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High-throughput imaging of adult fluorescent zebrafish with an LED fluorescence microscope

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Abstract

Zebrafish are a useful vertebrate model for the study of development, behavior, disease and cancer. A major advantage of zebrafish is that large numbers of animals can be economically used for experimentation; however, high-throughput methods for imaging live adult zebrafish had not been developed. Here, we describe protocols for building a light-emitting diode (LED) fluorescence microscope and for using it to simultaneously image up to 30 adult animals that transgenically express a fluorescent protein, are transplanted with fluorescently labeled tumor cells or are tagged with fluorescent elastomers. These protocols show that the LED fluorescence microscope is capable of distinguishing five fluorescent proteins and can image unanesthetized swimming adult zebrafish in multiple fluorescent channels simultaneously. The microscope can be built and used for imaging within 1 day, whereas creating fluorescently labeled adult zebrafish requires 1 hour to several months, depending on the method chosen. The LED fluorescence microscope provides a low-cost, high-throughput method to rapidly screen adult fluorescent zebrafish and it will be useful for imaging transgenic animals, screening for tumor engraftment, and tagging individual fish for long-term analysis.

INTRODUCTION

As a vertebrate model, zebrafish have long been valued for the optical clarity of their embryos and larvae, which allows visualization of complex developmental processes. Recently, the zebrafish has also proven to be an accurate model for the study of a broad range of human diseases, including hematological disorders¹, kidney disease², cancer^{3–5}, immunological disorders⁶ and complex behavioral problems such as alcoholism⁷, drug addiction⁸ and psychiatric disorders^{9,10}. The primary benefit of using zebrafish to study human disease, compared with mammalian models, is the economy in which very large-scale experiments can be performed. For example, our recent work quantifying the number

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of leukemia-initiating cells in zebrafish T-cell acute lymphoblastic leukemia (T-ALL) required nearly 1,500 transplant animals¹¹. For most researchers, such large-scale transplantation experiments are simply not economically feasible in rodent models.

Although experimental systems are beginning to take advantage of the high-throughput nature of zebrafish, imaging technologies to visualize adult zebrafish are lagging. Much like the mouse, zebrafish are imaged individually. Commonly, zebrafish are examined stereomicroscopically, or by using confocal or multiphoton microscopy. More sophisticated imaging techniques are also beginning to be used with the fish, including microcomputerized axial tomography¹², magnetic resonance imaging¹³ and high-frequency ultrasound¹⁴. The major benefits of most of these imaging techniques are the very high resolution that can be obtained and compatibility with fluorescent markers. However, in large-scale fish experiments, detail in imaging is not often a priority. In addition, image acquisition and processing using the above techniques can be labor intensive and time consuming, the associated costs can be high and the necessary imaging equipment may not be available at all institutions.

We have recently developed a LED fluorescence macroscope to address the current limitations in high-throughput zebrafish imaging¹¹. The macroscope can image up to 30 adult zebrafish at once, and is capable of distinguishing five fluorescent proteins: AmCyan, GFP, zsYellow, dsRed and mCherry. It can be equipped with four to six different LED lights (405- to 420-nm black light, 420- to 470-nm blue light, 500- to 570-nm green light and incandescent) and a selection of two or three optical filters, meaning that zebrafish can be imaged in multiple fluorescent channels simultaneously. In addition, imaging with the macroscope does not require that fish be anesthetized; hence, live videos can be captured of fluorescently labeled swimming animals. The macroscope was previously used to screen transgenic zebrafish that expressed a fluorescent protein in the muscle, with an accuracy rate of > 99% (140 of 141 transgenic fish detected)¹¹. The macroscope was also used to screen for engraftment of leukemias that expressed AmCyan, GFP, zsYellow or dsRed. When 1.5×10^4 tumor cells were transplanted, 100% of tumor-bearing fish were detected by the macroscope for all four fluorophores within 20 d after transplant. Solid tumor transplants were also reliably detected by the macroscope. Finally, the macroscope has been used to rapidly and accurately screen for engraftment of leukemia in zebrafish that had been transplanted with single tumor cells¹¹. To the best of our knowledge, the LED fluorescence macroscope is the first imaging technology available that facilitates high-throughput imaging of fluorescently labeled fish, and is also the first technique capable of imaging live, swimming zebrafish in multiple fluorescent channels.

Aside from its use in screening transgenic fish and those transplanted with fluorescently labeled tumor cells, the LED fluorescence macroscope can also distinguish adult zebrafish that have been tagged with fluorescent elastomers. An elastomer is a polymer compound commonly used to tag commercial fish stocks, but had not yet been used in zebrafish. In commercial fish species, animals are distinguished on the basis of the site of elastomer injection, combinations of color tags and numbers of tagging spots per animal^{15,16}. Zebrafish can also be tagged with fluorescent elastomers (see Step 5A in the PROCEDURE) and the macroscope can be used to identify and track tagged individual animals over time. Although exclusively used in fish for tagging purposes, elastomers have a history as a drug delivery vehicle in mammalian models¹⁷, making these reagents of potential benefit for assessing the effects of drugs on various disease processes in zebrafish. In addition, the LED fluorescence macroscope may also be used to track behaviors in adult zebrafish. Live videos can be made of unanesthetized, swimming fish, which either transgenically express a fluorescent protein or are tagged with a fluorescent elastomer. Because the macroscope is

capable of multispectral imaging, multiple fish can be labeled with different colored fluorescent tags, and behaviors of several individuals can be simultaneously monitored.

However, the macroscope has several important limitations that must be considered. For example, zebrafish are commonly used in cancer research for *in vivo* tracking of tumor cell invasion and angiogenesis; when built as described in this protocol, the macroscope is not capable of imaging fish with sufficient resolution to detect focal expression of fluorescent proteins, and is therefore not suitable for these types of experiments. In addition, when screening transgenic fish, any fluorescent protein expressed at low levels will not be detected by the Logitech Quickcam contained within the macroscope. Similarly, transgenic animals older than 45 d are large enough to be screened using the macroscope, whereas smaller larvae can be used only if the transgenic fluorescent protein is expressed highly enough. Some of these limitations may be overcome by using more specialized cameras; this protocol uses inexpensive cameras to keep the overall cost of the macroscope low.

In summary, the LED fluorescence macroscope fills an important gap in the imaging techniques currently available for zebrafish. This technology is capable of high-throughput imaging of fluorescently labeled adult fish, and it can image zebrafish in multiple fluorescent channels with or without anesthesia. Moreover, building the macroscope does not require special skills, and with a total cost for building supplies and software of less than \$500, it is also ideal for use in the classroom as a teaching tool. This protocol describes how to build and use both an inverted and upright macroscope, and researchers can customize either macroscope by choosing the types of LED lights, emission filters and cameras that suit their specific needs.

Experimental design

In addition to the discussion above, there are several other points to be considered when building and using the LED fluorescence macroscope. First, there are important differences between the inverted and upright macroscope models. Because cameras are positioned under the imaging area, the inverted macroscope (**Fig. 1a–c**) is ideal for live imaging of unanesthetized fish that have been intraperitoneally injected with fluorescently labeled tumor cells or a fluorescent tag. In addition, the lights and cameras are contained within the body of the inverted macroscope; accordingly, a dedicated darkroom for imaging is not required. The upright macroscope (**Fig. 1d–f**) does require a darkroom for optimal imaging, but it is watertight and much easier to construct and use than the inverted macroscope. Because the cameras are positioned immediately above the imaging area, the upright macroscope produces a brighter image than the inverted macroscope and thus can more readily detect fluorescent proteins that may be expressed at lower levels.

For both macroscope models, the number of lights and cameras can be adjusted depending on the experimental setup. For example, if GFP is the only fluorescent protein that will be detected, the macroscope can be built using only a black light and a camera equipped with a 535/45 filter. Additional cameras and lights can be easily added to both macrosopes as the experimental design changes. It is important to note that, in the macrosopes described in this protocol, only three different excitation lights were used in order to create a simple setup that can excite and detect five different fluorescent proteins. Although the excitation wavelengths described (**Table 1**) may be suboptimal for the fluorescent proteins being detected, fluorescence can often be excited across a broad range. For example, GFP can be excited by wavelengths ranging from 320 to 470 nm. Using the black light, which emits between 405 and 420 nm, a 20% excitation of GFP can be achieved, meaning that GFP protein expression must be high in order to be detected. To customize the macroscope, researchers can choose lights that emit more closely to the optimum excitation wavelength

for the desired fluorescent protein, which may allow fluorescence at lower levels to be detected.

The macroscope can be used to detect fluorescence in a variety of experimental systems, provided the fluorophore is expressed highly enough. To acquire images of fluorescent zebrafish using the digital camera, it is crucial that a wild-type (non-fluorescent) fish be used to set the exposure, gain, brightness and color; this ensures that negative fish are not incorrectly scored as positive because of autofluorescence. In general, the light and filter combinations used to detect AmCyan and dsRED produce less autofluorescence than GFP or zsYellow; this should be taken into consideration when designing experiments that use the macroscope.

MATERIALS

REAGENTS

- Zebrafish. Strain choice depends on the experimental design. For the experiments outlined in this protocol, the wild-type strain AB (Zebrafish International Resource Center, cat. no. ZL1) and the syngeneic strain CG1 (refs. 18,19) were used, as were transgenic fish expressing a fluorescent reporter under the *myl2* muscle promoter¹¹ **▲ CRITICAL** All zebrafish experiments should be performed in accordance with relevant guidelines and regulations approved by institutional animal care and use committees. Permission was obtained from the Massachusetts General Hospital Subcommittee on Research Animal Care for the treatment of zebrafish in our laboratory.
- Zebrafish *rag2*-driven mouse *Myc* expression vector²⁰
- Zebrafish *rag2*-driven human *KRASG12D* expression vector²¹
- Zebrafish *rag2*-driven fluorescent reporter²²
- KCl (5 M; Sigma-Aldrich, cat. no. P9541)
- Tris-EDTA (TE, 100×; Sigma-Aldrich, cat. no. T9285)
- PBS (1×; Gibco, cat. no. 10010-023)
- FBS (Gibco, cat. no. 16000-036)
- Liberase TM (Roche, cat. no. 0540119001)
- NotI restriction enzyme (New England Biolabs, cat. no. R0189)
- MassRuler High-Range DNA ladder (Fermentas, cat. no. R0621)
- Trypan Blue Vital Dye (1×; Gibco, cat. no. 15250-061)
- Phenol/chloroform (EMD Biochemicals, cat. no. 6805-OP)
- Tricaine-S (Western Chemical, cat. no. MS-222)
- Visible Implant Elastomer kit (Northwest Marine Technology, cat. no. IVIFE0004)
- Agarose HS (Denville Scientific, cat. no. CA3510-8)
- Ethidium Bromide (Sigma-Aldrich, cat. no. E1510)
- Ethanol (Pharmco-AAPER, cat. no. 111ACS200)
- Mineral oil (Fisher Scientific, cat. no. O121-1)
- Distilled water

EQUIPMENT

- Microsyringe (no. 701N; Hamilton, cat. no. 80366)
- Nylon cell strainer (40 μ m; BD Falcon, cat. no. 352340)
- Petri dishes (100 mm $\times$ 15 mm; BD Falcon, cat. no. 351029)
- Luer-slip syringe (1 ml; Fisher Scientific, cat. no. 03-377-20)
- Insulin needles (28 gauge; Fisher Scientific, cat. no. 20-023-377)
- Hemocytometer (Fisher Scientific, cat. no. 0261710)
- Stage Micrometer (Fisher Scientific, cat. no. 12561SM1)
- Common zebrafish equipment (tanks, mating chambers, egg strainers)
- Paramecia for feeding fish (Paramecia VAP Company, <http://www.parameciavap.com>)
- QuickTime Player, version 7.0 (Apple Freeware, <http://www.apple.com/quicktime/>)

For inverted microscope

- Pine board (12 inch $\times$ 1 inch $\times$ 8 foot, quantity 1)
- Pine board (4 inch $\times$ 1 inch $\times$ 8 foot, quantity 1)
- Pine board (2 inch $\times$ 1 inch $\times$ 8 foot, quantity 1)
- Plywood ($\frac{5}{8}$ inch; 2 foot $\times$ 2 foot, quantity 2)
- Plywood ($\frac{1}{4}$ inch; 2 foot $\times$ 2 foot, quantity 1)
- Waddell $\frac{5}{8}$ inch $\times$ $\frac{5}{8}$ inch $\times$ 36 inch hardwood square dowel (Waddell; model no. 8310U Internet/cat. no. 100548852; quantity 2)
- No. 6 wooden screws (1 $\frac{1}{4}$ inch; model no. 21032, Home Depot)
- Knob screws (1 $\frac{1}{2}$ inch, 8–32; model no. 28992, Home Depot)
- Wing nut (8–32)
- Screws ($\frac{1}{2}$ inch; 6–32)
- Lithonia Lighting single par holder floodlight (Model no. OFSH 150PR 120 WH M12, Home Depot; quantity 3)
- No.10 zinc-patted washers
- Wire connectors (Buchanan, cat. no. 73208, Home Depot)
- Indoor light-duty cord (16 gauge, 15 foot, cat. no. 0070296, Home Depot; quantity 3)
- Carlon Three Gang, nonmetallic switch and outlet box (44 cubic inch, Blue; Carlon, model no. B344AB; Home Depot)
- Leviton 15-A residential single pole toggle AC quiet switch (Leviton, model no. R56-01451-02T; Home Depot)
- Leviton ProGrade 2-gang switch Midway Nylon wallplate (Leviton, model no. 00PJ3R42000, Home Depot)

- PVC tube (1 inch, Charlotte Pipe 1 inch × 10 ft PVC schedule 40 solid-core plain end pipe, Charlotte Pipe, model no. PVC 04010 0600)
- Compound miter saw
- Circular saw
- Fixed base router with ¼ inch router bit
- Drill and drill bits ($\frac{1}{16}$ inch, ¼ inch and Irwin Speedbor 2000 6 Pc Wood boring bit set (½ inch; Irwin))
- Framing square (Empire 24 inch × 2 inch body, 16 inch × 1 ½ inch; Professional Tongue Framing Square, model no. 1110)
- Screwdrivers
- Hand-held Plexiglas cutter
- Wire cutters with stripper edge

For upright macroscope

- PVC pipe (¾ inch; Charlotte Pipe ¾ inch × 10 foot PVC Schedule 40 Solid-core Plain End Pipe, Charlotte Pipe, model no. PVC 04007 0600, Home Depot)
- PVC tube (1 inch; Charlotte Pipe 1 inch × 10 foot PVC Schedule 40 Solid-core Plain End Pipe, Charlotte Pipe, model no. PVC 04010 0600, Home Depot)
- Switch box (Type 2FSE, ¾ inch, 32 cubic inch, Gray, Double Gang FS Box, Carlon, model no. E9812E-CTN, Home Depot)
- TayMac multiapplication weatherproof receptacle cover (model no. MM1410C, Home Depot)
- PVC Schedule 40 reducing male adapter MIPT × slip (¾ inch × ½ inch model no. 436-074HC, Home Depot; quantity 6)
- PVC Schedule 40 90 degree elbow slip × slip (¾ inch; quantity 2)
- PVC Schedule 40 T configuration slip × slip × slip (¾ inch; quantity 9)
- PVC Schedule 40 reducing male adapter T (¾ inch × ½ inch; ½ inch MIPT threaded in the middle position, quantity 4)
- PVC 90 degree elbow with taped side outlet (¾ inch × ¾ inch × ½ inch; model no. 414-101HC, Home Depot; quantity 6)
- Knob screws (1 ½ inch, 8–32; model no. 28992, Home Depot)
- Wing nut (8–32)
- Screws (6–32 ½ inch)
- Lithonia Lighting single par holder flood light (model no. OFSH 150PR, 120 WH M12, Home Depot; quantity 3)
- No. 10 zinc-patted washers
- Wire connectors (Buchanan, cat. no. 73208, Home Depot)
- Indoor light-duty cord (16 gauge, 15 foot, cat. no. 0070296, Home Depot; quantity 3)

- Leviton 15 A residential single pole toggle AC quiet switch (Model no. R56-01451-02T, Home Depot)

Common to both

- LED fluorescent lights (E27-xLX3 LED bulb blue and green, <http://www.superbrightleds.com>)
- Ultra Violet 60 UV LED black light bulb PAR-20 (cat. no. GGL60SUV, <http://www.blacklight.com>)
- Logitech Quickcam Communicate S5500 (Logitech; quantity 3) **▲ CRITICAL** Drivers for other types of webcams may not support the simultaneous capture of images from multiple identical webcams.
- PVC tubing (3/4 inch)
- PVC tubing (1 inch)
- Optical interference filters (BJOMEJAG Ebuyer Store on Ebay (<http://www.ebay.com>); see **Table 1** for details)
- Black electrical tape
- Plexiglas
- Superglue
- Hot glue and hot glue gun (Arrow 4-inch 24-pack All-Purpose Clear Mini Glue Stix, model no. MG24-4; Arrow Professional Lever Feed Glue Gun, model no. TR550, Home Depot)
- QuickCam software (Logitech, supplied with Quickcam)
- AMCap software (download from <http://noeld.com>)
- Adobe Photoshop, CS4 Extended V11.0 (Adobe)
- Computer (Lenovo or similar)

PROCEDURE

Building and adjusting an LED fluorescence macroscope

- 1 Build the microscope. Detailed instructions, including step-by-step directions and photographs, for building both the inverted and upright macroscope can be found in **Supplementary Manual 1** and **Supplementary Figures 1–12**. Briefly, LED lights are positioned to illuminate a 100 mm × 15 mm Petri dish of fluorescently labeled fish. Digital cameras are equipped with optical filters that allow only light of specific wavelengths to be detected. In both macroscope models, the cameras have an 8-inch depth of field, from 2 inches to 10 inches above the camera, and a numerical aperture of 0.36. Cameras interface with a Lenovo computer, and images are captured and manipulated using AMCap software. A completed inverted macroscope is shown in **Figure 1a–c** and an upright macroscope is shown in **Figure 1d–f**.
- 2 Align the LED fluorescent lights so that light is evenly distributed across the sample; use option A for the inverted macroscope or option B for the upright macroscope.

A. Aligning LED lights on the inverted macroscope

- i. To align LED lights on the inverted macroscope, move lights from front to back by adjusting the wing nuts, and adjust the angle of the light by the screw mechanism on the light housing. The lights can also be adjusted from side to side.

B. Aligning LED lights on the upright macroscope

- i. To align LED lights on the upright macroscope, move the PVC fitting to adjust the angle of light and to move the lights from side to side.
- ii. To focus, place a white sheet of paper in the desired imaging area and move each fixture until the light is evenly distributed across a 10-cm space.
- iii. Lights cannot be adjusted in the z-plane; adjust the imaging area by inserting a platform to move the fish closer to the lights and camera.

- 3 Connect cameras to the computer and position them within the macroscope to align correctly with the imaging areas, using option A for the inverted macroscope or option B for the upright macroscope. Dedicated cameras should be affixed with permanent filter sets; however, filters can be changed by creating a modified filter housing described in detail in **Supplementary Manual 1**.

A. Adjusting cameras on the inverted macroscope

- i. For the inverted macroscope, camera housings can be built to facilitate movement of the camera by adjusting a wing nut and moving the camera as needed.

B. Adjusting cameras on the upright macroscope

- i. To adjust the camera in the upright macroscope, you should reposition the camera within drilled holes and secure the camera with a wing nut.

▲ CRITICAL STEP The camera lens should be covered when not in use.

- 4 Fit the 25-mm filters into a 1-inch PVC tube (trimmed to ¼ inch) to hold them in place over the camera.

▲ CRITICAL STEP Optimized filter and light combinations for detecting various fluorescent proteins are shown in **table 1**, but they can be adjusted to meet specific needs. For example, a 535/45-nm filter can be used to image both GFP and zsYellow and a 610/40 nm can discriminate both dsRED and mCherry. In general, filters of 480/30 nm, 535/45 nm, and either 610/40 nm or 640/35 nm will meet the needs of most users, and they are available from a variety of suppliers. Factory-rejected filters with imperfections are suitable for most imaging applications and can be purchased online through the BJOMEJAG Ebuyer Store on Ebay.

▲ CRITICAL STEP Water, fingerprints and scratches can damage the filters. If filters remain in the macroscope, they must be covered when not in use to prevent dust accumulation and moisture damage.

Creating adult fluorescent zebrafish

- 5 Fluorescent zebrafish can be prepared using several methods. For the imaging examples shown in this protocol (**Figs. 1–6**), fluorescently labeled zebrafish were prepared by tagging fish with fluorescent elastomers (option A); creating transgenic zebrafish bearing fluorescently labeled T-ALL cells (option B) or

fluorescently labeled rhabdomyosarcoma (RMS) (option C); or transplanting fluorescently labeled tumor cells into adult fish (option D).

A. Tagging zebrafish with fluorescent elastomers

- i. With the provided 1-ml syringe (in the visible implant elastomer kit—see REAGENTS), dispense 300 μ l of the fluorescent dye into a 1.5-ml flip-cap tube.
- ii. Next, dispense 30 μ l (1:10 volume) of the curing agent into the tube (use the provided 1-ml syringe). **▲ CRITICAL STEP** Avoid contaminating the curing agent syringe with the fluorescent dye to prevent curing the elastomer in the syringe.
- iii. Swirl with a pipette to mix, and centrifuge at 6,000g for 30 s at 25 °C.
- iv. Use a new 1-ml syringe (without a needle) to transfer the elastomer from the 1.5-ml tube to a 1-ml syringe equipped with a 28-gauge needle. **▲ CRITICAL STEP** The elastomer will solidify within 1 h; accordingly, prepare only enough for immediate application.
- v. Anesthetize zebrafish by placing them in a 100 mm $\times$ 15 mm Petri dish containing 30 ml of zebrafish system water with 300 μ l of 4 mg ml⁻¹ tricaine-S. Approximately 60 fish can be injected using the elastomer preparation outlined above, but fish should be anesthetized in groups of five to ensure that they are not killed as a result of prolonged exposure to tricaine.
- vi. Inject 1–5 μ l of elastomer into the peritoneum or tail muscle of the anesthetized zebrafish. Although not necessary, an epifluorescent dissecting microscope will aid in delivering the desired amount of elastomer to specific areas of interest on the fish.
- vii. Visualize the elastomers using the LED fluorescent macroscope using a black light and various filter combinations (**Table 1**). Blue, green, yellow and red elastomers can be easily distinguished by various filter sets, whereas pink and orange elastomers are less easily discerned by macroscopic observation. **? TROUBLESHOOTING**
- viii. Monitor the zebrafish using the macroscope as desired. Elastomers are stably incorporated into the fish; they do not affect viability ($n = 50$) and remain visible using the macroscope for at least 3 months.

B. Creating transgenic zebrafish bearing fluorescently labeled T-ALL cells

- i. Linearize 10 μ g each of *rag2-Myc* and *rag2-fluorescent reporter* expression constructs by digestion with 20 U of NotI and the appropriate buffer supplied by the manufacturer. We commonly perform digests in a total volume of 250 μ l and incubate them at 37 °C overnight.
- ii. Verify linearization by running 5 μ l of the digest on a 1% (wt/vol) agarose gel that contains 0.5 μ g ml⁻¹ ethidium bromide, at 150 V for 30 min. In addition, run 100 ng of uncut plasmid on the gel for comparison to ensure that DNA is fully linearized. Plasmid and linear DNA of the same total length will run at different speeds through the agarose, resulting in bands of different sizes when detected on an agarose gel.
- iii. Extract using phenol/chloroform and precipitate the linearized plasmid. Briefly, add 250 μ l of phenol/chloroform (1:1), vortex briefly and then

centrifuge at 14,000*g* for 5 min at 25 °C. Remove the upper aqueous layer and transfer to a new 1.5-ml centrifuge tube. Add 250 μ l of chloroform to the sample, vortex for 10 s and then centrifuge at 14,000*g* for 5 min at 25 °C. Remove the upper aqueous phase and again transfer to a new 1.5-ml centrifuge tube. Add 25 μ l of 3-M sodium acetate (pH 7.4) and 625 μ l of 100% ethanol. Vortex briefly to mix and then centrifuge at 14,000*g* for 20 min at 25 °C. Remove the supernatant and wash the DNA pellet with 500 μ l of 70% (vol/vol) ethanol. Vortex briefly and centrifuge at 14,000*g* for 10 min at 25 °C. Remove the supernatant and allow the DNA pellet to air-dry for 5–10 min. Resuspend in 20 μ l of distilled, autoclaved H₂O.

- iv. To quantify, dilute an aliquot of the resuspended DNA 1:1, 1:5 and 1:10 with distilled water, then run each dilution on a 1% (wt/vol) agarose gel with 20-, 10- and 5- μ l MassRuler High-Range DNA ladder. Quantify the amount of linearized DNA by comparing the band intensity of the linearized DNA with bands in the DNA ladder, according to the manufacturer's directions.
- v. Dilute the DNA for injection to a final concentration of 100 mM in KCl + 0.5 $\times$ TE, using a 1:1 mix of linearized Myc and fluorescent reporter plasmids, and freeze at – 20 °C until use.
- vi. For injection into wild-type fish, dilute DNA to 120 ng μ l^{–1} (60 ng μ l^{–1} *rag2-Myc* + 60 ng μ l^{–1} *rag2-fluorescent reporter*). For injection into syngeneic CG1 fish, use a total of 60 ng μ l^{–1} (30 ng μ l^{–1} *rag2-Myc* + 30 ng μ l^{–1} *rag2-fluorescent reporter*).
- vii. The night before the injection, place three female zebrafish and two male zebrafish in separate compartments of a mating chamber. In the morning of the day of injection, remove the plastic divider to combine the male and female fish. After 20–30 min, collect eggs using a strainer and briefly store in a Petri dish containing zebrafish system water.
- viii. Load the linearized DNA into the injection needle and adjust the pressure on the needle so that a volume of 500 pl is expelled. DNA volume size can be quantified by injecting into 50 μ l of mineral oil on a stage micrometer. The resulting bubble of DNA should be ~100 μ m in diameter, which is ~500 pl.
- ix. Inject one-cell-stage embryos with the appropriate concentration of plasmid DNA for the zebrafish strain selected, noted in Step 5B(vi), as previously described²³. Inject DNA directly into the cell, and not into the yolk of the zebrafish egg. Store injected embryos at 28.5 °C, and remove dead embryos after 24 h. Transfer larval fish to tanks at 5 d of age and feed with paramecia. Add water flow at 14 d of life, coincident with feeding larval fish with brine shrimp. **? TROUBLESHOOTING**
- x. At day 21 post injection, larvae can be examined for fluorescence in the thymus with an epifluorescent dissection microscope. Approximately 5% of injected larvae will have fluorescent T-cells within the thymus, and 100% of these fish will go on to develop fluorescently labeled T-ALL²⁰. Alternatively, fish can be screened using the macroscope ~45 d after injection with the appropriate excitation light and emission filter for the fluorescent reporter injected (**table 1**). At this time, a majority of the T-ALL cells will have progressed enough for the leukemias to be detected by the macroscope. **? TROUBLESHOOTING**

C. Creating transgenic zebrafish bearing a fluorescently labeled embryonal RMS (ERMS)

- i. Prepare the *rag2-kRASG12D* expression vector, as described in Steps 5B(i)–(iv), diluting linearized DNA to $120 \text{ ng } \mu\text{l}^{-1}$ in a final concentration of $100\text{-mM KCl} + 0.5\times \text{TE}$.
- ii. Set up male and female transgenic fish that stably express *mylz2-fluorescent reporter*¹¹ in mating chambers the night before injection, as described in Step 5B(vii).
- iii. Inject 500 μl of DNA into one-cell-stage embryos. Store injected embryos at 28.5°C and remove dead embryos after 24 h. Transfer larval fish to tanks at 5 d of age and feed with paramecia. Add water flow at 14 d of life, coincident with feeding larval fish with brine shrimp. ?

TROUBLESHOOTING

- iv. Monitor larvae in the tank for tumor growth in the tail, which will appear as a large mass in the muscle. Tumors will also be visible under the dissecting microscope by day 14 post injection, with $\sim 20\%$ of injected fish developing tumors²¹. The *mylz2-fluorescent reporter* transgenic fish will express the fluorescent protein in their muscle; because ERMS tumor cells express *mylz2*, they will also express the fluorescent reporter. ?

TROUBLESHOOTING

D. Transplantation of fluorescently labeled tumors into adult zebrafish • TIMING 20 d

- i. At 2 d before transplant, irradiate wild-type strain zebrafish with 23-Gy total body irradiation using a Cesium-137 source. **▲ CRITICAL STEP** Irradiation will only ablate the immune system for a maximum of 30 d. At this time, the immune system will recover and some transplanted tumors will regress. For long-term tumor transplants, consider using syngeneic zebrafish (Step 5D(iv)), which are genetically identical and therefore do not require immune ablation to allow tumor growth^{11,19}.
- ii. Euthanize one tumor-bearing fish by tricaine overdose and collect tumor cells. To collect leukemic cells, mince the fish with a razor blade in a Petri dish with 10 ml of $0.9\times \text{PBS} + 5\% \text{ (vol/vol) FBS}$. Pass the mixture through a $40\text{-}\mu\text{m}$ mesh filter into a 50-ml tube. Alternatively, to collect cells from the solid tumor, dissect the RMS from the fish, then mince with a razor blade in $250 \mu\text{l}$ of $0.9\times \text{PBS} + 2.5 \mu\text{l}$ of Liberase TM to allow tumor cell dissociation. Pipette the mixture up and down every 10 min for a total of 30 min to facilitate full dissociation. Add 5 ml of $0.9\times \text{PBS} + 5\% \text{ (vol/vol) FBS}$ to inactivate the enzyme, and pass the mixture through a $40\text{-}\mu\text{m}$ mesh filter into a 50-ml tube.
- iii. Count viable tumor cells by mixing $10 \mu\text{l}$ of tumor cells with $90 \mu\text{l}$ of Trypan blue. Count unstained cells using a hemocytometer. Multiply the number of unstained cells by the dilution factor (10), then by 10,000, as per the hemocytometer protocol, to determine the number of viable tumor cells per ml. If necessary, cells can be concentrated by centrifuging at $1,000g$ for 10 min at 25°C and the cell pellet can be resuspended in $0.9\times \text{PBS} + 5\% \text{ (vol/vol) FBS}$.
- iv. Dilute tumor cells for an injection volume of $5 \mu\text{l}$ per fish. For T-ALL transplant, inject 10^6 cells per fish to ensure tumor growth. For RMS

transplant, 10^4 cells per fish are routinely used to engraft disease. For transplants of primary tumors from the syngeneic strain CG1 into CG1 recipients, isolate and transplant tumor cells as in Steps 5D(ii)–(iv). Note that fewer tumor cells are required for tumor formation in syngeneic transplants. Typically, tumors will reliably form with an injection of 10^3 cells per fish, and even one cell per fish can induce tumor growth in a percentage of fish¹¹.

- v. Inject cells into the peritoneum of the fish using a no. 701N Hamilton syringe (5–10 μ l of injection volume per fish). Fish do not have to be anesthetized before injection.
- vi. Monitor the fish for tumor growth beginning at 20 d post transplant with the LED fluorescence macroscope. ? **TROUBLESHOOTING**

Imaging adult fluorescent zebrafish

- 6 Zebrafish can be imaged using a single fluorescent channel if only one fluorescent tag is used (option A) or multiple fluorescent channels can be used for multispectral imaging (option B).
 - A. Imaging adult zebrafish in a single fluorescent channel
 - i. Place up to 30 zebrafish in a 100 mm $\times$ 15 mm Petri dish. Turn on the appropriate LED light and equip the camera with the correct emission filter for the fluorescent protein being detected (**table 1**). Note that it is only necessary to turn on one light and use one filter to image fish in a single fluorescent channel.
 - ii. For live imaging, the upright macroscope is suitable for most applications. The inverted macroscope can be used to take live video of fish injected intraperitoneally with tumor cells or of fish tagged abdominally with elastomers. Otherwise, fish can be anesthetized with tricaine and imaged (while lying on their sides) using either the inverted or upright macroscope.
 - iii. Open the QuickCam software and click on the QuickCapture icon (a film reel) to view the images being captured by the camera. The captured image size can be changed by toggling the tab in the lower right corner of the window. Use the 4-megapixel setting for still photographs and use 1.3 megapixels for video capture. Photos and video can be taken by clicking on the icons in the QuickCapture window.
 - iv. Adjust the exposure, gain, color, zoom and brightness using the Webcam Settings window (shown by the gears). Use these settings to ensure that your filter sets are specific and are not detecting nonspecific background light. **▲ CRITICAL STEP** Exposure, gain and brightness must be optimized by visualizing a fluorescently labeled animal and a negative control (e.g., a wild-type AB strain fish). Adjust the camera settings so that fluorescence in the positive animals is detected, and background in the wild-type fish is minimized. ? **TROUBLESHOOTING**
 - v. Capture the desired images or movies, which can be opened from the gallery window, then rename and save each one.
 - vi. To sort fish, or to score them for tumor growth, lightly anesthetize them with tricaine, and then image them with either the inverted or upright

macroscope. Use a plastic spoon to retrieve and separate negative and positive fish, or to separate them by tumor stage.

B. Multispectral imaging of zebrafish with AMCap software

- i. Place up to 30 zebrafish in a 100 mm × 15 mm Petri dish. Turn on the appropriate combination of LED lights and use the correct emission filters for the two fluorescent proteins being detected (**table 2**). LED lights may need to be repositioned (Step 2) so that they can simultaneously and evenly illuminate the sample.
- ii. Start AMCap software by clicking on the icon. Repeat to open a second window.
- iii. Select 'Option' and then 'video capture filter'. In this window, there are three tabs. Select the 'Zoom/Face Tracking' icon to change the camera zoom. Set zoom to 180% if a 60-mm Petri dish is used. Turn the 'face tracking' feature off, and center the image using the positioning arrows.
- iv. Select 'Device Settings' in the Video Capture window to adjust brightness, contrast and saturation settings, which control hue and color. It is easiest to capture images in a black-and-white setting and then change the color setting in Adobe Photoshop after the video is captured.
- v. Select 'Advanced' in the Video Capture window to control the exposure and gain. Rather than alter the exposure setting, which influences the rate of capture of the video, it is better to alter the gain setting to digitally enhance the image or adjust brightness and contrast in 'Device Settings'.
▲ CRITICAL STEP Exposure rates should be the same on both cameras to ensure that the frame rate remains constant.
- vi. If images are inverted or upside-down, use the 'Advanced' control panel and toggle the image mirror icons as required.
- vii. Use the 'Devices' icon to move between cameras in the same window.
- viii. Select 'Capture' 'Start Capture' to capture movies of swimming fish. A box will appear showing where the file is saved. Do not start capturing yet.
- ix. Select the second AMCap window and repeat Step 6B(iv). With both windows open and poised to capture, start capturing in one window and then the second. After 10 s, go back to the first window and select 'Capture' 'Stop Capture'. Repeat in the second open window. Once the desired images are collected, verify that the capture rate is the same for both cameras, as denoted in the text below the video files. If the capture rates are different, adjust the exposure on each camera until the capture rates are equal. Further, ensure that no image frames were dropped in your acquisition. If so, image acquisition should be repeated. Dropped frames are common if the computer is slow or memory is limited.
- x. Open and view the video to ensure that the desired images are captured. Videos can be renamed at this point.

Processing multispectral videos in Adobe Photoshop extended

- 7 Open Photoshop (CS4 Extended Version 11.0) and import your .avi file by selecting 'File' 'Open' and selecting the first .avi file to be merged.

- 8 Using the Layer tab on the main tool bar at the top of the page, select 'Layer' → 'Video Layers' → 'New Video Layer' under File. Upload the second movie images as an .avi file. Both .avi files should now be integrated into the same Adobe file and can be visualized in the Layer window.
- 9 Click on each image layer followed by selecting 'Layer' → 'Layer Style' → 'Blending Options'. In this window, you can change the color of your images by selecting specific channels. For GFP, toggle only the G box; for dsRED/mCherry toggle the R box and for AmCyan the B box. This step ensures that colors are true and that images can be visualized simultaneously. For yellow, pseudocoloring is commonly used, wherein yellow is made to appear red by checking only the R box. Image frames can later be altered in Photoshop to correct for color. Alternatively, video imaging can be completed using the corrected colors in the AMCap software and merged by changing the opacity of the overlaying layer in the Blending Options window.
- 10 To begin to align the video frames, add an adjustment layer by toggling 'Layer' → 'New Adjustment Layer' → 'Levels'. Once the new layer appears in the Layer window, click on the layer. Next, move the far right arrow toward the left in the adjustments window to enhance the image and allow all the fish in the Petri dish to be seen, as detected by low-level autofluorescence.
- 11 To align the timing of the videos, click on the main tool bar icon 'Window' → 'Animation' to open the animation window. The 'Animation Timeframe' is now depicted in a bar below the image window. Move the blue triangle at the top of the time frame to 2 s (30 frames if the capture rate is 15 frames per second). Move the green time bars by clicking and holding the bars with the mouse. Images can be aligned by moving the bar to the left to ensure that the timing aligns between movie frames. Visualizing the same fish movements in both layers synchronizes the timing.
- 12 To align the videos by position, click on one of the layers in the Layer window and then use the Move tool to grab and align one layer with the second.
- 13 After aligning the movies with respect to both time and position, crop the image using the Rectangle Marquee tool. Select the area to crop and then go to 'Image' → 'Crop'.
- 14 Return to the Layer Window and delete the Adjustment Layer created in Step 10.
- 15 Make any final adjustments for intensity by clicking on the layer and selecting 'Image' → 'Adjustments' → 'Levels'. Do not move the middle arrow/toggle, as this inaccurately changes the exposure or colors of the image.
- 16 Go to 'File' → 'Export' → 'Render Video'. Change the name of the movie (i.e., Merged.mov). The frame rate reflects the rate at which images are captured in the AMCap software packages. Change frame rates as needed in the bottom box in this window.
- 17 Open the video in QuickTime Player (Version 10.0). Choose 'Edit' → 'Trim' and move the Yellow bar to trim the movie to eliminate frames in which only single color frames are shown. Once image frames have been selected to be included in the final movie, select 'Trim'. Name the trimmed file by selecting 'File' → 'Save As' and rename the video accordingly. The final merged movie is now complete.

Processing movies frames in adobe photoshop

- 18 To create Photoshop images that show individual frames of the movie created above, open the merged movie in Adobe Photoshop by selecting 'File' → 'Import' → 'Video Frames to Layers'. Select the .mov file to be opened.
- 19 Each video frame can now be imaged in its own layer and made into a final figure by creating a new file and dragging in the images from layers of interest. Specifically, go to 'File' → 'New' and create an RGB 8-bit image that is large enough to accept several frames.
- 20 Drag and drop layers into this new Photoshop file and save by using 'File' → 'Save As'. Recently added layers can be positioned in the new file by using the Move tool.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

• TIMING

Steps 1–4, Building and setting up the macroscope: 1–2 d for inverted model, 0.5 d for upright model

Step 5A, Tagging adult zebrafish with elastomers: 1 h

Step 5B, Generating fluorescently labeled T-ALL cells: 2 d, plus 2 months for tumor growth

Step 5C, Generating fluorescently labeled RMS: 2 d, plus 1 month for tumor growth

Step 5D, Transplanting tumors into wild-type fish: 3 d, plus 20 d for tumor growth

Step 5D, Transplanting tumors into syngeneic fish: 1–3 h, plus 20 d for tumor growth

Steps 6–20, Macroscopic imaging of zebrafish: 10 min to several hours, depending on experimental design and the number of animals to be assayed

ANTICIPATED RESULTS

The LED fluorescence macroscope described in this protocol provides the first high-throughput method to image adult fluorescent zebrafish. As shown in **Figure 2**, the macroscope can discriminate between at least four fluorescent colors, including AmCyan, GFP, zsYellow and mCherry. Transgenic zebrafish expressing four different fluorescent proteins under the control of the muscle-specific promoter *myl2* can be easily distinguished using combinations of excitation lights and emission filters (**Table 1**). As with most fluorescent imaging modalities, excitation lights and emission filters are often not specific for individual fluorescent proteins. For example, the black light and 545/35-nm filter used to detect GFP also detects AmCyan (**Fig. 2b**). Similarly, AmCyan and dsRED bleed into the zsYellow channel (**Fig. 2c**), and zsYellow can be detected using the green light and 640/35-nm filter used to image mCherry (**Fig. 2d**). Although light and filter sets detect overlapping fluorescence from multiple fluorescent proteins, one can easily distinguish individual animals expressing single fluorescent proteins when analyzed across numerous filter sets. The upright macroscope (**Fig. 2e–h**) is built so that LED lights directly illuminate the animals from above, and therefore produces a brighter and clearer fluorescent image than inverted macroscope (**Fig. 2a–d**). The inverted macroscope is also capable of live video imaging; shown is a single frame of a movie of live fluorescent transgenic zebrafish (**Fig.**

2m–p). In general, the upright microscope is easier to use and less expensive to build than the inverted microscope; however, the inverted microscope is ideal for live video imaging of fish with a peritoneal injection of tumor cells or fluorescent tags.

The microscope can also be equipped with two cameras and a combination of filter sets to simultaneously image fish in multiple fluorescent channels (**Table 2**). As shown in **Figure 3**, a single black light and two cameras equipped with either a 480/30-nm or a 535/45-nm emission filter were used for multispectral imaging of fish expressing *mylz2-AmCyan* and *mylz2-GFP* (**Fig. 3a–c**), whereas a blue light and cameras equipped with 535/45-nm and 640/35-nm filters were used to simultaneously detect combinations of transgenic fish expressing *mylz2-mCherry*, *mylz2-zsYellow* and *mylz2-GFP* (**Fig. 3d–l**). Further, in the examples of single light and filter combinations, it is important to note that there is some bleed-through of AmCyan and zsYellow into the GFP light and filter set (**Fig. 3b,d**), and of zsYellow into the dsRED channel (**Fig. 3k**). However, multispectral imaging clearly distinguishes the fluorescent proteins expressed by the transgenic fish.

Video capture software, such as AMCap, can also be used for live imaging of fluorescently labeled, swimming fish, using either a single emission filter or multispectral imaging. Live multispectral imaging is demonstrated in **Figure 4**, in which black and blue lights, in combination with 640/35-nm and 535/45-nm filters, were used for simultaneous video capture of stable transgenic *mylz2-AmCyan* and *mylz2-mCherry* fish. Still images (**Fig. 4a–l**) show a video capture rate of ~60 ms, indicating that the LED fluorescent microscope is suitable for imaging unanesthetized, swimming zebrafish (**Supplementary Movie 1**).

Besides fish transgenically expressing a fluorescent protein, the microscope can also detect fluorescent elastomers injected into the abdomen of the zebrafish. Elastomers are commonly used to tag marine fish for identification¹⁶, and have been used as a drug delivery vehicle in mammalian models¹⁷. As shown in **Figure 5**, elastomer injection can also permanently mark zebrafish, and the microscope can be used to accurately differentiate between fish tagged with blue, red, yellow and green fluorescent elastomers (**Fig. 5a–d, Table 1**). Because elastomers are brightly fluorescent, only a small quantity is necessary to tag the fish (**Fig. 5e–g**). These experiments suggest that it is possible to use the microscope in combination with fluorescent elastomer tags to track individual zebrafish in tanks containing large numbers of animals.

Zebrafish are also a useful model in cancer research. Fluorescently labeled tumors can be generated in fish by a variety of methods²⁴ and are readily transplanted into either syngeneic or immune-ablated adult zebrafish^{5,19}. The microscope facilitates rapid screening of animals that bear fluorescently labeled tumors. For example, in **Figure 6**, a blue light with a 535/45-nm filter can detect a fish bearing transplanted T-ALL cells that express zsYellow (**Fig. 6a**), and is also capable of staging tumor progression (**Fig. 6b**). A green light with a 640/35-nm filter can distinguish a fish transplanted with an *mCherry*-labeled ERMS cell from 30 non-tumor-bearing fish (**Fig. 6c**). Although zsYellow and mCherry are the only fluorophores shown, tumors expressing AmCyan, GFP and dsRED can also be readily detected in transplanted animals¹¹.

In summary, the LED fluorescence microscope is the only imaging technique currently available that is capable of taking still images or live video of up to 30 adult fluorescent zebrafish simultaneously and in multiple fluorescent channels. The microscope is a new imaging technology that exploits the high-throughput nature of adult zebrafish research, and it will be useful in a variety of experimental systems, including imaging adult transgenic zebrafish, identifying tagged individuals for long-term analyses and rapid screening for tumor growth in transplanted animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The LED fluorescence microscope. **(a)** Top view, **(b)** front view and **(c)** side view of the inverted microscope, which is ideal for live imaging of zebrafish that have been injected with fluorescently labeled tumor cells or fluorescent elastomers in the peritoneum. **(d)** Top view, **(e)** front view and **(f)** side view of the upright microscope, which is watertight and suitable for taking both still images and live video of fluorescently labeled zebrafish.

Supplementary Manual 1 and **Supplementary Figures 1–12** provide a detailed protocol for building both the inverted and upright microscope models.

Figure 2.

The LED fluorescence macroscope can distinguish five fluorescent proteins in adult transgenic zebrafish. Whole animal imaging of wild-type (control), *mylz2-AmCyan* (AmCyan), *mylz2-GFP* (GFP), *mylz2-zsYellow* (zsYellow) and *mylz2-mCherry* (mCherry) transgenic zebrafish. (a–h) Examples of images of anesthetized fish, imaged from the side, which were acquired with (a–d) the inverted macroscope and (e–h) the upright macroscope. AmCyan (a,e) was imaged with a black light and a 480/30-nm emission filter, GFP (b,f) with a black light and a 535/45-nm filter, zsYellow (c,g) with a blue light and 575/52-nm filter and mCherry (d,h) was imaged using a green light and 640/35-nm emission filter. dsRed (not shown) can be imaged using a blue light and 610/40-nm emission filter. Note that the excitation lights and emission filters are not completely specific for individual fluorescent proteins. (b) The black light and 545/35-nm filter used to detect GFP also detects AmCyan. (c) AmCyan and dsRED bleed into the zsYellow channel, and zsYellow can be detected using the green light and 640/35-nm filter used to image mCherry (d). This type of cross-talk between fluorescent channels is not unique to the macroscope, and is commonly seen in epifluorescence dissecting microscopes, confocal microscopes and fluorescence-activated cell sorting. (i–l) Single fish used in a–h were imaged using an epifluorescence-equipped dissecting microscope at $\times 0.7$ magnification. (m–p) Three unanesthetized swimming transgenic zebrafish, which expressed the indicated fluorescent protein, were imaged from above with the upright macroscope. Scale bar in a–h is 2 cm; scale bar in m–p is 1 cm.

Figure 3.

Multispectral imaging of adult transgenic zebrafish using the upright LED fluorescence macroscope. (a–d) Unanesthetized transgenic fish expressing *mylz2-AmCyan* and *mylz2-GFP* were imaged using a black light and a 480/30-nm filter alone (a), a 535/45-nm filter alone (b) or with two cameras (c), each equipped with a single filter (multispectral). An *mylz2-GFP* and an *mylz2-zsYellow* fish were imaged with a blue light and 535/45-nm (d) or 640/35-nm (e) filter or both (f). (g–i) *mylz2-mCherry* and *mylz2-GFP* fish were imaged using a blue light and 640/35-nm (g) or 535/45-nm (h) filter, or both in combination (i). (j–l) The *mylz2-zsYellow* and *mylz2-mCherry* fish were imaged using a blue light and a 535/45-nm filter (j), a 640/35-nm filter (k) or both (l). Scale bar, 1 cm.

Figure 4.

Still images from a live video of swimming transgenic zebrafish using the upright macroscope. Unanesthetized, swimming transgenic fish expressing *mylz2-AmCyan* and *mylz2-mCherry* were imaged using black and blue lights and 640/35-nm and 535/45-nm emission filters. (a–l) Still images from the video demonstrate an image capture rate of 60 ms. Scale bar, 1 cm. A multispectral video of *mylz2-mCherry* and *mylz2-GFP* transgenic zebrafish can be seen in **Supplementary Movie 1**.

Figure 5.

The macroscope differentiates between zebrafish tagged with four fluorescent elastomers. Adult zebrafish were tagged in the abdomen and skeletal muscle with blue, green, yellow or red elastomers and screened for fluorescence after 7 d. (**a–d**) Elastomers were detected using a black light and a 480/30-nm emission filter (blue; **a**), a 475/52-nm filter (yellow; **b**), a 535/45-nm filter (green; **c**) or a 610/40-nm filter (red; **d**). As previously shown in **Figure 2**, the light and filter sets detect overlapping fluorescence from multiple fluorescent colors. Individual animals tagged with a single fluorescent elastomer can be easily distinguished when analyzed across several filter sets. Single fish was examined under an epifluorescence dissecting microscope at $\times 0.7$ magnification. (**e–h**) Blue (**e**), yellow (**f**), green (**g**) and red (**h**) elastomers are shown. Images in **a–d** were captured using the upright macroscope. Scale bar for **a–d**, 2 cm.

Figure 6.

The LED fluorescent macroscope facilitates rapid screening of tumor-bearing zebrafish. Adult syngeneic zebrafish were transplanted with T-ALL cells that co-expressed *zsYellow* or with ERMS cells that co-expressed *mCherry*. (a,b) The macroscope (a) can distinguish two T-ALL-bearing fish from wild-type fish and (b) is also useful in tumor staging. (c) One ERMS tumor-bearing fish can be distinguished from 29 wild-type fish. (d,e) Single fish bearing T-ALL (d) or ERMS (e) were imaged using an epifluorescence-equipped dissecting microscope, at $\times 0.7$ magnification. Images in a and b were acquired using the inverted macroscope, and the image in c was captured with the upright macroscope. Scale bar for a–c, 2 cm.

TABLE 1

Excitation lights and emission filters used in the macroscope to detect common fluorophores and elastomers.

Fluorescent protein	Excitation light color	Excitation spectra (nm)	Emission filter (nm)
AmCyan	Black light	405–420	480/30
GFP	Black light	405–420	535/45
zsYellow	Blue	420–470	575/52
dsREDExpress	Blue	420–470	610/40
mCherry	Green	500–570	640/35
Elastomer-blue	Black light	405–420	480/30
Elastomer-green	Black light	405–420	535/45
Elastomer-yellow	Black light	405–420	575/52
Elastomer-red	Black light	405–420	610/40

TABLE 2
Filters and lights used for microscope multispectral imaging of two fluorescent proteins.

	mCherry		zsYellow		GFP	
	Lights	Filters (nm)	Lights	Filters (nm)	Lights	Filters (nm)
AmCyan	Black light	535/45	Not able to be imaged		Black light	480/30
	Blue	640/35				535/45
GFP	Blue	535/40	Blue	535/40		
		640/35		640/35		
zsYellow	Blue	535/40				
		640/35				

TABLE 3

Troubleshooting table.

Step	Problem	Possible reason	Solution
5A(vii)	Elastomer is not detected by the macroscope	The elastomer plug is too small	Inject a larger volume of elastomer
		The elastomer or fish is not optimally positioned for detection	Use a ventral injection site for imaging with the inverted macroscope or a dorsal injection site for use with the upright model
		Incorrect filter is being used to detect fluorescence	Change the filter in the macroscope
5B(ix), 5C(iii)	Dead or mutated embryos	Too much DNA is injected into the embryo	Lower the DNA concentration or decrease the injection volume
5B(x), 5C(iv)	No fluorescence in the thymus, no tumors form	Plasmid DNA is not completely linearized	Do not use DNA that has been partially digested
		Not enough DNA was injected into the embryo	Increase the DNA concentration
		DNA was injected into the yolk or the embryo was too old	Only inject into one-cell-stage embryos
5D(vi)	Transplanted tumors do not grow in recipient fish	Insufficient numbers of tumor cells were transplanted	Increase the number of viable cells injected (RMS) or carry out fluorescence-activated cell sorting to purify fluorescently labeled tumor cells from nonfluorescent stroma (T-ALL)
		Tumor cells were not resuspended in the correct solution	Use 0.9x PBS
		Recovered immune system kills the tumor cells	Screen for tumor growth earlier than 30 d after transplant, or use a syngeneic strain of zebrafish
6A(iv)	Background is high in nonfluorescent fish	The wrong emission filter is used	Use the correct filter for the fluorophore being detected (Table 1)
		Exposure, gain and brightness need to be adjusted	Settings may need to be readjusted for each fluorophore used
6A(iv)	Fluorescence cannot be detected by the macroscope	The fluorescent protein is not expressed at high enough levels	Use an alternative transgenic line or promoter that drives higher transgene expression
		Fluorescence is focal or confined to a small region of the fish	The macroscope may not be able to be used for imaging. Instead, use an epifluorescence dissecting microscope for these applications
		GFP is often harder to detect than other fluorescent proteins as a result of autofluorescence of animals	Consider using AmCyan or dsRED, which are more readily detected by the macroscope