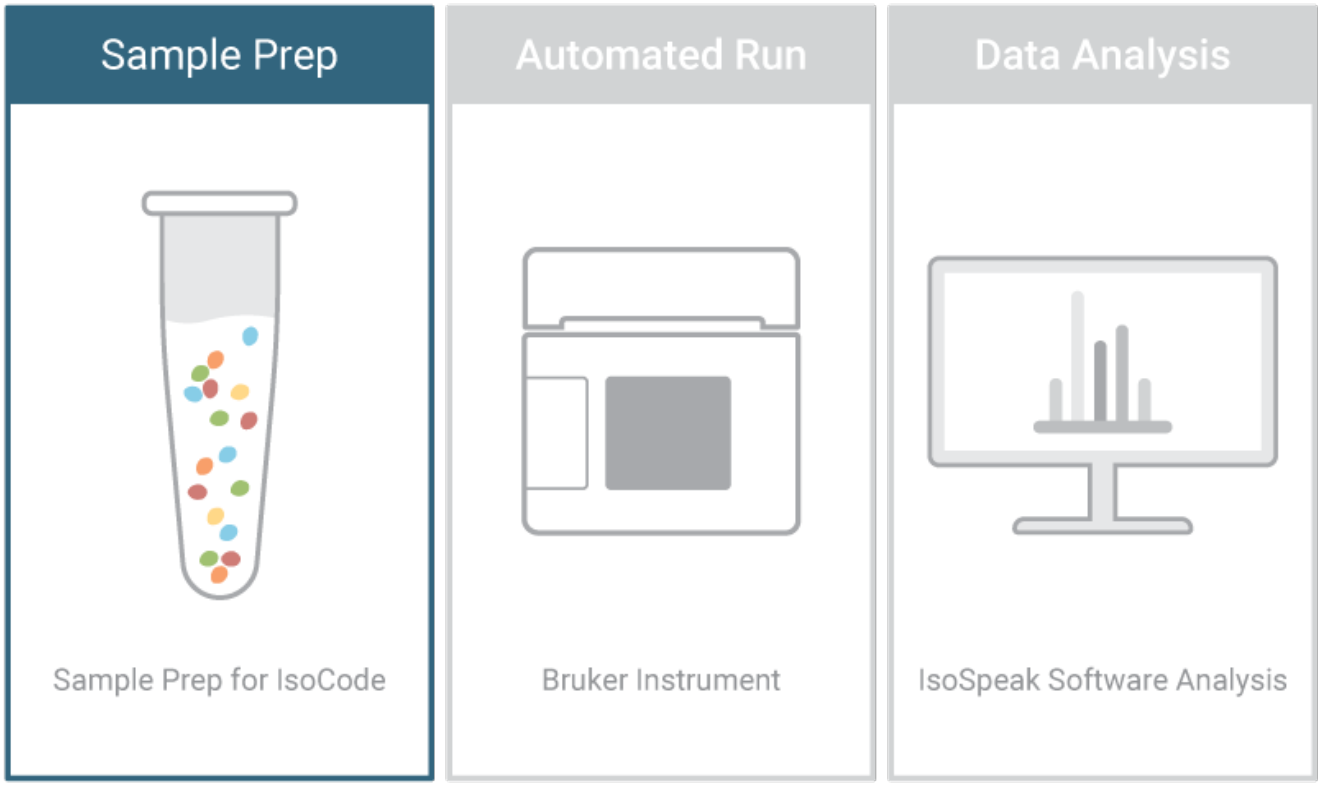
IsoCode Single-Cell Innate Immune: Mouse Monocyte Protocol

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



Key: ● TIP, ● CRITICAL, ● OPTIONAL

Contents

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A. Overview

Overview of Protocol

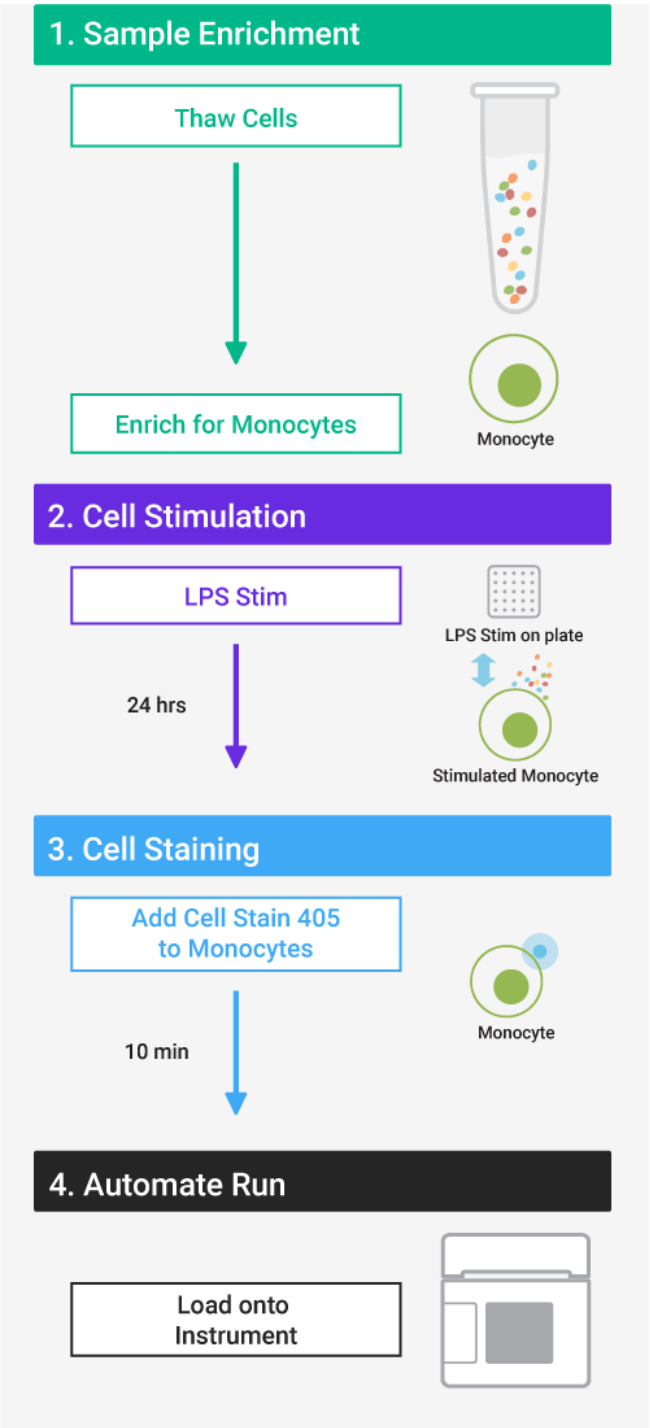
Day 1: Cryopreserved cells are thawed in complete RPMI media. Enrichment and Stimulation of Monocytes for 24 hours.

Day2: Staining and Loading of Monocytes onto IsoCode Chip.

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

NOTE:
It is recommended to use mouse bone marrow mononuclear cells as the starting material for this protocol. Splenocytes may be used, but the monocyte population is extremely low from spleens and will require greater starting sample material.

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.



Safety Warnings

Key: ● TIP, ● CRITICAL, ● OPTIONAL

- 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: Consumables Required to Perform Protocol but Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
T25 Flask	N/A	Corning	353108
T75 Flask	N/A	Corning	430641U
6 Well Nunc UpCell Dish	N/A	ThermoFisher	174901
24 Well Nunc UpCell Dish	N/A	ThermoFisher	174899
48 Well Nunc UpCell Dish	N/A	ThermoFisher	174898
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
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
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-Neomycin Solution Stabilized	100x	Sigma	P4083-100mL
Glutamax	100x	Thermo	35050061
FBS	1x	Sigma	F2442-6X500mL
2-Mercaptoethanol	55 mM	Gibco	21985-023 50mL
Phosphate Buffered Saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
RoboSep buffer	1x	StemCell Tech	20104
Ficoll Paque PREMIUM 1.084	N/A	Cytiva	17544602
Miltenyi Monocyte Isolation Kit (BM), Mouse	N/A	Miltenyi	130-100-629
Trypan Blue	0.4%	Gibco	15250-061
Accutase	1x	Innovative Cell Technologies, Inc	AT 104-500
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
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 and 50 mL conical 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 RPMI Recipe

- CRITICAL: Complete RPMI 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-Neomycin Solution Stabilized	100x	1x	5 mL	Sigma/P4083-100mL
Glutamax	100x	1x	5 mL	Thermo/35050061
FBS	100%	10%	50 mL	Sigma/F2442-6X500 mL
RPMI	1x	1x	440 mL	Fisher/MT10040CV

- CRITICAL: 2-Mercaptoethanol will be added to media used for all steps except cell staining quench media and final cell suspension volume to be loaded on IsoCode chip. 2-Mercaptoethanol should only be added to media right before use.

Note | Sterile-filter through 0.20 µm filter before use. Store complete RPMI Media at 4°C and warm up to 37°C in water bath prior to use.

Additional Reagents to Be Prepared

NOTE: 1X Accutase should be thawed overnight at 4°C upon receipt. 12 mL aliquots are recommended and storage is at -20°C. Thawed aliquots can be stored at 4°C for up to 1 month.

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 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]

Chapter 2: Recovery of Cryopreserved Cells

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
Cryopreserved Mouse Cells
15 mL Centrifuge Tube

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

NOTE: It is recommended to use fresh cells, if possible, due to higher cell viability from freshly enriched cells. In Bruker's experience, cryopreserved cells require more starting sample material. Proceed directly to Chapter 3 if fresh cells are being used.

Methods

1. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 50 µL of 55 mM 2-Mercaptoethanol into 50 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Pipette 5 mL of pre-warmed complete RPMI/2-Mercaptoethanol into a 15 mL centrifuge tube, labeled *Thawed Mouse Cells*.

- 3. Using proper PPE, remove cells from liquid nitrogen storage and thaw cells. TIP: Be careful of contamination.

4. 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.

5. 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.

- 6. Slowly pipette thawed cells into 5 mL of complete RPMI/2-Mercaptoethanol in 15 mL centrifuge tube, labeled *Thawed Mouse Cells*. TIP: Insert tip into complete RPMI when pipetting, be careful to not create bubbles.

- 7. Take 1 mL of complete RPMI/2-Mercaptoethanol and pipette into original thawed cell vial. Rinse inside the vial with the complete RPMI/2-Mercaptoethanol to recover additional thawed cells. TIP: Insert tip into complete RPMI, be careful to not create bubbles.

- 8. Draw up cell/complete RPMI/2-Mercaptoethanol mixture and pipette into the 15 mL centrifuge tube, labeled *Thawed Mouse Cells*. TIP: Insert tip into complete RPMI and pipette gently up and down. Be careful to not create bubbles.

9. Centrifuge cells for 10 minutes at 300 rcf.

10. After cells are centrifuged, check for cell pellet.

- 11. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.

12. Resuspend cell pellet in 1 mL of fresh complete RPMI/2-Mercaptoethanol.

- a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.

13. Slowly add additional complete RPMI/2-Mercaptoethanol to a final concentration of 1 x 10^6 cells/mL.

- 14. Mix with serological pipette. TIP: Gently pipet up and down 3-5 times, be careful to not create bubbles.

15. Proceed immediately to next chapter.

Chapter 3: Post-Recovery Sample Setup

Materials Required

Recovered Cells from Chapter 2 or Freshly Isolated Cells
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. Mix cell suspension in centrifuge tube well 5 times with 10 mL serological pipette. TIP: Be careful not to create bubbles.
- 2. 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.
- 3. 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.
- CRITICAL: If cells are less than 80% viable, proceed to Appendix D2 Dead Cell Depletion Using Ficoll.
4. Proceed immediately to next chapter.

Chapter 4: Pan Monocyte Sample Enrichment

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
RoboSep Buffer (4°C)
Miltenyi Monocyte Isolation Kit (BM), Mouse, (4°C)
MACS LS Column
Prepared Cells from Chapter 3
Enrichment Kit:
- MACS Metal Plate/Magnet Kit
- 2 x 15 mL Centrifuge Tubes (*Discard, Pan Monocyte fraction*)
- Lo-Bind Microcentrifuge Tube for Post-Enrichment Monocytes

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 5 x 10^7 cells, resuspend in 175 µL RoboSep (4°C), 25 µL FcR Blocking Reagent (4°C), and 50 µL Biotin-Antibody Cocktail (4°C).

3. Add 175 µL of cold RoboSep to centrifuge tube containing 5 x 10^7 or fewer cells. Mix well by pipetting up and down 15 times.
4. Add 25 µL of Miltenyi FcR Blocking Reagent and mix well by gently pipetting up and down 5 times.
5. Add 50 µL of Miltenyi Biotin-Antibody Cocktail and mix well by gently pipetting up and down 15 times.

- TIP: Be careful not to create bubbles.

- 6. Incubate in refrigerator (4°C) for 5 minutes. TIP: Don't incubate on ice as increased incubation times may be required.

- TIP: Keep RoboSep in refrigerator during enrichment process.

7. For every 5 x 10^7 cells, rinse with 10 mL of cold Robosep.
8. Centrifuge cells for 10 minutes at 300 rcf.
9. After cells are centrifuged, check for cell pellet.

- 10. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.

- CRITICAL: For every 5 x 10^7 cells, resuspend in 400 µL RoboSep (4°C) and 100 µL of Anti-Biotin MicroBeads (4°C).

11. Add 400 µL of cold RoboSep to centrifuge tube containing 5 x 10^7 or fewer cells. Mix well by gently pipetting up and down 15 times.
12. Add 100 µL of Miltenyi Anti-Biotin MicroBeads and mix well by gently pipetting up and down 15 times.

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

- 13. Incubate in refrigerator (4°C) for 10 minutes. TIP: Don't incubate on ice as increased incubation times may be required.

- TIP: Keep RoboSep in refrigerator during enrichment process.

- 14. Set up MACS sorting by setting metal plate in tissue culture hood and placing magnet on metal plate. Place LS column securely 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.

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

- 15. 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.

16. Unscrew and keep cap for "Pan Monocyte Fraction" tube. NOTE: This is in preparation for next step to ensure the column does not dry out during the transition.

17. When the last drop falls through to the "Discard" tube, move the rack over so the LS column is over the "Pan Monocyte Fraction" 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.
18. Increase volume of pipette to 800 µL to ensure all 500 µL of the cell suspension is drawn up.
19. Mix cell suspension by gently pipetting up and down 15 times. NOTE: This ensures that the cells are evenly dispersed after sitting.
20. Draw up all 500 µL of cell suspension and pipette carefully into the center of the LS column without touching the sides of the column.
21. Wash column 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 20. 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.
22. Centrifuge "Pan Monocyte Fraction" tube for 10 minutes at 300 rcf.
23. Discard LS column.
24. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 5 µL of 55 mM 2-Mercaptoethanol into 5 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

25. After cells are centrifuged, check for cell pellets.
26. Aspirate RoboSep buffer from "Pan Monocyte Fraction" tube. TIP: Be careful to not aspirate cell pellet.
27. Use pipette to aspirate the remaining supernatant from tube. TIP: Be careful to not aspirate cell pellet.
28. Add 1 mL complete RPMI/2-Mercaptoethanol to "Pan Monocyte Fraction" and resuspend cell pellet. TIP: Make sure there are no clumps or bubbles.
29. Add an additional 1 mL of complete RPMI/2-Mercaptoethanol and mix thoroughly by gently pipetting up and down 15 times. TIP: Make sure there are no clumps or bubbles.
30. Aliquot 10 µL of the Pan Monocyte fraction into a Lo-Bind Microcentrifuge tube and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.
31. Move "Pan Monocyte Fraction" tube to incubator until Cell Stimulation (Chapter 5).

Chapter 5: Cell Stimulation

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
LPS 1 mg/mL (-20°C)
15 mL Centrifuge Tube (*LPS Monocytes, Unstimulated Monocytes)*
T75 Flask, T25 Flask, 6 Well Nunc UpCell Dish, 24 Well Nunc UpCell Dish, or 48 Nunc UpCell Dish
1X Accutase (-20°C)
Incubated Pan Monocyte Tube in complete RPMI
1 x Lo-Bind Microcentrifuge Tubes for Cell Count (*label LPS Monocytes)*

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

Methods

1. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 5 µL of 55 mM 2-Mercaptoethanol into 5 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Thaw a vial of stock 1 mg/mL LPS at ambient temperature.
3. Vortex LPS for 5 seconds.
4. Prepare a working stock of LPS. Add 10 µL of 1 mg/mL LPS to 1 mL complete RPMI. Final concentration of LPS 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.

- 5. Vortex LPS working stock for 10 seconds. TIP: Ensure contents are well-mixed.

6. Take Pan Monocytes from the incubator.
7. Centrifuge Pan Monocytes for 10 minutes at 300 rcf.
8. After cells are centrifuged, check for pellet.

- 9. Aspirate supernatant. TIP: Be careful to not aspirate cell pellet.

10. Resuspend Pan Monocyte cells in complete RPMI/2-Mercaptoethanol to a cell density of 1 x 10^5 cells/mL.
11. Split cells into two separate 15 mL centrifuge tubes. One labeled “LPS Monocytes” and the other “Unstimulated Monocytes”.

- 12. Take 10 µL from the “LPS Monocytes” tube and transfer into a Lo-Bind Microcentrifuge tube. Proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

13. Centrifuge tube labeled “LPS Monocytes” for 10 minutes at 300 rcf.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

14. After cells are centrifuged, check for pellet.
15. Aspirate supernatant. TIP: Be careful to not aspirate cell pellet.
16. Resuspend the cell pellet with complete RPMI/2-Mercaptoethanol supplemented with 100 ng/mL of LPS to a density of 1 x 10^5 cells/mL. CRITICAL: Volume is dependent on number of cells.
    a. Add 10 µL of 10 µg/mL LPS working stock for every mL of complete RPMI/2-Merecaptoethanol. This yields a final concentration of LPS of 100 ng/mL.
    b. Use serological pipette to mix thoroughly. CRITICAL: Be careful not to create bubbles. This will maximize even stimulation of cell suspension.
17. Seed the monocyte suspensions into the appropriately sized dish or flasks depending on the volume of the suspension. For flasks, label one "LPS Monocytes" and the other "Unstimulated Monocytes". For a dish, note which wells have "LPS Monocytes" and which have "Unstimulated Monocytes" (See Table 9).

CRITICAL: Be careful not to scratch the bottom of the dish when seeding cells.

Table 9: Dish and Volume for Monocyte Seeding

Container	Volume
48 Well Dish	500 µL/well
24 Well Dish	1 mL/well
6 Well Dish	2 mL/well
T25 Flask	3-7 mL
T75 Flask	8-15 mL

18. Incubate dish or flasks for 24 hours at 37°C, 5% CO_2.
19. If flasks were used, place 1X Accutase at 4°C to allow 1X Accutase to thaw overnight.

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 Staining

NOTE: Please read before proceeding with cell staining.

There are two options for cell staining depending on container used during cell stimulation.

1. Cell staining cell suspension plated in 6 well dish, 24 well dish, or 48 well dish as described in Chapter 7a.
2. Cell staining cell suspension from T25 flask or T75 flask as described in Chapter 7b.

Chapter 7a: Cell Staining Cells from Dishes

Materials Required

LPS Stimulated and Unstimulated Pan Monocyte Cells in 6 Well Dish, 24 Well Dish, or 48 Well Dish from Chapter 5
4 x 15 mL Centrifuge Tubes (*LPS Monocytes, Unstimulated Monocytes, RPMI LPS Wash, RPMI Unstimulated Wash*)
5 x Lo-Bind Microcentrifuge Tubes (*LPS Bulk Assay, Unstimulated Bulk Assay, Stain Master Mix, LPS Monocytes, Unstimulated Monocytes)*
Sterile 1X PBS (Room Temperature)
Complete RPMI (37°C)
Complete RPMI (20°C)
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.

Steps below describe parallel workflow for stimulated and unstimulated monocytes.

Unless specified use pre-warmed RPMI (37°C).

Methods

1. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 25 µL of 55 mM 2-Mercaptoethanol into 25 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

● CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Remove dish containing “LPS Monocytes” and “Unstimulated Monocytes” from incubator.
3. Incubate the cells at room temperature for 15 to 40 minutes to allow the cells to detach. Observe the cells under the microscope, approximately every 15 minutes, until they appear detached.
4. Harvest monocytes in suspension by gently pipetting up the cell suspension.
    a. From “LPS Monocytes” dish, add this cell suspension to “LPS Monocytes” tube.
    b. From “Unstimulated Monocytes” dish, add this cell suspension to “Unstimulated Monocytes” tube

5. Centrifuge the tubes labeled “LPS Monocytes” and “Unstimulated Monocytes” for 10 min at 300 rcf.
6. Observe the wells under light microscope to ensure all cells have detached. If cells remain, gently rinse the wells with complete RPMI/2-Mercaptoethanol at 20°C. NOTE: 20°C media is used to ensure cells do not re-adhere to the dish.
    - For 6 Well Dish add 800 µL
    - For 24 Well Dish add 400 µL
    - For 48 Well Dish add 200 µL

● CRITICAL: Observe wells under light microscope to ensure all cells were removed from the dish. If not, repeat step 6.

7. Tilt dish and gently remove complete RPMI from dish.
    a. From dish labeled “LPS Monocytes”, remove complete RPMI with a pipette and add to a tube labeled “RPMI LPS Wash”.
    b. From dish labeled “Unstimulated Monocytes”, remove complete RPMI with a pipette and add to a tube labeled “RPMI Unstimulated Wash”.
8. After cells from step 5 are centrifuged, check for cell pellets.
9. Use a pipette and remove 1 mL of supernatant from each tube
    a. Remove 1 mL “LPS Monocytes” supernatant and add to Lo-Bind microcentrifuge tube labeled “LPS Bulk Assay”. Store at -80°C for bulk assay. Remaining cells/supernatant are used in step 10a.
    b. Remove 1 mL “Unstimulated Monocytes” supernatant and add to Lo-Bind microcentrifuge tube labeled “Unstimulated Bulk Assay.” Store at -80°C for bulk assay. Remaining cells/supernatant are used in step 10b.
10. RPMI wash from step 7 is combined with monocyte/supernatant remaining from step 9.
    a. Add “LPS RPMI Wash” from step 7a to the centrifuge tube labeled “LPS Monocytes” remaining from step 9a after aliquoting for bulk assay.
    b. Add “RPMI Unstimulated Wash” from step 7b to centrifuge tube labeled “Unstimulated Monocytes” remaining from step 9b after aliquoting for bulk assay.
11. Centrifuge the tubes labeled “LPS Monocytes” and “Unstimulated Monocytes” for 10 min at 300 rcf.
12. After cells are centrifuged, check for cell pellets.

● 13. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.

14. Add 1 mL of 1X PBS to each tube to dilute any remaining media and mix by pipetting up and down.

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

15. Centrifuge cells for 10 minutes at 300 rcf.
16. 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.

- 17. Prepare stain master mix by diluting 1 µL of cell stain 405 into 1 mL of 1X PBS in a Lo-Bind microcentrifuge tube (1:1000 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all cell stain 405 has been released. Depending on sample number and cell count, 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.**

18. After cells are centrifuged, check for cell pellets.

- 19. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.

20. Gently remix stain master mix.

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

21. For every 1 x 10^6 cells, add 100 µL of stain master mix to each tube labeled "LPS Monocytes" and "Unstimulated Monocytes". CRITICAL: Pipet to mix 15 times. Be careful not to create bubbles.

22. Incubate for 5 minutes at 37°C in the dark.

- 23. Gently pipet, to mix the cell suspension, 15 times. CRITICAL: Be careful not to create bubbles.

24. Incubate for an additional 5 minutes at 37°C in the dark.

- 25. After incubation, add 5 times the volume of complete RPMI **without 2-Mercaptoethanol**. CRITICAL: Pipet 15 times to gently mix the cells. Be careful not to create bubbles.

26. Incubate for 10 minutes at 37°C in the dark.

- 27. Take 10 µL of cells to count. 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.

28. Centrifuge stained cells for 10 minutes at 300 rcf.

29. After cells are centrifuged, check for cell pellets.

- 30. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.

31. Resuspend the cells with complete RPMI **without 2-Mercaptoethanol** to a cell density of **7.5 x 10^5 cells/mL**. Proceed to Chapter 8.

Chapter 7b: Cell Staining Cells from Flasks

Materials Required

LPS Stimulated and Unstimulated Pan Monocyte Cells in Flasks from Chapter 5
4 x 15 mL Centrifuge Tubes (*LPS Monocytes, Unstimulated Monocytes, PBS LPS Wash, PBS Unstimulated Wash*)
5 x Lo-Bind Microcentrifuge Tubes (*LPS Bulk Assay, Unstimulated Bulk Assay, Stain Master Mix, LPS Monocytes, Unstimulated Monocytes)*
Sterile 1X PBS (Room Temperature)
Complete RPMI (37°C)
1X Accutase (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.

Steps below describe parallel workflow for stimulated and unstimulated monocytes.

Methods

1. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 25 µL of 55 mM 2-Mercaptoethanol into 25 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.

- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.

2. Remove 1X Accutase from 4°C to allow 1X Accutase to come to room temperature. NOTE: 1X Accutase aliquots, after thaw, can be stored at 4°C for up to 1 month. Do not use 1X Accutase that has been at 4°C for longer than a month.
3. Remove flasks containing “LPS Monocytes” and “Unstimulated Monocytes” from incubator.
4. Harvest monocytes in suspension by gently pipetting up the cell suspension.
    a. From “LPS Monocytes” flask, add this cell suspension to “LPS Monocytes” tube.
    b. From “Unstimulated Monocytes” flask, add this cell suspension to “Unstimulated Monocytes” tube

- 5. Centrifuge cell suspensions from step 4 for 10 minutes at 300 rcf, to pellet non-adherent monocytes. TIP: This will ensure higher cell counts.

6. While cell suspension is centrifuging, rinse gently over the surface of the flask with 1X PBS.
    - For T75 Flask add 5 mL
    - For T25 Flask add 2 mL
7. Tilt flask and gently remove PBS from flask.

- a. From flask labeled "LPS Monocytes", remove PBS with a pipette and add to a tube labeled "PBS LPS Wash". TIP: PBS rinse will contain monocytes. These will be combined in a later step to ensure higher cell counts.
- b. From flask labeled "Unstimulated Monocytes", remove PBS with a pipette and add to a tube labeled "PBS Unstimulated Wash". TIP: PBS rinse will contain monocytes. These will be combined in a later step to ensure higher cell counts.

8. Pipet thawed 1X Accutase up and down 5 times to ensure it is well mixed. Detach adherent cells from the flask by gently adding 1X Accutase to the flask.
   - For T75 Flask add 5 mL
   - For T25 Flask add 2 mL
9. Rock the flask back and forth to ensure the 1X Accutase covers the entire bottom of the flask.
10. Incubate the cells with 1X Accutase for 10 minutes at room temperature to allow the cells to detach. During incubation, be sure to lightly tap the flask parallel to the benchtop once per minute. Observe the cells under the microscope until they appear detached.
11. After cells from step 5 are centrifuged, check for cell pellets.
12. Use a pipette and remove 1 mL of supernatant from each tube
    a. Remove 1 mL "LPS Monocytes" supernatant and add to Lo-Bind microcentrifuge tube labeled "LPS Bulk Assay". Store at -80°C for bulk assay. Remaining cells/supernatant are used in step 13a.
    b. Remove 1 mL "Unstimulated Monocytes" supernatant and add to Lo-Bind microcentrifuge tube labeled "Unstimulated Bulk Assay". Store at -80°C for bulk assay. Remaining cells/supernatant are used in step 13b.
13. PBS wash from step 7 is combined with monocyte/supernatant remaining from step 12.
    a. Add "LPS PBS Wash" from step 7a to the centrifuge tube labeled "LPS Monocytes" remaining from step 12a after aliquoting for bulk assay.
    b. Add "PBS Unstimulated Wash" from step 7b to centrifuge tube labeled "Unstimulated Monocytes" remaining from step 12b after aliquoting for bulk assay.
14. After incubation with Accutase, rinse the flask with complete RPMI/2-Mercaptoethanol to remove adherent monocytes. Use 5 mL of complete RPMI/2-Mercaptoethanol for a T75 or 2 mL of complete RPMI/2-Mercaptoethanol for a T25.

- a. Gently rinse the flask labeled "LPS Monocytes" with complete RPMI/2-Mercaptoethanol to remove LPS adherent fraction and add to the centrifuge tube labeled "LPS Monocytes". TIP: Rinse gently, be careful not to create bubbles.
- b. Gently rinse the flask labeled "Unstimulated Monocytes" with complete RPMI/2-Mercaptoethanol to remove the unstimulated adherent fraction and add to the centrifuge tube labeled "Unstimulated Monocytes". TIP: Rinse gently, be careful not to create bubbles.
- CRITICAL: Observe wells under light microscope to ensure all cells were removed from the flask. If not repeat step 14.

15. Centrifuge the tubes labeled "LPS Monocytes" and "Unstimulated Monocytes" for 10 min at 300 rcf.

16. After cells are centrifuged, check for cell pellets.
17. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.
18. Add 1 mL of 1X PBS to each tube to dilute any remaining media and mix by pipetting up and down.

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

19. Centrifuge cells for 10 minutes at 300 rcf.
20. 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.

21. Prepare stain master mix by diluting 1 µL of cell stain 405 into 1 mL of 1X PBS in a Lo-Bind microcentrifuge tube (1:1000 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all cell stain 405 has been released. Depending on sample number and cell count, 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.
22. After cells are centrifuged, check for cell pellets.
23. Remove the majority of the supernatant with a P1000 or P200 pipette. Switch to a P10 pipette to remove all media from the cell pellet. TIP: Be careful not to aspirate the cell pellets.
24. Gently remix stain master mix.

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

25. For every 1 x 10^6 cells, add 100 µL of stain master mix to each tube labeled “LPS Monocytes” and “Unstimulated Monocytes”. CRITICAL: Pipet to mix 15 times. Be careful not to create bubbles.
26. Incubate for 5 minutes at 37°C in the dark.
27. Gently pipet, to mix the cell suspension, 15 times. CRITICAL: Be careful not to create bubbles.
28. Incubate for an additional 5 minutes at 37°C in the dark.
29. After incubation, add 5 times the volume of complete RPMI without 2-Mercaptoethanol. CRITICAL: Pipet 15 times to gently mix the cells. Be careful not to create bubbles.
30. Incubate for 10 minutes at 37°C in the dark.

31. Take 10 µL of cells to count. 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.
32. Centrifuge stained cells for 10 minutes at 300 rcf.
33. After cells are centrifuged, check for cell pellets.
34. Aspirate supernatant with a pipette. TIP: Be careful not to aspirate the cell pellets.
35. Resuspend the cells with complete RPMI **without 2-Mercaptoethanol** 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
Stained Pan Monocyte 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 "LPS Monocytes" and "Unstimulated Monocytes" tubes by gently pipetting up and down. 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. 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 the 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
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 P10 pipette, 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 PBS or complete RPMI using a fresh sample aliquot.

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]

D2 Protocol: Dead Cell Removal Using Ficoll

Materials Required

Complete RPMI (37°C)
2-Mercaptoethanol (4°C)
Cells (Minimum $3x10^6$)
2 x 15 mL Centrifuge Tubes
Lo-Bind Microcentrifuge Tubes
Ficoll Paque PREMIUM 1.084

CRITICAL: It is recommended to start this protocol with a minimum of $3x10^6$ total cells.

1. Carefully add 6 mL of Ficoll to the bottom of the required number of 15 mL centrifuge tube(s) prior to harvesting stimulation cultures.

2. Centrifuge cells for 10 minutes at 300 rcf.
3. Remove cells from centrifuge, check for cell pellet.
- 4. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to aspirate remaining supernatant.
- 5. Resuspend the pellet(s) in 7 mL of complete RPMI. TIP: Be careful not to create bubbles.
- CRITICAL: Do not use more than 1 x 10^7 cells of your suspension per Ficoll.
- 6. Add the cell suspension(s) VERY SLOWLY to the tube(s) containing Ficoll. CRITICAL: Place the tip of your pipette on the wall of the tube, close to the Ficoll layer. Add cell suspension VERY SLOWLY.
- CRITICAL: This step must be done carefully and slowly to avoid mixing of the layers.
7. Centrifuge tubes for 20 minutes at 300 rcf without brake or acceleration.
- CRITICAL: Turn acceleration and brakes off to preserve the density layers established during centrifugation.
8. Prepare complete RPMI supplemented with 55 µM of 2-Mercaptoethanol.
    a. Dilute 6 µL of 55 mM 2-Mercaptoethanol into 6 mL of complete RPMI.
    b. Use serological pipette to mix thoroughly.
- CRITICAL: 2-Mercaptoethanol should only be added to media right before use. Discard any unused media.
9. While cells centrifuge, prepare appropriate number of 15 mL centrifuge tube(s) containing 6 mL of complete RPMI/2-Mercaptoethanol.
10. Remove cells from centrifuge, check for cloudy layer which are the viable cells.
- 11. Aspirate a small volume of the supernatant. CRITICAL: Be careful not to aspirate cloudy layer containing viable cells.
12. Using a P1000 pipette, collect the viable cells by recovering the cloudy layer between Ficoll and complete RPMI media
13. Transfer cells into the 15 mL centrifuge tube(s) containing complete RPMI.
- 14. Aliquot 10 µL of cell/complete RPMI mixture(s) into a Lo-Bind Microcentrifuge Tube(s) and proceed to cell count. CRITICAL: See Appendix D1 for cell counting instructions.

Key: ● TIP, ● CRITICAL, ● OPTIONAL

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 dish/flask	• Use recommended cell concentrations during incubation (**Chapter 5**) • 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 the cells • Follow closely the steps in **Chapter 7** (Cell Staining)
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 • Media not completely removed from cell pellet prior to staining	• Use complete RPMI media following recipe in **Table 8** • Use Bruker provided validated stain (**Table 4: Cell Staining Reagents**) • Follow staining steps as highlighted in **Chapter 7.** • Use only freshly prepared Cell Stain 405 per **Chapter 7** • Ensure all media is removed from cell pellet in **steps 7a.13** and **7b.17**

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 **8.3** 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 4** (Pan Monocyte 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 PREMIUM 1.084 • Low viable cells due to lack of 2-Mercaptoethanol supplementation to complete RPMI • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to complete RPMI to ensure viability of cells • Verify viability of cells is above 80% as stated in **Chapter 3** to ensure protocol is being performed with the highest quality of cells. Use Ficoll Paque PREMIUM 1.084 in **Appendix D2** if viability is less than 80% • Ensure that complete RPMI is supplemented with 2-Mercaptoethanol right before use • Use reagents at recommended temperatures (i.e. always use warmed media [37°C])
Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Stimulation step related*	• Recommended LPS stimulation concentration was not used • Recommended LPS stimulation duration was not used • Expired LPS used	• Use LPS concentrations listed in **Chapter 5** • Use LPS stimulation duration listed in **Chapter 5** • Do not use LPS stock older than 2 months • Use required vendors as listed in **Table 3**