

Video Article

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

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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 mammals¹.

Over the past 2 decades, transgenic *D. rerio* lines that label specific blood lineages and mutant lines deficient in these lineages have been created^{2,3,4,5}. One of these, the *lck:eGFP* transgenic line, uses the zebrafish lymphocyte protein tyrosine kinase (*lck*) promoter to drive GFP expression⁶. 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 FACS⁷. 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-ALL¹⁰, we discovered that B-lineage ALL also occur¹¹. 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 FACS¹¹. 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*, *blink*, *ltk*, *ighm*, *ighz*, and others, and can be purified from marrow, thymus, spleen, or peripheral blood¹¹. Therefore, both B- and T-lineage cells can be isolated from *lck:eGFP* zebrafish, and in the case of *rag2hMYC; lck:eGFP* animals, B- and T-ALL cells as well¹¹.

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 *rag2hMYC;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. Anesthetize the fish using 0.02% tricaine (MS-222) in fish system water.
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 teleosts¹⁴. Use an epifluorescence microscope (470/40 excitation wavelength and 525/50 emission filter) to detect GFP.
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.
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.
5. Place the euthanized fish in a Petri dish and dissect lymphoid organs of interest, using the fluorescent microscope to dissect the GFP⁺ thymi¹⁴, and bright field microscopy settings to dissect the kidney marrow and spleen¹⁵. 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).
6. Place each dissected organ in a 1.5 mL tube containing 500 μ L of cold sorting media.
7. Homogenize the tissue on ice using a pestle micro-tube homogenizer.
8. Pass homogenized tissue through a 35 μ m 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

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.
 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. Anesthetize and examine the fish as described in steps 1.1-1.2.
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.
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.
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.
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 technique¹⁶. 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).
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.
8. Peritoneal Wash Method
 1. Using a P1000 pipette, wash the fish peritoneal cavity with 500 μ L of cold sorting media, collecting the cells and media in a 5 mL tube.
 2. Using a fresh pipette tip, inject an additional 200-300 μ L 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.
 3. Repeat step 2.8.2 2-3 times. Keep the collected cells in sorting media on ice.
 4. Filter the cell suspension through a 35 μ m mesh filter prior to flow cytometry/FACS and keep the cells on ice until analysis.
9. Whole Body Homogenization
 1. After removing the fish head, place the body in a 1.5 mL tube containing 200 μ L of sorting media.
 2. Homogenize the body using a pestle micro-tube homogenizer.
 3. Add an additional 300 μ L of cold sorting media. Filter the cell suspension through a 35 μ m 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.

1. Set flow cytometric analysis and/or FACS parameters according to manufacturer's guide.
2. Acquire desired number of events to initially characterize the sample. Analyze 5×10^3 to 5×10^4 events prior to sorting to determining specific gates for subsequent sorting steps.

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 iodide (PI) or 7-aminoactinomycin D (7-AAD) viability staining. Previous experiments with PI typically demonstrate >95% viability of GFP⁺ cells after FACS.
2. Exclude the cell doublets, according to the parameters of the FACS machine being used.
3. Within the lymphoid and/or precursor gates, determine the number and percentage of GFP⁺ cells.
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**).
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.).
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-ALL¹¹. 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.

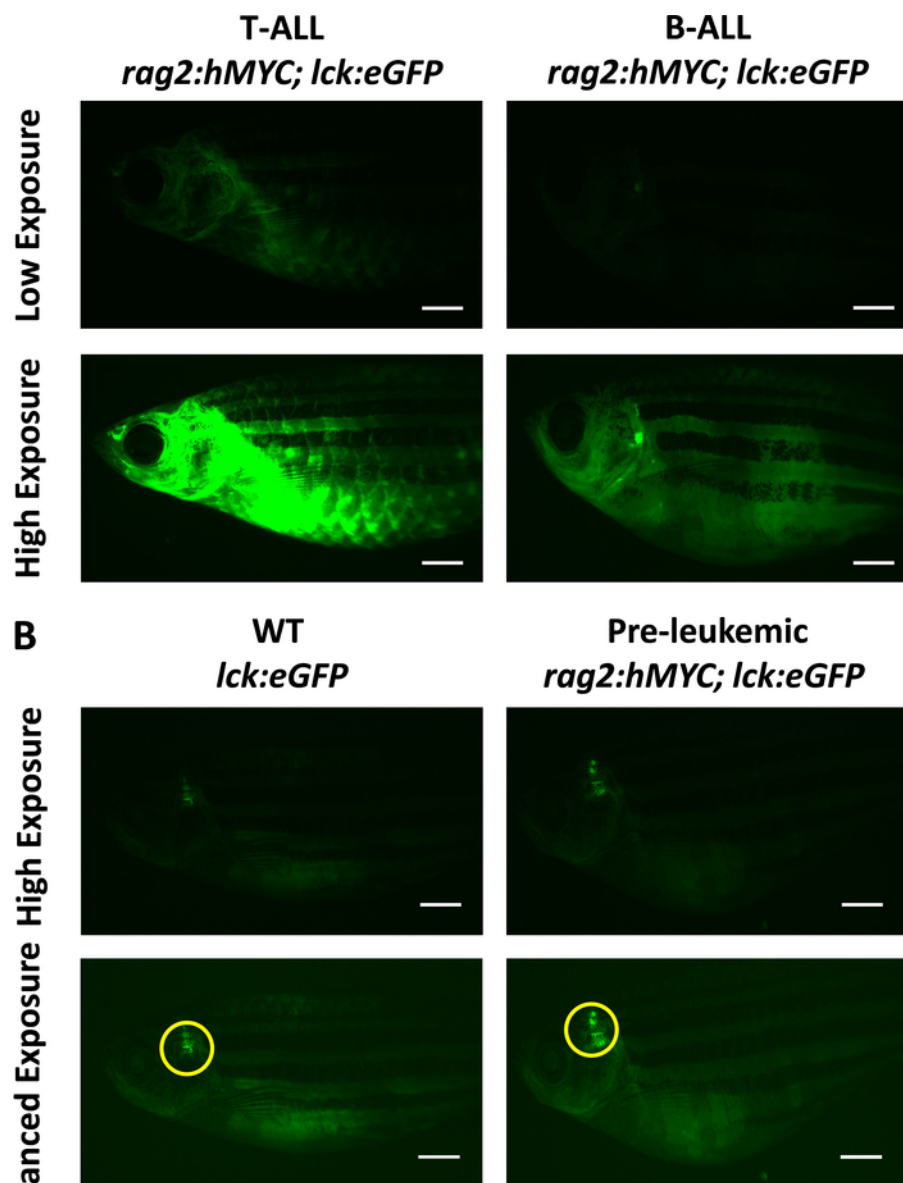


Figure 1: Distinct fluorescence patterns in *lck:eGFP* transgenic fish. (A) Fluorescent microscopy images of *rag2:hMYC; lck:eGFP* fish with brightly-fluorescent T-ALL (left) or dimly-fluorescent B-ALL (right). T-ALL is visible with low exposure settings; B-ALL can only be seen using high exposures. (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. [Please click here to view a larger version of this figure.](#)

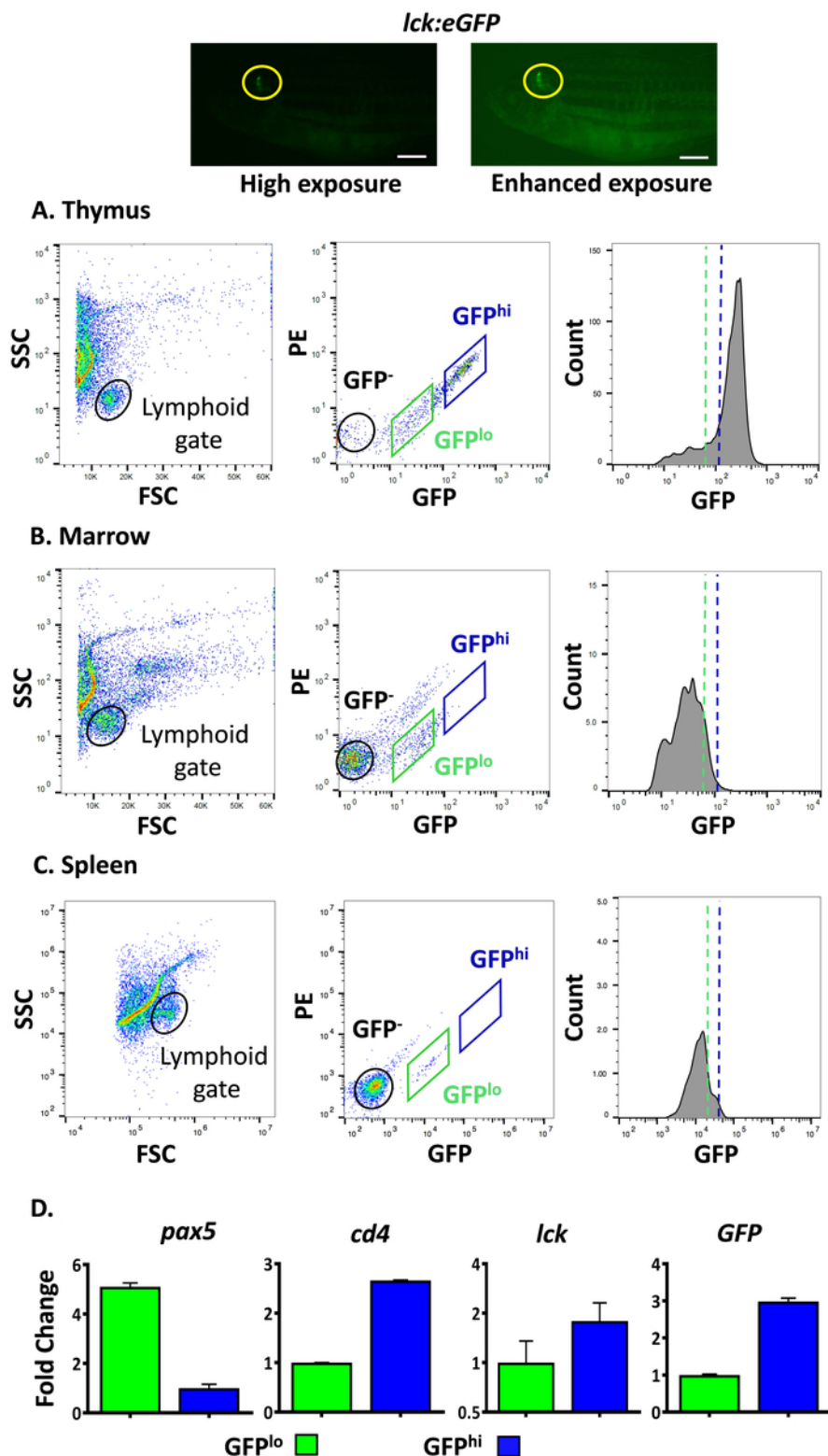


Figure 2: Lymphocyte populations in *lck:eGFP* zebrafish. Images at top show a 3-month-old WT, *lck:eGFP* fish with high exposure settings or computer-enhancement (to facilitate visualization). Scale bars = 20 mm. Flow cytometric analyses of thymus **A**, marrow **B**, and spleen **C**. Left panels show 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* thymus 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 *eef1a11*) and shown as fold-change $\pm$ Standard Error (S.E.). [Please click here to view a larger version of this figure.](#)

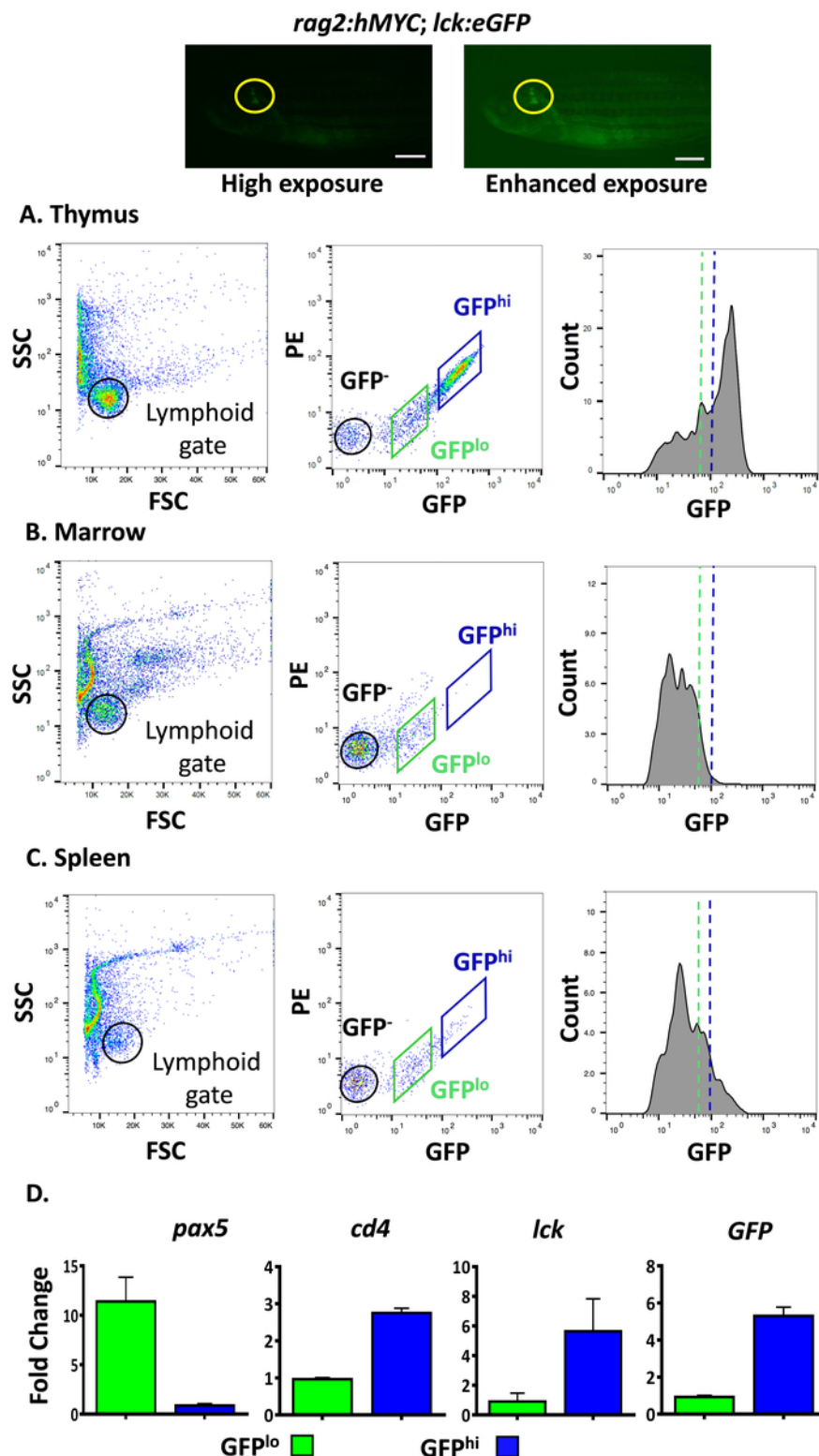


Figure 3: Lymphocyte populations in pre-leukemic *rag2:hMYC; lck:eGFP* zebrafish. Images at top show a 3-month-old *rag2:hMYC; lck:eGFP* fish with high and computer-enhanced exposures. Scale bar = 20 mm. Panels of parts A-D are depicted in identical format to **Figure 2**. (D) Expression of *pax5*, *cd4*, *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 $\pm$ S.E. [Please click here to view a larger version of this figure.](#)

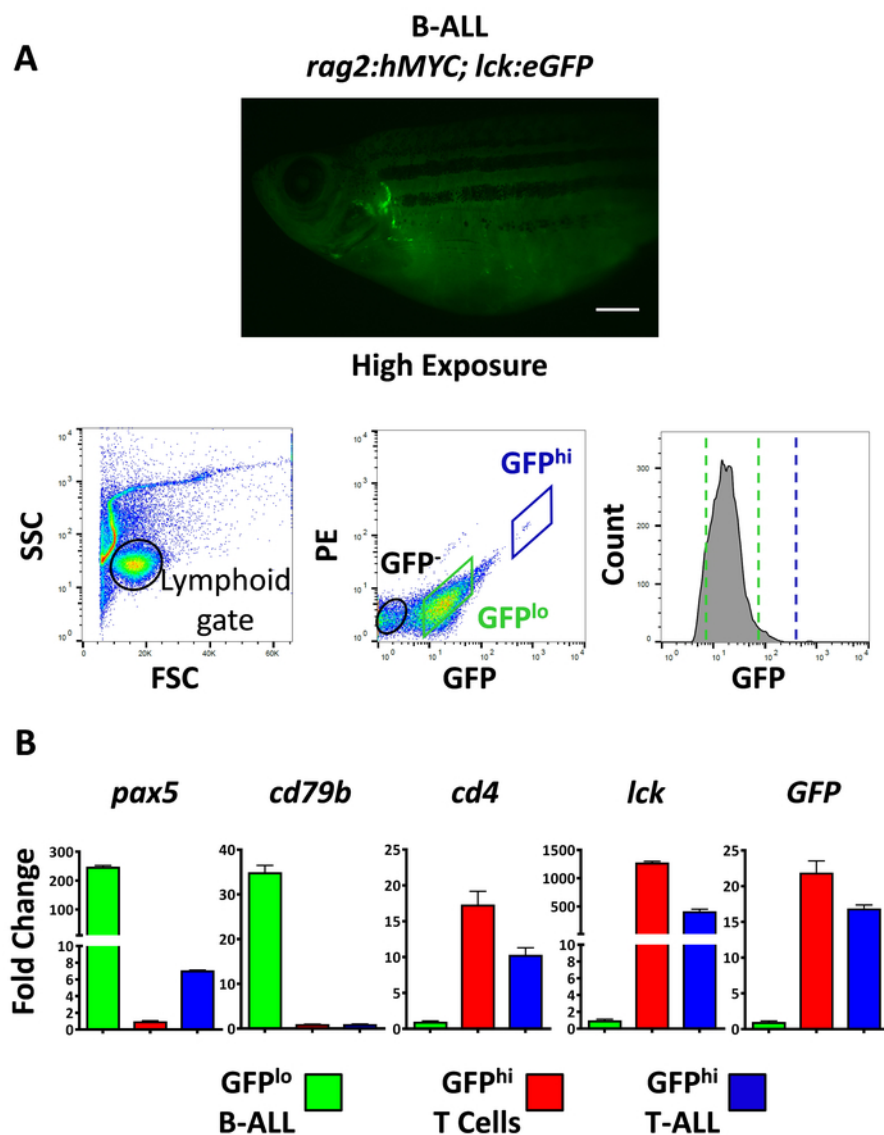


Figure 4: Analysis of B-ALL *rag2:hMYC; lck:eGFP* transgenic fish. (A) Top: 6-month-old *rag2:hMYC; lck:eGFP* fish with B-ALL using high exposure setting. Flow cytometric analyses of tumor cells isolated from fish body are identical format to **Figure 2**. (B) Gene expression in B-ALL GFP^{lo} cells (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 *eef1a1/1*) and shown as fold-change ± S.E. Scale bar=20 mm. [Please click here to view a larger version of this figure.](#)

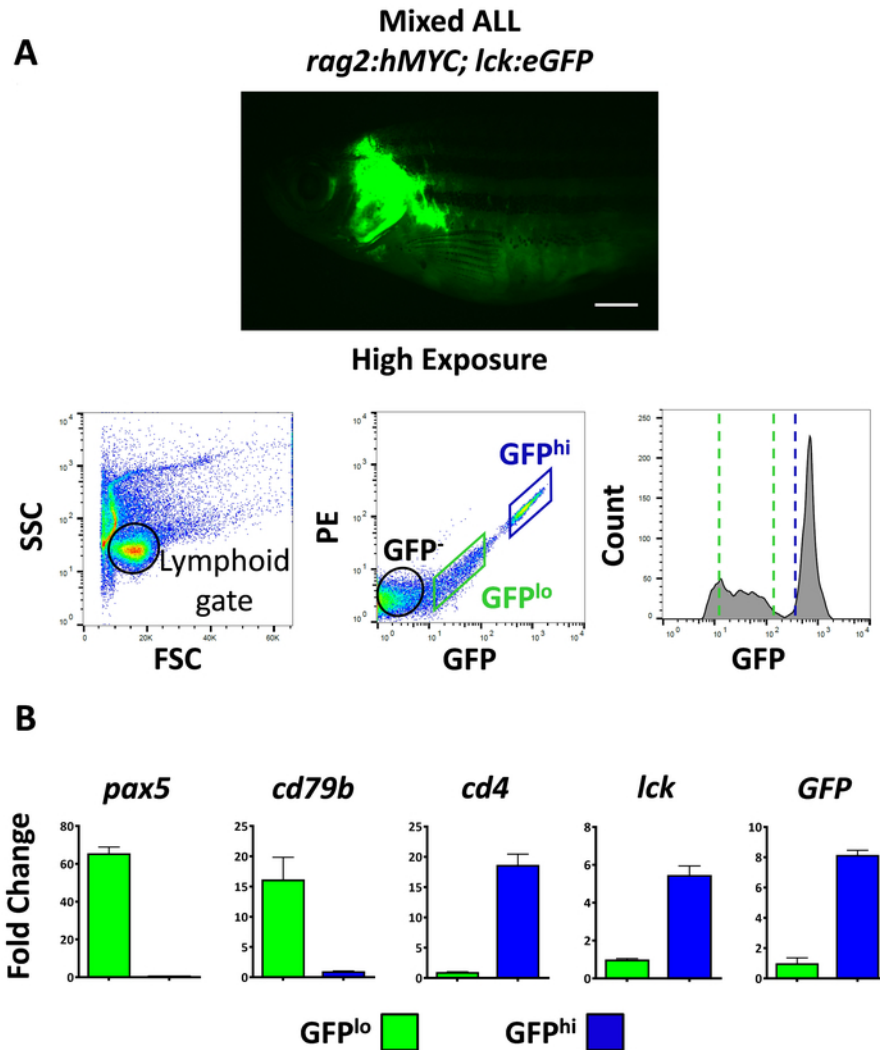


Figure 5: Analysis of mixed ALL *rag2:hMYC; lck:eGFP* transgenic fish. (A) Top: 6-month-old *rag2:hMYC; lck:eGFP* fish with mixed-ALL using high exposure setting. Flow cytometric analyses of tumor cells isolated from fish body are identical format to **Figure 2**. (B) GFP^{hi} (blue) and GFP^{lo} (green) 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 *eef1a11*) and shown as fold-change $\pm$ S.E. Scale bar = 20 mm. [Please click here to view a larger version of this figure.](#)

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-specific⁶, 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 work¹¹.

Recognizing B-ALL in *rag2:hMYC; lck:eGFP* 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 dpf⁶. 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 fish¹¹. Transgenic *rag2:hMYC* was known to induce T-ALL¹⁰ 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 simultaneously¹¹. 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.

Disclosures

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