University of Michigan Comprehensive Cancer Center	SOP-BCR-3.8	**Tumorspheres**	Author: S. Clouthier Sean McDermott Approved: M. Wicha	Rev: 0	Issued: 1/20/14 Revised:

1.0 Purpose

The purpose of SOP 3.8 is to describe how to create and plate tumorspheres from xenografts.

2.0 Scope

SOP 3.8 is intended to cover all resources, personnel and equipment in the BCR laboratory.

3.0 Primary Breast Cancer core samples – FACS staining

Channel	Marker	MAb	Clone	Company	Catalog	Lot	Conc	Stain vol (1X)
Alexa 750	Live-Dead	NA	NA	Invitrogen	L10119	1004203	1:10 dil	1 uL
V450	Mouse H2kd	Mouse IgG2a	SF1-1.1.1	eBioscience	48-5957-80	E14335-101	200ng/ul	1 uL
Cells								98 uL
							Total	100 uL

Channel	Marker	MAb	Clone	Company	Catalog	Lot	Conc	Stain vol (1X)
Alexa 750	Live-Dead			Invitrogen	L10119	1004203	1:10 dil	1 uL
V450	Mouse H2kd	Mouse IgG2a	SF1-1.1.1	eBioscience	48-5957-80	E14335-101	200ng/ul	1 uL
Cells								25 uL
Buffer								73 uL
							Total	100 uL

4.0 Materials/ Reagents needed:

4.1. 5 mL FACS tubes (12 x 75mm) with BLUE 35uM filter cap (BD 352235)
4.2. 5 mL FACS tubes (12 x 75mm) (BD 352058)
4.3. 500 uL microcentrifuge tubes [sterile]
4.4. Live-Dead Near IR (aka Alexa 750/APC-Cy7 channel) [Invitrogen L10119]
    a. Already diluted in 100% DMSO; may be frozen;
4.6. BD CompBead Plus Anti-Mouse Ig and Negative control set [BD 560497]
    a. Anti-Mouse Ig, k [Component 51-9006274; BLUE cap dropper]
    b. Negative control (BSA) [Component 51-9006227; WHITE cap dropper]
4.7. ArC Bead kit [Invitrogen A10346]
    a. Reactive Beads [Component A – GREEN cap]
    b. Negative Beads [Component B – white cap]
4.8. Antibodies. See tables for catalogs
4.9. PBS (or HBSS) with 3% FBS, sterile filtered
4.10. MCF7 (or SUM159) cells actively growing; at least 0.5x10^6 cells
4.11. Digested tumor xenograft cells in less than 3.5 mL volume
4.12. Cytometer with violet (i.e. ~405nm) laser

5.0 Procedure [for one xenograft sample and MCF7]:

5.1. Euthanize mouse with isoflurane and CO_2 and place it into 70% EtOH in a white bucket, and transfer to hood. Move mice to paper towel prior to dissection.
5.2. Take out fresh xenograft tumor tissue from mouse and place into labeled Petri dish (10 cm plate) for dissociation. *If the sample is too large for one plate, use multiple Petri dishes and combine after mincing*.
5.3. Cut one piece from the edge and place into corresponding cassette for Histology. Place cassette in 10% formalin.
5.4. Mince remaining tissue into very fine pieces by crossly using two sterile scalpels. Transfer the minced tissue into a 50-ml Conical tube, and add 10-15ml warm tumor Collagenase solution.
5.5. Either shake the tube on a rotary shaker for 45 minutes and check per 30 minutes until all larger tissue fragments are digested **OR** mix with a 5 mL pipette and place 50 mL tube(s) into a 37 degree C water bath, and every 15 minutes pipette each sample with a 5 mL pipette, then place back in water bath for a total of 45 minutes.
5.6. Add HBSS with 2% FBS to the 50 mL tube(s) and sieve each sample through a 40 uM cell filter into second labeled corresponding 50 mL tube.
5.7. Spin down at 1000 rpm for 5 minutes. Discard the supernatant.
5.8. Wash in 10 mL HBSS, centrifuge again and then aspirate the supernatant.
5.9. Resuspend pellet in 1 mL or so (depending on pellet size) of HBSS with 2% FBS.
5.10. Label 3 regular FACS tubes for compensation: “Unstained”, “Ax750”, “V450”
5.11. Label 6 regular FACS tubes: “Stain”, “DEAB”, and “Isotype”
    a. Label one set “Xenograft”, the other set “MCF7”
5.12. Label 2 blue-cap filter tubes: “xenograft” and “MCF7”
5.13. Label microcentrifuge tubes: “L/D”, “Stain”, “Isotype”
5.14. Filter xenograft and MCF7 cells into separate blue-cap FACS tubes:
    a. Add ~400 uL cells to filter (while still on FACS tube) and gently tap tube to get cells through filter.
    b. Add remaining cells to filter, which should pass through filter with relative ease
        i. Xenograft samples with a lot of clumps may clog the filter and it may take a bit of time to get whole sample through filter
    c. Add 1000 uL Aldefluor staining buffer to filter to exchange buffer
    d. Centrifuge tubes for 5min at 4C at 300-350g
    e. Aspirate supernatant, taking care to minimize disruption of cell pellet
    f. Resuspend MCF7 cells with 350 uL Aldefluor staining buffer
    g. Resuspend xenograft cells with 40 uL Aldefluor staining buffer
        i. Measure volume of cells
        ii. If needed, adjust volume with Aldefluor staining buffer to 100 uL
    h. Leave cells at RT (or ice if you are paranoid about cell death)
5.15. Dilute Live-Dead
    a. Add 18 uL Aldefluor staining buffer to “L/D” microcentrifuge tube
    b. Add 2 uL Live-Dead stock [screw-cap tube] into staining buffer
5.16. Add compensation beads to tubes:
    a. V450 tubes receive:
        i. One drop BD CompBead Plus Anti-Mouse Ig beads (BLUE cap dropper)
        ii. One drop BD CompBead Plus Negative control (BSA) [white cap dropper]
    b. Ax750 tube receives drop ArC Reactive beads [Component A – GREEN cap]
5.17. Add antibodies to beads in appropriate tube
    a. 1 uL Mouse H2Kd V450 to “V450” tube

5.18. Add 1 uL Live-Dead stock [NOT diluted] to “Ax750” tube
5.19. Add 100uL MCF7 cells to “unstained” and “ALDH” tubes [from Step 5f]
    a. Add 0.5 uL Aldefluor reagent to “ALDH” tube
5.20. Make “Stain” and “Isotype” master mixes in 500 uL microcentrifuge tubes:
    a. For 1 xenograft and MCF7, make 3X “Stain” mix

Channel	Marker	Stain (1X)	Stain (3X)
FITC	Aldefluor	0.5 uL	1.5 uL
PE	EpCAM	10 uL	30 uL
PE-Cy7	CD24	2 uL	6 uL
APC	CD44	10 ul	30 uL
Alexa 750	Live-Dead	1 uL “L/D”	3 uL “L/D”
V450	Mouse H2kd	1 uL	3 uL
	Total volume	24.5 uL	73.5 uL
	Vol/tube	24.5 uL	24.5 uL

    b. For 1 xenograft and MCF7, make 3X “Isotype” mix

Channel	Marker	Stain (1X)	Stain (3X)
FITC	Aldefluor	0.5 uL	1.5 uL
PE	Iso	2 uL	6 uL
PE-Cy7	Iso	2 uL	6 uL
APC	Iso	1 ul	3 uL
Alexa 750	Live-Dead	1 uL “L/D”	3 uL “L/D”
V450	Mouse H2kd	1 uL	3 uL
	Total volume	7.5 uL	22.5 uL
	Vol/tube	7.5 uL	7.5 uL

5.21. Aliquot xenograft and MCF7 cells to regular FACS tubes
    a. 75.5 uL cells [from Step 5g] to “Stain” tube
    b. 25 uL cells [from Step 5g] to “Isotype” tube
        i. Add 67.5 uL Aldefluor staining buffer to “Isotype” tube
5.22. Aliquot 5 uL diluted DEAB [from Step 6] to “DEAB” FACS tubes; recap tubes
5.23. Add 7.5 uL “Isotype” master mix to “Isotype” FACS tubes
5.24. Add “Stain” master mix to “Stain” FACS tube
    a. Add 24.5 uL master mix to cells
    b. Immediately transfer 50 uL of these cells to “DEAB” tube with the 5 uL diluted DEAB. Verify that cells and DEAB are mixed
    c. Proceed to next sample and repeat Steps 16a and 16b
5.25. Incubate all tubes in incubator at 37C for 45 min to 1 hour; cover with foil if paranoid or incubator is constantly being opened
5.26. Every 15 minutes, remove tubes from incubator and quickly shake tube rack to resuspend cells
5.27. Add 500 uL Aldefluor staining buffer to “Stain”, “DEAB”, “Isotype”, “Unstained”, “ALDH” tubes
5.28. Add 1000 uL PBS / 3%FBS to V450 compensation tubes
5.29. Add 2000 uL PBS / 3%FBS to Ax750 compensation tube [need to really dilute Live-Dead reagent, which will bind to ArC negative beads]

5.30. Centrifuge all tubes for 5 min at 4C at 300-350g
5.31. Aspirate supernatant from all tubes
    a. Compensation tubes (except Ax750) should have very small visible pellet
    b. Ax750 tube will have diffuse pellet
5.32. Add 1000uL Aldefluor staining buffer to “Stain”, “DEAB”, “Isotype” tubes and resuspend pellet.
5.33. Add 500 uL Aldefluor staining buffer to “Unstained” and “ALDH” tubes
5.34. Add 500 uL PBS / 3%FBS V450 compensation tubes
5.35. Add 500 uL PBS / 3%FBS to Ax750 compensation tube
    a. Add 1 drop of ArC NEGATIVE beads [Component B – white cap]
5.36. Place tubes on ice
5.37. Bring 6 blue-cap filter FACS tubes to filter samples right before sorting to minimize clogging
    a. Bring more if you have more than one xenograft and MCF7 sample
5.38. Use MCF7 cells to setup cytometer voltages
5.39. Collect viable and Lineage negative cells from CORE “Isotype” and “DEAB” for “bulk” tumor cells for comparison

Fraction	Default gates	Isotype	DEAB	Stain
Bulk	R1+R2+R3+R4			
ALDH+EpCAM+	R1+R2+R3+R4+R15+R20			
CD24-CD44+EpCAM+ALDH-	R1+R2+R3+R4+R5+R14			

5.40. Bring a labeled 24-well ultra-low attachment plate to plate 50K cells per well in .5 mL of media (use Sean’s media recipe on SOP 3.4). Place the plate, once sorted, into 10% CO2.
5.41. You should see growth within 5-6 days. Every week, add 300 uL of media to each well, the same day each week, until you passage 3 weeks after sorting/plating.
To passage: First spin down the tumorspheres from the wells, then add trypsin and leave in the incubator for 5 minutes, then add fresh media at 2X the volume of trypsin and filter through a 40 uM filter, then spin and plate again.

6.0 Applicable References

SOP 8.14
SOP 2.4
SOP 3.4

7.0 Change Description

Revision	Date	Reference	Description of Change