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Corresponding Author:	Takami Sato Thomas Jefferson University Philadelphia, PA UNITED STATES
Corresponding Author's Institution:	Thomas Jefferson University
Corresponding Author E-Mail:	Takami.Sato@jefferson.edu
Order of Authors:	Ken Kageyama
	Shinji Ozaki
	Takami Sato
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Thank you very much for reviewing our manuscript [manuscript number: **JoVE59941R0**] entitled "Generation of a liver orthotopic human uveal melanoma xenograft platform in an immunodeficient mouse".

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Specific major issues from editors' and reviewers' comments
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Sincerely yours,

Takami Sato, M.D., Ph.D.
K. Hasumi Professor of Medical Oncology
Department of Medical Oncology
Thomas Jefferson University

TITLE:

Generation of a Liver Orthotopic Human Uveal Melanoma Xenograft Platform in Immunodeficient Mice

AUTHORS & AFFILIATIONS:

Ken Kageyama[1,3], Shinji Ozaki[2,3], Takami Sato[3]

[1]Department of Diagnostic and Interventional Radiology, Osaka City University Graduate School of Medicine, Osaka, Osaka, Japan
[2]Department of Surgery, National Hospital Organization, Kure Medical Cancer Center, Kure, Hiroshima, Japan
[3]Department of Medical Oncology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, USA

Corresponding Author:

Takami Sato (Takami.Sato@jefferson.edu)

Email Addresses of Co-authors:

Ken Kageyama (kaeyama@med.osaka-cu.ac.jp)
Shinji Ozaki (sskozaki31@gmail.com)

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SHORT ABSTRACT:

Orthotopic human liver metastatic uveal melanoma xenograft mouse models were created using surgical orthotopic implantation techniques with patient-derived tumor chunk and needle injection techniques with cultured human uveal melanoma cell lines.

LONG ABSTRACT:

In recent decades, subcutaneously implanted patient-derived xenograft tumors or cultured human cell lines have been increasingly recognized as more representative models to study human cancers in immunodeficient mice than traditional established human cell lines in vitro. Recently, orthotopically implanted patient-derived tumor xenograft (PDX) models in mice have been developed to better replicate features of patient tumors. A liver orthotopic xenograft mouse model is expected to be a useful cancer research platform, providing insights into tumor biology and drug therapy. However, liver orthotopic tumor implantation is generally complicated. Here we describe our protocols for the orthotopic implantation of patient-derived liver-metastatic uveal melanoma tumors. We cultured human liver metastatic uveal melanoma cell lines into immunodeficient mice. The protocols can result in consistently high technical success rates using either a surgical orthotopic implantation technique with chunks of patient-derived uveal melanoma tumor or a needle injection technique with cultured human cell line. We also describe protocols for CT scanning to detect interior liver tumors and for re-implantation

techniques using cryopreserved tumors to achieve re-engraftment. Together, these protocols provide a better platform for liver orthotopic tumor mouse models of liver metastatic uveal melanoma in translational research.

INTRODUCTION:

Uveal melanoma is the most common intraocular malignant tumor among adults in the western world. During the past 50 years, the incidence of uveal melanoma (5.1 cases per million) has remained stable in the United States[1, 2]. Uveal melanoma arises from melanocytes in the iris, ciliary body, or choroid, and it is an extremely lethal disease when it develops metastasis. The death rate of patients with uveal melanoma metastasis was 80% at 1 year and 92% at 2 years after initial diagnosis of the metastases. The time between diagnosis of metastases and death is typically short, less than 6 months, without regards to therapy[3, 4]. The cancer spreads through the blood and tends to dominantly metastasize to the liver (89–93%)[4, 5]. An effective mouse model is urgently needed for further investigation of liver-metastatic uveal melanoma. For translational research, there is a clear demand to generate a liver-localized metastatic uveal melanoma mouse model.

Patient-derived tumor xenograft (PDX) mouse models are expected to provide individualized medicine strategies. These models might be predictive of clinical outcomes, be useful for preclinical drug evaluation, and be used for biological studies of tumors[6]. Representative PDX models are ectopically tumor-implanted xenograft mice, which have tumor at subcutaneous sites. Most researchers can do surgery for subcutaneous implantation without special practice [7, 8]. They can also monitor subcutaneous tumors easily. Although subcutaneous PDX models became popular in the research phase, they have some hurdles in moving to practical use. Subcutaneous implantation forces patient-derived tumors to engraft at a different microenvironment from the tumor origin, so that it leads to engraftment failure and slow tumor growth [9 – 14]. Orthotopic engraftment may be a more ideal and rational approach for a PDX model because it uses the same organ as the original tumor[15, 16].

Recently, we developed protocols for surgical orthotopic implantation techniques of patient-derived liver-metastatic uveal melanoma tumors and needle injection techniques with a cultured human liver-metastatic uveal melanoma cell line in NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice[17, 18]. The protocols result in consistently high technical success rates. We also established CT scanning techniques that are useful to detect interior liver tumors, and we developed re-implantation of cryopreserved tumors in the PDX platform. We found that uveal melanoma tumor xenograft models maintain the characteristics of the original patient liver tumor, including their histopathological and molecular features. Together, these techniques provide a better platform for liver orthotopic tumor models for uveal melanoma in translational research.

PROTOCOL:

Patients enrolled in the study should provide written consent allowing the use of discarded surgical samples for research purposes and genetic studies, according to an Institutional Review Board-approved protocol. This protocol was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National

Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC).

1. Collection of fresh patient-derived tumor tissue

1.1. Obtain patient-derived tumor tissue from surgery or a needle biopsy in a hospital operating room.

1.2. Put the tumor tissue in a 100 mL container containing Hanks' balanced salt solution (HBSS) solution on ice.

1.3. Transfer the tissue into a sterile hood (biosafety level 2) in a laboratory.

1.4. Proceed to step 2 as soon as possible.

NOTE: For safety reasons, exclude patients with known HIV or Hepatitis B or C infections.

2. Processing of fresh patient-derived tumor tissue

2.1. Put the tissue in a 50 mL tube containing phosphate-buffered saline (PBS) on ice. For washing the tissue, add PBS in the tube and discard PBS from the tube twice.

2.2. Transfer the tissue into a Petri dish containing PBS on ice.

2.3. Using sterile forceps and scissors, remove the necrotic parts of the tissue. Keep the tissue moist and cold during steps 2.3 to 2.5. For needle biopsy samples, skip step 2.3 and 2.5, and do not cut the samples.

NOTE: The necrotic tissue often breaks apart easily when touched.

2.4. Cut the tissue into 1 mm^3 cubes for surgical liver implantation.

2.5. Cut the rest of the tissue into 2 mm cubes in the Petri dish.

2.6. Transfer them to a 1.7 mm microtube with 4% formalin for histological analysis and to another tube for genomic and proteomic analysis.

2.7. Put the microtubes in a liquid nitrogen jar with liquid nitrogen. Transfer the tubes to a −80 °C freezer for permanent storage.

NOTE: The time between sample removal from the patient and tissue processing should not exceed 30 min.

3. Surgical liver implantation with patient-derived tumor tissue

3.1. Spray all objects coming into the hood for surgery with 70 % ethyl alcohol.

NOTE: This includes surgical instruments, heating pads, and anesthesia machines.

3.2. Measure the weight of a cotton swab and fabric sheet.

3.3. Anesthetize a mouse with a 3–5% isoflurane vaporizer by placing it in the induction chamber.

3.4. Once the mouse is fully anesthetized, place it in supine position on a heating pad. Place the isoflurane cone on the mouse's snout to inhale 1.5–3% isoflurane for maintenance of anesthesia.

NOTE: The mouse needs to be on the heating pad during the entire procedure. Lack of heating may cause hypothermia.

3.5. Confirm proper anesthesia by no reaction when the foot of the mouse is pricked with ultrafine forceps.

3.6. Inject buprenorphine (0.6 mg/kg) subcutaneously on the flank using a 27 G needle on a micro syringe before surgery.

3.7. Apply 70% ethyl alcohol to the abdomen and spread the fur upwards and downwards. After spreading the fur, confirm easier visualization of the skin below the left subcostal area for an easier cut. Do not shave off the fur from the abdomen.

NOTE: The fur will hide the incision site after surgery and prevent the mouse from scratching the incision post operation.

3.8. Apply iodine and let it be absorbed into the skin.

3.9. Place a sterile surgical drape with a 2 cm hole on the mouse.

3.10. Lift abdominal skin with curved ultrafine forceps and make a 1 cm transverse left subcostal skin incision with curved scissors.

3.11. Insert the tip of the curved scissors beneath the skin of the incision and slightly open them to separate peritoneum from skin. Retract the scissors from the incision with closed blades.

NOTE: Opening and closing scissors inside the mouse can cause damage and bleeding.

3.12. Locate the liver under the peritoneum. Confirm a dark reddish color through the peritoneum.

3.13. With curved scissors, make a 1 cm transverse incision in the peritoneum. If a peritoneal artery bleeds from the cutting edge, immediately stop the bleeding with cautery.

3.14. Grab fat tissue using curved ultrafine forceps with one hand, insert the edge of a cotton swab beneath the left liver lobe and roll the swab downward with the other hand to bring out the liver.

NOTE: Grabbing fat tissue is important to keep the fat tissue from sticking to the cotton swab.

3.15. Exteriorize the liver on the cotton swab and place the liver on a non-woven absorbent fabric sheet.

NOTE: The fabric sheet plays two essential roles in stabilizing the liver and absorbing hemorrhage.

3.16. Make an incision 5 mm in width and depth using a sterile No. 11 scalpel blade to form a pocket in the parenchyma while softly pressing the incision site with the cotton swab.

3.16.1. Insert the blade in parallel with the surface of the liver and cut horizontally.

3.16.2. Press the incision site with the cotton swab to stop any hemorrhage.

NOTE: Do not keep the blade vertical, otherwise you will break through the liver and injure large vessels in the middle of the liver.

3.17. Roll the cotton swab upward to open the incision site and implant a 1 mm^3 cube of tumor tissue into the pocket with curved ultrafine forceps. Retract the forceps while rolling the cotton swab in reverse rotation and pressing down.

NOTE: Pressing down on the incision site with the cotton swab while retracting the forceps helps to prevent displacement of the tumor inside the pocket.

3.18. Gently take the cotton swab off the incision site after implantation. Proceed to step 3.19. as soon as possible.

3.19. Put an absorbable hemostat on the incision site.

3.20. Confirm hemostasis. If bleeding continues, add more hemostat on the incision site.

3.21. Peel the liver off the fabric sheet with forceps (preferably blunt-ended) and put the liver back into the abdominal cavity.

3.22. Suture peritoneum with double ligature using 5-0 absorbable suture.

3.23. Suture skin with triple ligature using 5-0 absorbable suture.

NOTE: Triple ligature helps to prevent surgical incision dehiscence.

3.24. Observe the mouse until fully awake and put it back in the cage.

3.25. Measure the weight of the cotton swab and the fabric sheet with blood for bleeding volume during the surgery. Compare them with their original weights before surgery. Reduce bleeding during the surgery to less than 10% of circulating blood volume in mouse.

4. Collecting and processing of cultured human liver metastatic uveal melanoma cell line

4.1. Prepare cultured cells.

4.2. Collect cells and calculate the cell number using a cell counter.

4.3. Prepare an appropriate amount of cell suspension for 10.0 x 10^6 cells in a 15 mL tube.

4.4. Spin the tube at 300 x *g* for 5 min in a centrifuge at room temperature.

4.5. Remove the supernatant in the 15 mL tube. Leave the cell pellet at the bottom of the tube.

4.6. Add 50 µL of RPMI 1640 medium into a 1.7 mL tube.

4.7. Cut the tip of a 200 µL tip with scissors to enlarge the tip opening.

4.8. Add 60 µL of basement membrane matrix using a pipette with the cut tip into the 1.7 mL tube that has RPMI.

4.9. Mix RPMI and matrix in the 1.7 mL tube. Vortex it.

4.10. Add 110 µL of the mixture into the cell pellet in the 15 mL tube. Transfer the cell suspension into a new 1.7 mL tube.

4.11. Keep the tube on ice before needle injection.

5. Surgical needle implantation of cultured human liver metastatic uveal melanoma cell line into liver

5.1. Follow the above protocol from steps 3.1. to 3.15.

5.2. Collect the cell suspension with a microsyringe with a 27 G needle.

5.3. Insert the needle along the surface of the liver and advance the tip of the needle 5 mm deeper.

5.4. Inject 20 µL of cell suspension into the liver.

5.5. Cauterize the insertion point of the liver to prevent the injected cells from leaking out. Confirm hemostasis.

5.6. Follow the above protocol from steps 3.21 to 3.24.

6. CT scan

6.1. Place the mouse into a restrainer in the awake state.

6.2. Wipe the tail with a sterile alcohol pad for disinfection and vasodilation.

6.3. Inject 100 µL of CT contrast agent through the tail vein with a 27 G needle on a 1 mL syringe.

6.4. Wait for 4 h after injection before taking the CT scan.

NOTE: It takes 4 h until the agent is taken up by liver Kupffer cells.

6.5. Four hours after injection, anesthetize the tumor-bearing mouse with 3–5% vaporized isoflurane by placing it in the induction chamber.

6.6. Once the mouse is fully anesthetized, place it in the prone position on a CT. Place the isoflurane cone on the mouse's snout to inhale 1.5–3% isoflurane for maintenance of anesthesia.

6.7. Confirm proper anesthesia by no reaction when the foot of the mouse is pricked with ultrafine forceps.

6.8. Take a CT scan for 15 min.

6.9. Ensure that the mouse until it is fully awakened after the CT scan and put it back into the cage.

6.10. Evaluate for the existence of tumor and measure the tumor size on the CT images.

NOTE: The contrast agent enhances normal liver parenchyma so that it is easy to recognize unenhanced tumor. Do not misinterpret the gallbladder and stomach as tumor.

7. Harvesting and processing tissue

7.1. Euthanize mice using CO_2 followed by cervical dislocation by placing the index finger and thumb behind the skull and pulling the body by the base of the tail. Proceed to step 7.2. as soon as possible.

7.2. Place the mouse in a supine position and spray the abdomen with 70% ethyl alcohol.

7.3. Use sterile forceps and sterile scissors to make a 3-cm transverse incision below the xiphoid process to expose the abdominal organs.

7.4. Excise the tumor tissue and perform steps 2.1. to 2.2.

7.5. Cut the rest of the tumor into 2 mm cubes in the Petri dish.

7.6. Transfer them to a cryogenic tube with cryomedium for re-implantation after cryopreservation.

7.7. Put the tubes in a cryogenic freezing container filled with isopropanol.

7.8. Transfer the container to a −80 °C freezer for temporary storage. Do not put the cryotubes with cryomedium directly into a liquid nitrogen tank. Freeze them slowly at a cooling rate of -1 °C/min to preserve tumor tissue.

7.9. On the next day, transfer the tubes into a liquid nitrogen tank for permanent storage.

8. Re-implantation

8.1. Keep tubes frozen in a liquid nitrogen jar with liquid nitrogen until ready to implant tissue. Minimize exposure of the tissue to room temperature to maintain viability and enhance chances of engraftment.

8.2. Thaw cryopreserved tube in a 37 °C water bath.

8.3. Perform steps 2.2–2.4.

8.4. Implant the thawed tumor into mice as described in steps 3.1–3.24.

REPRESENTATIVE RESULTS:

Surgical orthotopic implantation using the liver pocket method can transplant human liver metastatic uveal melanoma tumor into the mouse liver with a high success rate of 80% within six months. The xenograft tumor engrafts in the liver as a solitary tumor without daughter nodules (**Figure 1** and **Figure 3A**). The surgical orthotopic injection technique into the liver using microneedles successfully engrafted cultured human liver-metastatic uveal melanoma cells in the liver in all cases (**Figure 2** and **Figure 3B**). However, some cases had dissemination around the main tumor. The contrast agent detects tumors in the liver on CT, including small tumors of 1 mm size (**Figure 3B**). Re-implantation of cryopreserved tumors successfully established them in the mouse liver with high success rates. The re-implanted xenograft tumors after cryopreservation retain the characteristics of the original patient tumors and pre-cryopreserved tumors.

FIGURE LEGENDS:

Figure 1: Patient-derived tumor xenograft mouse model by surgical orthotopic liver implantation. Mouse was euthanized after 6 months after tumor implantation. Pigmented black tumor (black arrow) is uveal melanoma. The tumor is engrafted in the left lobe of the liver.

Figure 2: Liver orthotopic human cell line-derived tumor xenograft mouse model using needle injection method. Mouse was euthanized 8 weeks after tumor injection. Pigmented black tumor (black arrow) is uveal melanoma. The tumor is engrafted in the left lobe of the liver.

Figure 3: CT images of liver tumors in the left lobe of the liver. Liver tumors are detected on enhanced CT. Normal liver tissue is enhanced by contrast agent. White arrows indicate the stomach next to the liver. (**A**) The tumor (black arrow) that was previously shown in **Figure 1**. Surgical orthotopic implantation forms a solitary tumor. (**B**) The tumors (black arrow) shown previously in **Figure 2**. Needle injection method forms a cluster of many small tumors.

Figure 4: Technical tips for the liver pocket method. (**A–C**) Left lobe (white arrows) of the liver can be exposed out of the abdomen using a cotton swab (black arrow) via a 1 cm incision. A retractor is not required to widen the incision. (**D**) Cotton swab presses on the incision softly. It obtains hemostasis after making an incision by the scalpel (green arrow). (**E**) Cotton swab rolls upward (curved red arrow). This lifts the liver parenchyma to open the incision. The tumor yellow arrow) is inserted into the liver pocket through the incision by ultrafine forceps (blue arrow). (**F**) Cotton swab rolls downward (curved red arrow) to prevent an inserted tumor in the pocket from backing out.

DISCUSSION:

The current orthotopic xenograft models are labor-intensive, time-consuming, and expensive to create. Orthotopic tumor xenograft mouse models for liver cancer were established more than two decades ago[19-21]. However, this technique is complicated and requires use of special equipment, such as a micro-needle holder and 6-0 to 8-0 fine sutures under a microscope. Tumor and normal liver tissue must be sewn up carefully so that the suture does not damage the fragile liver tissue. The conventional techniques lead to complications, such as hematoma and necrosis[22]. Recently, a modified technique was developed to solve these problems[23]. This modified technique uses absorbable hemostatic materials instead of suture to cover the tumor on the liver surface. However, this modified method does not completely cover the tumor within the liver parenchyma. A part of the tumor is exposed to the outside. We developed a surgical orthotopic implantation technique—the liver pocket method—to house the tumor entirely inside the parenchyma[18]. Our method makes a pocket in the liver to provide a natural environment for tumors. The liver pocket method is simpler than the conventional technique, allowing us to finish implantation into the liver within a few minutes from the beginning of the operation. This method results in formation of a solitary tumor in the liver and does not trigger metastases, at least for as long as we observed the mice, whereas needle injection of a single cell suspension tends to disseminate as intra-hepatic metastases[17]. A solitary tumor is more appropriate to evaluate tumor growth and would be useful to assess efficacy in a drug trial.

Compared to the original liver pocket method[18], we have modified our methods to enhance techniques of implantation. First, a retractor was not used during surgery to minimize the size of the incision. When the incision is smaller, we can shorten sewing time in surgery. With a 1 cm incision in the abdomen, we can easily bring the left lobe outside with a cotton swab (**Figure 4A–C**). Second, a cotton swab plays three important roles by stopping hemostasis after making the liver pocket, opening the liver pocket to be able to insert a tumor chunk and retaining the tumor chunk in the pocket without pushing the tumor back (**Figure 4D–F**). Average bleeding volume was approximately less than 10% of circulating blood volume in mice. Less bleeding provided great confidence in surgery. Third, a fabric sheet is useful for fixing the liver lobe outside the abdomen. The liver lobe sticks to the sheet and thus it prevents the lobe from sliding back into the abdomen (**Figure 4C**). One can easily cut the liver surface with a scalpel and inject a needle to the liver surface. As a result, fragile liver tissue is not injured.

We present have two troubleshooting tips for this method. First, when a small incision site is used, sometimes a left lobe is not visible. In this situation, the left lobe is likely sticking to the diaphragm. Insert blunted-edge forceps between the left lobe and the diaphragm to peel the lobe off. Second, when a tumor chunk is placed in the liver pocket with forceps, the tumor can stick to the forceps and pull back with it. Press the incision with a cotton swab while retreating the forceps. This works well to prevent the dislocation of the tumor out of the pocket.

Xenograft tumors are surrounded by mouse tissue, even though they are orthotopically implanted. Human stromal cells in patient-derived tumors are inevitably replaced by mouse stromal cells. Ideally, the mouse model had better provide human stromal tissue around tumors. Chimeric humanized liver mouse or humanized immune mouse models would be helpful to study the engraftment of uveal melanoma and to evaluate whether the drug metabolism is the same as a human-liver or human-immune environment[24, 25].

Orthotopic liver tumor xenograft mouse models require verification of tumor establishment with imaging studies. The commercially available CT contrast agent, developed for mouse liver CT images, allows detection of interior liver tumors in the live state on CT. The contrast agent specifically enhances normal liver on the CT. It is easy to distinguish the unenhanced site of the tumor[26]. The agent detects tiny tumors less than 1 mm (daughter nodules) around main tumors on CT. The agent can be tolerated by the mouse, and makes it possible to monitor liver tumors periodically. The agent would be used to evaluate efficacy of anti-cancer drugs against liver-localized xenograft tumors.

Generally, it is recommended to maintain PDX models at a relatively low passage number (less than 10) to conserve genetic and histological integrity of the original patient-derived tumor[27-29]. Most researchers refrain from making multiple passages of the PDX models to reduce the number of passages and animals. Once patient-derived tumors are temporarily preserved in a freezer, we are able to control PDX models at a lower passage number without wasting mice. This is called biobanking strategy. A cancer biobank is a rational approach to maintain tumor characteristics and to reduce the number of mice[28, 30]. Establishing a proper biobanking method can adjust the supply of PDX models to meet the patient's treatment plan or a mouse drug efficacy trial in the

future. We achieved re-implantation of cryopreserved tumors for cancer biobanking. We hope that this success facilitates PDX platform use in the near future.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

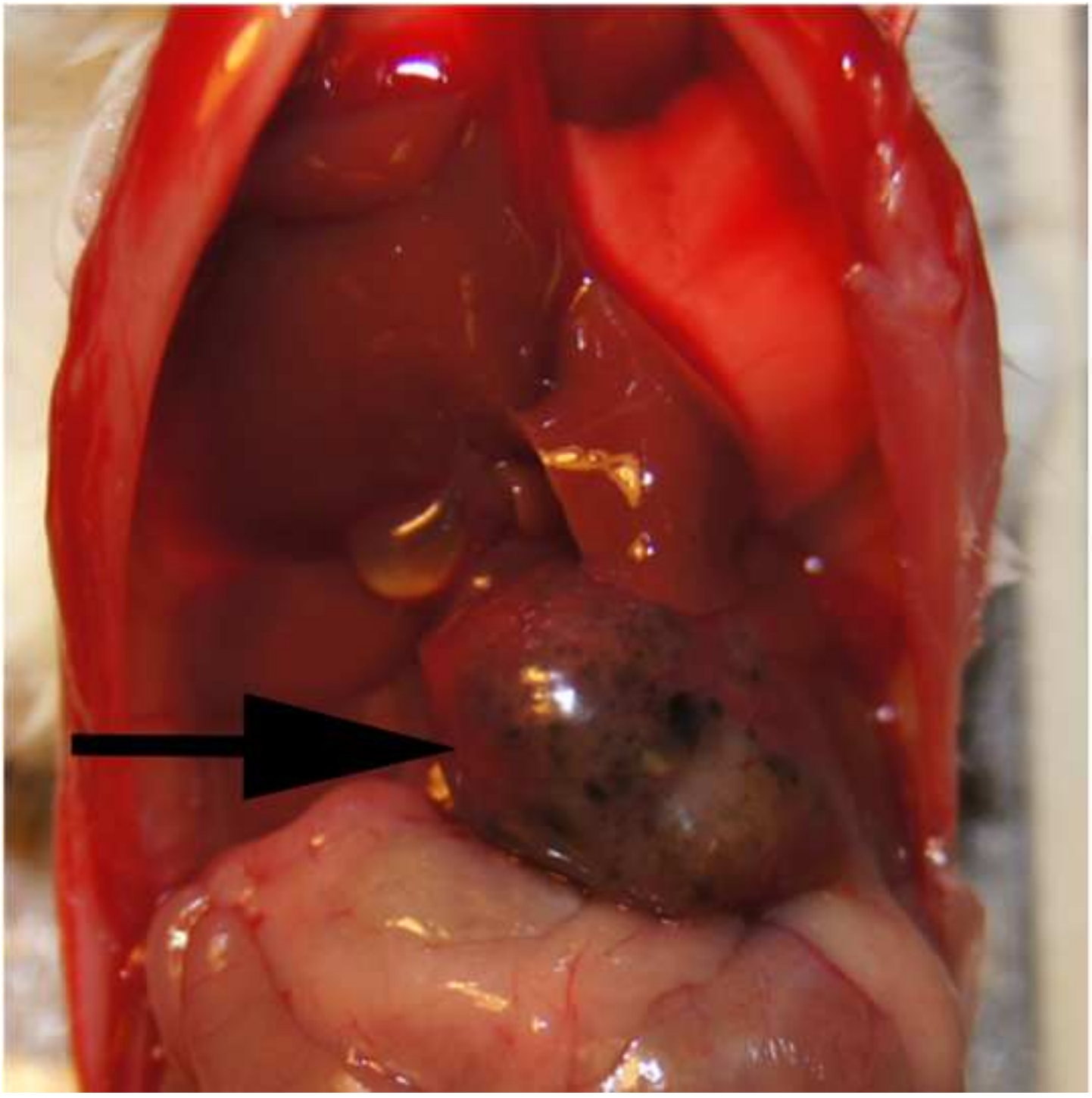


Figure 2

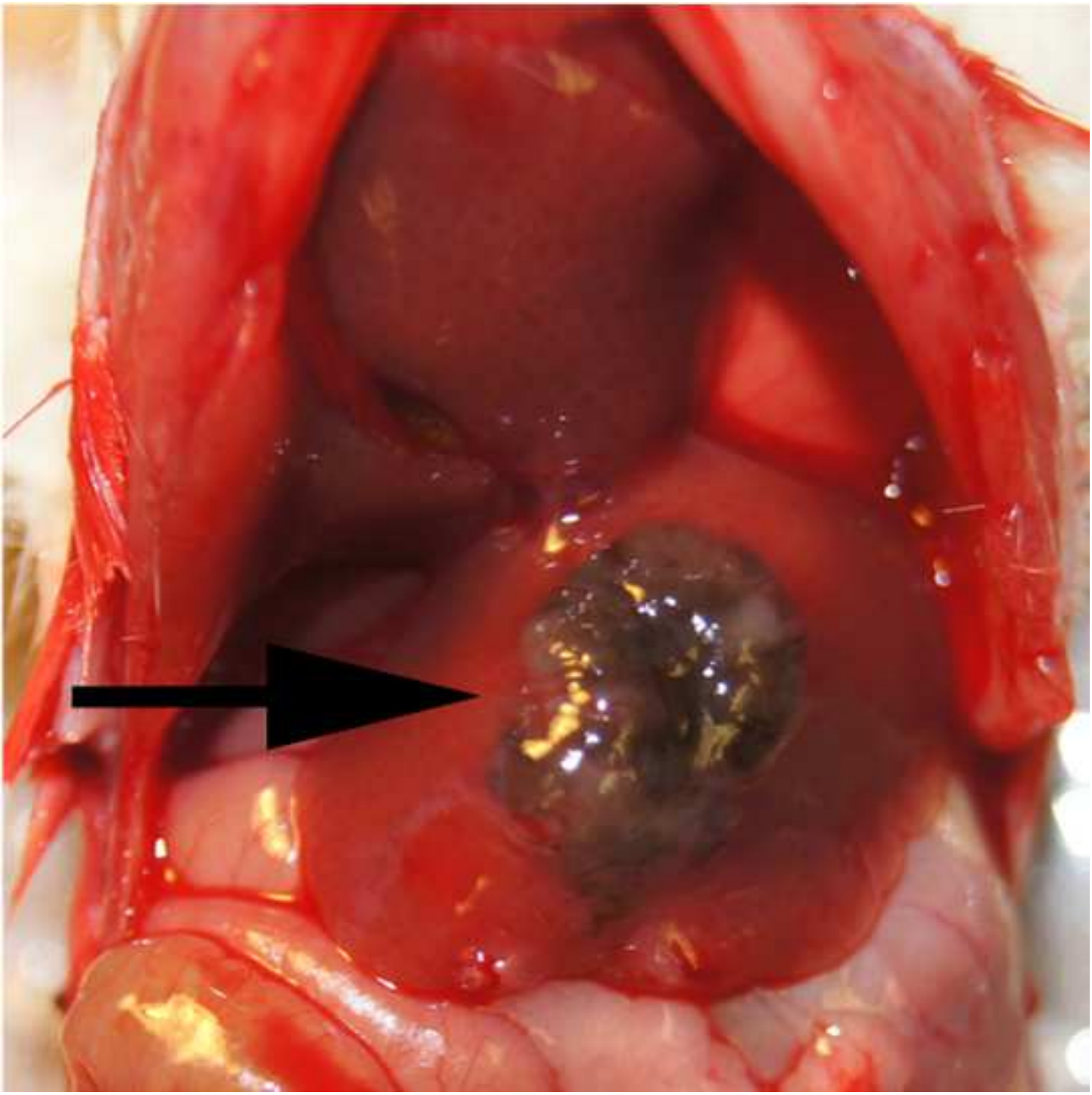


Figure 3

Click here to access/download;Figure;Figure 3.psd

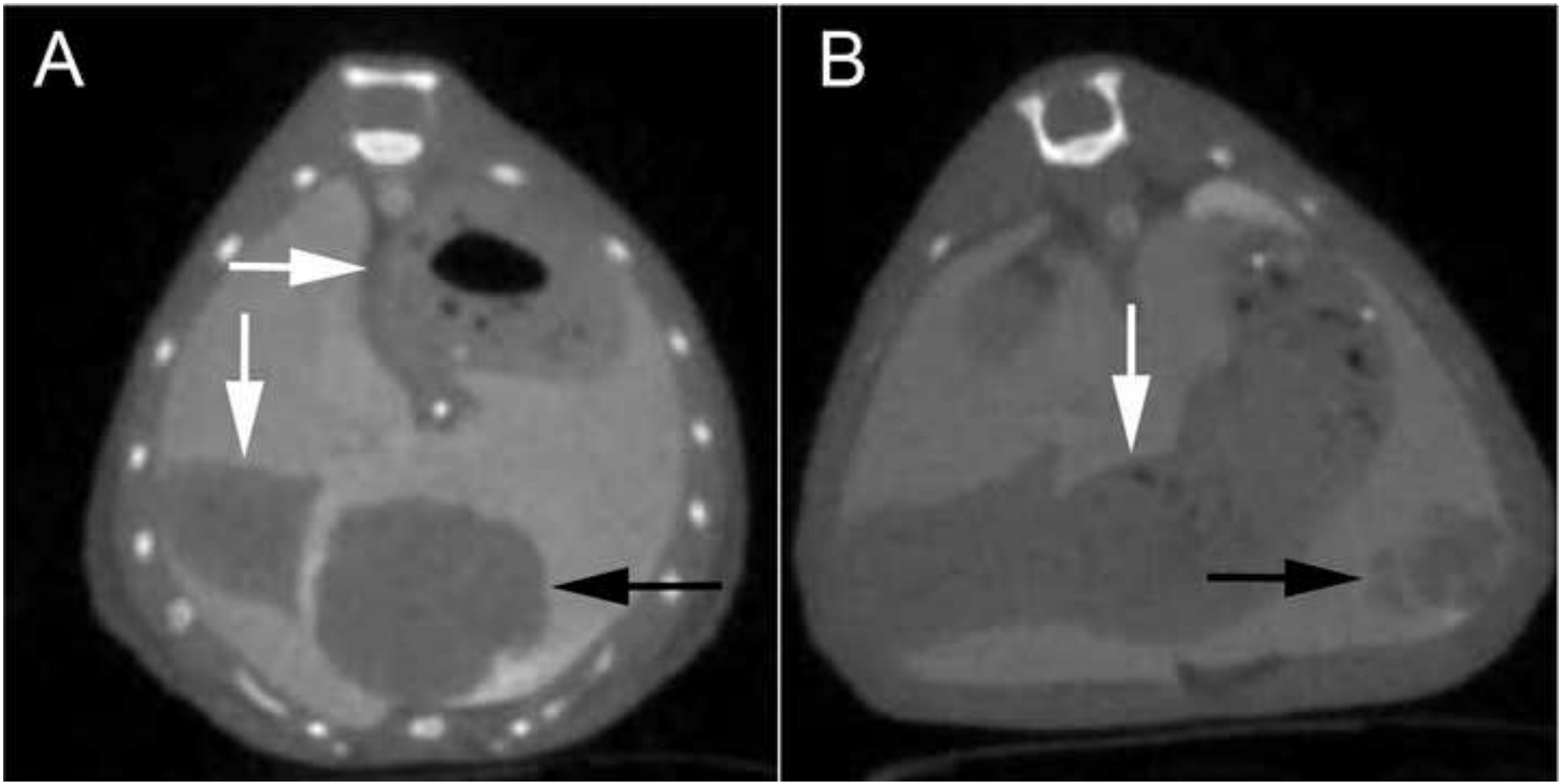
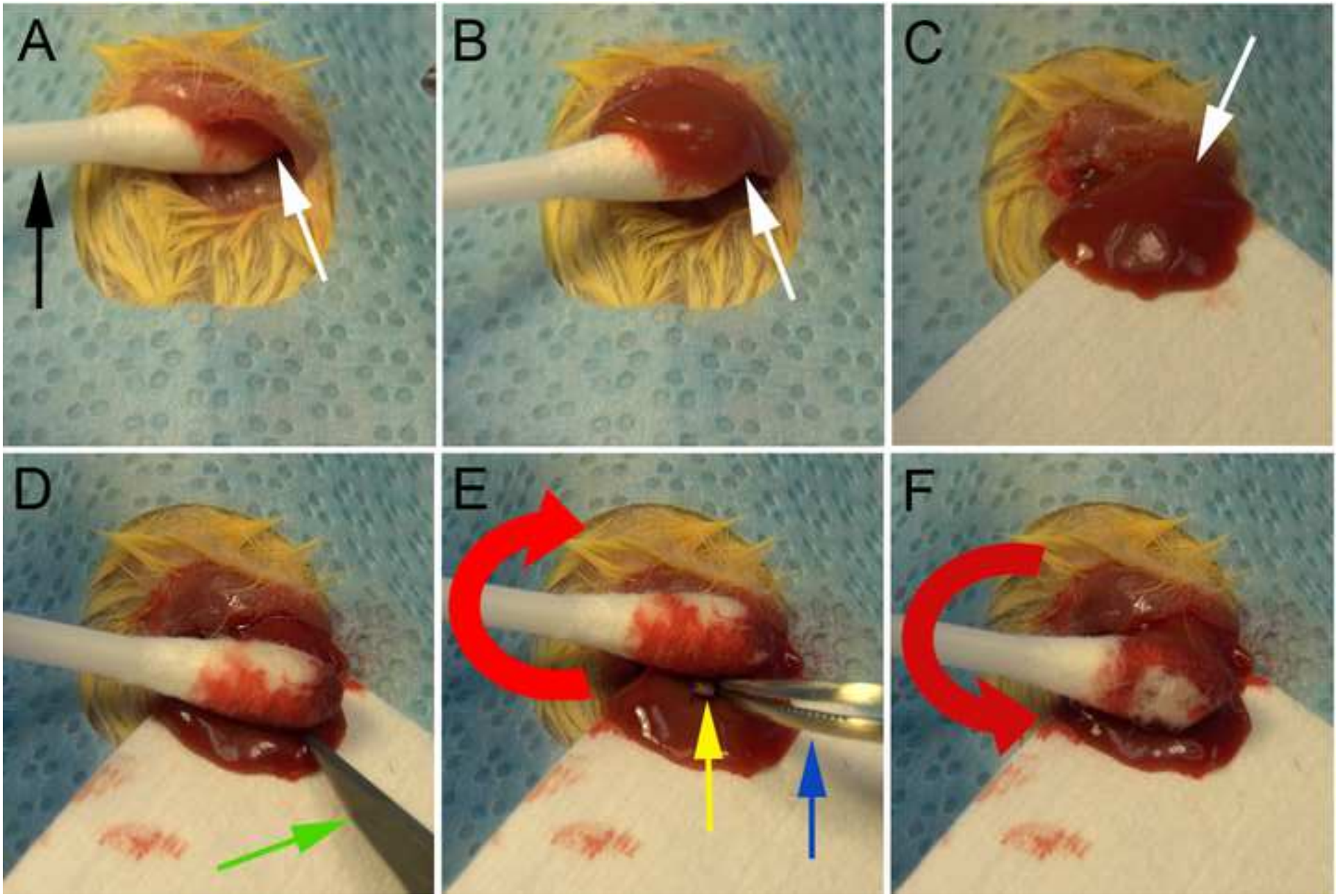


Figure 4

Click here to access/download;Figure;Figure 4.psd



Supplementary Video for jove-Liver pocket method

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Video or Animated Figure

Liver pocket 2 short version.mp4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Materials, tissues and animals			
Buprenorphine			
CO_2 tank			
Cryomedium			
Exitron nano 12000 (Alkaline earth metal-based na	Miltenyl Biotec	130-095-700	
HBSS 1X, with calcium & magnesium	Corning	21-020-CM	
Human liver metastatic uveal melanoma cell line			
Human uveal melanoma tissue in the liver			All tissue handling should be done in a Biosafety
Iodine			
Isoflurane	Purdue Products	67618-150-17	
Isopropanol	Fisher scientific	A416-1	Avoid direct contact to skin and eye and inhalat
Liquid nitrogen			
Matrigel HC	BD	354248	
NOD.Cg-*Prkdc* ^{scid} *Il2rg* ^{tm1Wjl} /SzJ (NSG) mice	Jackson Lab	5557	4 to 8 weeks old
PBS 1X, without calcium and magnesium	Corning	21-031-CM	
RPMI 1640	Corning	10-013-CV	
Sterile alcohol prep pad (70% isopropyl alcohol)	Nice-Pak products	B603	
4% paraformaldehyde phosphate buffer solution	Wako	163-20145	
70% Ethyl alcohol solution	Fisher Scientific	04-355-122	
Equipments			
Absorbable hemostat	Johnson and Johnson	63713-0019-61	
Autoclave			
Body weight measure			
Cautery	Bovie Medical	MC-23009	
Cell counter			
Centrifuzer			
Cotton swab			
Cryo freezing container	NALGENE	5100-0001	
Cryotube	SARSTEDT	72.379	
Curved scissors	World Precision Instruments	503247	

Curved ultrafine forceps	World Precision Instruments	501302	
Fabric sheet			
Freezer			
F/AIR Filter Canister	Harvard Apparatus	600979	
Heating pad			
Isoflurane vaporizer	Artisan Scientific	66317-1	
Liquid nitrogen			
Liquid nitrogen jar	Thermo Fisher Scientific	2123	
Micro-CT scan	Siemens		
Needle holder	World Precision Instruments	501246	
Petri dishes	Fisher Scientific	FB0875713	
Pipette			
Spray bottle			
Sterile hood			Biosafety level 2 cabinet
Sterile No.11 scalpel	AD Surgical	A300-11-0	
Straight forceps	World Precision Instruments	14226	
Surgical drape			
Tail vein restrainer	Braintree Scientific	TV-150-STD	
Water bath			
1 ml TB syringe with 27-gauge needle	BD	309623	
1.7 ml tube	Bioexpress	C-3260-1	
5-0 PDO Suture	AD Surgical	S-D518R13	
15 mL conical tubes	AZER SCIENTIFIC	ES-9152N	
27-gauge needle	BD	780301	
27-gauge needle	Hamilton	7803-01	
50 mL conical tubes	AZER SCIENTIFIC	ES-9502N	
50 µl micro syringe	BD	80630	
50 µl micro syringe	Hamilton	7655-01	
100 mL container	Fisher Scientific	12594997	
200µl tip			

/ Level 2 hood. Be careful when working with human tissue; always use gloves and avoid direct skin contact. Assume patients may have been in

ion of anesthetic agent.

fected with HIV or other highly transmissible organisms. Do not process samples known to carry infections.

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Author(s): Ken Kageyama, Shinji Ozaki, Takami Sato

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CORRESPONDING AUTHOR

Name:	Takami Sato
Department:	Medical Oncology
Institution:	Sidney Kimmel Cancer Center, Thomas Jefferson University
Title:	Proffesor
Signature:	Takami Sato
Date:	2/28/2019

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Dear editors and reviewers:

Thank you for giving us the opportunity to submit a revised version of our manuscript. We have modified the manuscript based on the helpful comments provided.

Editor:

Thank you for your kind consideration. We are grateful for your comments, which have helped us to improve our manuscript. As indicated in the responses that follow, we have taken your comments into account in the revision of our manuscript.

E-1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thank you for this helpful suggestion. According to your suggestion, we have had the manuscript checked for grammatical errors and ease of understanding by a native English speaker. The resulting changes are shown in color in the revised manuscript.

E-2. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 58-60, 65-71, 388-391, 404-408

Thank you for your kind consideration. According to your suggestion, we have used original language in the revised manuscript. Some sentences and words have been deleted from the manuscript, and then new sentences were inserted into the appropriate sections of the revised manuscript.

The following sentences have been deleted from the revised manuscript.

(Line 58 - 61)

"~~Uveal melanoma, which originates in the iris, ciliary body, or choroid, is highly fatal if it metastasizes. The mortality rate is over 90% within 2 years of initial diagnosis of metastasis, and median survival time is less than 6 months, regardless of treatment [3,4].~~"

The following sentences have been inserted into the appropriate section of the revised manuscript.

(Line 61 - 65)

"Uveal melanoma arises from melanocytes in the iris, ciliary body, or choroid, and it is an extremely lethal disease when it develops metastasis. The death rate of patients with uveal melanoma metastasis was 80% at 1 year and 92% at 2 years after initial diagnosis of metastases. The time between diagnosis of metastases and death is typically short, less than 6 months, without regard to therapy [3, 4]."

The following sentences have been deleted from the revised manuscript.

(Line 69 - 75)

"~~holds promise because it offers a personalized treatment approach and may be useful to predict clinical prognosis, drug efficacy, and tumor characteristics [6]. Most PDX models, including current uveal melanoma PDX models, are made by ectopic subcutaneous implantation. Subcutaneous implantation allows us to implant tumors quickly and to monitor tumors easily [7, 8]. However, subcutaneous PDX models have some limitations for translational research due to a different anatomic microenvironment from tumor origin, poor engraftment rate, and weak tumor growth [9–14].~~"

The following sentences have been inserted into the appropriate section of the revised manuscript.

(Line 75 - 84)

“s are expected to provide individualized medicine strategies. These models might be predictive of clinical outcomes, be useful for preclinical drug evaluation, and be used for biological studies of tumors [6]. Representative PDX models are ectopically tumor-implanted xenograft mice, which have tumor at subcutaneous sites. Most researchers can do surgery for subcutaneous implantation without special practice [7, 8]. They can also monitor subcutaneous tumors easily. Although subcutaneous PDX models became popular in the research phase, they have some hurdles in moving to practical use. Subcutaneous implantation forces patient-derived tumors to engraft at a different microenvironment from the tumor origin, so that it leads to engraftment failure and slow tumor growth [9 – 14].”

The following sentences have been deleted from the revised manuscript.

(Line 448 - 450)

“~~Patient-derived xenograft tumors are destined to be fostered by mouse vessels and tissues. If we demand a supreme mouse model, we need a chimeric mouse model with a humanized liver and a human-like immune environment in the mouse [24, 25]. These~~”

The following sentences have been inserted into the appropriate section of the revised manuscript.

(Line 450 – 453 and 455)

“Human stromal cells in patient-derived tumors are inevitably replaced by mouse stromal cells. Ideally, the mouse model had better provide human stromal tissue around tumors. Chimeric humanized liver mouse or humanized immune mouse”

“24, 25”

The following sentences have been deleted from the revised manuscript.

(Line 466 - 481)

~~"Most researchers refrain from making multiple passages of the PDX models in order to preserve the genetic and proteomic consistency of the original patient tumor, because these serial passages might lead to genomic rearrangement intrinsic to tumor adaptation [27 - 29]. A biobanking method would be theoretically useful to store patient tumors and xenograft tumors for re-implantation when required, and to keep PDX models at a lower passage number to retain genetic, proteomic, and histological consistency of original patient tumor [28, 30]. Establishing a proper biobanking method can reduce the number of animals needed and adjust the supply of PDX models to meet the patient's treatment plan or a mouse drug efficacy trial in the future. We achieved re-implantation of cryopreserved tumors. We hope that this success facilitates PDX platform use in the near future."~~

The following sentences have been inserted into the appropriate section of the revised manuscript.

(Line 466 – 481)

"Generally, it is recommended to maintain PDX models at a relatively low passage number (less than 10) to conserve genetic and histological integrity of the original patient-derived tumor [27 - 29]. Most researchers want to refrain from making multiple passages of the PDX models to reduce the number of passages and animals. Once patient-derived tumors could be temporarily preserved in a freezer, we are able to control PDX models at a lower passage number without wasting mice. This is called as biobanking strategy. A cancer biobank is a rational approach to maintain tumor characteristics and to reduce the number of mice [28, 30]. Establishing a proper biobanking method can adjust the supply of PDX models to meet the patient's treatment plan or a mouse drug efficacy trial in the future. We achieved re-implantation of cryopreserved tumors for cancer biobanking. We hope that this success facilitates PDX platform use in the near future."

E-3. Please sort the Materials Table alphabetically by the name of the material.

This has been done.

E-4. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Thank you for your valuable comments regarding our manuscript. Based on your comments, we have modified the sentences in the protocol in the revised manuscript. The resulting changes are shown in color in the revised manuscript.

E-5. What percentage isoflurane is used?

According to your suggestion, we have modified the Protocol, STEP 3.3., 3.4., 6.5., and 6.6., to address this question and enhance the reader's comprehension.

E-6. Please mention how proper anesthetization is confirmed.

Suggestion incorporated. According to your suggestion, we have modified the Protocol, STEP 3.5., and 6.7., to enhance reader's comprehension.

E-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, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Based on your comments, we have modified the sentences in the Protocol of the revised manuscript. The resulting changes are shown in color in the revised manuscript.

E-8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Thank you for these comments. Based on your comments, we have modified the sentences of the highlighted steps in the Protocol of the revised manuscript. We have actually changed all steps to the imperative tense in the protocol in the revised manuscript. However, in the NOTE of the steps, we have provided declarative sentences in the revised manuscript. The resulting changes are shown in color in the revised manuscript.

E-9. Please do not abbreviate journal titles.

We have reconfirmed journal titles. They don’t have abbreviation.

Miscellaneous points.

In the Acknowledgements, we have added two sources of support into the revised manuscript.

Reviewer 1:

None.

Reviewer 2:

Thank you for your kind consideration. We are grateful for your comments, which have helped us to improve the manuscript. As indicated in the responses that follow, we have taken your comments into account during the revision of our manuscript.

R2-1. Although conjunctival melanoma is a different tumor, the authors could mention and discuss Schlereth's conjunctival melanoma model: Invest Ophthalmol Vis Sci. 2015 Sep;56(10):5965-73. doi: 10.1167/iovs.15-17290. A Novel Model of Metastatic Conjunctival Melanoma in Immune-Competent Mice. Schlereth SL1, Iden S2, Mescher M2, Ksander BR3, Bosch JJ4, Cursiefen C1, Heindl LM1.

Thank you for your valuable comments regarding our manuscript. The above article is very important with respect to the method. The article showed that mouse-derived conjunctival melanoma (CM) was implanted in the conjunctiva of the immune-competent mouse. In our method, human uveal melanoma tumors were xenografted in the liver of immunodeficient mice. These two methods are quite different in type of mice, type of tumor cells, and location of implantation. We want to focus on our method in this revised manuscript. If we were to include the above article in the revised manuscript, it would be confusing and redundant for readers. And then, we have to provide one more paragraph in the discussion to explain the method of Schlereth's conjunctival melanoma model. However, if the editors and reviewers would like us to explain Schlereth's method, we would be pleased to do so.