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TITLE:

In Vivo Imaging and Quantitation of the Host Angiogenic Response in Zebrafish Tumor Xenografts

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SUMMARY:

The aim of this method is to generate an in vivo model of tumor angiogenesis by xenografting mammalian tumor cells into a zebrafish embryo that has fluorescently-labelled blood vessels. By imaging the xenograft and associated vessels, a quantitative measurement of the angiogenic response can be obtained.

ABSTRACT:

Tumor angiogenesis is a key target of anti-cancer therapy and this method has been developed to provide a new model to study this process in vivo. A zebrafish xenograft is created by implanting mammalian tumor cells into the perivitelline space of two days-post-fertilization zebrafish embryos, followed by measuring the extent of the angiogenic response observed at an experimental endpoint up to two days post-implantation. The key advantage to this method is the ability to accurately quantitate the zebrafish host angiogenic response to the graft. This enables detailed examination of the molecular mechanisms as well as the host vs tumor contribution to the angiogenic response. The xenografted embryos can be subjected to a variety of treatments, such as incubation with potential anti-angiogenesis drugs, in order to investigate strategies to inhibit tumor angiogenesis. The angiogenic response can also be live-imaged in order to examine more dynamic cellular processes. The relatively undemanding experimental technique, cheap maintenance costs of zebrafish and short experimental timeline make this model especially useful for the development of strategies to manipulate tumor angiogenesis.

INTRODUCTION:

Angiogenesis is one of the classic hallmarks of cancer and represents a target of anti-cancer therapy[1,2]. To study this process, xenograft models of cancer have been created by implanting mammalian tumor cells into animals such as mice[3]. A zebrafish xenograft model has also been developed, which involves the implantation of tumor cells into 2 days post fertilization (dpi)

zebrafish which results in rapid growth of zebrafish blood vessels into the xenograft[4].

This protocol describes an in vivo zebrafish embryo tumor xenograft model in which the angiogenic response can be accurately quantitated across the entire xenograft. This method allows the investigator to examine, in vivo, the molecular mechanisms that underpin the tumor angiogenic response. The genetic tractability of the zebrafish allows the host contribution to be interrogated, while selection of different tumor cell lines allows the tumor contribution to angiogenesis to also be examined[5-7]. In addition, as zebrafish larvae are permeable to small molecules, specific pathway inhibitors can be used or drug libraries can be screened to identify novel inhibitors of tumor angiogenesis[8-11].

The zebrafish embryo xenograft model presents unique advantages compared with other mammalian xenograft models. Zebrafish xenografts are cheaper and easier to perform, large numbers of animals can be examined and live cell imaging allows detailed examination of cell behaviour[4]. Unlike other in vivo models, which require up to several weeks to observe significant vessel growth, angiogenesis in zebrafish xenografts can be observed within 24 h following implantation[3,4]. However, the lack of an adaptive immune system in embryonic zebrafish, while beneficial to maintaining the xenograft, means that the adaptive immune response and its contribution towards tumor angiogenesis cannot be examined. In addition, the lack of tumor stromal cells, the inability to orthotopically implant the tumor and the difference in maintenance temperature between zebrafish and mammalian cells are potential weaknesses of this method. Nonetheless, the amenability of this model for live imaging and the ability to accurately quantitate the angiogenic response makes it uniquely beneficial for studying the cellular processes that regulate tumor angiogenesis in vivo.

PROTOCOL:

1. Preparation of microinjection needles

1.1. Turn on a micropipette puller and set the following parameters (calibrated for the micropipette puller model listed in **Table of Materials**): Heat, 680; Pull, 75; Velocity, 40; Time, 55; Pressure: 530.

1.2. Secure a borosilicate glass capillary into the micropipette puller and pull the capillary to make two needles. Repeat for as many needles as desired.

2. Cell culture for implantation

NOTE: When using this protocol, any mammalian cancer cell line can be used for implantation into zebrafish embryos as xenografts. However, there is much variation in the angiogenic response induced among different cell lines[5,11,12]. B16-F1 murine melanoma cells have been shown to induce a strong angiogenic response in zebrafish embryos[11] and are therefore appropriate for use in this protocol.

2.1. Grow B16-F1 cells at 37 °C in a 75 cm^2 flask using MEM-α media supplemented with Fetal Bovine Serum (FBS) to a final concentration of 10% (v/v) and Penicillin /Streptomycin, each at a final concentration of 100 µg/mL.

2.2. Remove the media from a 95-100% confluent 75 cm^2 flask of B16-F1 cells and wash the cells in 5 mL of room temperature phosphate-buffered saline (PBS).

2.3. Remove the 5 mL PBS, add 2 mL of room-temperature 0.25% Trypsin/ ethylenediaminetetraacetic acid (EDTA) and incubate at 37 °C for 60 s.

2.4. Tap the side of the flask to determine whether the cells are beginning to lose their attachment to the bottom of the flask.

2.5. Add 8 mL of room temperature MEM-α with 10% FBS into the flask, pipette against the inside of the flask to bring any cells that remain adhered to the bottom of the flask into suspension and pipette the cell suspension into a 15 mL tube.

2.6. Centrifuge the cell suspension at 800 x *g*, 4-8 °C for 5 min, aspirate the media and proceed to labelling the cells with dye.

3. Labelling B16-F1 cells with fluorescent dye

NOTE: In order to differentiate between the implanted tumor cells and other cells in the embryo, the tumor cells must be labelled with an appropriate fluorescent dye before implantation. This step can be skipped if the cells already express fluorescent reporters.

3.1. Incubate 2 mL of serum-free MEM-α media at 37 °C.

3.2. Prepare a stock solution of the chosen dye and dilute it in pre-incubated 2 mL of serum-free MEM-α media to make a workable concentration (examples of dyes and concentrations appropriate for B16 F1 cells are provided in **Table of Materials**).

3.3. Pipette 1 mL of the dye solution into the cell pellet (from step 2.6), mix thoroughly by pipetting, then add the other 1 mL of dye solution and mix.

3.4. Incubate the cells and dye mixture at 37 °C for 40 min, mixing by gentle shaking at 20 min.

3.5. Centrifuge the cell suspension at 800 x *g*, 4-8 °C for 5 min.

3.6. Aspirate the supernatant and wash the labelled cells by pipetting with 5 mL of PBS.

3.7. Centrifuge the cell suspension again at 800 x *g*, 4-8 °C for 5 min, aspirate the supernatant and place the cells on ice until ready for implantation.

NOTE: The final tumor cell pellet should have a volume of 20-40 µL.

4. Preparation of embryos for implantation

NOTE: Choose a transgenic zebrafish line that has fluorescently labelled blood vessels (e.g. *kdrl:RFP, fli1a:EGFP,* etc.)[13,14].

4.1. Two days prior to implantation, spawn the zebrafish as previously described and collect the embryos[15].

NOTE: As we are not injecting these fish at an early stage, they do not need to be collected within 20 min of spawning as described by Rosen et al.[15], but may be collected after a few hours of mating.

4.2. Place the embryos in 100 mm culture dishes at a density of approximately 100 embryos/dish.

4.3. Add 50 mL of E3[16] (supplemented with 5 µL of a 1% w/v aqueous stock solution of methylene blue to prevent contamination) to each dish, clean out any dead embryos or debris and keep the dish in a dark incubator at 28 °C until ready for injection.

4.4. At 1 day post fertilization (dpf), add 1-phenyl-2-thiourea (PTU) to each dish to produce a final concentration of 30 mg/L PTU in E3. PTU prevents pigmentation, which can interfere with the ability to view the tumor cells and blood vessels.

4.5. At 2 dpf, use needles or tweezers to manually dechorionate any embryos that are unhatched. Using a fluorescent microscope, select as many transgenic embryos as required for xenografting (50-200).

4.6. Prior to implantation, anaesthetize the selected embryos in a solution of 300 µg/mL Tricaine in E3 in order to prevent movement during the injection procedure.

4.7. Fill a 35 mm dish with a 2% methylcellulose in E3 solution to a quarter of the volume of the dish.

4.8. Use a transfer pipette to place approximately 50 embryos (minimizing the volume of E3 solution also transferred) onto the methylcellulose.

4.9. Use a microcapillary pipette tip to arrange the embryos so that they are all oriented laterally with their heads to the right, tails to the left and the side of the embryos facing up.

NOTE: Steps 4.7-4.9 can also be carried out by arranging the embryos on an agarose block as previously described[17], but in our experience, the embryos are maintained in a more stationary position when arrayed in methylcellulose.

5. Perivitelline injection of mammalian cancer cells into 2 dpf embryos

NOTE: To ensure the cells clump together as a graft when implanted, the cells must be mixed together with an extracellular matrix mixture (ECM). We have described the steps of making such a cell/ECM mixture when using an ECM mixture referenced in the **Table of Materials**. If an alternative matrix is used, the steps should be adjusted accordingly.

5.1. Divide a stock of ECM into 100 µL aliquots in 500 µL tubes and store at -20 °C until needed.

5.2. Thaw out an aliquot of ECM and add 100 µL of PBS to dilute the mixture to 50% (v/v).

NOTE: Diluted 50% ECM can be stored at 4 °C for up to 1 month.

5.3. Mix the 50% ECM well with the B16-F1 cell pellet (from step 3.7) by pipetting and stirring, to produce a mixture of cells/ECM in a 2:1 ratio and store the mixture on ice.

5.4. Using a microcapillary pipette, take up 3-10 µL of cells.

5.5. Carefully insert the pipette tip into a needle and eject the B16-F1/matrix mixture into the end of the needle.

5.6. Break the tip of the needle using tweezers to make a hole large enough for the cells to be ejected from the needle without squeezing the cells.

5.7. Insert the needle into the needle-holder and tilt at a 45° angle to the dish.

5.8. Turn on the pressurized air cylinder attached to the injection apparatus and turn the injector on “continuous” mode momentarily to push the cells to the tip of the needle.

5.9. Remove the needle out of the dish and replace the dish with a hemocytometer.

5.10. Place a drop of oil on the hemocytometer and inject the needle once onto this drop.

5.11. Count the number of cells ejected with a single pulse using the hemocytometer and calibrate the needle to eject approximately 150 cells per pulse by adjusting pulse duration.

NOTE: Ejecting this quantity of cells per pulse will require several pulses in order to implant a successful xenograft. This will give the researcher more control over the final size of the tumor xenograft by allowing them to adjust the number/location of pulses.

5.12. Point the needle towards the yolk sac of an embryo and push it through the yolk sac in a ventral direction until the tip of the needle has emerged from the yolk sac and is pushing the embryonic epidermis on the ventral side of the embryo just posterior to the heart.

NOTE: Position the needle so that it enters the embryonic yolk sac anterior to the final location where the cells will be ejected. This will ensure that the cells will be ejected in a direction away from the heart, decreasing the likelihood of injecting the cells into the common cardinal vein.

5.13. Carefully push the tip of the needle forward a little until it has created a space (perivitelline space) between the epidermis and the yolk sac membrane and then pulse the injector to eject some of the cell mixture into the perivitelline space.

5.14. Repeat the pulses until 500-800 cells have been injected into the perivitelline space, creating a visible bulge that extends at least half of the way along the bottom of the yolk sac, then remove the needle from the embryo.

NOTE: If the cells block the needle or are damaged during the injection, purge the needle by momentarily switching to "continuous" mode and then back to "pulse". This should unblock the needle and allow the cells to be ejected freely and undamaged.

5.15. Continue to do the same for all the embryos in the dish, loading a new needle with tumor cells when required.

5.16. After injecting all the embryos, remove the needle and push all the embryos together using a microcapillary pipette tip so that they can be pipetted out with as little methylcellulose as possible.

5.17. Transfer the embryos into a recovery dish containing E3 (with PTU and methylene blue) and wash them by gently pipetting E3 around the embryos.

NOTE: At this point, the xenografted embryos can be treated with drugs by separating them into different dishes and adding a drug solution to one dish and a control solution (such as the drug vehicle) to the other dish.

5.18. Incubate the embryos at 34 °C and perform twice daily checks to remove dead or oedematous embryos from the dish.

NOTE: 34 °C was found to be the optimal temperature for xenograft vascularization and has also been used by other studies employing the zebrafish embryonic xenograft angiogenesis model[18]. The xenografts can be imaged any time up to 48 hpi (hours post-implantation).

6. Live imaging

6.1. At 48 hpi, identify 3-5 healthy embryos without oedema. Anaesthetize them in 150 µg/mL Tricaine and mount them laterally in 1% Low Melting Point (LMP) agarose as previously described[19].

NOTE: As the agarose is solidifying, use a microcapillary pipette tip to keep the embryos positioned laterally.

6.2. Turn on the confocal microscope, the microscope controller, the lasers and the computer software for controlling the microscope.

6.3. Place the dish on a confocal microscope. Using a 20X magnification, water dipping objective lens, position the xenograft in the center of the field using brightfield imaging.

6.4. Select excitation lasers that are appropriate for detecting the dye used to label the tumor cells and the fluorophore used to label the zebrafish blood vessels.

6.5. Adjust the gain for each laser to a level that allows the detection of both the tumor cells and the blood vessels.

NOTE: Once confocal settings have been established, ensure these are kept unchanged throughout the experiment so that comparisons can be made between xenografts.

6.6. Using the laser channel appropriate for the tumor cells, determine a volume to be imaged that includes the entire volume of the xenograft, allowing for at least one or two optical sections either side of the graft. Use section intervals that are around 5 µm apart to create a z-stack.

NOTE: It is essential that the z-stack should contain the entire volume of the xenograft.

6.7. Using two channel imaging, image both the tumor xenograft and the blood vessels. Repeat steps 6.6 - 6.7 for each larva to be imaged.

7. Time lapse imaging

NOTE: This model is highly suited to imaging dynamic cellular processes due to its transparency and the availability of zebrafish transgenics that fluorescently label different cell types. This makes time-lapse imaging a key application of this model.

7.1. Turn on the environmental chamber and set to 34 °C at least 2 h before imaging. If the chamber is not humidified, add dishes of water.

7.2. Anaesthetize 3-5 embryos (using 120 µg/mL Tricaine at 2 dpf and 100 µg/mL Tricaine at 3 dpf) and mount them laterally in 0.8% LMP agarose for time-lapse imaging according to the previously described protocol[19].

NOTE: As the agarose is solidifying, use a microcapillary pipette tip to keep the embryos positioned laterally.

7.3. Set up the equipment and image the xenograft as described in steps 6.2-6.7, acquiring z-

stacks of the xenograft at 10 min intervals.

NOTE: The embryo must be maintained at 34 °C as it is being imaged and the E3 on top of the larvae in agar must be checked and supplemented occasionally to ensure that it does not dry out.

8. Quantitation of the angiogenic response to the zebrafish xenograft

NOTE: The following steps use 3D image analysis software. Specific steps will vary depending on the software used; the following is a general outline of the steps required.

8.1. Open the z-stack confocal image files of a tumor xenograft using the 3D image analysis software.

NOTE: In order to quantitate the levels of tumor vascularization, measurement protocols must be created with settings calibrated so that they can be used to measure either Tumor Volume or Tumor Vessel Volume for all xenografts.

8.2. Create a measurement protocol that will identify the tumor volume by selecting all the fluorescent "objects" in the tumor channel. Applying this protocol to a region of interest (ROI) that is drawn freehand around the tumor will ensure that no background fluorescence is included.

NOTE: Take care to ensure that none of the tumor is left out of the ROI. This will provide a region on which to perform the protocol.

8.3. Ensure that the values for minimum and maximum signal intensity as well as the minimum object size are thresholded appropriately to ensure only tumor cells are selected.

NOTE: This is important so that only the fluorescence from the tumor cells in the selected ROI (drawn in step 8.2) is detected and none of the background fluorescence is measured.

8.4. Apply the tumor volume protocol to identify the tumor volume. Use this to clip the freehand drawn ROI to a new ROI only containing the tumor volume.

8.5. Create a measurement protocol that will identify the tumor vessel volume by selecting all the fluorescent "objects" in the blood vessel channel. Apply this protocol to the tumor volume ROI identified in step 8.4.

8.6. Ensure that the values for minimum and maximum signal intensity as well as the minimum object size are thresholded appropriately to ensure only vascular cells are selected.

NOTE: Once the tumor volume and tumor vessel volume protocols have been established, do not alter the settings.

8.7. Divide vessel volume by the tumor volume and multiply the result by 100 to obtain a percentage value of graft vascularization.

8.8. Apply the tumor volume and tumor vessel volume measurement protocols for the remaining xenografts.

REPRESENTATIVE RESULTS:

By imaging an individual xenograft at 6, 24 and 48 hpi, the angiogenic response at different timepoints can be calculated as shown in **Figure 1A-C**. The largest angiogenic response is observed between 24-48 h post implantation, with the maximum levels of graft vascularization seen around 2 dpi (**Figure 1A-C**). A time-lapse movie of a typical angiogenic response to a B16-F1 xenograft from 20.75 hpi until 46 hpi is seen in **Supplementary Movie 1**. This movie was generated by imaging a xenograft implanted following this protocol into a 2 dpf *kdrl:EGFP* embryo. The vessels that grow through the xenograft form a tortuous network with vessels of irregular size and morphology, which is typical of the abnormal vascular network seen in mammalian tumors[20]. The angiogenic response in our model is a result of vessels that sprout from the common cardinal vein into the xenograft as opposed to previous studies, which have reported the sub-intestinal veins as the source of the vessels that grow into the xenograft[4]. The difference in vessel origin is due to the fact we have implanted our xenografts in a more ventral location in the perivitelline space, as this makes it easier to visualize the shape and size of the xenograft while injecting and imaging[20].

Figure 1D-F shows representative results of B16-F1 xenografts and their associated vasculature following treatment with either DMSO (control) or 50 nM of a VEGFR inhibitor (Tivozanib)[21,22]. Immediately after implantation, xenografted embryos were split into two groups and either 50 nM Tivozanib or DMSO (0.5% v/v) was applied to each group. They were maintained in these drugs before imaging at 2 dpi. These results show that the vessels associated with xenografts incubated in the VEGFR inhibitor (**Figure 1E**) are far less numerous than the vessels associated with xenografts incubated in DMSO (**Figure 1D**). In control embryos, there is an expansive vascular network that stretches across the entire xenograft region (**Figure 1D**), which is the typical angiogenic response observed following implantation of B16-F1 cells. In **Figure 1F**, the angiogenic response has been quantitated by following this protocol and it shows a clear reduction in graft vascularization in the VEGFR inhibitor-treated xenografts when compared to controls. Despite normalizing the levels of graft vascularization against graft volume, there remains a large variation in the levels of graft vascularization at 48 hpi (coefficient of variation of 54.2%). This is due to the variation in the total volume of the tumor vessels (coefficient of variation 73.3%); we observed that even similarly-sized grafts had large variation in the level of vascularization. Because of this, it is recommended that around 20 xenografts are used per treatment group.

The steps taken to quantitate the vessels associated with the xenograft from **Figure 1D** are shown in **Figure 2**. The tumor channel is shown alone in **Figure 2A**, where a mass of B16-F1 cells fluorescently-labelled (false-colored green) can be seen below the yolk sac, which displays autofluorescence. An ROI must carefully be drawn around the xenograft (**Figure 2A'**), taking care

not to include any of the autofluorescence from the yolk sac as the Tumor Volume protocol may then identify this autofluorescence as part of the tumor. Performing the Tumor Volume protocol identifies all the B16-F1 cells in the ROI, measures their volume and clips the ROI to their volume (**Figure 2A''**). Performing the Tumor Vessel Volume protocol in this new clipped ROI allows the identification of all the vessels associated with the mass of B16-F1 cells and measures their volume (**Figure 2A'''**). The values from the Tumor Volume and Tumor Vessel Volume protocols can be used to give a measure of graft vascularization which is then plotted in a graph as seen in **Figure 1F**.

FIGURE AND TABLE LEGENDS:

Figure 1: Zebrafish tumor xenograft vascularization is inhibited by a VEGFR signaling inhibitor. (**A-C**) Confocal images of a live embryo at 6 hpi (**A**), 24 hpi (**B**) and 48 hpi (**C**), with GFP-labelled blood vessels (magenta). The % graft vascularization was calculated and included in the corresponding panel. (**D-E**) Confocal images of live zebrafish larvae shown at 48 hpi with fluorescently-labelled B16-F1 xenografts (green) and GFP-labelled blood vessels (magenta) which were treated immediately after implantation with either 0.5% (v/v) DMSO in E3 (**D**) or 50 nm of a VEGFR inhibitor (Tivozanib) in E3 (**E**). The vessel channels for **D** and **E** are shown in isolation in **D'** and **E'**, respectively. (**F**) Graph displaying the data from quantifying the % graft vascularization at 48 hpi in these xenografts, *n = 42* (DMSO), *n = 20* (VEGFR inhibitor). **p>0.01 by Mann-Whitney test, error bars represent s.d. Scale bar = 50 μm. Data contained in **Figure 1C** is reproduced[11].

Figure 2: Quantifying graft vascularization. Confocal image of live 2 dpi zebrafish larvae with fluorescently-labelled B16-F1 xenografts (green) and GFP-labelled blood vessels (magenta). (**A**) Tumor channel prior to drawing of a ROI. (**A'**) An ROI is drawn around the xenograft region, ensuring that the autofluorescence in the yolk is not included. (**A''**) The "Tumor Volume" protocol is performed to identify the volume of xenograft inside the ROI and also creates a new ROI. (**A'''**) The "Tumor Vessel Volume" protocol is used to identify the volume of vessels inside the new ROI. Scale bar = 50 μm.

Supplementary Movie 1: Tumor xenograft angiogenesis. Confocal time-lapse imaging of a B16-F1 xenograft implanted into a 2 dpf zebrafish embryo with GFP-labelled blood vessels (*kdrl:EGFP*). Movie taken from 20.75 hpi to 46 hpi. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. Scale bar = 50 μm.

DISCUSSION:

The first critical step in the protocol is the implantation of tumor cells. It is essential that cells are injected into a location that will allow the xenograft to implant successfully in the embryo without making the embryo edematous. An injection that is too anterior can allow the cells to move towards the heart, blocking the bloodstream and leading to edema, while an injection that is too posterior will result in a poorly implanted xenograft. An anterior injection is best avoided by inserting the needle through the yolk sac in an anterior to posterior direction so that it is pointing away from the heart as it injects. A posterior injection is best avoided by injecting the cells at a location in the anterior half of the round part of the yolk sac. In addition, cells must be injected ventral to the yolk sac and not lateral to it, as the lateral location is more difficult to view and

subsequently image. Once the researcher is reasonably adept at this process, approximately 200-300 embryos can be implanted with xenografts each day. Imaging of the angiogenic response will take longer due to the time taken to mount and image the xenografts; it takes approximately one hour to image 15-20 xenografts using a standard laser-scanning confocal microscope.

The second critical step is the measurement of graft vascularization using 3D image analysis software. Quantitation by tumor volume is required as it is difficult to obtain consistently-sized xenografts through microinjection. It is necessary to calibrate the settings of the software protocols carefully and use them consistently in order to obtain accurate and unbiased quantitative measurements for all experiments. Setting the minimum threshold for intensity (for both Tumor Volume and Tumor Vessel Volume) is an important part of this step as it allows the protocol to correctly discriminate between fluorescence that is part of the tumor/vessels and background fluorescence. Drawing the ROI around the tumor xenograft to include only the tumor xenograft and not any bright autofluorescing parts of the embryo (such as the yolk sac) is also important; the accidental inclusion of any non-tumor autofluorescing regions or non-tumor blood vessels into the ROI will result in these parts being measured as part of "Tumor volume" or "Tumor Vessel volume", creating significant inaccuracies in the final calculation. There is always a large variation in the angiogenic response (see **Figure 1F**), however, rather than being a limitation of the model, this may merely reflect the natural variance in tumor vessel density observed in both human patients and murine xenografts[23,24]. The quantitation of graft vascularization in 60 xenografts will take approximately 1 h.

Careful selection of tumor cell line is also important as there is a wide variation in the angiogenic response induced between different mammalian cell lines, which may relate to the differing levels of pro-angiogenic molecules produced by these cells[4,11]. In our hands, B16-F1 mouse melanoma cells give a robust angiogenic response, possibly due to the high level of VEGFA secretion from these cells[11]. Possible modifications to this protocol would be the use of zebrafish melanoma cells, which would allow the assay temperature to be lowered to 28 degrees and therefore be optimal for both the host and tumor[25] or the use of cancer cells that overexpress pro-angiogenic growth factors such as FGF[4,7,20].

Some of the advantages of this model lie in its short time span, low cost, and the relative ease with which the xenotransplantation procedure can be conducted compared with other in vivo models such as murine xenografts, all of which make it highly amenable to be used for large scale studies (i.e., chemical screens for anti-angiogenic compounds[26]). The ability to live image the model and the availability of transgenic fish with fluorescently labelled blood vessels and other cell types allows researchers to study the details of the angiogenesis process (such as vessel sprouting) as well as the cell-cell interactions (such as macrophage-endothelial interactions) that take place during angiogenesis in this model[11,20,27]. As vessel normalization is also a key objective of antiangiogenic therapy, high-resolution live-imaging of the vascular network in the xenografts means that this model has the potential to identify treatments that are directed at this objective. Network tortuosity could be examined by counting the number of vessel branchpoints, while vessel morphology could be examined by measuring variation in vessel width, allowing pro-normalization treatments to be tested and evaluated in this model[28]. In addition, fluorescence

485 microangiography can be used to determine the patency and integrity of tumor vessels[29]. The
486 genetic tractability of the zebrafish also means that it can be genetically modified to examine the
487 role of various host signaling pathways in tumor angiogenesis[6]. This model can therefore be used
488 to identify novel anti-angiogenic compounds that are active in a tumor setting as well as studying
489 the cellular and molecular interactions that regulate tumor angiogenesis.
490
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502 **REFERENCES:**
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Figure 1

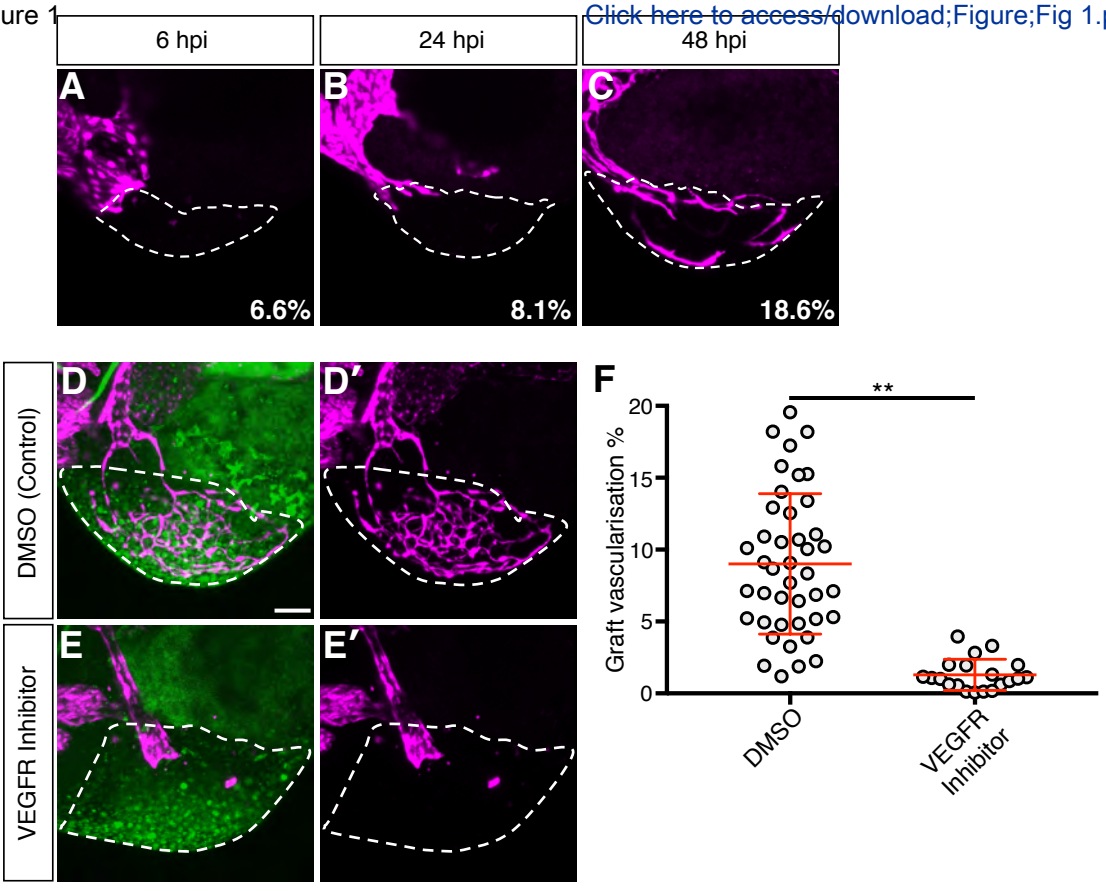
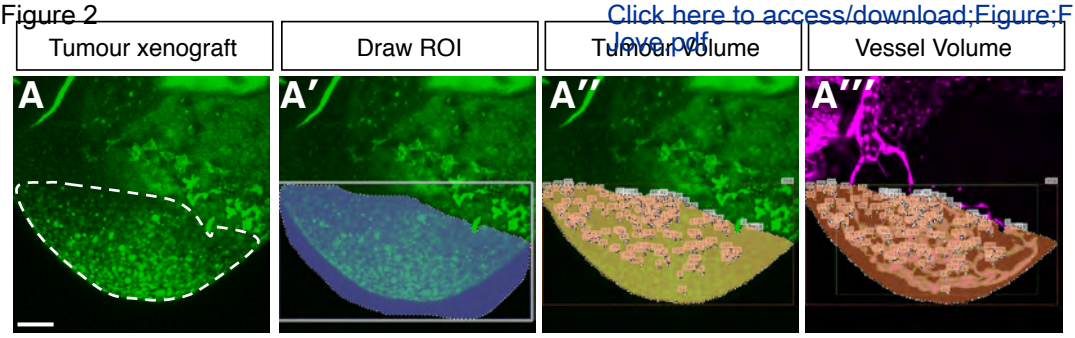


Figure 2



Movie 1

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Air cylinder	BOC	011G	Xenotransplantation
B16-F1 cells	ATCC		Cell culture
BD Matrigel LDEV-free (extracellular matrix mixture)	Corning	356235	Xenotransplantation
Borosillicate glass capillaries	Warner Instruments	G100T-4	OD=1.00 mm, ID=0.78 mm, Length =10 cm Cell injection
Cell culture dish -35 mm diameter	Thermofisher NZ	NUN153066	Fish husbandry
Cell culture dish -100 mm diameter	Sigma-Aldrich	CLS430167-500EA	Fish husbandry
Cell culture flask 75 cm^2	In Vitro Technologies	COR430641	Cell culture
CellTracker Green	Invitrogen	C2925	Cell labelling, Stock concentration (10 mM in DMSO), wor
Dimethyl sulfoxide	Sigma-Aldrich	D8418	Drug treatment, Cell labelling
E3 Media (60x in 2 L of water) 34.8 g NaCl 1.6 g KCl 5.8 g $CaCl_2 \cdot 2H2O$ 9.78 g $MgCl_2 \cdot 6H2O$ adjust to pH 7.2 with NaOH	In house [1]		Fish husbandry
Ethyl-3-aminobenzoate methanesulfonate (Tricaine)	Sigma-Aldrich	E10521	Xenotransplantation, Imaging
Filter tip 1000 µL	VWR	732-1491	Used during multiple steps
Filter tip 200 µL	VWR	732-1489	Used during multiple steps
Filter tip 10 µL	VWR	732-1487	Used during multiple steps
Fluorescence microscope	Leica	MZ16FA	Preparation of embryos

FBS (NZ origin)	Thermofisher Scientific		10091148	Cell culture
Gloves	Any commercial brand			Used during multiple steps
Haemocytometer cell counting chamber Improved Neubauer	HawksleyVet	AC1000		Xenotransplantation
Heraeus Multifuge X3R Centrifuge	Thermofisher Scientific		75004500	Cell culture, Cell labelling
Hoechst 33342	Thermofisher Scientific		62249	Cell labelling, Stock concentration (1 mg/ml in DMSO), wo
Low Melting Point, UltraPure Agarose	Thermofisher Scientific		16520050	Imaging
Methycellulose	Sigma-Aldrich	9004 67 5		Xenotransplantation
Methylene blue	sigma-Aldrich	M9140		Fish husbandry
Microloader 0.5-20 µL pipette tip for loading microcapillaries	Eppendorf		5242956003	Xenotransplantation
Micropipettes	Any commercial brand			Used during multiple steps
Micropipette puller P 87	Sutter Instruments			Xenotransplantation
Microscope cage incubator	Okolab			Time-lapse imaging
Microwave	Any commercial brand			Imaging
Mineral oil	Sigma-Aldrich	M3516		Xenotransplantation
Minimal Essential Media (MEM) - alpha	Thermofisher Scientfic		12561056	Cell Culture
MPPI-2 Pressure Injector	Applied Scientific Instrumentation			Xenotransplantation
Narishige micromanipulator	Narishige Group			Xenotransplantation
Nikon D Eclipse C1 Confocal Microscope	Nikon			Imaging
N-Phenylthiourea (PTU)	Sigma-Aldrich	P7629		Fish husbandry
PBS	Gibco		10010023	Cell culture

Penicillin Streptomycin	Life Technologies	15140122	Cell culture
S1 pipet filler	Thermoscientific	9501	Cell culture
Serological stripette 10 mL	Corning	4488	Cell culture
Serological stripette 25 mL	Corning	4489	Cell culture
Serological stripette 5 mL	Corning	4485	Cell culture
Serological stripette 2 mL	Corning	4486	Cell culture
Terumo Needle 22 gauge	Amtech	SH 182	Fish husbandry
Tissue culture incubator	Thermofisher Scientfic	HeraCell 150i	Cell culture
Tivozanib (AV951)	AVEO Pharmaceuticals		Drug treatment
Transfer pipette 3 mL	Mediray	RL200C	Fish husbandry
Trypsin/EDTA (0.25%)	Life Technologies	T4049	Cell culture
Tweezers	Fine Science Tools	11295-10	Fish husbandry
Volocity Software (v6.3)	Improvision/Perkin Elmer		Image analysis

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king concentration (0.2 µM in serum-free media)

rking concentration (6 μg/ml in serum-free media)

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17th April 2019

Editor, JoVE

JoVE59849 "*In vivo* imaging and quantitation of the host angiogenic response in zebrafish tumour xenografts."

Dear editor.

Thank you for allowing us to submit a revised version of our manuscript to *JoVE*. We have addressed all the comments raised by our reviewers as well as the required editorial changes.

The main changes are:

1) Addition of xenograft quantitation from different time-points post-injection. This new data has been added to Figure 1A-C

2) Discussion of the origin of the tumour vessels and the nature of the variation observed in graft vascularisation. This has been added to the representative results section.

3) Discussion on the throughput of the assay and the suitability of the assay to study tumour vessel normalisation. This has been added to the discussion section.

All textual changes as required by the reviewers are in red font. The editorial changes are unmarked.

We look forward to your response.

Yours sincerely

Dr. Jonathan Astin
Senior Research Fellow
Department of Molecular Medicine and Pathology
Faculty of Medical and Health Sciences
The University of Auckland

Referee #1:

Minor Concerns:

As the authors stated their method of using of 2% methylcellulose in line 153 for holding the embryos, which is different from many other researchers who simply use agarose block with V-shape mount for holding zebrafish embryos. Although it is the preference of the authors, it may be helpful for other audience to know if the authors would describe the advantage of their method of using methylcellulose instead of agarose block which was used by many other researchers.

We thank the reviewer for noting this and have added a comment on this alternative mounting method on page 5.

Also, their calibration of injector to deliver 40-50 cells per injection pulse, in order to deliver 300-400 cells per embryo, the authors will have to inject at least 6-8 times to the same location and same embryos, which seems excessive. It may be helpful if the authors explain why they choose to do like this.

This was a mistake, we actually inject around 150 cells per pulse. This have been corrected on page 6.

In addition, it will be very helpful if the authors could include the throughput of the number of xenograft embryos in one day and the throughput for imaging of the angiogenic response. These will help audience to evaluate if this method can be adopted for how many treatments and concentration in one experiment.

Comments on the throughput of the injection, imaging and analysis stages have been included in the discussion on page 12.

Referee #2:

Major Concerns:

1-Figure 1 and Movie 1 show the GFP-positive vessels invading the tumor mass. However, no information about the origin of these vessels is provided. When considering the site of injection and looking at the cropped image it seems that new vessels may come from the most rostral part of the Cuvier Duct. This origin is different from what described in the cited original paper from Nicoli at al.; 2007. This aspect has to be better clarified and discussed.

The reviewer is correct. The vessels in our grafts arise from the rostral portion of the duct of cuvier/common cardinal vein rather than the sub-intestinal vessels described in Nicoli et al (2007). This is because the site of graft injection differs from this previous study and we chose this more rostral injection site as it enables better imaging of the tumour. This is now explained in the results section on page 10.

2-In Figure 1 C, the graph shows the quantification of the xenograft vascularization after treatment with a known VEGFR inhibitor or DMSO as control. Is the large variation of the graft vascularization in DMSO treated embryos mostly due to the "Vessel Volume" values or there are also differences in "Tumor Volume"?
This could be an important point to be addressed if the method will be applied to evaluate a pharmacological compound that may affect both tumor and endothelial cells behavior.

We observe a large variation in both graft volume and in the amount of graft vascularisation. We normalise the levels of graft vascularisation against graft volume so the differences in overall graft vascularisation % are primarily due to the differences in graft vascularisation.

We have included a description of these variances in the results section on pages 10-11.

3-The authors present the model as highly suited to imaging dynamic cellular processes and show a time-lapse movie of the vascularization process occurring between 20.75 hours post injection (hpi) and 46 hpi. I suggest to show quantification of the angiogenic response at different time points (for example 1 hpi, 20.75 hpi and 46 hpi) taking advantage from the already produced z-stacks. This point is critical if the goal is to dynamically evaluate and quantitate the molecular mechanisms involved and the host vs tumor contribution to the angiogenetic process.

We thank the reviewer for this suggestion. We have included the images and quantitation of a representative xenograft at 6 hpi, 24 hpi and 48 hpi which illustrates that the angiogenic response is highest from 24-48 hpi. This data is now included in Figure 1A-C and in the results on page 10.

Minor Concerns:
1-Vessel normalization is a critical goal to be achieved in antiangiogenic therapy; an antiangiogenic approach that apparently does not reduce graft vascularization could instead cause vessel normalization, thus being suitable for combined therapy. The described method is based on confocal microscopy imaging so I wondering if it is possible to obtain some information regarding this phenomenon from the proposed method and to discriminate a molecule with a vessel normalizing activity from a non-effective one.

We have added some possible alternative analyses that may offer insight into vessel normalising activity. These include analysis of vessel integrity and tortuosity/branching. This has been added to the discussion on page 13.