Hypoxia-induced metastasis model in embryonic zebrafish

Pegah Rouhi[1,4], Lasse D Jensen[1,4], Ziquan Cao[2,4], Kayoko Hosaka[1], Toste Länne[2], Eric Wahlberg[2], John Fleng Steffensen[3] & Yihai Cao[1]

[1]Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, Sweden. [2]Institute of Medicine and Health, Linkoping University, Linkoping, Sweden. [3]Marine Biological Laboratory, Biological Institute, University of Copenhagen, Helsingor, Denmark. [4]These authors contributed equally to this work. Correspondence should be addressed to Y.C. (yihai.cao@ki.se).

Published online 4 November 2010; doi:10.1038/nprot.2010.150

Hypoxia facilitates tumor invasion and metastasis by promoting neovascularization and co-option of tumor cells in the peritumoral vasculature, leading to dissemination of tumor cells into the circulation. However, until recently, animal models and imaging technology did not enable monitoring of the early events of tumor cell invasion and dissemination in living animals. We recently developed a zebrafish metastasis model to dissect the detailed events of hypoxia-induced tumor cell invasion and metastasis in association with angiogenesis at the single-cell level. In this model, fluorescent DiI-labeled human or mouse tumor cells are implanted into the perivitelline cavity of 48-h-old zebrafish embryos, which are subsequently placed in hypoxic water for 3 d. Tumor cell invasion, metastasis and pathological angiogenesis are detected under fluorescent microscopy in the living fish. The average experimental time for this model is 7 d. Our protocol offers a remarkable opportunity to study molecular mechanisms of hypoxia-induced cancer metastasis.

INTRODUCTION

Invasion and metastasis are the hallmarks of malignant disease, and understanding the molecular mechanisms underlying cancer metastasis may define new therapeutic targets for anticancer drug development. Hypoxia is known to significantly facilitate tumor invasion and metastasis in association with angiogenesis[1]. However, mechanisms of hypoxia-induced tumor invasion and metastasis remain poorly understood. One of the initial steps of the metastatic cascade involves the dissemination of malignant cells from the primary site[2]. Tumor cell dissemination in cancer patients can occur at the early stage of tumor development, when primary tumors are relatively small[3]. Unfortunately, current cancer detection methods at both clinical and preclinical levels are not able to image early events of tumor cell dissemination from primary sites.

Owing to the transparent and immunoprivileged nature of zebrafish embryos, we have recently developed a tumor invasion and metastasis model in association with hypoxia-induced angiogenesis[2,4]. This metastatic model allows us to detect tumor cell dissemination at the single-cell level. Using this method, we demonstrated that VEGF- or hypoxia-induced angiogenesis facilitate tumor cell dissemination[2,4]. Implantation of fluorescently labeled mouse or human tumor cells into the perivitelline space of zebrafish embryos, which are subsequently placed in a hypoxic aquarium, allows us to study early events of metastasis while primary tumors are relatively small. This is the first animal model to study detailed events of cancer metastasis in relation to tumor hypoxia and angiogenesis. This protocol is complementary to other existing models that are primarily designed to study the later events of the metastatic cascade, including the formation of metastatic niches and the regrowth of tumors in distal tissues and organs[2,5,6]. Unlike other models, monitoring of tumor cell dissemination with respect to angiogenesis and hypoxia in embryonic zebrafish can be achieved in living animals, which allows kinetic analysis of tumor cell dissemination and formation of distal metastatic niches in the same animal.

The general advantages of using zebrafish to study pathological angiogenesis and disease development are described by Cao *et al.* in this issue[7]. Additionally, hypoxia-induced metastasis in zebrafish embryos provides an outstanding opportunity to define signaling components in the host, which facilitates tumor invasion, dissemination and metastasis using specific morpholino intervention technology. It should be emphasized that genetic manipulations of crucial components involving the hypoxia-induced host responses could also create global hypoxia in the host. For example, deletion of the von Hippel-Lindau tumor suppressor gene in zebrafish results in a systemic hypoxic response which recapitulates the key aspects of Chuvash polycythemia in humans[8]. These genetically altered hypoxic zebrafish models offer alternative options for studying the hypoxic effect on tumor angiogenesis and metastasis in zebrafish.

This protocol has broad applications for studying the mechanisms underlying cancer cell metastasis, invasion and spreading; for defining new molecular targets controlling tumor cell migration and hypoxia-regulated angiogenesis; for assessing antimetastatic effects of known anticancer drugs; and for discovering potential new drugs for the treatment of metastatic diseases. In addition to its use in the assessment of therapeutic efficacies of monotherapies, it is anticipated to be a powerful model to study the therapeutic effects of combination therapy containing two or more therapeutic molecules. This zebrafish metastatic model is complementary to existing mouse tumor and metastatic models, allowing the study of different aspects of cancer metastasis.

Advantages and limitations

Key advantages:

In addition to the general advantages and limitations of using zebrafish as an *in vivo* model to study hypoxia-induced angiogenesis (see the companion article by Cao *et al.*[7]), there are several other advantages of using zebrafish embryos for studying metastasis. These include:

- Effective knockdown of gene expression by morpholino technology in zebrafish embryos allows readers to study functions of specific gene products.
- The transparent nature of zebrafish embryos allows visualization of vascular changes and tumor cell dissemination under hypoxia and normoxia in living fish.
- Immunoprivilege of zebrafish embryos allows implantation of mammalian tumor cells, including human and mouse tumor cells[4].

Limitations:

- As the hypoxia chamber is designed for the whole embryo, it is difficult to create hypoxia only in the tumor tissue.
- As most mammalian tumors grow at 37 °C, it is difficult to study the process of xenograft tumor growth at the optimal temperature.
- Microinjection of tumor cells into the perivitelline space of a large number of zebrafish embryos is a tedious procedure and requires highly skillful micro-operations.

Experimental design

Aquaria. The general setup and design for the hypoxic and normoxic aquaria are described in the companion article in this issue[7]. For zebrafish embryos, we recommend using a nylon mesh (mesh size <0.25 mm) to separate zebrafish embryos from the magnetic stirring bar, as fish embryos are unable to swim against water currents.

Tumor cells. Monolayers of human, mouse or other mammalian tumor cells should grow in culture to subconfluency (about 70–80%) to ensure that they are at the proliferating phase. Although genetically manipulated tumor cells that express fluorescein-activated proteins such as red fluorescent protein or yellow fluorescent protein may be used for *in vivo* tracing, we do not recommend using enhanced green fluorescent protein (EGFP)-labeled tumor cells if *fli1:EGFP* or *flk-1:EGFP* zebrafish strains[9,10] are used. For convenience, we use 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), which labels cell membranes for a relatively long time (~2 weeks) without losing color[4].

Embryo production. While growing tumor cells, at least 5–6 pairs of adult zebrafish of breeding age should be placed in the breeding chamber (one pair per chamber) to ensure production of 200–300 eggs. We highly recommend choosing as many healthy embryos produced by the same pair of adult zebrafish as possible, as they are usually at the identical age.

Tumor cell injection. Approximately 100 DiI-labeled tumor cells should be injected into the perivitelline space and should not be implanted into the yolk sac. As considerable variation exists between individual zebrafish embryos in response to hypoxia, a relatively large number of zebrafish embryos should be included in each experimental group; in each experimental and control group, 100 zebrafish embryos should be implanted with tumor cells. Implantation of tumor cells into the perivitelline space of embryos at 48 h post fertilization (h.p.f.) is technically challenging; thus, a skillful and experienced person should perform injections. The investigator should not drink coffee or other caffeine-enriched beverages in order to avoid uncontrolled hand movement.

Embryo selection. Even after tumor cell implantation, fish embryos should undergo further selection under fluorescent microscopy to ensure that tumor cells are located only within the perivitelline space.

Experimental conditions. To obtain statistically meaningful results, at least 50 embryos should be used in each experimental and control group, as it is expected that some embryos will die during experimentation. Once placed in the hypoxic chamber, we do not recommend opening the lid and disturbing embryos until they are harvested for analysis. For analysis, each individual zebrafish embryo in both hypoxic and normoxic groups should be photographed using fluorescent microscopy. These data should be digitally deposited in Adobe Photoshop, ImageJ or an equivalent program for further analysis.

Drug treatment. In drug treatment experiments, chemical solvent is used as vehicle and should have as low toxicity as possible. Dissolve the orally active chemical compound such as sunitinib in a desirable solution such as distilled water in high concentrations to prepare the stock solution. According to the appropriate final concentration of the drug in the hypoxia and normoxia aquaria, add the appropriate volume of the stock solution. As an example, if the stock concentration is 10 mM, to reach a final concentration of 1 μM in 1 liter of hypoxic or normoxic aquaria embryo water, the drug should be diluted 10,000×; accordingly, 100 μl of stock should be added to the water.

Controls. In all experimental designs, appropriate controls should be used under normoxia and hypoxia. It is known that tumor cells, even though derived from the same origin of the same cell type, may have different potentials to trigger angiogenic responses and to metastasize. Thus, if applicable, tumor cells with high or low metastatic potentials from the same type of cancer cell should be considered to serve as positive and negative controls. If genetically manipulated tumor cells are used, it is essential to include two control groups under normoxia and hypoxia: (i) nonmanipulated wild-type tumor cells; and (ii) vector or mock-manipulated tumor cells.

MATERIALS

REAGENTS

- 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, cat. no. D3899)
- 1-Phenyl-2-thiourea (PTU; Sigma-Aldrich, cat. no. P7629)
- Agarose (SeaKem LE, cat. no. 50004)
- Calcium chloride ($CaCl_2$, Sigma-Aldrich, cat. no. C8106)
- Distilled water (dH_2O)
- Dulbecco Modified Eagle's Medium (DMEM (Sigma-Aldrich, cat. no. D6546)
- Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich, cat. no. D8537)
- Ethyl 3-aminobenzoate methanesulfonic acid salt ((98% purity) tricaine; Sigma-Aldrich, cat. no. E10521)
- FBS (Hyclone, Thermo scientific, cat. no. SV30160.03)
- HEPES buffer (1 M in water; Sigma-Aldrich, cat. no. 83264)
- L-glutamine solution (200 mM, Gibco, cat. no. G7513)
- Magnesium sulfate ($MgSO_4$, Sigma-Aldrich, cat. no. M7506)
- Nail polish, colorless
- Nitrogen gas (100%, AGA GAS AB)
- Penicillin-streptomycin solution (Gibco, cat. no. 15070-063)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405)
- Sodium chloride (NaCl; Merck, cat. no. 1.06404.1000)

- T241 mouse fibrosarcoma cell line (ATCC)
- Transgenic (*fli1:EGFP*) zebrafish were obtained from ZFIN (Zebrafish Model Organism Database; http://www.zfin.org/)
- Trypsin solution (2.5% (wt/vol), 10×, Gibco, cat. no. 15090-046)
- Vectashield mounting medium (Vector Laboratories, cat. no. H-1000)

EQUIPMENT

- Photoshop CS3 or later versions (Adobe)
- ImageJ software
- Bacterial culture dish (10 cm; Corning, cat. no. 391-1517)
- BD Falcon polypropylene conical tubes (50 ml; BD Biosciences, cat. no. 358206)
- CO_2 incubator (Thermo electron corporation, model 311)
- Confocal microscope (Nikon D-eclipse C1, Nikon)
- Confocal software (EZ-C1 3.9 Nikon digital eclipse, Nikon)
- Costar cell culture plates (48-well; Corning, cat. no. 3548)
- Dissecting stereomicroscope (Wild M3Z, Leica)
- Eppendorf microinjector (FemtoJet 5247, Eppendorf, cat. no. 5247000.013)
- Eppendorf polypropylene conical tubes (2 ml, VWR International, cat. no. 211-2120)
- Fish transfer nets (Fridhems Akvarier)
- Fluorescent software (NIS-Elements D 3.0, Nikon)
- Forceps superfine tips (Dumont's finest tip, AgnTho's AB, cat. no. 11254-20)
- Heraeus BK 800 refrigerated incubator (Thermo Scientific, cat. no. 50120350)
- Magnet bar (4 cm; VWR International, cat. no. 442-4527)
- Magnet stirrer with heating (VMS-A, VWR International, cat. no. 442-0185)
- Manipulator (MM33-Right, Märzhäuser Wetzlar, cat. no. 00-42-101-0000)
- Microloader tips (0.5–10 µl; Eppendorf, VWR, cat. no. 5242 965.003)
- Microscope cover slips (Thermo Scientific, cat. no. 631-0135)
- Microscope slides (Thermo Scientific, cat. no. 631-1303)
- Microsoft Office Excel 2003 or later version (Microsoft)
- Nonfilamentous borosilicate glass capillaries needles (1.0 mm in diameter; World Precision Instruments, cat. no. 1B100-4)
- Nonheating magnet stirrer (VS-C7-2 EU-1, VWR International, cat. no. 442-0551)
- Oxygen regulator (OxyReg, Loligo, cat. no. OX10000)
- P-97 Flaming/Brown-type micropipette puller (Automated Scientific, cat. no. SU-P-97)
- Pasteur pipette (150 mm, wide, Bergman Labora AB)
- Pipette pump (10 ml, Pi-pump, VWR, cat. no. 612-3756)
- Plastic containers (185 mm × 85 mm × 75 mm and 200 mm × 100 mm × 130 mm, Aqua-Schwarz)
- Plastic lid for zebrafish embryo hypoxia chamber (200 mm × 100 mm × 14 mm, and 245 mm × 145 mm, Aqua-Schwarz)
- Plastic pipette tips (10–1,000 µl; Sarstedt, cat. no. 70762.100)
- Reusable adhesive (Sense, Clas Ohlson, cat. no. 26-888)
- Pipette (1 ml; Sarstedt, cat. no. 861251.001)
- Tissue needle (straight tip, VWR, cat. no. 238-6250)
- Zebrafish spawn insert (175 mm × 80 mm × 63 mm, Aqua-Schwarz)

REAGENT SETUP

Tricaine stock (0.05% (wt/vol)) For convenience, a 25× stock solution in dH_2O (0.05% (wt/vol)) should be prepared. To prepare the 25× stock, tricaine powder (50 mg) is dissolved in 100 ml dH_2O; prepare 2-ml aliquots in plastic vials and store at −20 °C until use. This stock solution is diluted to a final concentration (2×, 0.004% (wt/vol)) immediately before use. ▲ **CRITICAL** Tricaine is unstable at low concentrations (2× concentration or lower) in aqueous solutions. Do not make a stock solution at a low concentration.

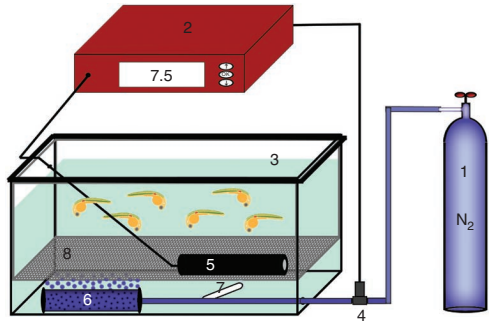

Figure 1 | Hypoxic setup for the embryonic zebrafish aquarium. (1) Nitrogen gas tank; (2) oxygen regulator; (3) embryonic hypoxia aquarium; (4) valves; (5) oxygen electrode; (6) air stone; (7) magnetic bar; and (8) nylon mesh.

Danieu's aquarium water (30×)
Prepare 1 liter sterile dH_2O containing 1.74 M NaCl, 21 mM KCl, 12 mM $MgSO_4$, 18 mM $CaCl_2$ and 150 mM HEPES buffer. This inorganic aquarium water can be kept at 22 °C for up to 12 months.

1-Phenyl-2-thiourea (PTU, 10×) Dissolve PTU powder in dH_2O to a final concentration of 2 mM. ▲ **CRITICAL** It usually takes several hours to dissolve PTU at room temperature with aid of a magnet stirrer. Once completely dissolved, PTU solution can be kept at 4 °C for a maximum of 2 weeks.

Agarose gel preparation To prepare a solution containing 2% (wt/vol) agarose, add 0.8 g agarose to 40 ml distilled water in a glass beaker and heat the solution in a microwave oven until all agarose particles are dissolved. Add 20 ml of the agarose solution into a Petri dish (10 cm diameter) and let it harden. About 15–20 min later, warm the rest of the solution in a microwave oven and add the solution on the top gel layer. Before solidification, place a mold in the center of the dish and leave it embedded in the agarose gel while it hardens. About 30 min later, remove the mold and add ~20 ml of 1% (vol/vol) sterile Danieu's aquarium water to immerse the gel. The agarose gel plate can be stored at 4 °C for ~30 d until use. Agarose plates should be pre-equilibrated to 26–28 °C before microinjection.

Preparation of DiI stock solution According to the manufacturer's instructions, add 97% (vol/vol) ethanol to the powder compound to constitute a concentration of 20 mg ml^{-1}; mix the solution to ensure that the DiI compound is completely dissolved. This stock solution can be stored in 5-µl aliquots at −20 °C.

DMEM cell culture medium preparation (FBS-DMEM) To prepare this medium, supplement the DMEM with 10% (vol/vol) FBS. Also add 1% (vol/vol) L-glutamine and 1% (vol/vol) penicillin-streptomycin. Store this medium at 4 °C. Before use, it should be pre-equilibrated to room temperature or, preferably, to 37 °C.

EQUIPMENT SETUP

Hypoxia aquarium for zebrafish embryos A hypoxia chamber for zebrafish embryos is created using a 1-liter standard plastic aquarium with a lid (**Fig. 1**). In general, the hypoxia chamber setup for zebrafish embryos is the same as for adult zebrafish (see Cao *et al.*[7]), with the exception that the bottom of the insert chamber is made of fine nylon mesh (mesh size <0.25 mm).

PROCEDURE

Tumor cell injection in zebrafish embryos ● TIMING ~3 d

1| *Day 0*: place a breeding insert inside a 1 liter breeding aquarium, fill the aquarium with aquarium water and put one male and one female adult *fli1:EGFP* zebrafish inside the breeding insert. Let the zebrafish mate overnight; fertilized eggs can be harvested the following morning.

2| *Day 1*: the next morning, collect eggs from fertilized *fli1:EGFP*-transgenic zebrafish and incubate them at 28.5 °C in the Danieu's solution (1%, dilute stock 1:100 in dH_2O) for 24 h.

? TROUBLESHOOTING

3| *Day 2*: add the PTU stock solution into the fish water to constitute a final concentration of 0.2 mM (10× dilution) and incubate the embryos for an additional 24 h.
! CAUTION PTU is used for preventing pigment formation in zebrafish embryos and is highly neurotoxic and carcinogenic. Thus, this chemical compound must be handled with a great caution in a chemical fume hood. PTU dishes and containers must be properly marked and handled only with gloves, and PTU waste should be kept in specific containers, which are disposed of separately.

4| *Day 2 (continued)*: while incubating zebrafish embryos with PTU for 24 h (incubation is usually started in the morning) prepare tumor cells labeled with DiI dye. Use monolayers of tumor cells cultured in 75 cm^3 culture flasks in DMEM supplemented with 10% (vol/vol) FBS, 1% (vol/vol) L-Glu and 1% (vol/vol) penicillin-streptomycin (FBS-DMEM, see REAGENT SETUP) at approximately 70–80% confluency. Add 1 µl of 20 mg ml^{-1} DiI stock into 10 ml of 1× DPBS (pre-equilibrated to room temperature) to achieve a final concentration of 2 µg ml^{-1}. Discard the conditioned medium from the flasks and add 5 ml of DPBS pre-equilibrated to room temperature to the flask shake gently and remove it. Add 10 ml of the DiI labeling solution into each of the 75 cm^3 culture flasks containing monolayers of tumor cells (**Fig. 2**); follow this by incubating cells at 37 °C for 30 min.
▲ CRITICAL STEP It is important to add the DiI into prewarmed 1× DPBS because DiI dye can solidify in cold DPBS. This is a crucial step to prevent blockage of capillary needles during the microinjection process of tumor cells into zebrafish embryos.

5| Collect all the medium containing the labeled tumor cells that become detached from the flasks as a result of DiI labeling and add it to a 50-ml Falcon tube; centrifuge at 1,000*g* for 3 min and discard the supernatant.
? TROUBLESHOOTING

6| Wash labeled cells with 1× DPBS twice: add ~5 ml of DPBS into the tube containing cell pellet and resuspend the cells in DPBS by pipetting up and down using a 1-ml pipette; centrifuge at 1,000*g* for 3 min and discard the supernatant. Repeat this step one more time.
▲ CRITICAL STEP To remove all free dye particles from the cell suspension, labeled tumor cells should be washed thoroughly.

7| Add 2 ml of 10% (vol/vol) FBS-DMEM (both fresh and pre-equilibrated to room temperature) and resuspend all the labeled cells with help of a pipette as described above; add the cell suspension into a new 75 cm^3 flask containing 13 ml of fresh 10% (vol/vol) FBS-DMEM without DiI, followed by incubation at 37 °C overnight.

8| *Day 3*: Use two pairs of microsurgical forceps to dechorionate 48 h.p.f. embryos from Step 6. Hold the chorion with one forceps, and, with the help of the other forceps, grip the chorion and tear it apart (**Fig. 2**).
? TROUBLESHOOTING

9| Anesthetize the dechorionated zebrafish embryos by transferring them to fish water containing 0.004% (wt/vol) tricaine and wait for ~2 min.

10| Place up to ten embryos in each well and add 0.004% (wt/vol) tricaine water to each well of the 2% (wt/vol) modified agarose gel plate to precisely fill the wells. Excessive water can be removed with a Pasteur pipette.
▲ CRITICAL STEP If an excessive amount of water is used, zebrafish embryos can float outside the agarose

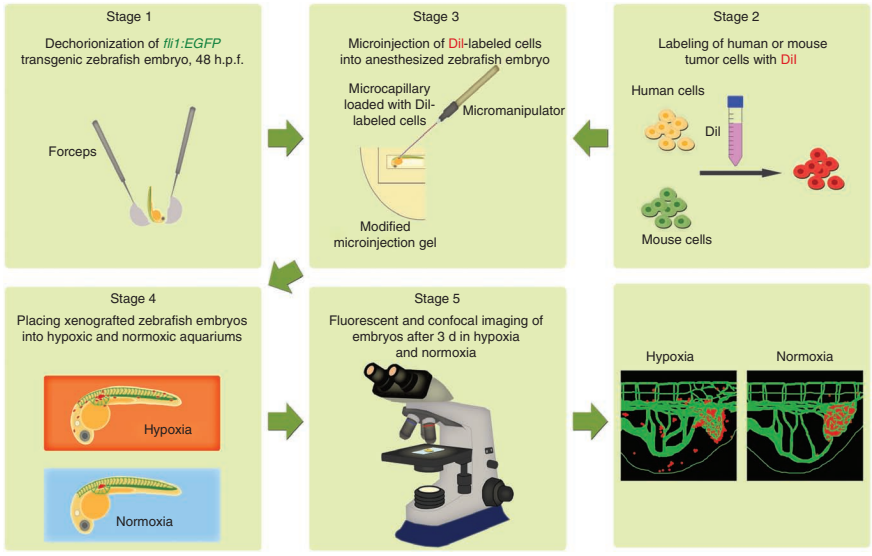

Figure 2 | Critical stages for tumor cell implantation into embryonic zebrafish and for analyzing tumor cell dissemination. *fli1:EGFP* zebrafish embryos at 48 h are dechorionized. Meanwhile, human or mouse tumor cells growing as monolayers are labeled with DiI dye and resuspended at an optimal concentration for microinjection. Approximately 100–150 tumor cells in 5 nl are injected into the perivitelline space of each zebrafish embryo. Tumor-implanted zebrafish embryos are incubated in normoxic and hypoxic water for 3 d. Dissemination of tumor cells can be detected using fluorescent microscopy at the single-cell level. Tumor cell (red) invasion and migration in association with tumor angiogenesis (green) or co-option of surrounding vasculatures in the host can be revealed using a two-channel fluorescent microscope.

well, leading to empty wells. Additionally, excessive water can result in difficulties in positioning the zebrafish embryos at a fixed position for injection.

11| To prepare tumor cells from Step 7 for implantation, remove the medium and wash attached monolayers of tumor cells twice with 1× DPBS by adding 5 ml of 1× DPBS into the flask, shaking the flask very gently and then removing the DPBS.

12| Add 2 ml of 0.25% (wt/vol) trypsin pre-equilibrated to room temperature into the flask containing attached monolayer of tumor cells and incubate at room temperature for 30 s. Remove the trypsin, tap the flask a few times very gently to help the cells to detach and stop trypsin activity by adding 2 ml of 10% (vol/vol) FBS-DMEM. Resuspend the tumor cells in the medium by pipetting up and down. Count the tumor cells under a phase contrast microscope. Centrifuge tumor cells at 1,000*g* for 5 min and resuspend cells at a concentration of 20–30 cells per nl in 0.5–1% FBS-DMEM (e.g., FBS-DMEM as prepared in REAGENT SETUP, but with 0.5–1% FBS instead of 10% FBS).
▲ **CRITICAL STEP** It is crucial to stop trypsin activity by adding serum-containing cell culture medium. Otherwise, viability of cells can be markedly reduced during the counting of cells.
? TROUBLESHOOTING

13| Place the cell suspension on ice during the entire microinjection procedure.

14| Pull nonfilamentous borosilicate glass capillary needles using a micropipette puller. Load the microcapillary glass needle with 4 µl of cell suspension and connect the needle to the micromanipulator. Point the needle tip to the injection site and gently insert the needle tip into the perivitelline space of the zebrafish embryo. Inject 5 nl of the cell suspension containing approximately 100–150 cells into the perivitelline space of each embryo from Step 10 (**Fig. 2**).
? TROUBLESHOOTING

15| Transfer the injected zebrafish embryos to PTU water. The death rate of zebrafish embryos after tumor cell injection is ~5%.

Hypoxia exposure period ● TIMING 3 d
16| Fill the hypoxia chamber for zebrafish embryos with 1% (vol/vol) Danieu's water to the two-thirds level of the aquarium and calibrate the oxygen electrode using the same procedure described in adult zebrafish hypoxia model (Cao *et al.*[7]). Switch on the nitrogen gas and set the air saturation level to 7.5% as shown on the oxygen regulator (**Fig. 1**).

17| If drug treatment is being tested, add the chemical compound to aquarium water at the concentration that functionally intervenes with hypoxia-induced host responses (optional; see Experimental design).

18| When the hypoxia reaches a steady-state level, transfer tumor-implanted zebrafish embryos from Step 15 to the hypoxia chamber and close the lid. Incubate up to 100 tumor cell–implanted zebrafish embryos in the hypoxia chamber at 28 °C.
▲ **CRITICAL STEP** A group of tumor-implanted zebrafish embryos should be placed in normoxia (regular water) to serve as a control.
▲ **CRITICAL STEP** Make sure that the lid is not completely sealed in order to avoid high pressure. If the lid is completely sealed, zebrafish embryos may die of anoxia.

19| Use a medium-sized magnet bar to mix the water and create a mild water current within the chamber to ensure that nitrogen gas is evenly distributed.

20| Maintain embryos in the hypoxia chamber for 3 d.
▲ **CRITICAL STEP** The death rate is quite high at this hypoxia level; therefore, it is very important to closely monitor the chamber during the course of the entire experiment. Unlike in adult zebrafish experiments, there is no need to remove dead zebrafish embryos. To ensure that all zebrafish embryos are evenly exposed to the same hypoxic level, embryos should be swirling gently around in the chamber.

21| After 3 d, transfer the tumor cell–implanted zebrafish embryos in water exposed to normoxia or hypoxia onto the surface of a 2% (wt/vol) agarose gel with help of a wide-tip Pasteur pipette for further analysis.
▲ **CRITICAL STEP** Analysis should be carried out immediately once the zebrafish embryos are placed on the agarose gel, as zebrafish embryos can only live for a few minutes in this condition.

Imaging ● TIMING 6–9 h

22| At room temperature, cut a piece (1 cm × 1 cm) of 2% (wt/vol) agarose gel and place it on a glass slide. Use a Pasteur pipette to pick up a zebrafish embryo and carefully place it onto the gel cushion. Remove excessive water and add a small drop of 0.04% (wt/vol) tricaine onto the embryo.

▲ **CRITICAL STEP** If embryos are not completely anesthetized, increase the concentration of tricaine. Imaging should be performed immediately after anesthesia to reduce the risk of embryo death due to liquid evaporation.

23| Collect an embryo with a wide-tip Pasteur pipette and transfer it onto a glass slide; remove the excess water around using a paper tissue. If the paper tissue touches the embryo, the embryo will get dry and cannot be used for imaging. Put a small drop of Vectashield on the embryo, place a cover glass on it and seal the edges of the cover slide with nail polish. Keep the glass slide in a horizontal position in the dark for a short while until the nail polish dries and then transfer immediately to −20 °C.

■ **PAUSE POINT** Mounted embryos at the horizontal position can be stored in dark at −20 °C for up to 2 weeks before microscopic examination.

24| Use fluorescent microscopy to analyze the number of disseminated tumor foci and cells. Perform imaging at low magnification (×4 objective) using the ultraviolet-activated filter at the wavelength of 488 nm. The fluorescent light filter allows excitement of EGFP in zebrafish embryos. Use filters for green fluorescence to visualize zebrafish embryo vasculature and filters for red fluorescence to image implanted tumor cells labeled with DiI. To illuminate the DiI-labeled tumor cells in zebrafish embryos, use green light at the wavelength of 555 nm. It is essential to capture green fluorescent–positive and red fluorescent–positive signals at the same position on the embryos in order to associate DiI-labeled tumor cells with blood vessels by superimposing green and red signals. Typically, for each zebrafish embryo, two different sets of images from the head region and the tail region are collected as the separate green fluorescent–positive signals, the red fluorescent–positive signals, and the merged signals. All disseminated tumor cell signals in each embryo should be counted. At least 13–20 embryos in each experimental group should be used to obtain statistically significant values.

25| Use three-dimensional (3D) confocal microscopy to study angiogenesis and metastasis in more detail. Confocal low magnification (×4 objective) can be used to image the whole body to get an overview of the tumor cell dissemination pattern, and higher magnifications (×20 and ×40 objectives) are suitable for studying intratumoral and peritumoral angiogenesis and also for precise localization of disseminated cells and foci inside the zebrafish embryo body. Use a 488-nm laser to scan the zebrafish embryo vasculature and a 543-nm laser to scan DiI-labeled implanted tumor cells. To achieve a high-quality 3D image, each embryo should be scanned in eight to ten layers and each layer should be scanned six times to remove the background and get a sharp picture (**Fig. 3**).

26| To quantify the number of disseminated cells and foci, count all the cells and foci that have disseminated from the tumor mass toward the fish embryo head and tail using fluorescence software, ImageJ or any other kind of useful software program. For further data analysis, use Excel or similar software to calculate the average number of disseminated foci and cells. In addition, to measure the distance of metastasis, choose the focus or cell that lies furthest away from the tumor mass and measure the distance between that focus and the primary tumor. The programs and software mentioned above can also be used for this purpose. Adobe Photoshop can be used to measure and analyze the ratio of tumor vessel density relative to size and tumor volume; see the accompanying paper[7] for more details.

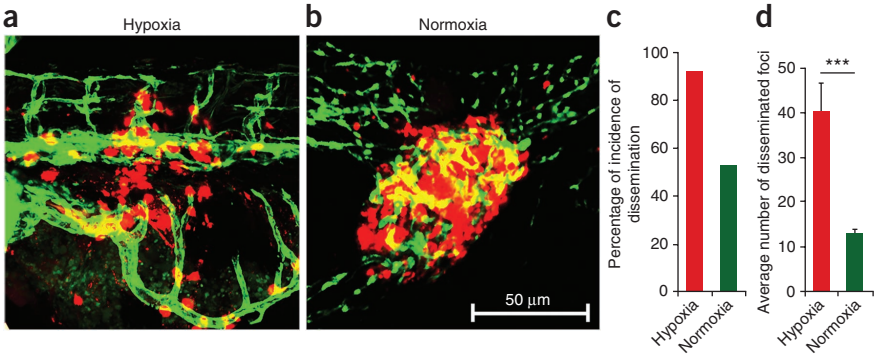

Figure 3 | Dissemination and quantification of disseminated mouse T241 fibrosarcoma cells. Approximately 100 DiI-labeled mouse T241 fibrosarcoma cells were injected into the perivitelline space of each 48 h.p.f. *fli1:EGFP* zebrafish embryo. The tumor cell-implanted zebrafish embryos are exposed to normoxic or hypoxic water at 28 °C for 72 h. Green color represents zebrafish embryo blood vessels, red color represents DiI-labeled tumor cells and yellow color shows overlapping positive signals. (**a**,**b**) Tumor cell dissemination and metastases under hypoxic conditions (**a**) and angiogenesis under normoxia (**b**) were detected using confocal microscopy. Scale bar applies to both panels **a** and **b**. (**c**) Percentage of zebrafish carrying disseminated tumor cells (total number of embryos studied was 13 for hypoxia and 15 for normoxia) under normoxia and hypoxia were quantified. All DiI-positive cells located outside the primary tumor mass were manually counted as disseminated cells or foci using the software mentioned in Step 26. (**d**) The average number of disseminated foci from primary tumor implanted in zebrafish embryos (13 for hypoxia and 15 for normoxia) were quantified. (Adapted from ref. 4 with permission.) Error bars indicate the standard deviation; asterisks stand for $P<0.001$ and show the significant difference between the two groups.

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Too few eggs	Nonproductive breeding pairs	Choose a few healthy, well-fed breeding pairs of zebrafish to ensure enough eggs
5	Tumor cells remain attached after 0.5-h incubation with labeling medium	Highly adherent cell line	Release cells by resuspending them in the labeling medium using a 1-ml pipette tip. Avoid using trypsin at this stage
8	Embryos are destroyed during dechorionization	Inexperienced and less skillful personnel	Practice by starting with a large number of embryos and choose perfect ones for injection
12	Formation of cell clusters	Dead cell clumps	Add 0.5% FBS to the medium to reduce trypsin-induced cell death
14	Blockage of injection capillaries	Formation of clumps and too much cell debris	Load the microcapillary needle with the desired volume of cell suspension, pipette up and down a couple of times, remove the suspension and load the needle again or increase cell density and reduce the injection volume

● TIMING

Step 1, Breeding (day 0): 15 h
Step 2, Incubation of embryos in Danieu's solution (day 1): ~24 h
Steps 3–7, Incubation of embryos in 0.2 mM PTU solution (day 2): ~24 h
Steps 8–15, Dechorionization and tumor cell injection in zebrafish embryo (day 3): 2–3 h per 100 embryos
Steps 16–21, Hypoxia exposure period: 3 d
Steps 22–26, Imaging: 6–9 h

ANTICIPATED RESULTS

Hypoxia-induced dissemination of tumor cells in embryonic zebrafish

Using this protocol, dissemination of DiI-labeled (T241, fibrosarcoma) tumor cells can be detected at single-cell level using a fluorescent microscope (stage 5 in **Figs. 2** and **3**). Moreover, hypoxia-induced tumor angiogenesis (green color) in relation to dissemination of tumor cells (red color) can be monitored using a two-channel confocal image analysis. Notably, a majority of disseminated tumor cells are associated with the vasculature, demonstrating the essential role of angiogenesis in tumor invasion and metastasis. As the primary tumor remains at a relatively small size during the entire experimental procedure, hypoxic exposure is unlikely to generate an uneven gradient that causes tumor cells to be superficially invasive owing to co-option to surrounding healthy vasculature. **Figure 3** shows an example of the expected results of metastasis under hypoxia using DiI-labeled mouse fibrosarcoma cells with quantification of metastases.

ACKNOWLEDGMENTS Y.C.'s laboratory was supported by research grants from the Swedish Research Council, the Swedish Cancer Foundation, the Karolinska Institute Foundation, the Karolinska Institute distinguished professor award, the European Union Integrated Project of Metoxia (Project no. 222741) and the European Research Council (ERC) advanced grant ANGIOFAT (Project no 250021). Z.C. is supported by the Swedish Heart and Lung Foundation.

AUTHOR CONTRIBUTIONS Y.C. designed the study. L.D.J., P.R. and Z.C. performed the experiments. L.D.J., P.R., Z.C. and Y.C. analyzed the data. K.H., T.L., J.F.S. and E.W. participated in designing and discussing this study. L.D.J., P.R., Z.C. and Y.C. wrote the paper.

COMPETING FINANCIAL INTERESTS The authors declare 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. Erler, J.T. *et al.* Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* **15**, 35–44 (2009).
2. Rouhi, P. *et al.* Pathological angiogenesis facilitates tumor cell dissemination and metastasis. *Cell Cycle* **9**, 913–917 (2010).
3. Cao, Y. Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. *Nat. Rev. Cancer* **5**, 735–743 (2005).
4. Lee, S.L. *et al.* Hypoxia-induced pathological angiogenesis mediates tumor cell dissemination, invasion, and metastasis in a zebrafish tumor model. *Proc. Natl. Acad. Sci. USA* **106**, 19485–19490 (2009).

5. Nissen, L.J. *et al.* Angiogenic factors FGF2 and PDGF-BB synergistically promote murine tumor neovascularization and metastasis. *J. Clin. Invest.* **117**, 2766–2777 (2007).
6. Cao, R. *et al.* PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* **6**, 333–345 (2004).
7. Cao, Z. *et al.* Hypoxia-induced retinopathy model in adult zebrafish. *Nat. Protoc.* **12**, 1903–1910 (2010).
8. van Rooijen, E. *et al.* Zebrafish mutants in the von Hippel-Lindau tumor suppressor display a hypoxic response and recapitulate key aspects of Chuvash polycythemia. *Blood* **113**, 6449–6460 (2009).
9. Lawson, N.D. & Weinstein, B.M. In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev. Biol.* **248**, 307–318 (2002).
10. Jin, S.W., Beis, D., Mitchell, T., Chen, J.N. & Stainier, D.Y. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* **132**, 5199–5209 (2005).