Cell tracking using a photoconvertible fluorescent protein

Kohei Hatta, Hitomi Tsujii & Tomomi Omura

Laboratory for Vertebrate Body Plan, Center for Developmental Biology, RIKEN Kobe 2-2-3 Minatojima Minamimachi, Chuo-ku, Kobe 650-0047, Japan.
Correspondence should be addressed to K.H. (khatta@cdb.riken.jp).

Published online 3 August 2006; doi:10.1038/nprot.2006.96

The tracking of cell fate, shape and migration is an essential component in the study of the development of multicellular organisms. Here we report a protocol that uses the protein Kaede, which is fluorescent green after synthesis but can be photoconverted red by violet or UV light. We have used Kaede along with confocal laser scanning microscopy to track labeled cells in a pattern of interest in zebrafish embryos. This technique allows the visualization of cell movements and the tracing of neuronal shapes. We provide illustrative examples of expression by mRNA injection, mosaic expression by DNA injection, and the creation of permanent transgenic fish with the UAS-Gal4 system to visualize morphogenetic processes such as neurulation, placode formation and navigation of early commissural axons in the hindbrain. The procedure can be adapted to other photoconvertible and reversible fluorescent molecules, including KikGR and Dronpa; these molecules can be used in combination with two-photon confocal microscopy to specifically highlight cells buried in tissues. The total time needed to carry out the protocol involving transient expression of Kaede by injection of mRNA or DNA, photoconversion and imaging is 2–8 d.

INTRODUCTION

To understand the embryogenesis of multicellular organisms, it is essential to be able to label cells noninvasively and to track cell fate, shape and migration at a high resolution. To achieve this, we have been exploring noninvasive labeling and imaging techniques in living vertebrates using fluorescent proteins whose color and intensity can be manipulated with light containing certain wavelengths[1–3]. One example of this type of protein is Kaede, isolated from the stony coral *Trachyphylia geoffroyi*: this protein is fluorescent green after synthesis but can be photoconverted red by violet or UV light[1]. The reversible green fluorescent protein Dronpa is a genetically engineered version of the green fluorescent protein cloned from Pectiniidae[2]. Its fluorescence can be switched on and off using two different light wavelengths: strong excitation at 488 nm bleaches Dronpa, and the bleached protein is able to completely regain its green fluorescence with minimal irradiation at 405 nm or 364 nm[4]. KikGR, a genetically engineered version of the green fluorescent protein KikG obtained from the coral *Favia favus*[3], is a green-to-red fluorescent protein similar to Kaede but more easily photoconverted.

Here we report a protocol that uses Kaede to track cells during embryogenesis in zebrafish by fluorescence microscopy as well as by confocal laser scanning microscopy, with technical tips for transient expression of Kaede by injection of mRNA or DNA and for efficient photoconversion and imaging of the embryo (**Figs. 1–4** and **Fig. 5a–g**). We inject mRNA for the ubiquitous expression of fluorescent proteins[4,5]. After cloning the cDNA encoding Kaede into an appropriate expression vector such as CS2+ (ref. 6), we synthesize capped mRNA *in vitro*. cDNA encoding Kaede may be cloned into an expression vector with tissue-specific[7], as well as inducible, regulatory elements (see ANTICIPATED RESULTS). In addition, the Gal4-UAS system[4,8–10] can be used to enhance expression and to improve specificity for transient mosaic expression (see below and ANTICIPATED RESULTS). Transgenic fish carrying UAS:Kaede (Tg(UAS:Kaede)) can be crossed with transgenic fish expressing Gal4 driven by a promoter of DeltaD (Tg(deltaD:Gal4))[11] to visualize neurons (**Fig. 5e,f**) and driven by a heat shock protein 70 promoter (Tg(hsp:Gal4))[12] to allow heat shock–inducible expression (**Fig. 5d,g**).

The technique involves changing the fluorescent color of cells in a pattern of interest defined by light, followed by time-lapse imaging. As well as allowing us to visualize previously known phenomena with improved visibility *in vivo*, the technique also has the potential to systematically uncover new cellular processes during vertebrate development (**Figs. 4, 6** and unpublished observation by K.H. and H.T.). Photoconversion is irreversible and stable, and resolution can be achieved at the single-cell level. The protocol also describes techniques for tracing neurons *in vivo* during imaging, including optical labeling of somas to visualize growth of neurites together with optical retrograde labeling that traces back from axons to somas. These basic procedures can be adapted to a number

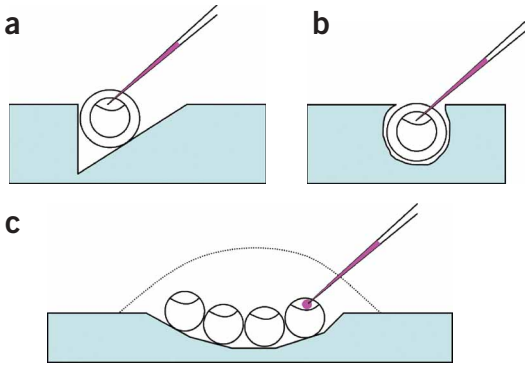

Figure 1 | Arranging eggs for microinjection. (**a**,**b**) An egg holder made of 4% agarose either sculpted by a razor blade (**a**) or made by using a glass microcapillary as a mold (**b**). (**c**) Dechorinated eggs arranged on a depression slide as a two-dimensional array. The appropriate diameter of the injected solution (shown in red) is approximately one-fourth the diameter of the egg.

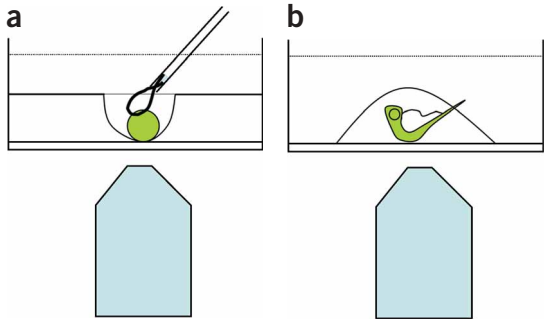

Figure 2 | Mounting embryos for photoconversion and observation. (**a**) A holder for younger embryos made of 1% LMP agarose using the bottom of a 0.5-ml PCR tube as a mold on a cover-glass-bottomed culture dish. A hair loop is used to carefully orient the embryo. (**b**) An older embryo mounted in a drop of 1% LMP agarose.

of other fluorescent proteins such as Dronpa[4] and KikGR (**Fig. 5h** and unpublished observations by K.H., H.T. and T.O.) and have the potential to be adapted in other vertebrates, including chicken and mice (unpublished observations by K.H. *et al.*).

Photoconversion techniques in a pattern of interest

Fluorescence microscope. Photoconversion of the fluorescence of Kaede from green to red can be easily achieved with a fluorescence microscope equipped with a filter set for DAPI. First, we made a spot of UV light at the center of the visual field by closing the aperture for excitation light. Photoconversion of circular area of diameter as small as 50 μm can be achieved using a 40× objective lens. To photoconvert a certain wide area of interest in a specific pattern, we moved the sample slowly, spending several seconds at each of the small overlapped parts, so that the area of interest was fully photoconverted (**Fig. 3a**). A pinhole[13] or a slit (100 μm × 4 mm) plate, set at the focal plane for the excitation light, might be able to replace the aperture in some microscopes. This allows photoconversion of a smaller area, or in a straight line if necessary.

Confocal laser scanning microscope. Photoconversion can be conveniently achieved by scanning with a 405-nm diode laser or a 364-nm UV laser. To photoconvert in the desired pattern, use the region of interest (ROI) and 'bleach' function to draw a pattern of interest programmed ahead of time (**Fig. 3b,c**). If the confocal microscope does not have such functions, simply scan the visual window with 405-nm (or 364-nm) laser. By doing this, the square area of the visual window can be specifically photoconverted. By magnifying the view, it is possible to cover an area as small as a single cell ($\sim 5 \times 5$ μm). Be aware of the bleaching of both green and red Kaede by strong excitation light, especially under higher magnification. Lowering the power of the laser and making the speed of scanning faster is helpful in avoiding bleaching.

Two-photon confocal laser scanning microscope. With conventional confocal microscopy, it is difficult to specifically highlight a single cell or cells that are buried in cell layers expressing the same photoconvertible proteins, because of difficulty in avoiding unintended photoconversion of the cells in the light path out with the focal plane. If it is possible, however, to use two-photon confocal microscopy for photoconversion, in which a laser whose wavelength is two times longer than usual is used, this allows the highlighting of cells buried inside of tissues without activating neighboring cells in the light path. While it has proven difficult to efficiently photoconvert Kaede with two-photon microscopy, we have been able to photoactivate Dronpa using two-photon microscopy with a laser tuned at 780 nm[4]. Although the use of two-photon microscopy required approximately 1,000 times more scanning than conventional microscopy (over several minutes) for photoactivation, it did improve resolution along the *z* (depth) axis, and it was possible to label a single cell[4]. KikGR was also photoconverted by the two-photon microscopy in a cell line[3] as well as *in vivo* (**Fig. 5h**) by using a longer scanning period than the one used in photoactivation of Dronpa.

Optical labeling of neurons

As photoconverted red Kaede diffuses in the cytoplasm, it is possible to label an entire neuron either in an anterograde or retrograde fashion.

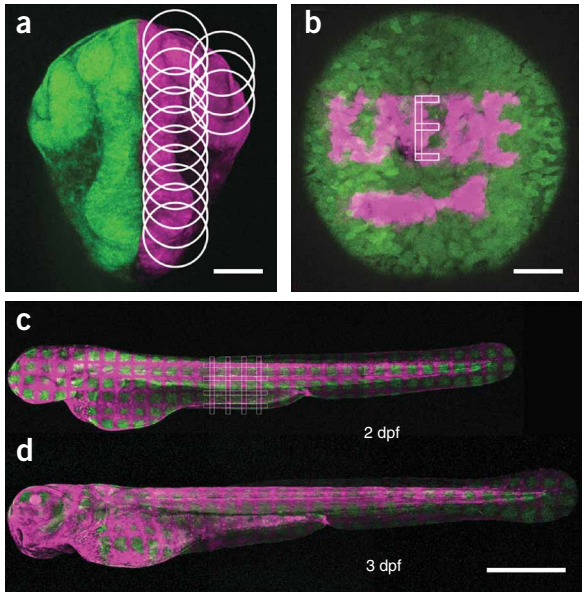

Figure 3 | Photoconversion in desired patterns. Kaede mRNA was injected at the one-cell stage. All images were taken by confocal microscope. (**a**) Schematic representation of the method for photoconverting the right side of the brain using a fluorescence microscope. Dorsal view of the brain at 1 d post-fertilization (dpf). Anterior is to the top. Aperture for the excitation light was closed and reduced in size. UV light was passed through a filter set for DAPI and was applied to the embryo; the stage for the embryo was slowly moved (several seconds at each white circle) to cover the whole area. Scale bar: 50 μm. (**b**) Imprinting the letters "KAEDE" and outline of fish by photoconversion on the embryo at the neurula stage (around 10 h post-fertilization (hpf)) using the ROI and bleach functions of the confocal microscope. Dorsal view; anterior is to the top. Scale bar: 30 μm. See **Supplementary Video 1** for movement of labeled cells over the next 1 h. (**c**) Schematic representation of the method for imprinting grids covering the larva to track morphogenesis by confocal microscopy (lateral view). A small grid (in white lines) was prepared by using the ROI function. At 2 dpf, the larva was scanned with a 405-nm laser using the bleach function. This procedure was repeated to cover the entire larva. Anterior is to the left. Dorsal is to the top. (**d**) The same larva at 3 dpf. Regional differences in growth and rearrangement can be detected by following the deformation of the grid. Scale bar: 500 μm.

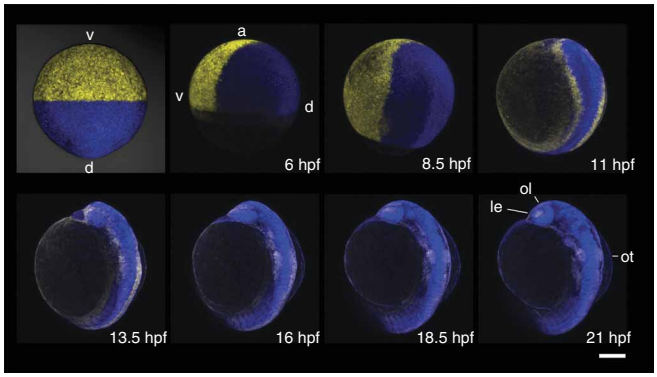

Figure 4 | Four-dimensional imaging of convergent-extension movement of neural plate and organogenesis derived from placodes in the zebrafish embryo. The embryo was injected with Kaede mRNA at the one-cell stage. At the early gastrula stage (at about 6 hpf), the ventral half (v) of the embryo was specifically scanned from the animal pole (a) with a 405-nm laser. The green fluorescence of Kaede at the dorsal side (d) was pseudocolored in blue, and the photoconverted red fluorescence at the ventral side (v), in yellow. Note that the dorsal side participated in the central nervous system. In contrast, the ventral side participated in placodal structures. During neurulation, cells derived from the ventral side accumulated at the periphery of the neural plate, then split, aggregated, and formed olfactory placode (ol), lens (le), otic vesicles (ot) and other sensory placodes. Scale bar: 100 μm (see also **Supplementary Video 2**).

Optical labeling from somas. Select a soma and irradiate it with UV or a 405-nm laser. Red fluorescence diffuses into axons, dendrites and fine structures such as growth cones and synapses (**Fig. 5b–d**). The time for diffusion is most likely dependent on the shape of the neuron and can range from seconds to hours in the zebrafish embryo. The red fluorescence is relatively stable *in vivo* and we have been able to track the same neuron usually for a few days. While the red Kaede is diffused away from the area of irradiation, green Kaede comes in to replace it. Moreover, green Kaede is often continuously synthesized in the cell. In this way, repetitive irradiation at intervals in the same region can increase the red-to-green ratio of the fluorescence.

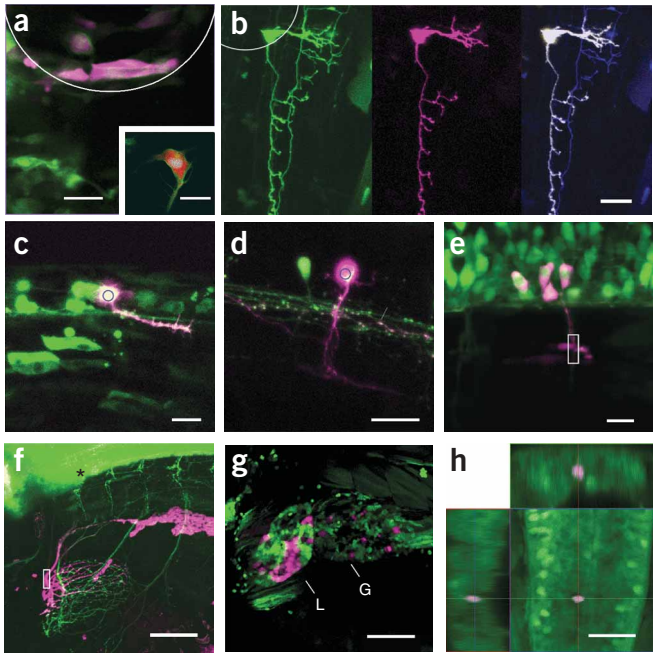

Figure 5 | Tracing neurons and other cell types using Kaede and KikGR combined with the Gal4-UAS system in either transient or permanent transgenic fish. (**a**) Coexpression of Kaede localized in the cytoplasm and nuclei, membrane-bound GFP (Lyn-GFP fusion protein), and nuclear-localized YFP (histone 2B (H2B)-YFP fusion protein, in green) in the neuroepithelial cells in the hindbrain at ~18 hpf. Anterior is to the top. The fertilized egg was coinjected with pEF:Gal4VP16-Ulyn-UH2B[10] and UAS:Kaede (see ANTICIPATED RESULTS). Gal4VP16 was driven by the promoter of α-elongation factor. A broad area on the top (white circle) was photoconverted by irradiation with UV light using a fluorescence microscope. See **Supplementary Video 3**. Images were taken every 2 min, and embryos were irradiated briefly with UV during each interval. Inset shows that these three fluorescent proteins could be discriminated using the META function (LSM510, Zeiss) in a neuron. GFP: green; red Kaede: red; YFP: pseudocolored in light blue. (**b**) Optical anterograde labeling of a single neuron in the hindbrain by a spot of UV at 2 dpf. pEF:Gal4VP16 (ref. 10) and UAS:Kaede were coinjected. Images of green fluorescence (left), red fluorescence (middle), and the merge of these two in pseudocolors (yellow in place of red and blue in place of green). (**c**) Optical anterograde labeling by 405-nm laser scanning at the soma (circled) of a single spinal neuron extending an axon with a growth cone and actively moving filopodia at ~26 hpf. A fertilized egg was coinjected with pEF:Gal4VP16 (ref. 10) and pUAS:Kaede-UAS:lynGFP so that Kaede was coexpressed with membrane-localized GFP. Anterior is to the right, dorsal is to the top (see **Supplementary Video 4**). The images captured every 2 min after photoconversion in the soma shows diffusion of the red Kaede along the axon and into the growth cone and filopodia. (**d**) Optical anterograde labeling of internal neurons expressing Kaede at the anterior spinal cord in the cross between Tg(hsp:Gal4)[14] and Tg(UAS:Kaede) (see ANTICIPATED RESULTS). It was possible to identify the origin of some synapses (indicated by a line). Gal4 was driven by the heat-shock protein 70 promoter. This expression was observed without heat shock in this line. Anterior is to the right; dorsal is to the top. (**e**) Optical retrograde labeling of spinal motoneurons from the ventral root in the cross between Tg(deltaD:Gal4)[11] and Tg(UAS:Kaede) at ~30 hpf. Gal4 was driven by the deltaD promoter. An axon bundle (enclosed by a rectangle) was scanned several times with intervals over 30 min. Note that the nuclei are a stronger green than the cytoplasm, suggesting that the diffusion of red Kaede into the nuclei from the cytoplasm is not as efficient as among the cytoplasm of the axon and the soma. Anterior is to the left. (**f**) Optical bidirectional (both anterograde and retrograde) labeling of motoneurons innervating the pectoral fin at 6 dpf in the cross between Tg(deltaD:Gal4) and Tg(UAS:Kaede). A portion of a thick bundle of nerves (enclosed by a rectangle) was repeatedly scanned every 10 min with a 405-nm laser over 2 h and 20 min. It visualized the soma of origins (indicated by asterisk) as well as the terminal field of the axons in the muscles in the pectoral fin. See **Supplementary Video 5**. Images were taken every 10 min after each photoconversion. Note the red autofluorescence by pigment cells on the left. Labeled fibers in the spinal cord were not background. They were due to the other photoconversion experiment prior to the time lapse in the CNS outside of the visual field. Anterior is to the left. (**g**) Liver (L) and gut (G) at 4 dpf visualized in a cross between Tg(hsp:Gal4) and Tg(UAS:Kaede) that was raised at 28.5 °C to 3 dpf, preincubated at 23 °C for 1 h, heat shocked at 37 °C for 1 h, then kept at 28.5 °C for 1 d. The organs were partially photoconverted by 405-nm laser scanning. Lateral view; anterior is to the left. (**h**) Photoconversion of KikGR using two-photon excitation microscopy with a laser tuned at 780 nm. A single cell was selectively labeled in the ventral spinal cord in the cross between Tg(UAS:KikGR) and Tg(deltaD:Gal4) after about 10 min of focused scanning at the center of the cell. An optical horizontal section of the dorsal view (anterior, to the top) with the reconstructed transverse section from the 3D data on the top (dorsal, to the top) and the reconstructed sagittal section on the left (dorsal, to the left). Surrounding cells were not labeled significantly. Scale bars: **a**–**e**,**h**, 20 μm; **f**,**g**, 100 μm.

Optical retrograde labeling from axons. Using retrograde labeling, the soma of origin can be traced back by irradiating part of the axon. The success of retrograde labeling is largely dependent on the ratio between the volume of axon in the area of irradiation and that of the whole cell. By repetitive irradiation, we have been able to enhance the strength of the red fluorescence (**Fig. 5e**).

Optical bidirectional labeling. The non-invasive nature of the labeling technique allows photoconversion of Kaede at a segment of axon (bundles) by repeated irradiation to trace the somas of origin and the terminal field simultaneously (**Fig. 5f**).

Use of Gal4-UAS system to express photoconvertible proteins
The system uses the yeast transcriptional activator Gal4 and UAS—the sequence of the DNA-binding motif of Gal4. In the activator lines, the gene for Gal4 is under the control of a promoter, while in the effector lines, multiple copies of the UAS sequence is fused to the gene to be expressed.

Transient expression by coinjection of a promoter:Gal4 construct and pUAS:Kaede. In general, we obtained stronger expression of the reporter by coinjection of a promoter and pUAS:reporter compared with the injection of a promoter:reporter. Even stronger expression was obtained if we used Gal4VP16 instead of Gal4 (ref. 10). For example, when expressing Kaede in neurons, either pDelta:Gal4, pHuC:Gal4 or (more ubiquitous) pEF:Gal4 with UAS:Kaede can be coinjected. This also allows coexpression of different fluorescent proteins (such as membrane-bound GFP with Kaede) to enhance visibility of the fine structure of interest (**Fig. 5a,c**) as well as functional proteins or their constitutively active or dominant-negative forms, in a tissue-specific or conditional manner in order to evaluate their function in development (K.H., S. Aramaki, M. Takeuchi and H.T., unpublished observations).

Transient expression in improved specificity by injection of a UAS construct into a Gal4-transgenic line. In general, transient expression of injected DNA tends to cause non-specific expression. The most dramatic case we found was that injection of pHSP:Kaede (heat shock protein 70 promoter[12]–driven Kaede) often caused abundant non-specific expression without heat shock. In such cases, a UAS construct can be injected to a Gal4 transgenic line to reduce the non-specific expression[4]. By injecting UAS:Kaede into Tg(hsp:Gal4)[14], we were able to dramatically reduce non-specific transient expression of Kaede (data not shown), and we were able to induce its expression after heat shock. This is probably due to the fact that fidelity of UAS as enhancer is much stricter than the HSP promoter.

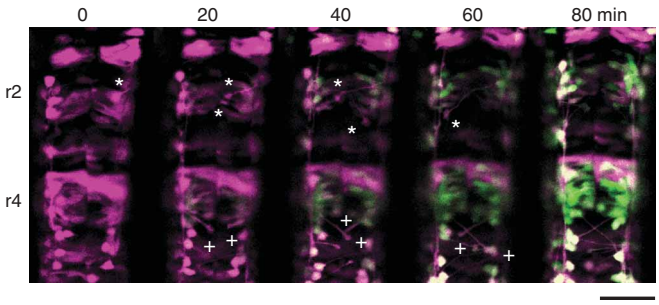

Figure 6 | Tracking some of the earliest commissural axons navigating in the hindbrain in the cross between Tg(deltaD:Gal4) and Tg(UAS:Kaede), dorsal view. Anterior is to the top. The embryo was photoconverted entirely with 405-nm laser scanning at about 19.5 hpf, so that the neurons differentiated and expressed Kaede driven by the deltaD promoter by then could be labeled in red. The neurons at rhombomere 4 (r4) extending red axons with prominent growth cones (+) and crossing at the midline are likely to be Mauthner cells, and the ones at rhombomere 2 (r2; *) are likely to be RoL2. Scale bar: 50 μm (see **Supplementary Video 6**, showing 2 h of development, at 10-min intervals).

Crossing of Gal4 and UAS transgenic lines. By crossing transgenic lines carrying UAS:Kaede (**Figs. 5d–g** and **6**), UAS:KikGR (**Fig. 5h**), or UAS:Dronpa[4] with appropriate Gal4 lines, a number of different organs and neural networks can be visualized.

MATERIALS

REAGENTS

- pKaede (commercially available from MBL)
- Embryo medium (EM) consisting of 10% HANKS' solution with Ca^{2+} at the concentration of the normal HANKS' solution (see REAGENT SETUP)
- Methylene blue (Sigma-Aldrich)
- Protease (Pronase; Sigma-Aldrich)
- 0.5% Phenol red (10–25×; Sigma-Aldrich)
- 100 mM KCl
- PTU (1-phenyl-2-thiourea; Sigma-Aldrich)
- Tricaine (MS222 or MESAB: 3-amino benzoic acidethylester, Sigma-Aldrich)
- LMP (low–melting point) agarose
- Agarose
- Agar
- RNase free water

REAGENT SETUP

Artificial fresh water Either a bottle of commercial "mineral water" or 60 mg sea salt (e.g., Instant Ocean) in 1 l distilled water.

Embryo medium (EM or E2) Dissolve 17.5 g NaCl, 0.75 g KCl, 4.9 g $MgSO_4 \cdot 7H_2O$, 0.41 g KH_2PO_4, 0.36 g $Na_2HPO_4 \cdot 12H_2O$ in 1 l water (20×E2 stock). Dissolve 7.35 g $CaCl_2 \cdot 2H_2O$ in 100 ml water (500 × $CaCl_2$ stock). Dissolve 3 g $NaHCO_3$ in 100 ml water (500 × $NaHCO_3$ stock). Autoclave the stock solutions and store them at 4 °C. Mix 50 ml E2 stock, 2 ml of $CaCl_2$ stock, 2 ml of $NaHCO_3$ stock and distilled water to make them up to 1 l. pH should be 7 to 7.5.

Methylene blue stock solution (333×) Dissolve at 0.01% (wt/vol) in water. Add 3 ml stock solution to 1 l EM to prevent growth of bacteria and mold during culture.

HEPES-buffered EM 10 mM HEPES (pH 7.2) may be added to stabilize pH during extended time-lapse recordings[15]. Add 2.38 g HEPES to 1 l EM. Adjust pH to pH 7.2 using 1 M NaOH.

Protease stock solution (2.5–10 ×) Dissolve 5 mg protease in 1 ml E.M. and store at –20 °C.

Tricaine stock solution (40–50 ×) Dissolve 0.4 g Tricaine, 2 g $Na_2HPO_4 \cdot 12H_2O$ in 50 ml water. pH should be 7 to 7.5. Store at 4 °C.

PTU stock solution (50 ×) Dissolve 150 mg PTU in 100 ml water with a stirrer; it may take several hours to dissolve. Store at 4 °C. Use gloves, since PTU is toxic.

1% agarose in EM to coat culture dishes.

4% agarose in water to make egg holders for microinjection.

1% LMP agarose in EM to mount embryos for observation.

0.1% agar in EM to prevent rotation of embryos at early stages.

EQUIPMENT

- Necessary equipment for raising fish and collecting eggs (see the *Zebrafish Book*[16] for details)

- Necessary equipment to clone DNA and synthesize RNA
- DNA purification kit (Qiagen)
- mMESSAGE mMACHINE kit (Ambion)
- Fluorescence dissection microscope equipped with a filter set for GFP
- Either inverted fluorescence microscope equipped with filter sets for DAPI, GFP and rhodamine, or inverted confocal microscope equipped with lasers for 405 nm (or 364 nm), 488 nm and 543 nm.
- Micropipette puller (Model P-97/IVF, Sutter Instrument Co.)
- Microinjector using air pressure (IM-30 (Narishige)) with micropipette holder and tubing
- N_2 tank with a regulator
- Micromanipulator (Narishige)
- Fine pairs of forceps
- Glass microcapillary with or without an internal glass fiber (external diameter of 1 mm)
- Depression slide glass
- Humidity chamber (a glass jar or a Petri dish with capillary holder containing some water at the bottom)
- Hair loop made with a human hair attached with super glue to a microcapillary tube (**Fig. 2a**)
- Cover-glass-bottomed culture dish (e.g., Lab-Tek Chambered Coverglass (Nunc))

PROCEDURE

Expressing Kaede in the zebrafish embryo

1| Prepare mRNA (option A) or DNA (option B) for injection.

(A) mRNA

(i) For mRNA injection to achieve ubiquitous expression, clone the cDNA encoding Kaede from pKaede into an appropriate expression vector such as CS2+ (ref. 6).

(ii) Synthesize capped mRNA *in vitro* using the mMESSAGE mMACHINE kit (Ambion) following the manufacturer's instructions.

(B) DNA

(i) For DNA injection to achieve transient mosaic expression, clone the cDNA encoding Kaede into a vector with a ubiquitous, tissue-specific, or inducible promoter/enhancer. See ANTICIPATED RESULTS for references.

■ **PAUSE POINT**

▲ **CRITICAL STEP** Purify DNA using a Qiagen plasmid purification kit following the manufacturer's instructions. In case the DNA solution proves toxic to the embryo, prepare plasmid DNAs using an Endofree plasmid kit (Qiagen)[10]. For transient expression, inject either circular or linearized DNA. To obtain permanent transgenic lines, we often inject linearized DNA into eggs; the rate of germline transmission is approximately 2–5%.

2| One day before carrying out the experiment, set the male and female fish as a pair or as a group in a tank with marbles or a net at the bottom to keep the fish from eating their eggs.

3| Construct micropipettes for injection by pulling a microcapillary with an internal fiber using a puller.

▲ **CRITICAL STEP** A glass capillary lacking an internal fiber may be used. In this event, the tip of the injection needle should be first broken and the solution sucked through the broken end, either by the reduced air pressure function equipped on the injector, by mouth pipette, or by piston and syringe, instead of Steps 4 and 7.

4| Fill micropipettes with either DNA (option A) or mRNA (option B) solution.

(A) DNA

(i) Apply 1–3 µl DNA solution (20 ng/µl) in 100 mM KCl containing 0.02% phenol red at the flat end of the microcapillary in a humid moist chamber. The tip of the micropipette should gradually fill with the solution.

(B) mRNA

(i) Alternatively, fill the micropipette with the mRNA (500 ng/µl) in RNase-free H_2O containing 0.02% phenol red.

5| Collect eggs using the siphon and net and clean them by transfer with a pipette (zebrafish will lay eggs at dawn within 1 h after the light is turned on).

6| Inject eggs retaining chorion (option A), or remove the chorion first and then inject them (option B).

(A) Eggs with chorion

(i) Arrange the eggs on an egg holder made with 4% agarose in a dish (**Fig. 1a,b**). These eggs can be manipulated and raised either in the fish tank water (artificial fresh water) or in EM (see MATERIALS for recipe) containing 0.3 ppm methylene blue.

(B) Eggs without chorion

(i) Alternatively, dechorionate fertilized eggs before injection at the one-cell stage either by tearing manually with a pair of fine forceps, or by digesting with 0.5–2.0 mg/ml protease in EM for 1–6 min at room temperature followed by washing three times with EM or fish tank water.

(ii) Raise dechorinated embryos in the EM containing 0.3 ppm methylene blue on a 1% agarose-coated culture dish. Arrange dechorinated eggs on a depression slide (**Fig. 1c**).

▲ **CRITICAL STEP** Construct the egg holder by either sculpting a groove using a laser blade (**Fig. 1a**) or using a mold such as a 1-mm-diameter capillary (**Fig. 1b**).

7| Using a fine pair of forceps, break the tip of the DNA- or mRNA-filled micropipette just before injection under a dissection microscope. Then attach the micropipette to the micropipette holder connected to the tubing from the microinjector and N_2 tank. Use a manipulator to move the injection needle to penetrate the egg. Subsequently, inject DNA or mRNA into the cytoplasm of one- to four-cell-stage embryos using air pressure. The pressure and duration of injection will vary considerably depending on the micropipette. Adjust them so that the diameter of the injected solution is approximately one-fourth the diameter of the egg (**Fig. 1c**).

8| Raise the injected embryos at 28.5 °C in an incubator to obtain a standard developmental speed[17] or at 23 °C to obtain an approximately 1.6-times slower developmental speed. Remove dead embryos with a Pasteur pipette or by replacing the dish occasionally to ensure it is kept clean.
▲ **CRITICAL STEP** Dead embryos may cause delayed development of their neighbors.

9| Monitor the expression of green Kaede in the embryo using a dissection microscope equipped with a GFP filter set. The exact time for the start of expression after injection of DNA depends on the promoter/enhancer used in the expression vector. Expression of the fluorescence after injection of mRNA will be evident before gastrulation (4–5 h post-fertilization (hpf)).
▲ **CRITICAL STEP** Conventional interior lighting, including regular fluorescent lamps and halogen lamps used in dissection and microscopic observation, will not photoconvert Kaede. Researchers should be aware, however, that natural light can convert the color of Kaede[1].

10| Transfer embryos expected to have pigmentation during observation (beyond 24 hpf) to a solution of EM containing 0.003% PTU before 24 hpf to inhibit pigmentation. Alternatively, mutant strains with less pigmentation such as golden (gol^-/gol^-)[18] can also be used.

Mounting embryos

11| A range of options exist for mounting embryos, depending on their age and purpose: option A (for embryos under 10 hpf), option B (for embryos under 10 hpf to avoid unintended rotation during observation), or option C (for embryos older than 10 hpf) can be used, depending on your requirements.
▲ **CRITICAL STEP** Embryos that have already started moving (after 16 hpf) should be anesthetized with 0.16–0.2 mg/ml Tricaine in EM, both prior to mounting and during the entire period of observation.

(A) Embryos that have not reached 10 hpf (Fig. 2a)

(i) Set the dechorinated embryo free in a small hole made in a layer of 1% LMP agarose using a 0.5-ml PCR tube as a mold on a cover-glass-bottomed culture dish filled with (HEPES-buffered) EM.

(ii) Carefully rotate the embryo with a hair loop so that the desired aspect of the embryo faces the bottom.

(B) Avoiding unintended rotation of embryos under 10 hpf (Fig. 2a)

(i) Place the embryo in melted and cooled 0.1% agar in EM, transfer it into the hole together with the agar, and quickly rotate the embryo with a hair loop to fix its orientation. This prevents the embryo from undergoing any unexpected rotation without disturbing epiboly and gastrulation.

(C) Embryos older than 10 hpf (Fig. 2b)

(i) Place the embryo into melted and cooled 1% LMP agarose in EM and transfer the embryo directly to a cover-glass-bottomed culture dish in a drop of LMP agarose before quickly rotating the embryo with a hair loop to fix its orientation so that the area of interest faces the bottom. Wait for a few minutes. Carefully fill the dish with (HEPES-buffered) EM.

Photoconversion

12| Photoconversion can be achieved conveniently with an inverted fluorescence microscope (option A) or with an inverted confocal microscope (option B).
? TROUBLESHOOTING See **Table 1** to solve commonly occurring problems during photoconversion and observation.
▲ **CRITICAL STEP** See INTRODUCTION for techniques for photoconversion in a pattern of interest and techniques used to trace neurons.

(A) Fluorescence microscope

(i) First, use excitation light from a mercury lamp passed through the GFP filter set to observe green fluorescence. Adjust the focal plane on the cells of interest. Close the aperture for the excitation light and make a small circular window so that the cells to be labeled are within that spot, and then switch to the filter set for DAPI.

(ii) Photoconvert green Kaede to red Kaede by irradiating with UV light passed through the filter set for DAPI. The exposure time required for photoconversion varies (from a few seconds to a few minutes) depending on the intensity and the magnitude of the objective lens. In our standard procedure, using UV light at the maximum strength and a 20× objective lens, it took about 10 s for complete photoconversion.

(iii) To observe photoconverted red Kaede, use the filter set for rhodamine. Switch to the rhodamine filter set to monitor the appearance of red color, and then switch back to the GFP filter set to see whether the green color has diminished.

(iv) Repeat UV irradiation until the green fluorescence has been eliminated and the increase in the brightness of red fluorescence reaches a plateau.
▲ CRITICAL STEP It is recommended to monitor the change of color at the beginning of the new experiment in order to obtain the maximum efficiency of photoconversion but to avoid excessive exposure to UV.

(B) Confocal microscope

(i) Image embryos expressing green Kaede as for GFP with a 488-nm argon laser and BP505-530 filter, and select the ROI.
(ii) To photoconvert Kaede, scan the region with a 405-nm diode laser. We found that the time for scanning varied (from a fraction of second to a few minutes) depending on the strength of the laser, the size and complexity of selected region, and the magnitude of the objective lens.
(iii) Image the signal of red Kaede as for rhodamine with a 543-nm helium-neon laser and a LP560 filter.
(iv) Repeat scanning with 405 nm until the green fluorescence has been eliminated and the increase in the brightness of red fluorescence reaches a plateau.

Observation and imaging

13| Capture images of both green and red fluorescence regularly to trace the shape and movement of the cells labeled in red. Images of *z*-slices at multiple time points (four-dimensional imaging or time-lapse imaging) of several embryos (multi-point imaging) can be obtained by laser confocal microscopy equipped with the appropriate software. Kaede may be as harmless as GFP, although extremely strong expression of either can damage cells. The photoconversion of Kaede is irreversible, and both green Kaede and red Kaede are fairly stable in the embryo. Usually, we were able to track red cells over 1 d and sometimes over 5 d. If the interval for observation is too long, then the mounted embryo can be taken out of the agarose and transferred to the culture medium (EM) until the next imaging, since the embryo can often be deformed without extending its body axis properly in the agarose.

● TIMING

After construction of plasmids, 1 d for preparing DNA or RNA for injection and the crossing fish to obtain fertilized eggs; 1 to 7 d for photoconversion, observation and imaging.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	SOLUTION
Some fluorescent cells die and become fragmented.	Extremely high level of expression may kill cells. Lower the expression of the fluorescent proteins by injecting a decreased amount of DNA or mRNA.
Cells have red fluorescent color from the beginning.	1. *Specific signal from red Kaede.* When the expression of Kaede is very high, background red fluorescence by residual red Kaede may become evident. This can be addressed by lowering the gain for imaging of red fluorescence and merging with green fluorescence. 2. *Nonspecific autofluorescence by pigment cells or by other cell types.* Cells often show strong red and/or green fluorescence. As the color of fluorescence cannot be converted after irradiation with UV or violet light, then these cells must be ignored (**Fig. 5f**).
Spontaneous photoconversion from green to red occurs during observation.	Check the filters used for observation of green and red fluorescence. Some filters may allow light containing unwanted short wavelengths (with the potential to cause photoconversion) to pass through.
Both green and red signals gradually decrease during observation or photoconversion.	If the excitation light is too strong, both green Kaede and red Kaede are susceptible to bleaching. Lower the strength of light or reduce the time needed for observation.

ANTICIPATED RESULTS

Various applications of the photoconversion technique are illustrated in **Figures 3–6**. See also **Supplementary Videos 1–6** online for time-lapse illustration of the methods. We used the Gal4-UAS system[9,10] to enhance expression of Kaede or other photoconvertible fluorescent proteins in a transient expression as well as to increase its specificity[4] (see INTRODUCTION for details). We also generated permanent transgenic lines carrying UAS:Kaede (**Figs. 5d–g** and **6**), UAS:KikGR (**Fig. 5h**), or UAS:Dronpa[4]. These lines will be available from the Zebrafish International Resource Center (ZIRC; Eugene, Oregon, USA). By crossing these lines with appropriate Gal4 lines, a number of different organs and neural networks can be visualized (**Figs. 5d–h** and **6**).

The stages of zebrafish embryos were determined by morphology and by time after fertilization (in hours post-fertilization (hpf)) at 28.5 °C (ref. 17). The protocol concerning the practical use of Kaede *in vivo* was developed directly by the authors listed on the title page, while other procedures were adapted from methods developed by the zebrafish community. See ref. 16 (*The Zebrafish Book*), ref. 19 (*Zebrafish*) and the Zebrafish Information Network (Zfin) website (http://mirror.zfin.org/index.html) for basic procedures concerning zebrafish. See ref. 15 for basic procedures concerning imaging. See ref. 4 for the use of Dronpa *in vivo*. KikGR was used technically in the same way as Kaede in zebrafish. pUAS:Kaede, pHSP:Kaede, pUAS:Kaede-UAS:lynGFP (pUKUlyn), and Tg(UAS:Kaede) were generated by H.T. and K.H. Tg(UAS:KikGR), pHuC:Gal4 (Gal 4 driven by HuC promotor [20,21]) were generated by T.O. and K.H. These transgenic fish were generated using T2-transposon-mediated gene transfer[4,22,23].

Some of the data for application of Kaede *in vivo* were previously presented at the 6th International Meeting on Zebrafish Development and Genetics at Madison, Wisconsin, USA, by K.H. and H.T. in 2004. Part of the original data from which **Figure 4** and **Supplementary Video 2** were generated were used in reference 24 (*Developmental Biology*) with the permission of the authors and the publishers.

Note: Supplementary information is available on the Nature Protocols website.

ACKNOWLEDGMENTS We thank S. Aizawa for support; S. Hayashi for encouragement; S. Aramaki, H. Togashi, S. Yonemura, M. Hibi and K. Agata for help; M. Royle for comments and J. Clarke for encouraging us to publish this protocol. pKaede[1] and pKikGR[3] were kindly provided by A. Miyawaki; pEF:Gal4VP16, pUlyn and pEF:Gal4VP16-UlynU2B[17] by R. Köster and S. Fraser; pDeltaD:Gal4 (ref. 11) by N. Sheer and J. Campos-Ortega; the HuC promoter[20,21] by S. Higashijima; the HSP promoter[12] by W. Shoji; vectors for T2 transposon–mediated gene transfer[22,23] by K. Kawakami and Tg(deltaD:Gal4)[11] and Tg(hsp:Gal4)[14] by the Zebrafish International Resource Center. K.H. received grants-in-aid for Scientific Research from The Ministry of Education, Culture, Sports, Science, and Technology of Japan.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 12651–12656 (2002).
2. Ando, R., Mizuno, H. & Miyawaki, A. Regulated fast nucleocytoplasmic shuttling observed by reversible protein highlighting. *Science* **306**, 1370–1373 (2004).
3. Tsutsui, H., Karasawa, S., Shimizu, H., Nukina, N. & Miyawaki, A. Semi-rational engineering of a coral fluorescent protein into an efficient highlighter. *EMBO Rep.* **6**, 233–238 (2005).
4. Aramaki, S. & Hatta, K. Visualizing neurons one-by-one *in vivo*: Optical dissection and reconstruction of neural networks with reversible fluorescent proteins. *Dev. Dyn.* **235**, 2192–2199 (2006).
5. Ciruna, B., Jenny, A., Lee, D., Mlodzik, M. & Schier, A.F. Planar cell polarity signalling couples cell division and morphogenesis during neurulation. *Nature* **439**, 220–224 (2006).
6. Turner, D.L. & Weintraub, H. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434–1447 (1994).
7. Sato, T., Takahoko, M. & Okamoto, H. HuC:Kaede, a useful tool to label neural morphologies in networks *in vivo*. *Genesis* **44**, 136–142 (2006).
8. Fischer, J.A., Giniger, E., Maniatis, T. & Ptashne, M. GAL4 activates transcription in *Drosophila*. *Nature* **332**, 853–856 (1988).
9. Scheer, N. & Campos-Ortega, J.A. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. *Mech. Dev.* **80**, 153–158 (1999).
10. Köster, R.W. & Fraser, S.E. Tracing transgene expression in living zebrafish embryos. *Dev. Biol.* **233**, 329–346 (2001).
11. Scheer, N., Groth, A., Hans, S. & Campos-Ortega, J.A. An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* **128**, 1099–1107 (2001).
12. Halloran, M.C. *et al.* Laser-induced gene expression in specific cells of transgenic zebrafish. *Development* **127**, 1953–1960 (2000).
13. Girdham, C.H. & O'Farrell, P.H. The use of photoactivatable reagents for the study of cell lineage in *Drosophila* embryogenesis. *Methods Cell Biol.* **44**, 533–543 (1994).
14. Scheer, N., Riedl, I., Warren, J.T., Kuwada, J.Y. & Campos-Ortega, J.A. A quantitative analysis of the kinetics of Gal4 activator and effector gene expression in the zebrafish. *Mech. Dev.* **112**, 9–14 (2002).
15. Cooper, M.S., D'Amico, L.A. & Henry, C.A. Confocal microscopic analysis of morphogenetic movements. *Methods Cell Biol.* **59**, 179–204 (1999).
16. Westerfield, M. *The Zebrafish Book: a Guide for the Laboratory Use of Zebrafish (Danio rerio)* 4th edn. (Univ. of Oregon Press, Eugene, Oregon, 2000).
17. Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B. & Schilling, T.F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995).
18. Streisinger, G., Coale, F., Taggart, C., Walker, C. & Grunwald, D.J. Clonal origins of cells in the pigmented retina of the zebrafish eye. *Dev. Biol.* **131**, 60–69 (1989).
19. Nüsslein-Volhard, C. & Dahm, R. *Zebrafish*. Practical Approach Series No. 261 (Oxford University Press, Oxford, 2002).
20. Park, H.C. *et al.* Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Dev. Biol.* **227**, 279–293 (2000).
21. Higashijima, S., Masino, M.A., Mandel, G. & Fetcho, J.R. Imaging neuronal activity during zebrafish behavior with a genetically encoded calcium indicator. *J. Neurophysiol.* **90**, 3986–3997 (2003).
22. Kawakami, K., Shima, A. & Kawakami, N. Identification of a functional transposase of the Tol2 element, an Ac-like element from the Japanese medaka fish, and its transposition in the zebrafish germ lineage. *Proc. Natl. Acad. Sci. USA* **97**, 11403–11408 (2000).
23. Kawakami, K. *et al.* A transposon-mediated gene trap approach identifies developmentally regulated genes in zebrafish. *Dev. Cell* **7**, 133–144 (2004).
24. Gilbert, S. *Developmental Biology* 8th edn. (Sinauer Associates, Sunderland, Massachusetts, 2006).