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Using Flow Cytometry to Detect and Quantitate Altered Blood Formation in the Developing Zebrafish

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Cover letter

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February 9, 2020

Dear *JoVE* editors:

I am pleased to present to you our research article entitled “Utilizing flow cytometry to detect and quantitate altered blood formation in the developing zebrafish.” In this manuscript, we describe the development of a technique to quantitate fluorescent blood cells in the developing zebrafish embryo. This assay is exciting because it adds to the growing toolbox of assays available to make zebrafish hematopoietic studies more rigorous. More importantly, we believe that they will appeal to the wide and diverse readership of *JoVE*; these assays are novel and versatile, so people can adapt them to their specific experimental questions, even outside of the hematopoietic field.

We have corrected the overlap that the original manuscript had with previous articles written by our laboratory. We have also addressed all the Reviewer’s concerns, expanding Figure 1 and adding two Supplemental Figures to address issues.

Included in our submission is a document containing an abstract of 299 words, main text of 3872 words, as well as one figure, one table, and two supplemental figures.

I believe that you will find the manuscript much improved, and I look forward to hearing from you.

Sincerely,

David Stachura

TITLE:

Using Flow Cytometry to Detect and Quantitate Altered Blood Formation in the Developing Zebrafish

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KEYWORDS:

blood cells, zebrafish, morpholinos, CRISPR, hematopoiesis, development, stem cells, flow cytometry, FACS

SUMMARY:

This assay is a simple method to quantitate hematopoietic cells in developing embryonic zebrafish. Blood cells from dissociated zebrafish are subjected to flow cytometry analysis. This allows the detection of blood defects in mutant animals and after genetic modification.

ABSTRACT:

The diversity of cell lineages that comprise mature blood in vertebrate animals arise from the differentiation of hematopoietic stem and progenitor cells (HSPCs). This is a critical process that occurs throughout the lifespan of organisms, and disruption of the molecular pathways involved during embryogenesis can have catastrophic long-term consequences. For a multitude of reasons, zebrafish (*Danio rerio*) has become a model organism to study hematopoiesis. Zebrafish embryos develop externally, and by 7 days postfertilization (dpf) have produced most of the subtypes of definitive blood cells that will persist for their lifetime. Assays to assess the number of hematopoietic cells have been developed, mainly utilizing specific histological stains, in situ hybridization techniques, and microscopy of transgenic animals that utilize blood cell-specific promoters driving the expression of fluorescent proteins. However, most staining assays and in situ hybridization techniques do not accurately quantitate the number of blood cells present; only large differences in cell numbers are easily visualized. Utilizing transgenic animals and analyzing individuals with fluorescent or confocal microscopy can be performed, but the quantitation of these assays relies on either counting manually or utilizing expensive imaging software, both of which can make errors. Development of additional methods to assess blood cell numbers would be economical, faster, and could even be automated to quickly assess the effect of CRISPR-mediated genetic modification, morpholino-mediated transcript reduction, and the effect of drug compounds that affect hematopoiesis on a large scale. This novel assay to quantitate blood cells is performed by dissociating whole zebrafish embryos and analyzing the

amount of fluorescently labelled blood cells present. These assays should allow elucidation of molecular pathways responsible for blood cell generation, expansion, and regulation during embryogenesis, which will allow researchers to further discover novel factors altered during blood diseases, as well as pathways essential during the evolution of vertebrate hematopoiesis.

INTRODUCTION:

Blood production (hematopoiesis) is an essential developmental process that first starts in the early embryo. This process begins by generating primitive red blood cells and macrophages directly from mesoderm, and later shifts towards the production of hematopoietic stem and progenitor cells (HSPCs). These stem cells, which are multipotent, generate all of the varieties of mature blood cells in the organism. Capable of self-renewal, the system is continually replenished through these HSPCs. While this process starts early in development, hematopoiesis continues for the life of the animal, providing the ability to transport oxygen to distant sites of the body, to stop bleeding after injury, and to protect the body from infection. The development of this complex system is controlled temporally and spatially during development and any perturbations in blood cell production can be catastrophic for the organism, resulting in anemia, thrombocytopenia, leukopenia, and leukemia.

A popular animal model used for hematopoietic research is the zebrafish (*Danio rerio*) because they have similar blood development when compared to humans. In fact, many of the genes and molecular pathways used during hematopoiesis are conserved throughout vertebrate evolution, allowing us to learn about human genes by studying zebrafish. Importantly, zebrafish embryos develop outside the body and within 7 days have generated most mature blood cell types, allowing for direct visualization of the hematopoietic system in a short amount of time. Zebrafish are also extremely fecund, which allows researchers to observe a larger number of samples in a short time frame, which is also important for generating reproducible data. The zebrafish's short generation time and external development provides for easier manipulation and observation during mutagenesis studies[1-5] and drug screening[6-10]. This allows a panel of promising therapeutic compounds for human blood disorders to be quickly and efficiently tested.

Importantly, zebrafish are also genetically amenable, and the genome is sequenced and annotated. This tractability allows reverse genetics techniques such as morpholino- (MO-) mediated knockdown and CRISPR-mediated genetic ablation to be performed. Zebrafish have also proven their utility as a model to perform forward genetic screens; many essential genes and pathways involved in vertebrate blood formation have been discovered in this manner. Numerous methods of observing blood cells have also been developed in zebrafish. While traditional histological staining techniques exist, it is also possible to perform in situ hybridization for blood-specific transcripts. Importantly, numerous transgenic lines of fish also exist whereby fluorescent proteins are expressed by lineage-specific promoters, allowing the labelling of specific blood cells with fluorescent proteins[11]. This allows researchers to perform up-to-the-minute observation of blood cell genesis, expansion, and regulation in a living organism over time.

Overall, conservation of the hematopoietic system, the presence and easy development of transgenic lines, easy visualization, and short generation time has made the zebrafish an economical, fast, and adaptable model of hematopoiesis. To improve upon the toolbox of techniques available for zebrafish researchers, we developed this assay to robustly quantitate the number of blood cells in embryos. The method involves digesting transgenic animals and performing flow cytometry for fluorescent blood cells. In this way, blood cells from mutant animals, the effect of MO and CRISPR modification, and the effect of small molecules can be quantitatively analyzed in a quick and reproducible manner. These assays are user-friendly and economical ways to enumerate blood cells, allowing examination of their generation, proliferation, and maintenance over time.

PROTOCOL:

The Institutional Animal Care and Use Committee (IACUC) advisory board at California State University, Chico, approved all methods described below.

NOTE: It is advised to treat embryos with 1-phenyl 2-thiourea (PTU; **Table 1**) at 24 hours postfertilization (hpf) to prevent pigmentation which negatively affects fluorescence discrimination by flow cytometry. All procedures listed below are acceptable for flow cytometry and fluorescence-activated cell sorting (FACS). However, if one's goal is to culture the hematopoietic cells after FACS, then adhere to sterile practices when processing samples. The protocol for bleaching and preparing embryo samples in a sterile manner has been described previously[12].

1. Dechorionation of 48 hpf zebrafish embryos

1.1. With embryos (at least 5, but no more than 200 embryos) in a 10 cm plastic Petri dish, tilt the dish so that the embryos sink to the bottom edge.

NOTE: It is easy to tilt the dish by removing its lid and placing the lid underneath one edge of the Petri dish.

1.2. Remove and discard as much E3 medium (**Table 1**) as possible and add 500 µL of dechorionation protease (10 mg/mL). Incubate at room temperature for 5 min.

1.3. After 5 min, pick up the Petri dish (keeping all the embryos at the bottom edge of the plate), and gently tap the side of the Petri dish, allowing embryos to gently rub against the bottom of the plate to completely remove chorions.

1.4. With a squeeze bottle, add ~20 mL of E3 medium to dilute protease. Allow embryos to settle and remove the E3 medium by decanting.

1.5. Repeat step 1.4 3x to remove all traces of the dechorionation protease.

NOTE: For each individual sample to analyze, at least 5 embryos are required. This dechorionation procedure can accommodate up to 200 embryos per Petri dish. Samples can be grouped at this stage and separated later if desired. Alternatively, manual dechorionation can be performed by gently tearing the chorions with watchmaker's forceps. This is advantageous when there are small numbers of embryos. Usually zebrafish will hatch from their chorions by 72 hpf. Severely malformed embryos may not, however, and will require dechorionation (either manual or chemical) after that time.

2. Preparation of embryo samples for dissociation

2.1. Using a P1000 pipette, place 5–10 embryos into a single 1.5 mL microcentrifuge tube.

2.2. Remove E3 medium with a pipette and discard.

CAUTION: Pipette with care as embryos can easily be discarded during the following steps.

2.3. Add 1 mL of 10 mM dithiothreitol (DTT) in E3 medium to remove the mucus coating surrounding the embryonic zebrafish[11-14]. Lay the microcentrifuge tube horizontally, and incubate at room temperature for 30 min.

3. Embryo dissociation

3.1. Wash the embryos 3x with 1 mL of Dulbecco's phosphate buffered saline (DPBS) with Ca^{2+} and Mg^{2+} to remove traces of DTT. After the last wash add 500 µL of DPBS with Ca^{2+} and Mg^{2+} and 5 µL of 5 mg/mL (26 U/mL) dissociation protease.

NOTE: Other isotonic buffered solutions may be used, but they must contain Ca^{2+} and Mg^{2+} for the dissociation enzyme to function properly.

3.2. Incubate samples at 37 °C on a horizontal orbital shaker at 185 rpm for 60 min. Triturate embryos with a P1000 pipette until samples are fully dissociated.

CAUTION: Take care not to overdigest the embryos; some solid tissue should be present, and it should not be completely homogenous. It is possible to overdigest, which will destroy the target blood cells to be observed on the flow cytometer (**Supplemental Figure 1**).

NOTE: This procedure works best for 48–72 hpf embryos. Later stages may require longer incubation with dissociation protease. Timing for different stages must be determined empirically. There are quicker alternative methods to dissociate embryonic tissue, but it is found empirically that this method, although it takes 60 min, is gentle and thorough enough to allow efficient isolation of live hematopoietic cells.

4. Preparation of dissociated embryos for flow cytometry

4.1. Pipette the 5 dissociated embryos onto the top reservoir of a 5 mL polystyrene round bottom tube with a 35 µmM cell strainer cap.

4.2. Rinse cells by adding 4 mL of phosphate buffered saline (PBS) with no Ca^{2+} or Mg^{2+} to the strainer cap of the 5 mL polystyrene tube containing the filtered cells.

NOTE: Other isotonic buffered solutions may also be used, but they should lack Ca^{2+} or Mg^{2+} to dilute and render the dissociation enzyme ineffective.

4.3. Centrifuge tubes at 4 °C and 300 x *g* for 5 min to pellet the cells. With a pipette, remove and discard supernatant. Resuspend the cells in 500 µL of PBS (with no Ca^{2+} or Mg^{2+}).

5. Flow cytometry

5.1. Gently vortex the cell suspension and add 1:1,000 red dead cell stain (**Table of Materials**).

NOTE: The red dead cell stain is a cell-permeable dye that allows exclusion of dead cells and debris from target cells and is an excellent choice of dye for dead cell discrimination, because it is excited by the 633 nm laser, which is different from the 488 nm laser used to excite common fluorophores such as green fluorescent protein (GFP) and DsRed. In this way, there is no spectral overlap from dead cells and transgenic blood cells. Propidium iodide (PI) at 1 mg/mL is an inexpensive alternative to the red dead cell stain, but make sure to reserve some samples stained only with PI as well as samples only with the fluorophore of interest to set the instrument compensation. Otherwise, the signals of PI and fluorophores such as GFP will overlap, resulting in false-positive results. If the flow cytometer also has a 405 nm laser, other dyes such as blue dad cell stain are also an excellent choice.

CAUTION: When analyzing blood cells, note that after 30 min certain cell types will begin to phagocytose the red dead cell stain. Keep cells protected from light and on ice until analysis and perform flow cytometry within 30 min of adding the dye.

5.2. Flow cytometry analysis

NOTE: Every flow cytometer is a little different, but the following steps will assist in preparing samples for analysis on most machines. For users new to flow cytometry, seek the advice of someone that is an expert on the particular piece of equipment. The following instructions pertain to the BD FACSAria Fusion flow cytometer.

5.2.1. Turn on the cytometer and allow lasers to warm up for 20 min. Empty waste tank, fill sheath tank with appropriate solution (varies based on flow cytometer) and start the fluidics system.

NOTE: There is usually a particular startup procedure for each type of flow cytometer; follow that startup procedure carefully.

5.2.2. In the analysis software, draw five plots (**Figure 1A**).

5.2.2.1. Have the first dot plot measure forward scatter (FSC) on the x axis and side scatter (SSC) on the y axis. Set FSC as **linear** and SSC as **log**.

NOTE: FSC is a measurement of cell size, and SSC is a measurement of granularity; this will allow the discrimination of cells from debris. Embryonic zebrafish blood cell populations do not separate by size and granularity in the same manner as adult zebrafish blood cells described previously[15]. Instead they will appear in the same population with all the other digested embryonic cells.

5.2.2.2. Set the second plot to examine FSC height (H) versus FSC width (W). Set the third plot to measure SSC (H) versus SSC (W).

NOTE: This step is used to exclude doublets (cells that are stuck together when they pass through the laser).

5.2.2.3. Set the fourth dot plot to measure the red dead cell stain on the x axis (depending on the cytometer this is usually equivalent to the filter used for allophycocyanin [APC]) and SSC on the y axis. This will allow the discrimination of live cells from dead cells.

NOTE: Filter sets of each cytometer can be different. Make sure to use the correct detection filter for the dead cell discrimination dye.

5.2.2.4. The fifth plot measures the fluorophore of choice. Visualize this as a histogram of the fluorophore (**Figure 1**) or use a dot plot (**Supplemental Figure 2**).

NOTE: When examining more than one fluorophore in the same animal, it is recommended to use a dot plot to view both parameters at the same time. If there is any chance that the fluorophores have spectral overlap with each other (**Supplemental Figure 2**), a dot plot is also recommended. Use the **Area (A)** settings to calculate the area under the curve generated by the laser pulse of the cytometer for all parameters, because they are more accurate.

5.2.3. Load the sample and reduce the flow rate so that the sample does not run out rapidly. Adjust the FSC and SSC settings so that the bulk of the cell population can be clearly seen (**Figure 1A**).

5.2.4. Draw a gate around the cell population and label it **cells**. Making sure that the second dot plot is gated on **cells**, exclude doublets by first gating on FSC and then on SSC singlets.

5.2.5. On the fourth plot adjust the red dead cell stain settings so that there is clearly a negative population. Draw a gate around these cells, and label them **live cells**. On the fifth plot, gate on

live cells and examine fluorophore negative and positive cells. Draw a gate around the positive cells.

NOTE: This hierarchical gating strategy examines single fluorophore$^+$ cells that reside in the **live cells** gate, which also reside in the **cell** gate. Take care to set gates properly. These gates can be customized depending on the experiment.

5.2.6. Run each sample, collecting at least 25,000 live cell events.

5.2.7. Follow the shutdown procedure to turn off the fluidics. Refill sheath tank and empty the waste tank.

NOTE: It is often difficult to visualize fluorophore$^+$ cells when the protein expression is low, or the cell population is rare. To ensure the parameters are set properly, fluorophore$^-$ sibling embryos should be prepared alongside to properly draw gates and adjust laser voltages (**Supplemental Figure 2**). If the target cell population is rare, collect more than 25,000 events. With 5−10 digested animals, one should be able to collect millions of events. The total number of fluorophore$^+$ cells can also be obtained with these data. Simply count the total number of cells in the sample with a hemocytometer and multiply the percentage of fluorophore$^+$ cells to obtain the absolute number of fluorophore$^+$ cells per fish.

REPRESENTATIVE RESULTS:

To enumerate red blood cells in embryonic zebrafish, *lcr*:GFP[16] embryos were injected at the one-cell-stage with PBS, 7.0 ng/nL *ism1* MO, or 7.0 ng/nL *ism1* MO with 7.0 ng *ism1* mRNA[12]. At 48 hpf they were digested and subjected to flow cytometry analysis. After analyzing the percentage of GFP$^+$ red blood cells from each sample (each sample is 5 randomly selected embryos; **Figure 1A**), the average of all the control group was calculated. This average was set as **1**, and all percentages were calculated from that reference point. These data indicate that reducing *ism1* transcript with a specific MO reduced the number of GFP$^+$ red blood cells present in the 48 hpf embryo. Additionally, rescuing this reduction in *ism1* levels with exogenous mRNA returned the number of red blood cells to normal.

FIGURE AND TABLE LEGENDS:

Figure 1: *ism1* MO reduces the number of red blood cells produced during embryogenesis. (**A**) *lcr*:GFP[16] embryos were injected with PBS and subjected to flow cytometry analysis. First, size and granularity were determined, and a gate was drawn around the cell population of interest (**cells**, left panel). Then, FSC H and FSC W was evaluated to eliminate cells stuck together (**singlets FSC**). SSC H and W was also evaluated to reduce the possibility of evaluating cells stuck together (**singlets SSC**). Red fluorescence was then examined to determine live cells (**live cells**). Finally, live cells were examined for GFP fluorescence (right panel). (**B**) *lcr*:GFP[12] embryos were injected with PBS (control, circles), *ism1* MO (*ism1* MO, squares), or *ism1* MO + *ism1* mRNA[12] (rescue, triangles) at the one-cell-stage of development. After 48 h, they were collected, digested, and subjected to flow cytometry as shown in panel A. Each point represents five randomly chosen individual embryos. Fold change is calculated by taking the average percentage of GFP$^+$ cells of the control

sample and setting that as **1**. All other samples are compared to that average. *p < 0.005, and N.S. = not significant.

Table 1: Recipes for solutions.

Supplemental Figure 1: Digestion of embryos with dissociation protease. Images of 10 embryos not properly digested (left; undigested), after adequate digestion (middle; digested), and after excess digestion (right; overdigested).

Supplemental Figure 2: Evaluation of two fluorophores in 5 dpf embryos. Gates were drawn to evaluate FSC and SSC to determine the cell population of interest (**cells**, left column), to evaluate singlets (not shown), to determine live cells (**live**, middle column), and fluorophore expression (*mpx*:GFP[17] and *gata1*:DsRed[15]). First, gates were set on an animal that has no fluorophores (top row). Then *mpx*:GFP$^+$ (with no *gata1*:DsRed) animals were evaluated to set the gates and compensation for GFP (second row). *gata1*:DsRed$^+$ (with no *mpx*:GFP) animals were evaluated to set the gates and compensation for DsRed (third row). Finally, the two colors can be evaluated in double positive animals that are *mpx*:GFP$^+$ and *gata1*:DsRed$^+$.

DISCUSSION:

Zebrafish are an excellent model system for studying primitive and definitive vertebrate hematopoiesis. Over the past few decades, multiple assays have been developed and refined, allowing zebrafish to become a quick and economical model for testing drugs, generating and testing genetic mutants, and overall allowing researchers to analyze molecular pathways essential for hematopoiesis. This protocol utilizes embryonic zebrafish which allows quick data collection, the use of less physical space than adult animals, and the use of less drugs for large-scale chemical screens. It also allows quantitation after overexpression of mRNA, generation of CRISPR mutants, and MO-induced transcript reduction to alter specific genetic pathways that occur during early embryonic development. Importantly, it allows sensitive, robust quantitation of blood cells, which is difficult to do with in situ hybridization or histological staining techniques.

This assay is flexible and can be modified to answer many research questions. One modification of this protocol can be performed whereby the developmental stage of the animal is manipulated to quantitate different blood cell types. For example, red blood cells arise early in zebrafish development, and with transgenic animals such as the *lcr*:GFP[16] transgenic line can be detected as early as the 16-somite stage. If one is interested in studying thrombocytes, however, the *itga2b*:GFP[18] (also known as *cd41*:GFP) transgenic line starts expressing GFP at 48 hpf, with mature circulating thrombocytes observed at 72 hpf. If one desires to quantitate B cells with this assay then it can be performed with *ighm*:EGFP[19] transgenic animals closer to 20 dpf. Importantly, this assay can also be used to follow blood cells over time. For example, by analyzing the numbers of *lcr*:GFP$^+$ cells at 24 hpf, 48 hpf, 72 hpf, one could determine if a genetic modification (or drug) is regulating the maintenance of red blood cells versus just their generation.

These assays allow researchers to reduce gene expression with CRISPR or MOs or overexpress gene expression by injecting mRNA at the one-cell-stage of development and accurately compare the number of blood cells produced in these modified embryos. Care should always be taken to perform proper controls for these experiments at the same time by injecting sibling animals with non-specific guide RNAs or mismatched MOs and examining their blood alongside the experimental groups. The developmental stage is critical; alterations of only a few hours during development could show vastly different numbers of blood cells present in an embryo. In other words, make sure to carefully monitor the hours of development when comparing embryos. To ensure that embryos are age-matched, morphological cues should be utilized. In early embryos, consider counting somites to age-match. Later in development, other markers such as the beginning of the heart beating, the size of the otic vesicle, or length of the body can be used to match the stages of modified fish to control fish. Furthermore, the numbers of total cells can be enumerated from digested embryos to ensure the same number of cells are present in experimental and control animals. In addition to using these assays to examine genetic modification, these assays can also be modified to perform large scale drug screens. Embryos can be exposed to different concentrations of compounds temporally during development to see if the chemicals in question have any effect on hematopoiesis. If the experiment is designed to test the effect of a particular drug, then animals treated with vehicle only should also be included as a control. In these ways, researchers can determine if gain- or loss-of-function of specific genes or signaling pathways play an essential role in hematopoiesis.

It is essential that when examining different transgenes that the number of animals digested per sample is optimized; too few animals may generate little or no fluorescence. This is especially true when examining HSPCs which are not abundant at a particular timepoint. It is also critical when examining transgenics that have weak promoters driving fluorescence. To counteract these issues more animals (and hence, more cells) are required for accurate counts. When altering developmental stages, it is also critical to optimize the digestion time. This protocol is optimized for 5 individual 48 hpf embryos per tube. Digesting more mature embryos will likely require longer time.

Some potential problems can arise during the procedure. If there is no fluorescence observed, there could be a technical issue with the cytometer that needs to be resolved. For this reason, it is essential to verify that the embryos are fluorescent before beginning the procedure by quickly checking them under a microscope. Another common issue is over-digesting the embryos, which destroys the cells. Take care at the digestion step and alter the timing if too many dead cells are observed.

These assays have a few limitations. The largest issue is that these assays are relying on the expression of a transgenic marker. Potentially the change of the transgenic marker's expression may not reflect the biology of what is occurring in the embryo. Additionally, flow cytometry may not be the best method to quantitate cells if the target cell population is extremely low. To deal with these possibilities, other assays such as in situ or histological staining techniques could be utilized. If specific antibodies exist for the cell type of interest immunohistochemistry can also be performed. Embryos could also be subjected to qRT-PCR to measure lineage specific genes, and

if these assays are performed on a FACS machine, the cells could be isolated and studied individually with even more sensitive methods. Excluding these issues, this quantitative flow cytometry assay can generate a lot of useful information for researchers.

With these assays, researchers can easily observe hematopoietic defects in vertebrate animals. Modifying genetic pathways with MOs or CRISPR and then performing flow cytometry to elucidate if the gene plays a role in hematopoiesis can be done quickly and is quantitative. Additionally, forward genetic screens (as long as the animals have a fluorescent tag) can be performed and defects assessed. Zebrafish have also become an excellent model for large scale drug screens[6-10], allowing efficient drug screening assays on living organisms to observe if the drugs are efficacious and if they have negative effects on development/survival. Coupling this assay with automated flow cytometry technology enhanced by robotics would make it even more efficient[20-22], allowing truly large-scale analysis and screening of pathways important in blood cell genesis, growth, and regulation that remain obscure.

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DISCLOSURES:

D.L.S. is a scientific consultant and has received compensation from Finless Foods, Inc. and Xytogen Biotech, Inc. K.F.R. declares no competing interests.

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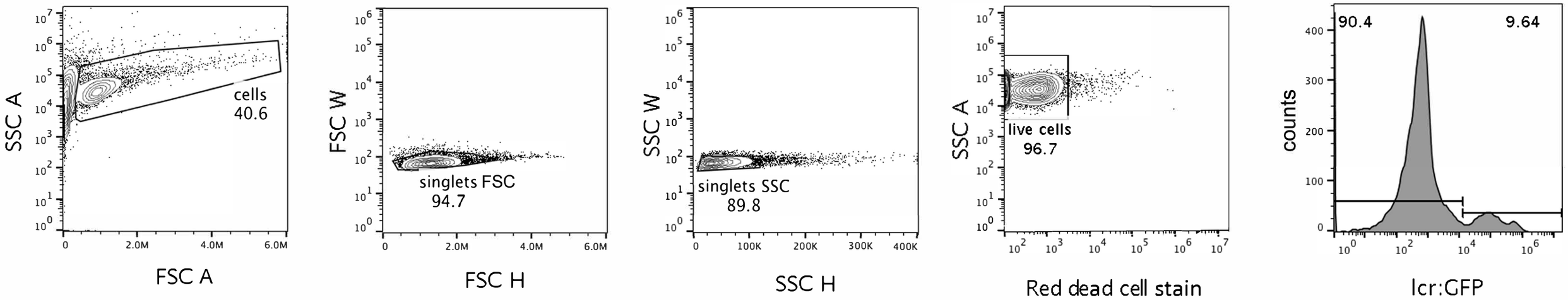
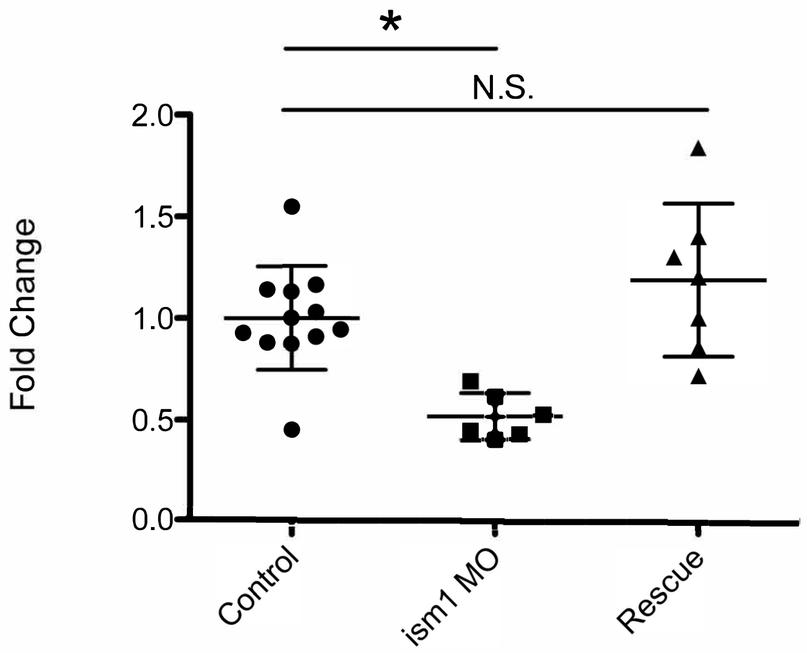
A



B



Solution	Ingredients
E3 medium (50x)	14.61 g of NaCl, 0.63 g of KCl, 1.99 g of $MgSO_4 \cdot 7H_2O$, 1.83 g of $CaCl_2 \cdot 2H_2O$
E3 medium (1x)	40 mL of 50x E3
1-Phenyl-2-thiourea (PTU) in E3 medium	40 mL of 50x E3 in a 2 L bottle, 40 mg of PTU

Notes
To a 2 L graduated cylinder, add the ingredients and enough distilled water to bring the total volume to 1 L.
To a 2 L graduated cylinder, add enough distilled water to bring the total volume to 2 L.
To a 2 L graduated cylinder, add enough distilled water to bring the total volume to 2 L. Stir for at least 2 days to completely dissolve PTU.

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1.5 ml MCF tube	FisherBrand	05-408-129	
10 mm Polystyrene easygrip Petri dish	Corning Falcon	351008	
5 ml Polystyrene round bottom tube with cell strainer cap	Corning Falcon	352235	
BD FACSAria Fusion flow cytometer	BD Biosciences		
Dithiolthreitol (DTT)	Sigma-Aldrich	646563	
DPBS (10x) with Ca^{2+} and Mg^{2+}	Life Technologies	14080-055	
FBS 500 mL	Gemini Bio-Products	100-108	
HyClone PBS (1x)	GE Healthcare Life Sciences	sh30256.01	
Librease	Roche Sigma-Aldrich	5401119001	dissociation protease
Pronase	Roche Sigma-Aldrich	11459643001	dechorionation protease
SYTOX Red Dead Cell Stain	Invitrogen	S34859	

Editorial comments:
Please note that the manuscript has been modified to include line numbers and minor formatting changes. The updated manuscript is attached and please use this updated version for future revisions.

We apologize, but the manuscript was not attached. We edited our version and submitted it. Hopefully that is ok.

All our responses are italicized, and additions/changes to the text are in red.

Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript.

2. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

We have revised statements to be in the imperative tense in the actual protocol. We assumed that "discussion about the protocol" referred to the discussion in the "Representative Results" section. As many of those points are also brought up in the Discussion, we have deleted that paragraph.

3. 1.1: How many embryos are placed in one dish?

We have revised this section, and added the numbers of embryos:

"With embryos in a 10 cm plastic petri dish, tilt the dish so that the embryos sink to the bottom edge. Use at least 5, but no more than 200 embryos at this stage. It is easy to tilt the dish by removing its lid and placing the lid underneath one edge of the petri dish."

4. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

We have amended these issues throughout.

5. Please upload Table 1 to your Editorial Manager account as an .xlsx file. Avoid any coloring or formatting in the tables.

We have uploaded Table 1 as an .xlsx file.

6. Table of Materials: Please sort the materials alphabetically by material name.

We have sorted the revised table alphabetically.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
Here the authors describe a method to dissociate embryos and use flow cytometry as a robust way to compare haematopoietic cell populations labelled with specific transgenes. This is a complementary method to other used in the field (in situ hybridization, qPCR, RNAseq, etc) and is very useful provided the researchers have access to the

required haematopoietic cells. I think stimulating the use of flow cytometry alongside other methods to study haematopoiesis should be encouraged. The manuscript reads well and it's easy to follow; it also looks somewhat limited in scope, so I would suggest a few changes to strengthen the interest and widen the applicability of the method for others in the field.

We thank the reviewer for their comments, which are addressed below.

Major Concerns:
It would be nice to see a sorting strategy to isolate both GFP and Cherry-labelled cells, where the authors also show the gating strategy, including a non-fluorescent control and a single-colour control.

We have added double-positive embryos with GFP and DsRed-labeled cells, and have included these data in Supplemental Figure 1. We have also added gating strategies for non fluorescent fish and single color controls. We would have added it into Figure 1, but we do not have lcr:GFP fish lines that also have other fluorophores present.

Minor Concerns:
Protocol, step 1: would be useful to mention a range of stages where this is applicable, as well as adding a note clarifying that manual dechorionation is also an option.

We have added the following clarification:

"Note: For each individual sample to analyze, at least 5 embryos are required. This dechorionation procedure can accommodate up to 200 embryos per petri dish. Samples can be grouped at this stage and separated later if desired. Alternatively, manual dechorionation can be performed by gently tearing the chorions with watchmaker forceps. This is advantageous when there are small numbers of embryos.

Note: Usually zebrafish will usually hatch from their chorions by 72 hpf. Severely malformed embryos may not, however, and will require dechorionation (either manual or chemical) after that time."

Step 2.1 - perhaps mention a range of embryos (5-??) per tube.

We have added the following clarification:

"Using a P1000 pipette, place 5-10 embryos into a single 1.5 mL microcentrifuge (mcf) tube."

Step 3.1 - using the DTT to remove the mucus coating is new to me; is there a reference that could be used for this? Alternatively, another line of explanation of this step would be useful (or a note in this step).

Our group has described this before, but we have also included references from others.

We have added the following references:

Berrun, A. C. & Stachura, D. L. Development of an In Vitro Assay to Quantitate Hematopoietic Stem and Progenitor Cells (HSPCs) in Developing Zebrafish Embryos. J Vis Exp, doi:10.3791/56836 (2017).

Westerfield M, Z. L., Detrich HW Essential Zebrafish Methods: Cell and Developmental Biology. 517 (Academic Press, 2009).

Drummond I.A, D. A. J. Methods in Cell Biology. Vol. 100 (Elsevier, 2010).

Step 4 - there are other methods out there that will take considerably less time to dissociate the embryos, so perhaps it would be worth to mention that in the note at the end of step 4.

We have added an explanation to the end of step 4:

"Note: There are quicker alternative methods to dissociate embryonic tissue, but we found empirically that this method, although it takes 60 minutes, is gentle and thorough enough to allow efficient isolation of live hematopoietic cells."

Representative results - as mentioned above, would be nice to see the gating strategy for dual color isolation of haematopoietic cells and their respective negative and single-color controls.

We have added these data to the Supplemental Figure 1.

Discussion - not sure about the applicability of this method in high throughput screens; the bottleneck here would be the time it takes for each sample to be processed in the flow cytometer. Is there a concrete example the authors could use to justify this? At the end of the discussion the authors mention again flow cytometry linked to robotics, which might help with the throughput issue. Perhaps re-write this section to better clarify how the flow cytometry could be used for large scale screens.

We have revised this section and added a few references that speak to the applicability of high throughput flow cytometry screens.

"With these assays, researchers can easily observe hematopoietic defects in vertebrate animals. Modifying genetic pathways with MOs or CRISPR and then performing flow cytometry to elucidate if the gene plays a role in hematopoiesis can be done quickly and is quantitative. Additionally, forward genetic screens (as long as the animals have a fluorescent tag) can be performed and defects assessed. Zebrafish have also become an excellent model for large scale drug screens[6-10], allowing efficient drug screening assays on living organisms to observe if the drugs are efficacious and if they have negative effects on development/survival. Coupling this assay with automated flow cytometry technology enhanced by robotics would make it even more efficient[18-20], allowing truly large-scale analysis and screening of pathways important in blood cell genesis, growth, and regulation that remain obscure."

Reviewer #2:

Manuscript Summary:

As zebrafish become a convenient animal model to study hematopoiesis, more specific and effective assays for zebrafish blood cells is important. With this protocol, authors provided an easy way to quickly quantify the blood cells by flow cytometer. On the other hand, some limitation shows with this assay and need to be addressed before publishing. However, the authors do offer a good way to quickly quantify the defect of hematopoiesis in some cases.

Major Concerns:

1. Flow cytometer is better for defining large number of cells rather than small number of target cells. When the target cell population is small, it is better to perform staining rather than flow cytometry. The authors need to highlight this point.

Unfortunately, this isn't true. Flow cytometry is an excellent technique for defining and quantitating rare cell populations. In fact, it is usually the tool of choice for detecting extremely low numbers of cells and has been instrumental to the identification of stem cells and rare circulating tumor cells; advancements in the technique, computational processing speed, and instrumentation has been reported to identify rare cells at frequencies as low as 0.0001% in cell populations. When looking for these rare populations, many sample events must be collected (1-10 million cells is usually sufficient), but that many cells are present in 5-10 48hpf embryos. Furthermore, FACS (basically performing the same experimental procedures with a different machine) is the only way to identify and isolate these cells for further study, especially in zebrafish, which lack antibodies to identify and isolate cells in other ways (such as magnetic separation). Regardless, we do agree that there are other ways of observing rare cells, and have mentioned this in the Discussion.

"These assays have a few limitations. The largest issue is that these assays are relying on the expression of a transgenic marker. Potentially the change of the transgenic marker's expression may not reflect the biology of what

is occurring in the embryo. Additionally, flow cytometry may not be the best method to quantitate cells if the target cell population is extremely low. To deal with these possibilities, other assays such as in situs or histological staining techniques could be utilized. If specific antibodies exist for the cell type of interest immunohistochemistry can also be performed. Embryos could also be subjected to qRT-PCR to measure lineage specific genes, and if these assays are performed on a FACS machine, the cells could be isolated and studied individually with even more sensitive methods. Excluding these issues, this quantitative flow cytometry assay can generate a lot of useful information for researchers."

2. The authors need to show how to make sure that the dissociation of embryos is good. It is better to show a picture of cells after digestion.

We have added Supplemental Figure 1 to highlight this.

3. It is better to gate the lcr:gfp positive cells on a two dimensional plot (For example, APC vs GFP). Sometimes the fluorescent background of embryos could interfere with the one dimensional plot of GFP.

APC and GFP cannot overlap, as APC is excited by the 633nm laser and GFP is excited by the 488nm laser and they have little spectral overlap. To address the issue of multiple colors, however, we have added Supplemental Figure 2.

4. The authors need to provide more details about flow cytometry. For example, which model of flow cytometry machine they are using? How is the start up procedure for their machine. So that people could follow their procedures exactly.

While we understand the desire to include all of this information, we're not sure it really helps the reader perform flow cytometry, especially with complex FACS machines. The operating manual for the BDFACSAria Fusion, which is the machine we utilize, is 2 3-ring binders and requires a week long operator training in San Jose to be a certified operator. We feel that this is simply too much information to include in this protocol. We have mentioned the machine that we utilize, however, and hope the Reviewer also notes all the additional descriptions of flow cytometry that we have added to the revised manuscript.

Reviewer #3:

Manuscript Summary:

Rueb and Stachura have established a flow cytometry-based quantification method to determine the phenotypic effect in zebrafish embryos. Although the method is easy and useful to achieve better quantification of target cells in embryos compared with WISH-based quantification, a few steps should be added or modified before publication.

Major Concern:

The authors enumerated red blood cells based on the percentage of GFP+ cells in lcr:GFP embryos injected with PBS or ism1 MO with or without ism1 mRNA. The percentage of GFP is relative to the live cells within the sample, and therefore, it is largely dependent on the effect on other types of cells. For instance, if MO reduces the number of neural cells, but not blood cells, the percentage of GFP may relatively increase, leading to a false positive result. In addition, it is also difficult by this method to compare the number of fluorophore+ cells between different stages of embryos due to different sizes and cell numbers, while the authors discussed this point in Discussion. The author should add a step to confirm if absolute numbers of live cells collected from each embryo type were equivalent using a hemocytometer with trypan blue staining or flow cytometry-based quantification. In addition, the authors should also discuss a method to compare the absolute number of fluorophore+ cells between phenotypes.

We agree that these are limitations to using relative percentages of fluorophore+ cells. We have added more to the Discussion on these issues and information about quantitating total numbers of cells in fluorophore+ animals.

We added this to the protocol:

"Note: Total numbers of fluorophore$^+$ cells can be obtained with these data. Simply count the total number of cells in the sample with a hemocytometer and multiply the percentage of fluorophore$^+$ cells to get the absolute number of fluorophore$^+$ cells per fish."

And this to the Discussion:

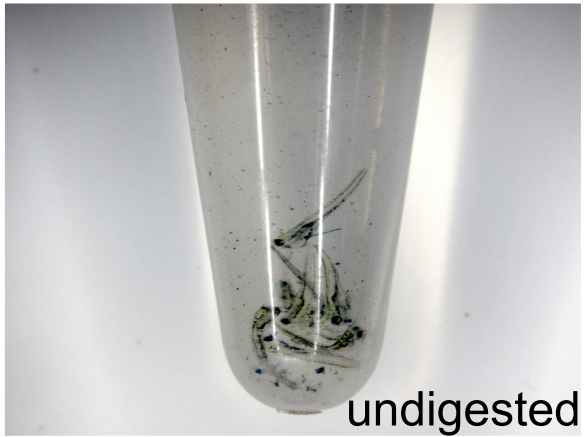
"To ensure that embryos are age-matched, morphological cues should be utilized. In early embryos, consider counting somites to age-match. Later in development, other markers such as the beginning of the heart beating, the size of the otic vesicle, or length of the body can be used to match the stages of modified fish to control fish. Furthermore, the numbers of total cells can be enumerated from digested embryos to ensure the same number of cells are present in experimental and control animals. In addition to using these assays to examine genetic modification, these assays can also be modified to perform large scale drug screens. Embryos can be exposed to different concentrations of compounds temporally during development to see if the chemicals in question have any effect on hematopoiesis. If the experiment is designed to test the effect of a particular drug, then animals treated with vehicle only should also be included as a control. In these ways, researchers can determine if gain- or loss-of-function of specific genes or signaling pathways play an essential role in hematopoiesis."

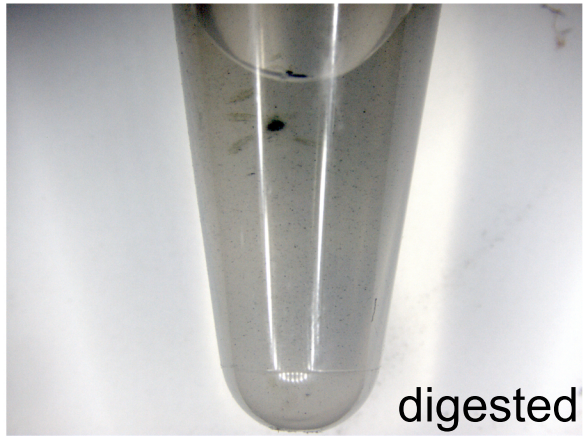
Minor Concern:
It is quite standard in flow cytometric analysis that doublets and triplets can be excluded by using the "width" of FSC and/or SSC in combination with the "area" of these parameters. This is particularly important to enumerate cells expressing multiple fluorophores. The authors should discuss this point as well.

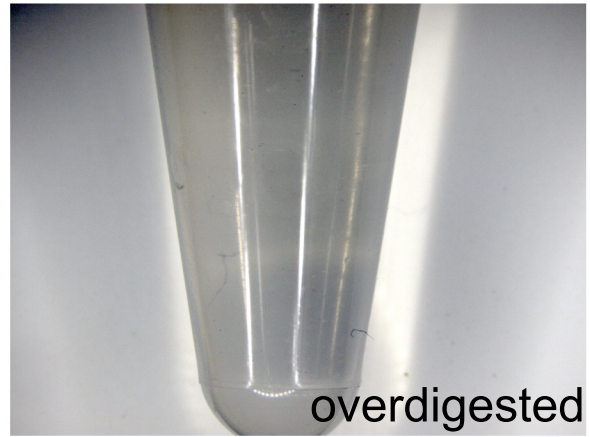
We agree. While this is more essential when actually sorting the cells, we have added these discussions into the protocol and added to Figure 1 to address this issue.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Rueb et al., Figure S1.pdf







Supplemental Figure 2

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