Axolotl blastema dissociation into single cell suspension

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Nicholas Leigh[1]

[1]Brigham and Women's Hospital/Harvard Medical School

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Nicholas Leigh

ABSTRACT

This protocol is designed to obtain blastema cells (and wound epidermis if left attached) into a single cell suspension. It was specifically designed for single cell RNA sequencing, but can be used for other applications in which a single cell suspension is desired. The goal is to obtain a single cell suspension with near 100% viability in a rapid fashion. This protocol does not use centrifugation, but instead uses a gradient replacement strategy pioneered by Briggs et al, 2018 Science. This strategy washes away free-floating RNA from cells by using progressively denser solutions to "lift" off the solution in which the cells are currently emersed. This protocol could be can be modified to utilize centrifugation instead and likely should be if planning on anything other than single cell RNAseq on inDrops as a next step.

EXTERNAL LINK

https://doi.org/10.1038/s41467-018-07604-0

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Leigh ND, Dunlap GS, Johnson K, Mariano R, Oshiro R, Wong AY, Bryant DM, Miller BM, Ratner A, Chen A, Ye WW, Haas BJ, Whited JL, Transcriptomic landscape of the blastema niche in regenerating adult axolotl limbs at single-cell resolution. Nature Communications doi: 10.1038/s41467-018-07604-0

PROTOCOL STATUS

Working

GUIDELINES

It is critical that all steps be performed in the absence of RNases and away from any potential RNA contamination. All tools used should be cleaned with RNase Zap prior to touching tissue. All tips and tubes should be RNase-free. Ideally, all steps downstream of tissue harvest should occur in a hood.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Tricaine methanesulfonate MS222		Sigma Aldrich
Sulfamerazine sodium salt	S0800	Sigma – Aldrich
Optiprep (Iodixanol)	D1556-250ML	Sigma Aldrich
10X HBSS	14185-052	Thermo Fisher Scientific
UltraPure™ DNase/RNase-Free Distilled Water	10977023	Thermo Fisher Scientific
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PDS Kit, Papain Vial	LK003178	Worthington Biochemical Corporation
RNase Zap	R2020-250ML	Sigma Aldrich
Falcon™ Cell Strainers	08-771-2	Fisher Scientific
Costar® 6 Well Clear TC-Treated Multiple Well Plates, Individually Wrapped, Sterile	3516	Westnet
10X HBSS	14185-052	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
SYRINGE 3ML LL 200 S/C	309657	Westnet
25 G BD™ Needle 1 1/2 in. single use, sterile	305127	BD Biosciences
Optiprep (Iodixanol)	D1556-250ML	Sigma Aldrich
10X HBSS	14185-052	Thermo Fisher Scientific
BSA, molecular biology grade, 20 mg/ml	B9000S	New England Biolabs

Tissue collection and enzymatic digestion

1 Narcotize animals in 0.1% Tricaine prepared in 40% Holtfreter's.

Tricaine methanesulfonate MS222
by Sigma Aldrich

NOTE
After survival surgeries always keep animals overnight in 40% Holtfreter's supplemented with 0.5% sulfamerazine.

Sulfamerazine sodium salt
by Sigma – Aldrich
Catalog #: S0800

2 Dilute 10X HBSS with UltraPure H_2O to 0.7X HBSS. Dilute Optiprep to 5% v/v, 10% v/v, and 15% v/v using 0.7X HBSS. Place all solutions on ice. These must be cold prior to use.

Optiprep (Iodixanol)
by Sigma Aldrich
Catalog #: D1556-250ML

10X HBSS
by Thermo Fisher Scientific
Catalog #: 14185-052

UltraPure™ DNase/RNase-Free Distilled Water
by Thermo Fisher Scientific
Catalog #: 10977023

3 Dilute 10X DPBS with UltraPure H_2O to 0.7X HBSS. Add 2.5mL of 0.7X HBSS to the papain and put bottle in a 37°C water bath for 10 minutes to dissolve papain into solution. After 10 minutes, remove 400ul of papain solution and add to a RNase-free 1.5mL microcentrifuge. Papain should be at room temperature prior to use.

UltraPure™ DNase/RNase-Free Distilled Water
by Thermo Fisher Scientific
Catalog #: 10977023

PDS Kit, Papain Vial
by Worthington Biochemical Corporation
Catalog #: LK003178

4 With iridectomy scissors cut parallel to the plane of the limb around the circumference of the blastema where it interfaces with the stump. Slide a forcep between the WE and blastema, and slowly peel away the wound epidermis. At this point you can either discard the wound epidermis or put the wound epidermis and blastema (post-separation) into the microcentrifuge tube with papain. Start a timer counting up.

NOTE
Thoroughly clean iridectomy scissors and forceps with RNase Zap prior to use.

RNase Zap
by Sigma Aldrich
Catalog #: R2020-250ML

5 Place microcentrifuge tube on a nutating rocker and rock for 10 min. Make sure that tissue does not stick to the side of the tube throughout rocking.
00:10:00

6 Using a p1000 pipette 20-30 times, making sure that the tissue enters the pipette each time. The first few pipettes may need to break down the tissue a bit so that it can enter the tip and may need to be forceful. After the tissue can easily enter, pipette gently. Generally this should take about 1 min. Place back on rocker for 5 min.
00:05:00

7 At the 15 min post-harvest, use a p1000 to gently pipette the tissue 20-30 times and place back on nutating rocker for 5 min.
00:05:00

8 Repeat step 7 after the tissue has been rocking for 20, 25, and 30 min post-harvest. In total, gently pipettting should occur at 10, 15, 20, 25, and 30 min post-harvest.

Removal of free-floating RNA

9 After final pipette step, use a p1000 pipette to pass cells through a 70µm cell strainer into the bottom of a sterile, tissue culture-treated 6-well plate. Wash microcentrifuge tube with 600µl of 0.7X HBSS to collect any cells that remained in the tube and pass this through the cell strainer into the 6-well plate. With 2mL 0.7X HBSS wash the cell strainer, making sure that 0.7X HBSS passing through the strainer enters the 6-well plate. After all straining/washes, there should be 3mL of cell containing solution in one well of a 6-well plate

Falcon™ Cell Strainers
by Fisher Scientific
Catalog #: 08-771-2

Costar® 6 Well Clear TC-Treated Multiple Well Plates, Individually Wrapped, Sterile
by Westnet
Catalog #: 3516

10X HBSS
by Thermo Fisher Scientific
Catalog #: 14185-052

10 Place the plate on ice and let cells settle to bottom of 6-well plate for 10 min.
00:10:00

11 Towards the end of the incubation fill a 3mL syringe with 25 1/2 gauge needle with 5% Optiprep solution.

SYRINGE 3ML LL 200 S/C
by Westnet
Catalog #: 309657

25 G BD™ Needle 1 1/2 in. single use, sterile
by BD Biosciences
Catalog #: 305127

Optiprep (Iodixanol)
by Sigma Aldrich
Catalog #: D1556-250ML

10X HBSS
by Thermo Fisher Scientific
Catalog #: 14185-052

12 After 10 min. has passed use the 3mL syringe to slowly layer 3mL of 5% Optiprep down the side of the well trying not to disturb the cells. Let cells re-settle for 1-2 min.

13 Using a p1000 remove ~3mL of 0.7X HBSS, which should have risen to the top after it was replaced with denser 5% Optiprep. Do this in the same location as where the optiprep was layered in, this area should be the most devoid of cells.

14 Let cells sit for ~1 min to resettle, during this time fill the next syringe with 10% optiprep, before repeating steps 12 and 13 with 10% Optiprep. This time the layer that will be removed is the 5% Optiprep.

15 Repeat steps 12 and 13 with 15% optiprep for layering in and removing the 10% Optiprep layer.

16 Using a p1000 remove ~5.5mL of 10%/15% Optiprep, leaving ~200ul or less of 15% Optiprep which contains the cells.

17 Make sure to wash the bottom of the plate thoroughly by both swirling and a few pipettes with the p1000, move cells into an RNase-free microcentrifuge tube.

Preparing cells for inDrops

18 Count cells using trypan blue to determine viability. Cells should be >90% viable and will range in concentration based on the size of the blastema taken and the final volume.

19 Estimate the total volume of cell containing 15% Optiprep using a p1000 and then add BSA to 0.5% (i.e. 0.5µl of 20mg/mL solution to 100µl cells)

BSA, molecular biology grade, 20 mg/ml
by New England Biolabs
Catalog #: B9000S