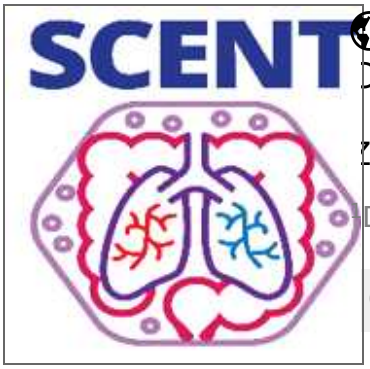

U54 SCENT Intracellular Staining (ICS) Senescence Flow Cytometry Panel

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Cellular Senescence Network (SenNet) Method Development Community

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OPEN ACCESS

ABSTRACT

This protocol describes the Intracellular Staining (ICS) Senescence Flow Cytometry Panel

MATERIALS

Reagents & Materials:

- **Pipettes and Tips for 1-1000uL**
- **96 Well Round Bottom Plates:** Costar Cat #3799
- **Bullet Tubes:** Costar Cat #4401
- **BSB Plus:**BD Cat #566385
- **TruStain FcX:** BioLegend Cat #422302
- **Monensin:** BD Cat # 51-2092KZ, Store undiluted at 4°C.
- **GolgiPlug - Brefeldin A Solution ("BFA"):** BD Cat #555029, Store @ 4°C
- **dPBS:** Invitrogen Cat #: 41190-250

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Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 86602

FBS Aliquot Prep

1 **FBS** (hiFBS): Gemini Bio Products Cat #100-106 1L
Prepare FBS Aliquots:

1.1 Thaw heat-inactivated FBS (hiFBS):
- Thaw a 500mL Bottle of FBS at 4°C. This may require more than overnight so the 500mL Bottle may be removed 2 or 3 days prior to use. Do not leave the FBS at room temperature overnight.

1.2 Aliquot the 500mL FBS Bottle in 50mL aliquots (a total of ten 50mL hi-FBS Aliquots)

1.3 Label the aliquots with Batch#, Expiration Date, Aliquot Date

1.4 Store 50mL aliquots @ -20ºC until expiration date or for up to 2 freeze/thaw cycles

FACS Wash

2 **FACS Wash w/ EDTA (D-PBS with 0.5% FBS + 2 mM EDTA):** Invitrogen Cat # 41190-250

2.1 Remove 4.5mL PBS from a 500mL bottle

2.2 Add 2.5mL of thawed FBS

2.3 Add 2mL of 0.5M EDTA solution (Catalog #: E7889-100ML)

2.4 Label the bottle with preparer's initials and expiration date (one month from preparation)

2.5 Store @ 4°C

Pen-Strep-Glut (PSG)

3 **Pen-Strep-Glut (PSG) (L-Glutamine-Penicillin-Streptomycin Soln):** Sigma Cat#: G6784-100ML

3.1 Thaw 100mL bottle

3.2 Aliquot into 10mL into 15mL conicals (total of ten 15mL conicals)

3.3 Label the aliquots with Batch#, Expiration Date, Aliquot Date

3.4 Store @ -20°C

R10FBS Media Preparation

4 **R10FBS Media Preparation (“R10”)**

4.1 Remove 55mL RPMI from a 500mL bottle of RPMI

4.2 Add 50mL aliquot of thawed, hiFBS

4.3 Add 5mL aliquot of thawed Pen-Strep-Glut

4.4 Label the bottle with preparer's initials and expiration date (one month from preparation)

Cytofix/Cytoperm

5 **Cytofix/Cytoperm Solution (10X) with GolgiStop (monensin):** BD Biosciences Cat # 554715

Kit Contents :

- 125mL 10X Perm/Wash Buffer

- 100mL Cytofix Solution
- 0.7mL GolgiStop (monensin)

To prepare 1X Perm Wash:
1. Dilute 10X BD Perm/Wash buffer in distilled H2O to make a 1X solution prior to use

Formaldehyde Solution

6 **1% Formaldehyde Solution ("1% Fix") ("PFA")**

Reagents:
- 10% Formalin
- PBS

6.1 Add 5 mL 10% Formalin to a sterile 50 mL centrifuge conical tube

6.2 Add 45 mL PBS

6.3 Label the bottle with reagent name, initials & expiration date (one month from preparation)

6.4 Store 1% Fix at Room Temperature (18-25°C) for up 1 month

PepMixes

7 **PepMixes:**

- PepMix CEFX Ultra SuperStim Pool MHC-II Subset, JPT Technologies Cat #:PM-CEFX-3
- PepMix CEF Pool (extended), JPT Technologies Cat #: PM-CEF-E-1

7.1 Resuspend peptide pellet [25ug] in 50uL of DMSO [500ug/mL]

- Add 10uL DMSO at a time, with vortexing to resuspend

7.2 Aliquot and store at -20°C until day of use

7.3 On day of use, use 0.45uL per test

Protocol

8 Overview

Day 1: Thaw cells and distribute to 2 plates. Rest plates at 37C/5% CO2 for >6 hours
Overnight: Stim for 6 hours
Day 2: 1 Stim + 1 Unstim plate stain for surface and ICS then acquire flow data
Time experiment steps according to lab and anticipated acquisition schedule

8.1 Day 1 – Thawing

1. Prepare 20ml of R10 in 50mL conical tube per sample (1-4 vials per tube)

2. Warm the R10 for 30 min at 37C prior to use

3. Place cryovials in a 37C bath for 3-5 sec at a time. Withdraw, examine and repeat (usually 3-4 rounds) until small, pea-sized amount of ice remains

4. Spray with 70% EtOH and wipe off before returning to the hood

5. To each cryovial, add 1ml of R10 dropwise to each cryovial

6. Transfer the 2ml PBMC sample from the cryovials into the 50ml conicals

7. Invert 3x to mix

8. Centrifuge at 350g for 10 min

9. Pour off the supernatant, do not shake to allow some volume to remain

10. Gently swirl the 50mL conical in remaining volume to loosen pellet

11. Add 10mL pre-warmed R10 and resuspend by pipetting 10 times. Mix sample carefully but thoroughly to break up any cell clumps.

12. Centrifuge at 350g for 10 min

13. Pour off the supernatant, do not shake to allow some volume to remain

14. Gently swirl the 50mL conical in remaining volume to loosen pellet

15. Add 10mL pre-warmed R10 and resuspend by pipetting 10 times. Mix sample carefully but thoroughly to break up any cell clumps

16. **COUNTING & VIABILITY:**

a) Perform a cell count using the Countess II to determine PBMC viability & recovery
b) Add 10uL Trypan Blue to well of mixing plate
c) Add 10uL Cells, pipette up and down
d) Remove 10uL of cell mix and dispense into Countess slide
e) Wait 30 sec
f) Calculate total cells and viability
g) Insert into Countess II to calculate total cells and viability

8.2 Overnight – Stim

1. Cells have been plated at 2x10^6 cells/well in 200uL R10 and rested for >6hours

2. For all wells (Stim and Unstim), add BFA/Monensin at

a. BFA: 0.23 uL per test
b. Monensin: 0.16 uL per test
c. CD107a antibody: 0.313uL per test

3. For Stim plates, add CEF PepMix at

a. PepMix CEFX Ultra SuperStim Pool MHC-II Subset: 0.45uL per test
b. PepMix CEF Pool (extended) : 0.45uL per test

4. Gently plate by vortexing at medium speed for 3 sec

5. Incubate cells at 37º C, 5% CO2 for 6 hours

6. At 6 hours (9am), wrap plate in Parafilm and place in 4C refrigerator and proceed with staining

	A	B	C	D

	A	B	C	D
	Stim Mixes	# Tubes:	16	16
		uL per test	Stim	UnStim
	BFA	0.23	4.232	4.232
	Monensin	0.16	2.944	2.944
	CEFX ultra MHC II	0.45	8.28	-
	CEF Pool Ext (MHC I)	0.45	8.28	-
	CD107a	0.313	5.7592	5.7592
	R10	23.5	432.4	432.4
	Total	25.103		
	Add 25uL Mix to 200uL Cells for total 225uL			

8.3 Day 2 – Stain

- Keep everything as cold as much as possible
- Keep everything covered as much as possible; work in dark or incandescent light

Surface staining:

1. Remove plates from refrigerator

2. Centrifuge 400g x 3 min

3. Flick off supernatant and vortex gently

4. Add 47.5uL FACS wash to each well

5. Add 2.5uL TruStain FCX blocking to each well

6. Incubate 4C for 15 min

7. Prepare Surface Stain Mix in PBS with 10uL BSB Plus, set aside

8. Add 50uL of Surface Stain Mix in PBS to each well and gently mix

9. Incubate in 4C fridge for 30 min, covered in foil

10. Add 100uL cold FACS wash & mix by pipetting up and down x3

11. Centrifuge 400g x 3 min

12. Flick off supernatant and vortex gently

13. Add 200uL cold FACS wash to each well

14. Centrifuge 400g x 3 min

15. Flick off supernatant and vortex gently

Intracellular cytokine staining (ICS):

Surface / ICS Stain Steps:

Reagent	Vol Added/tube	Init
Surf Stain Mix	50uL	
FACS Wash	100uL	
FACS Wash	200uL	
CytoFix/CytoPerm	100uL	
1X Perm Wash	100uL	
1X Perm Wash	200uL	
ICS Stain Mix	50uL	
1X Perm Wash	150uL	
1X Perm Wash	200uL	
1X Perm Wash	200uL	
1% PFA	200uL	

16. Add 100uL BD Cytofix/Cytoperm solution to each well.
a. **NOTE:** mix well with cells

17. Incubate on ice for 20 min, covered in foil
a. **NOTE:** do not over-incubate (Cytofix is toxic to cells)

18. Prepare ICS Stain Mix in 1X Perm Wash with 10uL BSB Plus, set aside

19. Add 100uL cold 1X Perm Wash solution

20. Centrifuge 400g x 3 min

21. Flick off supernatant and vortex gently

22. Add 200uL cold 1X Perm Wash solution

23. Centrifuge 400g x 3 min

24. Flick off supernatant and vortex gently

25. Add 50uL of the ICS Mix in Perm Wash to each well and gently mix

26. Incubate at 4° in refrigerator x 30 min, covered in foil

27. Add 150uL cold 1X Perm Wash solution

28. Centrifuge 400g x 3 min

29. Flick off supernatant and vortex gently

30. Add 200uL cold 1X Perm Wash solution

31. Centrifuge 400g x 3 min

32. Flick off supernatant and vortex gently

33. Add 200uL cold 1X Perm Wash solution

34. Centrifuge 400g x 3 min

35. Flick off supernatant and vortex gently

36. Add 200uL 1% PFA

37. Transfer samples to bullet tubes, cover with aluminum foil, store at 4°C, & acquire within 6 hours

	A	B	C	D	E	F	G	H	I	J
	Staining Step	Specificity	Fluor	Vendor	Cat#	Clone	Isotype	Conc ug/mL	MRA uL	Titered uL

	A	B	C	D	E	F	G	H	I	J
	Stim	CD107a	PE	BL	328608	H4A3	IgG1 k	400	5	0.313
	Surface	KLRG1	BV421	BL	367706	SA231A2	IgG2a k	100	5	1.25
	Surface	CD45RA	Pacific Blue	BL	304118	H100	IgG2b k	500	1	0.5
	Surface	CD8	BV570	BL	301038	RPA-T8	IgG1 k	100	5	2.5
	Surface	CD127	BV605	BL	351334	A019D5	IgG1 k	100	5	2.5
	Surface	CD56	BV650	BL	362532	5.1H11	IgG1 k	100	5	1.25
	Surface	CCR7	BV711	BL	353228	G043H7	IgG2a k	100	5	5
	Surface	CD27	BV750	BL	302850	O323	IgG1 k	100	5	2.5
	Surface	PD1	VioBright 515	Miltenyi	130-120-386	REA1165	rHu IgG1		2	2
	Surface	NKG2A	PE-Vio615	Miltenyi	130-120-035	REA110	rHu IgG1		2	1
	Surface	CD16	PerCP-Cy5.5	BL	302028	3G8	IgG1 k	200	5	2.5
	Surface	CD38	PerCp-eFluor710	TF	46-0388-42	HB7	IgG1 k	120	5	5
	Surface	CD19	SparkNIR 685	BL	302270	HIB19	IgG1 k	100	5	1.25
	Surface	CD14	SparkNIR 685	BL	367150	63D3	IgG1 k	200	5	0.156
	Surface	Zombie nIR	Zombie nIR	BL	423105	-	-	-	1	0.4

	A	B	C	D	E	F	G	H	I	J
	Surface	HLA-DR	APC-Fire810	BL	307674	L243	IgG2a k	50	5	2.5
	ICS	CD3	BV480	BD	566105	UCHT1	IgG1 k	200	5	2.5
	ICS	CD4	PerCP	BD	550631	L200	IgG1 k	6.3	20	5
	ICS	IFN-g	PE-Cy7	BL	502528	4S.B3	IgG1 k	50	5	0.313
	ICS	IL-2	APC	BL	500310	MQ1-17H12	Rat IgG2a k	25	5	0.313
	ICS	TNFa	Alexa700	BL	502928	MAb11	IgG1 k	100	5	0.63