Isolating and growing fibroblast cells from threespine stickleback (Gasterosteus aculeatus) V.2

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This protocol goes through the process of isolating and growing fibroblast cells from threespine stickleback.

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Reagents:

- Recombinant bFGF, 10ug/ml, Stemcell Tech 78003.1 **(-80°C)**
- Recombinant IGF-I, 100ug/ml, Stemcell Tech 78022.1 **(-80°C)**
- Collagenase II, 100mg/ml, Stemcell Tech 07418 **(-20°C)**
- Hyaluronidase, 50,000 U/mL, Stemcell Tech 07461 **(-20°C)**
- N-acetylglucosamine hydrochloride (NAG), 10 mg/mL, Sigma Aldrich A3286 **(-20°C)**
- Carboxymethyl-chitosan, 10 mg/mL, ChemCruz 358091 **(4°C)**
- Antibiotic/Antimycotic Solution 100x, Sigma A5955 **(-20°C)**
- FBS, Thermo-Fisher, Gibco 16140-063 **(-20°C)**
- L-15 (Leibovitz Medium, liquid with L-glutamine), Sigma L1518 **(4°C)**
- 1X TrypsinLE Express, Gibco 12605-010 **(room temp)**
- 1X DPBS, without magnesium or calcium **(4°C)**
- Cell culture water **(4°C)**
- Iodine (PVP-Iodine) **(room temp)**
- Bleach/Sodium Hypochlorite, Fisher SS290-1 **(room temp)**

Disposables:

- Sterile 35 mm petri dishes (CellTreat 229638)
- 6-Well culture plates (CellTreat 229105)
- Pasteur pipettes, autoclaved
- Sterile serological pipettes (5mL- Fisher, 13-676-10H; 10 mL- Fisher, 13-678-11E ; 25 mL- Fisher 13-676-10K; 50mL- Fisher, 13-678-11F)
- Micropipette tips with filters
- Sterile conical vials- (50mL- Celltreat, 229420; 15mL- any)
- Sterile cell culture flasks (Celltreat 229331)
- Sterile syringe filters (Whatman, 6870-2502)
- Sterile syringes with Luer-Lock (10 mL- VWR, 53584-023; 20 mL- VWR, 53584-025)
- Sterile single-use syringe needles (BD Precision Glide, 305196)

Materials:

- Autoclaved tweezers
- Autoclaved shears/dissecting scissors
- Pipette controller
- Micropipettes

Recipes and Other Reagent Info

Reconstitution of cytokines and reagents

- bFGF - 10ug/ml in sterile water. **Do not filter.** (stable up to 3 months at -80C; good for 1 week at 2-8C after being defrosted)
- Recommended aliquoting is 25 uL
- IGF-I - 100ug/ml in sterile water. **Do not filter**. (stable up to 3 months at -80C; good for 1 week at 2-8C after being defrosted)
- Recommended aliquoting is 15 uL
- Collagenase II - 100mg/ml in 0.9x PBS. **Do not filter**. (-20°C; do not use after defrosting)
- Recommended aliquoting is 110 uL
- Hyaluronidase - 50,000 U/mL in 0.9x PBS. **Do not filter.** (-20°C; do not use after

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defrosting)
- Recommended aliquoting is 15 uL
- N-acetylglucosamine hydrochloride (NAG) - 10 mg/mL in 0.9x PBS. **Sterile filter with media**. (4°C, up to 2 weeks after added to PBS)
- Recommended to just make 1 mL worth
- Carboxymethyl-chitosan - 10 mg/mL in 0.9x PBS. **Sterile filter with media**. (4°C, up to 2 weeks after added to PBS)
- Recommended to just make 1 mL worth
- 0.9x PBS - 555 mL. Add 55 mL of water to each new bottle of 500 mL 1X PBS
- 0.9x L-15 - 555 mL. Add 55 mL of water to each new bottle of 500 mL 1X L-15

Media recipes

- 5% FBS media (30 mL)
- 1.5 mL FBS
- 28.5 mL L-15
- 100 uL of antibiotic/antimycotic
- **Needs to be sterile filtered**
- 20% FBS initial media (~10 mL)
- 2 mL FBS
- 8 mL L-15
- 100uL antibiotic/antimycotic solution
- **Needs to be sterile filtered**
- 20% FBS full media (~10 mL)
- 2 mL FBS
- 8 mL L-15
- 50 uL NAG
- 10 uL Carboxymethyl-chitosan
- 100 uL antibiotic/antimycotic solution
- 10 uL bFGF
- 4 uL IGF-I
- **Needs to be sterile filtered**

Digestion Recipes

- TrypsinLE (10 mL)
- 9 mL 1X TrypsinLE
- 1 mL 0.9x PBS
- **Needs to be sterile filtered**
- Hya + Coll. II (5 mL)
- 5 uL Hya
- 100 uL Coll. II
- ~5 mL 0.9x PBS
- **Needs to be sterile filtered**

Tissue Sterilization Recipes

- 1% Bleach (50 mL)
- .5 mL bleach/sodium hypochlorite
- 49.5 mL 0.9x PBS

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- .2% Iodine
- .002 g IVP-Iodine
- 1 mL 0.9x PBS

Obtaining tissue

1 Prepare and sterile filter 30 mL of 5% FBS media.

2 Prepare and sterile filter 10 mL of 20% FBS media.

3 Prepare two petri dishes on ice for collecting the tissues. Fill the petri dish half of the way with the 5% FBS media.

4 Prepare 2x 6 well culture dishes: 4 wells filled respectively with .2% iodine, 1% bleach, 0.9x PBS, and 0.9x PBS.

5 Euthanize the fish following standard lab protocols and place body on ice.

6 Using tweezers and mini dissecting scissors, cut as much of the pectoral, pelvic and caudal fins and place in the petri dish. (Note that this should be done outside the biosafety cabinet to avoid later contamination). Place remaining fish back on ice and continue this step until all fins have been removed from all fish.

7 Take petri dish to biosafety cabinet and wash with each of the 4 washes for 30 seconds-1 minute each.

8 In the last PBS wash, use the scissors to cut the tissues as small as possible.

9 Using a pipette, remove all liquid and tissue from last PBS wash well and transfer to sterile 15mL tube.

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10 Begin peritoneal tissue removal, using autoclaved dissecting tubes, open fish ventrally and pin body open. Wash inside of fish with iodine solution for 1 minute each.

11 Remove peritoneal tissue using tweezers and mini dissecting scissors. Place tissue in second petri dish and repeat for each fish. Once done with dissections, repeat steps f-h for peritoneal tissue.

Digesting tissue

12 Centrifuge tubes of PBS + tissue for 10 minutes at 90 g at 4C.

13 Prepare solution of .9x TrypsinLE in a small conical tube.

14 Aspirate the PBS from tissue tubes, leaving approximately 1.5 mL of liquid + tissue while avoiding disturbing the pellet.

15 Fill to 11mL (by adding approximately 10 mL) with TrypsinLE solution, and gently mix.

16 Let the suspension incubate on the vial's side at ~17 degrees C for 20 minutes.

17 While incubating, prepare sterile collagenase and hyaluronidase solution.

18 Remove tubes from incubator and centrifuge at 90 g for 10 minutes at 4C.

19 Aspirate to remove the TrypsinLE solution, leaving leaving approximately 1.5 mL of liquid + tissue while avoiding disturbing the pellet.

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20 Resuspend cells with the 5 mL of collagenase/hyaluronidase solution and gently mix.

21 Incubate for 2 hours at ~17 degrees C (17C).

22 Centrifuge again at 90g for 10 min at 4C.

23 Aspirate to remove the collagenase/hyaluronidase solution, leaving approximately 1.5 mL of liquid + tissue while avoiding disturbing the pellet.

24 Wash cells with 5% FBS solution; gently mix to resuspend pellet.

25 Centrifuge again at 90 g for 10 min at 4C.

Cell culture flask preparation and care

26 Aspirate out the majority of the remaining liquid, leaving approximately 1.5 mL of liquid + tissue while avoiding disturbing the pellet.

27 Resuspend pellet in ~5mL the 20% FBS media and gently mix.

28 Pipette 5 mL of solution into each cell culture flask while they are positioned vertically. If there is any medium on the neck of the flask, aspirate it off.

29 CRITICAL! Make sure there is no liquid remaining on the interior flask neck or cap in order to avoid contamination. If there is liquid, aspirate to remove.

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30 Carefully transfer flasks to incubator and lay them horizontally on the side that ensures the neck of the flask is facing up. Again, do not let liquid splash on the cap while in motion.

31 Optional: check flasks under microscope for bacterial and fungal contamination.

32 Incubate at ~17 C for 12-24 hours.

33 Prepare the full 20% FBS media (with growth factors, chitosan, NAG, antibiotic- antimycotic). Make sure to sterile filter final solution.

34 Remove flasks from the incubator, check for bacterial/fungal contamination, and transfer to biosafety cabinet. Carefully remove cap and tilt flask mostly upright so that cells fall out of suspension to bottom of flask.

35 Aspirate out as much media as possible without removing cells.

36 Add 5 mL of full 20% FBS media to each flask making sure no liquid is left on the neck or cap of the flask. Aspirate any liquid which may have splashed on the neck of the flask.

37 Incubate at ~17 degrees C for three-five days.

38 Monitor cells daily. Fibroblasts should be released 2-4 days after receiving growth factors. Replace full growth medium every 3-5 days and keep them incubated at ~17 C.

Cell culture media change

39 Remove flasks from the incubator, check for bacterial/fungal contamination, and transfer to biosafety cabinet.

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40 Carefully remove cap and tilt flask mostly upright so that cells fall out of suspension to bottom of flask.

41 Aspirate out as much media as possible without removing cells.

42 Add 5 mL of full 20% FBS media to each flask making sure no liquid is left on the neck or cap of the flask.

43 Aspirate any liquid which may have splashed on the neck of the flask.

Cell culture passaging

44 Do not ever let the confluency reach over 80%.

45 Once cells have reached 60-75% confluency, they are ready to be passaged.

46 Prepare sterile .9x TrypsinLE (make 5 mL for each flask you passage), 20% FBS media with additives, and 20% FBS media (no additives).

47 When transfering flasks into biosafety cabinet, always tilt the cap side away from yourself and then spray with ethanol to avoid spraying cap with ethanol.

48 Carefully remove cap and tilt flask mostly upright so that cells fall out of suspension to bottom of flask. Aspirate old media and put in 5 mL of the TrypsinLE.

49 For the very first passage, leave trypsin in for about 5 minutes. You may want to take flask from the biosafety cabinet and look under microscope while doing this to make sure most cells have dislodged from the flask. Gently move the flask side to side throughout the time frame. After about a minute, return it to upright position, and firmly hit the side of the flask to dislodge cells into suspension. Repeat as needed to resuspend most cells, but know there will probably

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still be hundreds of cells that will remain adhered. Don't go past the 5 minute mark or else you risk further harming cells.

50 In all subsequent passages, it may only take 2-3 minutes for cells to dislodge.

51 Take flask back to the biosafety cabinet and place the flask upright.

52 Add 8 mL of the 20% FBS media. Use the same pipette to thoroughly mix and resuspend any cells remaining adhered to the bottom of the flask.

53 Place the media and the cells into a new 15mL conical tube and centrifuge at 90 g for 10 minutes at 4C.

54 Aspirate as much of the liquid as possible, leaving just enough to ensure that the pellet is not disturbed. Add ~10mL of L-15 media to thoroughly rinse the cells.

55 Centrifuge again at 90 g for 10 minutes at 4C.

56 While waiting for centrifuge to finish, prepare and label new flasks (include date, name and passage #).

57 Add 4 mL of 20% FBS media with additives to each flask.

58 Once centrifuging is complete, aspirate each tube until there is about 2 mL left.

59 Using a pipette, thoroughly mix remaining liquid to dislodge the cells and resuspend.

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60 Take 25%-50% of the 2mL and put them into a new flask.

61 Continue to change media every three-five days, and monitor until cells are ready for next passage.

Cryopreservation

62 Ideally cells should be ready for passage (minimum 60% confluency); and have been through several passages prior to freezing.

63 Prepare the Mr. Frosty using the steps listed on the container.

64 Prepare sterile .9x TrypsinLE (make 5 mL for each flask you freeze), and 20% FBS media (no additives).

65 When transfering flasks into biosafety cabinet, always tilt the cap side away from yourself and then spray with ethanol to avoid spraying cap with ethanol.

66 Carefully remove cap and tilt flask mostly upright so that cells fall out of suspension to bottom of flask. Aspirate old media and put in 5 mL of the TrypsinLE.

67 Follow steps for passaging to dislodge cells from the flask.

68 Add 8 mL of the 20% FBS media. Use the same pipette to thoroughly mix and resuspend any cells remaining adhered to the bottom of the flask.

69 Place the media and the cells into a new 15mL conical tube and centrifuge at 90 g for 10 minutes at 4C.

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70 Aspirate as much of the liquid as possible, leaving just enough to ensure that the pellet is not disturbed. Add ~10mL of 20% FBS media to thoroughly rinse the cells.

71 Centrifuge again at 90 g for 10 minutes at 4C.

72 While waiting for centrifuge to finish, prepare and label new vials (include date, name and passage #).

73 Fill vials carefully about halfway with DMSO.

74 Once centrifuging is complete, aspirate each tube until there is about 2 mL left.

75 Using a pipette, thoroughly mix remaining liquid to dislodge the cells and resuspend.

76 Take 2 mL of suspension and pipette into vial.

77 Transfer vial into Mr. Frosty and let freeze overnight in -80.

78 Transfer all vials to vapor stage of a liquid nitrogen freezer as soon as possible for long term storage.

Cell revival from cryopreservation

79 Prepare a 37C water bath.

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80 Pipette 10mL 5% FBS into a sterile conical tube; place in incubator to warm to 18C.

81 Transport vial to lab on dry ice.

82 Thaw vial QUICKLY (<1 min) in an 18C water bath (hotter if necessary).

83 Transfer contents to warmed 18C conical tube.

84 Spin at 90G for 10 minutes.

85 Aspirate liquid.

86 Wash with 5mL 5% FBS.

87 Spin at 90G for 10 minutes.

88 Resuspend in 5mL full 20% media.

89 Transfer to new flask & incubate overnight.

90 Monitor cells daily for viability.

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