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Ultrasound-Guided Approaches to Improve Orthotopic Mouse Xenograft Models for Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death worldwide. While curative approaches for early stage HCC exist, effective treatment options for advanced HCC are lacking. Furthermore, there are no efficient chemopreventive strategies to limit HCC development once cirrhosis is established. One challenge for drug development is unsatisfactory animal models. In this article, we describe an orthotopic xenograft mouse model of human liver cancer cell lines through image-guided injection into the liver. This technique provides a less invasive yet highly efficient approach to engraft human HCC into mouse liver. Similarly, image-guided injections are used to deliver chemotherapeutics locally, enabling reduction in potential systemic adverse effects, while reducing the required dose for a therapeutic effect. In summary, this image-guided strategy provides a novel and convenient approach to improve current HCC mouse models.

Keywords

doxorubicin; echography; echo-guided injection; hepatocarcinoma; Huh-7; orthotopic

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Conflicts of Interest
The authors declare no conflicts of interest.

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Introduction

Hepatocellular carcinoma (HCC) is the most common cause of cancer-related deaths worldwide (Ferlay, Forman, Mathers, & Bray, 2012) and is the leading cause of death among patients with cirrhosis related to viral hepatitis (El-Serag, 2012). Survival rates remain poor —generally not more than 5 years—when HCC is diagnosed at an advanced stage (Davila et al., 2010). Early diagnosis of HCC using biomedical imaging allows improved prognosis. Until now, surgery has been the most effective treatment. From a pharmacologic point of view, chemotherapy and the multikinase inhibitor sorafenib enable survival improvements but demonstrate numerous adverse effects, especially in advanced or metastatic HCC (Llovet et al., 2008). Relevant preclinical animal models are crucial for the development of innovative approaches to HCC (i.e., electroporation, gene therapy, and immunotherapy). Various murine models (chemical induction, genetic modification, or xenogeneic tumor cell transplantation) have already contributed to defining the pathogenesis and the current knowledge of HCC (Heindryckx, Colle, & Van Vlierberghe, 2009). However, murine models that reproduce and recapitulate all HCC etiologies and steps are not available and will be difficult to obtain. For this reason, during drug development different complementary preclinical models (Wu et al., 2016) are used to assess efficacy and safety of innovative interventional therapies, as well as diagnostic modalities.

Early detection and confirmation of HCC using ultrasound imaging with or without contrast agent (Anton et al., 2017), computed tomography, and/or magnetic resonance imaging is a key goal to improve patient outcomes. Here, we describe a protocol to establish a xenogeneic orthotopic model of HCC through intrahepatic echo-guided injection of a hepatoma tumor cell line and the effect of local intratumoral delivery of the cytotoxic treatment doxorubicin on tumor progression. Basic Protocol 1 describes the technique of echo-guided injection. Basic Protocol 2 describes tumor size and volume monitoring.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to local regulations regarding the care and use of laboratory animals.

Preparation and Echo-Guided Injection of Tumor Cells and Cytotoxic Drugs

The Huh-7-Luc human tumor cell line is transplanted into anesthetized 6-week-old mice weighing between 25 and 30 g by echo-guided intrahepatic injection with a high-resolution ultrasound imaging system. Injection of drugs into the tumor uses the same protocol. In this protocol one can expect that a tumor will be visible and measurable by ultrasounds 2 weeks after injection. One can follow growth until the ethical endpoint is reached, leading to sacrifice. Treatment efficacy is also discussed.

Materials

Doxorubicin hydrochloride powder (e.g., Sigma-Aldrich, cat. no. D1515-10MG)

0.9% (w/v) NaCl (saline), sterile

Huh-7-Luc cells

Cell culture medium (see recipe)

0.25% Trypsin-EDTA (e.g., Thermo Fisher Scientific, cat. no. 25200072) Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium (e.g., Eurobio, cat. no. CS1PBS01-01)

0.4% Trypan blue (e.g., Bio-Rad, cat. no. 1450021)

Isoflurane, for anesthesia induction 6-week-old, 25 to 30 g, female NMRI-nu *(Rj:NMRI-foxn1$^{nu/nu}$)* nude mice

(e.g., Janvier Labs)

Hair removal cream

Lubricating eye gel

Ultrasound gel (e.g., EDM Medical Imaging)

Buprenorphine

1.5-ml microcentrifuge tubes

37°C, 5% CO_2 cell culture incubator

37°C water bath

Centrifuge and microcentrifuge

75-cm^2 cell culture flask (e.g., Falcon, part no. 353024)

Light microscope

15-, 30-, or 50-ml centrifuge tubes

Automated cell counter (e.g., Bio-Rad TC20) with corresponding counting slides (e.g., Bio-Rad, cat. no. 1450011)

Rodent anesthesia induction system with vaporizer/manifold

High-resolution ultrasound system with imaging station (e.g., VisualSonics Vevo), including microinjection apparatus, mouse handling thermally regulated table with real-time monitoring of key physiologic parameters (respiration, heart rate), rectal temperature probes, and anesthesia line connected to anesthesia face mask

Ultrasound transducer, 25 to 55 MHz for abdominal scanning (e.g., MS550D)

1-ml syringe with 30-G, 1-in. needle

Computer running ultrasound imaging software (e.g., Vevo LAB VisualSonics imaging software version 3.0.0)

Heating pad

Prepare doxorubicin

1. Dissolve 10 mg doxorubicin powder in 5 ml of 0.9% sterile NaCl to obtain a soluble, clear solution at a final concentration of 5 mg/ml. Prepare 5 aliquots with 1 ml stock solution, and store at –20°C for up to 12 months.

Doxorubicin requires handling under a chemical extractor with adequate personal protective equipment (e.g., laboratory coat, gloves, masks).

Prepare Huh-7-Luc cell line

2. Produce 4×10^6 viable Huh-7-Luc cells per mouse.

This represents the target number of cells for this protocol. With the goal of injecting 20 mice, this protocol should be started ~10 days before planning the injection. The results of this protocol will be 20 1.5-ml microcentrifuge tubes (1 per mouse) with a viable cell suspension that is ready for injection.

Huh-7-Luc cells were generated in Thomas Baumert's laboratory as previously described (Wu et al., 2016). Briefly, HuH-7 cells (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan) were transduced with a retrovirus produced from a luciferaseencoding pCLNCX vector and cloned by limited dilution. These cells are available upon request to Thomas Baumert (thomas.baumert@unistra.fr).

3. Cultivate Huh-7-Luc cells as adherent cells in culture medium in a 37°C incubator with 5% CO_2. To keep cell growth dynamic, do not allow cells to grow to complete confluency. Renew culture medium every 2 to 3 days. Perform trypsinization when cells reach around 90% confluency.

4. Thaw cryovial of 2 million (2×10^6) Huh-7-Luc cells in a 37°C water bath until only a small block of ice remains (around 30 sec). Transfer cells as quickly as possible to culture medium, and centrifuge 3 min at 300 × *g*, 21°C to 23°C, to remove DMSO. Resuspend cell pellet into 15 ml culture medium, and transfer to a 75-cm^2 culture flask. Incubate flask in a cell culture incubator.

5. Check cell growth and morphology at least every 2 days under a microscope.

Huh-7-Luc cells have the same morphology as HuH-7 cells, whose representative images can be found at https://clsgmbh.de/p7178_HuH7.html. *We view the cells under the microscope to check confluency of cells and to verify that no contamination (e.g., bacteria, fungi) has occurred.*

6. Remove culture medium, and wash with around 5 ml PBS. When cells reach -90% confluency (generally after 3 days), harvest cells by adding 3 to 5 ml warmed trypsin to the side wall of the flask, and gently swirl to cover the cell layer. Pipette up and down with a

10-ml pipette to detach cells and obtain a unicellular suspension. Add 5 to 10 ml culture medium to inactivate trypsin.

Cells can be harvested earlier than 3 days to keep on a weekend-free schedule if convenient.

7. Transfer cell suspension to a centrifuge tube (15-, 30-, or 50-ml volume, as convenient), and centrifuge 3 min at 300 × *g*, room temperature. Remove supernatant and gently resuspend cells in 10 ml culture medium.

8. Determine number of viable cells. To do so, take a 10-µl aliquot of cell suspension, and add 10 µl Trypan blue. Load 10 µl of this mixture on a counting slide, and proceed to counting. Determine percentage of viable cells and number of viable cells per ml. Multiply this number by 10 (volume in ml of cell suspension) to obtain the number of viable cells in the flask.

The percentage of viable cells should be >95%, and it is generally 99%.

In our hands, the expected number of viable cells is 8–12 × 10^6 per 75-cm^2 flask at this first passage after thawing. For subsequent passages, the expected number of cells is around 15–20 × 10^6 per 75-cm^2 flask depending on confluency.

9. Split cells in as many 75-cm^2 flasks as needed considering that you add 2–2.5 × 10^6 cells per 75-cm^2 flask. Incubate in cell culture incubator.

10. Repeat steps 4 to 9 until enough cells are produced, considering that 4 × 10^6 cells need to be produced per injected mouse.

It is recommend to produce more cells than needed (around 10% more), in order to ensure you have enough cells the day of injection.

As an example, a weekend-free schedule for injecting 20 mice would begin with mouse injection on a Thursday, which then allows for starting tumor imaging during the week (4 to 5 days later). To prepare cells for injection on a Thursday, one would begin 10 days prior: On day 0 (Tuesday), thaw 2 × 10^6 cells and transfer to a 75-cm^2 flask. On day 3 (Friday), transfer cells to 3 different 75-cm^2 flasks with 2 × 10^6 cells per flask (throw away remaining cells). On day 6 (Monday), transfer cells to 7 to 8 different 75-cm^2 flasks with 2–2.5 × 10^6 cells per flask (throw away remaining cells). On day 9 (Thursday), perform injection (see step 14).

11. Repeat steps 4 to 9, when enough cells are produced, the day of mouse injection. Then, resuspend Huh-7-Luc cells in culture medium at a concentration of 20 × 10^6 cells/ml.

12. Aliquot cell suspension into sterile 1.5-ml microcentrifuge tubes (4 × 10^6 cells in 200 µl culture medium). Prepare, at a minimum, 1 microcentrifuge tube per mouse to inject (generally 20).

This volume of cell suspension takes into account the dead volume of the injection syringe/needle. For each mouse, only 50 µl cell suspension (1 × 10^6 cells) will be injected.

13. Store tube on ice, and immediately proceed to step 14.

Cells stored on ice can be kept for up to 4 hr, which is roughly the time required to inject all 20 mice.

Prepare nude NMRI mouse

14. Set up heating platform at 37°C.

15. Place animal platform in an orientation that allows for correct placement of the MS550D imaging probe in front of the abdomen of the mouse and the isoflurane anesthesia nose clamp (Fig. 1A,B).

16. Anesthetize mouse in induction chamber with 3% to 5% isoflurane, and turn on flow meter between 500 and 1000 ml/min.

These parameters do not need to be adjusted based on body weight.

We recommend a minimum of 8 mice per condition to have statistically relevant results.

17. Place mouse on the platform, and maintain anesthesia with a nose cone delivering 1% to 3% isoflurane at a rate of 100 to 200 m/min to prevent movement during the imaging session.

18. Remove abdominal hair with hair removal cream, and apply lubricating gel to both eyes to prevent drying of the sclera.

19. Gently insert rectal temperature probe, and tape paws to the echography electrodes after having applied ultrasound gel for electrical contact.

Prepare coordinate of injection using the imaging station

20. Prepare 1-ml syringe with 30-G, 1-in. needle.

21. Place empty syringe with sheathed needle, bevel side oriented upward, in syringe clamp, and secure ultrasound transducer probe in the scan head clamp (Fig. 1C).

22. Align transducer parallel to the axis of the needle under visual control using fine manipulation.

23. Fix scan head position by tightening the clamp (Fig. 1C).

24. Cover head of the transducer tip with ultrasound gel along the ultrasound emission beam.

Be sure not to move the needle/transducer horizontally to ensure that you do not disturb horizontal alignment.

25. Scan superior abdominal area with the transducer to obtain a coronal section of the liver.

26. Move transducer in the anterior direction from the animal platform using the scan height control (Fig. 1C).

This will not disrupt the x-axis alignment of the transducer to the long axis of the needle (Fig. 1B).

Specific areas of the liver (right lobe) can be targeted for injection by changing the vertical (y-axis) position of the needle and/or by moving the animal platform. This part of the liver offers suitable accessibility for injection by keeping the mouse in front of the operator.

27. Load a new needle and syringe with injectate (see cell preparation in steps 2 to 14) to the final target volume. Be sure to remove air bubbles.

28. Place syringe into the syringe clamp without adjusting the *x*-axis alignment.

Inject mouse

29. Lower transducer onto the abdominal area using the scan head height control of the animal platform.

30. Use animal platform adjustment controls (Fig. 1C) to adjust the field of view and to target any desired injection site in the liver.

31. With syringe in the fully retracted syringe clamp, slowly advance syringe towards the mouse's abdomen by turning the injection control clockwise (Fig. 1D). To permit clear ultrasound visualization of both the liver and the needle tip as it approaches the abdomen, use ultrasound gel over the left side of the abdomen, and optimize the acoustic window by setting a wide field of view on the echography control panel.

32. Set focal point/zone at the target site for injection.

Minor adjustments to the needle mount controls can optimize the image of the needle along its length.

33. Activate needle guide software function to digitally extend a line along the long axis of the needle through to the target. Check that the tip of the needle is not in a large vessel (portal vein or inferior vena cava; Fig. 2A).

It is safer to place the needle far from large vessels.

34. When tip is in the desired location, deliver injectate by pushing on the syringe plunger. Deliver 50 µl injectate (1×10^6 cells/ml complete medium) slowly over 5 to 30 sec.

In preliminary experiments, the site of injection was optimized and checked by injection of the same volume of Evans blue. This enabled verification that the product remained in the right lobe of the liver and that there was no leakage.

35. Once injectate has been administered, slowly withdraw needle by counter-clockwise rotation of the injection control knob.

36. Gently remove ultrasound gel from the tissue, and remove mouse from the anesthesia unit.

37. Put mouse in cage placed on a heating pad until full recovery (i.e., normal movements and response to noise).

38. Inject 0.1 mg/kg buprenorphine to minimize postprocedural pain.

39. Place mouse into its home cage with full access to water and food.

40. At 2 weeks after cell injection and when the tumor reaches 50 mm^3, dilute doxorubicin stock solution to 4 different concentrations (4, 2, 1, and 0.5 mg/kg), and inject solution following the same technique as detailed in steps 14 through 39.

Tumor Size and Volume Imaging and Postacquisition Volumetric Analysis

At 4 to 5 days after tumor cell injection, we image the organ of interest using 2D and 3D imaging modes. We scan the changes in liver tissue, indicative of a potential tumor. The 3D mode allows for measurement of area, height, length, and volume of the tumor.

Materials

Injected mouse (see Basic Protocol 1)

Isoflurane, for anesthesia induction

Ultrasound gel (e.g., EDM Medical Imaging)

Rodent anesthesia induction system with vaporizer/manifold

High-resolution ultrasound system with imaging station (e.g., VisualSonics Vevo), including mouse handling thermally regulated table with real-time monitoring of key physiologic parameters (respiration, heart rate), rectal temperature probes, and anesthesia line connected to anesthesia face mask

Ultrasound transducer, 25 to 55 MHz (e.g., MS550D for abdominal scanning) 3D motor assembly (see Fig. 1C)

Computer running ultrasound imaging software (e.g., Vevo LAB VisualSonics imaging software version 3.0.0)

1. Place anesthetized mouse on the animal platform in a similar way as described in Basic Protocol 1, steps 14 to 17, for injection, and visualize liver tissue, vessels, and abdominal organs.

As in human HCC, tumors formed after Huh-7-Luc cell injection appear as nodules that are more echogenic (brighter) than surrounding normal tissue (Figs. 2B and 3A).

In some cases, areas of necrosis appear as black holes in the tissue, which can be confirmed by histology (Fig. 3B). An acoustic interface (i.e., a difference in contrast between two regions due to a shift in the speed of the ultrasound in the tissue) can also be observed between normal and tumor tissues. This difference is

converted into brightness (white versus black) and can be converted by the system in acoustic intensity (e.g., 1400 W/cm^2 in a region of interest in a tumor versus 100 W/cm^2 in surrounding normal tissue).

2. Locate a putative tumor, and adjust the *x*- and *y*-axis for optimal image quality. Then, scan the entire tumor volume.

3. Clip transducer onto the 3D motor. After adjusting the *x*- and *y*-axis positions, locate the tumor region, placing it in the center of the image display. Use the micrometer adjustors for fine adjustment (Fig. 1C).

The 3D motor assembly for imaging of the tumor (see Fig. 1C) is used for 3D reconstruction. Briefly, the probe is placed in a fixed arm that is then displaced by a step-by-step motor in a way to obtain a 2D image every 0.076 mm on a scan of 15-mm length. Then,3D reconstruction is performed by superimposing these pictures on a z-axis using the Vevo LAB VisualSonics imaging software version 3.0.0.

4. Observe tumor image from distal to proximal, and note length and position of the transducer at the midpoint.

5. Scan a distance equal to the tumor length plus 3 to 4 mm on both sides.

6. Review images and save the scan only if the whole tumor was scanned.

7. Return mouse into its home cage.

8. Use the software study management function for 3D volumetric analysis. Load desired 3D scan file for analysis, and initiate the volumetric analysis function.

9. Trace tumor/tissue border around the perimeter of the tumor, and left-click to anchor specific points. When the entire border is traced, right-click to close the circle

10. Add the region of interest on every each slice (*z*-axis) through the tumor depth in both directions.

11. Complete volumetric analysis to derive a final, calculated tumor volume, and view as a solid surface.

Due to an absence of tumors, it is not possible to quantify tumors in control mice. Nevertheless, a qualitative classification scheme can be designed: 0 (absence of tumor), + (small tumor), ++ (medium-sized tumor), and +++ (large tumor). This qualitative classification is based on the calculation of the tumor volume. We estimate a volume between 10 and 200 mm^3 as small tumor, one between 200 and 500 mm^3 as a medium tumor, and one >500 mm^3 as a large tumor.

We use B-Mode imaging to acquire 2D and 3D images of an area of interest and for identification of anatomical structures. 2D quantification of tumor sizes is based on tracing the tumor/tissue border around the perimeter of the tumor (Figs. 2A and 3A). Two diameters (D_1 and D_2) on both sides of the tumor are obtained, and

the volume is extrapolated based on the following formula that assumes the tumor as a sphere: tumor volume = $\pi/6 \times (D_1 \times D_2)^{3/2}$. 3D quantification of the tumor is obtained as described above using the tomographic reconstruction process (see postacquisition volumetric analysis; Fig. 2C). A linear regression between 2D and 3D measures showed a very good correlation up to a tumor volume of 1000 mm^3 ($n = 22$, $r = 0.9569$, $p < 0.0001$).

Reagents and Solutions

Cell culture medium

Dulbecco's Modified Eagle Medium with high glucose and HEPES (e.g., Thermo Fisher Scientific, cat. no. 42430-025) supplemented with:

Non-essential amino acids (e.g., Thermo Fisher Scientific, cat. no. 11140050 or 11140035)

35 µg/ml gentamycin (e.g., Duchefa Biochemie)

10% (v/v) fetal bovine serum (e.g., GE Healthcare, cat. no. SV30160.03) Maintain sterile technique when preparing

Store at 4°C for up to 1 month

Warm to at least room temperature before use.

Commentary

Background Information

Echo-guided injection in mice was previously developed for organs, such as the heart, and in embryos (Pierfelice & Gaiano, 2010; Zhou & Zhao, 2014). It is a technique currently employed in clinics. Nevertheless, no paper has standardized the method for the mouse liver. Moreover, no study has investigated the effect of cytotoxic compounds injected using the same route into the mouse liver. To develop and standardize the method, we employed the same number of cells that was previously administered in the liver parenchyma following laparotomy. This procedure was long lasting and painful for the animals, and a high mortality rate was observed likely because of vessel injury (Wu et al., 2016). As a consequence, we decided to use echography as a way to precisely locate the site of injection far from a vessel and to administer drugs locally. Compared to the surgical method (i.e., laparotomy implantation), this new method offers a better way to follow the 3R criteria (replace, refine, reduce) by improving the rate of success and reducing mortality. The tumor engraftment rate is 95% with image-guided injection versus 86% with intrahepatic injection in the left lobe after laparotomy (Wu et al., 2016). A lower mortality rate is also obtained (0.01% versus 5.2% for echo-guided injection and laparotomy, respectively).

In this article, we described injection of doxorubicin, but this method can be used for many other medications (e.g., other drugs, biotherapeutics, RNA).

Critical Parameters

Several critical points must be taken into consideration. Consider using additional mice for late tumor onset. For the injection itself, the size of the needle should not exceed 30-G because larger gauges induce tissue injury and cell leakage outside the liver when the needle is removed. Mouse placement on the heating station must allow for proper alignment of the needle and the ultrasound beam, as the experimenter should be able to see the whole needle on the screen together with the site of injection in the liver. Settings of the ultrasound machine must be adjusted by changing contrast and gain to obtain the proper contrast between the tumor and the surrounding liver parenchyma (Fig. 4A,B). Vessels must be well localized to avoid intravascular injection that could provoke metastasis or hemorrhage. Nevertheless, some small vessels cannot be easily visualized. An injection in such vessel has no significant consequence on the mouse and future outcome of tumor development. Otherwise place the mouse in an optimal position for the experimenter.

Troubleshooting

In some cases (-2%), we have observed a lack of tumor engraftment in the liver but surprisingly some tumors in ovaries and pancreas. Conversely, we have not observed tumors in lungs. Thus, we assume that our injection was outside the liver lobe. This could be prevented by placement of the tip of the needle not too deeply in the liver tissue. Nevertheless, when the tip is too close to the cutaneous surface, tumor development can be observed in subcutaneous tissue. Bleeding (immediate or delayed) causing the death of the mouse can be prevented by avoiding vessels at the time of needle placement.

Understanding Results

Tumorigenicity of the Huh-7 cell line was confirmed by less-invasive echo-guided intrahepatic injection into nude mice. We observed the presence of solid tumors 2 weeks after injection in 56 of 58 injected mice (95%) with no evidence of infection. The tumor grows quickly to reach ethical limits (1000 mm^3) by 5 weeks after injection (Fig. 5A).

Sensitivity to doxorubicin of Huh-7 cells was compared using direct intratumoral (Fig. 5B) versus intraperitoneal injection of doxorubicin with increasing doses. The effect of intratumoral 4 mg/kg doxorubicin injection was more pronounced than in mice treated by intraperitoneal injection and in the control saline-treated group. A dose-dependent decrease in tumor size was observed by this local route of delivery (Fig. 5B). All data were extracted from longitudinal follow-up of tumor development. At each time point the volume of the tumor was measured as described in Basic Protocol 2. Doxorubicin-treated mice were compared to their respective controls. Results are expressed as mean of the tumor volume at each time point among mice of a given group. For each mouse, the area under the curve of tumor development is used as a single value giving information on both size and speed of growth.

Time Considerations

Cell culture—Timing is calculated for producing 80×10^6 cells, the quantity needed for injecting 20 mice. Thawing the cells requires 15 min. Culturing Huh-7-Luc cells requires

about 20 to 40 min each day of cell passaging (days 3 and 6) and 5 min for other days. Preparing Huh-7-Luc cells the day of injection (day 9) requires about 1 hr. We generally start at 9:00 AM, and the cells are ready for injection around 10:00 AM. Cells are kept on ice until the beginning of the afternoon, when the last mouse is injected.

Mouse injection—Upon delivery, nude immunodeficient mice are housed in a maximum barrier facility with pressurized, sterile, individually ventilated cages and sterile food, water, and bedding. Animal caretakers should wear protective equipment, sterile scrubs, frocks, gloves, masks, and hair coverings at all times. Mice should be tagged or otherwise labeled using approved procedures 1 week after acclimation. The time required for these steps is 4 hr for care upon delivery and 1 hr for tagging/labeling. The injection procedure requires around 25 min per mouse, which takes into account preparation of the mouse (5 min), preparation of the materials (10 min), and injection (10 min). The same experimenter can prepare, anesthetize, and inject each mouse.

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All protocols were reviewed and approved by the Institutional Animal Care and Ethical Committee of the French Research Ministry and conform to French and European regulations regarding the care and use of laboratory animals (authorization number: 2018032911411188; 14314).

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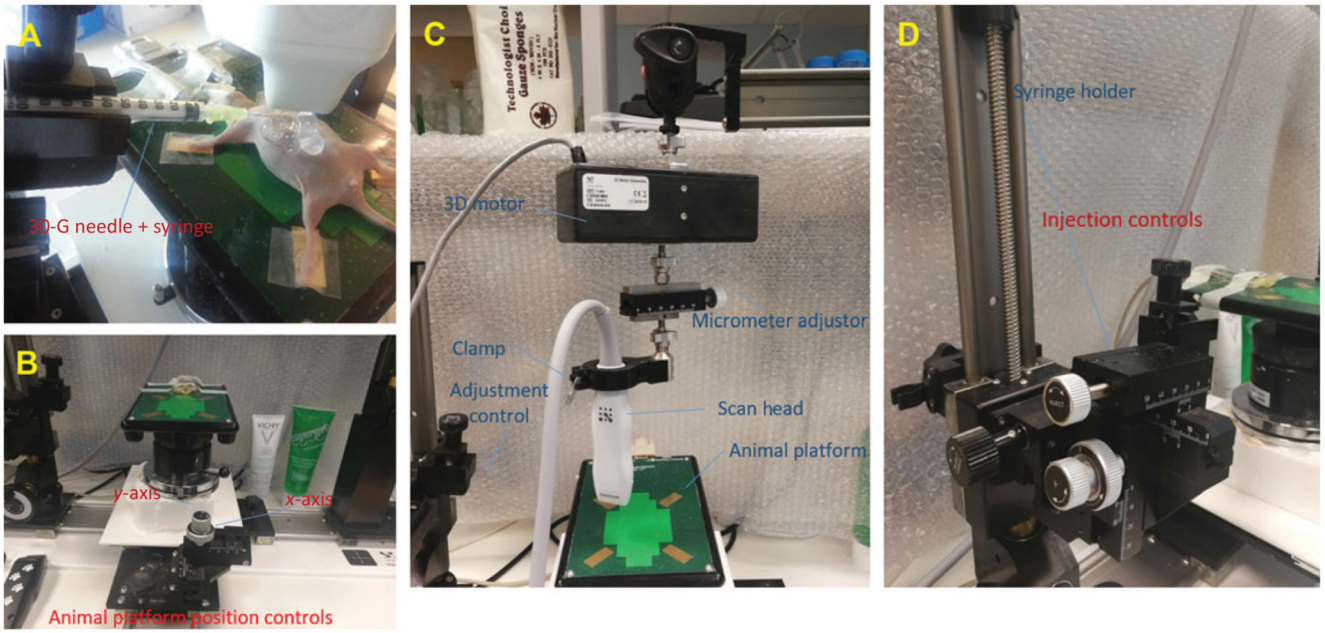

Figure 1. Ultrasound-guided injection setup and optimal positioning of mouse.
(**A**) Mouse position and needle orientation. (**B**) Integrated rail system necessary for alignment of injection syringe and ultrasound scan head. (**C**) Animal platforms and ultrasound scan head controls. (**D**) Injection syringe controls.

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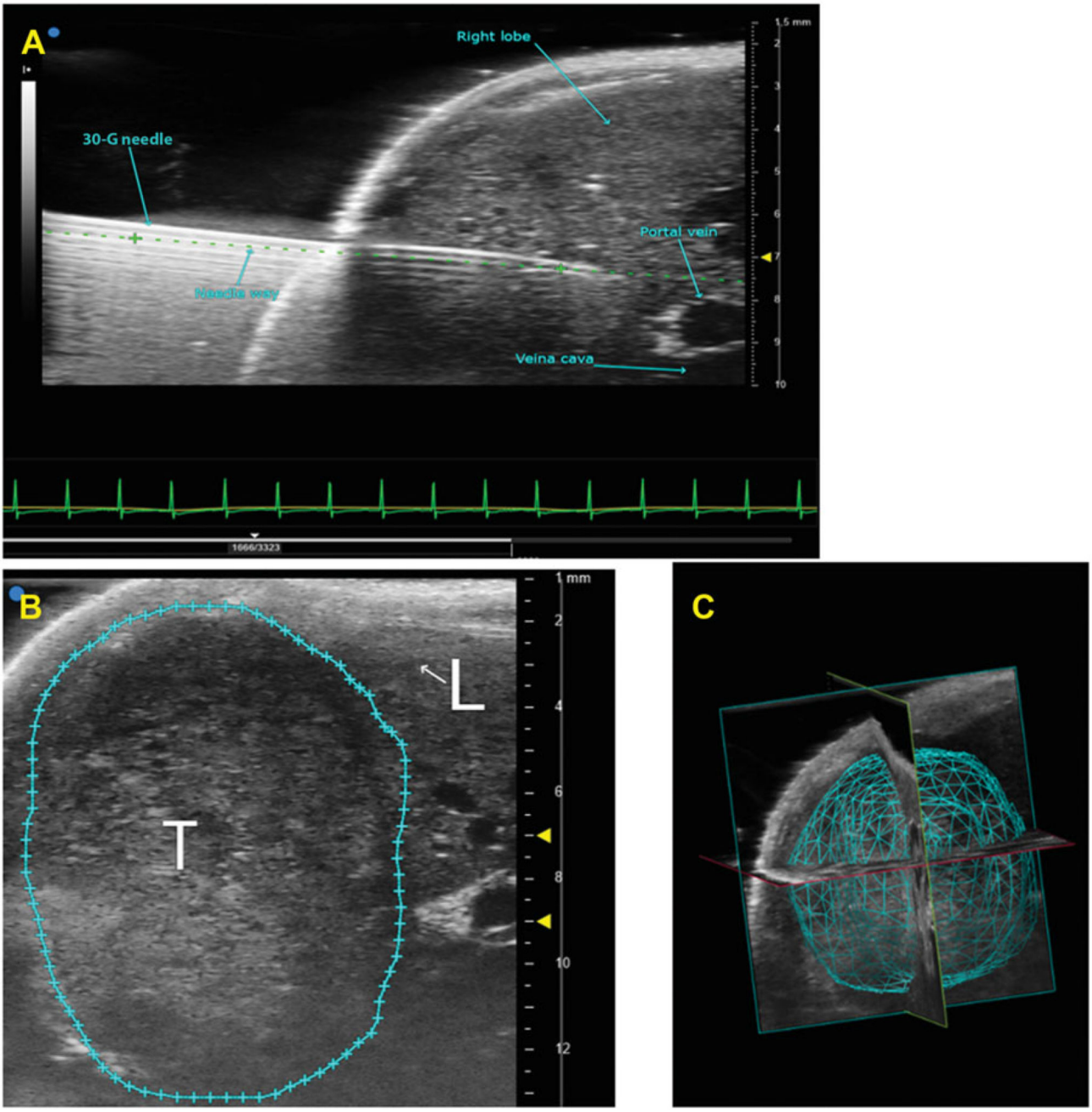

Figure 2. Representative ultrasound images of echo-guided injection and tumor.

(**A**) Optimal position and alignment of the needle for injection of Huh-7-Luc tumor cells with 30-G needle buried within the right lobe of the liver. (**B**) Completed contour of tumor formation at 2 weeks after cells injection (2D area in blue). (**C**) Once contours have been drawn around the tumor in each slice, the software can reconstruct the tumor and quantify the volume.

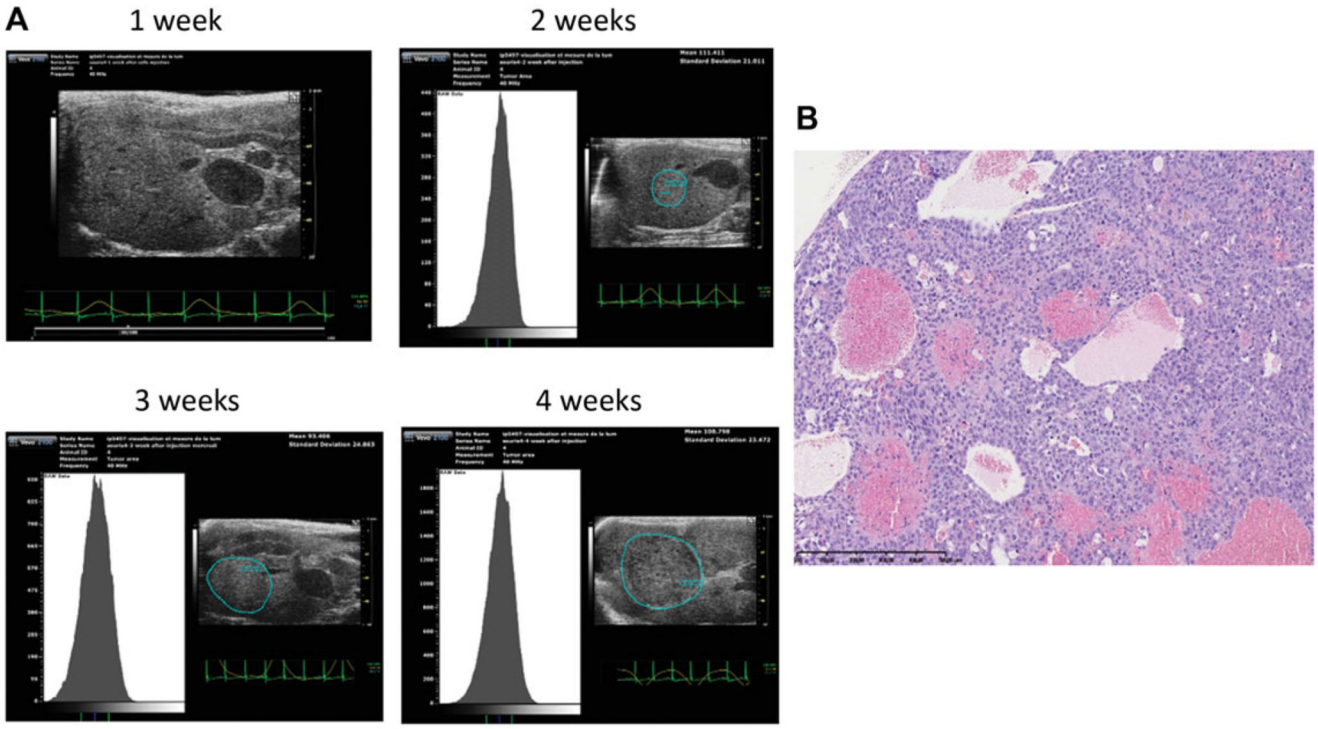

Figure 3. Longitudinal follow-up of tumor growth.

(**A**) Tumor formation at 1, 2, 3, and 4 weeks after injection (2D area in blue) and histogram for the surrounding area representative of the pixel count. (**B**) Histological stain of the tumor.

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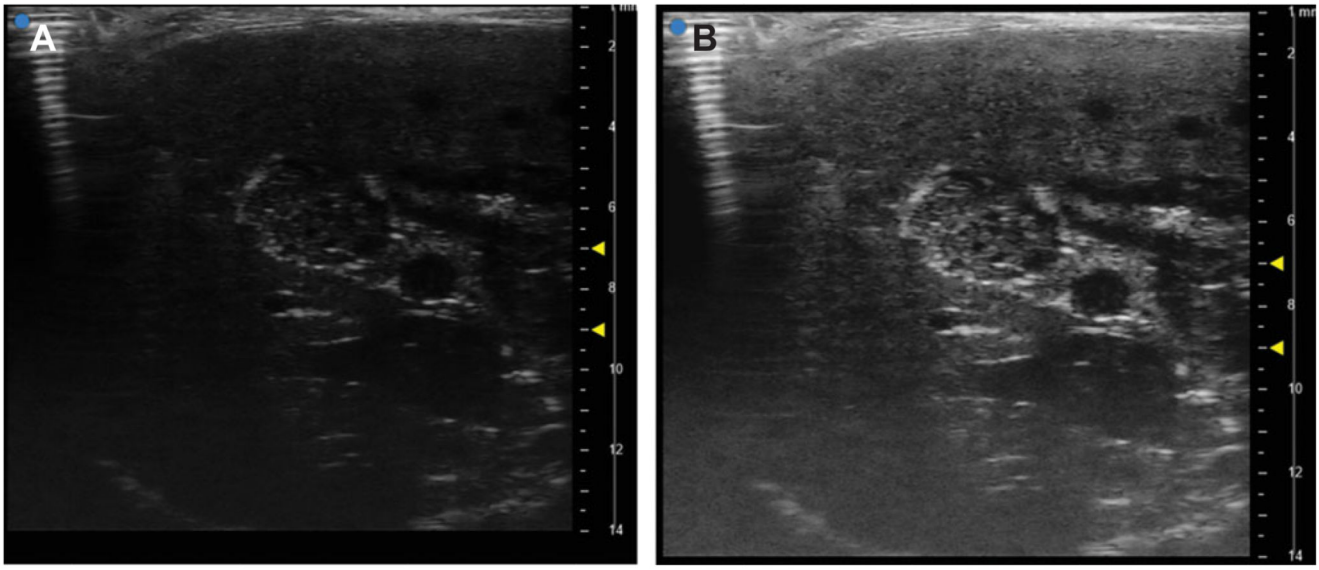

Figure 4. Suboptimal and optimal ultrasound imaging of tumor.

(**A**) Suboptimal tumor imaging without adjustment of brightness and contrast. (**B**) Optimal imaging after modification to visualize the proper contrast between the tumor and the surrounding liver parenchyma.

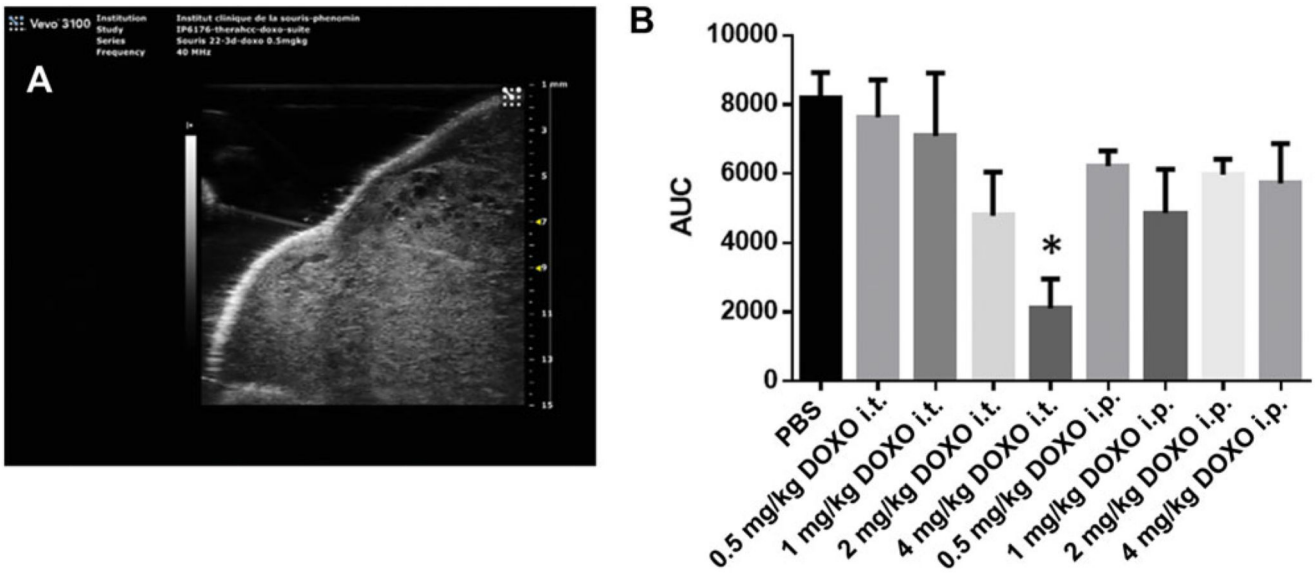

Figure 5.

(**A**) Representative ultrasound image of echo-guided intratumoral (i.t.) injection of doxorubicin (DOXO). Optimal position and alignment of the needle before doxorubicin injection. (**B**) Area under the curve (AUC) calculated from the curves of tumor volume (n = 6 per condition) during follow-up after doxorubicin treatment delivered i.t. or intraperitoneally (i.p.). Data were compared to control mice treated with phosphate-buffered saline (PBS) using an ANOVA and Bonferroni test. $*p < 0.05$.

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