November 16, 2018

Re: Revision, JoVE59191 manuscript

Dear Dr. Wu & Reviewers,

Here, we submit a revised manuscript "Isolating Malignant and Non-Malignant B Cells from *lck:eGFP* Zebrafish" by Burroughs-Garcia, et al., for consideration of publication by *JoVE*. We appreciate the thorough and insightful critiques of our reviewers, and now provide an amended manuscript addressing their concerns. Their suggestions, while numerous, were straightforward. We believe this new version improves our paper by answering their queries. As directed, changes have been tracked so reviewers can easily identify relevant sections with edits.

The remainder of this letter lists each editorial or reviewer comments in *italics*, followed by replies to their queries with line numbers pertaining to the specific sections where the requested changes can be found:

**Editorial comments:**

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

We believe all errors have been corrected.

2. *Please remove the brackets enclosing the reference numbers.*

Superscripted numeric footnotes now replace bracketed references in text.

3. *Please define all abbreviations before use.*

Abbreviations have been defined.

4. *JoVE cannot publish manuscripts containing commercial language…*

Commercial language, trademarks, registered symbols, and company names are now replaced by generic terms, apart from the Table of Materials and Reagents.

5. *Please revise the protocol text to avoid the use of any personal pronouns.*

Personal pronouns have been removed from the protocol.

6. *Please revise the protocol to contain only action items that direct the reader to do something…. The actions should be described in the imperative tense.... Please move the discussion about the protocol to the Discussion.*

The protocol is now amended as specified, using the proper tense. Aspects of the protocol requiring special consideration are now located in the Discussion.

7. 1.4: *Please specify the concentration of tricaine used.*

Tricaine concentration is now listed at 0.02% (**line 104**).

8. 1.5: *Please describe how to dissect lymphoid organs….specify the tools used. Alternatively, add references specifying how to perform the protocol action.*

We now list references describing these dissections (**lines 118-119**).

9. 2.1, 2.7: *Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a “Note.”*

As mentioned above, the revised protocol now incorporates these changes, with Notes added at **lines 151, 171-173, 217-221, and 230-233**.

10. *JoVE articles focus on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol* AND *b) Any modifications and troubleshooting of the technique*

Both topics discussed in the 3rd paragraph of the Discussion (**lines 332-343**).

*c) Any limitations of the technique*

Not explicitly detailed, as the Discussion section is already rather lengthy.

*d) The significance with respect to existing methods*

Discussed in the 1st (**lines 318-324**) and 2nd Discussion paragraphs (**lines 326-330**).

*e) Any future applications of the technique*

Listed in the 4th (**lines 352-360**), 5th (**lines 365-370**), and 6th Discussion paragraphs (**lines 372-377**).

11. *Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.*

Scale bars are now added to fluorescence microscopy images in Figures 1-5 as described in their legends (**lines 280, 284, 295, 306, and 313.**

12. *Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.*

The ordering of items in the Table of Equipment and Materials has been corrected as directed.

13. *References: Please do not abbreviate journal titles. If there are six or more authors, list the first author and then “et al.”.*

References are now formatted in this manner (**lines 393-459**).

*14. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc., and h, min, s for time units*.

Standard units have been used throughout the protocol.

*15. Please split some long steps into two or more sub-steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.*

Long paragraphs have been split (**lines 137-142 and 145-148**).

*16. What’s the composition of lysis buffer?*

We have corrected the sentence making a more general statement. The reader to choose the appropriate lysis buffer according to analysis of interest (Lines **240-243**).

*17. Step 2.3: Please write this step in imperative tense…* *Please write each step in complete sentence and in imperative tense.*

The step is now amended as specified, using the proper tense and complete sentences. With note added at lines **254-258.**

*18. 2.7: This step does not contain action items that direct the reader to do something. Please rewrite it as a note.*

Statement have been rewrite as a note (**line 173-176**)

*19. 3.3: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense may be added as a “Note.”*

This step has been modified to the proper tense. A note was added (**lines 235-238**)

**Reviewer #1 (listed only** Minor Concerns):

*In step 1.5, although this is discussed at the end of the protocol, a note on which tissue is best to isolate B cells vs T cells would be helpful.*

At the editor’s request (see editor comment #6), we discuss this key consideration in **lines 347-353** of the Discussion. We agree with Reviewer 1 that readers may be curious about this topic when reading the protocol, so we now specifically address this point in step 1.5 (**lines 119-120**) and refer readers to the Discussion for further information.

*In step 1.8, creating a filter unit, disposable mesh strainers that fit into 50mL tubes are widely available for purchase from somewhere like Fisher and VWR, and might be more straightforward than creating one. As written, it wasn't clear where the hole was supposed to be poked into the tube. Into the lid?*

We were unaware these filters are commercially available, having always made them ourselves. In view of this, we added these to the Table of Materials and Reagents and deleted this from the protocol (**lines 131-135**). In answer to Reviewer 1’s question, the hole is made at the side of the 50 ml conical, just below the threads for the lid. It serves to release pressure as cells and media pass through the filter.

*In step 2.3.1, if the fish is only thymus-positive, is this a tumor, or pre-malignant cells?*

This is an important issue. Admittedly, it is challenging to detect early-stage ALL involving only thymus (in fact, at this stage, it is probably more accurately termed lymphoblastic lymphoma). Complicating matters further, in our experience, *rag2:hMYC* fish have larger thymi than WT fish, making it even more difficult to discern pre-malignant vs. early LBL/ALL. To definitively prove malignancy, allo-transplantation is necessary, which is beyond the scope of this paper. To clarify this confusing—and debatable—issue, we have refined our definition of ‘Level 1’ tumors in “note” section 2.3 (**lines 154-158)**. We apologize that we cannot provide convincing criteria to identify LBL/ALL at its earliest onset; we would very much like to.

*In step 2.8.6, homogenizing an entire adult zebrafish with a pestle in a 1.5mL microtube seems messy, and not very efficient (unless there is a step missing?). It is also possible to mince the fish in FACS media on a petri dish then filter, and these methods have been published elsewhere.*

Homogenizing an entire fish (after removing the head; see section 2.8) is messy, but this is the method we use. We utilize the entire body when we wish to purify the maximum number of ALL cells possible. We typically use ‘Level 3’ fish (section 2.3.3), where disease is extensive, for this. We will demonstrate this procedure via video. When fewer cells are needed, we favor peritoneal washing. We realize other laboratories use other techniques, such as mincing tissue in a petri dish, but we do not customarily do this. To include this as an alternative, we now add this option to the Discussion (**lines 350-353**).

*In step 3.2.1, are FACS gates set using wild type tissue as a control, so true GFP-negative cells can be gated. Otherwise is could be difficult to determine negative from low from high.*

Fortunately, in both WT fish and *rag2:hMYC* fish with ALL, differentiating GFP- vs. GFPlo vs. GFPhi cells is not difficult, as these have >10-fold differences in GFP intensity (see Figures 2-5 for examples). We do not find it necessary to ‘calibrate’ with GFP- WT tissue prior to analyzing or sorting. To clarify this for readers, we have added a note to our protocol step 3.2.2 (**lines 218-225**).

*In figure 2, 3, 4, instead of 2^-ddCT, fold change can just be listed for clarity sake. Also, what is the sample expression compared to? GFP-negative cells? If the lymphoid gate is set in FACS using forward or side scatter, what are the GFP- cells, if not T or B cells?*

We appreciate this suggestion, and have changed the y-axis of all qRT-PCR data to fold-change values rather than 2^ddCT in Figures 2-5. In each case, we have set the lowest-expressing sample = 1, with other samples expressed as fold-change relative to 1. We normalized expression of each target gene (*pax5*, *cd79b*, *cd4*, *lck*, or *GFP*) to that of two housekeeping genes (*-actin*, *eef1a1l1*). Each comparison is between GFPlo vs. GFPhi cells; GFP- cells were not analyzed. We do not know the identities all GFP- cells in the lymphoid gate (and these probably vary between kidney marrow, thymus, spleen, and homogenized whole fish), but the GFP- fraction does contain some B cells (i.e., we detect *pax5*, *cd79b*, and other B cell mRNAs) and—based on other transcripts analyzed—likely also contains NK cells. We suspect additional cell types are also present in the GFP- fraction.

**Reviewer #2 (also listed only Minor Concerns):**

*The authors tend to hyphenate "B cell" and "T cell" as "B-cell" and "T-cell." This isn't necessary, and should be removed. The same can be said for "kidney-marrow." However, "3 months-old" (and other ages) should read "3-months-old."*

We have modified hyphenations as suggested throughout the manuscript.

*Line 87: should a "respectively" be added after "transgenic lines?"*

For clarity, we have replaced the sentence in question (**lines 90-91**) with a new sentence (**lines 88-90**) where “respectively” is unnecessary.

*Step 1.2: Could the authors describe the anatomical location of the thymi or list a reference for readers/viewers unfamiliar with these organs in teleosts?*

We have incorporated these suggestions (**lines 106-107**).

*Step 1.5: Could the authors describe (or reference) how to dissect the lymphoid organs of interest?*

This information has been added (**lines 119-120**).

*Lines 130-131: Is it necessary to list the specific make and model of microscope and the settings? I assume that anyone could universally do this procedure, so it might be more useful to describe this in a more general way.*

We have removed details pertaining to our microscope (Nikon AZ100), as required by *JoVE* guidelines (see editor comment #4). We retained our ‘low exposure’ (200 ms, 2.4x gain) and ‘high exposure’ (1.5s, 3.4x gain) settings as examples for readers to have an idea of how fish with B-ALL will vary in appearance from fish with T-ALL. We agree with Reviewer 2 that the specific settings will likely vary based upon the specific fluorescence microscopy equipment used.

*Step 2.5: Doesn't the yield of cells depend on the fish size? Could the authors somehow qualify this by saying how large the fish are?*

Yields of ALL cells obtained by FACS vary based several factors: (1) extent of disease involvement, which we describe in steps 2.3. (2) age and size of the fish, which are inter-related, and (3) preparation method (now included as a Note to step 2.6, and discussed in detail in **lines** **341-350** of the Discussion). In our experience, disease spread is the best predictor of yield, which is why we cite this in the protocol. Even small 2-3-month-old Level 3 fish often have >10 million ALL cells.

*Line 154: What does "higher sorting efficiency" actually mean? On the BD sorters, this can mean something different that what the average reader might assume (does this refer to low "conflict rate?" It could refer to many gating and sorting parameters like drop "masking" that the average reader is not familiar with). Can the authors describe this in a different way? And, why in line 157 would lower "sorting efficiency" be "more costly?"*

Thank you for bringing this to our attention, these considerations were not at all what we were trying to say. Stated simply, peritoneal washing typically procures a higher % of GFP+ cells, but lower total yield of GFP+ cells. In a short time (lower FACS cost), ~2 million GFP+ cells can be purified. Homogenization of the entire fish is the opposite: lower % of GFP+ cells, but higher total # of GFP+ cells (often >20 million), but it requires much greater FACS time, and hence is more costly. We believe Discussion **lines 341-350** now clearly makes these points.

*In step 2.8.4 and 2.8.7, can you reference "step 1.8" for making the filter?*

We learned from Reviewer 1 that filters are commercially available, so we now list these in our Table of Materials and Reagents instead. I learned how to make them from scientists in the Traver lab, and then taught people in my lab; I was unable to find the original reference describing their construction.

*Line 189: should "FAC-sorting" just be "FACS?"*

Corrected (**line 209**).

*Line 198: can "refer to" be removed?*

Removed (**line 219**).

*Line 201: Usually FSC and SSC H and W are compared to eliminate doublets. Can the authors be more general and just say "be sure to eliminate doublets" in some way?*

We added a general statement as Protocol step 3.2.3 to make this point (**lines 227-228**).

*Line 206: Can the gates just be referred to by their actual excitation and emission parameters? It is confusing throughout the descriptions and Figures- it seems like the authors use "PE" consistently, but oscillate between "GFP" and "FITC" fluidly. Most people performing these studies will understand, but for clarity, maybe just list the filters and call them "PE" and "GFP." For example, on line 260, the authors say "FITC," but it is "GFP" in the figure.*

Simpler is better. We have replaced every mention of FITC in the manuscript with GFP.

*Line 216: How many cells are need for different analyses?*

This is difficult to answer, as there are so many conceivable downstream applications, it is not possible to envision every possible analysis. In our lab, we use FACS-purified cells for allo-transplant, single-cell and bulk qRT-PCR, single-cell and bulk RNA-seq, and protein extraction. Each requires different numbers of cells. We think it reasonable for readers to have an idea of the number of cells they will need for their experiments. Our 3rd Discussion paragraph (**lines 341-350**) addresses this topic.

*Convention-wise, should the Figure Legends be before the Discussion?*

The *JoVE* template places Figure Legends prior to the Discussion section. We agree it is unusual.

*In the beginning of the Discussion, the authors discuss how lck is not specific for T cells, but in the figure legends of Figure 4 and 5, they claim that lck is T cell specific. I think that needs to be rectified to avoid confusion.*

Thank you for catching this—on occasion, we still forget that *lck* is not truly T cell specific. Legends for Figures 4 and 5 have been corrected (**lines 311 and 318**).

*In line 294, should LCK be italicized? I think these studies likely deal with the protein, and not the gene.*

Human data pertain to RNA microarray results, so we believe *LCK* is correct in this case. These data are shown in Supp. Fig. 2B-C of the *Borga* et al. *Leukemia* paper; original human data derive from:

Novershtern N*, et al.* Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell* 2011 **144**(2)**:** 296-309.

Haferlach T, *et al.* Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the International Microarray Innovations in Leukemia Study Group. *J Clin Oncol* 2010 **28**(15)**:** 2529-2537.

*Figure 4 and 5: should "Cd4" be "cd4?"*

Thank you, we have corrected these errors.

**Reviewer #3 (also listed only Minor Concerns):**

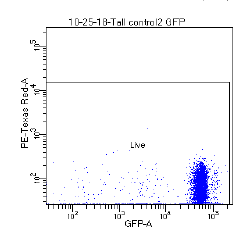
*Lines 71-73. Please add a citation for reference 11 at the end of this sentence. When I read this, I thought at first that reference 10 had been cited in error as the B-lineage ALLs are from reference 11. The reader will be less confused if reference 11 is cited here and not just in the subsequent sentences.*

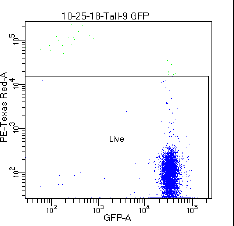
Reference 11 is now cited here. Thank you for the suggestion, it was confusing as originally written.

*Lines 112-113. It would be helpful to include references for the dissection methods for these tissues. Perhaps refer to other JoVE papers?*

References (#14, #15) have been added to the protocol in **lines 106-107** and **119-120**.

*Line 195. Section 3.2.1. You have not used any live-dead discrimination (with PI or 7-AAD) in this protocol. Perhaps this was a conscious decision for specific reasons? Could you please add a few lines to comment on this choice and/or discuss how live/dead discriminators might be included in the protocol if desired? In your experience, what is the viability of GFPlo and GFPhi cells after sorting from the various tissues?*

We used to assess cell viability with PI, but viability was always >95% after FACS (see examples of two *rag2:hMYC* T-ALL below), so we eliminated this from our standard protocols. For this reason, and in the interest of brevity, we opted not to include this. As suggested, we now add a note in the protocol mentioning the possibility of assessing viability by PI or 7-AAD (**lines 223-225**).



*Line 206. FTIC should be FITC*

As suggested by Reviewer 2, we replaced all mentions of FITC with GFP, eliminating this error.

*Figures 2D, 3D, 4B, 5B. It would be helpful to explain more clearly in the figure legends what 2^Dct means.*

In response to Reviewer 1’s suggestion, we reformatted qRT-PCR data in Figures 2-5 to now depict fold-change relative to the lowest-expressing sample, rather than 2^ddCT. We believe this greatly simplifies interpretation of these data.

We believe these changes improve our manuscript, and hope it will now meet with the satisfaction of our editor and reviewers for publication in *JoVE*. We thank you once again for your thorough reviews and insightful comments. Please contact me if you require additional materials or information to evaluate our revised submission.

Sincerely,



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