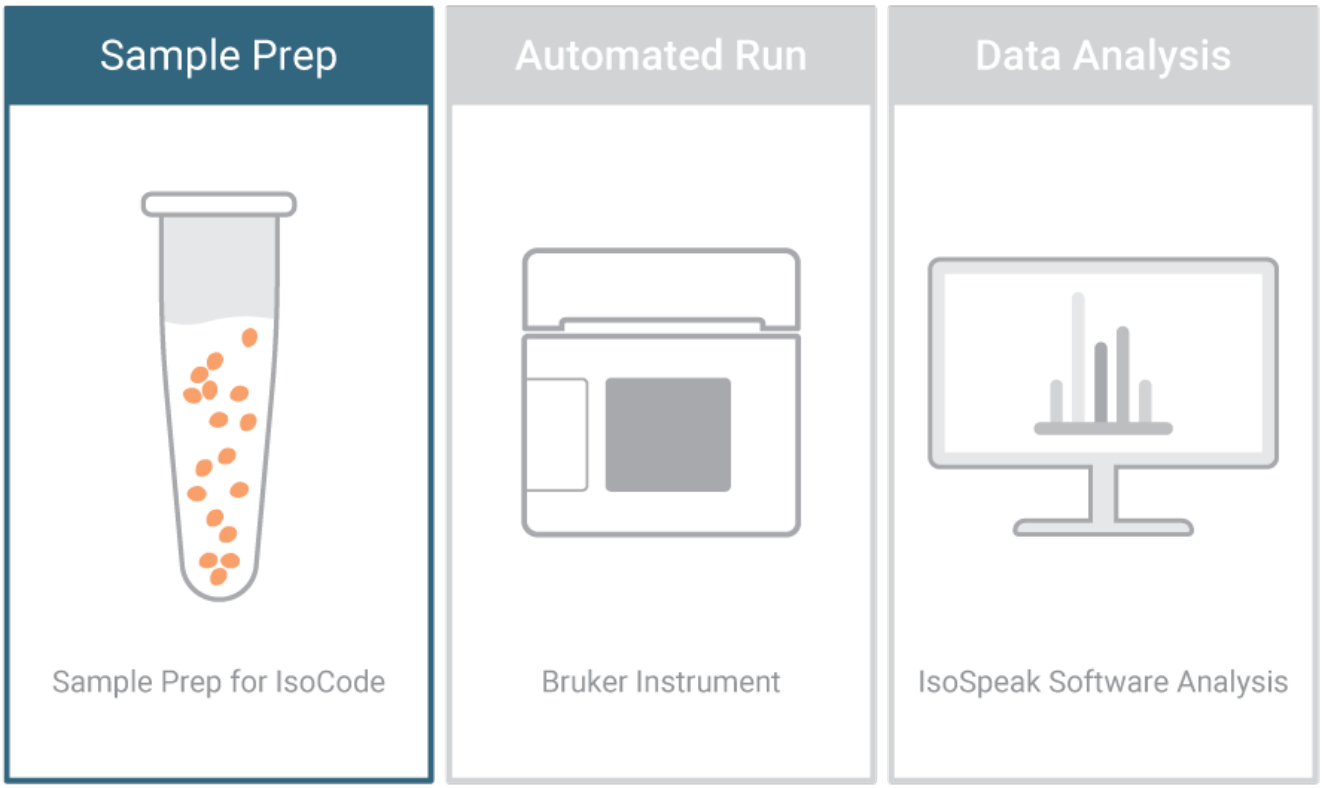
IsoCode Single-Cell Adaptive Immune: Mouse Astrocytes Protocol

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

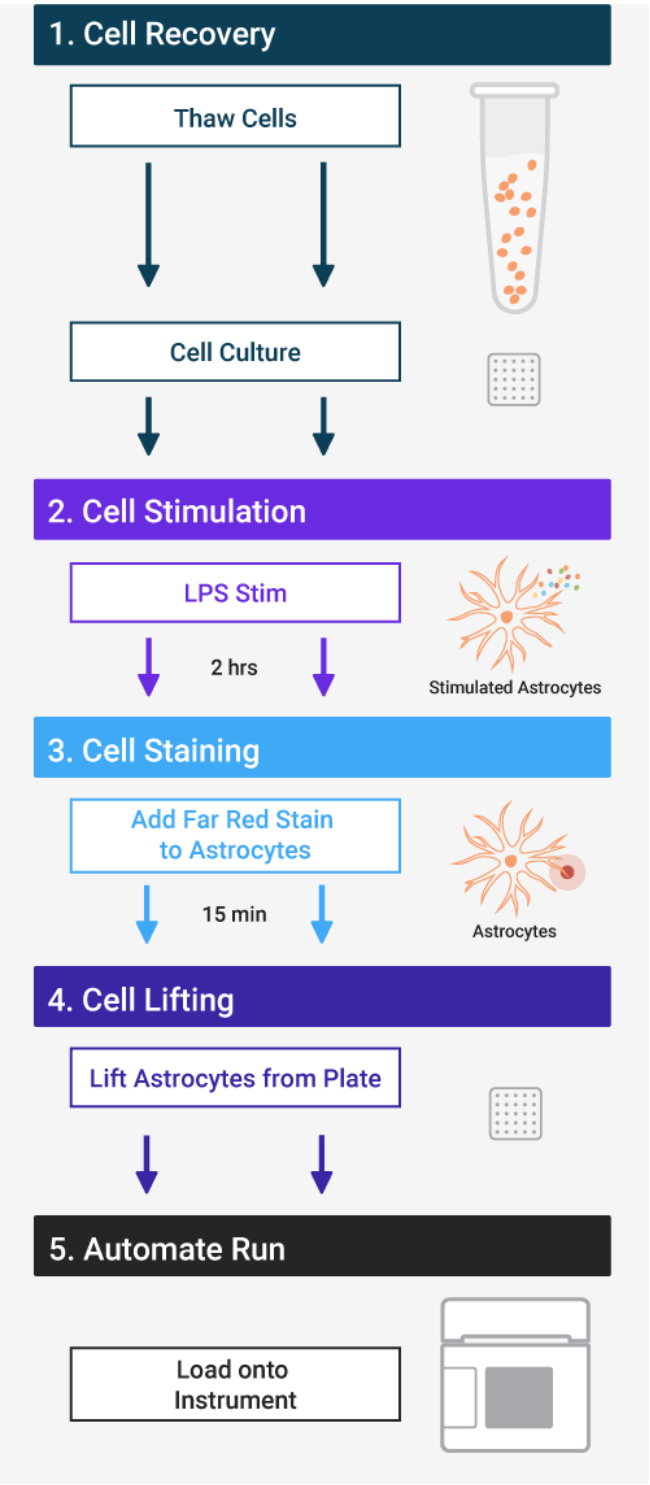
Overview of Protocol

Day 1: Cryopreserved cells are thawed. Culture for 3 to 5 days.

Day 3: Cell stimulation for 2 hours. Staining and loading onto IsoCode Chip.

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

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
PRO-41 REV 4.0

- 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 Reagents and 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

Table 2: Required Consumables Not Supplied by Bruker

Consumable	Type	Source	Catalog Number
6 Well Plate Flat Bottom	N/A	Corning	353046
Centrifuge Tubes*	Polypropylene, 15 mL	VWR	CA62406-200
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	2 mL 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
Distilled Water	N/A	Gibco	15230-147
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
DMEM – High Glucose	1x	Sigma	D5796-500ML
Penicillin-Streptomycin Solution	100x	Gibco	15140122
FBS	1x	Sigma	F2442-6X500mL
Phosphate buffered saline (1XPBS) without Calcium or Magnesium	1x	Gibco	10010072
Poly-D-Lysine	1 mg/mL	Millipore Sigma	A-003-E
TrypLE Express Enzyme (1X), no phenol red	1x	Gibco	12604021
CellTrace Far Red Cell Proliferation Kit	N/A	Invitrogen	C34564
DMSO	N/A	Invitrogen	C34564
LPS (lyophilized powder)	N/A	Sigma	L2654-1MG
Trypan Blue	0.4%	Gibco	15250-061
Reagent alcohol 70%	N/A	Lab Grade	N/A

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

Table 4: 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

Table 5: 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 15mL conical tubes or 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 should 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 comes 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 6: mAstro Media Recipe

- CRITICAL: mAstro 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
FBS	100%	10%	50 mL	Sigma/F2442-6X500 mL
DMEM – High Glucose	1x	1x	440 mL	Sigma/D5796-500ML

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

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.

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

Chapter 2: Coat Culture Plate with Poly-D-Lysine

Materials Required

Sterile 1X PBS (Room Temperature)
Poly-D-Lysine (PDL) (4°C)
Distilled (DI) Water
15 mL Centrifuge Tube (*PDL Dilution*)
6 Well Plate Flat Bottom (*Mouse Astrocytes, Date, Time*)

All the following steps should take place in a sterile tissue culture hood.

Methods

NOTE: Complete this chapter 1 to 2 hours prior to thawing cells.

1. Prepare a Poly-D-Lysine (PDL) in 1X PBS 1:1 mixture in a 15 mL centrifuge tube.
   a. Add 5 mL of 1X PBS to a 15 mL centrifuge tube.
   b. Add 5 mL of PDL to the 15 mL centrifuge tube.
   c. Mix well 5 times with 10 mL serological pipette.

2. Pipette 1.5 mL of the PDL in 1X PBS mixture into each well of a 6 well flat bottom plate.
3. Incubate covered plate for 1 hour at room temperature.
4. Aspirate PDL in 1X PBS mixture from each well.
5. Rinse each well with 1 mL of DI water. NOTE: Pipette water down the wall of the well.
6. Aspirate DI water with a pipette.
7. Leave the uncovered plate to dry in the hood for 1 hour or until dry.

Chapter 3: Recovery of Cryopreserved Cells

Materials Required

mAstro Media (37°C)
Cryopreserved Mouse Astrocytes or Fresh Mouse Astrocytes in Suspension (Proceed to Step 8)
15 mL Centrifuge Tube
PDL Coated 6 Well Plate

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 mAstro media into a 15 mL centrifuge tube, labeled *Thawed Mouse Astrocytes*.
- 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 under 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 mAstro media in 15 mL centrifuge tube, labeled *Thawed Mouse Astrocytes*. TIP: Insert tip into mAstro media when pipetting, be careful to not create bubbles.
- 6. Take 1 mL of mAstro media and pipette into original thawed cell vial. Rinse inside the vial with the mAstro media to recover additional thawed cells. TIP: Insert tip into mAstro media, be careful to not create bubbles.
- 7. Draw up cell/mAstro media mixture and pipette back into the 15 mL centrifuge tube, labeled *Thawed Mouse Astrocytes*. TIP: Insert tip into mAstro 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 5 minutes at 200 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
11. After cells are centrifuged, check for cell pellet.
12. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
13. Resuspend cell pellet to a density of 1 x 10^5 cells/mL in fresh mAstro media.
    a. Mix well to resuspend. TIP: Make sure to pipette around the tube to ensure there are no clumps or bubbles.
14. Add 1.5 to 2.5 mL of fresh mAstro media to each well on the pre-prepared PDL coated plate.
15. Transfer 500 µL of cell suspension to each well on plate. TIP: Slowly pipette down the side of the flask as to not create bubbles.
    a. Note which wells on plate will be for "Stimulated Astrocytes" and which will be for "Unstimulated Astrocytes". At least 1 well should be plated for each condition.
16. Spread out cell suspension by rocking the plate carefully to fully cover the bottom of the container. TIP: Be careful to not make bubbles.
17. Move to incubator for recovery at 37°C, 5% CO_2.
18. Cells should be 85-95% confluent before proceeding to Chapter 5. NOTE: Check the cells periodically, typically 3 to 5 days is required to reach desired confluency. After 2-3 days complete Chapter 4 for media change.

Chapter 4: Media Change

Materials Required

mAstro media (37°C)
6 Well Plate with Mouse Astrocytes

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

Methods

NOTE: Complete this chapter if mouse astrocytes have been on plate for 2-3 days without a media change.

1. Take cells out of the incubator.
2. Aspirate one half of the mAstro media from one well. Add the same volume of fresh mAstro media to the well. NOTE: Gently pipette media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.
3. Repeat step 2 until media has been changed for all wells.
4. Incubate plate at 37°C, 5% CO_2 until additional media change is required or 85-95% confluency is reached. Once 85-95% confluency is reached, proceed to Chapter 5.

Chapter 5: Cell Stimulation

Materials Required

mAstro Media (37°C)
LPS 1 mg/mL (-20°C)
15 mL Centrifuge Tubes
Confluent Mouse Astrocytes in 6 Well Plate

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 at ambient temperature.
2. Vortex LPS for 5 seconds.
3. Prepare LPS mAstro media mixture. Aliquot 10 mL mAstro media into a centrifuge tube labeled "LPS mAstro". CRITICAL: Volume required is dependent on number of cells.
    a. Aliquot 10 mL mAstro media into a 15 mL centrifuge tube labeled "LPS mAstro."
    b. Add 5 µL of LPS (1 mg/mL) to the mAstro media. Final concentration is 500 ng/mL.
    c. Vortex LPS mAstro media mixture for 5 seconds.
    d. Use serological pipette to mix thoroughly.

CRITICAL: Discard remaining/unused LPS—aliquots are single use only and cannot be refrozen.

4. Prepare mAstro unstimulated media.
    a. Aliquot 10 mL mAstro media into a 15 mL Centrifuge Tube labeled "mAstro Unstimulated" —set this mAstro media aside as it will serve as the media used for the **unstimulated** (negative control) condition.
5. Take cells out of the incubator.
6. Aspirate media from one well. CRITICAL: Be careful not to disturb adherent astrocytes on plate.
7. Add 2 mL of "mAstro Unstimulated" or "LPS mAstro" to well.
    a. For well(s) for the "Unstimulated Astrocytes" condition, add 2 mL of "mAstro Unstimulated" media. **NOTE: Gently pipette media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**
    b. For well(s) for the "Stimulated Astrocytes" condition, add 2 mL of "LPS mAstro" media. **NOTE: Gently pipette media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**

    NOTE: Change media one well at a time.
8. Repeat steps 6 and 7 until media has been changed for all wells.
9. Incubate plate for 2 hours at 37°C, 5% CO_2.

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 and Cell Lifting

Materials Required

mAstro Media (37°C)
TrypLE Express (Room Temperature)
Sterile 1X PBS (Room Temperature)
15 mL Centrifuge Tube (*Stain Master Mix*)
5 mL Lo-Bind Microcentrifuge Tubes (*Unstimulated Astrocytes, Stimulated Astrocytes*)
1.5 mL Lo-Bind Microcentrifuge Tubes (*Working Stock, Cell Count*)
Stimulated and Unstimulated Mouse Astrocytes in 6 Well Plate
CellTrace Far Red (-20°C)
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 CellTrace Far Red stock.
    a. Thaw tube of DMSO at room temperature.
    b. Spin tubes of CellTrace Far Red and DMSO in a mini centrifuge for 10 seconds to collect the contents at the bottom of the tubes.
    c. Add 20 µL of DMSO directly to the tube of CellTrace Far Red. Pipet up and down 15 times gently to resuspend.

- CRITICAL: CellTrace Far Red must be prepared fresh. Discard remaining stain—do not store.

  NOTE: DMSO tube is stored at -20°C after preparing the CellTrace Far Red Stock.

2. Prepare a working stock by diluting 2 µL of CellTrace Far Red into 398 µL of 1X PBS in a 1.5 mL Lo-Bind microcentrifuge tube (1:200 dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red has been released.

a. With a P100 set to 50 µL, gently pipette the stain working stock up and down **15 times**.

b. **Gently vortex** the stain working stock for **5 seconds**.

c. **Ensure working stock is mixed well.**

- 3. Prepare stain master mix by diluting 100 µL of CellTrace Far Red working stock into 10 mL of 1X PBS in a 15 mL centrifuge tube (1:100 dilution of working stock, 1:20,000 final dilution). With the same pipette tip, pipette up and down 10 times to ensure all CellTrace Far Red has been released. Depending on number of wells 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 5 mL serological pipette, 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.**

4. Take astrocytes out of the incubator.

5. Remove supernatant with a P1000 pipette*. CRITICAL: Be careful not to disturb adherent astrocytes in plate. Ensure all media is removed.

*NOTE: Supernatants may be stored at -80°C for bulk assay.

6. Add 1 mL of 1X PBS to each well. **NOTE: Gently pipette 1X PBS down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**

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

7. Remove 1X PBS from 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 1 mL of stain master mix to each well. **NOTE: Gently pipette stain master mix down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**

10. Incubate for 15 minutes at 37°C in the dark.

11. After incubation, add 1 mL of mAstro media to each well. **NOTE: Gently pipette mAstro media down the wall of the well, not directly onto the cells, to avoid displacing adherent cells.**

12. Remove stain master mix/mAstro media mixture from each well with a pipette.

13. Detach adherent cells from plate by gently adding 500 µL of 1X TrypLE to each well.

14. Incubate the cells with 1X TrypLE for 5 minutes at 37°C to allow cells to detach.

15. Rock the plate back and forth.

16. Observe the cells under the microscope to ensure cells have less projections and are starting to detach from the plate.

17. After incubation, add 500 µL of mAstro media to each well. Pipette up and down to rinse the entire surface of each well and to lift the cells from the plate.

18. Collect cells/TrypLE/mAstro media mixture and transfer to a 5 mL Lo-Bind microcentrifuge tube.
    a. From well(s) containing "Unstimulated Astrocytes", transfer mixture to 5 mL Lo-Bind microcentrifuge tube labeled "Unstimulated Astrocytes".
    b. From well(s) containing "Stimulated Astrocytes", transfer mixture to 5 mL Lo-Bind microcentrifuge tube labeled "Stimulated Astrocytes".
19. Gently rinse each well with 500 µL of 1X PBS.
20. Collect cells/1X PBS mixture and transfer to a 5 mL Lo-Bind microcentrifuge tube.
    a. From well(s) containing "Unstimulated Astrocytes", transfer mixture to 5 mL Lo-Bind microcentrifuge tube labeled "Unstimulated Astrocytes".
    b. From well(s) containing "Stimulated Astrocytes", transfer mixture to 5 mL Lo-Bind microcentrifuge tube labeled "Stimulated Astrocytes".
- 21. Mix well 5 times with P1000 set to 1000 µL. TIP: Be careful not to create bubbles.
- 22. 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.
- 23. Centrifuge cells for 5 minutes at 200 rcf. While cells are centrifuging, use hemocytometer to count cells. CRITICAL: See Appendix D1 for cell counting instructions.
24. After cells are centrifuged, check for cell pellet.
- 25. Aspirate supernatant. TIP: Be careful not to aspirate cell pellet.
    a. Use pipette to remove last bit of supernatant.
26. Resuspend each cell pellet with mAstro media to a cell density of 1 x 10^6 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 Stimulated and Unstimulated Astrocytes at 1 x 10^6 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 Astrocytes" and "Unstimulated Astrocytes" 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 30 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 30 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
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 1X PBS or mAstro media 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]

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	• Use recommended cell concentrations during incubation • 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 • Thoroughly rinse cells in well with pipette prior to transferring to tube (**Chapter 7**)
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 • CellTrace Far Red not stored at -20°C prior to use • Media not completely removed from cells prior to staining	• Use mAstro media following recipe in **Table 6** • Use Bruker validated stain • Follow staining steps as highlighted in **Chapter 7** • Use only freshly prepared CellTrace Far Red per **Chapter 7** • Ensure all media is removed from cells in **step 7.6**

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 possibly due to:
	  ○ Pipetting wrong concentration
	  ○ 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 **8.2** and **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.
		• Load recommended number of cells (30,000 cells per chip) (**Chapter 8**)
		• Use low protein binding centrifuge tubes

Limited frequency of stimulated cells, i.e. those with cytokine signal, on chip *Viability related*	• Leaving thawed cells in DMSO for an extended period • Decreased viability due to cell shock	• After thaw, quickly transfer cells from DMSO to mAstro media to ensure viability of cells. • 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	• Use LPS concentration listed in **Chapter 7** • Use LPS timing listed in **Chapter 7** • Use recommended vendor as listed in **Table 3**

Key: ● TIP, ● CRITICAL, ● OPTIONAL