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Tissue disaggregation and isolation of specific cell types from transgenic *Xenopus* appendages for transcriptional analysis by FACS

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Abstract

Xenopus embryos and tadpoles are versatile models for embryological, cell biological, and regenerative studies. Genomic and transcriptomic approaches have been increasingly employed in these frogs. Most of these genome-wide analyses have profiled tissues in bulk, but there are many scenarios where isolation of single cells may be advantageous, including isolation of a preferred cell type, or generation of a single-cell suspension for applications such as scRNA-Seq. Here we present a protocol for the disaggregation of complex tail and limb bud tissue, and use cell type-specific fluorescence in transgenic *X. tropicalis* appendages to isolate specific cell populations using fluorescence activated cell sorting (FACS). Our protocol addresses a specific challenge in *Xenopus* embryos and tadpoles: the storage of maternal yolk platelets in each cell, which can introduce light scatter and thereby false positives into FACS analysis. Here we gate against both non-transgenic and ubiquitously transgenic animals to reduce both false positives and false negatives. We use the Xtr.Tg(pax6:*GFP*,cryga:*RFP*,actc1:*RFP*)^{Papal} transgenic line as a test case to demonstrate that nucleic acid preparations made from sorted cells are high quality and specific. We anticipate this method will be adaptable to study various cell types that have transgenic reporter lines to better profile cell types of interest.

Keywords

Xenopus; FACS; transgenic; ATAC-Seq; limb bud; tail regeneration

Introduction

Xenopus tropicalis and *Xenopus laevis* are leading models for embryonic development, appendage regeneration, and human disease modeling (reviewed in^{1–4}). Next generation sequencing approaches are used routinely in both these species to profile changes in gene expression, transcription factor occupancy, chromatin conformation and chromatin accessibility (reviewed in^{2,5,6}). More recently, scRNA-Seq has also allowed interrogation of transcriptional changes in specific cell types^{7–9}. However, there remain many scenarios where it may be advantageous to isolate a cell population of interest prior to sequencing

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or other downstream applications. Among other reasons, in situations where one seeks to identify transcriptional changes in a specific or rare cell type, profiling gene expression in all the other cell types within that tissue may be both superfluous and cost-prohibitive. Other applications where capture of defined cell types may be useful include assaying simple abundance of a target cell type, capturing a cell based on a reportable state transition (such as cell cycle state or signaling pathway reporter), or capturing a subset of cell types with shared expression of a single marker. For most of these applications, fluorescent transgenic reporters can be used to identify the target cell population, and in many other species, fluorescence activated cell sorting (FACS) based on transgenic fluorescent reporters or fluorescent cell surface markers is routinely used to capture the cell population of interest^{10–15}.

A specific challenge to FACS in *Xenopus* species is that for the first seven days after fertilization each cell has its own maternal yolk store¹⁶. The yolk is light scattering, thus potentially making it more difficult to confidently recover true positive fluorescent cells. Several groups have successfully used FACS in *Xenopus* embryos or tadpoles, demonstrating that the technique can be leveraged in these species to recover diverse cell types ranging from blood cells to neurons^{9,17–21}. However, there is not yet a consensus protocol available that specifically addresses yolk light scatter and how to recover true fluorescence from a yolk background. As the library of cell type-specific fluorescent reporter lines continues to expand in both *Xenopus* species, a standardized protocol for FACS will be advantageous. The tadpole tail and limb bud represent useful starting tissues for this analysis. In addition to their widespread value as models for regeneration^{4,22–24}, both appendage types comprise a variety of cell types from ectodermal, mesodermal, and neural crest lineages. Moreover, a prerequisite for FACS as well as other techniques that require cell disaggregation, like scRNA-Seq, is generation of a single-cell suspension. At younger embryonic stages, this can be achieved by using CMFM or PhoNaK media^{25,26}, but as the tadpole matures, the extracellular matrix becomes denser, and enzymatic methods are required. There are a wide array of enzymatic approaches for tissue disaggregation, many of which have been optimized for cell culture or mammalian tissues at 37 degrees celsius. These include trypsin, papain, and collagenase mixtures^{27,28}. Because over-aggressive disaggregation strategies may lead to cell damage or death, it is valuable to assay the performance of enzymatic cell disaggregation strategies in new species or tissues. The viability and recovery of *Xenopus* cells in these reagents has not been thoroughly compared and reported.

Here we introduce a FACS strategy for *Xenopus* that accommodates light scatter due to yolk by gating both against non-fluorescent wild-type cells and against cells expressing GFP under the control of a ubiquitous promoter (Xtr.Tg(*eef1a1*:GFP)^{Krieg}²⁹. We previously reported a specific application of FACS for isolating neural progenitors during tail regeneration⁹. Here we expand on that strategy to assay multiple enzymatic approaches, and efficacy in the limb bud as well as tail. Our cell disaggregation and FACS method (Figure 1) is optimized to retrieve only true positive cells while excluding light scattering cells. Using neural progenitor cells with the Xtr.Tg(*pax6*:GFP;*cryga*:RFP;*actc1*:RFP)^{Papal}^{30,31} transgenic line as a test case, we evaluated the quality of RNA or chromatin extracted from cells that have gone through the sorting process by comparing gene expression and

chromatin accessibility to cells that were not sorted. We also used expression analysis to validate the neural progenitor identity of sorted cells. These assay measures present a potential guideline for assaying cell type fidelity and transcriptional stress response signatures in *Xenopus* sorted cells. Our hope is that this optimized protocol will be applicable to a variety of cell types represented by new and established transgenic lines, and be adjustable for several forms of downstream analysis.

Results and Experimental Procedures

Transgenic lines used

Our primary goal was to articulate FACS conditions that would use transgenic expression of fluorescent proteins to let us confidently recover specific cell types from the complex tissues of the tadpole tail and limb. We therefore selected comparable *Xenopus tropicalis* lines that were either non-transgenic (wildtype), ubiquitously expressed GFP [Xtr.Tg(eef1a1:GFP)^{Krieg}, hereafter *eef1a:GFP*], or had tissue-specific expression of GFP [Xtr.Tg(pax6:GFP;cryga:RFP;actc1:RFP)^{Papal}, hereafter *pax6:GFP*]^{29–31}. Wildtype parent frogs were purchased from Nasco, and transgenic parent frogs from the National *Xenopus* Resource (<https://www.mbl.edu/xenopus/>)³². Information on the *eef1a:GFP* line and *pax6:GFP* line can be found with the reference ids RRID:NXR_1.0008 and RRID:NXR_1.0021, respectively. The *eef1a:GFP* line was used as a positive control for sorting, as *eef1a* is stably expressed during development and used in *Xenopus* as a reference gene for RT-PCR studies³³. In an *eef1a:GFP* transgenic animal, we observed robust reporter expression in most tissues in the head, the gut, and the axial tissues (Figure 2A). This transgenic line was used to evaluate how well our gating returned true positive reporter cells. Transgenic *pax6:GFP* tadpoles were utilized to isolate a rare cell type (neural progenitor cells) from the tail. The GFP reporter is expressed specifically in the forebrain, hindbrain, eye, and spinal cord of these tadpoles (Figure 2B). Transgenic matings were performed by crossing a heterozygous transgenic frog to a wild-type frog. These matings yielded clutches with 50/50 wt/*pax6:GFP*+ populations.

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Tadpole tail and limb isolation

Tadpoles were reared in 1/9 modified ringer's solution (1/9 MR)³⁴ to 3 days post fertilization or stage 41^{35,36}, at which time tail collections were performed. For limb bud isolation, tadpoles were reared to stage 53/54. Tadpoles were anesthetized using 0.5% MS222 in 1/9 MR, and tested for the loss of the escape reflex and other touch responses prior to amputation surgery. Once fully anesthetized, a sterilized scalpel was used to amputate the posterior third of the tail by cutting the tail from dorsal to ventral. Amputated tails were collected by pipetting with a p200 from the collection dish to an Eppendorf tube. Numbers of tails collected were noted. Following surgery, tadpoles were either removed from anesthetic media within 10 minutes of amputation into new 1/9x MR for regeneration studies or left in anesthetic for euthanasia.

For limb bud collection, NF stage 53 tadpoles were euthanized by anesthetic immersion in MS-222, and limb buds were removed using forceps to pinch off limb buds where the limb connects to the body (Figure 2D–E). Single limbs were collected into their own eppendorf tube.

Tissue dissociation to single cell suspension

Successfully dissociating tissue to a single cell suspension full of intact and viable cells is the first major check-point in the cell sorting process. To this end, we compared three different enzyme systems for tissue dissociation. Our goal was to break apart the tissue while preserving reporter expression and cell integrity. We optimized our protocol for speed and gentleness, to minimize disruptions to the gene expression or chromatin accessibility profile through time and stress. We interrogated three commonly-used enzymes for our dissociation protocol: liberase, trypsin, and papain. We describe the performance of each below, but selected liberase for downstream experiments.

Liberase—Liberase (a proprietary collagenase mixture from Roche, cat 5401020001) was resuspended from a 5mg lyophilized pellet to 0.35mg/ml solution in 1xPBS. The working concentration was made by diluting the stock ten fold in 1xPBS to a concentration of 0.035mg/ml. Once tails were collected into Eppendorf tubes, tails were spun down for 1 minute at 500g to pellet the tails. The supernatant was discarded and replaced with 200µl of 0.035 mg/ml Liberase solution. Tails were left to incubate for 20 minutes at room temperature. To preserve reporter fluorescence, incubations were performed in dark conditions. Following the incubation period, tails were manually dissociated using a p200 pipette and gently pipetted up and down until no visible tissue chunks were apparent (~2–5 minutes). Once cells were dissociated, the suspension was spun down for 5 minutes at 1000g. The supernatant was discarded, and the pellet was then washed in 180µl of 1xPBS. The washed pellet was then spun down for 1 minute at 1000g. The supernatant was discarded, and the pellet was resuspended in 250ul of 1xPBS (or volume suitable for tail amount and FACS analysis). The cell suspension was then passed through a 70µm cell strainer (Fisher 22-363-548) into an appropriate round bottomed polystyrene tube adaptable to the BD FACSAria III cell sorter (hereafter referred to as FACS collection tube) (Corning 352008). The cell suspension was placed on ice and in the dark until ready to be sorted. Resulting cell suspensions retained GFP expression (Figure 2C).

For limb disaggregation, media was discarded and replaced with 200µl of 0.035mg/ml liberase for one limb. The same procedures for disaggregation of the tail tissue were followed to bring limb buds to single cell suspension. Resulting suspensions yielded well disaggregated tissue (Figure 2F).

Trypsin—After spinning down tails in 1/9 MR as described previously, supernatant was discarded, and the pellet was resuspended in 100µL of 0.25% trypsin (ThermoFisher 25200056). Tails were incubated in trypsin for 20 minutes at room temperature. Tails were then gently mechanically dissociated with a p200 pipette until no visible tissue chunks were apparent (~2–5 minutes). Once tissue was fully dissociated, 100µL of newborn calf serum (NBCS) (ThermoFisher 26010066) was added to the cell suspension and mixed to stop

enzymatic activity. The cell suspension was then spun down for 5 minutes at 1000g and supernatant was discarded. The pellet was washed with 180µl of 1xPBS and spun down for 1 minute at 1000g. The pellet was then resuspended in 250µl of 1xPBS and passed through a 70µm cell strainer into a flow cytometry tube. The cells were kept on ice and in the dark until sorting.

Papain—As with the liberase preparation, tails in 1/9 MR were spun down for 1 min at 500g to collect tails at bottom of Eppendorf tube and media was replaced with 100µl of papain solution (750 µL papain and 75 µL DNase, following instructions in the Worthington Papain Dissociation System: catalog LK003160). The concentrations of papain and DNase in solution are 20units/ml papain and 0.005% DNase. Tails were incubated in papain solution for 10 minutes at room temperature followed by gentle agitation by pipetting up and down with a p200 for about 20 rounds. The tails were then left for an additional 10 minutes to incubate at room temperature. Tails were pipetted with a p200 gently until no more visible tissue chunks were apparent (~2–5 minutes). Following dissociation, 100µL of albumin-ovomucoid inhibitor solution was added to cell suspension to inactivate enzyme activity. Cell suspension was then spun down at 1000g for 3 minutes. Supernatant was discarded and the pellet was washed with 180µL of 1xPBS. The suspension was then spun down at 1000g for 1 minute, and the supernatant was replaced with 150µL of 1xPBS. The cell suspension was then pipetted through a 70µm cell strainer into a flow cytometry tube and kept on ice and in the dark until sorting.

For each enzyme preparation tested, cells were passed through the flow cytometer and the number of single cells were recorded and divided by the number of tails used for cell preparation (see Figure 3B-2). All three disaggregation systems resulted in a mean number of cells per tail recovered that were not significantly different from one another (Figure 2G). We did not find significant advantages for any one enzyme. We moved forward with Liberase because it had fewer components (no inactivating solution) and had less variability in cells per tail recovered.

Viability analysis with Trypan blue

To evaluate how many live cells we recovered from the tissue disaggregation process, we measured the cell viability from the single cell population. Trypan blue staining was used to distinguish dead cells from live cells; dead cells would stain with blue whereas live cells remain colorless. To do this analysis, 14µL of cell suspension was mixed with 7µL of 0.4% Trypan blue (Thermo 15250061). 10µL of solution was loaded on each side of a hemocytometer and cells were counted in a designated 25 squares corresponding to a cell count conversion of 1 cell counted to 10,000 cells/ml solution. First all cells were counted, followed by only dead cells being counted. The percent viability was calculated by the following equation: $(\text{all cells} - \text{dead cells}) / \text{all cells} * 100$. We found that on average, disaggregation resulted in ~86% cell viability in suspensions collected by all three enzymatic methods (Figure 2H).

Isolation of GFP⁺ single cells using FACS

Setting gates for isolation of fluorescent cells from transgenic animals—The next step for purification of GFP⁺ cells was to use the BD FACS Aria III cell sorter to collect cells. One issue arising from sorting cells for GFP fluorescence from early stage *Xenopus* embryos is that each individual cell has a maternal yolk store for nutrition. This results in light scatter, which could skew the sorting toward false-positive cells. An example of the light scatter that occurs in wildtype tadpoles of this stage of tadpole can be seen in the GFP channel of Figure 2B. To mitigate the number of false-positives picked up as a result of the light scatter, we first used wild-type tadpole cells to set our gates (Figure 3A). A gating sample (wild-type cells) was prepared in addition to transgenic samples for collection in parallel. These cells were flowed through the BD FACS Aria III sorter at a flow rate of 3–6 μ l/min, through a 70 μ m nozzle. Once cells were flowing, cells were first gated by forward scatter (cell size) and side scatter (granularity) light to select against cellular debris (Figure 3B-1). Next, the cells from the “cell” population were gated by forward scatter to select for single cells apart from cells that had not been dissociated (Figure 3B-2). The cells selected by this gate were then visualized on a histogram where the x-axis represents GFP cells and the y-axis shows the count of them (Figure 3B-3). Two vertical gates were set, one selecting GFP[−] cells and one selecting GFP⁺ cells. Because no wild-type cells should have GFP reporter, all cells were selected for in the GFP[−] group. A second gate was then set to be the GFP⁺ group. This step can also be visualized by a scatter plot, where the x-axis represents GFP and the y-axis represents side scatter light (Figure 3B-4). Here, we draw a gate encompassing all clustered cells representing the GFP[−] cells and a second gate slightly offset to capture GFP⁺ cells. Our goal was to use this gating scheme and apply it to transgenic samples, resulting in true GFP⁺ cells being captured.

Sorting and collecting transgenic cells—To be sure the gates were representing GFP⁺ signal and not excluding true positive cells from collection samples, a transgenic gating sample was also prepared. This sample was again flowed through the sorter with the same flow rate, and the gates from the wild-type gating sample were applied. Using the exact same gates as for wild-type cells, we saw an increase in the number of cells captured by the GFP⁺ gate (Figure 3C/D). In the case of *eef1a*:GFP, this gating scheme recovered 88.1% of cells as GFP⁺, with a clear division between the positive and negative cells (Figure 3C). This demonstrates that the FACS protocol was able to recover most cells, and therefore most cell types, counted by the cytometer. The strong split in signal between GFP⁺ and GFP[−] cells also suggests that a small proportion of cells in this transgenic line may not express detectable GFP at this stage. In addition to the sorting parameters, the collection action was now activated to collect GFP⁺ and GFP[−] cells. Cells were collected into 1.5ml Eppendorf tubes containing 100–200 μ l of chilled 1xPBS. Alternatively, cells destined for RNA extraction could be sorted into 1.5ml Eppendorf tubes containing 200 μ l of RNA-lysis buffer. Collected cells were kept on ice until nucleic acid extraction.

Downstream library preparation and analysis

The downstream use of isolated cells will vary among users. For the purpose of this paper, we extracted RNA for RT-qPCR and transposed DNA for ATAC-Seq, as described previously⁹. Specific alterations to the previously-described methods are noted.

Strategies for validating cell type recovery and quality

Sorted vs unsorted vs undissociated genomic library preparations—Our primary interest was in isolating specific cell types for analysis of gene expression and chromatin accessibility. A concern both when disaggregating tissue from its native state and when putting individual cells through the process of flow cytometry is that the cells may undergo damage as a consequence of either mechanical stress or of the time the procedure takes. While transcriptomic studies in leukocytes and mouse mammary tissue have suggested that the overall transcriptional profile is minimally changed by FACS^{37,38}, there is still relatively little information about how other tissues and cell types may be changed, particularly in *Xenopus*. Indeed, work conducted in mouse macrophages and cultured astrocytes suggests that there may be significant alterations to the metabolome and redox state of cells when subjected to FACS^{39,40}. Finally, the impact of FACS on the epigenome, and specifically on chromatin accessibility, has not been systematically interrogated. These motivations led us to optimize our disaggregation and FACS protocol for speed and gentleness, but to assess its performance we sought to test whether gene expression and chromatin accessibility were significantly disrupted in cells subjected to FACS when contrasted to comparable cells not subjected to FACS.

ATAC-seq library data analysis in sorted vs unsorted cells suggests that FACS does not disrupt chromatin accessibility—To test whether the physical force of flowing cells through a flow cytometer changes chromatin structure, we made ATAC-Seq libraries from cells that have been through a flow cytometer (sorted) and cells from the exact same tissue that have not (unsorted). For ATAC-Seq experiments, tadpole tails were amputated and the 0 hour post amputation tail tips were pooled (n=4 for ATAC-Seq) (Figure 4A). The pooled tails were then dissociated to single cell suspension. After washing, the cells were resuspended in 300µl of 1xPBS and split into two 150µl aliquots in separate FACS tubes; the two aliquots served as “sorted” and “unsorted” partner replicates. “Sorted” cells were flowed through the flow cytometer only utilizing gates 1:“Cells” and 2:“Single Cells” from Figure 3. All single cells were collected directly into chilled 1xPBS and placed on ice until all samples were collected. “Unsorted” samples were held in the FACS tubes on ice while its replicate pair was flowed through the flow cytometer (~7–10 minutes) (Figure 4A–5). When the sorted partner was finished running, the unsorted partner was transferred from the FACS tube to an Eppendorf tube containing chilled 1xPBS. We made ATAC-Seq libraries from these cells. When we ran libraries out on a bioanalyzer, we found that ATAC-Seq libraries made from either sorted or unsorted cells have a comparable single nucleosome peak at 180bp (Figure 4B). The presence of this peak is an important quality control step for efficient tagmentation and library amplification. We then went on to sequence these libraries.

Sequencing results were returned as *fastq* files and the pre-processing pipeline was performed as previously described⁹. Both types of libraries had comparable total read pairs sequenced, greater than 90% alignment to the genome, and a minimum of 25000 peaks called. These samples are within range of ENCODE’s strictest QC parameters (<https://www.encodeproject.org/atac-seq/>). Next we ran a correlation analysis to test how well correlated the counts per peaks called were between the sorted and unsorted libraries. Correlation analysis between sorted and unsorted 0hpa libraries was performed by using the

R command “lm” to fit a linear model to the counts per million of peaks in each library type. Linear regression analysis called an R^2 of 0.98 showing that each sample has a similar number of counts per peak (Figure 4C). This suggests that sorted and unsorted libraries are not significantly divergent when evaluated by their peak calls.

Sorted vs unsorted vs undissociated gene expression—In addition to testing the differences in chromatin accessibility for sorted and unsorted cells, we added in one more technical consideration by also collecting RNA from whole-tail lysates without cell dissociation. For these experiments, 25 uninjured tails were pooled per sample. Sorted and unsorted samples were collected in the same manner as the ATAC-Seq samples, however cells were sorted or added to 200 μ l RNA-lysis buffer. In contrast, the “undissociated” tail tissue was spun down after amputation, supernatant was removed, and RNA-lysis buffer was added directly to tails. We elected to lyse the whole tails immediately after collection rather than incubating on ice, reasoning that cells in the context of whole tissue would be more vulnerable to long-range signals that might trigger apoptosis. Once cells were in lysis buffer, they were treated the same from RNA extraction to cDNA synthesis. To assure we controlled for cell loss in each condition, RNA concentration was normalized prior to cDNA synthesis.

To test whether gene expression is altered by the dissociation and FACS process, we extracted RNA from grouped cell populations that were FACS-sorted or not sorted and from cells collected from whole-undissociated tissue and made cDNA from each population. We collected sorted, unsorted, and undissociated cell populations in triplicate and used qRT-PCR to measure relative gene expression of commonly-used housekeeping genes, using previously reported primers³³. To test whether there were significant changes in gene expression between the different conditions, the fold change for each gene was calculated in the sorted and undissociated samples with respect to the unsorted samples. When evaluating the differences in gene expression we used a t-test between sorted and unsorted samples and undissociated and unsorted samples and found that there was not a significant change in gene expression for housekeeping genes in either of these conditions. (Figure 4D). This analysis suggests that expression of these high-abundance housekeeping genes is not significantly changed when tissue is dissociated or sorted through the flow cytometer.

Validation of specific sorted cell identity using *pax6:GFP*—To test whether GFP-positive sorted cells from the *pax6:GFP* transgenic line had identity consistent with the transgenic reporter, we compared chromatin accessibility of sorted transgenic cells against cells from aggregate tissue. To assess whether ATAC-Seq could detect cell-type specific chromatin signatures we analyzed 0hpa *pax6:GFP* and 0hpa aggregate tissue samples from⁹ (GEO accession GSE146830) by identifying differentially accessible peaks enriched in *pax6:GFP*+ 0hpa samples relative to aggregate tissue 0hpa samples with edgeR⁴¹. Each peak was annotated to the nearest transcription start site. We then filtered the differentially accessible peaks to those within 15000 bases of a transcription start site (218 regions) and input the gene annotations to gProfileR2⁴² for gene ontology analysis. Gene ontology analysis revealed that the *pax6:GFP*+ libraries are enriched for regions annotated to GO terms such as “Nervous System Development” (p=0.0019), “Regulation of postsynaptic specialization assembly” (p=0.0145), and “Regulation of nervous system development”

($p=0.0280$) (Figure 5C). These GO terms confirm that the *pax6:GFP*⁺ cells have a neural signature when compared to the aggregate tissue cells. If we look specifically at regions more accessible in the *pax6* libraries called in the GO term “Nervous system development”, we see that known neural markers are more accessible (Figure 5C), thus indicating that purifying cells from a transgenic reporter by FACS presents a method to increase the resolution of bulk ATAC-Seq for a cell type of interest.

Conclusions

In this article we articulate a protocol to disaggregate *Xenopus tropicalis* tail and limb tissue to a single cell suspension and sort the cell suspension by transgenic fluorescent reporter expression. By gating against wild-type nonfluorescent cell populations, our protocol allows recovery of true positive cells, even in situations where a clear break-point in the fluorescent signal distribution is hard to identify. Downstream validation through Gene Ontology analysis of chromatin signatures from ATAC-Seq libraries confirms that this protocol can recover a rare target cell population, the neural progenitors, from small amounts of starting tissue.

In comparing multiple enzymatic approaches to disaggregate these tissues, we did not find a significant difference in performance between liberase, trypsin and papain. While we chose liberase for our downstream analysis, researchers using other starting tissues or with different target cells may wish to compare all three enzymes for performance in their specific application.

FACS offers the opportunity to isolate a population of interest and then sequence it in bulk, which is more cost effective than scRNA-Seq for situations where all members of a specific cell type are the target. For situations where single cell analysis is needed, our disaggregation protocol also offers a jumping-off point with high cell viability. We find that the FACS process does not drastically disrupt expression of well-known housekeeping genes, in agreement with existing literature that FACS has minimal effect on the transcriptome. Importantly, we find that FACS also causes minimal disruption to the epigenome as assayed by ATAC-Seq peak locations genome-wide. With the growing library of transgenic animals and expanding toolbox of sequencing techniques, we expect this protocol will be a useful resource for *Xenopus* researchers wanting to characterize specific cell types and may also offer a starting point for researchers in emerging aquatic vertebrate systems seeking to develop their own protocols.

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References

1. Duncan AR, Khokha MK. *Xenopus* as a model organism for birth defects-Congenital heart disease and heterotaxy. *Semin Cell Dev Biol.* 2016;51:73–79. doi:10.1016/j.semcdb.2016.02.022 [PubMed: 26910255]
2. Harland RM, Grainger RM. *Xenopus* Research: Metamorphosed by Genetics and Genomics. *Trends Genet.* 2011;27(12):507–515. doi:10.1016/j.tig.2011.08.003 [PubMed: 21963197]
3. Nenni MJ, Fisher ME, James-Zorn C, et al. Xenbase: Facilitating the Use of *Xenopus* to Model Human Disease. *Front Physiol.* 2019;10:154. doi:10.3389/fphys.2019.00154 [PubMed: 30863320]
4. Phipps LS, Marshall L, Dorey K, Amaya E. Model systems for regeneration: *Xenopus*. *Development.* 2020;147(6). doi:10.1242/dev.180844
5. Grainger RM. *Xenopus tropicalis* as a Model Organism for Genetics and Genomics: Past, Present and Future. *Methods Mol Biol.* 2012;917:3–15. doi:10.1007/978-1-61779-992-1_1 [PubMed: 22956079]
6. Kakebeen A, Wills A. Advancing genetic and genomic technologies deepen the pool for discovery in *Xenopus tropicalis*. *Dev Dyn.* 2019;248(8):620–625. doi:10.1002/dvdy.80 [PubMed: 31254427]
7. Aztekin C, Hiscock TW, Marioni JC, Gurdon JB, Simons BD, Jullien J. Identification of a regeneration-organizing cell in the *Xenopus* tail. *Science.* 2019;364(6441):653–658. doi:10.1126/science.aav9996 [PubMed: 31097661]
8. Briggs JA, Weinreb C, Wagner DE, et al. The dynamics of gene expression in vertebrate embryogenesis at single-cell resolution. *Science.* 2018;360(6392). doi:10.1126/science.aar5780
9. Kakebeen AD, Chitsazan AD, Williams MC, Saunders LM, Wills AE. Chromatin accessibility dynamics and single cell RNA-Seq reveal new regulators of regeneration in neural progenitors. *Elife.* 2020;9. doi:10.7554/eLife.52648
10. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. *Rev Sci Instrum.* 1972;43(3):404–409. doi:10.1063/1.1685647 [PubMed: 5013444]
11. Cerda GA, Hargrave M, Lewis KE. RNA profiling of FAC-sorted neurons from the developing zebrafish spinal cord. *Dev Dyn.* 2009;238(1):150–161. doi:10.1002/dvdy.21818 [PubMed: 19097188]
12. Gallardo VE, Behra M. Fluorescent Activated Cell Sorting (FACS) Combined with Gene Expression Microarrays for Transcription Enrichment Profiling of Zebrafish Lateral Line Cells. *Methods.* 2013;62(3):226–231. doi:10.1016/j.ymeth.2013.06.005 [PubMed: 23791746]
13. Gerber T, Murawala P, Knapp D, et al. Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science.* 2018;362(6413). doi:10.1126/science.aag0681
14. Hadjantonakis AK, Nagy A. FACS for the isolation of individual cells from transgenic mice harboring a fluorescent protein reporter. *Genesis.* 2000;27(3):95–98. doi:10.1002/1526-968x(200007)27:3<95::aid-gene10>3.0.co;2-a [PubMed: 10951501]
15. Manoli M, Driever W. Fluorescence-Activated Cell Sorting (FACS) of Fluorescently Tagged Cells from Zebrafish Larvae for RNA Isolation. *Cold Spring Harb Protoc.* 2012;2012(8):pdb.prot069633. doi:10.1101/pdb.prot069633
16. Selman GG, Pawsey GJ. The utilization of yolk platelets by tissues of *Xenopus* embryos studied by a safranin staining method. *J Embryol Exp Morphol.* 1965;14(2):191–212. [PubMed: 4159802]
17. Gilbert DM. Temporal order of replication of *Xenopus laevis* 5S ribosomal RNA genes in somatic cells. *Proc Natl Acad Sci U S A.* 1986;83(9):2924–2928. [PubMed: 3458252]
18. Noelanders R, Vleminckx K. Cell Cycle Analysis of the Embryonic Brain of Fluorescent Reporter *Xenopus tropicalis* by Flow Cytometry. In: Vleminckx K, ed. *Xenopus: Methods and Protocols. Methods in Molecular Biology.* New York, NY: Springer; 2018:243–250. doi:10.1007/978-1-4939-8784-9_17
19. Ny A, Vandevelde W, Hohensinner P, et al. A transgenic *Xenopus laevis* reporter model to study lymphangiogenesis. *Biol Open.* 2013;2(9):882–890. doi:10.1242/bio.20134739 [PubMed: 24143274]
20. Sato K, Uehara A, Kinoshita S, et al. Flow cytometric analysis of *Xenopus laevis* and *X. tropicalis* blood cells using acridine orange. *Scientific Reports.* 2018;8(1):16245. doi:10.1038/s41598-018-34631-0 [PubMed: 30390005]

21. Tsujioka H, Kunieda T, Katou Y, Shirahige K, Kubo T. Unique Gene Expression Profile of the Proliferating *Xenopus* Tadpole Tail Blastema Cells Deciphered by RNA-Sequencing Analysis. *PLoS One*. 2015;10(3). doi:10.1371/journal.pone.0111655
22. Beck CW, Izpisua Belmonte JC, Christen B. Beyond early development: *Xenopus* as an emerging model for the study of regenerative mechanisms. *Dev Dyn*. 2009;238(6):1226–1248. doi:10.1002/dvdy.21890 [PubMed: 19280606]
23. Takebeen AD, Wills AE. More Than Just a Bandage: Closing the Gap Between Injury and Appendage Regeneration. *Front Physiol*. 2019;10:81. doi:10.3389/fphys.2019.00081 [PubMed: 30800076]
24. Suzuki M, Yakushiji N, Nakada Y, Satoh A, Ide H, Tamura K. Limb Regeneration in *Xenopus laevis* Froglet. *ScientificWorldJournal*. 2006;6:26–37. doi:10.1100/tsw.2006.325 [PubMed: 17205185]
25. Godsave SF, Slack JM. Clonal analysis of mesoderm induction in *Xenopus laevis*. *Dev Biol*. 1989;134(2):486–490. doi:10.1016/0012-1606(89)90122-x [PubMed: 2744244]
26. Sargent TD, Jamrich M, Dawid IB. Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Developmental Biology*. 1986;114(1):238–246. doi:10.1016/0012-1606(86)90399-4 [PubMed: 3956863]
27. Freshney RI. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. John Wiley & Sons; 2015.
28. Reichard A, Asosingh K. Best Practices for Preparing a Single Cell Suspension from Solid Tissues for Flow Cytometry. *Cytometry Part A*. 2019;95(2):219–226. doi:10.1002/cyto.a.23690
29. Johnson AD, Krieg PA. pXcX, a vector for efficient expression of cloned sequences in *Xenopus* embryos. *Gene*. 1994;147(2):223–226. doi:10.1016/0378-1119(94)90070-1 [PubMed: 7926804]
30. Hartley KO, Hardcastle Z, Friday RV, Amaya E, Papalopulu N. Transgenic *Xenopus* embryos reveal that anterior neural development requires continued suppression of BMP signaling after gastrulation. *Dev Biol*. 2001;238(1):168–184. doi:10.1006/dbio.2001.0398 [PubMed: 11784002]
31. Hirsch N, Zimmerman LB, Gray J, et al. *Xenopus tropicalis* transgenic lines and their use in the study of embryonic induction. *Dev Dyn*. 2002;225(4):522–535. doi:10.1002/dvdy.10188 [PubMed: 12454928]
32. Horb M, Wlitzla M, Abu-Daya A, et al. *Xenopus* Resources: Transgenic, Inbred and Mutant Animals, Training Opportunities, and Web-Based Support. *Front Physiol*. 2019;10:387. doi:10.3389/fphys.2019.00387 [PubMed: 31073289]
33. Dhome-Pollet S, Thlie A, Pollet N. Validation of novel reference genes for RT-qPCR studies of gene expression in *Xenopus tropicalis* during embryonic and post-embryonic development. *Dev Dyn*. 2013;242(6):709–717. doi:10.1002/dvdy.23972 [PubMed: 23559567]
34. Kay B, Peng H. Appendix A: solutions and protocols. In: *Xenopus Laevis: Practical Uses in Cell and Molecular Biology*. Vol 36. ; 1991:661–662.
35. Nieuwkoop PD, Faber J. *Normal Table of Xenopus Laevis (Daudin)*. New York: Garland Publishing Inc; 1994.
36. Nieuwkoop PD, Faber J. *Normal Table of Xenopus Laevis (Daudin): A Systematical and Chronologica Survey of the Development from the Fertilized Egg till the End of Metamorphosis*. North Holland, Amsterdam; 1956.
37. Beliakova-Bethell N, Massanella M, White C, et al. The effect of cell subset isolation method on gene expression in leukocytes. *Cytometry A*. 2014;85(1):94–104. doi:10.1002/cyto.a.22352 [PubMed: 24115734]
38. Richardson GM, Lannigan J, Macara IG. Does FACS perturb gene expression? *Cytometry A*. 2015;87(2):166–175. doi:10.1002/cyto.a.22608 [PubMed: 25598345]
39. Binek A, Rojo D, Godzien J, et al. Flow Cytometry Has a Significant Impact on the Cellular Metabolome. *J Proteome Res*. 2019;18(1):169–181. doi:10.1021/acs.jproteome.8b00472 [PubMed: 30362351]
40. Llurio EM, Wang L, Naser FJ, Patti GJ. Sorting cells alters their redox state and cellular metabolome. *Redox Biol*. 2018;16:381–387. doi:10.1016/j.redox.2018.03.004 [PubMed: 29627745]

41. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010;26(1):139–140. doi:10.1093/bioinformatics/btp616 [PubMed: 19910308]
42. Raudvere U, Kolberg L, Kuzmin I, et al. g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res*. 2019;47(W1):W191–W198. doi:10.1093/nar/gkz369 [PubMed: 31066453]

Highlights

- A protocol for tissue disaggregation in tadpole tails and limb buds.
- Setting gates with cells from non-transgenic animals eliminates light scattering and false positive cells.
- Sorted cells do not have significantly different gene expression relative to unsorted cells of the same type.
- Sorted cells have similar chromatin accessibility profiles to unsorted cells of the same type.

Liberase	5mg (working: 0.035 mg/ml)	Roche 5401119001
PBS	1x	in house
Cell Strainer	70um	Fisher 22-363-548
Polystyrene round bottom tube	12x75mm, 5 mL	Corning 352008
Trypan Blue	0.4%	Thermo 15250061

Figure 2. Disaggregation of *Xenopus tropicalis* tails and limbs.

(A-B) NF stage 41/42 live tadpoles imaged for reporter fluorescence. (A) *Eef1a:GFP* transgenic tadpole. (B) *Pax6:GFP* transgenic tadpole. (C) Dissociated cells from a *pax6:GFP* animal. Images were taken in DIC and in the GFP channel. (D) Wild-type NF stage 53 tadpole trunk and (E) the isolated limb bud from that tadpole. (F) Cells from dissociated limb bud in (E). (G) Boxplot showing cells per tail for different enzyme preparations measured from FACS treated wild-type tail tissue. Liberase, n=7; Papain, n=4; Trypsin, n=6. A pairwise t-test was performed between any two enzymes. ns = not significant. (H)

Boxplots showing the % cell viability from disaggregated tissue prior to FACS for different enzyme types (n=3). The number reported above the plot is the mean viability for n=3.

Figure 3. FACS gating settings for GFP⁺ cell isolation.

(A) Gating plan for isolating true positive GFP cells from transgenic animals using wild-type tadpoles as controls. (B-D) The first column (1.Cells) refers to cells sorted by forward-scatter light (FSC-A) and side-scatter light (SSC-A) to select against cellular debris. Cells from this selected population were re-sorted by FSC-H and FSC-W to exclude multiples of cells (2.Single Cells). Single cells were then visualized by a histogram with GFP on the x-axis and counts on the y-axis (3.GFP-Histogram). The single cells were also visualized by a scatter plot with GFP on the x-axis and SSC-A on the y-axis (4. GFP-Scatter Plot). (B) Example of gating in wild-type cells. In the GFP gating, all cells are selected against

to restrict light scattering cells from being picked up by the GFP gate. The gating from (B) was applied to transgenic cells from the (C) *eef1a:GFP* transgenic line and (D) *pax6:GFP* transgenic line. Cells in the GFP⁺ gate in (C/D) represent true GFP positive reporter cells.

Figure 4. Sorting by FACS does not significantly change gene expression or chromatin quality.

(A) Experimental design to test gene expression and chromatin architecture of cells that have been sorted and cells that have not been sorted and RNA from undissociated tails. (B) Bioanalyzer traces for the sorted and unsorted libraries. Arrow points to characteristic ~180bp. (C) Correlation plot of counts in counts per million (cpm) from ATAC-Seq samples that were sorted or unsorted. The samples correlated with an r^2 of 0.938. (D) RT-qPCR results from RNA extracted from cells that have been undissociated (n=3), unsorted (n=3), or sorted (n=3). We tested three house-keeping genes and quantified fold change expression between sorted or undissociated to the unsorted condition. Expression was independently normalized to *odc*, *eef1a*, or, *smn2*. T-tests between sorted and unsorted conditions were used to identify if FACS significantly altered gene expression. Error bars represent standard deviation. ns= not significant

Figure 5. Sorting cells by FACS enriches specific cell types.

(A) Experimental design for collection of *pax6:GFP⁺* cells, *pax6:GFP⁻* cells, and wild-type, aggregate tissue cells (B) Reported $-\log(p\text{value})$ for gene ontology terms returned for regions more accessible in *pax6:GFP⁺* ATAC-Seq libraries (n=3) than aggregate tissue libraries (n=3). (C) Heatmap showing accessibility of regions of the chromatin annotated to the nearest transcription start site shown in aggregate tissue or sorted *pax6*, Ohpa ATAC-Seq libraries. Regions shown called the GO term “Nervous system development” in (B).