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Large-Scale Production of Human iPSC-Derived Macrophages

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We use this protocol and it's working

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Disclaimer

This protocol needs prior approval by the users' institutional review board (IRB) or equivalent ethics committee(s).

Abstract

To differentiate iPSC-derived macrophages, we adopted and modified a previously published protocol. Starting from the pluripotent state, we induced mesoderm and subsequent hemogenic endothelium differentiation in preformed and size-controlled embryoid bodies (EBs) for four days by supplementing the culture medium with recombinant human bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor (VEGF), and stem cell factor (SCF). On day 5, floating EBs are reseeded for attachment to culture vessels in “factory” medium containing human macrophage colony-stimulating factor (M-CSF) and interleukin 3 (IL-3). A key modification to some previously published protocols is the reseeding of the EBs on growth factor-reduced (GFR) Matrigel-coated culture vessels.

Materials

Reagents

	A	B	C
	Reagents	Supplier	Cat#
	BMP4	Peprotech	120-05ET
	SCF	Peprotech	300-07
	VEGF	Peprotech	100-20
	X-VIVO 15	Lonza	BE02-053Q
	M-CSF	Peprotech	300-25
	IL-3	Peprotech	200-03
	Y-27632 dihydrochloride	Miltenyi Biotech	130-106-538
	mTeSRTM Plus	STEMCELL Technologies	100-0276
	GlutaMAX	Life Technologies	35050061
	2-mercaptoethanol	Life Technologies	31350-010
	Matrigel® hESC-Qualified Matrix	Fisher Scientific	08-774-552
	Matrigel® Growth factor reduced	Fisher Scientific	CB-40230
	Aggrewell 800 well (24 wells)	STEMCELL Technologies	34811
	Anti-Adherence Rinsing Solution	STEMCELL Technologies	7010
	Nunc™ EasYFlask™ Cell Culture Flasks	Thermo Fisher	156499
	Suspension Flat Bottom 6-well Cell Culture Plate	Sarstedt	83.3920.500

	A	B	C
	Suspension Flat Bottom Tissue culture dish, 100 × 20 mm	Sarstedt	83.3902.500
	Strainers, 40 mm	Corning	352340
	Strainers, 70 mm	Fisher Scientific	22-363-548
	Gentle Cell Dissociation Reagent	STEMCELL Technologies	100-0485
	DPBS (Without Mg2+ or Ca2+)	Gibco	14190144
	0.5 M EDTA	Invitrogen	15575-038
	DMEM/F12, HEPES	Gibco	11330032

Complete Media: mTeSR+ with BMP4 (50 ng ml−1), VEGF (50 ng ml−1), SCF (20 ng ml−1)

Factory Media: X-VIVO 15 Supplemented with 100 ng/mL M-CSF, 25 ng/mL IL-3, 2 mM GlutaMAX, 0.050 mM b-mercaptoethanol

Macrophage media: X-VIVO 15 supplemented with 100 ng/mL M-CSF, 2 mM GlutaMAX and 0.050 mM b-mercaptoethanol

EDTA solution: 0.05 mM EDTA in DPBS (Without Mg2+ or Ca2+)

Human BMP-4 Recombinant Protein, PeproTech® **Thermo Fisher Scientific Catalog #**120-05ET-10UG

Recombinant Human SCF **peprotech Catalog #**300-07

Recombinant Human VEGF **peprotech Catalog #**100-20

X-Vivo 15 with Recomb Transf Gent L-Gln Phen Red 1 L **Lonza Catalog #**BE02-053Q

Recombinant Human M-CSF **peprotech Catalog #**300-25

Recombinant Human IL-3 **peprotech Catalog #**200-03

StemMACS™ Y27632 **Miltenyi Biotec Catalog #**130-106-538

mTeSR plus media kit **STEMCELL Technologies Inc. Catalog #**100-0276

GlutaMAX™ Supplement **Thermo Fisher Scientific Catalog #**35050061

2-Mercaptoethanol (50 mM) **Gibco - Thermo Fisher Scientific Catalog #**31350010

- Corning™ Matrigel™ hESC-Qualified Matrix **Fisher Scientific** **Catalog #**08-774-552
- Corning™ Matrigel™ GFR Basement Membrane Matrix **Thermo Fisher Scientific** **Catalog #**CB-40230
- AggreWell™800 24-well Plate, 1 pack 1 Plate **STEMCELL Technologies Inc.** **Catalog #**34811
- Anti-Adherence Rinsing Solution **STEMCELL Technologies Inc.** **Catalog #**07010
- Nunc™ EasYFlask™ Cell Culture Flasks, T75, filter **Thermo Fisher** **Catalog #**156499
- Cell culture plate 6 well surface: Suspension flat base **Sarstedt** **Catalog #**83.3920.500
- 10 cm Petri Dishes SureGrip **Sycamore Life Sciences** **Catalog #**Sarstedt 83.3902.500
- Falcon 40 µm Cell Strainer **Corning** **Catalog #**352340
- Fisherbrand™ Sterile Cell Strainers **Fisher Scientific** **Catalog #**22-363-548
- Gentle Cell Dissociation Reagent **STEMCELL Technologies Inc.** **Catalog #**100-0485
- DPBS, no calcium, no magnesium **Thermo Fisher Scientific** **Catalog #**14190144
- UltraPure 0.5M EDTA, pH 8.0 **Thermo Fisher Scientific** **Catalog #**15575-038
- Gibco™ DMEM/F-12 HEPES **Thermo Fisher Scientific** **Catalog #**11330032

Culturing of iPSC

1 Maintain pluripotent hiPSC on Matrigel hESC in mTeSR+ medium.

2 Passage the cells in aggregates every 4 days with Gentle Cell Dissociation Reagent, followed by re-seeding with ROCK inhibitor ([M] 10 micromolar (µM) Y-27632) included in the medium for the first 24:00:00 after plating.

3 Authenticate cells by STR profiling upon receipt and check monthly for mycoplasma contamination by PCR.

Differentiation protocol: Preparation of Aggrewell 800 plates (Day 1) 8m

4 Warm basal media (DMEM/F12) and complete media.

5 Open AggreWell plates in a biosafety cabinet.

> Note
>
> NOTE: Do not expose AggreWell plates to organic solvents, including ethanol or isopropanol.

6 Pre-treat wells with Anti-Adherence Rinsing Solution by adding 500 µL for a well of 24-well plate.

7 Centrifuge plate at 1300 x g, 00:03:00 in a swinging bucket rotor fitted with plate holders. 3m

> Note
>
> NOTE: Plates must be well balanced. Prepare a balance plate using a standard plate filled with water to match the weight and position of the AggreWell plate.

8 Observe the plate under a microscope to ensure that bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 5m

1300 x g, 00:05:00 . Aspirate Anti-Adherence Rinsing Solution from the wells.

9 Rinse each well with 2 mL of warm basal medium. Aspirate medium from the well.

10 Add 1 mL of warm complete medium with ROCK inhibitor (10 µM Y-27632) to each well to be used for a 24-well plate.

Differentiation protocol: Generation of Embryoid Bodies (EBs)

1d 0h 5m

11 Prepare a single-cell suspension in the desired medium (mTeSR+).

12 To achieve EBs size of 10,000 cells per EB, plate 4.0 x 10^6 in each well and add complete medium with ROCK inhibitor ([M] 10 micromolar (µM) Y-27632) to each well to achieve a final volume of 2 mL/well.

13 Prepare a centrifuge balance plate using a standard plate filled with water to match the weight and position of the AggreWell plate.

14 Pipette cells up and down gently several times to ensure even distribution of cells throughout the well. Be careful not to introduce bubbles into the microwells.

15 Immediately centrifuge the AggreWell plate at 100 x g, 00:05:00 to capture cells in the microwells, using the balance plate prepared.

5m

16 Observe the plate under a microscope to verify that cells are evenly distributed among the microwells.

17 Incubate the plate at 37°C with 5% CO_2 and 95% humidity for 24:00:00 .

1d

Differentiation protocol: Changing medium in Aggrewell plate (Day 1-4)

18 Warm complete medium (no need for ROCK inhibitor at this point).

19 Perform a 50 - 75% medium change by removing slowly 1 - 1.5 mL of medium from each well.

20 Replace with 1 - 1.5 mL of the fresh complete medium by slowly pipetting down the wall of the well. Slowly dispensing the medium helps to prevent displacement of EBs/spheroids from the microwells.

Differentiation protocol: Harvesting EBs from Aggrewell plates (Day 4)

21 Warm basal and completed medium.

22 Using a serological pipette:

Note

Alternative option: This step can be achieved by using P1000 cut tips.

22.1 Remove approximately half of the culture medium from the well.

22.2 Dispense the medium firmly back onto the surface of the plate to dislodge the EBs/spheroids from the microwells. Repeat if necessary.

23 Use the 40 µm strainer and a 50 ml conical tube for separation of EBs/spheroids from single cells.

24 Place strainer on top of the tube with the arrow pointed upward. Add 1 mL of PBS to wet the surface of the strainer.

25 Gently aspirate the dislodged EBs/spheroids. Pass the EB/spheroid suspension through the strainer.

Note

NOTE: The aggregates will remain on the filter; any unincorporated single cells will flow through.

26 Using a serological pipette, dispense 1 mL (24-well plate) of the warm basal medium across the entire surface of the well to dislodge any remaining EBs/spheroids.

26.1 Collect wash and pass over the strainer.

26.2 Repeat this wash step 3 times. Wash can be repeated until you do not see more EBs under the microscope in the AggreWell.

27 Invert the strainer, and place over a new conical tube of the same size. Collect the EBs/spheroids by washing with 2 – 5 mL of complete medium per well harvested.

28 Transfer correct number of EBs to Matrigel-GFR coated plates/flasks. Swirl the plate/flask gently to distribute the EBs around the whole surface.

Note

NOTE: EBs are differentiated in either 6-well plates (15 EBs/well), T75 (75 EBs), or T175 flasks (175 EBs).

Differentiation protocol: Differentiation to Monocytes and Macrophages: (Day 4 onwards)

29 Differentiate EBs in X-VIVO 15 Supplemented with 100 µL M-CSF, 25 µL IL-3, [M] 2 millimolar (mM) GlutaMAX, [M] 0.050 millimolar (mM) b-mercaptoethanol with fresh medium added weekly.

Note

Cells can be maintained in this medium for up to 6 months.

30 After approximately 4 weeks, collect weekly the monocytes emerging into the supernatant and replenish differentiation cultures with fresh medium. Strain (70 um) harvested cells. Use it either directly or plate onto ultra-low attachment tissue culture plates.

31 Differentiate cells in suspension (monocytes) into macrophages by culturing in X-VIVO 15 supplemented with 100 µL M-CSF, [M] 2 millimolar (mM) GlutaMAX and [M] 0.050 millimolar (mM) b-mercaptoethanol for 7 days until cells are adherent and elongated.

Note

NOTE 1: We usually plate 4.0 x 10^6 monocytes per 10 cm dish. Scale up or down accordingly.
NOTE 2: On day 3 after plating do a half media change of macrophage media.

32 Detach macrophages by washing in 1X PBS -Ca, -Mg and then incubating the plate at 37 °C for 10-15 min in [M] 0.05 millimolar (mM) EDTA solution followed by gently scraping.

33 Count cells and stain them for flow cytometry or plate them for experiments in ultra-low attachment plates.

Quality control for iPSC-derived macrophages

34 Flow cytometry for CD14+, CD11b+, CD45+.

Protocol references

1. Adapted from Gutbier, Cowley, Patsch et al. Int. J. Mol. Sci. 2020, 21, 4808; doi:10.3390/ijms21134808.