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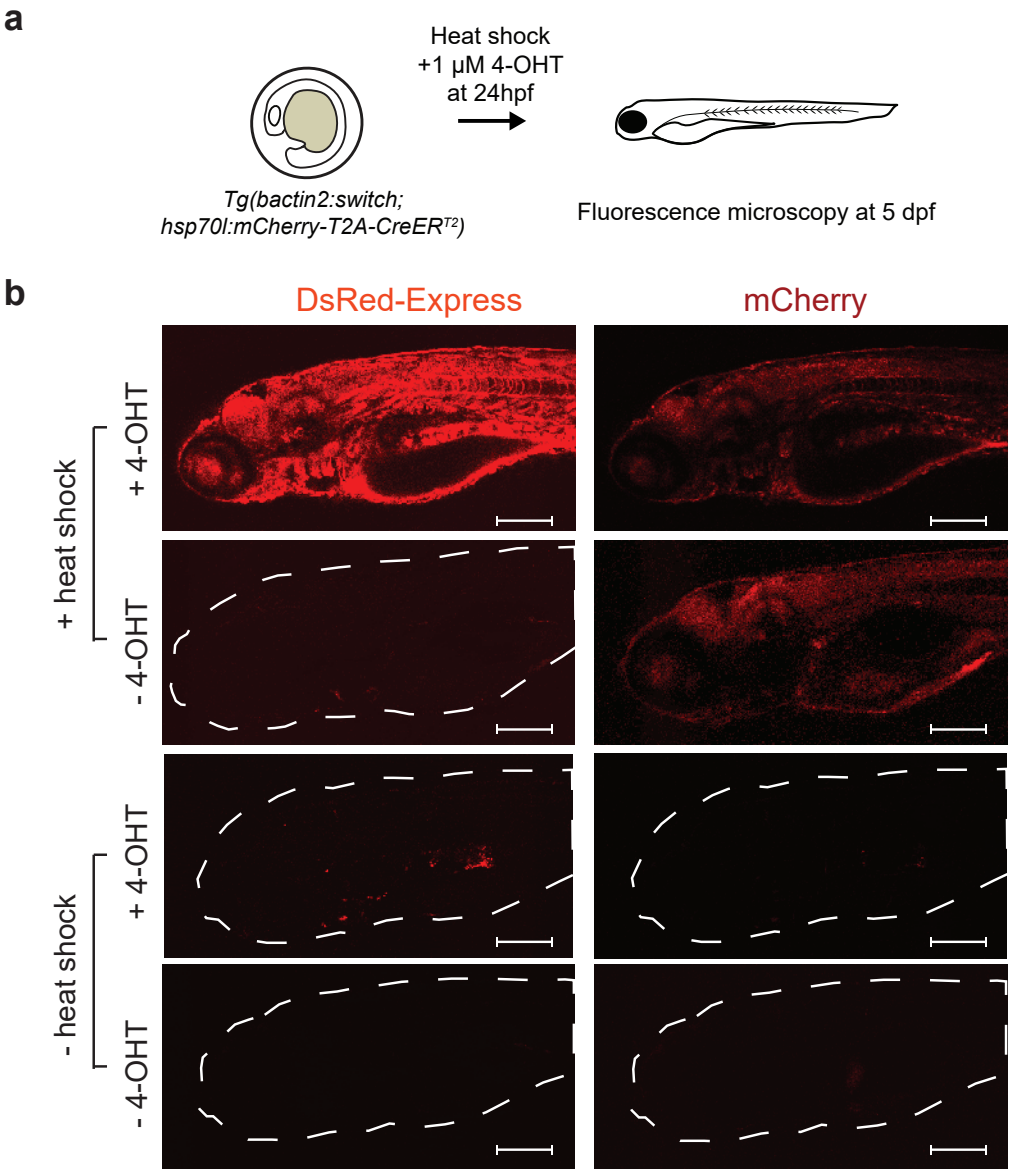
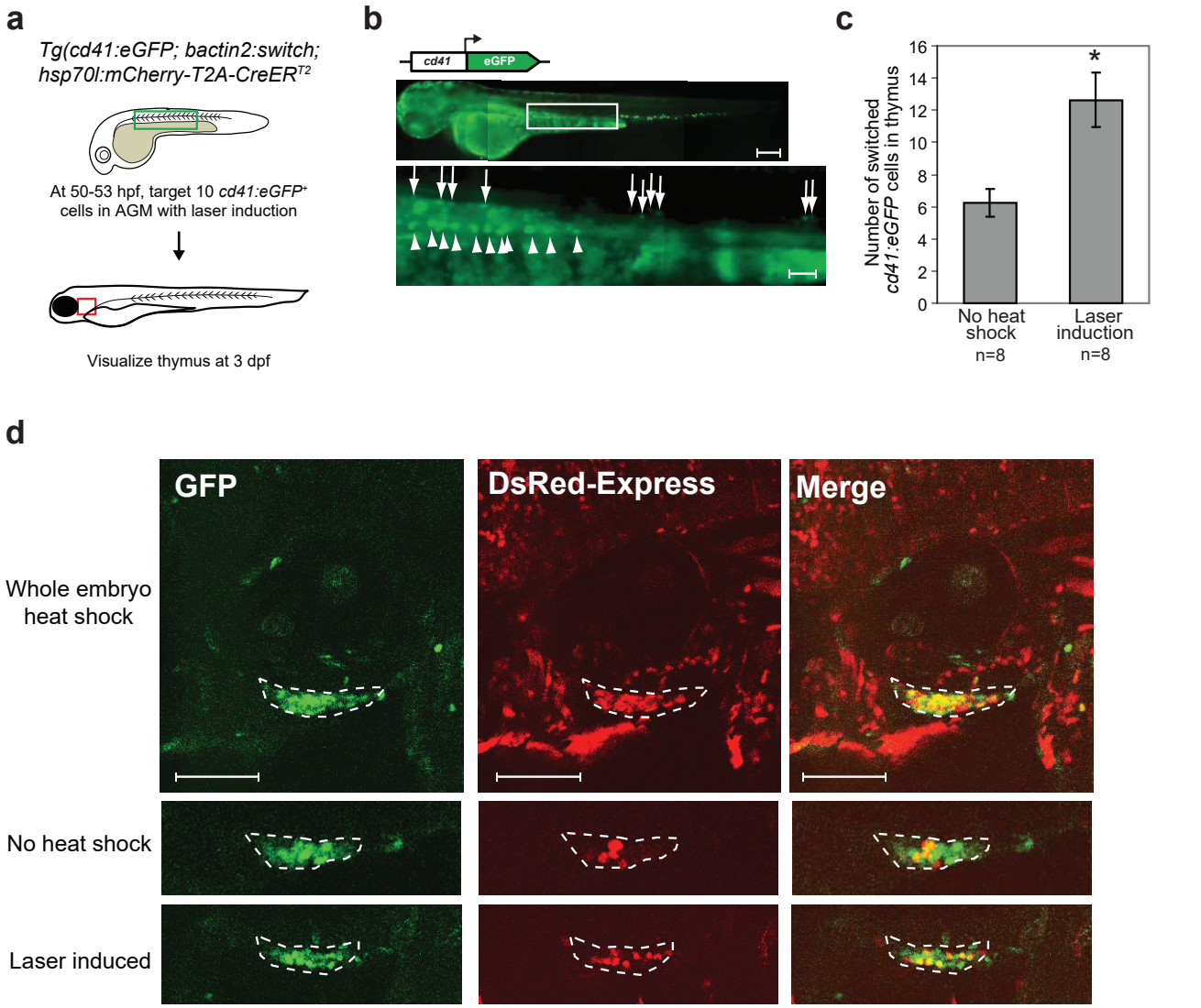
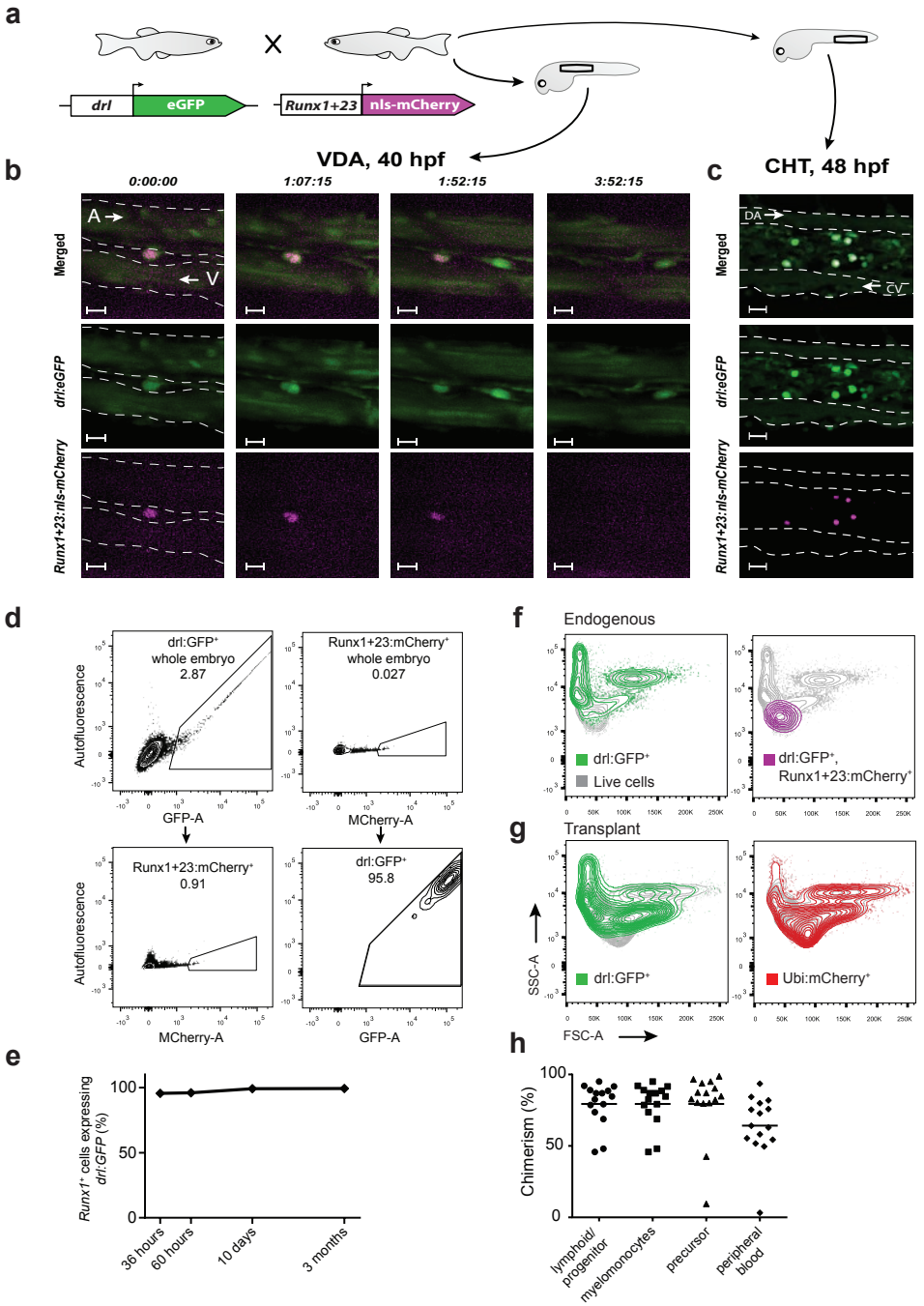


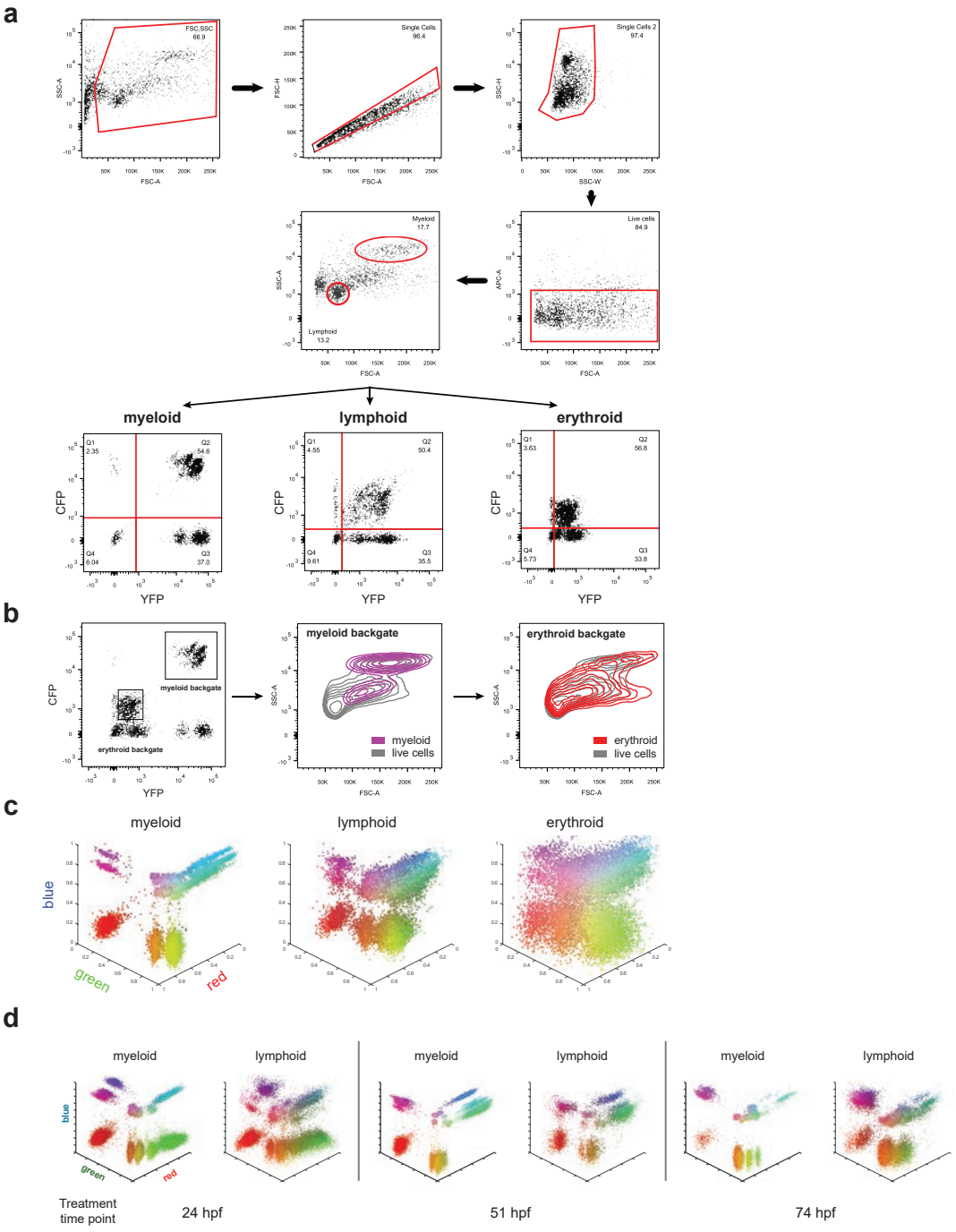
Supplementary Figure 1 Heat-shock and 4-OHT requirement for DsRed-Express labeling. (a) Strategy to validate dual-inducible fate mapping system. (b) DsRed-Express and mCherry expression in double transgenic embryos Tg(bactin2:switch; hsp70l:mCherry-T2A-CreERT2) in the presence or absence of heat shock at 24 hours post-fertilization (hpf), and in the presence or absence of 4-hydroxytamoxifen (4-OHT). Top, representative brightfield image of an embryo. Box, region of embryo in fluorescence images. Left column, DsRed-Express signals. Right column, mCherry signals. Dash, outline of embryo. Embryos were imaged at 5 days post-fertilization (dpf). Scale bar is 200 μm.



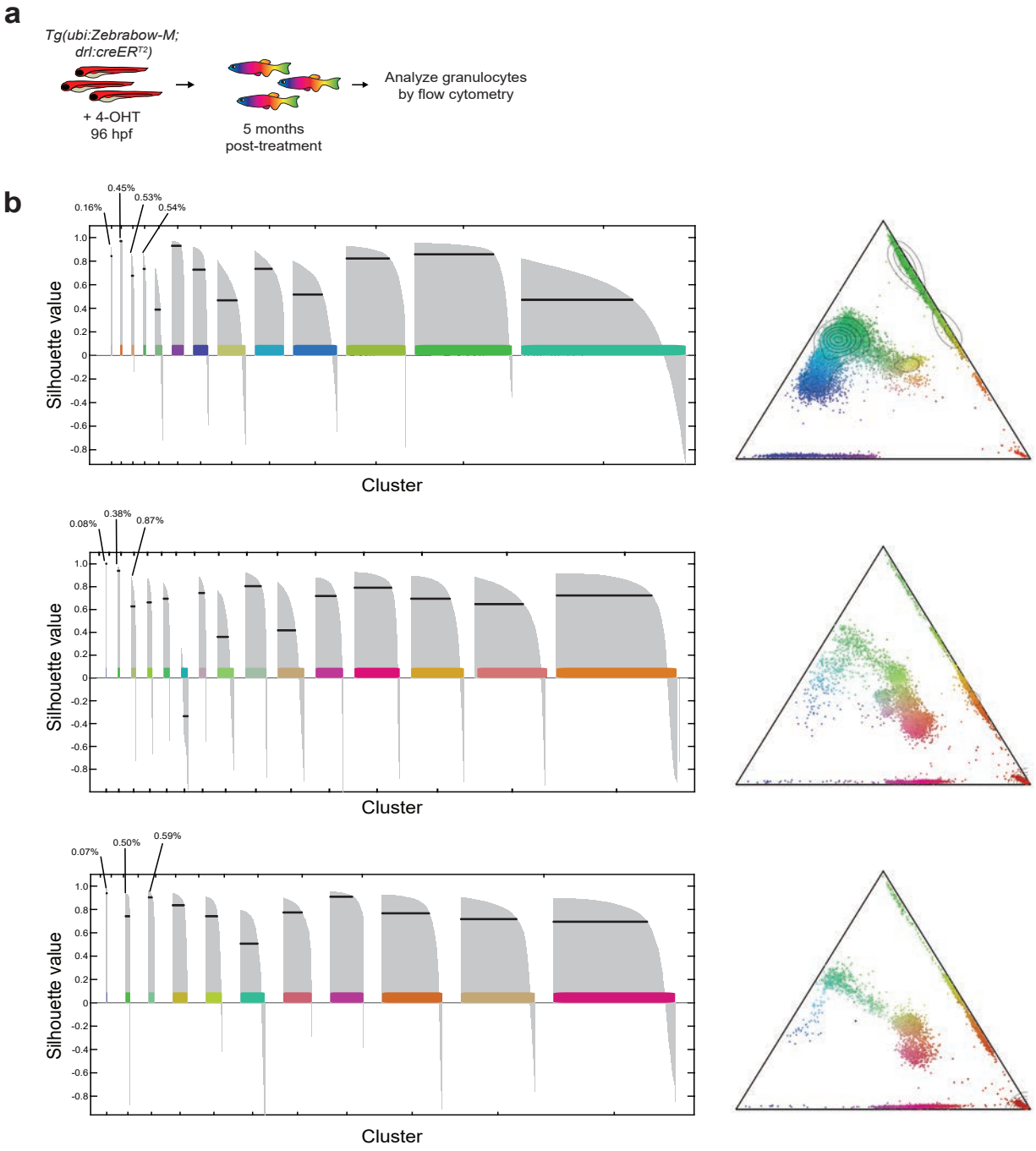
Supplementary Figure 2 Migration of labeled cd41:eGFP+ cells from VDA to thymus. (a) Scheme of laser induction targeting 10 cd41:eGFP+ cells in VDA of triple transgenic Tg(cd41:eGFP; bactin2:switch; hsp70l:mCherry-T2A-CreERT2). (b) cd41:eGFP+ target cells in VDA. Top, transgene. Middle, transgenic embryo at 50 hpf (scale bar = 200 μm).; box, enlarged region (Scale bar is 50 μm). Bottom, close up of VDA. Arrows, cd41:eGFP+ cells in the VDA. Arrowheads, non-haematopoietic cd41:eGFP+ cells in pronephric duct. (c) Quantification of cd41:eGFP+DsRed-Express+ cells in one thymic lobe, with or without laser induction. All z-planes were analyzed. Error bars, s.e.m. *P=0.036, two-tailed t-test (n = 8 for no heat shock, n = 8 for laser induction). (d) Representative confocal images (one z-plane shown) of thymi of 3-dpf embryos. Top row, whole embryo heat shock. Middle row, no heat shock. Bottom row, laser induction. Dash, outline of thymus. Left column, cd41:eGFP. Middle column, DsRed-Express. Right column, merged images (scale bar = 50 μm).



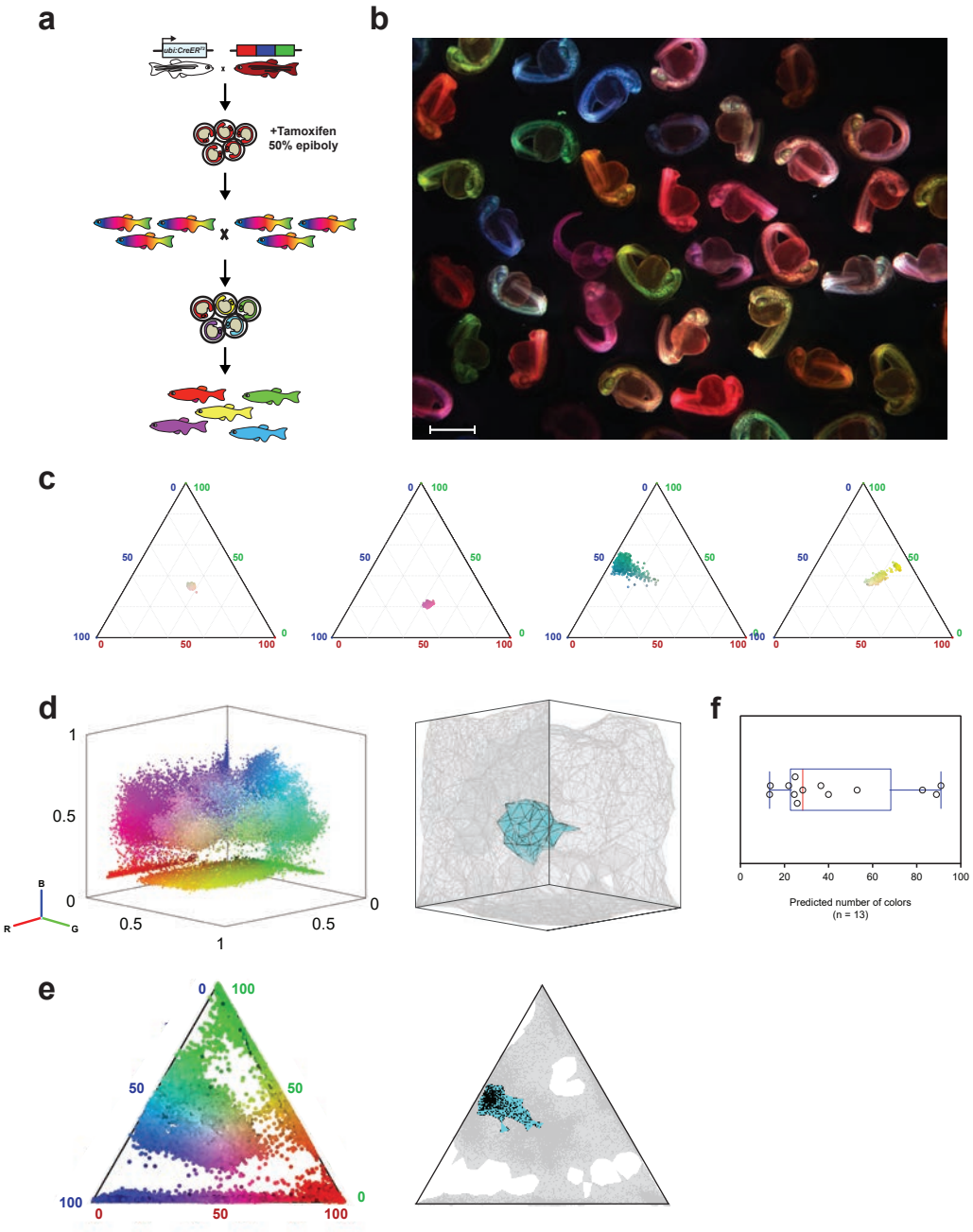
Supplementary Figure 3 Characterization of draculin reporter activity in HSPCs. (a) Strategy to measure co-expression of drl:eGFP and Runx1+23:nls-mCherry, a stem cell reporter. (b) Time-lapse imaging of the VDA, showing a single drl:eGFP+;Runx1+23:nls-mCherry+ cell budding from the ventral wall of the dorsal aorta at 40 hpf and entering circulation four hours later (A = dorsal aorta, V = cardinal vein, arrows denote blood flow, scale bar = 10 μm). (c) Confocal image showing the co-expression of drl:eGFP+ and Runx1+23:nls-mCherry in five stem cells that have engrafted in the CHT at 48 hpf (DA = dorsal aorta, arrows = blood flow, scale bar = 25 μm). (d) Representative flow cytometry plots showing expression and co-expression of drl:eGFP and Runx1+23:nls-mCherry in pooled whole embryos at 36 hpf. (e) Quantification of Runx1+23:nls-mCherry cells that are positive for drl:eGFP expression by flow cytometry at various developmental stages in pooled embryos. (f) Representative flow cytometry plot of FSC/SSC characteristics and back-gating of drl:eGFP+ and drl:eGFP+Runx1+23:mCherry+ cells from whole kidney marrow. (g) Transplant of 6x104 drl:eGFP+ubi:mCherry+ cells from the lymphoid/progenitor and precursor gates into lethally irradiated recipients. Recipient marrow was analyzed at 3 months post-fertilization. Here we show a representative FACS plot, demonstrating recapitulation of drl:eGFP expression and multilineage reconstitution demonstrated by the presence of ubi:mCherry in all populations. (h) Quantification of chimerism levels in recipient zebrafish. Each marker corresponds to an individual animal. (green cells = drl:eGFP+, purple cells = drl:eGFP+Runx1+23:mCherry+, red cells = ubi:mCherry+, and grey = total live cells, n = 15 engrafted recipients)



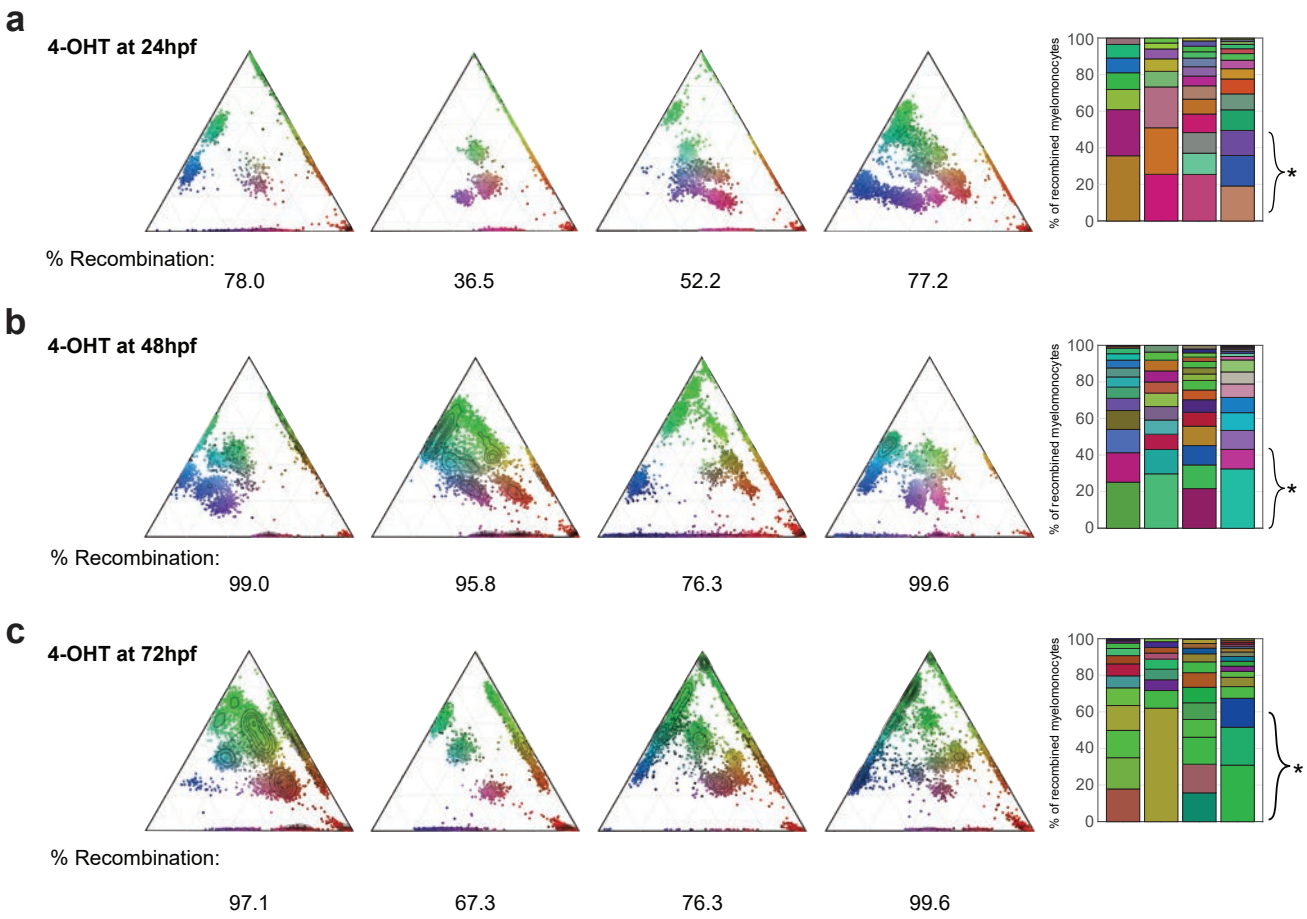
Supplementary Figure 4 Flow cytometry of the Zebrabow fluorophores and lineage analysis of labeled marrow. (a) Gating strategy to analyze Zebrabow fluorophores in multiple lineages. Each lineage (myeloid, lymphoid, erythroid) expresses the Zebrabow transgenes at different levels, but the relative expression of each fluorophore between populations remains the same (gates drawn based on negative, non-fluorescent controls). (b) Erythrocytes contaminate the myeloid population after filtering live, single cells. The myeloid and erythroid populations can be easily distinguished by intensity level of the fluorophores. (c) Plotting the three Zebrabow colors in three dimensions demonstrates the similar color barcodes present in all three lineages. Because the erythrocytes are relatively dim, there is much more variation in the signal. (d) The myeloid and lymphoid populations were isolated from the marrow of adults that were treated at various developmental time points by the gating strategy in (a). Three-dimensional plots of the three Zebrabow fluorophores shows that the color distribution in the myeloid and lymphoid lineages are almost identical. These are representative examples of the adults used to quantify lineage output in Fig. 3e.



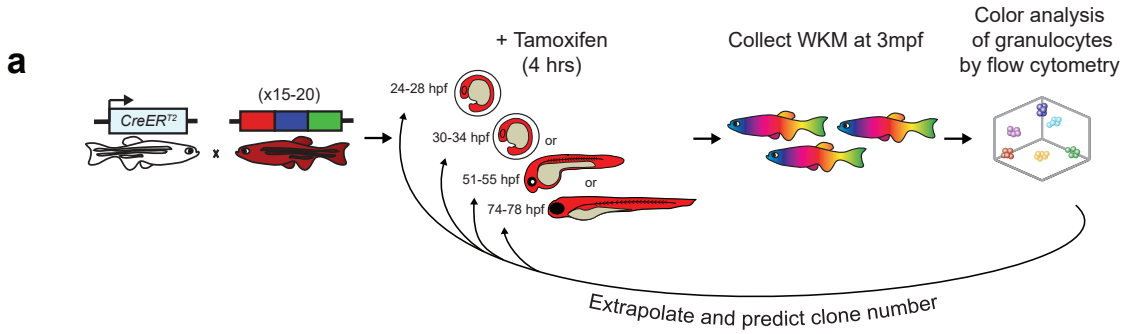
Supplementary Figure 5 Evaluation of clustering quality for small clones. (a) Tg(drl:creERT2;ubi:Zebrabow-M) embryos were treated at 96 hpf, and their marrow was analyzed by flow cytometry at 5 months to measure small clonal contributions. (b) Silhouette plots from three representative marrows evaluate the quality of the clustering solutions. Small clones that contribute <1% of the granulocyte population were detected with high silhouette values, demonstrating the low threshold for detection in this system.

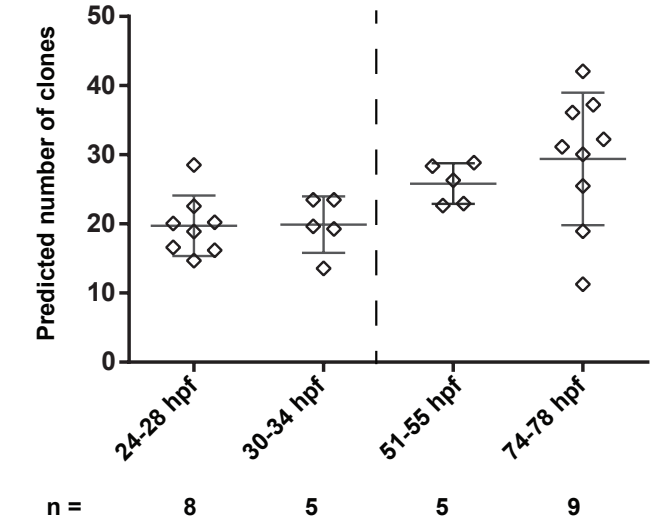


Supplementary Figure 6 Analysis of single color Zebrabow animals. (a) Breeding and treatment strategy to carry the Zebrabow recombination through the germline. (b) Example image of single color Zebrabow fish from a single clutch. Individual channels (dTomato, CFP, YFP) were imaged and merged to generate the RGB colors (scale bar = 20 mm). (c) Example ternary plots of granulocytes from marrows of adult single color zebrafish. (d) Digital combination of 100,000 labeled granulocytes from 12 individual marrows from adult fish treated at 24 hpf with 4-OHT. The 3D scatter plot shows the normalized intensities of these combined cells. The right graph shows the alpha shape volume of these cells in color space (grey), with an example single color marrow (blue) mapped into the color space. (e) These ternary graphs show the same example from (d) in 2D space. (f) Boxplot that shows quantification of the predicted number of Zebrabow colors from mapping individual single colored marrows into 2D color space (red line is median and box represents inner quartile range, n = 13 fish).



Supplementary Figure 7 Example ternary plots from fate mapping at defined developmental time points. This figure provides example ternary diagrams that depict color barcodes in the granulocyte lineage from adult fish treated during embryogenesis. Each ternary diagram depicts an individual marrow at 24 hpf (a), 48 hpf (b), or 72 hpf (c) with labeling efficiency (% recombination) below each plot. Bar graphs on the right show the quantification of clonal contribution for each ternary diagram. Brackets (*) correspond to large clusters that are likely to be polyclonal populations, which were ignored for predicting clonal numbers.





c

Embryonic stage	Predicted number of clones	95% C.I.
24-28 hpf	20	16.1-23.4
30-34 hpf	20	14.8-25.0
51-55 hpf	26	22.2-29.5
74-78 hpf	29	22.0-36.8

Supplementary Figure 8 Repeat of Zebrabow fate mapping at defined developmental time points with brief 4-OHT treatment. (a) Strategy to fate map HSCs at defined developmental time points. In this experiment, embryos were exposed to 4-OHT for only 4 hours until they were switched to fresh water. (b) Color barcodes in granulocytes were quantified and extrapolated to predict the number of clones present during label induction that contribute to adult blood (dashed line marks the beginning of definitive haematopoiesis). Each marker represents one animal. Error bars show mean and s.e.m. (n = 8, 5, 5, and 9 fish for 24, 30, 51, and 74 hpf, respectively) (c) Table showing quantification of these predicted values with 95% confidence intervals. Cluster data is available in the Supplementary Table 1.

Supplementary Table Legend

Supplementary Table 1 (Statistics Source Data). Cluster data, including the color cluster information for each individual fish treated at distinct stages, is provided in this table. The data includes experiments from Figure 5 and Supplementary Figure 8. The Average cluster sizes of each fish are provided, and predicted clone number is calculated by dividing 100% by the average cluster size.

Supplementary Movie Legends

Supplementary Movie 1 Time lapse imaging of drl:eGFP embryos. This movie demonstrates the activity of the drl regulatory elements during definitive blood development. drl:eGFP embryos were imaged by confocal microscopy beginning at 40 hpf. GFP+ cells can be seen in the vasculature and haematopoietic cells in both the ventral dorsal aorta and the caudal haematopoietic tissue (CHT).

Supplementary Movie 2 Time lapse imaging of Tg(Zebrabow-M; drl:creERT2) embryos. Zebrabow embryos were treated with 4-OHT at 24 hpf and imaged by confocal microscopy beginning at 48 hpf. This movie shows Zebrabow labeling in the CHT in both the vasculature and haematopoietic cells. A putative stem cell resides in the center of the frame, and it eventually divides to yield progeny with identical color.