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Microglia differentiation from human pluripotent stem cells (v2;optimised)

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We use this protocol and it's working

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Abstract

Protocol for the directed differentiation of hPSCs towards erythro-myeloid progenitors and further to microglia. Based on Washer et al, Sci Rep, 2022 with input from Bioneer A/S. Option to use "De Strooper" protocol variant (Fattorelli et al, Nature Protocols, 2021) is described.

Attachments

PDF
Optimised microglia ...
1.1MB

PDF
Protocol for coating...
608KB

Materials

	Reagent	Vendor	Cat. no
	mTeSR1 Basal Medium and 5x supplement kit	STEMCELL Technologies	85850
	X-VIVO 15	Lonza	BE04-418
	Advanced DMEM/F12	Gibco	12634010
	GlutaMAX	Life Technologies	35050-061
	β-mercaptoethanol	Life Technologies	31350-010
	EDTA	Thermo Fisher	15575020
	DPBS	Thermo Fisher	A1285601
	Y-27632 dihydrochloride	Miltenyi	130-106-538
	KnockOut‰ Serum Replacement	Thermo Fisher	10828028
	Pen/Strep	Life Technologies	17502-048
	BMP4	Miltneyi	130-111-165
	VEGF	Miltneyi	130-109-385
	SCF	Miltneyi	130-096-692
	M-CSF	Miltneyi	130-096-492
	IL-3	Miltneyi	130-095-069
	GM-CSF	Miltneyi	130-093-864
	IL-34	Miltneyi	130-108-977

	Reagent	Vendor	Cat. no
	TGFb1	Peprotech	100-21C-100UG
	TPO	Thermo Fisher (Peprotech)	300-18
	FLT3L	Thermo Fisher (Peprotech)	300-19
	Aggrewell 800 (24 wells)	STEMCELL Technologies	34811
	Reversible Strainer	StemCell	27250
	Anti-Adherence Rinsing Solution	STEMCELL Technologies	7010
	Matrigel	Corning	354230

Media Compositions

1 **EB formation (Aggrewell) medium** (Day 0-6)

	Reagent	Stock Concentration	Final Concentration	Final Volume = 50ml	Dilution
	mTeSR1			49.7ml	
	VEGF	50 µg/ml	50 ng/ml	50µl	1:1000
	SCF	20 µg/ml	20 ng/ml	50µl	1:1000
	BMP4	10 µg/ml	50 ng/ml	250µl	1:200
	Pen/Strep	5,000 U/mL	100 U/mL	100µl	1:500

1.1

2 **preMACS differentiation (Factories) medium - "De Strooper" protocol, Fattorelli et al, 2021** (Day 6-12)

	Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
	X-VIVO15			98.2ml	
	M-CSF	100 µg/ml	50 ng/ml	50µl	1:2000
	IL-3	25 µg/ml	50 ng/ml	200µl	1:500
	SCF	20 µg/ml	50 ng/ml	250µl	1:400
	TPO	5 µg/ml	5 ng/ml	100µl	1:1000
	FLT3L	50 µg/ml	50 ng/ml	100µl	1:1000
	GlutaMAX	200 mM	2 mM	1ml	1:100

	Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
	β-mercaptoethanol	50 mM	50 µM	100µl	1:1000
	Pen/Strep	5,000 U/mL	100 U/ml	200µl	1:500

preMACS differentiation (Factories) medium - "De Strooper" protocol, Fattorelli et al, 2021 (Day 13-)

	Reagent	Stock Concentration	Final Concentration	Final Volume = 100ml	Dilution
	X-VIVO15			98.2ml	
	M-CSF	100 µg/ml	50 ng/ml	50µl	1:2000
	GM-CSF	10 µg/ml	25 ng/ml	250µl	1:400
	FLT3L	50 µg/ml	50 ng/ml	100µl	1:1000
	GlutaMAX	200 mM	2 mM	1ml	1:100
	β-mercaptoethanol	50 mM	50 µM	100µl	1:1000
	Pen/Strep	5,000 U/mL	100 U/ml	200µl	1:500

3 **preMACS differentiation (Factories) medium- "Cowley" protocol, Washer et al, 2022** (Day 6-)

	Reagent	Stock Concentration	Final Concentration	Final Volume	Dilution
	X-VIVO15			98.2ml	
	M-CSF	100 µg/ml	100 ng/ml	100µl	1:1000
	IL-3	25 µg/ml	25 ng/ml	100µl	1:1000

	Reagent	Stock Concentration	Final Concentration	Final Volume	Dilution
	GlutaMAX	200 mM	2 mM	1ml	1:100
	β-mercaptoethanol	50 mM	50 µM	100µl	1:1000
	Pen/Strep	5,000 U/mL	100 U/mL	200µl	1:500

4 **Microglia Maturation Medium**

	Reagent	Srock Concentration	Final Concentration	Final Volume	Dilution
	ADMEM/F12			98ml	
	Glutamax	200mM	2mM	1ml	1:100
	M-CSF	100ug/ml	25ng/ml	25ul	1:4000
	GM-CSF	10ug/ul	10ng/ml	100ul	1:1000
	IL-34	100ug/ul	100ng/ml	100ul	1:1000
	TGFb1	50ug/ml	50ng/ml	100ul	1:1000
	Pen/Strep	5,000 U/mL	100 U/mL	200ul	1:500

Embryo Body (EB) Generation

5 **Preparation of Aggrewell-800 plates**

5.1 Pre-treat wells with Anti-Adherence Rinsing Solution. For 24-well plates, add 500 µL per well

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

5.2 Centrifuge plate at 1300 x g for 3 minutes in a swinging bucket rotor fitted with plate holders

NOTE: Plates must be well-balanced. Prepare a balanced plate using a standard plate filled with water to match the weight and position of the Aggrewell plate

5.3 Observe the plate under a microscope to ensure bubbles have been removed from microwells. If bubbles remain trapped in any microwells, centrifuge at 1300 x g for an additional 3 minutes

5.4 Aspirate Anti-Adherence Rinsing Solution from the wells

5.5 Rinse each well with 500 µL of DMEM/F12 medium

5.6 Centrifuge at 1300 x g for an additional 3 minutes

5.7 Remove media and add 1ml of fresh DMEM/F12 medium (second wash)

6 hPSC dissociation: DIV 0

6.1 Cultures should be 70–80% confluent:
- homogeneous-appearing colonies with clear borders
- absence of obvious differentiating zones

6.2 Prepare appropriate volume of EB medium + 10µM Y-27632 ROCK inhibitor

6.3 Aspirate medium from hPSC cultures

6.4 Wash cells with appropriate PBS volume

6.5 Add the appropriate volume of 50uM EDTA in PBS

	Plate	Seeding area (Sarstedt)	EDTA 50uM (75µL/cm2)
	6-well	9.07 cm2	1.9ml
	12-well	3.65 cm2	700ul
	24-well	1.82 cm2	300ul

6.6 Incubate at 37°C for 7 min

NOTE: If very confluent leave cells in the incubator for up to 10min

6.7 Add 1 mL of wash medium to the well and pipette off the colonies from all sides of the well using a P1000 pipette
- The cells should easily be removed by washing the well with a medium
- Prepare a single-cell suspension

6.8 Transfer the colonies to the 15 mL tube containing 10ml Wash Buffer

6.9 Count cells with Nucleocounter:
- Determine the concentration of cells/ml
- Determine the total volume of cell suspension to initiate differentiation

6.10 Transfer the appropriate cell suspension volume to a new tube

6.11 Centrifuge cells at 400g for 5min at RT

7 EB generation: DIV 0

7.1 To achieve EB size of 15,000 cells per EB, plate 4 × 10^6 cells in each well

7.2 Aspirate medium and resuspend cells in 1ml of EB medium per well

7.3 Add 1ml of cell suspension to each 24-well already containing 1ml of EB medium

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

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

7.6 Centrifuge the AggreWell plate at 100 x g for 3 minutes to capture cells in the microwells

7.7 Incubate the plate at 37°C with 5% CO2 and 95% humidity

8 **EB medium change: DIV 1 - DIV 6**

8.1 Perform 75% medium change (EB medium) every day

8.2 Slowly remove 1.5ml of medium from each well

8.3 Replace with 1.5m of the fresh EB medium by slowly pipetting down the wall of the well; Slowly dispensing the medium prevents displacement of EBs/spheroids from the microwells

preMACS - "Cowley" version

9 **Coat Flasks with Matrigel**

9.1 Remove a pre-diluted aliquot of matrigel (5ml of 5% matrigel) from the freezer and thaw in the fridge overnight

9.2 Add 5ml of DMEM/F12 (w/ Glutamax) to each aliquot
- Matrigel should always be handled on ice

9.3 Add 10ml of the matrigel in the flask

9.4 Incubate the flask at 37°C with 5% CO2 and 95% humidity for at least 1h

9.5 Remove coatingbefore seeding the EBs

10 **Transfer EBs into matrigel coated T75 flask**

10.1 Remove EBs carefully from Aggrewells using a 5ml stripette

10.2 Transfer EBs to a 40µm invertible cell strainer; Use a 50ml Falcon tube as liquid waste beneath the strainer
- This step ensures that only EBs are transferred into the flask
- Single & dead cells are captured on the strainer membrane

10.3 Flash the remaining EBs from the Aggrewell using 2ml PBS and transferto the strainer

10.4 Repeat step 3

10.5 Wash dead cells away from the EB in the strainerusing 4 mL of PBS

10.6 Invert the strainer over a new 50ml Falcontube and wash off EBs off using 4 mL of preMACS differentiation medium

10.7 Divide EBs evenly into two matrigel-coated T75 flasks, each containing 18ml of preMACS differentiation medium (total V = 20ml)

10.8 Incubate the plate at 37°C with 5% CO2 and 95% humidity

11 **preMACS Factoriesmedia change: ~ till DIV35**

11.1 Factories are fed weekly by **adding**
10ml of preMACS differentiation media until macrophage/microglia precursors (preMACS) cells start to appear in the supernatant; This process usually takes ~5 weeks

NOTE: At this point,preMACS can be used for microglia maturation experiments

11.2 Factories can be maintained successfully for at least 4mo:

- Factories are fed weekly by **adding** 10ml of preMACS differentiation media
- This is okay for most cell lines, including RC17, but it needs to be adjusted to twice a week for other lines, like KOLF2.1; Keep an eye on the media color and adjust as necessary
- When the flask becomes too full, transfer the supernatant containing the preMACS in the suspension into a 50ml Falcon tube, centrifuge for 5min at 400g, remove supernatant, resuspend preMACS into 10ml of preMACS differentiation media, and return the freshly resuspended preMACS into the relevant flask

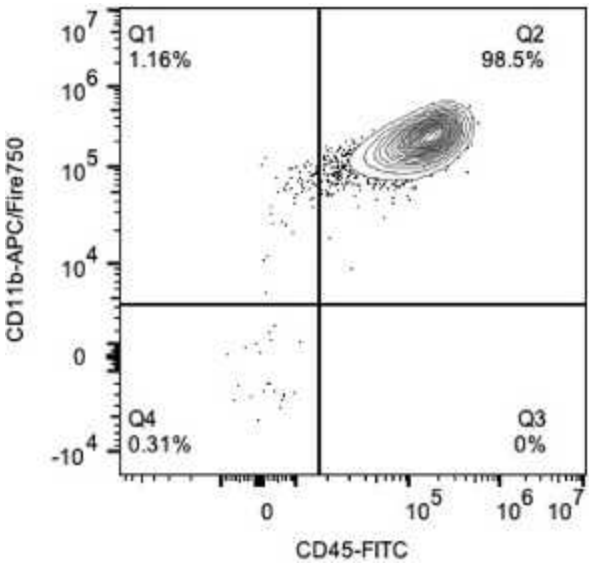
preMACS - "De Strooper" version

12 Follow the steps described for the "Cowley" protocol - the only difference is the preMACmedia composition (see table above)

QC preMACS by FACS

13 From around DIV30 onwards,preMACs can be analysed by flow cytometry for the co-expression of CD45 and CD11b using the protocol and antibodies linked below. The time of QC depends on the cell line; for example, RC17s are typically not ready before DIV40.

	Antibody	Cat. no	Company	Flurophore	Dilution
	CD45	555482	BD Biosciences	FITC	1:100
	CD11b	301352	Biolegend	APC/Fire750	1:500



Microglia monolayer differentiation

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- From ~DIV35 onwards,free-floating preMACS in the Factories supernatant can be collected and matured into microglia with/ Microglia Maturation Medium (MMM).
- preMACS can be seeded on plastic plates or glass coverslips.
- Microglia can be used between preMACS seeding day +7d of MMM until preMACS seeding day +2mo of MMM.
- preMACs for maturation into microglia are seeded at 100,000 cells/cm2

15 **Seeding monolayermicroglia**

15.1 Aspirate all medium from a preMACsFactory containing flask

15.2 Transfer into a 50ml Falcon througha 40um strainer

15.3 Count cells using a nuclecounter to determine the concentration of cells/ml

15.4 Determine the total volume of cell suspension needed and transferthe appropriate volume into a new
Falcon tube

NOTE: Do not leave the preMACsin the tube for too long, as cells tend to stick to everything, including the tube walls

15.5 Centrifuge cells at 400g for 5min at RT

15.6 Aspirate medium and resuspend cells in MMM (according to the table below)

	Plate	Seeding area	MMM (V)
	6-well	9.07 cm2	2.5ml
	12-well	3.65 cm2	1ml
	24-well	0.64 cm2	500ul
	48-well	1.82 cm2	300ul
	96-well	0.29 cm2	100ul

15.7 Incubate the plate at 37°C with 5% CO2 and 95% humidity

16 Monolayer microglia media change

16.1 Perform 50% medium change(MMM) every other day

16.2 Microglia monolayers can be maintained successfully for up to 2mo

QC microglia by qRT-PCR

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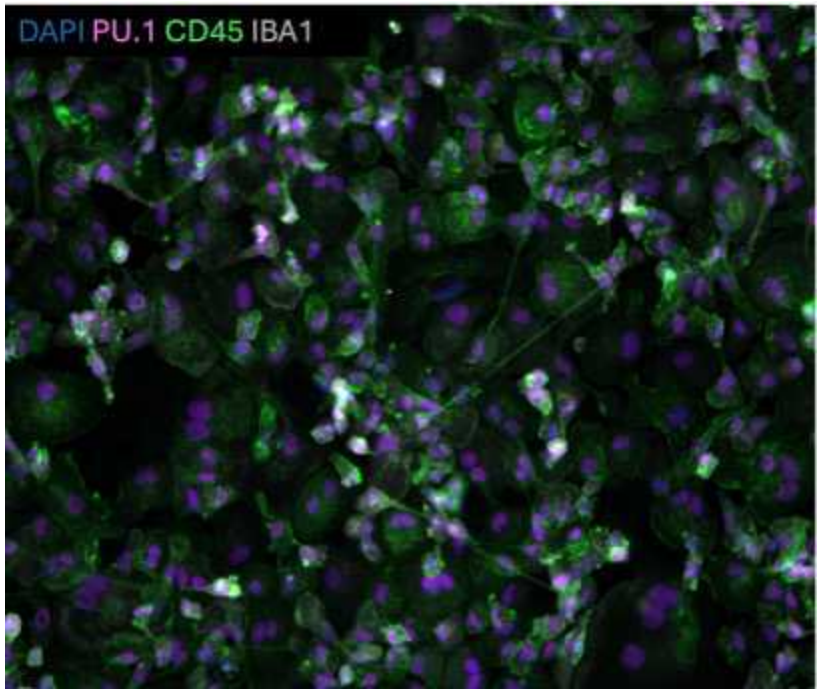
	Gene name	Primer_Fw	Primer_Rv
	P2RY12	TGCCAAACTGGGAACAGGACCA	TGGTGGTCTTCTGGTAGCGATC
	TMEM119	AGTCCTGTACGCCAAGGAAC	GCAGCAACAGAAGGATGAGG
	TREM2	ATGATGCGGGTCTCTACCAGTG	GCATCCTCGAAGCTCTCAGACT
	CSF1R	TCCAAAACACGGGGACCTATC	CGGGCAGGGTCTTTGACATA
	CX3CR1	CACAAAGGAGCAGGCATGGAAG	CAGGTTCTCTGTAGACACAAGGC
	IRF8	TGGCTGATCGAGCAGATTGACAGT	AAGGGATCCGGAACATGCTCTTCT
	PU.1	GAGCCCCCCACTGGAGGT	TGGTACAGGCGGATCTTCTTCT
	CD68	CGAGCATCATTCTTTCACCAGCT	ATGAGAGGCAGCAAGATGGACC
	AIF1/IBA1	CCCTCCAAACTGGAAGGCTTCA	CTTTAGCTCTAGGTGAGTCTTGG
	ITGAM/CD11b	GGAACGCCATTGTCTGCTTTCG	ATGCTGAGGTCATCCTGGCAGA
	CD44	GGACACCATGGACAAGTTTTGG	CACGTGGAATACACCTGCAAAG
	MERTK	CAGGAAGATGGGACCTCTCTGA	GGCTGAAGTCTTTCATGCACGC
	CD45	CTTCAGTGGTCCCATTGTGGTG	CCACTTTGTTCTCGGCTTCCAG
	CCR2	TGG CTG TGT TTG CTT CTG TC	TCT CAC TGC CCT ATG CCT CT
	GAPDH	TTGAGGTCAATGAAGGGGTC	GAAGGTGAAGGTCGGAGTCA
	ACTB_v2	ATGTGGCCGAGGACTTTGATTG	ATGGCAAGGGACTTCCTGTAAC

QC microglia by ICC

18 After 7 days in MMM, monolayer microglia should be positive for the following markers:

	Antibody	Cat. no	Company	Concentration
	CD45	304002	BioLegend	1:300
	PU.1	2258S	Cell Signaling	1:300
	IBA1	434200	Thermo Fisher	1:500



Protocol references

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