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TITLE:

In Vivo Targeting of Xenografted Human Cancer Cells with Functionalized Fluorescent Silica Nanoparticles in Zebrafish

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Zebrafish, *Casper*, transplantation, in vivo targeting, nanoparticles, cancer

SUMMARY:

Described here is a method for utilizing zebrafish embryos to study the ability of functionalized nanoparticles to target human cancer cells in vivo. This method allows for the evaluation and selection of optimal nanoparticles for future testing in large animals and in clinical trials.

ABSTRACT:

Developing nanoparticles capable of detecting, targeting, and destroying cancer cells is of great interest in the field of nanomedicine. In vivo animal models are required for bridging the nanotechnology to its biomedical application. The mouse represents the traditional animal model for preclinical testing; however, mice are relatively expensive to keep and have long experimental cycles due to the limited progeny from each mother. The zebrafish has emerged as a powerful model system for developmental and biomedical research, including cancer research. In particular, due to its optical transparency and rapid development, zebrafish embryos are well suited for real-time in vivo monitoring of the behavior of cancer cells and their interactions with their microenvironment. This method was developed to sequentially introduce human cancer

cells and functionalized nanoparticles in transparent *Casper* zebrafish embryos and monitor in vivo recognition and targeting of the cancer cells by nanoparticles in real time. This optimized protocol shows that fluorescently labeled nanoparticles, which are functionalized with folate groups, can specifically recognize and target metastatic human cervical epithelial cancer cells labeled with a different fluorochrome. The recognition and targeting process can occur as early as 30 min postinjection of the nanoparticles tested. The whole experiment only requires the breeding of a few pairs of adult fish and takes less than 4 days to complete. Moreover, zebrafish embryos lack a functional adaptive immune system, allowing the engraftment of a wide range of human cancer cells. Hence, the utility of the protocol described here enables the testing of nanoparticles on various types of human cancer cells, facilitating the selection of optimal nanoparticles in each specific cancer context for future testing in mammals and the clinic.

INTRODUCTION:

The development of nanoparticles that are capable of detecting, targeting, and destroying cancer cells is of great interest to both physicists and biomedical researchers. The emergence of nanomedicine led to the development of several nanoparticles, such as those conjugated with targeting ligands and/or chemotherapeutic drugs[1,2,3]. The added properties of nanoparticles enable their interaction with the biological system, sensing and monitoring biological events with high efficiency and accuracy along with therapeutic applications. Gold and iron oxide nanoparticles are primarily used in computed tomography and magnetic resonance imaging applications, respectively. While the enzymatic activities of gold and iron oxide nanoparticles allow the detection of cancer cells through colorimetric assays, fluorescent nanoparticles are well suited for in vivo imaging applications[4]. Among them, ultrabright fluorescent nanoparticles are particularly beneficial, due to their ability to detect cancers early with fewer particles and reduced toxicities[5].

Despite these advantages, nanoparticles require experimentation using in vivo animal models for the selection of suitable nanomaterials and optimization of the synthesis process. Additionally, just like drugs, nanoparticles rely on animal models for preclinical testing to determine their efficacy and toxicities. The most widely used preclinical model is the mouse, which is a mammal whose upkeep comes at a relatively high cost. For cancer studies, either genetically engineered mice or xenografted mice are typically used[6,7]. The length of these experiments often spans from weeks to months. In particular, for cancer metastasis studies, cancer cells are directly injected into the circulatory system of the mice at locations such as tail veins and spleens[8-10]. These models only represent the end stages of metastasis when tumor cells extravasate and colonize distant organs. Moreover, due to visibility issues, it is particularly challenging to monitor tumor cell migration and the targeting of tumor cells by nanoparticles in mice.

The zebrafish (*Danio rerio*) has become a powerful vertebrate system for cancer research due to its high fecundity, low cost, rapid development, optical transparency, and genetic conservations[11,12]. Another advantage of the zebrafish over the mouse model is the fertilization of the fish eggs ex utero, which allows the embryos to be monitored throughout their development. Embryonic development is rapid in zebrafish, and within 24 h postfertilization (hpf), the vertebrate body plane has already formed[13]. By 72 hpf, eggs are hatched from the

chorion, transitioning from the embryonic to the fry stage. The transparency of the zebrafish, the *Casper* strain in particular[14], provides a unique opportunity to visualize the migration of cancer cells and their recognition and targeting by nanoparticles in a living animal. Finally, zebrafish develop their innate immune system by 48 hpf, with the adaptive immune system lagging behind and only becoming functional at 28 days postfertilization[15]. This time gap is ideal for the transplantation of various types of human cancer cells into zebrafish embryos without experiencing immune rejections.

Described here is a method that takes advantage of the transparency and rapid development of zebrafish to demonstrate the recognition and targeting of human cancer cells by fluorescent nanoparticles in vivo. In this assay, human cervical cancer cells (HeLa) cells genetically engineered to express a red fluorescent protein were injected into the vascularized area in the perivitelline cavity of 48 hpf embryos. After 20–24 h, HeLa cells had already spread throughout the embryos through the fish circulatory system. Embryos with apparent metastasis were microinjected with ~0.5 nL of a nanoparticle solution directly behind the eye, where the rich capillary bed is located. Using this technique, the ultrabright fluorescent silica nanoparticles can target HeLa cells as quickly as 20–30 min postinjection. Due to its simplicity and effectiveness, the zebrafish represents a robust in vivo model to test a variety of nanoparticles for their ability to target specific cancer cells.

PROTOCOL:

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston University School of Medicine under the protocol #: PROTO201800543.

1. Generation of *Casper* zebrafish embryos

1.1. Choose adult *Casper* fish that are at least 3 months of age for natural breeding to generate transparent *Casper* zebrafish embryos.

1.2. Fill two-chamber mating tanks with fish water in the evening, separate the upper tanks using dividers, place one male fish into one side of the chamber and one or two female fish into the other side of the chamber, and leave the fish separated overnight by dividers.

1.3. Pull out the dividers the next morning at 8:00 AM when the lights are on. Add artificial enrichment plants and tilt the top chamber slightly to create a shallow area of water. Allow the fish to breed for 3–4 h.

1.4. Lift the top chambers of the mating tanks that contain the fish and return them to their original tanks.

1.5. Collect eggs located in the bottom chambers by pouring the water through a mesh net. Transfer eggs to a sterile Petri dish at a density no greater than 200 eggs per dish. Remove any dead or unfertilized eggs and fill the dish 2/3 full with fresh fish water.

NOTE: Fertilized and healthy eggs should be translucent and round. Any eggs that are cloudy, white, or disfigured should be removed. Fish water is obtained from fish tanks in the fish facility.

1.6. Incubate embryos in the incubator at 28.5 °C overnight.

1.7. Bleach the embryos the next morning using the standard protocol as described in *The Zebrafish Book*[16], and put the embryos back to the incubator (optional step).

1.8. Take the 24 hpf embryos out of the incubator in the afternoon, and dechorionate the embryos using pronase.

1.8.1. Remove as much fish water as possible from the embryos in the Petri dish and add a few drops of the pronase solution (1 mg/mL in fish water) to the dish. Gently swirl the Petri dish. Once the chorions show signs of disintegration, pipette the embryos up and down a few times to break down the chorions to release the embryos.

1.8.2. Add fresh fish water immediately into the Petri dish to terminate the process once a majority of the embryos are out of the chorions. Rinse the embryos 3x more using fish water to remove the floating chorions. Return the embryos to the incubator.

2. Preparation of human cancer cells for transplantation

2.1. Set the incubator temperature to exactly 35.5 °C. Monitor the incubator to ensure a consistent and stable temperature using a thermometer inside the incubator.

2.2. Autoclave 1 L of fish water in a glass bottle. Make a 3% agarose solution by adding 3 g of electrophoresis-grade agarose to 100 mL of autoclaved fish water and microwaving until the agarose is completely dissolved.

2.2.1. Pour hot agarose solution into a Petri dish until it is ¾ full. Place the microinjection mold on the agarose. Ensure that the mold is not in contact with the bottom of the Petri dish and no bubbles form underneath.

2.2.2. Allow the solution to solidify and carefully remove the mold from the plate. Fill the plate with autoclaved fish water and store the plate at 4 °C. Prewarm the agarose plate and fish water in the 35.5 °C incubator before harvesting HeLa cells.

2.3. Pull 1.0 mm O.D. x 0.78 mm borosilicate glass capillaries on a pipette puller using the following settings: pressure at 500, heat at 560, pull at 100, velocity at 100, and time/delay at 200. Store needles on putty in a large Petri dish that has been wiped with an ethanol towel.

CAUTION: Pulled needles are very sharp and fragile. Use caution when handling.

2.4. Prepare a stock solution of tricaine methanesulfonate (MS222, 4 mg/mL) by dissolving

MS222 into autoclaved fish water. Vortex well before use. Dilute MS222 stock solution 1:100 in fish water (i.e., add 200 µL of MS222 stock solution to 20 mL of fish water to a final concentration of 40 µg/mL) to anesthetize embryos for the following procedures.

2.5. Culture hLabel HeLa cells by transducing them with PLenti6.2_miRFP670 lentivirus using the protocol described[17]. Harvest RFP+ HeLa cells 30 min–1 h before the transplantation.

NOTE: Human HeLa cells have been cultured in a tissue culture incubator up to 70% confluency in complete growth medium (DMEM medium with 10% FBS) at 37 °C supplemented with 5% CO_2.

2.5.1. Remove the HeLa cell medium by aspiration in a tissue culture hood. Briefly rinse the cell layer with sterile PBS to remove all traces of the serum.

2.5.2. Add 3.0 mL of sterile Trypsin-EDTA solution to a T-75 flask and place the cells back into the 37 °C tissue culture incubator for 3–5 min to facilitate enzymatic digestion. Observe the flask under the microscope until ~80% of the cells become suspended.

2.5.3. Add 6–8 mL of complete growth medium into the flask. Collect the cells into a 15 mL sterile tube by gently pipetting. Centrifuge at 135 x *g* for 5 min.

2.5.4. Aspirate the supernatant and resuspend HeLa cells in 3 mL of complete growth medium. Repeat the above washing step 2x. Resuspend the cells in 1 mL of complete growth medium and count the cells under the microscope using a hemocytometer.

2.5.5. Spin the cells down again, remove the supernatant, and resuspend the cells in a 1.5 mL microcentrifuge tube at a concentration of 5 x 10^7 cells/mL. Keep the cells warm by holding the tube in one hand when transporting to the fish facility.

NOTE: Always keep the cells warm by storing the cells inside the 35.5 °C incubator before or when injecting the embryos.

3. Transplantation of human cancer cells

3.1. Clean up the work area using ethanol towels before transplantation (e.g., scissors, tweezers, plastic pipettes, razor blades).

3.2. Align embryos within the grooves of the agarose plate using a plastic pipette. Lay embryos on the side with the anterior facing forward.

NOTE: Make sure that the fish water covers the embryos. Set some embryos aside to not inject cancer cells and use as controls.

3.3. Turn on the air source and microinjector. Take HeLa cells out of the incubator and pipette the cells up and down 20–30x using a P200 tip. Load 3 µL of cell mixture immediately into a needle

using a gel loading tip with a cut end. Carefully insert the tip toward the sharp bottom end of the needle. If needed, shake the needle to ensure the cell mixture moves down the needle to fill up the sharp end.

3.3.1. Insert the needle into the needle holder. Use a pair of tweezers to carefully break open the tip of the needle. Adjust the pressure and duration of time on the microinjector to push out all of the air bubbles inside the needle tip. Reduce the pressure and injection duration time until the size of the injection droplets is ~1 nL.

3.3.2. Place the injection plate under the microscope in an appropriate position to have the yolk side of embryos facing the needle. Anesthetize the embryos by adding five drops of the diluted MS222 solution (40 µg/mL).

3.3.3. Position the injector and allow the needle to touch the perivitelline cavity of each embryo.

3.4. Inject the cell mixture into the embryos at the vascularized area under the perivitelline cavity by pressing the foot pedal.

3.5. Use the nondominant hand to move the injection plate to the next embryo. Use the dominant hand to extend and retract the injector while pressing the foot pedal simultaneously to continue the injection. Pipette a few drops of sterile fish water onto embryos that have been injected. Once the injection of all embryos on the plate is completed, wash the embryos off with sterile fish water, put them on a sterile Petri dish and immediately move them to the 35.5 °C incubator.

3.6. After 3 h, examine the injected embryos and remove the dead ones.

3.7. Return the live embryos to the 35.5 °C incubator and incubate them for 20–24 h to allow the HeLa cells to spread from the injection site to other parts of the body.

NOTE: Embryos are kept in 35.5 °C incubator to allow the survival and migration of human cancer cells because the cells do not do well at 28.5 °C, the temperature fish embryos are normally incubated.

4. Injection of nanoparticles or vehicle

4.1. Anesthetize the transplanted embryos the next morning with five drops of diluted MS222 solution. Do not to add too much MS222, because this will kill the embryos. Under a fluorescent microscope, carefully pick up embryos with tail metastasis of RFP+ HeLa cells and place them into a new Petri dish with sterile fish water.

4.2. Make the injection needles as previously described in section 2 using the following settings: pressure at 500, heat at 645, pull at 60, velocity at 50, and time/delay at 100.

4.3. Follow the procedures in steps 3.1–3.3 to align embryos and load vehicle (e.g., H_2O) or the nanoparticle solution into the needle.

4.4. Inject 0.5 nL of 1 mg/mL nanoparticle solution behind the eye and continue the injection as described in steps 3.5–3.6 (**Figure 1B**). This location behind the eyes is enriched with capillaries, allowing the nanoparticles to enter circulation.

4.5. Following a similar procedure, inject the vehicle (e.g., H_2O) that was used to suspend the nanoparticles into embryos with HeLa cells transplanted and those without (i.e., controls) (**Figure 1A**,**C**).

4.6. Incubate all injected embryos at 35.5 °C.

5. Imaging and tracking of nanoparticles and cancer cells

5.1. Examine injected embryos under a fluorescent microscope at 0, 30, 60, 90, 120, 180, and 210 min postinjection of nanoparticles to monitor their distribution in circulation and the degree of cancer cell targeting. The targeting of cancer cells by nanoparticles can be observed as early as 30 min postinjection depending on the type of nanoparticle tested.

5.2. Pipette 2–3 embryos into a Petri dish and immobilize them by adding five drops of diluted MS222 solution (40 µg/mL). Once the embryos stop swimming, remove most of the water to allow the embryos to lie on their sides.

5.3. Use a pipette with a thin, soft brush attached to its end to align the embryos so they lie on their sides with the anterior facing forward and only one eye visible.

5.4. Image the embryos in red, blue, and brightfield channels at low magnification (2x) to capture the whole embryo and repeat at higher magnification (6.4x) to capture the tail area. Focus the embryo under the red channel to avoid the bleeding of nanoparticles.

NOTE: The embryos must not move during imaging. Any movement will lead to blurry images and the inability to overlap images from different channels.

5.5. Add fresh fish water to the embryos immediately after imaging and return them to the incubator. Repeat steps 5.2–5.4 to image the embryos at different time points.

REPRESENTATIVE RESULTS:

The protocol schematic in (**Figure 1**) illustrates the overall procedures for this study. Transparent *Casper* male and female adult fish were bred to generate embryos (section 1). RFP+ HeLa cells were injected into the vascularized area under the perivitelline cavity of the zebrafish embryos at 48 hpf, with uninjected embryos as controls (section 3). For individuals experienced in microinjection, the survival rate of embryos is often high, with at least 50% of embryos transplanted with cancer cells surviving in the 35.5 °C incubator, a temperature suboptimal for

zebrafish embryos but required for the survival and migration of human cancer cells. HeLa cells are highly invasive and can intravasate and spread to the tail region of the embryos as quickly as 8 h postinjection. By 20–24 h post-transplantation, ~50% of embryos transplanted showed signs of metastatic spread of HeLa cells. Those embryos with cancer cell tail metastases were selected for downstream experiments. At 72 hpf, these embryos were subsequently injected behind the eyes either with blue fluorescent nanoparticles (section 4 and **Figure 1B**) or solely with the vehicle as controls (**Figure 1A**). Age-matched embryos injected with nanoparticles but without cancer cell transplantation were the second group of controls (**Figure 1C**). For more detailed information on nanoparticle synthesis, preparation, and characterization see Peerzade et al.[18].

At 0, 30, 60, 90, 120, 180, 210 min postinjection of nanoparticles, the injected embryos were monitored by imaging to determine the interaction of nanoparticles with RFP+ HeLa cells, using the vehicle-injected embryos as controls. Specifically, the zebrafish tail areas where RFP+ HeLa cells had spread to were imaged at red, blue, and brightfield illumination using a fluorescent microscope (section 5). The detailed characterization of the ability of the ultrabright nanoparticles to target xenografted cancer cells in zebrafish over time is shown in Figure 5 of Peerzade et al.[18]. The red dots seen in the tail of the embryos are metastatic human cervical cancer cells that were visible in both vehicle- and nanoparticle-injected embryos (**Figure 2A**,**D**; **Figure 3A**,**D)**. As expected, no specific blue fluorescent signals were detected in embryos with the vehicle-only injection (**Figure 2B**,**E**). Additionally, when the images captured in the red and blue channels were merged, only red cancer cells in the tail region without any blue signals were observed (**Figure 2C**,**F**). However, in embryos that were injected with ultrabright fluorescent silica nanoparticles, there were blue dots in the tails, concentrated near and around the cancer cells at 3.5 h (**Figure 3B**,**E**). In the overlaid images captured from both red and blue channels, red HeLa cells and blue nanoparticles colocalized, seen as pink dots (**Figure 3C**,**F**). In those embryos that were injected solely with nanoparticles but not transplanted with HeLa cells, the blue fluorescent particles did not concentrate into any particular cells or areas, but distributed relatively evenly into the circulatory system of the embryos, highlighting blood vessels (**Figure 4B**,**E**). As expected, no specific red fluorescent signals were detected in these embryos despite some weak background fluorescent signals (**Figure 4A**,**C**,**D**,**F**).

This protocol was subsequently used to test different types of nanoparticles[18-20]. Colocalization of cancer cells with certain types of nanoparticles were observed as early as 30 min postinjection depending on the properties of the nanoparticle tested. By 120 min, there was >80% targeting of cancer cells by these nanoparticles in the tail region of the fish. However, for other nanoparticles, minimal targeting of cancer cells was observed, consistent with their lack of cancer-specific ligand. The detailed results and analysis are included in Peerzade et al. (see Figure 3 and Figure 4, Supplementary Figures S12–S16, and Supplementary Table S6)[18]. These results demonstrated differential targeting of nanoparticles to xenografted HeLa cells in zebrafish. Thus, using this protocol, one should be able to efficiently select nanoparticles based on their ability to recognize and target metastatic human cancer cells in vivo.

FIGURE AND TABLE LEGENDS:

Figure 1. Protocol schematic for studying the ability of nanoparticles to target human cancer cells. Transparent *Casper* embryos were generated through breeding male and female adult fish. Fertilized embryos were collected in a Petri dish. At 48 hpf, RFP+ HeLa cells were injected into zebrafish embryos at the perivitelline cavity, leaving some age-matched embryos uninjected as controls. At 72 hpf, embryos with metastatic RFP+ HeLa cells were selected and split into two groups: (**A**) injected with vehicle (H_2O) as control and (**B**) injected with nanoparticles suspended in H_2O. The third group was age-matched embryos that were injected with nanoparticles alone (**C**). All three groups were imaged under a fluorescent microscope. The boxed area shown is where images were captured (see **Figure 2**–**Figure 4**). Scale bars for adult fish = 1 mm and for embryos = 500 μm.

Figure 2. **Zebrafish transplanted with metastatic HeLa cells without nanoparticles.** Only red fluorescent HeLa cells were visible in the individual (**A**,**D**) or overlaid images of the red channel and blue channel (**C**,**F**). No specific blue fluorescent signals were detected in the embryo with vehicle injection control (**B**,**E**). Images in (**A**-**C**) show the fish tail region boxed as in **Figure 1A**. Images in (**D**-**F**) are enlarged views of the boxed areas in (**A**-**C**). Scale bars in (**A**-**C**) = 200 μm and in (**D**-**F**) = 100 μm.

Figure 3. **Colocalization of red fluorescent HeLa cells and blue fluorescent nanoparticles in zebrafish.** The zebrafish tails were imaged at both low (**A**-**C**) and high (**D**-**F**) magnification in the red and blue channel. Red fluorescent signals revealed metastatic HeLa cells (**A**,**D**), whereas blue fluorescent signals showed the nanoparticles (**B**,**E**). The overlaid images from both red and blue channels (**C**,**F**) show colocalization of HeLa cells and nanoparticles. The images were taken after 3.5 h from injection with ultrabright silica nanoparticles. Images in (**A**-**C**) show the fish tail region as boxed in **Figure 1B**. Images in (**D**-**F**) are enlarged views of the boxed areas in (**A**-**C**). Scale bars in (**A**-**C**) = 100 μm and in (**D**-**F**) = 50 μm.

Figure 4. Zebrafish injected with nanoparticles without human HeLa cells. Blue fluorescent nanoparticles were distributed into the circulatory system of the embryos in the individual (**B**,**E**) and overlaid images of the red and blue channel (**C**,**F**). No specific red fluorescence was visible at either low or high magnification (**A**,**D**) except some background fluorescence common to zebrafish embryos. Images in (**A**-**C**) show the fish tail region as boxed in **Figure 1C**. Images in (**D**-**F**) are enlarged views of the boxed areas in (**A**-**C**). Scale bars in (**A**-**C**) = 100 μm and in (**D**-**F**) = 50 μm.

DISCUSSION:

The protocol described here utilizes the zebrafish as an in vivo system to test the ability of nanoparticles to recognize and target metastatic human cancer cells. Several factors can impact the successful execution of the experiments. First, embryos need to be fully developed at 48 hpf. The correct developmental stage of the embryos enables them to endure and survive the transplantation of human cancer cells. Embryos younger than 48 hpf have a significantly lower survival rate compared to older and more developed embryos. Second, cancer cells should be kept as healthy as possible by ensuring they are: 1) in the exponential growth phase[21], 2) freshly harvested 30 min–1 h immediately before transplantation, and 3) kept warm at all times. Third,

the needle must not be clogged. Pipette the HeLa cells up and down at least 20x before loading the cell mixture into the needle. Fourth, different types of needles must be used for transplantation of human cancer cells and injection of nanoparticles. The needle for human cell transplantation is relatively wide, with an angle to avoid cell clogging, whereas the needle for nanoparticle injection is sharp and thin. Fifth, the location of the injection differs. The location for transplantation of human cells is the perivitelline cavity, but for nanoparticle injection, the needle should be inserted behind the eye, where there are enriched capillaries. Finally, the skill of the individual who performs transplantation matters. An experienced individual can accurately inject HeLa cells into the perivitelline cavity space, while an inexperienced person often injects tumor cells into the yolk area where tumor cells barely spread into the fish body. Similarly, the embryos' survival rate is much higher when handled by an experienced individual, with at least 50% of embryos transplanted with cancer cells surviving.

Although zebrafish embryos are usually incubated at 28.5 °C, human cancer cells require higher temperatures to survive and migrate[22,23]. To allow the survival of both fish embryos and human cancer cells, the embryos transplanted with human cancer cells are incubated at 35.5 °C instead. Although it may be easier to deliver cancer cells into the yolk sac, they barely spread into the circulatory system. Therefore, it is critical to inject the cancer cells into the vascularized area under the perivitelline cavity to ensure intravasation and spread of cancer cells. Additionally, one must take care not to add too much or too concentrated MS222 when anesthetizing the embryos during injection and imaging. To aid in imaging and visualization of nanoparticles, the *Casper* zebrafish was chosen instead of AB fish. The *Casper* fish is a double mutant for *Nacre* and *Roy* that lacks melanocytes and iridophores, resulting in increased transparency compared to AB fish[14]. The transparency of the *Casper* zebrafish enables monitoring the spread of nanoparticles in circulation and the targeting of the nanoparticles to cancer cells. The challenge of this protocol is the relatively high mortality rate of the embryos if an unexperienced individual performs the transplantation of human cancer cells. Interestingly, the injection of nanoparticles slightly behind the eyes is relatively well tolerated. This is likely due to the use of thinner needles compared to the needles used for tumor cell transplantation. To avoid damaging human cancer cells, one must use needles with wide openings, but these can lead to damage of the embryos if handled inappropriately.

This protocol utilizes the *Casper* zebrafish to visualize targeting of metastatic cancer cells with functionalized nanoparticles in vivo. A major advantage of this assay is that it allows the researchers to perform real-time imaging over the course of zebrafish development to monitor the interaction of cancer cells with nanoparticles. In fact, due to its high fecundity and rapid development, the zebrafish allows the researcher to obtain results in just a few days[18-20,24]. Moreover, this assay also allows the elimination of toxic nanoparticles from further research if most embryos die after injection of a particular type of nanoparticle. Although the zebrafish is not a mammal, it facilitates the selection of a large number of nanoparticles in a rapid and economical manner, providing useful information for downstream studies in large animals and clinical testing. Taken together, the zebrafish is making an impact in nanomedicine and nanotechnology by helping select suitable nanoprobes for early detection and potential destruction of cancer cells through cancer-specific targeting.

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DISCLOSURES:

I.S. declares interest in NanoScience Solutions, LLC (recipient of STTR NIH R41AI142890 grant). All other authors declare no conflicts of interest.

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22. Rao, P. N., Engelberg, J. Hela Cells: Effects of Temperature on the Life Cycle. *Science.* **148** (3673), 1092-1094 (1965).
23. Avdesh, A. et al. Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction. *Journal of Visualized Experiments.* (69), e4196 (2012).
24. Spence, R., Gerlach, G., Lawrence, C., Smith, C. The behaviour and ecology of the zebrafish, Danio rerio. *Biological Reviews of the Cambridge Philosophical Society.* **83** (1), 13-34 (2008).

Figure1

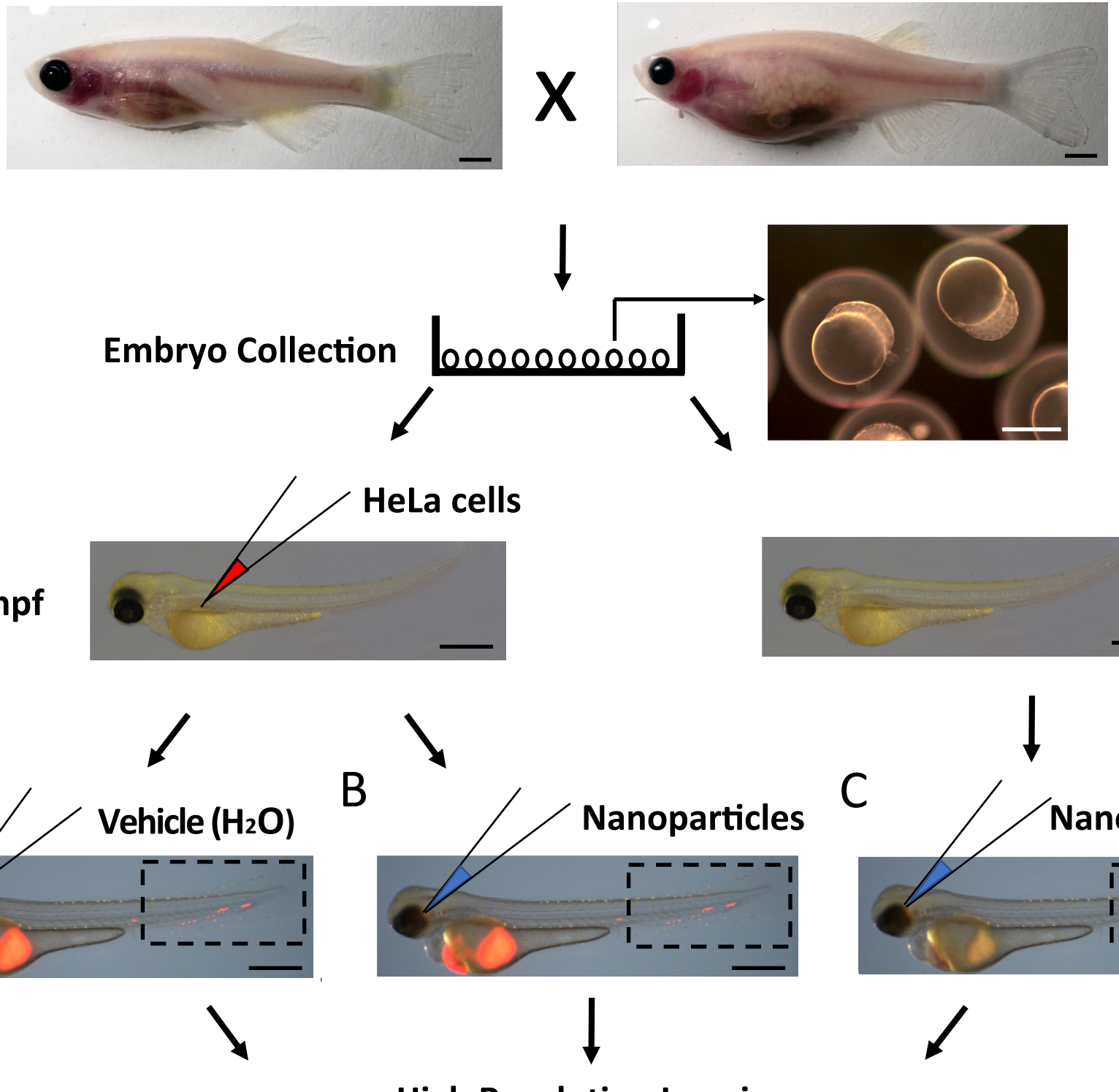

Figure 2

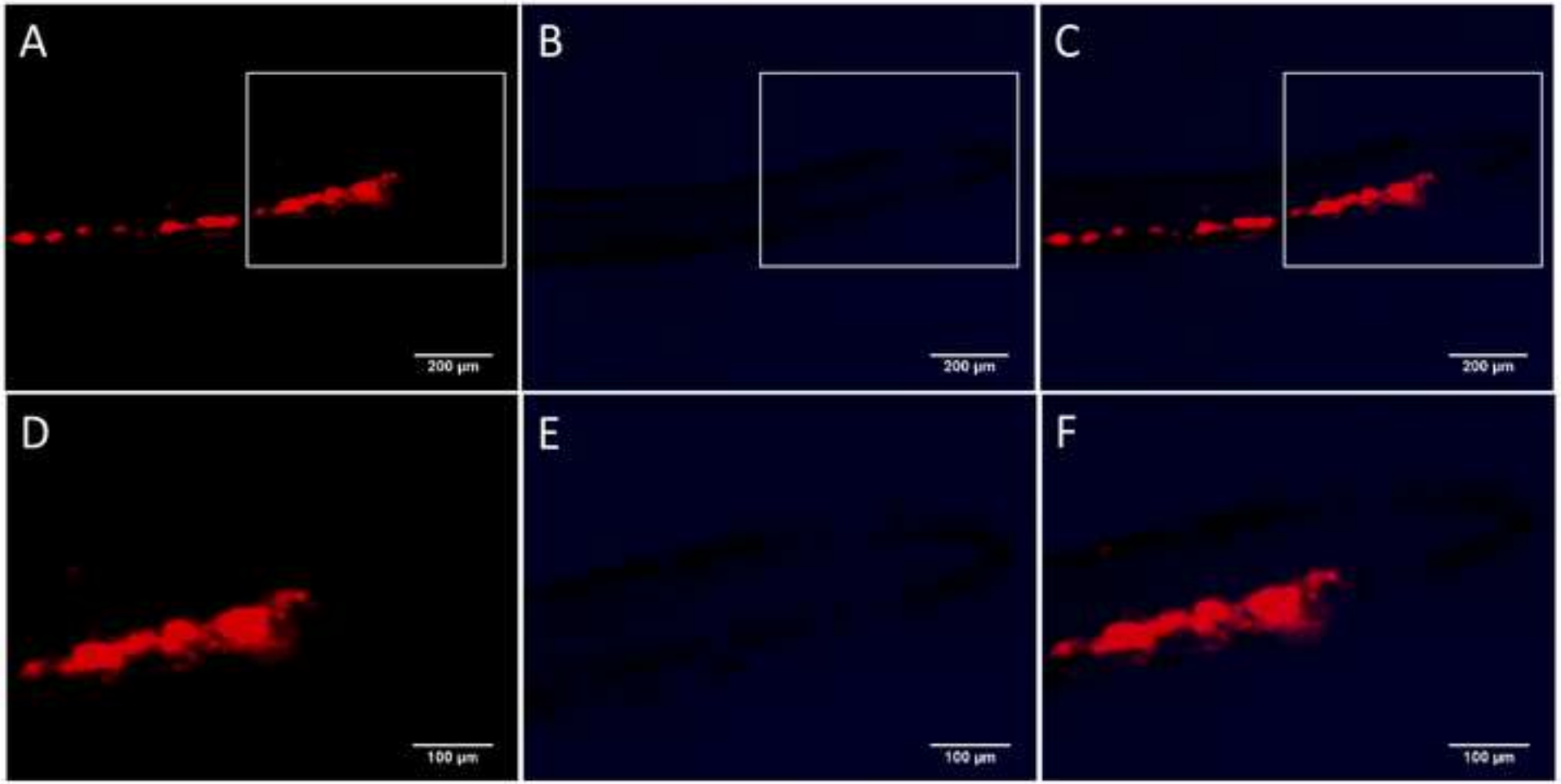

Figure 3

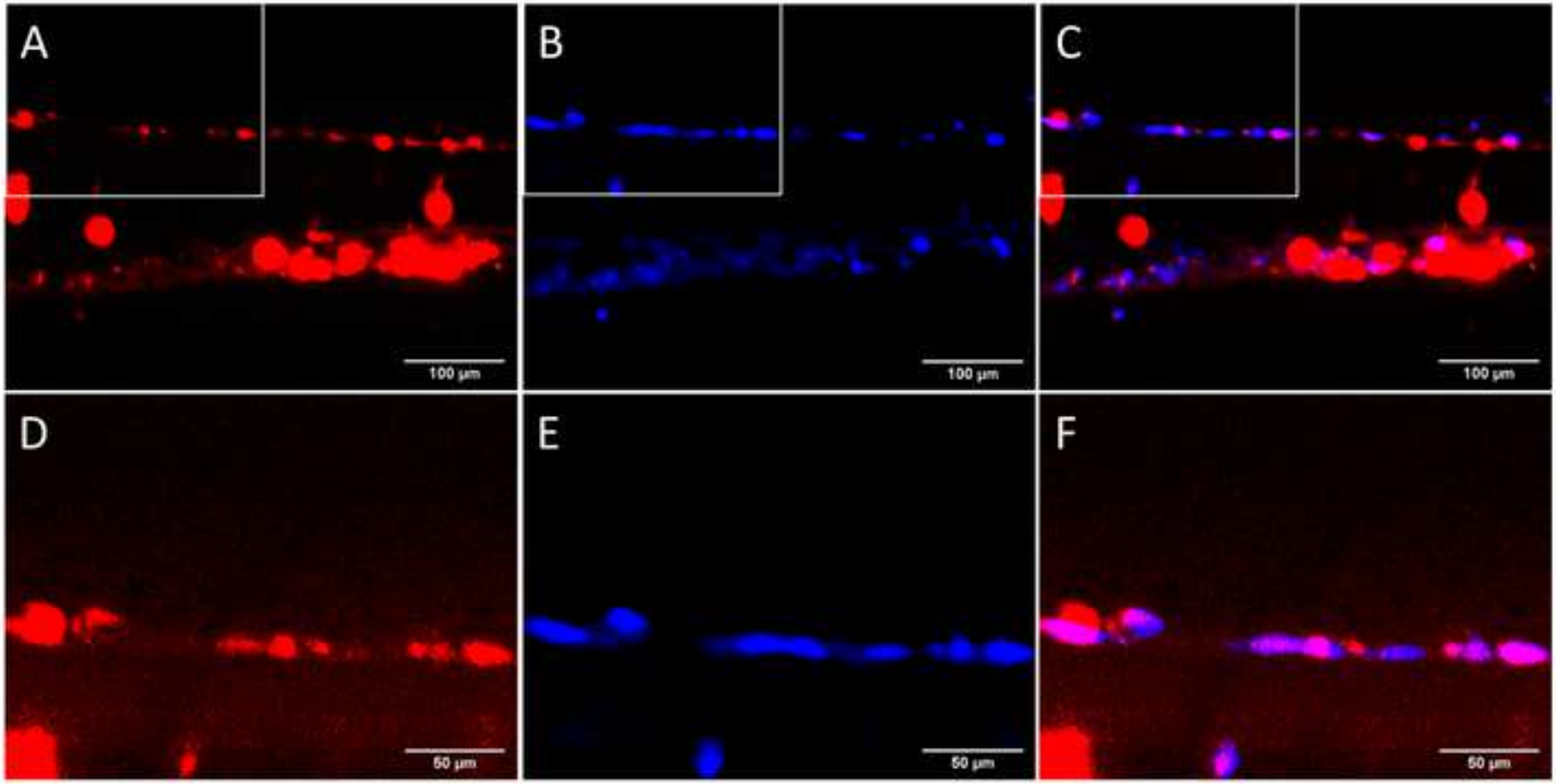

Figure 4

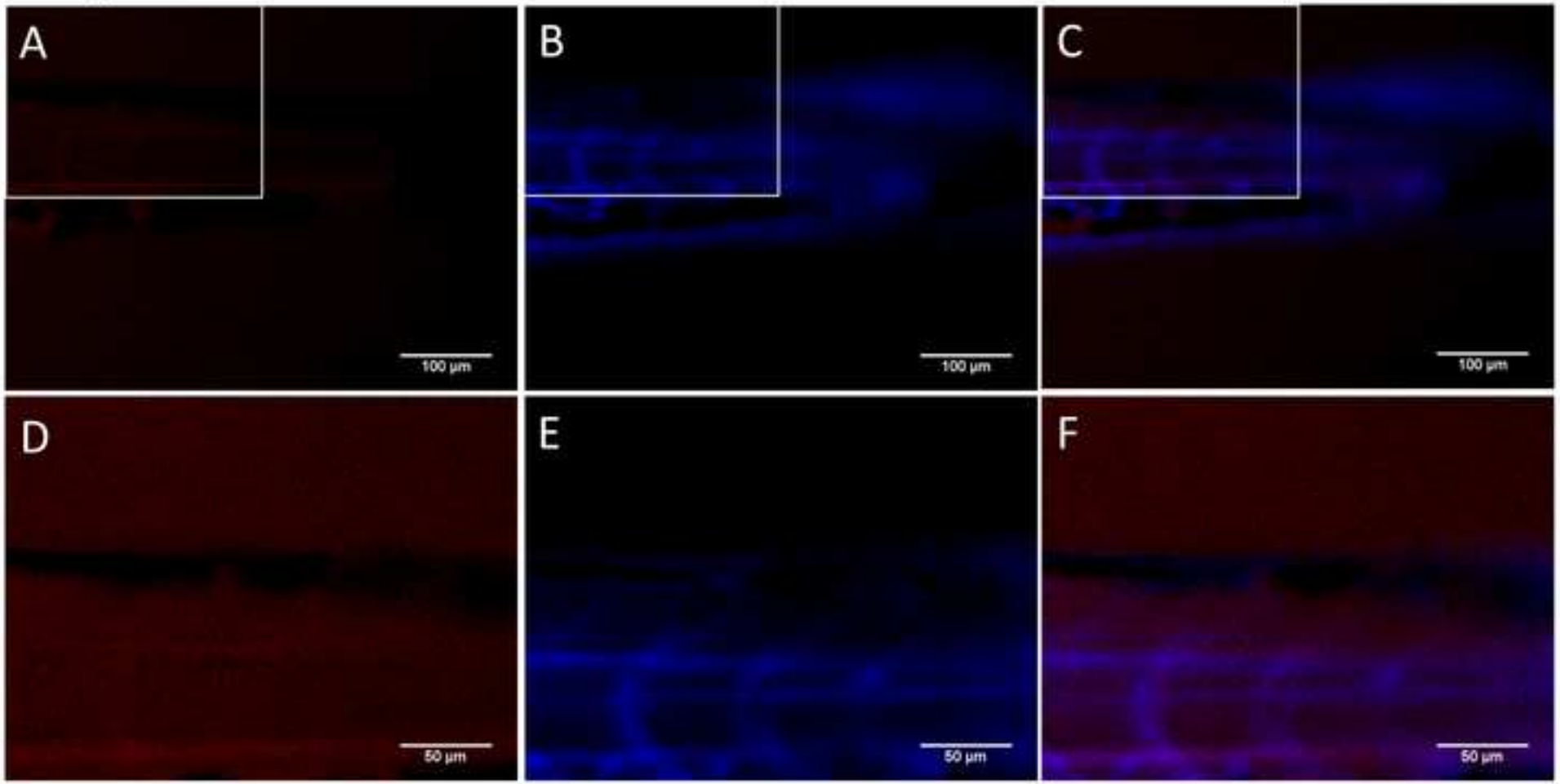

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comment/Description
Agarose	KSE scientific	BMK-A1705	
Borosilicate glass capillaries	World Precision Instruments	1.0 mm O.D. x 0,78 mm	
Computer and monitor	ThinkCentre	X000335	
DMEM (Dulbecco's Modified Eagle's Medium)	Corning	10-013-CV	sold by Fisher
Fetal Bovine Serum	Sigma-Aldrich	F0926	
Fish incubator	VWR	35960-056	
Hemocytometer	Fishersci brand	02-671-51B	
Magnetic stand	World Precision Instruments	M10	
Microloader tip	Eppendorf	E5242956003	sold by Fisher
Micromanipulator	Applied Scientific Instrumentation	MMPI-3	
Needle Puller	Sutter instruments	P-97	
Olympus MVX-10 fluorescent microscope	Olympus	MVX-10	
P200 tip	Fishersci brand	07-200-293	
PBS (Dulbecco's Phosphate-Buffered Salt Solution 1X	Corning	21-030-CV	sold by Fisher
Petri dish	Corning	SB93102	sold by Fisher
Plastic pipette	Fishersci brand	50-998-100	
pLenti6.2_miRFP670	Addgene	13726	
Pneumatic pico pump	World Precision Instruments	SYSPV820	
Pronase	Roche-Sigma-Fisher	50-100-3275	Roche product made by Sigma- sold by Fisher
Razor blade	Fishersci brand	12-640	
SZ51 dissection microscope	Olympus	SZ51	
Tricaine methanesulfonate	Western Chemicals	NC0872873	sold by Fisher
Trypsin-EDTA	Corning	MT25053CI	sold by Fisher
Tweezer	Fishersci brand	12-000-122	

Nandita Singh, Ph.D., Senior Science Editor
Vineeta Bajaj, Ph.D., Review Editor
JoVE
1 Alewife Center Suite 200
Cambridge, MA 02140

Dear Dr. Singh and Dr. Bajaj,

We thank the Editors and Reviewers from the JoVE for the favorable review of our manuscript and valuable suggestions. Based on their comments, we have carefully revised the manuscript. We believe that the revised manuscript is much improved and hope that it now warrants publication in the JoVE.

Sincerely,

Hui Feng, M.D., Ph.D.

We thank both the Editors and the Reviewers for their helpful comments to improve the manuscript. We have addressed all comments point-by-point as listed below.

EDITORS' COMMENTS:

1. "*…thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*"

Author's Response: We have thoroughly proofread the manuscript and ensured that there are no errors in spelling and grammar in the revised manuscript.

2. "*Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points*."

Author's Response: We have formatted the manuscript according to the Editors' requirements.

3. "*…Please remove all commercial language from your manuscript and use generic terms instead*."

Author's Response: We have removed all commercial language from the manuscript and listed them in the Table of Materials.

4. "*Please convert centrifuge speeds to centrifugal force (x g)...*"

Author's Response: Based on the Editors' suggestion, we have converted the centrifugal speeds to relative centrifugal force "rcf" in the revised manuscript.

Page 4, line 199:

"… Centrifuge at 135 rcf for 5 min."

5. "*Please ensure that all text in the protocol section is written in the imperative...*"

Author's Response: We have changed all text in the protocol section to the imperative tense.

6. "*Please ensure you answer the 'how' question, i.e., how is the step performed?"*

Author's Response: We have clarified the vague steps and elaborated on how these steps are performed.

7. "*…Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video…"*

Author's Response: We have highlighted the essential steps in the protocol section and ensured that they are less than 2.75-page long.

8. "*Please bring out clarity between HeLa and Nanoparticle injection together. What is the vehicle in this case? Please present the results for vehicle as well.*"

Author's Response: We thank the Editors' comments. The vehicle we used to resuspend nanoparticles was de-ionized water (H_2O). We did present the results for vehicle injection alone (see Figures 1A, 2 and the result section).

Page 6, line 270-271

"Follow the procedures as described in 3.1-3.3 to align embryos and load vehicle (H_2O) or the nanoparticle solution into the needle."

Page 6, line 277-279:

"Follow a similar procedure, inject the vehicle (H_2O) that was used to resuspend nanoparticles into embryos with or without HeLa cells transplanted as controls (**Figure 1A**,**C**)."

Page 7, line 321:

"…using embryos injected with the vehicle as controls (**Figure 1A**)."

Page 7, line 334-337:

"As expected, we failed to detect any specific blue fluorescent signals in embryos with the vehicle injection (**Figure 2B**,**E**). Additionally, when we merged the images captured in the red and blue channels, we only observed red cancer cells in the tail region without any blue signals (**Figure 2C**,**F**)."

9. "*How did you use nanoparticles to reduce or destroy the cancer formation in your experiment? Please include figures.*"

Author's Response: The editors raised an interesting question; however, in this study, we did not investigate the ability of our nanoparticles to destroy cancer cells *in vivo*. We do plan to apply our established methodology to address this question in the future.

10. "*Please obtain explicit copyright permission to reuse any figures from a previous publication…*"

Author's Response: All figures included in this manuscript have not been published elsewhere, thus there is no copyright issue involved.

11. "*As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:*

a) *Critical steps within the protocol*
b) *Any modifications and troubleshooting of the technique*
c) *Any limitations of the technique*
d) *The significance with respect to existing methods*
e) *Any future applications of the technique*"

Author's Response: We thank the Editors for the suggestion and have ensured that the discussion covers the required aspects in the revised manuscript.

12. "*Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, (YEAR).] For more than 6 authors, list only the first author then et al*."

Author's Response: We have downloaded JoVE endnote style and formatted the references accordingly.

REVIEWER COMMENTS:

Reviewer #1:

1. "*There is no image provided to show healthy fertilized eggs that must be transferred into petri dish...*"

Author's Response: We thank the Reviewer for the suggestion, and have modified Figure 1 to

include the step for embryo collection together with an image showing healthy fertilized eggs.

2. "*The anesthetization of embryos using MS222 have high potential to kill the embryos, if not careful. So more discussion should be provided.*"

Author's Response: We thank the Reviewer for pointing this out, and have included more details in the protocol and in the discussion about the use of MS222.

Page 4, line 180-183:

"Prepare a stock solution of tricaine methanesulfonate (MS222, 4 mg/mL) by dissolving MS222 into autoclaved fish water. Vortex well before use. Dilute MS222 stock solution 1:100 in fish water (add 200 μL MS222 stock solution to 20 mL of fish water, final concentration 40 μg/mL) to anesthetize embryos in the following procedures."

Page 5, line 234-235:

"Anesthetize the embryos by adding five drops of the diluted MS222 solution (40 μg/mL)."

Page 9, line 420-422:

"Additionally, one must take care not to add too much or too concentrated MS222 when anesthetizing the embryos during injection and imaging."

3. "*During the injection of nanoparticles into embryo, it is done behind the embryo eye which is enriched with capillaries. There may be risk here to cause harm or potential death*…"

Author's Response: The Reviewer makes a good point. When we perform the injection, we use a very thin needle that is typically for DNA microinjection. We insert the needle behind the eye quickly for a short period time. This ensures minimal damages to the embryos that tolerate this procedure well. We have delivered nanoparticles through injection into other head areas; however, injection behind the eye allows nanoparticles to enter the circulatory system of the embryos efficiently.

4. "*They should provide clear quantified data, e.g.: should be provided a graph against time that shows the distribution of nanoparticles in circulation in the embryos & a graph against time that shows the degree of targeting of the nanoparticles to the cancer cells."*

Author's Response: Indeed, we have performed detailed characterization and analysis of the distribution and targeting of different nanoparticles to cancer cells. This information was recently published in *Nanoscale* (Peerzade S et al., Ultrabright fluorescent silica nanoparticles for in vivo targeting of xenografted human tumors and cancer cells in zebrafish, 2019 – reference 18 in the manuscript). We have included this information in the revised manuscript.

Page 7, line 326-332:

"At 0, 30, 60, 90, 120, 180, 210 min post-injection of nanoparticles, we monitored the injected embryos by imaging to determine the interaction of nanoparticles with RFP+ HeLa cells, using the vehicle injected embryos as controls. Specifically, we imaged the zebrafish tail areas where RFP+ HeLa cells had spread to at red, blue, and brightfield using a fluorescent microscope (Protocol Section 5). The detailed characterization for the ability of our ultra-bright nanoparticle to target xenografted cancer cells in zebrafish over time is shown in Figure 5 of our paper recently published in *Nanoscale* [18]."

Page 7, line 347-353:

"We subsequently applied this protocol to test different types of nanoparticles [18-20]. Depending on the properties of the nanoparticle tested, we observed co-localization of cancer cells with certain types of nanoparticles as early as 30 min post-injection. By 120 min, there was >80% targeting of cancer cells by these nanoparticles in the tail region of the fish. However, for other nanoparticles, minimal targeting of cancer cells was observed, consistent with their lack of cancer-specific ligand. The detailed results and analysis are included in our recent publication in *Nanoscale* (see Figures 3-4, supplementary Figures S12-S16, and supplementary Table S6) [18]."

5. "*The margin of the survival rate for the embryos is too large (10% to 50%)...*"

Author's Response: We apologize for the confusion caused by our writing. The survival rate for the embryos is actually high when microinjection is performed by an experienced individual. We have revised our manuscript to clarify this.

Page 9, line 408-413:

"Finally, the skill of the individual who performs transplantation matters. An experienced individual can accurately inject HeLa cells into the perivitelline cavity space, while an inexperienced person often injects tumor cells into the yolk area where tumor cells barely spread into the fish body. Similarly, the embryos survival rate is much higher for the experienced individual, with at least 50% of embryos transplanted with cancer cells surviving."

Reviewer #2:

Major Concerns:

1. "*...injection of a large number of embryos (>100) for each experiment seems to be a bit challenging*."

Author's Response: Although we appreciate the Reviewer's concern, it is rather straightforward and fast to inject a large number of embryos. As a matter of fact, one experienced individual can inject ~500 embryos within 3 hr.

2. "*I am not convinced why there is a need to use such young embryos (48 hpf)? ...*"

Author's Response: One of the reasons we use 48 hpf embryos is to avoid rejection of human cancer cells by the fish's immune system. Although we could use embryos older than 48 hpf, their transparency decreases as fish develop over time. Therefore, 48 hpf embryos are commonly used for transplantation of human cancer cells.

Page 2, line 93-96:

"Finally, zebrafish develop their innate immune system by 48 hpf, with the adaptive immune system lagging behind and only becoming functional at 28 days post-fertilization [15]. This time gap is ideal for the transplantation of various types of human cancer cells into zebrafish embryos without experiencing immune rejections."

3. "*Where NPs are localized in absence of cancer cells? It is possible that these NPs are located in the same region of zebrafish tail with and without cancer cells...*"

Author's Response: We thank the Reviewer for this outstanding suggestion. We have now included the additional control in the revised manuscript, showing that different from the distribution of nanoparticles in fish transplanted with cancer cells (see Figure 3), nanoparticles in fish without xenografted human cells distribute relatively evenly into the circulatory system of embryos, highlighting blood vessels (see new Figure 1C and Figure 4).

Page 7, line 341-345:

"We also injected nanoparticles into the embryos that were not transplanted with HeLa cells. Instead of concentrating into particular cells or areas, the blue fluorescent particles in these embryos distributed relatively evenly into the circulatory system of embryos, highlighting blood vessels (**Figure 4B**,**E)**. As expected, we could not detect any specific red fluorescent signals in these embryos despite some weak background fluorescent signals (**Figure 4A**,**C**,**D**,**F)**."

Page 8, line 385-391:

"**Figure 4. Zebrafish injected with nanoparticles without human HeLa cells.** Blue fluorescent nanoparticles are distributed into the circulatory system of the embryos in the individual (**B**,**E**) and overlaid images of the red and blue channel (**C**,**F**). No specific red fluorescence is visible at either low or high magnification (**A**,**D**) except some background fluorescence common to zebrafish embryos. Images in (**A**-**C**) show the fish tail region as boxed in Figure 1C. Images in (**D**-**F**) are enlarged views of the boxed areas in (**A**-**C**). Scale bars in (**A**-**C**) = 100 µm and in (**D**-**F**) = 50 µm."

4. "*Although authors claim that this protocol is suitable for various NPs testing and they did such experiments, there are no results (images) are presented in the paper…*"

Author's Response: Indeed, we have performed detailed characterization and analysis of the distribution and targeting of different nanoparticles to cancer cells. This information was recently published in *Nanoscale* (Peerzade S et al., Ultrabright fluorescent silica nanoparticles for in vivo targeting of xenografted human tumors and cancer cells in zebrafish, 2019 – reference 18 in the manuscript). We have included this information in the revised manuscript.

Page 7, line 326-332:

"At 0, 30, 60, 90, 120, 180, 210 min post-injection of nanoparticles, we monitored the injected embryos by imaging to determine the interaction of nanoparticles with RFP+ HeLa cells, using the vehicle injected embryos as controls. Specifically, we imaged the zebrafish tail areas where RFP+ HeLa cells had spread to at red, blue, and brightfield using a fluorescent microscope (Protocol Section 5). The detailed characterization for the ability of our ultra-bright nanoparticle to target xenografted cancer cells in zebrafish over time is shown in Figure 5 of our paper recently published in *Nanoscale* [18]."

Page 7, line 347-353:

"We subsequently applied this protocol to test different types of nanoparticles [18-20]. Depending on the properties of the nanoparticle tested, we observed co-localization of cancer cells with certain types of nanoparticles as early as 30 min post-injection. By 120 min, there was >80% targeting of cancer cells by these nanoparticles in the tail region of the fish. However, for other nanoparticles, minimal targeting of cancer cells was observed, consistent with their lack of cancer-specific ligand. The detailed results and analysis are included in our recent publication in *Nanoscale* (see Figures 3-4, supplementary Figures S12-S16, and supplementary Table S6) [18]."

Reviewer #3:

1. "*Is the fish water also used for zebrafish eggs and embryos? How to prepare fish* water?"

Author's Response: The Reviewer is correct. The fish water is used for zebrafish at all developmental stages. We did not make the fish water ourselves, instead we simply obtained fish water from fish tanks housed in our fish facility. The aquatic system sold to fish facilities is pre-programmed by manufacturers to generate fish water with suitable salt concentration and pH. Therefore, researchers do not need to prepare fish water manually. We have clarified this information in the revised manuscript.

Page 3, line 135-136:

"Fish water is obtained from fish tanks in the fish facility."

2. "*As Casper zebrafish is transparent, what is the purpose to bleach embryos in 1.7? provide the information on how to bleach the embryos*."

Author's Response: Embryo bleaching is an optional procedure in many fish facilities. The reason for bleaching embryos is to potentially kill some pathogens, and may improve the survival rates of the embryos. We used the common procedure described in *The Zebrafish Book* and have included this information in the revised manuscript.

Page 3 and line number 140-141:

"Bleach the embryos the next morning using the standard protocol as described in *The Zebrafish Book* [16], and put the embryos back to the incubator. (optional step)"

3. "*Provide the information in detail for anesthetizing embryos, such as how many drops of MS222 to what volume of media, what is the final concentration of MS222?*"

Author's Response: We thank the Reviewer for the comment. We have now updated our manuscript to include a more detailed explanation of how to prepare MS222.

Page 4, line 180-183:

"Prepare a stock solution of tricaine methanesulfonate (MS222, 4 mg/mL) by dissolving MS222 into autoclaved fish water. Vortex well before use. Dilute MS222 stock solution 1:100 in fish water (add 200 μL MS222 stock solution to 20 mL of fish water, final concentration 40 μg/mL) to anesthetize embryos in the following procedures."

Page 5, line 234-235:

"Anesthetize the embryos by adding five drops of the diluted MS222 solution (40 μg/mL)."

4. "*How to label the cells? Why are the cells left in 35.5 degrees before injection?*"

Author's Response: We labeled HeLa cells by transducing PLenti6.2_miRFP670 lentivirus, and have now included this information in the revised manuscript. We store cancer cells at 35.5 °C before and during injection so they can stay healthy to migrate in fish.

Page 4, line 185-186:

"Label HeLa cells by transducing them with PLenti6.2_miRFP670 lentivirus using the protocol as described [17]."

Page 9, line 415-416:

"Although zebrafish embryos are usually incubated at 28.5 °C, human cancer cells require higher temperatures to survive and migrate [22,23]."

5. "*After cell injection, the embryo survival rate is low. Is it because of the temperature? Did the authors grow the embryos at 28 degree? ...*"

Author's Response: We apologize for the confusion caused by our writing. The survival rate for the embryos is actually high when microinjection is performed by an experienced individual. We have revised our manuscript to clarify this. Although embryos may not like higher temperature, the embryo lethality is not caused by incubating them at 35.5 °C. We also incubated the uninjected embryos at 35.5 °C as controls. These embryos tolerated the temperature well and showed little lethality. We kept embryos in this study at 35.5 °C to allow the survival and migration of human cancer cells.

Page 9, line 408-413:

"Finally, the skill of the individual who performs transplantation matters. An experienced individual can accurately inject HeLa cells into the perivitelline cavity space, while an inexperienced person often injects tumor cells into the yolk area where tumor cells barely spread into the fish body. Similarly, the embryos survival rate is much higher for the experienced individual, with at least 50% of embryos transplanted with cancer cells surviving."

Page 9, line 415-417:

"Although zebrafish embryos are usually incubated at 28.5 °C, human cancer cells require higher temperatures to survive and migrate [22,23]. To allow the survival of both fish embryos and human cancer cells, we incubate the embryos transplanted with human cancer cells at 35.5 °C instead."

6. "*Provide the information of nanoparticles. How to prepare and characterize them? How to label them?*"

Author's Response: This information was recently published in *Nanoscale* (Peerzade S et al., Ultrabright fluorescent silica nanoparticles for in vivo targeting of xenografted human tumors and cancer cells in zebrafish, 2019 – reference 18 in the manuscript). We have included this information in the revised manuscript.

Page 7, line 323-324:

"Please see our recent publication for the detailed information for nanoparticle synthesis, preparation, and characterization [18]."

7. " *Protocol 1, the title could be 'Generation of Casper zebrafish embryo' "*

Author's Response: We thank the Reviewer for the suggestion, and have changed the subtitle accordingly.

8. "*Protocol 2, the title could be 'Preparation of human Hela cell microinjection' "*

Author's Response: We thank the Reviewer for the suggestion, and have changed the subtitle accordingly.