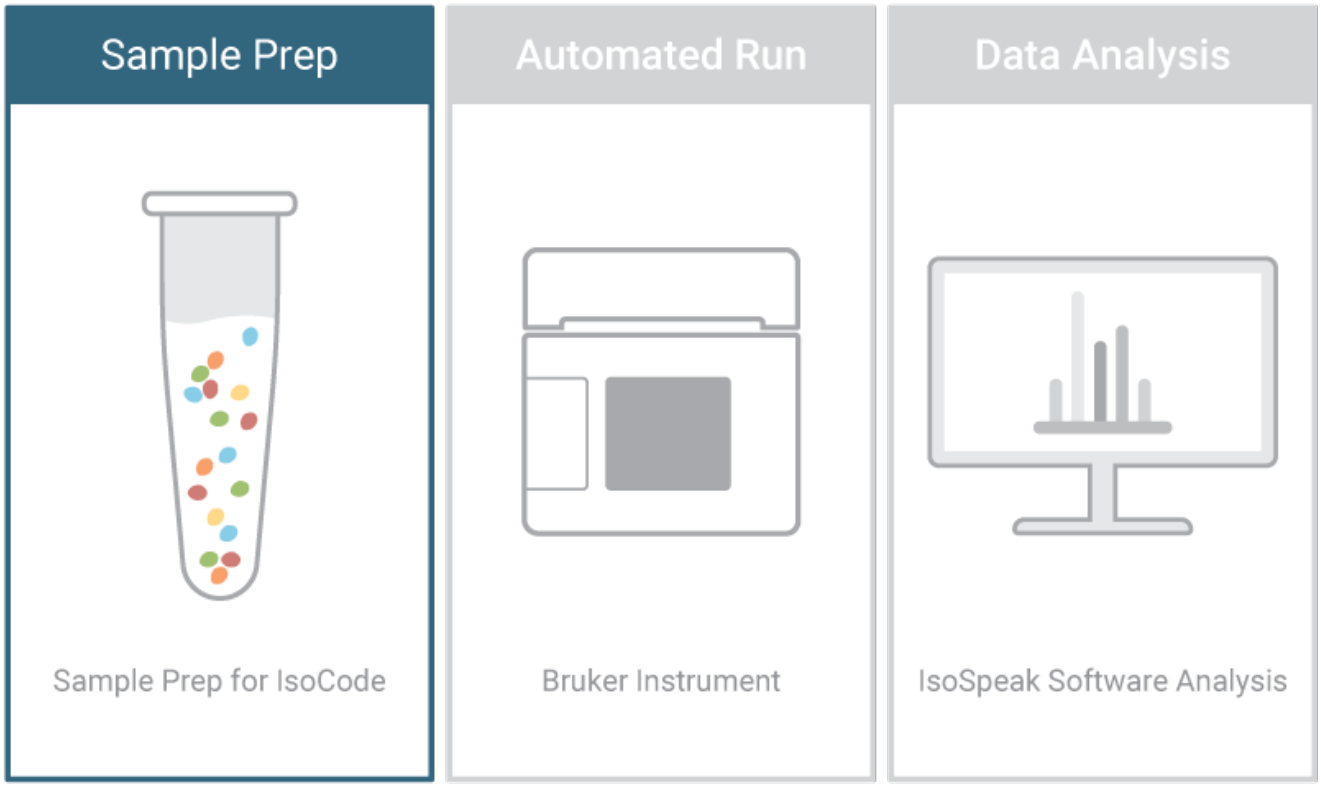
IsoCode Single-Cell Innate Immune: Human Monocyte-Derived Microglia-Like (MDMi) Cells Protocol

Ensure you achieve the maximum benefit from the Bruker systems and generate impactful data as quickly as possible



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

Key: ● TIP, ● CRITICAL, ● OPTIONAL

A. Overview

Overview of Protocol

Day 1: Cryopreserved cells are thawed and immediately enriched for monocytes.

Day 7: Stimulation of MDMi for 24 hours.

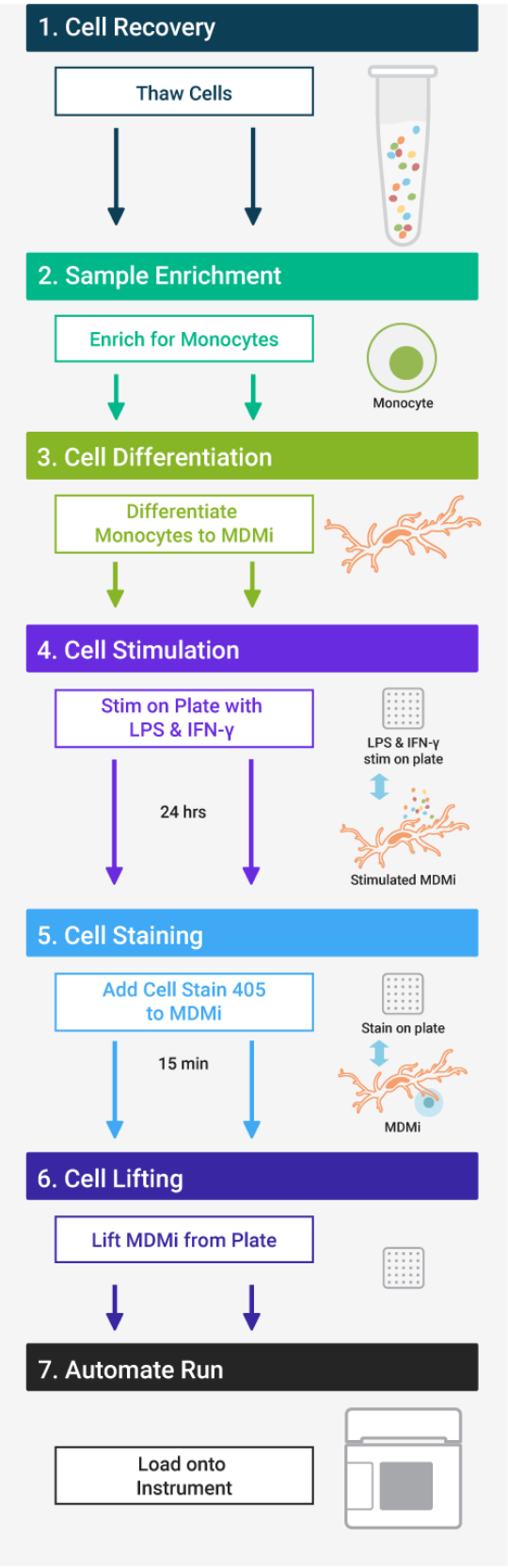
Day 8: Staining and Loading of MDMi onto IsoCode Chip.

NOTE:
This protocol outlines the standard method for thawing and culturing of human MDMi only and may not be valid for other species or cell types.

NOTE:
Cryopreserved PBMC utilized for this protocol should be stored for no longer than 3 months. Length of storage may have an impact on MDMi secretions.

NOTE:
This protocol requires a high number of monocytes to be plated due to cell death during the differentiation process. It is suggested to plate a minimum of 25 million monocytes for differentiation.

NOTE:
Using stains and protocols other than the included kit surface stains and protocols might result in failed runs. Stains and staining procedures not approved by Bruker will require validation prior to use. Please consider Bruker's IsoPACE program to assist in custom marker and protocol validation.



Key: ● TIP, ● CRITICAL, ● OPTIONAL
PRO-36 REV 5.0

Safety Warnings

- Read MSDS documents of all materials prior to use.

- Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.

Required Reagents, Consumables and Equipment

Table 1: Required Consumables Provided by Bruker

Item	Catalog Number	Quantity	Comment
IsoCode Kit	Please see website (https://brukercellularanalysis.com/) for available kits or talk to Bruker's Customer Service team for details	One chip per sample/cell type/condition	Subcomponents stored at 4°C and -20°C

IsoCode Kit Components

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- 50 mL Tubes containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
- 1 Bag of Disposable Reagent Sippers

IsoSpark IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- Cartridge containing Reagents 1, 2, 3, and 4

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 per box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
96 Well Plate Flat Bottom	N/A	Corning	353072
24 Well Plate Flat Bottom	N/A	Corning	3524
6 Well Plate Flat Bottom	N/A	Corning	353046
MACS LS Column	N/A	Miltenyi	130-042-401
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
Centrifuge Tubes*	50 mL	VWR	21008-242
Pipette tips (filtered)	10 µL Graduated Filter Tips 100 µL Graduated Filter Tips 1000 µL XL Graduated Filter Tips	USA Scientific	1181-3710 1183-1740 1182-1730
Pipette Tips (Filtered, Low-Adhesion)	100 µL Tips	Oxford Lab Products	OAR-100-SLF
Pipette Tips (Filtered, Low-Adhesion)	1000 µL XL Graduated Filter Tips	USA Scientific	1182-1830
Serological Pipette	2mL Pipette 5 mL Pipette 10 mL Pipette	USA Scientific	1072-0510 1075-0110 1071-0810
Lo-bind Microcentrifuge Tubes, sterile	1.5 mL	USA Scientific	4043-1081
Lo-Bind Microcentrifuge Tubes	5 mL	Sigma	EP0030108302
Syringe with BD Luer-Lok Tip	50 mL	VWR	BD309653
0.22 µm Syringe Filter with Acrylic Housing	N/A	VWR	76479-024
Fisherbrand Disposable PES Filter Units (0.20 µm)	500 mL	Fisher Scientific	FB12566504

*Bruker strongly recommends that low protein binding centrifuge tubes are used for cell culture work to ensure optimal cell pelleting.

Table 3: Required* Reagents Not Supplied by Bruker

Reagent	Stock Concentration	Source	Catalog Number
RPMI	1x	Fisher	MT10040CV
Penicillin-Streptomycin Solution	100x	Gibco	15140122
Amphotericin B (AmpB)	1x	Gibco	15290026
Phosphate buffered saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
GM-CSF	N/A	R&D Systems	215-GM-010/CF
β-NGF	N/A	Biolegend	577906
M-CSF	N/A	Biolegend	574806
CCL2	N/A	Biolegend	571406
IL-34	N/A	R&D Systems	256-GF-100
RoboSep buffer	1x	StemCell Tech	20104
Ficoll Paque Plus	N/A	GE Healthcare	17-1440-03
Miltenyi CD14 MicroBeads, Human	N/A	Miltenyi	130-050-201
Trypan Blue	0.4%	Gibco	15250-061
Bovine Serum Albumin (BSA), lyophilized powder	N/A	Sigma-Aldrich	A9647-10G
TrypLE Express Enzyme (1X), no phenol red	1x	Gibco	12604021
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
IFN-γ	N/A	R&D Systems	285-IF-100/CF
Reagent alcohol 70%	N/A	Lab Grade	N/A

*Reagents have been validated by Bruker and no alternatives may be used.

Table 4: Cell Staining Reagents

Test Material	Catalog Number	Color
Cell Stain 405	STAIN-1001-1	Violet

Table 5: Required Equipment

Equipment	Source	Catalog Number/Requirements
IsoLight, IsoSpark, or IsoSpark Duo Instrument	Bruker	ISOLIGHT-1000-1, ISOSPARK-1000-1, or ISOSPARK-1001-1
Culture Hemocytometer	(Fisher) Hauser Levy	02-671-55A
Hemocytometer Cover Glass	(Fisher) Hauser Levy	02-671-53
MidiMACS separator	Miltenyi	130-042-302
MACS MultiStand	Miltenyi	130-042-303

Table 6: General Equipment

Equipment	Requirements
Pipette	P10, P100, P200, P1000
Pipettor	Ability to pipette between 1 and 10 mL
Incubator	37°C, 5% CO_2
Tabletop Centrifuge	Temperature controlled*; swinging bucket rotor; ability to centrifuge 15 mL conical tubes, 50 mL conical tubes and 5 mL microcentrifuge tubes
Microcentrifuge	Temperature controlled*; fixed rotor; ability to centrifuge 1.5 mL microcentrifuge tubes
Mini centrifuge	Ability to spin micro sample sizes
Water Bath	Ability to heat to 37°C
Microscope	Inverted light microscope with 10x and 20x objectives
Vortex Mixer	Ability to vortex vials and microcentrifuge tubes; adjustable speed

*Temperature controlled centrifuges are required so that centrifuging steps can be conducted at room temperature without risk of overheating. Temperature on centrifuges should be set to 21°C.

B. Before Getting Started

1. Important Precautions

Read MSDS documents of all materials prior to use.

Working with Biohazardous Reagents

Please refer to your institute's guidelines and obtain proper training to handle potentially biohazardous samples. It is also strongly recommended that any lab personnel handling human samples be vaccinated against HBV if the individual does not have sufficient HBV antibody titer.

Additional precautions need to be taken when working with samples that potentially contain an EID agent:

1. Laboratory workers should wear standard PPE, including disposable gloves, protective eyewear, and laboratory coats.
2. Any procedure or process that cannot be conducted in the designated EID BSC should be performed while wearing gloves, gown, goggles and a fit tested N-95 mask.
3. Work surfaces should be decontaminated on completion of work with appropriate disinfectants. This includes any surface that potentially came in contact with the specimen (centrifuge, microscope, etc.).
4. All liquid waste produced in the processes must be treated to a final concentration of 10% bleach prior to disposal.

2. Reagents to Be Prepared Before Starting

Table 7: LPS Recipe

- CRITICAL: LPS has been validated for use by Bruker. Using alternative stimulant may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 1 mL	Vendor/Catalog
LPS	N/A	1 mg/mL	1 mg	Sigma/L2654-1MG
PBS	1x	1x	1 mL	Gibco/ 10010072

- CRITICAL: Prepare 10 µL LPS aliquots and freeze at -20°C for no longer than 2 months. Aliquots are single use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Table 8: Complete MDMi Media Recipe

- CRITICAL: Complete MDMi media has been validated for use by Bruker. Using alternative media may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 500 mL	Vendor/Catalog
Penicillin-Streptomycin-Solution	100x	1x	5 mL	Gibco/15140122
RPMI	1x	1x	495 mL	Fisher/MT10040CV

Note | Sterile-filter through 0.20 µm filter before use. Store complete MDMi media at 4°C aliquoted in 50 mL tubes and warm up to 37°C in water bath prior to use. If MDMi media in 50 mL tubes is not closed or stored properly, the media may turn fuchsia indicating that it is not acceptable for use. MDMi media when stored properly is a light peach color.

Table 9: Complete MDMi Differentiation Media Recipe

- CRITICAL: Complete MDMi differentiation media has been validated for use by Bruker. Using alternative media may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 500 mL	Vendor/Catalog
Penicillin-Streptomycin-Solution	100x	1x	5 mL	Gibco/15140122
Amphotericin B (AmpB)	250 µg/mL	2.5 µg/mL	5 mL	Gibco/15290026
RPMI	1x	1x	490 mL	Fisher/MT10040CV

Note | Sterile-filter through 0.20 µm filter before use. Store complete MDMi media at 4°C aliquoted in 50 mL tubes and warm up to 37°C in water bath prior to use. If MDMi media in 50 mL tubes is not closed or stored properly, the media may turn fuchsia indicating that it is not acceptable for use. MDMi media when stored properly is a light peach color.

Table 10: IFN-γ Recipe

- CRITICAL: IFN-γ has been validated for use by Bruker. Using alternative stimulant may result in failed runs. Please contact your Field Application Scientist for additional information.

Ingredient	Stock Concentration	Final Concentration	Amount for 500 µL	Vendor/Catalog
IFN-γ	N/A	200 µg/mL	100 µg	R&D Systems/285-IF-100/CF
Sterile deionized water	1x	1x	500 µL	N/A

- CRITICAL: Prepare 20 µL IFN-γ aliquots and freeze at -20°C for no longer than 3 months. Aliquots are single use only and are to be thawed immediately prior to their usage. If there is any remaining volume in an aliquot, do not refreeze but discard.

Additional Reagents to Be Prepared

NOTE: M-CSF concentration is lot specific. Follow vendor instructions to determine concentration. Add additional 1X PBS to obtain a final concentration of 100 µg/mL. 10 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: CCL2 concentration is lot specific. Follow vendor instructions to determine concentration. Add additional 1X PBS to obtain a final concentration of 200 µg/mL. 25 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: IL-34 concentration is lot specific. Follow vendor instructions to determine concentration. Add additional 1X PBS to obtain a final concentration of 200 µg/mL. 25 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: GM-CSF should be reconstituted in 100 µL of 1X PBS upon receipt from vendor. Final concentration is 100 µg/mL. 10 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: β-NGF should be reconstituted in 1 mL of 1X PBS upon receipt from vendor. Final concentration is 100 µg/mL. 5 µL aliquots are recommended and storage is at -20°C for up to 3 months.

NOTE: AmpB is recommended to be divided into 5 mL aliquots and storage is at -20°C for up to 12 months.

C. Protocol

Chapter 1: Getting Started

Kit Contents

IsoLight IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tube A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- 50 mL Tubes Containing Reagents 1, 2, 3, 4, 5, 6, 7, 8
- 1 Bag of Disposable Reagent Sippers

IsoSpark IsoCode Reagent Box (4°C)

- 15 mL Tube A
- 15 mL Tube B
- 1.5 mL Tubes A/B: Cocktail A in Micro-Tube (Green Cap) and Cocktail B in Micro-Tube (Red Cap)
- Cartridge containing Reagents 1, 2, 3, and 4

IsoCode Chip Set (-20°C)

- Boxes of IsoCode Chips (2 Per Box)
  - IsoSpark: 4 chip kits
  - IsoLight: 4 or 8 chip kits
- Cell Stain 405 [ordered separately]
- Cell Stain 405 Diluent (DMSO) [part of cell stain 405 kit]

Chapter 2: Thawing of Cryopreserved Cells

Materials Required

Complete MDMi Differentiation Media (37°C)
Cryopreserved PBMC
15 mL Centrifuge Tube
Lo-Bind Microcentrifuge Tube for Cell Count

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods

1. Pipette 5 mL of pre-warmed complete MDMi differentiation media into a 15 mL centrifuge tube, labeled *Thawed PBMC*.

2. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.
3. Quickly move vials into a water bath (37°C) to thaw. While thawing, swirl the vial in the water until a single ice crystal remains in the vial. Be sure to prevent (to the best of your ability) any of the water from the water bath from getting underneath the cap and into the sample.
4. When the sample is nearly thawed, remove the vial and immediately spray vial with 70% alcohol before bringing into the hood. It is important to allow the alcohol to evaporate before opening the vial.
5. Slowly pipette thawed cells into 5 mL of complete MDMi differentiation media in 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete MDMi differentiation media when pipetting, be careful to not create bubbles.
6. Take 1 mL of complete MDMi differentiation media and pipette into original thawed cell vial. Rinse inside the vial with the complete MDMi differentiation media to recover additional thawed cells. TIP: Insert tip into complete MDMi differentiation media, be careful to not create bubbles.
7. Draw up cell/complete MDMi differentiation media and pipette into the 15 mL centrifuge tube, labeled *Thawed PBMC*. TIP: Insert tip into complete MDMi differentiation media and pipette gently up and down. Be careful to not create bubbles.
8. Mix well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
9. Take a 10 µL aliquot of your cells and transfer to a Lo-Bind Microcentrifuge Tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
10. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
11. Proceed immediately to next chapter.

Chapter 3: CD14 Sample Enrichment and Differentiation

Materials Required

Complete MDMi Differentiation Media (37°C)
GM-CSF 100 µg/mL (-20°C)
β-NGF 100 µg/mL (-20°C)
M-CSF 100 µg/mL (-20°C)
CCL2 200 µg/mL (-20°C)
IL-34 200 µg/mL (-20°C)
T75 Flasks, T25 Flasks, 6 Well Plate Flat Bottom, 24 Well Plate Flat Bottom, or 96 Well Plate Flat Bottom
RoboSep Buffer (4°C)
Miltenyi CD14 Microbeads, Human, 2 mL (4°C)
MACS LS Column
Prepared Cells from Chapter 2
Enrichment Kit:
- MACS Metal Plate/Magnet Kit
- 3 x 15 mL Centrifuge Tubes (*Discard, Flow Through, CD14 fraction*)
- Lo-Bind Microcentrifuge Tube for Post-Enrichment CD14

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods

1. Remove the centrifuged cells and check for cell pellet.
2. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.

CRITICAL: For every 1 x 10^7 cells, resuspend in 80 µL RoboSep (4°C) and 20 µL of CD14 beads (4°C).

3. Add 80 µL of cold RoboSep to 15 mL centrifuge tube containing 1 x 10^7 or fewer cells.
4. Vortex the Miltenyi CD14 Microbeads at a slow speed for 10 seconds.
5. Add 20 µL of Miltenyi CD14 Microbeads and mix well by gently pipetting up and down 5 times.

TIP: Make sure to eliminate clumps so that beads are evenly distributed among cells. Be careful not to create bubbles.

6. Incubate in refrigerator (4°C) for 15 minutes. TIP: Don't incubate on ice as increased incubation times may be required.
7. After 15 minutes, add 2 mL of cold RoboSep. TIP: Not necessary to mix for this step.
8. Centrifuge cells for 10 minutes at 300 rcf.

TIP: Keep RoboSep in refrigerator during enrichment process.

9. Set up MACS sorting by setting metal plate in tissue culture hood and placing magnet on metal plate. Place LS column in magnet with wings facing out and align the 15 mL centrifuge tube labeled "Discard" under the LS column. CRITICAL: LS Column should not touch the tubes.
10. After cells are centrifuged, check for cell pellet and continue with MACS separation.
11. Aspirate RoboSep from cell pellet. TIP: Since it is a small volume, use pipette for this step to prevent accidental aspiration of the cell pellet.
12. For 1 x 10^8 or fewer cells, resuspend with 500 µL of cold RoboSep.
    a. Mix well to resuspend by gently pipetting up and down 5 times. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.

CRITICAL: Be careful not to let column dry out. Do not add liquid when there is already liquid in the LS Column.

13. Start with the LS column over the "Discard" tube, add 3 mL of cold RoboSep to LS Column. CRITICAL: Be careful not to create bubbles or touch sides of LS column. Let all the RoboSep flow through before moving on to the next step. As a reminder, be careful to not let the column dry out.
14. Unscrew and keep cap for "Flow Through" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.
15. When the last drop falls through to the "Discard" tube, move the rack over so the LS column is over the "Flow Through" tube. CRITICAL: Be careful not to let column dry. If there is one drop remaining that will not fall, move on to the next step.
16. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
17. Mix cell suspension by gently pipetting up and down 5 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
18. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching sides of the column.
19. Wash 3 times with 3 mL of cold RoboSep.
    a. First wash: Rinse inside walls of cell suspension tube with 3 mL of cold RoboSep before transferring the mixture to LS Column. NOTE: This is to retrieve any cells that have been left behind.
        i. Pipette all the mixture into LS Column after last drop passes through or does not fall from step 18. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
    b. Second wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
    c. Third wash: Add 3 mL of RoboSep into LS Column after last drop passes through or does not fall. CRITICAL: Be careful not to let LS Column dry out or allow pipette to touch sides.
20. After the last drop of the third wash passes through or does not fall, remove the LS Column carefully from the magnet, and place carefully on the tube labeled for "CD14 fraction".
21. Cap the "Flow Through" tube. Keep if performing further enrichments. Otherwise, discard.
22. Add 5 mL of cold RoboSep to the LS column. CRITICAL: Be careful not to touch the sides.

- 23. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
24. Set LS Column back on the "CD14 fraction" tube.
- CRITICAL: Do not allow the plunger to interact with external contaminants. It will be used for one more step.
25. Loosen up plunger. Remove plunger briefly from column and hold in one hand.
26. Add another 2 mL of cold RoboSep to the LS Column.
- 27. Take plunger, smoothly push down on the plunger to push the RoboSep buffer through the LS Column. TIP: Lift up at the end of the plunging action so that the liquid does not splash back onto the LS Column tip.
28. Discard LS Column and plunger.
29. Centrifuge "CD14 fraction" tube for 10 minutes at 300 rcf.
30. After cells are centrifuged, check for cell pellet.
- 31. Aspirate RoboSep buffer from "CD14 fraction" tube. TIP: Be careful to not aspirate cell pellet.
- 32. Use pipette to aspirate the remaining supernatant from the tube. TIP: Be careful to not aspirate cell pellet.
- 33. Add 1 mL complete MDMi differentiation media to "CD14 fraction" and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.
- 34. Aliquot 10 µL of the "CD14 fraction" into a Lo-Bind Microcentrifuge tube for cell counting. CRITICAL: See Appendix D1 for cell counting instructions.
- 35. Centrifuge cells for 10 minutes at 300 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
36. While cells are centrifuging prepare 100 mL of complete MDMi differentiation media with cytokines in two 50 mL centrifuge tubes.
    a. Dilute 5 µL of GM-CSF (100 µg/mL) per 50 mL of complete MDMi differentiation media. Final concentration is 10 ng/mL.
    b. Dilute 5 µL of β-NGF (100 µg/mL) per 50 mL of complete MDMi differentiation media. Final concentration is 10 ng/mL.
    c. Dilute 5 µL of M-CSF (100 µg/mL) per 50 mL of complete MDMi differentiation media. Final concentration is 10 ng/mL.
    d. Dilute 25 µL of CCL2 (200 µg/mL) per 50 mL of complete MDMi differentiation media. Final concentration is 100 ng/mL.
    e. Dilute 25 µL of IL-34 (200 µg/mL) per 50 mL of complete MDMi differentiation media. Final concentration is 100 ng/mL.
    f. Use serological pipette to mix thoroughly.
37. Remove the centrifuged cells and check for cell pellet.
- 38. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.

39. Using complete MDMi differentiation media with cytokines, prepared in step 36, resuspend all the CD14 cells to a cell concentration of 2 x 10^6 cells/mL.
40. Based on half of the cell number from step 35, determine the appropriately sized plate or flask for use based on the cell number range in Table 11. **NOTE: The total cell number is divided in half in preparation for splitting the cells into an unstimulated and stimulated condition in a later step.**
41. Seed 2 x 10^6 cells/mL CD14 cell suspension into appropriately sized flask or plate determined in step 40.
    a. Seed one half of the CD14 cell suspension into well(s) or flask for the stimulated condition.
    b. Seed the other half of the CD14 cell suspension into another flask or well(s) on another plate for the unstimulated condition.
    c. Label one flask or plate as "Stimulated MDMi" and the other "Unstimulated MDMi".
42. Add additional fresh complete MDMi differentiation media with cytokines to each well or flask to reach the final volume in Table 11.

    NOTE: For example, if there are 3.2 x 10^7 CD14 cells then one half would be 1.6 x 10^7 CD14 cells. 1.6 x 10^7 CD14 cells is not within any range in Table 11 and therefore will need to be divided into multiple containers for both the stimulated and unstimulated conditions. Two T75 flasks would be seeded with 4 mL each of 2 x 10^6 cells/mL cell suspension (8 x 10^6 cells/flask) for the unstimulated condition. Two T75 flasks would be seeded with 4 mL each of 2 x 10^6 cells/mL cell suspension (8 x 10^6 cells/flask) for the stimulated condition. Additional complete MDMi differentiation media would be added to each flask to bring the final volume in each flask to 30 mL.

Table 11: Plate/Flask and Volume for CD14 Fraction Seeding

Plate/Flask	Range of Cell Number (2.5 x 10^5 cells/cm^2 – 5 x 10^5 cells/cm^2)	Final Volume in Flask or Well (mL)
96 Well Plate	7.5 x 10^4 – 1 x 10^5 cells/well	0.2
24 Well Plate	2.5 x 10^5 – 3 x 10^5 cells/well	1
6 Well Plate	1 x 10^6 cells/well	4
T25 Flask	2.5 x 10^6 – 3 x 10^6 cells/flask	10
T75 Flask	7.5 x 10^6 – 8 x 10^6 cells/flask	30

43. Incubate plates or flasks for 7 days at 37°C, 5% CO_2 without a media change. **NOTE: Do not disturb cells during differentiation process.**

Chapter 4: Cell Stimulation

Materials Required

Complete MDMi Media (37°C) LPS 1 mg/mL (-20°C) IFN-γ 200 µg/mL (-20°C) 2 x 1.5 mL Lo-Bind Microcentrifuge Tubes *(LPS Working Stock, IFN-γ Working Stock)* Differentiated MDMi in Flasks or Plates

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods

1. Thaw a vial of stock 1 mg/mL LPS and a vial of stock 200 µg/mL IFN-γ at ambient temperature.
2. Vortex LPS and IFN-γ for 5 seconds.
- 3. Prepare a working stock of LPS. Add 10 µL of 1 mg/mL LPS to 990 µL complete MDMi media. Final concentration of LPS working stock is 10 µg/mL. CRITICAL: Working 10 µg/mL LPS stock should be made fresh. If there is any remaining 1 mg/mL LPS stock, discard and do not re-freeze.
- 4. Prepare a working stock of IFN-γ. Add 20 µL of 200 µg/mL IFN-γ to 980 µL complete MDMi. Final concentration of IFN-γ working stock is 4 µg/mL. CRITICAL: Working 4 µg/mL IFN-γ stock should be made fresh. If there is any remaining 200 µg/mL IFN-γ stock, discard and do not re-freeze.
- 5. Vortex LPS working stock and IFN-γ working stock for 10 seconds. TIP: Ensure contents are well-mixed.
6. Prepare complete MDMi unstimulated media.
    a. Set aside a 50 mL centrifuge tube of complete MDMi media and label it "Complete MDMi Unstimulated". This MDMi media will serve as the complete MDMi media used for the unstimulated (negative control) condition.
7. Prepare 50 mL "Complete MDMi Stimulated" media by supplementing complete MDMi media with LPS and IFN-γ.
    a. Label a 50 mL centrifuge tube with complete MDMi media "Complete MDMi Stimulated".
    b. Remove and discard 1 mL of complete MDMi media from the 50 mL centrifuge tube labeled "Complete MDMi stimulated".
    c. Add 0.5 mL of LPS (100 µg/mL working stock concentration) to complete MDMi media. Final concentration is 100 ng/mL.
    d. Add 0.5 mL of IFN-γ (4 µg/mL working stock concentration) to complete MDMi Media. Final concentration is 40 ng/mL.
    e. Use serological pipette to mix thoroughly.
8. Take MDMi out of the incubator.

9. Observe plates or flasks under light microscope, using a 10X objective, to ensure cells are exhibiting MDMi morphology. See Figure 1.

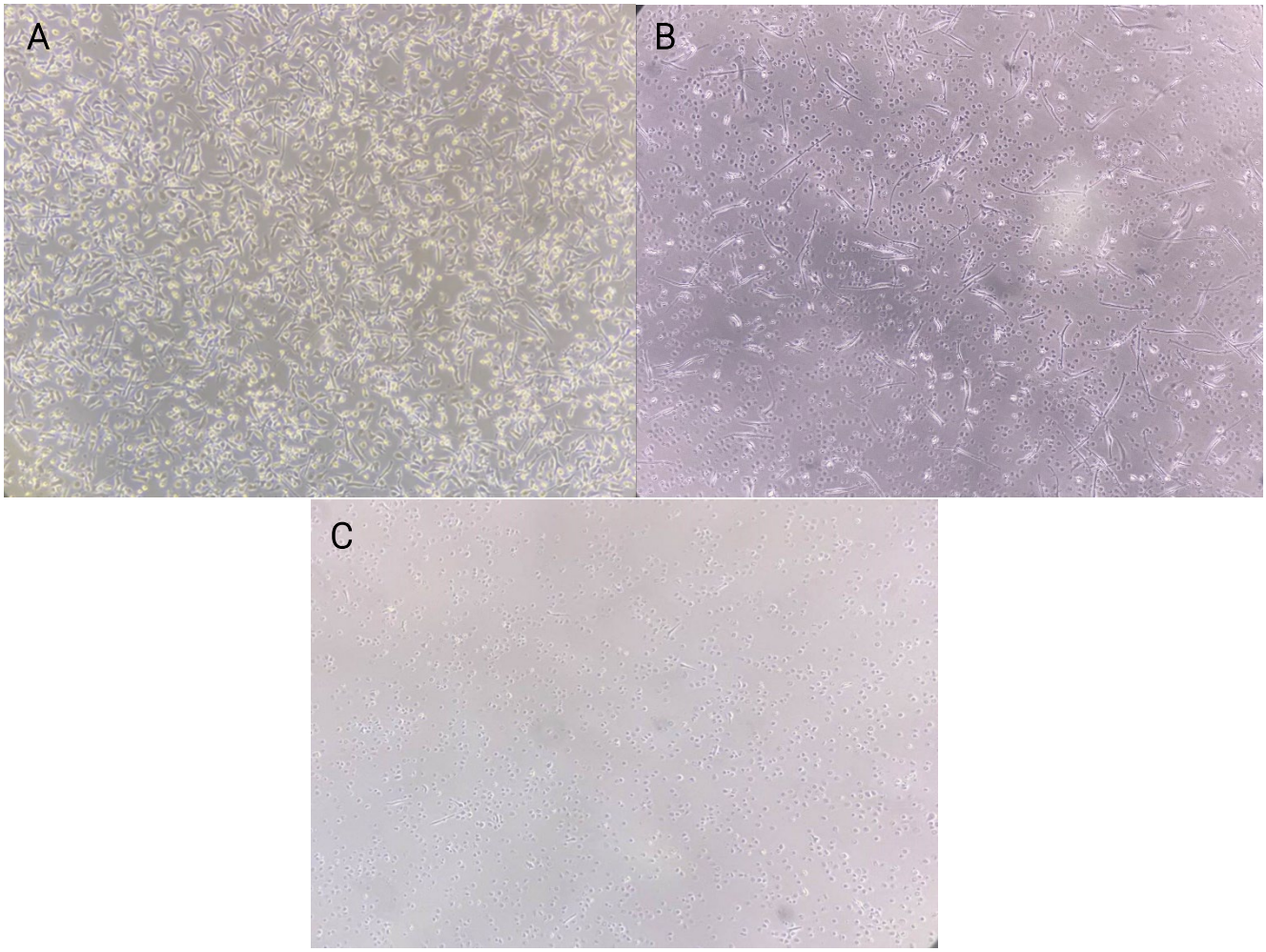


Figure 1. Example cell morphologies at day 7. (A) An example of donor cells that differentiate extremely well into MDMi. (B) An example of donor cells that differentiated into MDMi. (C) An example of donor cells that did not respond to differentiation. Example cells shown in (A) and (B) are acceptable for use in this protocol.

NOTE: Differentiated MDMi should have long and ramified cell morphology and present spiny processes. A heterogenous mixture of differentiated MDMi, monocyte or macrophage-like cells, and dead cells is expected after differentiation. Bright circles are monocyte or macrophage-like cells. These cells are the minority. Dark circles are monocytes that did not survive the differentiation. Dead cells will remain on the plate or flask or will not pellet in later steps.

10. Aspirate supernatant from flasks or plates. CRITICAL: Be careful not to disturb adherent MDMi on flask or plate.

11. Add "Complete MDMi Unstimulated" media to flask or wells labeled for the unstimulated condition according to volumes in Table 12.

12. Add "Complete MDMi Stimulated" media to flask or wells labeled for the stimulated condition according to volumes in Table 12.

Table 12: Plate/Flask and Volume for Adding Media

Plate/Flask	Range of Cell Number (5×10^5 cells/cm^2)	Volume per Flask or Well (mL)
96 Well Plate	$7.5 \times 10^4 - 1 \times 10^5$ cells/well	0.2
24 Well Plate	$2.5 \times 10^5 - 3 \times 10^5$ cells/well	0.5
6 Well Plate	1×10^6 cells/well	2
T25 Flask	$2.5 \times 10^6 - 3 \times 10^6$ cells/flask	5
T75 Flask	$7.5 \times 10^6 - 8 \times 10^6$ cells/flask	15

13. Incubate plates or flasks for 24 hours at 37°C, 5% CO_2.

Chapter 5: Cell Staining

Materials Required

LPS/IFN-γ Stimulated & Unstimulated MDMi from Chapter 4
2 x 15 mL Centrifuge Tubes (*Stimulated Bulk Assay, Unstimulated Bulk Assay*)
3 x Lo-Bind Microcentrifuge Tubes (*Cell Count, Stimulated Bulk Assay, Unstimulated Bulk Assay)*
Sterile 1X PBS (Room Temperature)
Cell Stain 405 (-20°C)
Cell Stain 405 Diluent (DMSO) (-20°C)

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods

1. Prepare cell stain 405 stock.
    a. Thaw tube of cell stain 405 diluent (DMSO) at room temperature.
    b. Spin tubes of cell stain 405 and cell stain 405 diluent (DMSO) in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
    c. Add 20 µL of cell stain 405 diluent (DMSO) directly to the tube of cell stain 405. Pipet up and down 15 times gently to resuspend.

- CRITICAL: Cell stain 405 must be prepared fresh. Discard remaining stain—do not store.

- 2. Prepare stain master mix by diluting 3 µL of cell stain 405 into 1 mL of 1X PBS in a Lo-Bind microcentrifuge tube (1:333 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all cell stain

405 has been released. Depending on plate or flask used, additional tubes of stain master mix may need to be prepared. CRITICAL: Failure to follow these steps will negatively impact cell counts.

a. With a P1000 set to 500 µL, gently pipette the stain master mix up and down **15 times**.

b. **Gently vortex** the stain master mix for **5 seconds**.

c. **Ensure master mix is mixed well before adding stain to cells.**

3. Remove plates or flasks labeled "Stimulated MDMi" and "Unstimulated MDMi" from incubator.

4. Remove supernatant from plates or flasks with a P1000 pipette. CRITICAL: Ensure all media is removed.

OPTIONAL: Transfer supernatant to 15 mL centrifuge tube. Pool wells if there are replicates. Centrifuge supernatant for 5 minutes at 200 rcf set to a brake of 1. Transfer supernatant to 1.5 mL Lo-Bind microcentrifuge tube. Supernatant may be stored at -80°C for bulk assay.

5. Gently rinse over the surface of the plate or flask with 1X PBS using a P1000.
   - For T75 flask use 5 mL
   - For T25 flask use 2 mL
   - For 6 well plate use 1 mL
   - For 24 well plate use 500 µL
   - For 96 well plate use 100 µL

6. Rock flask or plate back and forth to ensure any residual media is rinsed off.

CRITICAL: Failure to remove excess media will result in poor staining.

7. Remove 1X PBS wash from flask or plate with a P1000 pipette.

8. Gently remix stain master mix.

CRITICAL: Failure to remix stain master mix will result in poor staining.

9. Add stain master mix to plate or flask according to the volumes shown in Table 13.

Table 13: Plate/Flask and Volume for Adding Stain Master Mix

Plate/Flask	Volume of Stain Master Mix per Flask or Well (mL)
96 Well Plate	0.1
24 Well Plate	0.5
6 Well Plate	1
T25 Flask	2
T75 Flask	5

10. Incubate for 7.5 minutes at 37°C in the dark. **NOTE: Ensure plates or flasks are completely flat. Do not stack plates or flasks during incubation.**

11. Gently rock the plate or flask back and forth to ensure even coating of the stain master mix.

12. Incubate for an additional 7.5 minutes at 37°C in the dark. NOTE: Ensure plates or flasks are completely flat. Do not stack plates or flasks during incubation.

Chapter 6: Chip Thaw

Materials Required

IsoCode Chips in Vacuum Sealed Bag (-20°C)

Methods

- 1. Take vacuum sealed bag containing IsoCode chips from -20°C. CRITICAL: Chips must stay sealed until Chip Loading (Chapter 8).
2. Place on bench to thaw at ambient temperature 30 - 60 minutes prior to use.
3. While chips thaw, prepare liquid reagents and setup in the Bruker instrument. Refer to your instrument's system guide for detailed instructions.

Chapter 7: Cell Lifting

Materials Required

Stained Stimulated & Unstimulated MDMi from Chapter 5
50 mL Centrifuge Tube
2 x 1.5 mL Lo-Bind Microcentrifuge Tubes (*Unstimulated Cell Count, Stimulated Cell Count)*
5 mL Lo-Bind Microcentrifuge Tubes *(Unstimulated MDMi, Stimulated MDMi)*
BSA
Sterile 1X PBS (Room Temperature)
0.22 µm Syringe Filter
50 mL Syringe with BD Luer-Lok Tip
P100 Low-adhesion Pipette Tips
Complete MDMi Media (37°C)
TrypLE Express (Room Temperature)

All the following steps should take place in a sterile tissue culture hood. Centrifugation steps should be performed at 21°C.

Methods

- CRITICAL: Use only low-adhesion pipette tips and centrifuge tubes when working with MDMi for this chapter.

1. Prepare 50 mL of 1% BSA in PBS.
    a. Add 500 µg of BSA into 49 mL of 1X PBS in a 50 mL centrifuge tube.
    b. Rotate solution until BSA powder is dissolved and then bring final volume up to 50 mL with 1X PBS.

c. Sterile-filter through 0.22 µm filter.

2. Remove stained MDMi from incubator.

● 3. Aspirate stain master mix from flasks or plates. CRITICAL: Be careful not to disturb adherent MDMi on flask or plate.

4. Detach adherent cells from flasks or plates by gently adding 1X TrypLE.

- For T75 Flask add 3 mL
- For T25 Flask add 1 mL
- For 6 well plate add 800 µL
- For 24 well plate add 500 µL
- For 96 well plate add 100 µL

5. Gently rinse the cells with 1X TrypLE using a serological pipette.

6. Incubate the cells with 1X TrypLE for 10 minutes at 37°C to allow cells to detach. NOTE: Incubating cells for additional time does not improve cell detachment and may cause a decrease in viability.

7. Gently rinse the cells with 1X TrypLE using a serological pipette.

● a. Rinse in a "Z-like" pattern across the flask or well. CRITICAL: Cells will not lift unless the stream from the serological pipette hits the cells directly.

8. Observe wells or flask under light microscope to determine if at least 80% of cells have been lifted from the plate or flask. If not, repeat step 7.

9. Collect cells/1X TrypLE mixture and transfer to a 5 mL Lo-Bind centrifuge tube.

a. From the wells or flask(s) labeled "Unstimulated MDMi", transfer the cells/1X TrypLE mixture to a 5 mL Lo-Bind centrifuge tube labeled "Unstimulated MDMi". NOTE: More than one 5 mL Lo-Bind centrifuge tube may need to be used depending on number of flasks or plates and volume.

b. From the wells or flask(s) labeled "Stimulated MDMi", transfer the cells/1X TrypLE mixture to a 5 mL Lo-Bind centrifuge tube labeled "Stimulated MDMi". NOTE: More than one 5 mL Lo-Bind centrifuge tube may need to be used depending on number of flasks or plates and volume.

10. Gently rinse plate or flask with 1% BSA in PBS.

- For T75 Flask add 2 mL
- For T25 Flask add 2 mL
- For 6 well plate add 800 µL
- For 24 well plate add 500 µL
- For 96 well plate add 100 µL

11. Observe wells or flask under light microscope to ensure the majority of cells remaining after rinsing with TrypLE have been removed from the plate or flask. NOTE: Some cells will not be lifted and will remain on the plate or flask.

12. Gently remove 1% BSA in PBS from plate or flask.
    a. From plate or flask labeled "Unstimulated MDMi", transfer 1% BSA in PBS to 5 mL Lo-Bind centrifuge tube labeled "Unstimulated MDMi".
    b. From plate or flask labeled "Stimulated MDMi", transfer 1% BSA in PBS to 5 mL Lo-Bind centrifuge tube labeled "Stimulated MDMi".
13. Centrifuge 5 mL Lo-Bind centrifuge tubes for 5 minutes at 200 rcf set to a brake of 1.
14. Remove the centrifuged cells and check for cell pellet.
15. If cells appear clumpy or if there are cells still in suspension, centrifuge 5 mL Lo-Bind centrifuge tubes again for 5 minutes at 200 rcf set to a brake of 1. Otherwise, proceed to step 17.
16. Remove the centrifuged cells and check for cell pellet.
17. Aspirate supernatant. TIP: Be careful not to aspirate the cells.
    a. Use pipette to aspirate remaining supernatant.
18. Resuspend cell pellet in each 5 mL Lo-Bind centrifuge tube with 100 µL of complete MDMi media using a low-adhesion P100 pipette tip. NOTE: Pool suspensions from Lo-bind microcentrifuge tubes if there are replicates.

    NOTE: The cells can be sticky and challenging to resuspend. Resuspend cell pellet as much as possible and use pipette tip to break up clumps.
19. Take a 10 µL aliquot of your cells using a P100 with a low-adhesion tip set to 10 µL. Transfer to a Lo-Bind microcentrifuge tube for cell counting. Count cells using a hemocytometer and determine percent of viable cells as described in Appendix D1. TIP: Cell counting can be done while cells are incubating.
20. Using cell count from step 19, increase volume of each cell suspension with complete MDMi media to a cell density of **7.5 x 10^5 cells/mL**. Proceed to Chapter 8.

Chapter 8: Chip Loading

Materials Required (Pre-prepared)

Pre-Thawed IsoCode Chips in Vacuum Sealed Bag from Chapter 6
P100 Low-adhesion Pipette Tips
Stained MDMi Cells at 7.5 x 10^5 cells/mL

Methods

1. Remove IsoCode chips from vacuum sealed bag and place on a flat surface. CRITICAL: Keep protective blue film on bottom of chip.
2. Resuspend "Stimulated MDMi" and "Unstimulated MDMi" tubes by gently pipetting up and down using a P100 with a low-adhesion tip. Immediately proceed to chip loading. CRITICAL: Pipet to mix 30 times to reduce cell clumping. Be careful not to create bubbles.

- 3. Pipette 40 µL of cell suspension into IsoCode chip using a P100 with a low-adhesion tip. CRITICAL: Be careful not to create bubbles. Insert pipette tip vertically into inlet port until tip lightly touches bottom, and slowly pipette 40 µL into inlet port. Be careful not to eject second step of pipette–it will cause bubbles.
4. Let IsoCode chips sit for one minute on a flat surface.
- 5. Check bottom of chip to ensure liquid has entered the chip. TIP: If liquid has not flowed, tap IsoCode chip on flat surface lightly.
6. When inserting IsoCode chip into instrument, make sure the logo is facing up and towards you with the magnet facing the instrument. Take the blue film off while inserting each IsoCode chip into the instrument.

   NOTE: Please refer to your instrument's loading instructions for details.

D: Appendix

D1 Protocol: Cell Quantification & Viability

Materials Required

Hemocytometer
10 µL aliquot of cells
P100 Low-adhesion Pipette Tips
Trypan Blue

NOTE: Automated cell counters can be used in this protocol EXCEPT prior to loading cells on chip due to spectral overlap of the stains. Manual cell counting is required prior to loading on the chip.

NOTE: To obtain an accurate representation of cell viability, cells should be counted within 15 minutes of staining as cell viability will drop over time because Trypan Blue is toxic.

1. Quick spin the Trypan Blue to pellet potential debris. Remove aliquot from the top of Trypan Blue.
2. Using a P100 pipette with a low-adhesion tip, add equal volume of Trypan blue solution to 10 µL of sample. Mix gently to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
3. Load onto hemocytometer. CRITICAL: Be careful not to overfill or create bubbles.
4. Count and record viable (clear) and dead cells (blue) of all four 16-square corners.

CRITICAL: If more than 200 cells/16 squares were counted, repeat count using a 1:5 or 1:10 dilution with complete MDMi media or complete MDMi differentiation media using a fresh sample aliquot. If count is occurring in Chapter 3 or before use MDMi differentiation media. If count is occurring in Chapter 4 or after use complete MDMi media.

5. Calculate the concentration of cells as follows:
    a. Concentration (cells/mL) = Average per square cell count x 10^4 x dilution factor
6. Calculate the number of cells as follows:
    a. Number of cells = Cell concentration (cells/mL) from D.1.5 x total volume of cell suspension (mL)
7. Calculate percent viable cells:
    a. % Viable cells = 100 x number of viable cells / [number of viable cells + number of dead cells]

Troubleshooting & References

Please contact Support at 844-476-7539 (toll free) or 475-221-8402 or email support@isoplexis.com with specific troubleshooting questions.

Problem	Possible Reason	Solution
Low quality cell count on chip *Cell Counting & Concentration related*	• Recommended cell concentrations not used • Issue with Cell Counting procedure • Trypan Blue may have debris • Trypan Blue is toxic • Poor cell removal from plate • Low-adhesion pipette tip was not used	• Use recommended cell concentrations during incubation (**Chapter 4**) • Use appropriate dilutions recommended in **Appendix D1**. • Do a recount if initial count does not seem accurate • Quick spin Trypan Blue to pellet potential debris, remove aliquot from top of Trypan Blue. Start with fresh aliquot of Trypan Blue. • Count within 15 minutes of staining cells • Thoroughly rinse cells in well or flask with pipette using low-adhesion tip prior to transferring to tube (**Chapter 7**) • Use low-adhesion pipette tips as recommended in **Appendix D1**
Low quality cell count on chip *Stain Process related*	• Use of media other than the recommended media in protocol which could interact with cell stain • Use of stains not recommended in protocol • Recommended stain concentration, incubation time and/or incubation temperature not used • Cell stain 405 not stored at -20°C prior to use • Stain was not evenly distributed • Media not completely removed from cells prior to staining	• Use complete MDMi media and complete MDMi differentiation media following recipes in **Tables 8 and 9** • Use Bruker provided validated stain (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapter 5.** • Use only freshly prepared cell stain 405 per **Chapter 5** • Ensure flask or plate is completely flat and not stacked in incubator during stain incubation. • Ensure all media is removed from cells in **step 5.4**

Low quality cell count on chip *Technique Detail related*	• Bubbles loaded onto chip, especially at Chip Loading
	• Detection of potential artifacts such as debris, cell clumping, inefficient enrichment possibly due to:
	  ○ Pipetting wrong concentration
	  ○ Not fully inserting column into MACS separator
	  ○ Reagents not stored at recommended temperatures
	• Recommended number of cells not loaded on chip
	• Cell pellet or cells lost during centrifuging
		• Follow Critical step in **Chapter 8 step 3** to avoid introduction of bubbles on chip
		• Ensure use of a sterile space to reduce introduction of potential contaminants. Use dedicated pipettes, tips, and tubes for sterile work. Pipette up and down gently and throughout protocol to reduce clumps.
		• Follow closely the Critical steps and tips in **Chapter 3** (CD14 Sample Enrichment)
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 8**)
		• Use low protein binding centrifuge tubes

Key: ● TIP, ● CRITICAL, ● OPTIONAL

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Viability related*	• Leaving thawed cells in DMSO for an extended period • Low viable cells due to low viability input sample and lack of utilization of Ficoll-Paque • Decreased viability due to cell shock • Decreased viability due to scraping	• After thaw, quickly transfer cells from DMSO to complete MDMi differentiation media to ensure viability of cells. • Verify viability of cells is above 80% to ensure protocol is being performed with the highest quality of cells. • Use reagents at recommended temperatures (i.e. always use warmed media [37°C]) • Do not scrape to lift cells
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended LPS and IFN-γ stimulation concentration was not used • Recommended LPS and IFN-γ stimulation duration was not used • Expired LPS or expired IFN-γ used • LPS or IFN-γ working sticks were not vortexed prior to use	• Use LPS and IFN-γ concentrations listed in Chapter 4 • Use LPS and IFN-γ stimulation duration listed in Chapter 4 • Do not use LPS stock older than 2 months • Do not use IFN-γ stock older than 3 months • Use required vendors as listed in Table 3 • Follow LPS and IFN-γ preparation instructions listed in Chapter 4
Low/no differentiation of cells observed	• Recommended differentiation cytokine concentrations were not used • Recommended differentiation duration was not followed • Expired differentiation cytokines used • Use of improperly stored media • Cells were disturbed during differentiation	• Use differentiation cytokine concentrations listed in Chapter 3 step 36 • Use differentiation duration listed in Chapter 3 • Do not use differentiation cytokines older than 3 months • Only use media that is a light peach color. Do not use media that is fuchsia. • Ensure flask or plate is not disturbed during differentiation