~400 μl and for 12 well, 1 ml solution is preferable for the final volume.

6. Clusters will adhere to the bottom of the well or cover slip by next day, or latest by 3 days. The cells gradually expand.
Expansion of cells from the cluster. 10 days after culture.

7. Culture for 1~2 weeks, and then subject the samples to RT-PCR or immunocytochemistry.

<For RT-PCR>
Use following kits for isolation of small scale mRNA and for reverse transcription.

- NucleoSpin RNA XS : Macherey-Nagel Cat#740 902.10
- SuperScript VILO cDNA Synthesis Kit : Invitrogen Cat#11754050
- TaKaRa Ex Taq : TaKaRa Cat#RR01A

human RT-PCR primer
β-actin F: 5’-GGCGGACTATGACTTAGTTCGTTACACC-3’
   R: 5’-AAGTCTCTCGGCCACATTTGAACTTTG-3’
Nkx2.5 F: 5’-GGGACTTGAATGCGGTTCAG-3’
   R: 5’-CTCCACAGTTGGGTTCATCTGTAA-3’
α-fetoprotein F: 5’-CCACTTGTGCACTACATGAAC-3’
   R: 5’-TGCAGGGAGGACATATGCTCA-3’
MAP-2 F: 5’-ACTACAGTTTCACACCCCATTT-3’
   R: 5’-AAGGGTGACAGAGACACAGATAC-3’
GATA6 F: 5’-CCTGCGGGCTCTACAGCAAGTGAAC-3’
   R: 5’-CGCCCCTGAGGTGTTGTTGTT-3’

<For immunocytochemistry>
Fix the sample with 4% (v/v) paraformaldehyde / 0.01M PBS

Antibodies for use
- anti-SMA (Lab Vision, MS-113-P0, 1:100)
- anti-Neurofilament-M (Chemicon, AB1987, 1:200)
- anti-α-fetoprotein (DAKO, N1501, 1:100)
- anti-desmin (BD Biosciences, 550626, 1:100)
- anti-cytokeratin 7 (Chemicon, MAB3226, 1:100)

Blocking solution : 20% (vol/vol) BlockAce / 5% (wt/vol) BSA / 0.3% (vol/vol) Triton X-100 / 0.02 M D-PBS
Antibody diluent : 5% (vol/vol) BlockAce / 1% (wt/vol) BSA / 0.3% (vol/vol) Triton X-100 / 0.02 M D-PBS