Journal of Visualized Experiments

Is My Mouse Pregnant? High-Frequency Ultrasound Assessment: Utility and Pitfalls

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61893R1
Full Title:	Is My Mouse Pregnant? High-Frequency Ultrasound Assessment: Utility and Pitfalls
Corresponding Author:	Colin Phoon UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	Colin.Phoon@nyulangone.org
Order of Authors:	Colin Phoon
	Mindong Ren
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	New York, NY, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please specify the section of the submitted manuscript.	Developmental Biology
Please provide any comments to the journal here.	We plan to shoot our own video, with input from the Editorial team. However, I was not given this option when I logged on to resubmit. Also, I could not upload 2 large videos.

TITLE:

Is My Mouse Pregnant? High-Frequency Ultrasound Assessment

AUTHORS AND AFFILIATIONS:

Colin K.L. Phoon[1], Mindong Ren[2,3]

[1]Department of Pediatrics, New York University Grossman School of Medicine, New York, NY, USA

[2]Department of Anesthesiology, New York University Grossman School of Medicine, New York, NY, USA

[3]Department of Cell Biology, New York University Grossman School of Medicine, New York, NY, USA

Email address of co-author:

Mindong Ren mindong.ren@nyulangone.org

Corresponding author:

Colin K. L. Phoon colin.phoon@nyulangone.org

KEYWORDS:

Mouse models; Ultrasound biomicroscopy; Pregnancy; Uterus; Embryos; Resorption

SUMMARY:

High-resolution ultrasound can help streamline experiments requiring timed-pregnant mice by determining the state of pregnancy, gestational age, and pregnancy losses. Presented here is a protocol to illustrate methods to assess mouse pregnancies as well as potential pitfalls (image artifacts) that may mimic pregnancy.

ABSTRACT:

The mouse is the mammalian animal model of choice for many human diseases and biological processes. Developmental biology often requires staged-pregnant mice to determine evolving processes at various timepoints. Moreover, optimal and efficient breeding of model mice requires an assessment of timed pregnancies. Most commonly, mice are mated overnight, and the presence of a vaginal plug is determined; however, the positive predictive value of this technique is suboptimal, and one needs to wait to know if the mouse is truly pregnant. High-resolution ultrasound biomicroscopy is an effective and efficient tool for imaging: 1) Whether a mouse is pregnant; 2) What gestational stage the mouse has reached; and 3) Whether there are intrauterine losses. In addition to the embryos and fetuses, the investigator must also recognize common artifacts in the abdominal cavity so as not to mistake these for a gravid uterus. This article provides a protocol for imaging along with illustrative examples.

INTRODUCTION:

The mouse is the preferred mammalian model for many human diseases and biological processes[1-4]. Research in developmental biology often requires staged-pregnant mice to

determine evolving processes at various timepoints[5-8]. Moreover, optimal and effective breeding of model mice requires an assessment of timed pregnancies, particularly when investigators are studying the effects of a gene mutation on development. Typically, investigators mate heterozygous mice overnight, look for a vaginal plug early the next morning, and hope that a pregnancy ensues[9]. Determining intrauterine loss typically starts with checking a newborn litter for Mendelian ratios of genotypes, then working backwards by sacrificing pregnant mice at various gestational stages, and recovering the embryos. Investigators may determine weight gain as a metric of a positive pregnancy[10-11]; however, especially with genetically-engineered mice, the litters may be very small and subsequently resorbed when there is intrauterine loss due to which the weight gain may not be obvious (particularly early in pregnancy, ~E6.5–8.5). A mouse may appear falsely pregnant due, for example, to a benign abdominal tumor. In essence, one works "blind".

High-resolution ultrasound biomicroscopy allows for direct visualization of the gravid uterus and developing mouse embryos[12-16]. Although we had initially developed methods to assess embryonic mouse cardiovascular physiology[16,17], we recognized the utility of this imaging modality to streamline our mouse breeding. Specifically, we no longer had to wait to "see" if a mouse were pregnant, based on either the obvious weight gain or delivery of a litter; we could determine the gravid state and re-mate mice quickly if the dam was not pregnant. Moreover, intrauterine losses could also be easily imaged, and a timeline of loss could be determined without sacrificing the mouse (see **Figure 1** for a schematic). Time, valuable model mice, and funds can thus be saved.

PROTOCOL:

All the steps in this protocol follow the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and have been approved by the Institutional Animal Care and Use Committee of New York University Grossman School of Medicine.

1. Mating of mice for timed pregnancies

1.1. Pair the appropriate female mouse (usually a heterozygote) in a cage with the appropriate male mouse (usually a heterozygote) for overnight mating.

1.2. Separate the mice the next morning. Alternatively, continuously mate the female and male mice, thus increasing the chances of pregnancy.

NOTE: However, an accurately timed pregnancy cannot be assured with the alternative, and staging of mouse embryos by ultrasound is not clear, especially when the disease process results in intrauterine growth retardation. If the embryos carrying the gene variant are assumed to be small, look for the larger wildtype littermates to gauge the gestational age.

1.2.1. Optional: Look for the vaginal plug. If there is no vaginal plug, the female mouse has not been mated. If there is a vaginal plug, it is still likely that the female mouse does not become pregnant.

1.3. Time the day after the overnight mating as E0.5.

1.4. Perform imaging at E6.5–E.8.5 to determine pregnancy, and re-mate the mice if the mouse is not pregnant (see step 1.1).

NOTE: At this stage, the female dam is not obviously pregnant to the eye; hence, the ultrasound imaging allows early determination and re-mating.

2. Anesthesia and preparation of mouse

2.1. Place the pregnant mouse in the anesthetic induction chamber.

2.2. Mix isoflurane with either room air or medical oxygen, at a concentration of 2%–3% at 1 L/min flow rate to induce sedation of the pregnant mouse in the induction chamber.

NOTE: Sedation typically occurs within 1–2 min. The mouse will be lying still, and her breathing will have slowed.

2.3. Quickly transfer the mouse to the imaging platform. The imaging platform typically has heating elements as well, which can help keep the mouse warm.

2.4. Place the nose of the mouse into the anesthetic nosecone.

2.5. Quickly re-route the isoflurane/oxygen mixture to the imaging platform nosecone. Maintain isoflurane at 2%–3% at 1 L/min flow.

2.6. Determine the level of sedation by paw pinch, corneal reflex, level of respiration, and any movement.

NOTE: The corneal reflex can be initially determined by applying moisturizing ointment to the eyes to keep them from drying out while the mouse is anesthetized (the mouse does not close her eyes).

2.7. With the mouse lying supine (on its back), tape the paws to the imaging platform's electrocardiogram (EKG) pads.

NOTE: However, an EKG is not necessary for this kind of imaging.

2.8. Remove the fur from the pregnant dam's abdomen as follows:

2.8.1. Wet the abdominal fur thoroughly with 70% ethanol, including up to the lateral edges. Do not apply so much that there is run-off onto the platform.

NOTE: Ethanol works better as a shaving lubricant than water.

2.8.2. Use the razor blade to carefully shave the abdomen. Be careful not to cut the nipples.

2.8.3. Wipe the shaved fur off the abdomen with gauze or some wipes.

2.8.4. Alternatively, use a depilatory cream after using the fur clippers to remove most of the fur.

3. Transabdominal imaging of the (presumed) pregnant mouse

3.1. After the abdomen is shaved, reduce the isoflurane to 1%–1.5%, still maintaining a flow rate of 1 L/min. Monitor the level of sedation by the level of respiration and any movements as well as paw pinch and/or corneal reflex.

NOTE: The heart rate of the anesthetized mouse will typically be 400–500/min, depending on the core (rectal) temperature. For the purpose of a rapid pregnancy check, do not warm the mouse to a physiological core temperature; the heart rate will be closer to 400–450/min, but may drop further with an irregular rhythm should the mouse become cold with prolonged imaging.

3.1.1. Importantly, make sure that the respirations are moderate in depth and regularity, not erratic or agonal (gasping: deep, slow, and erratic, with deep intercostal and subcostal retractions).

NOTE: The respiratory rate of the non-ventilated, anesthetized mouse will typically be 60–100/min. See https://ahcs.ninds.nih.gov/ACUC_pages/pg_003_anesth_animals.html and https://az.research.umich.edu/animalcare/guidelines/guidelines-anesthesia-and-analgesia-mice.

3.2. Apply ultrasound (acoustic coupling) gel to the abdomen generously.

3.3. Place the imaging transducer on the abdomen to orient it in a horizontal plane: orient the probe to obtain a left-right orientation on the imaging system; the “dot” or ridge indicated on the side of the imaging probe should be facing right.

NOTE: Sliding the imaging probe to the mouse’s right should shift the corresponding image on the ultrasound system to the mouse’s right (imagine looking “up” from the mouse’s tail—the mouse’s right will be left on the monitor).

3.3.1. Typically, use the imaging system’s transducer mount and rail-manipulator system. Here, “free hand” imaging has been used in which the imaging transducer is hand-held (two hands are steadier than one) and which allows for more rapid movements around the abdomen. These movements, as will be outlined below, include both rotational and translational movements. However, this requires more practice.

3.4. Identify the bladder on the screen (**Video 1**).

3.5. Scanning caudally from the bladder, identify the vagina. Then, scanning slowly and smoothly in a cranial direction, identify the bifurcation of the vagina into the left and right uterine horns (**Figure 2**; **Video 1**).

3.6. Begin the survey of the uterus (left and right uterine horns)[13] (**Figure 3**; **Video 2**).

NOTE: Up to mid-gestation (E10.5 or E11.5), the mouse embryos will be positioned along the right and left peripheries. As they grow, the more distal portions of the uterus and their corresponding embryos will turn outwards and posteriorly. As the embryos grow further (E15.5 and later, generally), the mouse fetuses will be positioned almost randomly in various directions, and it becomes difficult to "track" a uterus from proximal to distal.

3.6.1. Scan quickly simply to check if a mouse is pregnant or not. This rapid method requires only recognition of a gravid uterus and mouse embryos.

3.6.2. Alternatively, take extra time to enumerate the embryos (live, dead, resorbed) in each uterine horn.

NOTE: In general, a live embryo will exhibit distinct organs such as a heart, limbs, head with ventricles, and eyes. A dead embryo takes on a homogeneous, "mushy" appearance unless just dead. Resorbed embryos have a pinpoint echogenic spot in the middle of a gravid-looking uterus (**Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**).

3.6.3. To determine if an embryo is truly alive, look for the heartbeat and/or blood flow.

NOTE: Color Doppler flow mapping can aid in determining the presence of blood flow, both in the embryo and in the umbilical cord. In general, this may be applied to embryos older than E8.5.

3.6.4. Recognize potential artifacts that may mimic a gravid uterus (**Figure 9**, **Figure 10**). In addition, as bowel gas and other ultrasound "shadow" artifacts may obscure segments of the uterine horn, perform the imaging from multiple vantage points to ensure adequate visualization of the uterus.

3.7. After the survey is complete, wipe the gel from the abdomen with gauze or wipes. Try to remove as much as possible as the gel tends to cool the mouse down.

3.8. Untape the paws, and remove the mouse from the anesthetic nosecone.

3.9. Gently move the mouse back into her cage. She should wake up and start moving around within a minute or so.

NOTE: Genetically modified mice may take a little longer to recover from anesthesia and need to

be watched more closely.

REPRESENTATIVE RESULTS:

This protocol will allow an investigator to determine confidently whether a mouse is pregnant, including during the early stages and to determine whether there are obvious prenatal embryonic or fetal losses without needing to sacrifice the pregnant dam. This protocol is especially useful when breeding genetically engineered mice; typically, heterozygous x heterozygous crosses to yield homozygous offspring leads to failure of proper development, which causes prenatal lethality. **Figure 1** depicts a representative situation in which embryos progressively die and then are resorbed through mid-gestation. **Figure 2** shows how to find the left and right uterine horns by following the vagina up through its bifurcation. **Figure 3**, **Figure 4**, **Figure 5**, **Figure 6**, **Figure 7**, **Figure 8**, and **Video 3** show mouse embryos at various stages of development. Early-stage mouse embryos, dead embryos, or resorbed embryos may resemble other organs in the abdomen or feces in the intestines, or conversely, intestinal loops may mimic the non-gravid uterus. **Figure 9** and **Figure 10**, as well as **Video 4** and **Video 5**, demonstrate such potential imaging artifacts that may mimic the gravid uterus, for which the investigator must be on alert.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic diagram of a theoretical pregnant mouse abdomen, imaged at E11.5, then again at E14.5. Up to mid-gestation (E10.5 or E11.5), the mouse embryos will be positioned along the right and left peripheral aspects of the abdomen. As the embryos grow, the more distal portions of the uterus and their corresponding embryos will turn outwards and posteriorly. As the embryos grow further (E15.5 and later, generally), the mouse fetuses will be positioned almost randomly in various directions, and it becomes difficult to "track" a uterus from proximal to distal. When there is prenatal lethality in a genetically engineered mouse model, the embryos (open circles) may die; the dead embryos (hatched circles) will eventually become resorbed (solid circles).

Figure 2. Once one finds the vagina (**A**), immediately to the right of the bladder, sweeping cranially will demonstrate the bifurcation (**B**) to the right and left uterine horns (**D**)–(**F**). Scale bar (**A**) = 2 mm.

Figure 3. Images of non-gravid (non-pregnant) uterus (identified by the rows of arrows). The uterus may vary in thickness: thicker in (**A**), (**B**), (**E**); thin with a central thin echogenic line (**C)**, very thin (**D**), or may even contain small, cystic structures that should not be mistaken for concepti (**B**) and (**E**) especially, although this may be difficult to determine. (**A**) is a right uterine horn; this is more difficult to follow distally in our experience due to bowel gas. (**B**)–(**F**) are left uterine horns; (**F**) is quite distal/lateral and so becomes more difficult to image due to increasing bowel gas artifact. Scale bar (**A**) = 2 mm.

Figure 4. Resorbed and dead embryos have distinct appearances. Resorbed embryos, which are very commonly found, are encased within a round (gravid) uterine sac that appears relatively homogeneous except for a central echogenic (very bright) "spot"—arrows in (**A**) and (**B**). (**C**)

shows resorbed or dead embryos; there is an entirely homogeneous, "mushy" appearance to the uterus, and we see probably 3–4 dead embryos in this frame. (**D**) shows a recently dead embryo, which still shows some structures; there also appears to be cellular debris in the amniotic sac. In (**E**), the dead embryo is much shrunken and still connected to the placenta ("P"). (**F**) shows a homogeneous, "mushy" appearance of an embryo that probably died 1–2 days previously, but is not yet completely resorbed. Scale bar for (**A**) and (**D**) = 2 mm.

Figure 5. Early-stage embryos, from approximately E5.5 (**A**) and E6.5 (**B**) to E8.5 ((**C**) and (**D**)). There are variations in appearances, and the estimated stages here were based on timing of mating as well as the appearance of the embryos themselves. Scale bar (**A**) = 2 mm.

Figure 6. E9.5 embryos are considerably larger than E8.5 embryos and are beginning to take form. Representative images, showing adjacent embryos, are shown in (**A**) and (**B**). Scale bars = 2 mm.

Figure 7. E10.5 embryos exhibit even clearer organs such as the head, spine, and heart. Representative images, showing adjacent embryos, are shown in all panels; in (**D**), a dead/resorbing embryo lies adjacent to a live embryo. Scale bar = 2 mm.

Figure 8. Older embryos, approximately E12.5 (**A**), E14.5 (**B**), and E15.5 (**C**). Oblique planes of imaging obscure the precise anatomy somewhat, but the heart (arrow) is in the central portion of each embryo; in (**C**), the myocardium is now more echogenic than the blood.

Figure 9. The bowel, which is the organ most likely to be confused with the uterus. In (**E**), a resorbed embryo (arrowheads) overlies a segment of bowel (arrows). In (**F**), a non-gravid uterus (arrowheads) overlies a short segment of bowel (arrows)

Figure 10. Additional imaging artifacts in the gravid abdomen include the kidneys, spleen, and liver. (**A**) Right kidney; (**B**) Right kidney with renal artery (arrows); (**C**) Left kidney; (**D**) Left kidney with renal artery (arrows); (**E**) Spleen; (**F**) Liver overlying a segment of bowel; (**G**) Kidney overlying segment of bowel; (**H**) Spleen, liver, and left kidney seen in one plane of imaging. B = bowel; K= kidney; L= liver; S= spleen. Scale bar (**A**) = 2 mm.

DISCUSSION:

The most important first step in the imaging is to identify the vagina and then to determine the bifurcation of the uterine horn to the left and right. By following each uterine horn, the imager is less likely to mis-identify loops of the bowel as the uterus. Moreover, understanding the variations in the appearance of the bowel (with/without fecal matter) is important to distinguish these from the uterus; occasionally, fecal "balls" in bowel loops may mimic a gravid (pregnant) uterus. Although other authors have described the diagnosis of pregnancy and staging of mouse embryonic development[17-19] including the detection of resorbed embryos[20], this study is the first to outline the steps and potential pitfalls in imaging the gravid murine uterus.

The imager must recognize potential artifacts that may mimic an early pregnancy or gravid uterus or that may interfere with the imaging of the uterus and embryos. Following the uterine horns

laterally will reduce the likelihood of mistaking other organs and artifacts in the abdomen for the uterus (and small embryos). Potential artifacts that may be mistaken for uterus, embryos, and/or obstructive items include the bowel and bowel gas, feces, spleen, liver, and stomach.

This method requires general anesthesia, and we are careful to limit: 1) time of imaging and 2) frequency of pregnancy checks, to reduce any chance of intrauterine loss due to anesthesia. Although anesthetics and analgesics appear to be safe overall during pregnancy[21], significant exposure may have consequences on mouse embryonic growth[22]. As mouse knockout models often demonstrate prenatal or early perinatal death, exposure of the embryos to general anesthesia during this imaging may (at least theoretically) increase their risk of demise or influence their biology in unknown ways. While an absolute time limit is unknown, we try to limit each imaging session to no more than 15 minutes, and to 2–3 imaging sessions (maximum) per pregnancy. The "ALARA principle" is prudent here: As Low As Reasonably Achievable.

This method allows for more efficient breeding as well as rapid determination of intrauterine demise. This is especially important in experiments using knockout models that die early; other examples include toxicological studies. While a few studies have detailed the weight gain during pregnancy, it is quite clear that the weight gain early (prior to E8.5) is small and may not be different from diurnal weight changes. Furthermore, the data were derived from first-time pregnant mice only and may not reflect the confounding effects of multi-gravid mice[10,11]. Timed pregnancies may not be evident early on, and especially with genetically-engineered mice, intrauterine losses may be common or even affect the entire litter. Thus, simply because a mouse does not deliver a litter does not mean she was never pregnant. Mice can be re-mated in a week if the female is not pregnant; otherwise, researchers will simply have to wait it out to see if the female has become pregnant. After skills are developed to do more than simply check for pregnancy, this method will also allow mapping and monitoring the embryos as the pregnancy progresses. In this way, the optimal timing for embryo harvest can be determined if tissues must be harvested prior to demise[23].

ACKNOWLEDGMENTS:
None.

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Bogue M. A. et al. Mouse Phenome Database: an integrative database and analysis suite for curated empirical phenotype data from laboratory mice. *Nucleic Acids Research*. **46** (D1), D843–D850 (2018).
2. Ito, R., Takahashi, T., Ito, M. Humanized mouse models: Application to human diseases. *Journal of Cellular Physiology*. **233** (5), 3723–3728 (2018).
3. Law, M., Shaw, D. R. Mouse Genome Informatics (MGI) is the international resource for information on the laboratory mouse. *Methods in Molecular Biology*. **1757**, 141–161 (2018).
4. Rydell-Törmänen, K., Johnson, J. R. The applicability of mouse models to the study of

353 human disease. *Methods in Molecular Biology*. **1940**, 3–22 (2019).
354 5. Hinton, R. B., Yutzey, K. E. Heart valve structure and function in development and disease.
355 *Annual Reviews of Physiology*. **73**, 29–46 (2011).
356 6. Dickinson, M. E. et al. High-throughput discovery of novel developmental phenotypes.
357 *Nature*. **537** (7621), 508–514 (2016).
358 7. Tam, P. P. L. et al. Formation of the embryonic head in the mouse: attributes of a gene
359 regulatory network. *Current Topics in Developmental Biology*. **117**, 497–521 (2016).
360 8. Palis J. Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid,
361 megakaryocyte, and myeloid potential in the mammalian embryo. *FEBS Letters*. **590** (22), 3965–
362 3974 (2016).
363 9. Behringer, R., Gertsenstein, M., Nagy, K. V., Nagy, A. Selecting female mice in estrus and
364 checking plugs. *Cold Spring Harbor Protocols*. **2016** (8), doi:10.1101/pdb.prot092387 (2016).
365 10. Heyne, G. W. et al. A simple and reliable method for early pregnancy detection in inbred
366 mice. *Journal of the American Association for Laboratory Animal Science*. **54** (4), 368–371 (2015).
367 11. Finlay, J. B, Liu, X., Ermel, R. W., Adamson, T. W. Maternal weight gain as a predictor of
368 litter size in Swiss Webster, C57BL/6J, and BALB/cJ mice. *Journal of the American Association for*
369 *Laboratory Animal Science*. **54** (6), 694–699 (2015).
370 **12.** Zhou, Y. Q. et al. Applications for multifrequency ultrasound biomicroscopy in mice from
371 implantation to adulthood. *Physiological Genomics*. **10** (2), 113–126 (2002).
372 13. Ji, R. P., Phoon, C. K. L. Noninvasive localization of nuclear factor of activated T cells c1-/-
373 mouse embryos by ultrasound biomicroscopy-Doppler allows genotype-phenotype correlation.
374 *Journal of the American Society of Echocardiography.* **18** (12), 1415–1421 (2005).
375 14. Kulandavelu, S. et al. Embryonic and neonatal phenotyping of genetically engineered
376 mice. *ILAR Journal*. **47** (2), 103–117 (2006).
377 15. Mu, J., Slevin, J. C., Qu, D., McCormick, S., Adamson, S. L. *In vivo* quantification of
378 embryonic and placental growth during gestation in mice using micro-ultrasound. *Reproductive*
379 *Biology and Endocrinology*. **6**, 34 (2008).
380 16. Phoon, C. K. L., Turnbull, D. H. Cardiovascular imaging in mice. *Current Protocols in Mouse*
381 *Biology.* **6** (1), 15–38 (2016).
382 17. Phoon, C. K. L., Turnbull, D. H. Ultrasound biomicroscopy-Doppler in mouse cardiovascular
383 development. *Physiological Genomics.* **14** (1), 3–15 (2003).
384 18. Peavey, M. C. et al. A novel use of three-dimensional high-frequency ultrasonography for
385 early pregnancy characterization in the mouse. *Journal of Visualized Experiments.* **128**, 56207
386 (2017).
387 19. Greco A. et al. High frequency ultrasound for in vivo pregnancy diagnosis and staging of
388 placental and fetal development in mice. *PLoS One*. **8** (10), e77205 (2013).
389 20. Flores, L. E., Hildebrandt, T. B., Kühl, A. A., Drews, B. Early detection and staging of
390 spontaneous embryo resorption by ultrasound biomicroscopy in murine pregnancy.
391 *Reproductive Biology and Endocrinology*. **12**, 38 (2014).
392 21. Norton, W. B. et al. Refinements for embryo implantation surgery in the mouse:
393 comparison of injectable and inhalant anesthesias - tribromoethanol, ketamine and isoflurane -
394 on pregnancy and pup survival. *Laboratory Animal*. **50** (5), 335–343 (2016).
395 22. Thaete, L. G., Levin, S. I., Dudley, A. T. Impact of anaesthetics and analgesics on fetal
396 growth in the mouse. *Laboratory Animal*. **47** (3), 175–183 (2013).

23. Phoon, C. K. L. et al. Tafazzin knockdown in mice leads to a developmental cardiomyopathy with early diastolic dysfunction preceding myocardial noncompaction. *Journal of the American Heart Association*. **1** (2), jah3-e000455 (2012).

Figure 1

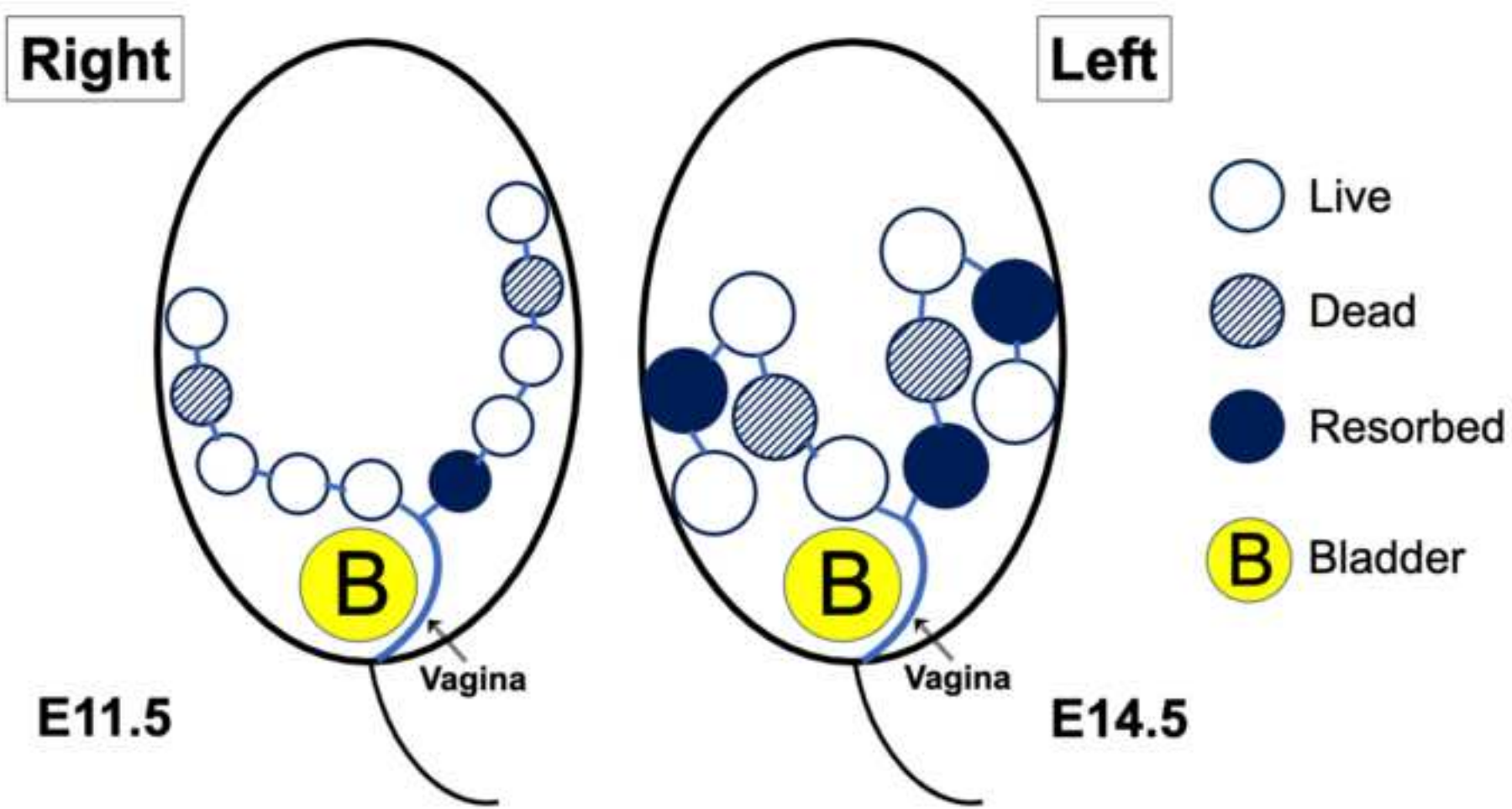


Figure 1


Click here to access/download
Video or Animated Figure
FIGURE 1-schematic.svg

Figure 2


Click here to access/download
Video or Animated Figure
FIGURE 2.svg

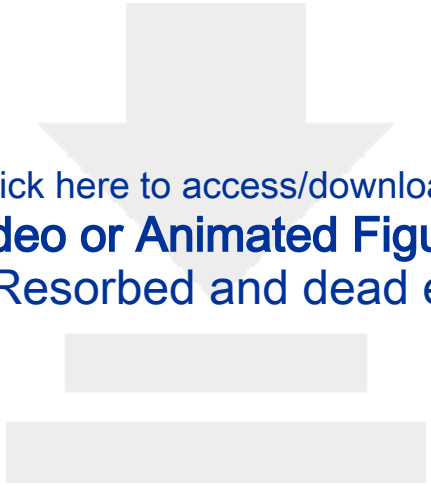
Figure 3

Click here to access/download

Video or Animated Figure

FIGURE 3- Non-gravid uterus.svg

Figure 4

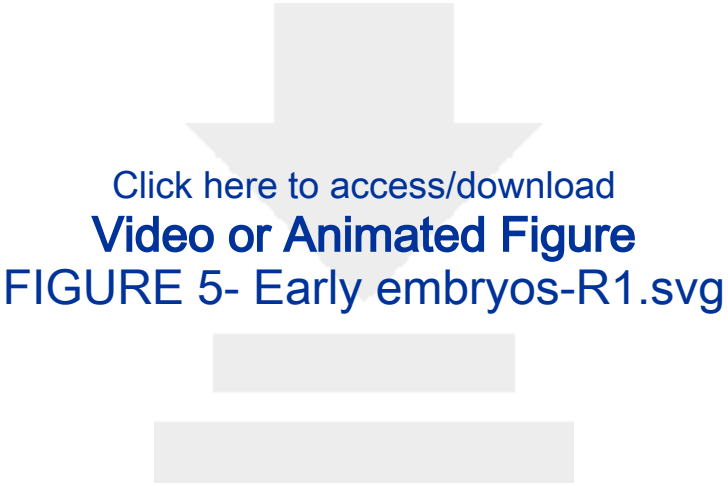


Click here to access/download

Video or Animated Figure

FIGURE 4- Resorbed and dead embryos.svg

Figure 5

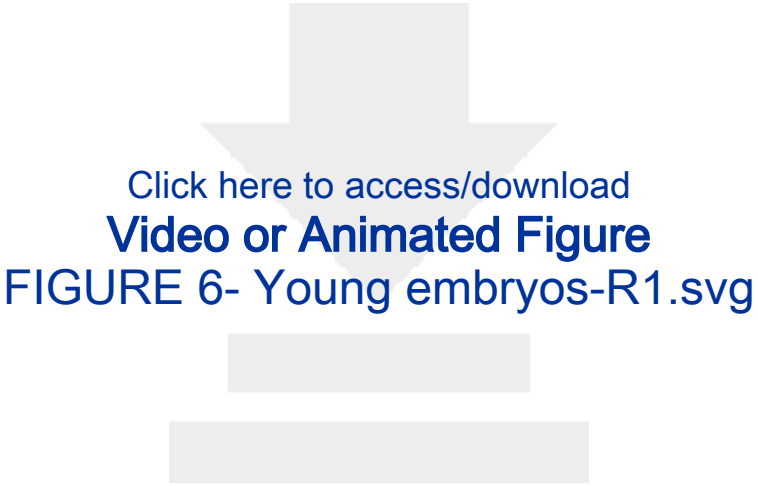


Click here to access/download

Video or Animated Figure

FIGURE 5- Early embryos-R1.svg

Figure 6

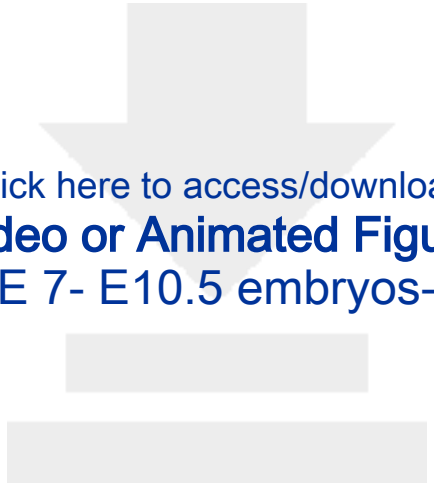


Click here to access/download

Video or Animated Figure

FIGURE 6- Young embryos-R1.svg

Figure 7



Click here to access/download

Video or Animated Figure

FIGURE 7- E10.5 embryos-R1.svg

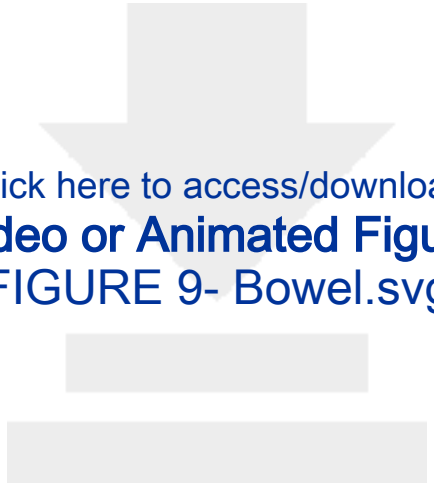
Figure 8

Click here to access/download

Video or Animated Figure

FIGURE 8- Older embryos-R1.svg

Figure 9

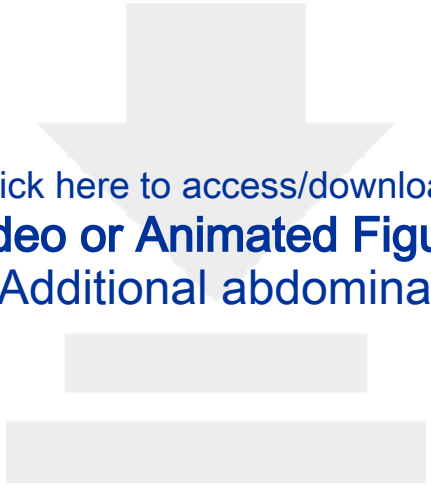


Click here to access/download

Video or Animated Figure

FIGURE 9- Bowel.svg

Figure 10



Click here to access/download

Video or Animated Figure

FIGURE 10- Additional abdominal artifacts.svg

VIDEO 1

Click here to access/download

Video or Animated Figure

VIDEO 1a-VAGINA SWEEP UTERUS BIFURC_2020-02-25-11-48-27-484.avi

VIDEO 3

Click here to access/download

Video or Animated Figure

VIDEO 3-EMBRYO E9.5_2019-06-20-10-57-38-328.avi

VIDEO 4

Click here to access/download

Video or Animated Figure

VIDEO 4-RT BOWEL_2019-06-27-10-22-38-937A.avi

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Depilatory cream			
Ethanol, 70%			
Fur clippers			
Gauze or KimWipes			
Isoflurane			
Medical oxygen (optional)			
Medical tape			
Mouse imaging system (including anesthesia set-up and imaging platform)	Fujifilm Visual Sonics	Various	Any system with 40 MHz center-frequency ultrasound transducer pro
Razor blade (not a safety razor)			
Scale (to weigh mouse)			
Ultrasound gel			

be

September 29, 2020

RE: JoVE61893
"Is My Mouse Pregnant? High-Frequency Ultrasound Assessment: Utility and Pitfalls"

Dear Editors and Reviewers,

Thank you for your thorough review of our manuscript, "Is My Mouse Pregnant? High-Frequency Ultrasound Assessment: Utility and Pitfalls". We acknowledge the significant improvements as a result of this thoughtful feedback. Please find below our detailed responses to the comments.

Sincerely yours,

Colin Phoon, MPhil, MD
Associate Professor
Pediatrics and Pediatric Cardiology

Mindong Ren, PhD
Research Associate Professor
Anesthesiology and Cell Biology

Editorial comments:

Changes to be made by the Author(s):

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.
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 response: I believe we have adhered to the guidelines. The only item I was not sure about was adding an *additional* line space between *substeps* but I did make sure that each substep was started on a new line.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

Author response: We have used this recommended wording "Presented here is a protocol…" and also specified that we will illustrate imaging pitfalls that may mimic pregnancy, which we had not previously included in the Summary. The word count is 42.

4. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

Author response: Word count is 152, and we clearly state the goals of the ultrasound imaging protocol.

5. Please ensure the Introduction include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Author response: We believe all elements above are addressed. In addition, we have responded to the Reviewers' comments relevant to the above items.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Author response: This statement has been added.

7. Please include the strain of mice used for the study.

Author response: In this protocol, the strain is not relevant, and depends on the mouse model or experimental mouse that the investigator's laboratory is using.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Author response: We believe the protocol is written in this manner, except in very occasional circumstances when the protocol allows for alternatives. The phrases "could be," "should be," and "would be" are avoided.

9. The Protocol should contain only action items that direct the reader to do something.

Author response: The protocol is written in this manner.

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

Author response: We believe each step and substep answers the "how" question.

11. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Author response: We have taken care to keep each step continues this limited number of action sentences.

12. Please move the potential artefact part to the discussion section in paragraph style.

Author response: We have done this, and added substep 3.6.4 to alert the reader to watch for potential artifacts.

13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 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.

Author response: To be honest, we are not sure about this step, mostly because we would like to work with the Journal on the video portion of this manuscript. We are still in discussion with our Animal Facility staff about creating a brief video of imaging the anesthetized mouse. We have included now videos of ultrasound imaging, the relevant parts of which in the Protocol are highlighted. Highlighted text is in yellow.

14. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Author response: We have now included a Representative Results paragraph that refers to all the figures.

15. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

Author response: We have removed the embedded figures from the manuscript.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Author response: No Figures were reused from previous publications; all are new Figures acquired specifically for this manuscript.

17. 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 response: We believe we cover these items in the Discussion.

18. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the table in alphabetical order.

Author response: When we submitted this manuscript, we were explicitly advised by the editorial team we did not need to provide the name, company, and catalog numbers of most of the relevant materials, since these are generic items. We feel that providing specific product names (e.g., “Nair” for the depilatory cream, as suggested by one reviewer) would give the impression of favoring certain company’s products over others, which is not the case. We feel strongly that there is no valid reason to provide specific company product information for most of the materials. The only reason we have specified Visual Sonics for the imaging platform company is that this is almost the only company that supplies such equipment; even here, they market several different platforms, with different catalog numbers, and their platforms are subject to upgrades and advances with new catalog numbers. Thus, we have not included any catalog numbers for the Visual Sonics imaging equipment.

The Table has been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors present a protocol for determining, during early gestation, if a mouse is pregnant using high frequency ultrasound. This is a very important question and has potential significance for a wide range of basic research scientists that use timed pregnancies. The ability to identify if a mouse is pregnant (and the number of fetuses) will improve efficiency of experiments and will decrease unnecessary euthanasia. The detailed description of what to look for during screening is both accurate and useful. I also liked the practical examples of things you might mistake for the uterus.

Major Concerns:
I have no major concerns.

Minor Concerns:
- From experience, weight gain alone is not a great metric of positive pregnancy. A mouse might only have 1-2 fetuses and not appear pregnant. On the other hand, a mouse might appear to have gained weight but have a benign tumour. In both cases, knowing the pregnancy status would be very useful. Could you address this in the motivation of the protocol?

Author response: We have incorporated this additional motivation in the Introduction. Moreover, we have now cited a couple of articles on weight gain during pregnancy. We have discussed the disadvantages of gauging pregnancy by weight alone also in the Discussion.

Protocol:
- 2.8.4 - please identify Nair as a depilatory cream that is typically used

Author response: There are a number of depilatory creams available, and Nair is but one of many. We would prefer not to specify a single brand, which might be perceived as an endorsement or some sort of favoritism. An Amazon.com search turned up 852 results, with the following brands: Veet, Nair, Neomen, Segminismart, Blitzby, Tomiya, Toulifly, Cidbest, Scobuty, and Surgi-Cream. We would appreciate any advice or suggestions from the editorial staff and reviewers if they still would like us to name a brand.

- do you use eye salve to prevent drying of the eyes?

Author response: Thank you for pointing out our omission. We have added this in as Protocol item 2.6.1.

- 3.1 -what range of heart rate and resp rate would you expect for that isoflurane level?

Author response: We have added in an additional comment about heart rate and respiratory rate ranges, as well as respiratory effort.

- 3.6.3 - specify colour Doppler for how to look at blood flow

Author response: We thank the Reviewer for this suggestion, which has been added as 3.6.3.1.

Figures:
- please add the gestational ages to Figure 5 and Figure 8

Author response: This has been done.

- for Figure 6 and 7, can you add labels to identify the structures (ie. head, spine, heart)

Author response: We thank the Reviewer for this suggestion. At these stages and unless the embryo is in a standard plane of imaging (e.g., sagittal plane), it may be difficult to “see” the various landmarks on the still images. The younger embryos (E9.5) are even more difficult. We also felt the labels and arrows might further obscure the landmarks. We therefore labeled Figure 7 but left Figure 6 unlabeled; Figure 6 also does not clearly show these organs well at all (and perhaps, only the head of one embryo). We also took an earlier suggestion to label with the gestational stages, which are now included in Panel A of each Figure.

Discussion:
- second paragraph: can you recommend the maximum length of time you think the dams should be under anesthesia?

Author response: We appreciate the Reviewer’s asking for recommendations! While there are no data, we have provided a recommendation in the Discussion.

Reviewer #2:

Manuscript Summary:
This manuscript outlines a protocol for assessing murine pregnancy efficacy using ultrasound assessment. The protocol explains how to mate, anesthetise and image a (presumed) pregnant mouse. Representative images are presented to indicate how to identify the uterine horns and both viable and non-viable pups. There are also images included that demonstrate what can often be misinterpreted as concepti.

Major Concerns:
No major concerns

Minor Concerns:
This protocol is written in a way that could easily be followed by others. The inclusion of the figures to show what investigators should expect to see is good. However, identifying true concepti will need practise for any individual who wants to use this protocol. Although images showing the uterine horn, concepti and organs that are mistaken for concepti or the uterus, it would have been advantageous to make reference to those figures in the protocol as well as in the discussion where reference is made to the importance of discriminating between the bowel and fecal matter with a gravid uterus.

Author response: We thank the Reviewer for suggesting references in the text to the Figures, and have incorporated these. We also refer to the Figures in the new Representative Results section.

Quite rightly the authors have stated that this method is the quickest way to establish successful pregnancies and also enables determination of fetal demise without the need to end the pregnancy. In addition, it enables quicker turn arounds to re-mate mice who are not pregnant. The current method to establish "non-pregnant" in a cohort assumed to be pregnant is to assess weight gain. From 7.5 days gestation weight gain will identify pregnancy with a false positive of up to 10%. Obviously this method gives no indication of fetal demise, but it is a simple and inexpensive means of determining whether a mouse is pregnant or not. Overall, those researchers who have access to an ultrasound machine and gain the experience to use it and interpret the images will be able to not only determine pregnancy success, but will also be able to time fetal demise.

Author response: We have added additional references to and discussion about, weight gain during pregnancy.

Reviewer #3:
Manuscript Summary:
The manuscript describes the use of ultrasound to image live , dead and resorted embryos. It provides a useful tool for minimizing animals in reproductive experiments.

Major Concerns:
This technique has already been published in JoVe Peavey et al JOVE doi: 10.3791/56207 and it is not clear this manuscript adds significantly new information. The manuscript does not show how to stage the embryos . Most figures do not show the stage of pregnancy or show any new quantifiable approaches.

Author response: We appreciate the Reviewer's bringing to our attention the article by Peavey et al. While this is similar to our manuscript, Peavey's protocol focuses on imaging early-stage embryos in known pregnancies. What our manuscript adds is advice on how to recognize artifacts, and therefore, how to specifically tell if a mouse is pregnant at all. The purpose of this article is not to show stages of pregnancy or to guide quantification or staging approaches. Nevertheless, in response to this Reviewer's comment and another above, we have added additional stage data to our Figures.

Minor Concerns:
Most of the figures do not show the stage of pregnancy that is being imaged.

Author response: Please see our response above.