FACS-array protocol

Striatal Dissociation Protocol for FACS

Dissection

Set up equipment for dissection and vibratome for cutting (make sure to pack vibratome with ice to keep brain cold)

1. Anesthetize animal and dissect out brain (use 3-4 EGFP mice and 1 wt mouse)
2. Immediately place brains in slice solution (bubbled with carbogen)
3. Place brains on petri dish(w/ filter paper) and cut off cerebellum and tip of forebrain with razor blade
4. Crazy glue brain to vibratome, cover in slice solution and section at 400µm (EGFP brains can be cut together)
5. Remove sections to Petri dish (keep in slice solution) and dissect out striatum under dissecting microscope
6. Place striatal sections in papain mix

Dissociation

Prior to dissection:

1. If using a new albumin ovomucoid inhibitor (AOI) vial then add 32 ml EBSS and equilibriate with carbogen
2. Add 5 ml EBSS to papain mix and split mix into separate tubes for wt and EGFP tissue
3. Incubate papain mix at 37º C while equilibriating with carbogen (95% O_2/5% CO_2)
4. Add 500µl EBSS to DNase vial and mix gently (keep DNase on ice until ready to use)

After dissection:

1. Add DNase to papain mix (50µl per 1 ml papain mix) **DNase is added to prevent the cells from clumping together**
2. Place striatal sections in papain mix and displace air in vial with carbogen
3. Incubate at 37º C for 40min
4. Periodically shake tissue sections by hand (ideally a rocker platform should be used)
5. Triturate with 3 glass pipettes of decreasing tip diameter
6. Centrifuge @ 1200rpm for 5min
7. Prepare EBSS/AOI solution: 2.7ml EBSS , 300µl reconstituted AOI, and 150µl DNase
8. Remove supernatant and resuspend cell pellets in EBSS/AOI solution
9. Prepare discontinuous density gradient: Add 2-3ml AOI to a 15 ml tube and carefully layer cell suspension atop
10. Centrifuge @ 900rpm for 6min

11. Discard supernatant and resuspend pellet in 2ml FACS Buffer
12. Pass cell suspension through 70µm mesh and collect in a 50 ml tube
13. Centrifuge at 1200rpm for 5 min
14. Remove supernatant and resuspend in 1 ml FACS Buffer
15. Transfer to sterile FACS tube and add 2µl PI (propodium iodide) (1mg/ml) to stain dead cells red
16. Transfer samples on ice to FACS core for sorting

*__keep cells on ice in between centrifugation and use a 4°C centrifuge if possible__

note: addition of phenol red can interfere with the lasers' ability to properly detect fluorescence

FACS Buffer recipe (50ml)
-50mg BSA
-0.5ml 1M Hepes
-0.5ml 100x Pen-Strep
-1.25mg DNase (2,020 units/mg)
-Bring up to 50ml with L15-CO2 w/ no Phenol Red

Slice Solution

Agent	mM	MW (mols)	2L	4L
NaHCO3	26	84.01	4.3686g	8.7372g
NaH2PO4 . H20	1.25	137.99	.3450g	.6900g
Glucose	10	180.2	3.6040g	7.2080g
5M NaCL	130	58.44	52mL	104mL
2M KCl	3	74.56	3mL	6mL
1 M MgCl2	5	95.21	10mL	20mL
1M CaCl2	1	147.02	2mL	4mL

ph=7.2-7.4
Osmo=290-310

Papain Dissociation Kit Vials
Vial 1: EBSS
Vial 2: Papainwith L-cysteine and EDTA
Vial 3:DNase
Vial 4: AOI

FACS/RNA extraction

1. FACS cells immediately- prolonging FACS has resulted in decreased EGFP+ cell recovery

2. We use the UCLA Flow Cytometry Core: There are multiple technicians sorting on multiple machines but it is best to use the same technician/machine for consistency
3. Use wt (non-EGFP) cells to set parameters
4. FACS set up should be set to detect PE (PI signal) and FITC (EGFP signal)
5. Keep cells at 4°C during FACS
6. Collect cells in FACS buffer at 4°C

We collect two populations of cells: Low EGFP expression- FITC signal 10^3-$10^{3.5}$, High EGFP expression- FITC signal >$10^{3.5}$ (Total High EGFP recovery is typically 5,000-12,000 cells)

7. Spin down cells immediately following FACS and proceed with Arcturus Picopure RNA extraction protocol (w/ Qiagen DNase treatment)
8. Check RNA integrity on Bioanalyzer picochip (Geschwind Lab)

We use high EGFP expressing cells for further study- this population shows consistent RT-PCR results for our known genes.

Two Round RNA Amplification

Amplify and label with Agilent Low RNA Input Fluorescent Linear Amplification Kit (starting RNA concentration is approximately **3ng**- a relative concentration was obtained from the picochip. 2 rounds of amplification is necessary.)

* *For RT-PCR one round of amplification is sufficient.

DAY I

cDNA Synthesis from Total RNA

Annealing Rxn

	uL
RNA soln (3ng)	10.3
T7 Promoter Primer	1.2
Total Vol	11.5

Incubate 65C 10 min, ice 5 min.

cDNA Rxn

Annealing Rxn	11.5
5X First Strand Buffer	4
0.1 M DTT	2
dNTP Mix (10mM)	1
MMLV RT	1
RnNase Out	0.5
Total Vol	20

Incubate 40C
2hrs.
Inactivate 65C 15 min., ice 5 min.

IVT Amplification - Round 1

IVT Amplification Rxn

cDNA rxn	20
Nuclease free water	12.1
4X Transcription Buffer	20
0.1 M DTT	6
NTP Mix	8
CTP	5.6
50% PEG	6.4
RnNase Out	0.5
Inorganic Phosphate	0.6
T7 RNA Polymerase	0.8
Total Vol	80

Incubate 40C for 16 hours **(Protocol suggested 4 hours to overnight. I did 16 hours the first time and used the same time for all my samples.)**

Purification by Qiagen RNAeasy Mini Kit, Elute in 30 uL Water
Vacuum centrifuge down to 10.5uL
(Recently I've been using Qiagen Rneasy mini elute kit to avoid the vacuum centrifuge.)

DAY II

cDNA Synthesis from cRNA

First Strand Synthesis
Annealing Rxn

cRNA Soln	10.5
Random Hexamers	1
Total Vol	11.5

Incubate 65C 10 min, ice 5 min.

cDNA Rxn - First Strand

Annealing Rxn	11.5
5X First Strand Buffer	4
0.1 M DTT	2
dNTP Mix (10mM)	1
MMLV RT	1
RnNase Out	0.5
Total Vol	20

Incubate 40C for 2 hrs.

Second Strand Synthesis
Annealing Rxn

cDNA Soln	20
T7 Promoter Primer	1.2
Total Vol	21.2

Incubate 65C 10 min, ice 5 min.

cDNA Rxn - Second Strand

Annealing Rxn	21.2

Nuclease free water	1.8
5X First Strand Buffer	8
0.1 M DTT	4
dNTP Mix (10mM)	2
MMLV RT	2
RnNase Out	1
Total Vol	40

****Some of this sample can be used for RT-PCR validation**

Incubate 40C
2hrs.
Inactivate 65C 15 min., ice 5 min.

DAY III

IVT Amplification - Round 2

IVT Amplification Rxn	**Cy3**	**Cy5**
cDNA rxn	20	20
CTP-Cy3	2.4	2.4
Nuclease free water	15.3	15.3
4X Transcription Buffer	20	20
0.1 M DTT	6	6
NTP Mix	8	8
50% PEG	6.4	6.4
RnNase Out	0.5	0.5
Inorganic Phosphate	0.6	0.6
T7 RNA Polymerase	0.8	0.8
Total Vol	80	80

Incubate 40C for 5 hours
(Protocol suggested 4 hours to overnight. I did 5 hours the first time and used the same time for all my samples.)

Purification by Qiagen RNAeasy Mini Kit, Elute in 60 uL (2 X 30 uL) Water

14. Prior to hybridization check concentration on Nanodrop and Bioanalyzer nanochip (Geschwind lab)
15. Hybridize to mouse and mouse developmental Agilent arrays (Microarray core)

FACS-array reagents/kits/etc

Product	**cat#**	**company**	**price ($)**
Cell Dissociation/FACS			
Dissociation			
Papain Dissociation System (1bx)	LK003150	Worthington Biochem	160.00
Slice solution (see protocol for recipe)			

glass pipettes			
FACS Buffer reagents			
Leibovitz-L15 media (no phenol)	21083-027	Invitrogen	
BSA			
Pen-Strep 100x	P0781	Sigma	
Dnase I	D 4527	Sigma	
Hepes			
Other reagents			
Propidium Iodide			
70µm mesh cell strainer	21008-952	VWR	
FACS (UCLA Flow cytometry core)			$70/hour?

RNA

RNA extraction			
Picopure RNA Extraction	KIT0204	Arcturus	
RNA quality			
Picochip (Agilent Bioanalyzer)	5065-4473	Agilent	598.00
Nanochip (Agilent Bioanalyzer)	5065-4476	Agilent	487.00

RNA Amplification

Low Input Linear Amp kit (20 rxns)	5184-3523	Agilent	798.00
Cy-3 CTP (100nmol)	NEL580	Perkin Elm	214.20
Cy-5 CTP (100nmol)	NEL581	Perkin Elm	214.20
Rneasy Mini kit	74104	Qiagen	203.00
	(alternative : order Rneasy mini elute kit so RNA doesn't have to be vacuum centrifuged after 1st round)		

Microarray Hybridization

Agilent Whole mouse oligo Arrays (5 slides)	G4122A	Agilent	2875.00
Hybridization kit Plus (for 10 slides)	5184-3568	Agilent	226.00
Stabilization and Drying Solution, 500 mL	5185-5979	Agilent	95.00
Surehyb backings (5 slides)	G2534-60003	Agilent	100.00
Other			
Scanning (per array)	Array Core	Agilent	25.00