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TITLE:

Isolating Malignant and Non-Malignant B Cells from *lck:eGFP* Zebrafish

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SUMMARY:

Transgenic *lck:eGFP* zebrafish express GFP highly in T lymphocytes, and have been used to study T cell development and acute lymphoblastic leukemia. This line can be used to study B cells, which express *lck* at lower levels. This protocol describes purification of malignant and non-malignant B cells from *lck:eGFP* zebrafish.

ABSTRACT:

Zebrafish (*Danio rerio*) are a powerful model to study lymphocyte development. Like mammals, *D. rerio* possess an adaptive immune system that includes B and T lymphocytes. Studies of zebrafish lymphopoiesis are difficult because antibodies recognizing *D. rerio* cell surface markers are generally not available, complicating isolation and characterization of different lymphocyte populations, including B-lineage cells. Transgenic lines with lineage-specific fluorophore expression are often used to circumvent this challenge. The transgenic *lck:eGFP* line has been used to study *D. rerio* T cell development, and has also been utilized to model T cell development and acute lymphoblastic leukemia (T-ALL). Although *lck:eGFP* fish have been widely used to analyze the T-lineage, they have not been used to study B cells. Recently, we discovered that many zebrafish B cells also express *lck*, albeit at lower levels. Consequently, *lck:eGFP* B cells likewise express low levels of GFP. Based on this finding, we developed a protocol to purify B-lineage cells from *lck:eGFP* zebrafish, which we report here. Our method describes how to utilize a fluorescent-activated cell sorter (FACS) to purify B cells from *lck:eGFP* fish or related lines, such as double-transgenic *rag2:hMYC*; *lck:eGFP* fish. In these lines, B cells, particularly immature B

cells, express GFP at low but detectable levels, allowing them to be distinguished from T cells, which express GFP highly. B cells can be isolated from marrow, thymus, spleen, blood, or other tissues. This protocol provides a new method to purify *D. rerio* B cells, enabling studies focused on topics like B cell development and B lymphocyte malignancies.

INTRODUCTION:

Zebrafish offer powerful attributes, such as genetic manipulability, high fecundity, optical translucency, and rapid development that facilitate studying vertebrate development using genetic approaches. These advantages, together with the shared features of teleost and mammalian hematopoiesis, make *D. rerio* ideal for *in vivo* analyses of lymphopoiesis and lymphocyte function, from their earliest appearance in larvae throughout adulthood. Blood development in zebrafish relies upon well-conserved genetic processes that are shared with mammals, and these extend to the adaptive immune system. Additionally, molecular mechanisms governing lymphoid development are remarkably conserved between zebrafish and mammals[1].

Over the past 2 decades, transgenic *D. rerio* lines that label specific blood lineages and mutant lines deficient in these lineages have been created[2-5]. One of these, the *lck:eGFP* transgenic line, uses the zebrafish lymphocyte protein tyrosine kinase (*lck*) promoter to drive GFP expression[6]. This gene, which is highly expressed by both T-lineage precursors and mature T lymphocytes, allows *in vivo* tracking of thymic T cell development and *ex vivo* purification of T-lineage cells by FACS[7]. Previously, we used this line in a forward-genetic ENU mutagenesis screen to identify germline mutants prone to T-ALL and to study somatically-acquired genetic events linked to T cell oncogenesis[8,9].

Recently, our laboratory further extended the utility of *lck:eGFP* zebrafish. In double-transgenic *rag2:hMYC* (human MYC), *lck:eGFP D. rerio* that are known to develop T-ALL [10], we discovered that B-lineage ALL also occur[11]. Unlike T-ALL in this model, which fluoresce brightly due to high GFP expression, B-ALL are dimly-fluorescent due to low GFP levels, allowing fish with B-ALL to be distinguished grossly from those with T-ALL by fluorescent microscopy. This differential GFP expression also permits the separation of GFP^{lo} B-ALL cells from GFP^{hi} T-ALL cells using FACS[11]. Moreover, low *lck* expression is not unique to zebrafish B-ALL, as human B-ALL also express low levels of *LCK*[11,12]. Likewise, normal B-lineage cells of *D. rerio*, mice, and humans also express low levels of *lck*/*Lck*/*LCK*, with immature B cells having the highest expression[11,13]. On a per cell basis, B-lineage cells in *lck*:*eGFP* zebrafish or derivative lines express 1-10% as much GFP as T lymphocytes. These GFP^{lo} cells express characteristic B cell mRNAs such as *pax5*, *cd79b*, *blnk*, *btk*, *ighm*, *ighz*, and others, and can be purified from marrow, thymus, spleen, or peripheral blood[11]. Therefore, both B- and T-lineage cells can be isolated from *lck*:*eGFP* zebrafish, and in the case of *rag2*:*hMYC*, *lck*:*eGFP* animals, B- and T-ALL cells as well[11].

Here, we present our protocol to efficiently FACS-purify non-malignant B cells from *lck:eGFP* zebrafish, and non-malignant or malignant B cells of *rag2*:*hMYC*;*lck:eGFP* fish, using various source tissues. Such cells can likewise be quantified by flow cytometry without FACS isolation, if desired. Discovery of low *lck* expression—and consequently, low GFP expression—by B cells

opens new doors of experimental possibilities for *lck*:*eGFP* zebrafish, such as *in vivo* B cell developmental studies. Thus, this transgenic line, first reported in 2004, has new life as we seek to utilize it to glean fresh insights concerning zebrafish adaptive immunity.

PROTOCOL:

All procedures involving zebrafish were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Oklahoma Health Sciences Center.

1. Isolating Non-malignant B and T Lymphocytes from Transgenic *lck:eGFP* Fish

1.1. Anesthetize the fish using 0.02% tricaine (MS-222) in fish system water.

1.2. Examine 2-6 month old fish for fluorescent thymi, which are located at the dorsomedial aspect of the branchial cavity of zebrafish and other teleosts[14]. Use an epifluorescence microscope (470/40 excitation wavelength and 525/50 emission filter) to detect GFP.

1.3. Prepare in advance 50 mL of filter-sterilized 1x Roswell Park Memorial Institute Medium (RPMI) containing 1% fetal bovine serum and 1% penicillin-streptomycin (sorting media). Unused sorting media can be stored at 4 °C for up to 2 months.

1.4. Euthanize the fish by placing it in a beaker containing 0.2% Tricaine for approximately 5 min, followed by ice bath immersion. Confirm death by the cessation of opercular (gill) movement.

1.5. Place the euthanized fish in a Petri dish and dissect lymphoid organs of interest, using the fluorescent microscope to dissect the GFP^+ thymi[14], and bright field microscopy settings to dissect the kidney marrow and spleen[15]. Results presented here were obtained using 3-month-old fish. Lymphocyte proportions and absolute numbers vary by age and genotype (see discussion for further detail).

1.6. Place each dissected organ in a 1.5 mL tube containing 500 µL of cold sorting media.

1.7. Homogenize the tissue on ice using a pestle micro-tube homogenizer.

1.8. Pass homogenized tissue through a 35 µm mesh filter to generate a single cell suspension. Keep the cells on ice until analysis.

2. Isolating Malignant Lymphocytes from Double-Transgenic *rag2:hMYC;lck:eGFP* Zebrafish

2.1. Beginning at 2-4 months, microscopically screen *rag2:hMYC*; *lck:eGFP* fish for abnormal GFP patterns.

NOTE: B cells are GFP^{lo} in the *lck:eGFP* background, so B-ALL requires practice to recognize; T-ALL is obvious, because T cells are GFP^{hi}. Consequently, the *rag2:hMYC*, *lck:eGFP* dual-transgenic line has two phenotypes: brightly-fluorescent T-ALL, which usually arise from the thymus and extend into the body, and dimly-fluorescent B-ALL.

2.1.1. Using a fluorescent microscope, screen the fish using "low exposure" settings (200 ms, 2.4X gain) to identify bright T-ALL (**Figure 1A**) and "high exposure" (1.5 s, 3.4X gain) to identify dim B-ALL (**Figure 1A**). WT controls and pre-leukemic fish have GFP localized only in the thymus (**Figure 1B**).

2.2. Anesthetize and examine the fish as described in steps 1.1-1.2.

2.3. Categorize the fish based on the extent of GFP fluorescence, using a simple three category system:

NOTE: Level 1: Fluorescence appears as a thymic tumor with only limited local spread.
Level 2: Fluorescence appears beyond the thymus, involving <50% of the body.
Level 3: Fluorescence extends beyond 50% of the body.

2.4. Separate the fish with ALL from those without cancer. Monitor pre-leukemic fish (i.e., fish without GFP^+ tumors) once monthly for the development of new ALL. By ~9 months, all *rag2:hMYC*, *lck:eGFP* fish develop T-ALL, B-ALL, or both.

2.5. For T- or B-ALL cell isolation, select Level 3 fish (ALL involving >50% of the body), which yield more than 2×10^6 ALL cells, and typically many more.

2.6. Euthanize the fish as in step 1.4.

NOTE: There are two methods to obtain ALL cells: whole body homogenization and a peritoneal wash technique[16]. The methods differ in the absolute number of cells collected for sorting, and thus, the amounts of FACS time required and cost (see discussion for further detail).

2.7. For either method, first place the euthanized fish in a Petri dish and using a razor blade, remove the head including the thymic region. This can be processed separately, if desired, or used for histological staining.

2.8. Peritoneal Wash Method

2.8.1. Using a P1000 pipette, wash the fish peritoneal cavity with 500 µL of cold sorting media, collecting the cells and media in a 5 mL tube.

2.8.2. Using a fresh pipette tip, inject an additional 200-300 µL of cold sorting media into the body cavity. Then, using the tip of the pipette, apply gentle pressure to the fish body to extrude the cells out of the body cavity. Collect this media and add to the 5 mL tube.

2.8.3. Repeat step 2.8.2 2-3 times. Keep the collected cells in sorting media on ice.

2.8.4. Filter the cell suspension though a 35 µm mesh filter prior to flow cytometry/FACS and keep the cells on ice until analysis.

2.9. Whole Body Homogenization

2.9.1. After removing the fish head, place the body in a 1.5 mL tube containing 200 µL of sorting media.

2.9.2. Homogenize the body using a pestle micro-tube homogenizer.

2.9.3. Add an additional 300 µL of cold sorting media. Filter the cell suspension though a 35 µm mesh filter. Add sorting media as needed to wash all cells through the filter, until only tissue debris remains on the filter. Keep the cells on ice until analysis.

3. Cytometric Analysis of Normal or Malignant B Lymphocytes.

3.1. Set flow cytometric analysis and/or FACS parameters according to manufacturer's guide.

3.2. Acquire desired number of events to initially characterize the sample. Analyze 5 x 10^3 to 5 x 10^4 events prior to sorting to determining specific gates for subsequent sorting steps.

3.2.1. Determine gates: Define lymphocyte and progenitor cell gates using forward scatter (FSC) and side scatter (SSC) parameters, excluding cellular debris (**Figure 2A-C**)[17]. FSC and SSC correspond to cell size/diameter and granularity, respectively.

NOTE: GFP^-, GFP^{lo}, and GFP^{hi} cells differ 10-to-100-fold in terms of their GFP fluorescence intensity, making separation of these populations straightforward (**Figures 2-5**). Live-dead discrimination can be assessed at this point using propidium iodine (PI) or 7-aminoactinomycin D (7-AAAD) viability staining. Previous experiments with PI typically demonstrate >95% viability of GFP^+ cells after FACS.

3.2.2. Exclude the cell doublets, according to the parameters of the FACS machine being used.

3.2.3. Within the lymphoid and/or precursor gates, determine the number and percentage of GFP^+ cells.

3.3. Use phycoerythrin (PE) and GFP intensities, define gate for GFP^- vs. GFP^{lo} vs. GFP^{hi} cells.

NOTE: B cells exhibit dim GFP fluorescence and T cells show bright GFP in *lck:eGFP* lines. Many lymphoid organs or tumor samples contain both GFP^+ populations. B-lineage cells/B-ALL are GFP^{lo} and T-lineage cells/T-ALL are GFP^{hi}. Define gates to distinguish between GFP^-, GFP^{lo}, and GFP^{hi}

cells, and collect these populations separately (**Figure 2A-C**, **Figure 3A-C**, **Figure 4A** and **Figure 5A**).

3.4. Sort each cell population into different 15 mL polypropylene tubes containing 2 mL of sorting media, or directly into different 1.5 mL tubes containing an appropriate buffer for further analyses (e.g., RNA, DNA, or protein extraction, allo-transplantation, etc.).

3.5. Keep purified cells on ice prior to further analyses.

REPRESENTATIVE RESULTS:

We used flow cytometry to analyze and FACS to isolate GFP^{lo} and GFP^{hi} cells from thymus, kidney marrow, and spleen of *lck:eGFP* transgenic zebrafish. Analysis of 3-month-old fish revealed the thymus contained mostly GFP^{+} lymphocytes. GFP^{+} cells were largely confined to the lymphoid gate previously described by Traver et al.[17]. Two distinct GFP^{+} populations, GFP^{lo} and GFP^{hi}, can be observed in the thymus. GFP^{hi} lymphocytes represented a higher percentage, ~60%, while GFP^{lo} cells were less abundant, representing ~40% of total thymic lymphocytes (**Table 1**). Unlike mammals, fish hematopoietic marrow is localized within the kidney, rather than within bone. We determined B cells residing in kidney marrow also express low levels of GFP (**Figure 2B** and **Figure 3B**). GFP^{lo} cells in marrow were abundant, while GFP^{hi} cells were scarce (**Figure 2B** and **Figure 3B**)), indicating that only a small percentage of T lymphocytes were present in the marrow at 3 months of age. Splenic samples likewise showed higher percentages of GFP^{lo} than GFP^{hi} cells (**Figure 2C** and **Figure 3C**). We also analyzed non-malignant lymphocytes from thymus, marrow, and spleen of double-transgenic *lck:eGFP; rag2:hMYC* zebrafish, which are prone to both B- and T-ALL[11]. In fish that had not yet developed ALL, results from thymus, marrow, and spleen were similar to single-transgenic *lck:eGFP* fish. However, the numbers of lymphocytes per organ were increased (**Figure 3**), presumably due to MYC-driven expansion of immature B and T cell populations where the *rag2* promoter is active.

We also analyzed double-transgenic fish with B- and/or T-ALL that had developed fluorescent cancers by 6 months. Predictably, B-ALL fish with dimly-fluorescent cancers contained mostly GFP^{lo} cells (**Figure 4A**). In contrast, brightly-fluorescent fish can harbor either isolated T-ALL or mixed populations of both GFP^{lo} B-ALL and GFP^{hi} T-ALL cells (**Figure 5**). An example of mixed ALL, which contains distinct B- and T-ALL, is shown here (**Figure 5**). To confirm the identities of GFP^{lo} and GFP^{hi} cells as B- and T-lineage, respectively, we analyzed B- and T-cell-specific transcripts by quantitative real-time PCR (qRT-PCR). Our results show GFP^{lo} cells express higher levels of B cell transcripts and GFP^{hi} cells express higher levels of T cell genes (**Figure 2D**, **Figure 3D**, **Figure 4B** and **Figure 5B**). Furthermore, expression of *lck* and *GFP* in GFP^{lo} ALL correspond to the dim *in vivo* GFP fluorescence of B-ALL (**Figure 2D**, **Figure 3D**, **Figure 4B** and **Figure 5B**; qRT-PCR conditions and primers sequences previously described by Borga et al.)[11].

FIGURE LEGENDS:

Figure 1: Distinct fluorescence patterns in *lck:eGFP* transgenic fish. (**A**) Fluorescent microscopy images of *rag2:hMYC; lck:eGFP* fish with brightly-fluorescent T-ALL (left) or dimly-fluorescent B-ALL (right). T-ALL is visible with low exposure settings; B-ALL can only be seen using high

exposures. (**B**) High exposure settings show faint thymic fluorescence (yellow circles) in wild-type *lck:eGFP* fish (left) and *rag2:hMYC; lck:eGFP* double-transgenic fish without ALL (right). Scale bars = 20 mm.

Figure 2: Lymphocyte populations in *lck:eGFP* zebrafish. Images at top show a 3-month-old WT, *lck:eGFP* fish with high exposure settings or computer-enhancement (to facilitate visualization). Scale bars = 20 mm. Flow cytometric analyses of thymus **A**, marrow **B**, and spleen **C**. Left panels show FSC (x-axis) and SSC (y-axis), with black ovals indicating lymphocyte gates. Middle panels depict fluorescence-based gating with GFP (x-axis) and PE (y-axis). GFP^{hi} (blue rectangle), GFP^{lo} (green rectangle) and GFP^{-} (black ovals) populations are shown. Right panels display histograms of GFP^{hi} and GFP^{lo} cells. Dashed lines indicate gating criteria in middle panels. (**D**) WT *lck:eGFP* thymi sorted for GFP^{hi} (blue) and GFP^{lo} (green). Expression of B cell gene (*pax5*), T (*cd4*) cell-specific genes), *lck* and *GFP*. Results are normalized to housekeeping genes (β-*actin* and *eef1a1l1*) and shown as fold-change ± Standard Error (S.E).

Figure 3: Lymphocyte populations in pre-leukemic *rag2:hMYC; lck:eGFP* zebrafish. Images at top show a 3-month-*old rag2:hMYC; lck:eGFP* fish with high and computer-enhanced exposures. Scale bar = 20 mm. Panels of parts A-D are depicted in identical format to **Figure 2**. (**D**) Expression of *pax5*, *cd4*, *lck*, and *GFP* in FACS-purified thymic GFP^{lo} (green) GFP^{hi} (blue) cell populations of *lck:eGFP* fish. qRT-PCR results are shown as fold-change ± S.E.

Figure 4: Analysis of B-ALL *rag2:hMYC; lck:eGFP* transgenic fish. (**A**) Top: 6-month-old *rag2:hMYC; lck:eGFP* fish with B-ALL using high exposure setting. Flow cytometric analyses of tumor cells isolated from fish body are identical format to **Figure 2**. (**B**) Gene expression in B-ALL GFP^{lo} cells (green gate in **Figure 4A**), *rag2:hMYC; lck:eGFP* GFP^{hi} thymocytes (blue gate in **Figure 3A**), and T-ALL GFP^{hi} cells (blue gate in **Figure 5A**). Expression of B (*pax5*, *cd79a*) and T (*cd4*) cell-specific genes, *lck*, and *GFP*. Results are normalized to housekeeping genes (β-*actin* and *eef1a1l1*) and shown as fold-change ± S.E. Scale bar=20 mm.

Figure 5: Analysis of mixed ALL *rag2:hMYC; lck:eGFP* transgenic fish. (**A**) Top: 6-month-old *rag2:hMYC; lck:eGFP* fish with mixed-ALL using high exposure setting. Flow cytometric analyses of tumor cells isolated from fish body are identical format to **Figure 2**. (**B**) GFP^{hi} (blue) and GFP^{lo} (green) FACS gates. Expression of B (*pax5*, *cd79a*) and T (*cd4*) cell-specific genes, *lck*, and *GFP*. Results are normalized to housekeeping genes (β-*actin* and *eef1a1l1*) and shown as fold-change ± S.E. Scale bar = 20 mm.

DISCUSSION:

We developed and provide a protocol to isolate B cells from *lck:eGFP* transgenic zebrafish, adding this to other *D. rerio* models with B-lineage labels[3,4]. Somewhat surprisingly, the identification of GFP^{lo} B cells in this line went unnoticed since its description in 2004. Generally, *lck* is considered to be T cell-specific[6], but recent studies found unexpected *lck* expression by natural killer and myeloid cells, as well as in B cells as shown here[18,19]. In agreement with our discovery that zebrafish B cells are GFP^{lo}, pre-B, naïve, and mature B cells in humans also express low levels of *LCK*[11,13].

Due to the differing GFP levels in B and T cells of these fish, cells of both lineages can now be isolated from this line. Although these animals have classically been used to study thymic T lymphocyte development and tracking, we demonstrate that similar studies are also possible with B cells. To this end, we identify B cells in thymus, kidney marrow, and spleen here, and in peripheral blood and elsewhere in prior work[11].

Recognizing B-ALL in *rag2:hMYC*; *lck:eFFP* fish requires a keen eye, but with practice, is straightforward. Next, choosing which method to use to purify ALL cells is critical. Here, we present two methods: peritoneal wash and whole body homogenization. Peritoneal washing yields a much lower number of total cells, but a very high percentage of GFP^+ cells. Consequently, little FACS time is required, minimizing the cost. For downstream applications, where total yield is less important (e.g., qRT-PCR), this is more efficient. Alternatively, whole body homogenization results in larger single cell suspensions containing many more cells, but a lower percentage of cells will be GFP^+. Thus, more FACS time is required to collect the same number of GFP^+ cells as with peritoneal washing, but millions more cells can be purified. For downstream studies, where high yield is important (e.g., Western blot), this may offset the added cost. In addition, for ALL studies, total body homogenization captures the diversity of cancer cells present, more accurately representing tumor heterogeneity. Although not listed in our protocol, it is also feasible to dissect only GFP^+ body regions/organs from fish with ALL for homogenization by mincing in a Petri dish, thus decreasing total FACS time and cost, akin to peritoneal washing.

Thymic GFP expression in *lck:eGFP* fish showed that GFP is detectable as early as 5 dpf[6]. Our studies here were performed in adult fish 3 months of age or older, and show that at this age, B cells are abundant in kidney marrow and spleen, but T cells are rare. Subsequent studies with fish of different ages will be required to determine if T cells are more prevalent at different time points. Likewise, at 3 months, T cells are enriched in the thymus, but a considerable number of thymic B cells are also present. If these lymphocyte populations vary at different ages is currently unknown, but overall, thymic fluorescence fades in *lck:eGFP* fish beginning at sexual maturity (~3 months), so it is likely that T cell numbers diminish with increasing age, and B cells may also. Likewise, when GFP^{lo} B cells colonize the zebrafish thymus is currently unknown. Using *lck:eGFP* fish, it is now possible to monitor thymic B and T cell development, influx, efflux, and the specific kinetics of these events. In addition, such questions are also pertinent to other anatomic sites where B cells reside, such as kidney marrow, spleen, blood, and intestine (data not shown).

Besides B cell developmental studies, we recently found B-ALL in *lck:eGFP*; *rag2:hMYC* double-transgenic fish[11]. Transgenic *rag2:hMYC* was known to induce T-ALL[10] but B-ALL had gone unrecognized. Due to the high penetrance of this oncogene, both types of ALL are prevalent in these fish, and many fish actually develop both ALL types simultaneously[11]. Since B-ALL are GFP^{lo}, while T-ALL are GFP^{hi}, varying GFP expression allows these two entities to be isolated by FACS, even when occurring in the same animal (**Figure 5A**). Crucially, in both normal and malignant lymphocytes, qRT-PCR results demonstrate that GFP^{lo} and GFP^{hi} cells consistently correspond to the B- and T-lineages, respectively (**Figure 2** and **Figure 3**).

The untapped potential of *lck:eGFP* fish was central to this work, highlighting the fact that many pre-existing transgenic lines likely have utility beyond their intended purpose. Here, we present a novel protocol to isolate B and T cells from *D. rerio* with transgenic *lck:eGFP*, opening the door to new investigational avenues concerning normal and malignant B lymphocytes. The results of such studies will undoubtedly re-vitalize what has already been a valuable resource to the hematopoiesis, lymphopoiesis, and cancer biology fields.

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DISCLOSURES:

The authors declare no conflict of interests.

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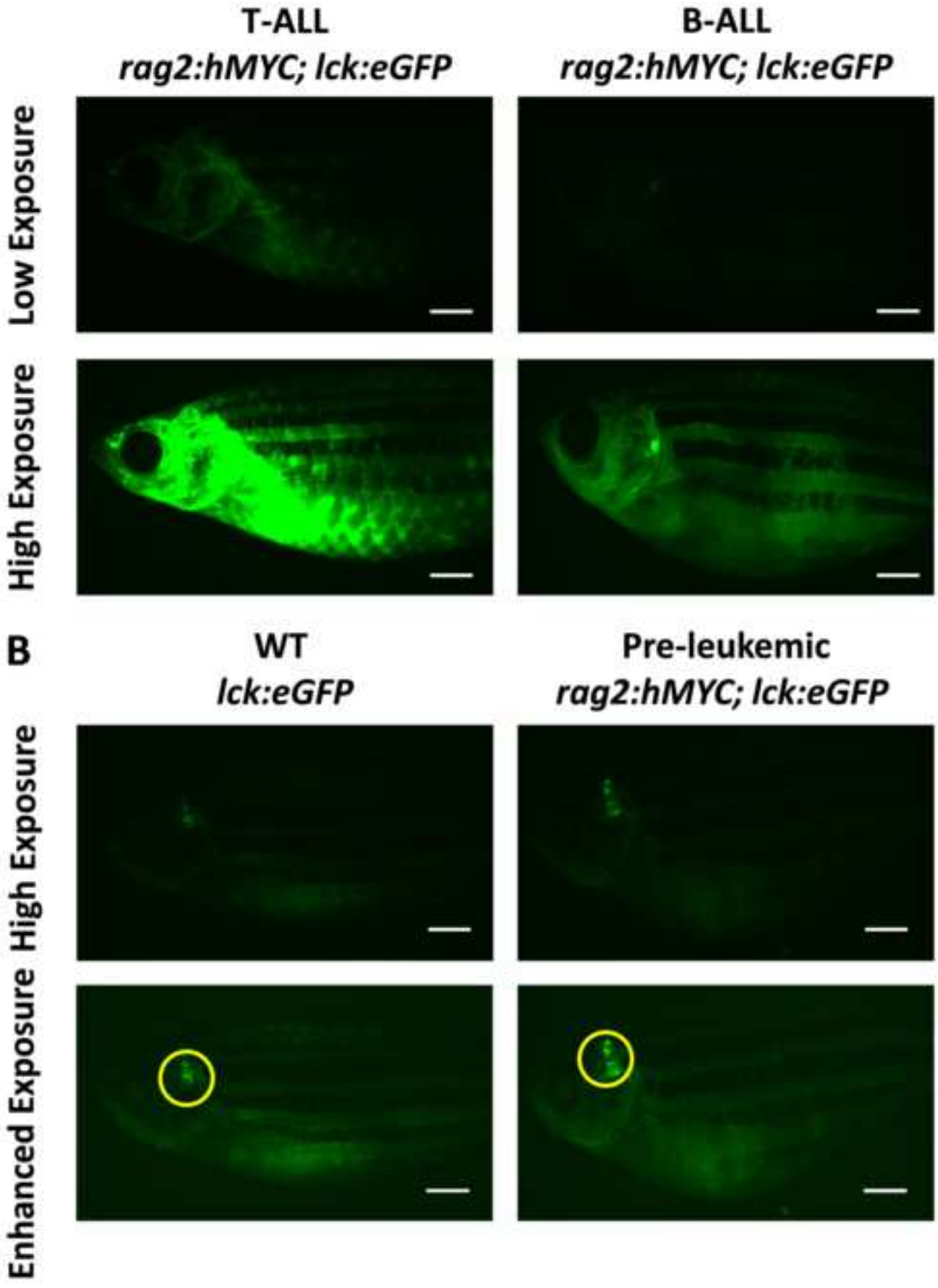
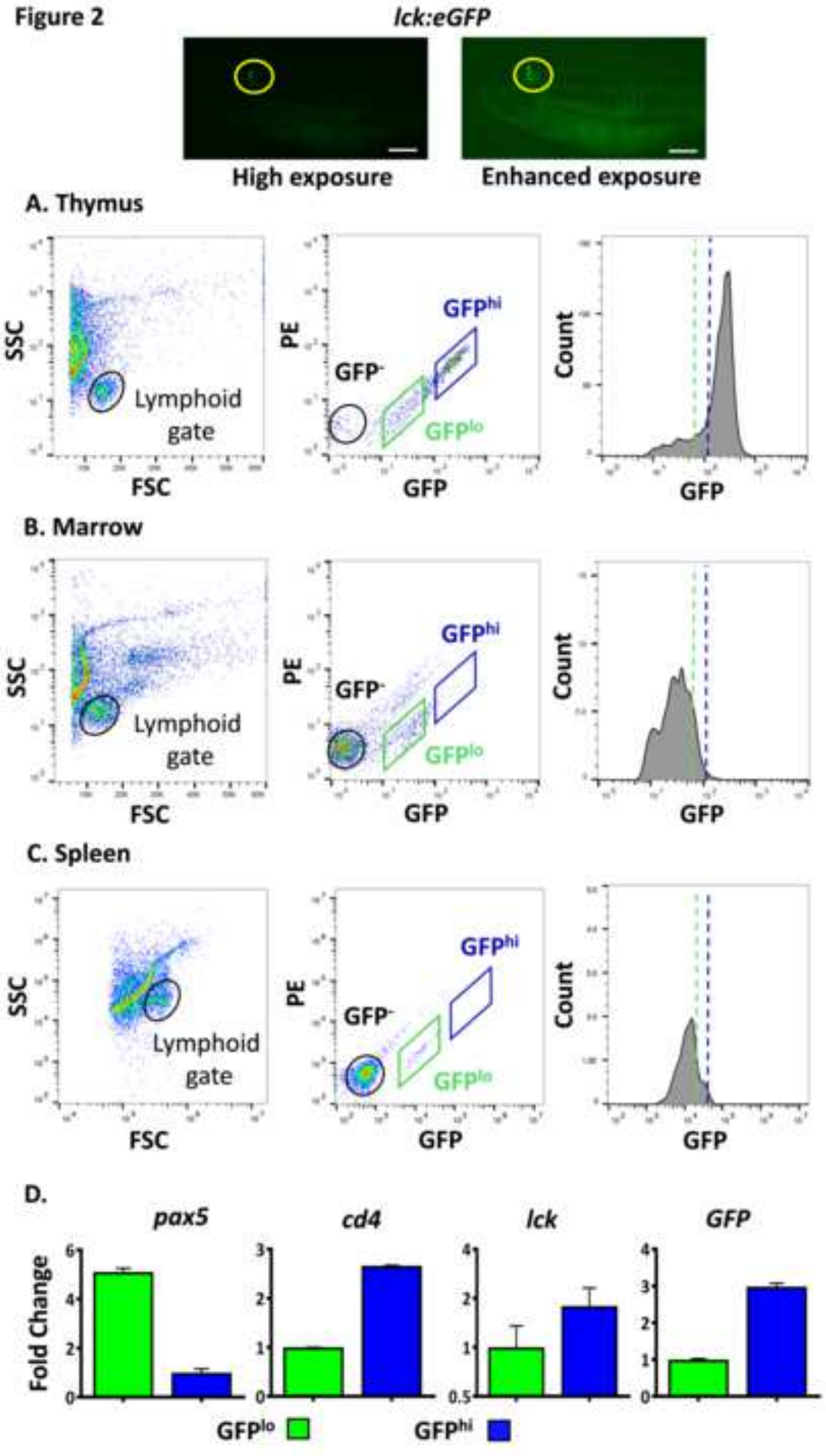
Figure 1





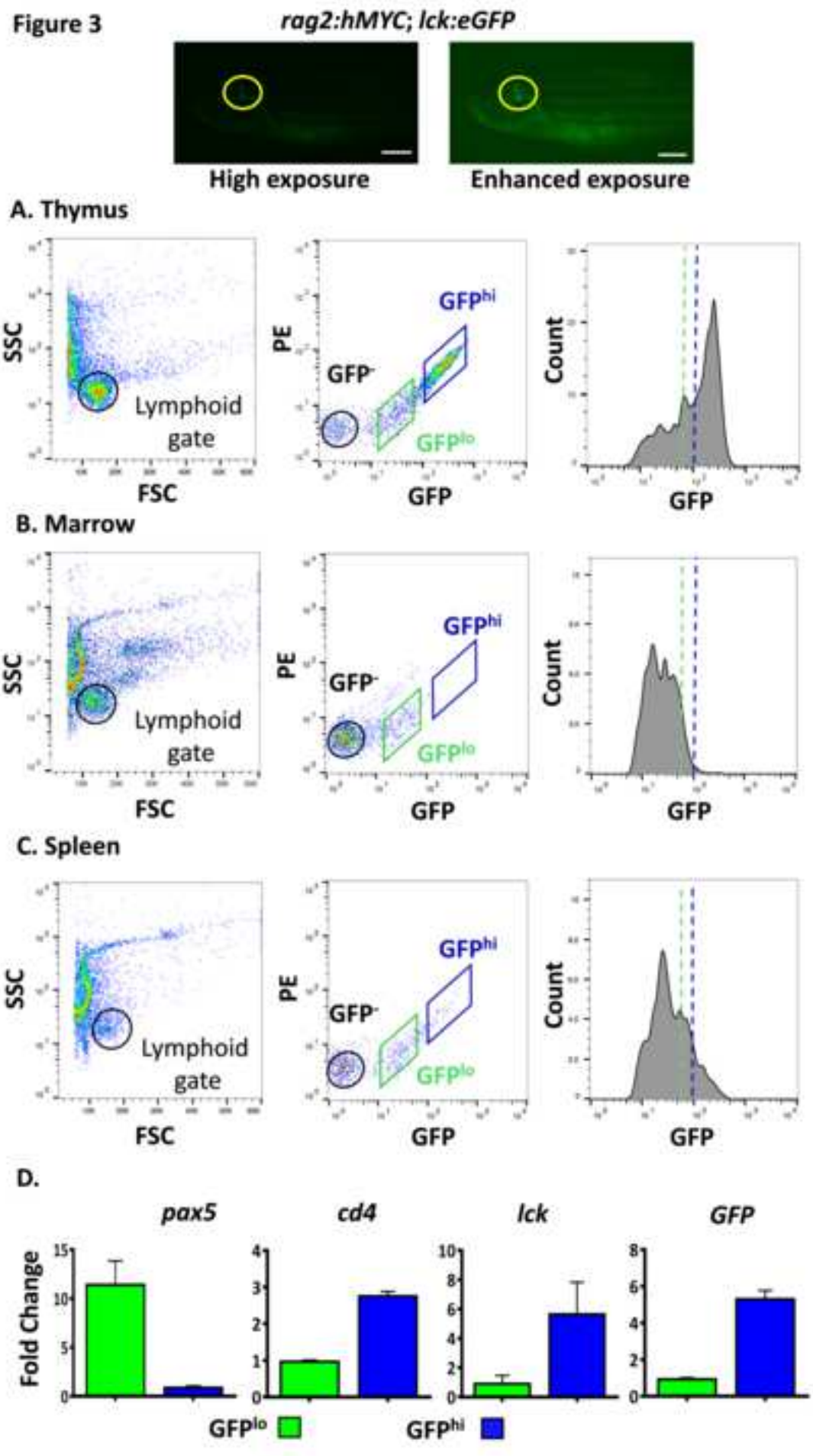


Figure 4

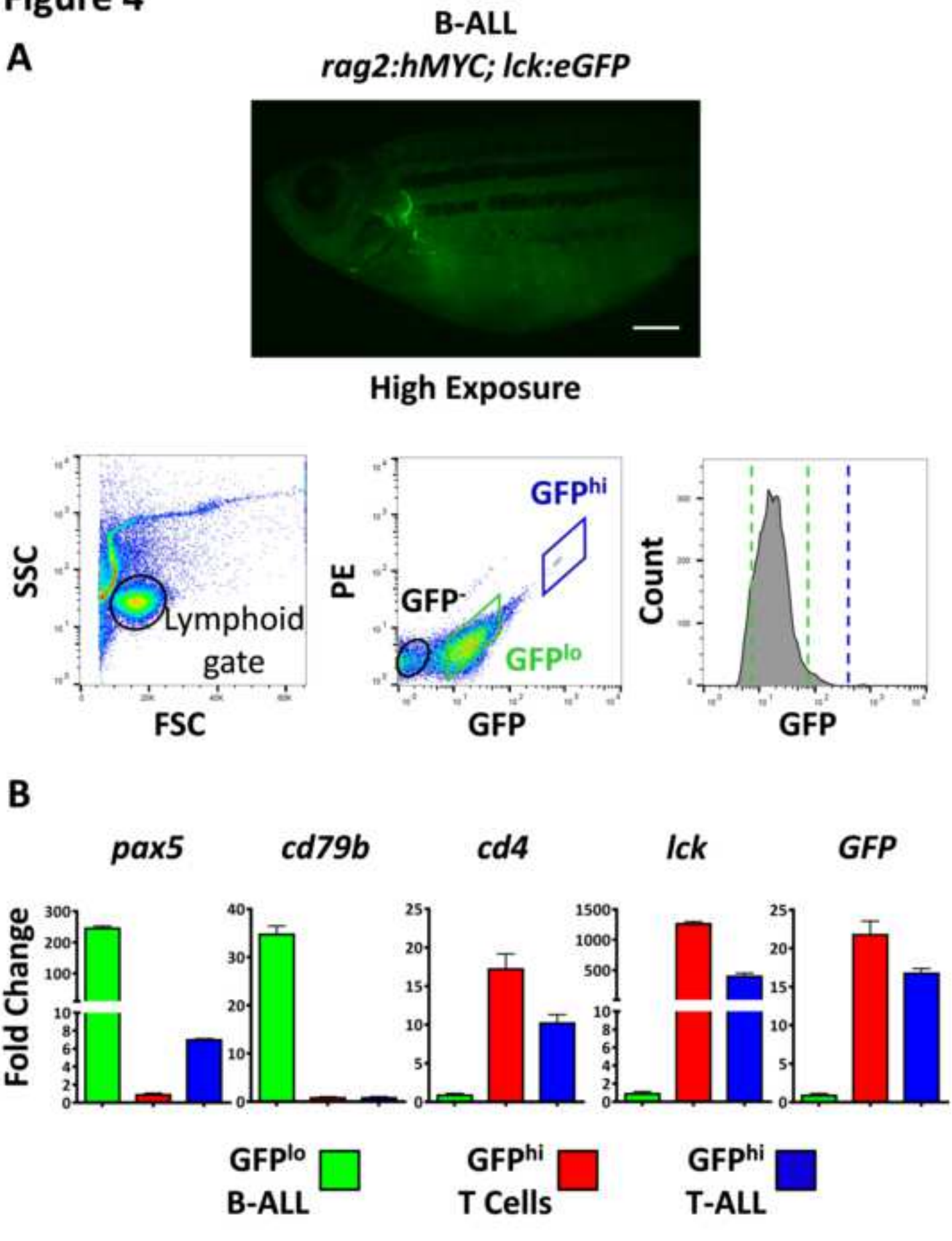
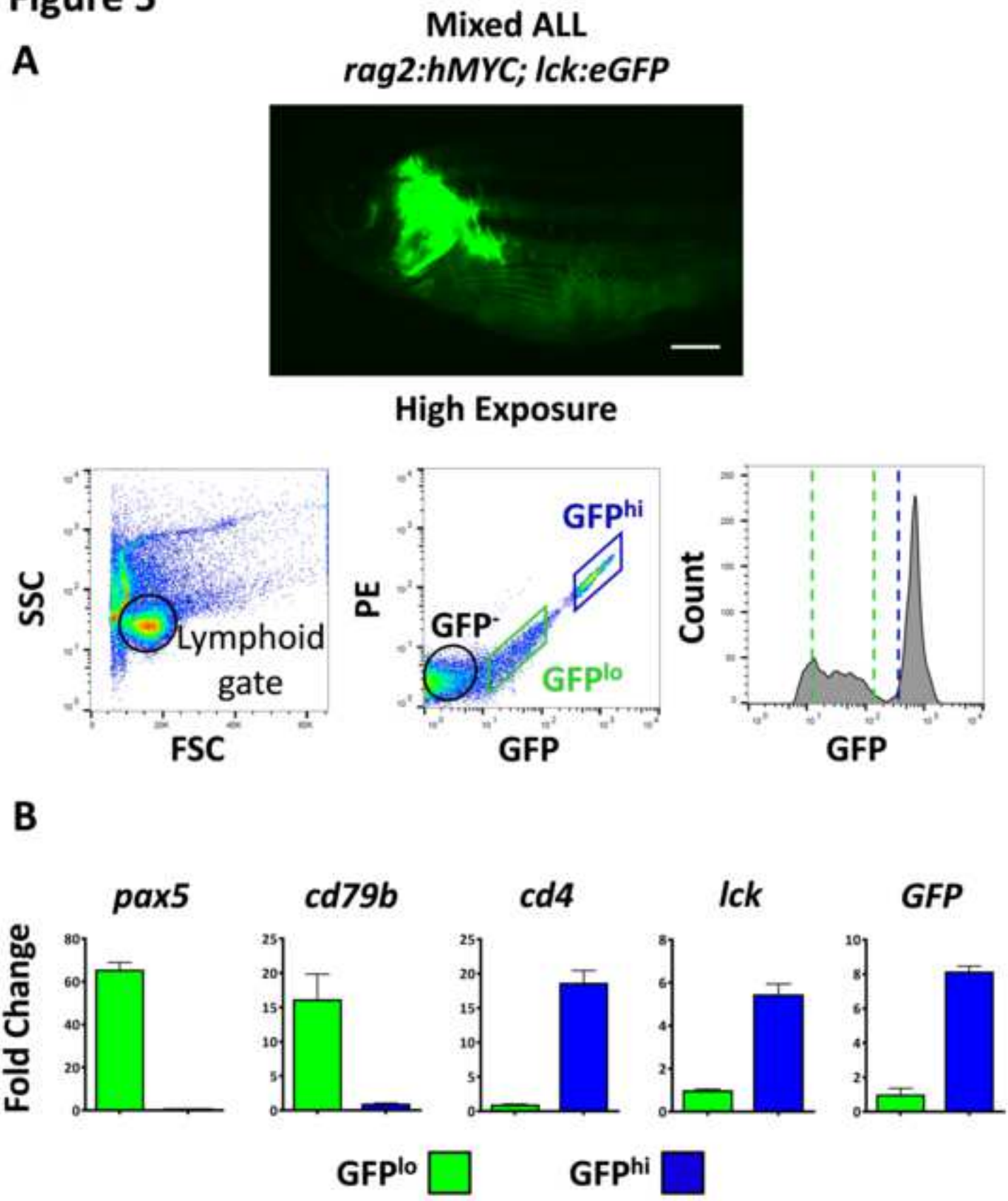


Figure 5



lck:GFP
rag2:hMYC; lck:eGFP **pre-leukemic lymphocytes**
rag2:hMYC; lck:eGFP **Mixed ALL**
rag2:hMYC; lck:eGFP **B-ALL**

Thymus		Kidney		Spleen		Bod
GFP^{hi}	GFP^{lo}	GFP^{hi}	GFP^{lo}	GFP^{hi}	GFP^{lo}	GFP^{hi}
60%	40%	2%	98%	6%	94%	-
81%	19%	1%	99%	12%	88%	-
-	-	-	-	-	-	43%
-	-	-	-	-	-	0.3%

y
GFP^{lo}
-
-
57%
99.7%

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials-tables.xls

Name of Material/ Equipment
35 µm mesh
5 ml Polystyrene round-Bottom tube with cell-strainer cap
50 ml conical tube
AZ APO 100 Fluorescent microscope
Cytoflex
DS-Qi1MC camara
Ethyl 3-aminobenzoate methansesulfonate; MS-222
FACSJazz
Fetal bovine Serum
FlowJo v10.2
lck:eGFP
NIS Elements software
Penicilin -Streptomycin
Pestle micro-tube homogenizers
Plastic Transfer pippetes

rag2:hMYC -ER
RPMI Media 1640 1X

Company	Catalog Number
Sefar Filter technology	7050-1220-000-13
Falcon Corning Brand	352235
VWR international	525-0448
Nikon	
Beckman Coulter	
Nikon	
Sigma	E-10521
BD Biosciences	
Thermo Fisher	10437028
FlowJo, LLC	
Nikon	Version 4.13
Sigma	P4333
Electron Microscopy Sciences	64788-20

Life Technologies	11835-030

Comments/Description
See Langeneu et al., 2004

See Gutierrez et al., 2011

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Author(s): Jessica Burroughs-Garcia, Ameera Hasan, Gilseung Park, Chiara Borga, and J. Kimble Frazer

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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. GFP^{lo} vs. GFP^{hi} cells is not difficult, as these have $\geq$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 (*β-actin*, *eef1a1l1*). Each comparison is

between GFP^{lo} vs. GFP^{hi} 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.

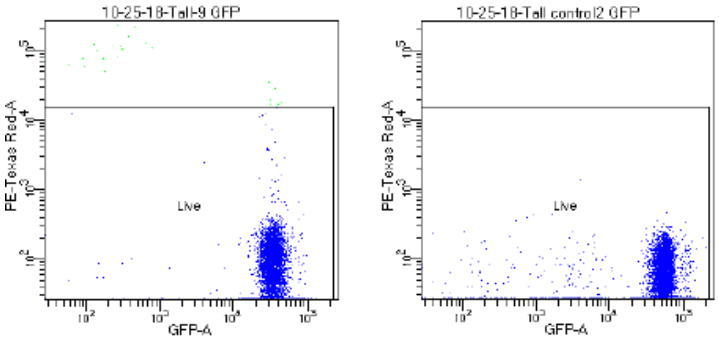
Reference 11 is now cited here. Thank you for the suggestion, it was confusing as originally written.

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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