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Surgical preparation of rats and mice for intravital microscopic imaging of abdominal organs

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A B S T R A C T

Intravital microscopy is a powerful research tool that can provide insight into cellular and subcellular events that take place in organs in the body. However, meaningful results can only be obtained from animals whose physiology is preserved during the process of microscopy. Here I discuss the importance of preserving the overall state of health of the animal, methods of anesthesia, surgical techniques for intravital microscopy of various abdominal organs, methods to maintain and monitor the physiology of the animal during microscopy and associated peri- and post-operative recovery considerations.

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1. Introduction

Intravital microscopy has afforded researchers the ability to better understand cellular and subcellular processes within multiple organs in the body [1–4]. The utilization of the rat and mouse models can be advantageous in that organ structure and function in these species mimics humans. Therefore, models of disease specific to these organs may be studied in the rat and mouse, potential treatment modalities may be tested thereafter, and, the application of such treatment modalities may potentially be transferred to humans [2,4–7]. However, meaningful results can only be obtained from animals whose physiology is preserved during microscopy. Here, I describe methods of anesthesia, surgical techniques, and methods to maintain and monitor animal physiology that we have developed for intravital microscopy of the kidney, liver, spleen and pancreas of rats and mice.

The methods described here were optimized to provide access to abdominal organs such that the tissues are sufficiently immobilized to support high-resolution imaging, while preserving the animal and organ physiology. These methods were designed in accordance with guidelines provided by Indiana University's Institutional Animal Care and Use Committee (IACUC). Prior to any animal studies, appropriate institutional approval must be obtained. In the United States, each research institution must have an IACUC that reviews proposals for research, teaching or testing activities involving vertebrate animals, and approval is required prior to initiating any such activities. IACUCs follow federal regulations as they pertain to the Animal Welfare Act, National Research Council's Guide for the Care and Use of Laboratory Animals, and the Public Health Services Policy on Humane Care and Use of Laboratory Animals.

Intravital microscopy studies may consist of a single imaging session or of a sequence of multiple imaging sessions, as for longitudinal studies in which an animal is repeatedly imaged over periods of days or weeks. Since the animal is euthanized at the end of a single-session imaging study, the surgery is considered a non-survival surgery. Studies involving multiple imaging sessions involve survival surgeries, which incur additional considerations for anesthesia, surgical preparation, and pain management, as described below.

2. Anesthesia and pain management

There are several key factors to consider when choosing an anesthetic agent and pain management during intravital imaging: 1) physiologic state of the animal, 2) whether the session is non-survival or survival, 3) rat versus mouse, 4) personal preference of the investigator that is either performing or supervising the procedure, and 5) length of the procedure/duration of action of the agent. The overall goal is to achieve a surgical plane of anesthesia prior to manipulating the animal. A surgical plane of anesthesia is described as a state of medically-induced unconsciousness in

which the animal does not produce protective reflexes to stimuli [8]. In general, we tend to utilize Isoflurane as our number one agent of choice because it is easy to titrate to effect, which is critical in physiologically unstable animals, and emergence and recovery from the surgical plane of anesthesia is relatively rapid compared to injectable agents that are available (Figs. 1 and 2). The physiologic state of the animal refers to the temperature, blood pressure, heart rate and respiratory rate. Regardless of whether a rat or mouse model is used, it is important to inspect the animal's physiologic state regularly throughout the course of the imaging session. Observation of respiratory rate, color of the mucosa and response to stimulation (ear pinch) are appropriate for determining proper depth of anesthesia and are indirect indicators of blood pressure and heart rate. However, monitoring of blood pressure and heart rate with an intra-arterial catheter or tail vein blood pressure cuff transducer system, and rectal thermometer, to measure core temperature, afford the investigator a more accurate determination of the physiologic state of the animal [1,5,9].

2.1. Single imaging session studies: non-survival surgeries

Intravital imaging using non-survival surgery requires that the animal will be euthanized once the imaging session is complete. The animal, therefore, does not need to emerge from anesthesia. The vital characteristic of any anesthetic modality in the non-survival setting, therefore, is that it must induce a state of unconsciousness in the animal in which painful stimuli are not sensed by the animal. It is in this surgical plane of anesthesia that the animal must be maintained prior to skin incision and even at the time of euthanasia. Analgesia in the non-survival setting is, therefore, not imperative if the surgical plane of anesthesia is maintained throughout.

2.1.1. Rat anesthesia in non-survival studies

In non-survival studies in the rat, agent selection is influenced most heavily by the individual investigator's personal preference and underlying physiologic state of the animal (Table 1). The following agents are those most commonly used in our facility. The advantages and limitations of each are discussed.

Thiobutabarbital (Inactin), administered intraperitoneally, at a dose of 100–160 mg/kg, is an agent that we utilize in the male rat in only non-survival settings given its relatively long duration of action [9–11]. It is a barbiturate that is described as short-acting, however the male rat remains in a surgical plane for more than 4–6 h. Therefore, redosing is not required for non-survival studies of that length. It's especially useful in cases in which the underlying physiology of the rat has not been compromised.

Female rats present a unique problem from an anesthetic standpoint in our experience, because they do not consistently respond to Thiobutabarbital. Previously, sodium pentobarbital has been used in females [9], however, given its lack of availability, it is more likely that Isoflurane or ketamine cocktail will now be utilized.

Sodium Pentobarbital is a popular injectable agent for imaging procedures, in physiologically stable rats, that are less than 60 min long [12,13]. It is a shorter acting barbiturate, injected intraperitoneally at 30–60 mg/kg, that usually requires re-dosing every 45–60 min. However, pentobarbital in female rats has been used in non-survival sessions of long-duration because it has a significantly longer duration of action compared to males [12]. Unfortunately, it's availability in the US is rather limited currently and for that reason it may not be a viable option.

Isoflurane is an inhalational agent that is most valuable in rats that have compromised physiologic states due to disease models employed [14–17]. Use of Isoflurane does require a closed anesthesia circuit with a pressurized vaporizer, metered oxygen flow, and utilizing a gas waste scavenging system. It can be more cumbersome to manage the circuit on the microscope stage. However, it is quite easy to adjust the level of agent administered, and therefore, appropriately titrate the depth of anesthesia in those animals who would be susceptible to overdose with a standard injectable agent.

Ketamine cocktails, are also viable injectable options for non-survival imaging in the rat. Ketamine and xylazine mixture,
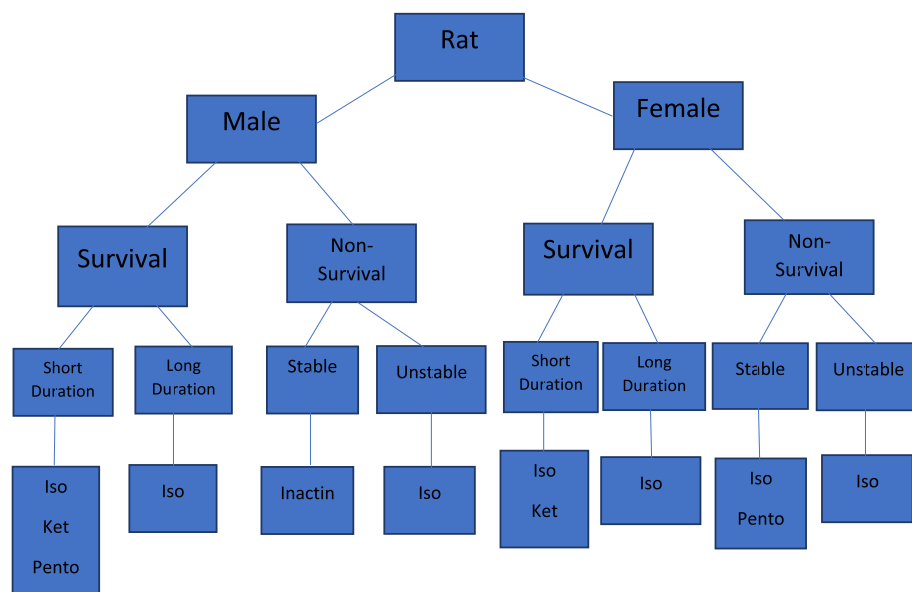
Fig. 1. Flow diagram for selection of anesthetic agent in rats. Isoflurane is generally the agent of choice because it is easy to titrate to effect for cases of long or short duration, or in rats that are physiologically unstable. In addition, emergence and recovery from Isoflurane are rapid in comparison to injectable agents. Inactin is ideal in non-survival cases in male rats that are physiologically stable because it has a long duration of action that eliminates the need for redosing. Iso = Isoflurane 1–2% vapor with a flow of Oxygen at 1 L/min. Ket = Ketamine cocktail 60–100 mg/kg Intraperitoneal injection (typically with Xylazine 5–10 mg/kg +/- Acepromazine 2.5 mg/kg). Pento = Sodium Pentobarbital 30–60 mg/kg Intraperitoneal injection. In survival cases Isoflurane is combined with Buprenorphine HCl subcutaneously at a dose of 0.01–0.05 mg/kg for analgesic purposes.
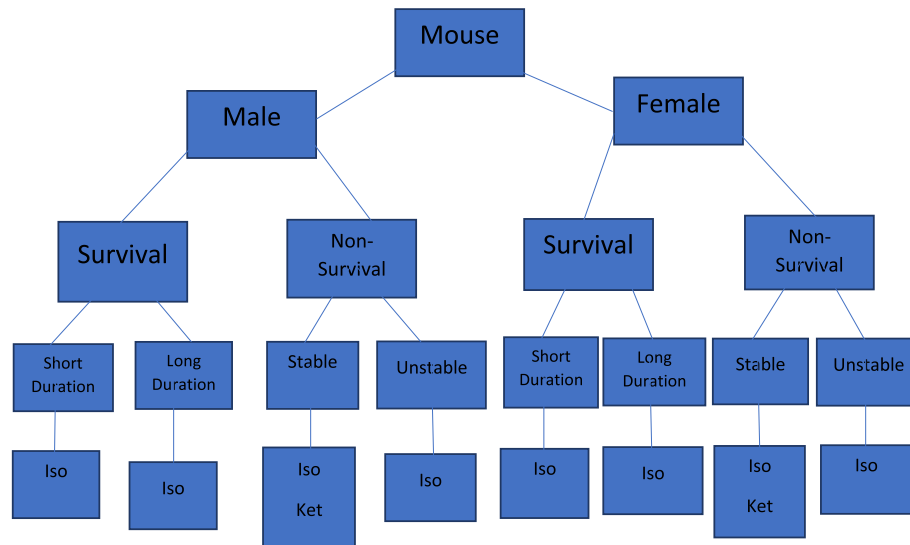
Fig. 2. Flow diagram for selection of anesthetic agent in mice. Isoflurane is generally the agent of choice because it is easy to titrate to effect for cases of long or short duration, and emergence and recovery from Isoflurane are rapid in comparison to injectable agents. Iso = Isoflurane 1–2% vapor with a flow of Oxygen at 1 L/min. Ket = Ketamine cocktail 90–100 mg/kg intraperitoneal injection (xylazine 2.5–5.0 mg/kg +/- acepromazine 1.0–2.5 mg/kg). In survival cases Isoflurane is combined with Buprenorphine HCl subcutaneously at a dose of 0.05–2.0 mg/kg for analgesic purposes.

Table 1
Anesthetic agent options for rats and mice in non-survival procedures performed within our research group. The agents have similar profiles, in terms of maintaining surgical plane of anesthesia. In general, the choice of agent heavily depends on individual preference and availability of the agent. Isoflurane is predominantly the first choice because it is easy to titrate to effect, especially in animals that are physiologically unstable. Inactin is a viable choice in stable male rats because its long duration of action requires only a one-time intraperitoneal injection, and no rodent anesthesia circuit is required to maintain a surgical plane of anesthesia.

	Species	Dose and Route of Administration	Pros	Cons
Isoflurane	Rat and Mouse	Inhalational administration 1–2% vapor with 1 L/min flow of O_2	Easy to titrate volume of delivery in physiologically compromised animals; Redosing not required. Effective in procedures in male and female rats and mice	Associated equipment is expensive, and can be cumbersome when dealing with microscope stage
Ketamine cocktail	Rat and mouse	Rat: Intra-peritoneal injection 60–100 mg/kg ketamine, 5–10 mg/kg, 2.5 mg/kg acepromazine. Mouse: Intraperitoneal injection 90–100 mg/kg ketamine, 2.5–5 mg/kg xylazine, 1–2.5 mg/kg acepromazine	Effective in procedures of short duration (less than 60 min) in both male and female rats and mice. No cumbersome equipment that interferes with the set up on the microscope stage,	Redosing required in procedures that last longer than 60 min. More of a risk of overdose in animals with compromised physiology
Sodium Pentobarbitol	Rat and Mouse	Rat: Intraperitoneal injection 30–60 mg/kg. Mouse: Intraperitoneal injection 50–90 mg/kg	Effective in procedures of short duration (less than 60 min) in both male and female rats and mice. No cumbersome equipment that interferes with the set up on the microscope stage	Not readily available in the US. Redosing required in procedures that last longer than 60 min. More of a risk of overdose in animals with compromised physiology
Thiobutabarbital	Male rat	Intraperitoneal injection 100–160 mg/kg	Effective in procedures of long duration (4–6 h). No need for redosing. No cumbersome equipment that interferes with the set up on the microscope stage	More of a risk of overdose in animals with compromised physiology. Not effective in mice and female rats

administered intraperitoneally at 60–100 mg/kg and 5–10 mg/kg, provides adequate anesthesia for approximately 45–60 min before redosing is required. Some also add acepromazine at a dose of 2.5 mg/kg to enhance sedation in the animal.

2.1.2. Mouse anesthesia in non-survival studies

Mice do not respond consistently to thiobutabarbital, so we do not consider it as an option in such cases. A higher preference exists for Isoflurane [15,18,19] and Ketamine cocktails [20–22] in non-survival sessions in mice (see Table 1).

2.2. Multiple imaging session studies: survival surgeries

In contrast to single imaging session studies, studies of animals conducted over multiple survival procedures involve anesthetics, whose effects are temporary, and analgesia to be provided to animals during recovery from the surgical/imaging procedures.

2.2.1. Rat anesthesia and analgesia in survival studies

Anesthetic agents in the setting of survival procedures are listed below (Table 2). While Isoflurane creates a state of unconsciousness

Table 2

Anesthetic agent options for rats and mice in survival procedures performed within our research group. The agents have similar profiles, in terms of maintaining surgical plane of anesthesia. In general, the choice of agent heavily depends on individual preference and availability. Isoflurane is predominantly the first choice because it is easy to titrate to effect and emergence and recovery from the surgical plane of anesthesia is relatively rapid compared to injectable agents.

	Species	Dose and Route of Administration	Pros	Cons
Isoflurane + Buprenorphine HCl	Rat and Mouse	Inhalational administration 1–2% vapor with 1 L/min flow of O_2 Buprenorphine Rat: subcutaneous 0.01–0.05 mg/kg Mouse: subcutaneous 0.05–2 mg/kg	Easy to titrate volume of delivery in physiologically compromised animals; Redosing not required Effective in procedures in male and female rats and mice	Associated equipment is expensive, and can be cumbersome when dealing with microscope stage
Ketamine cocktail	Rat and mouse	Rat: Intraperitoneal injection 60–100 mg/kg ketamine, 5–10 mg/kg, 2.5 mg/kg acepromazine Mouse: Intraperitoneal injection 90–100 mg/kg ketamine, 2.5–5 mg/kg xylazine, 1–2.5 mg/kg acepromazine	Effective in procedures of short duration (less than 60 min) in both male and female rats and mice No cumbersome equipment that interferes with the set up on the microscope stage,	Redosing required in procedures that last longer than 60 min More of a risk of overdose in animals with compromised physiology
Sodium Pentobarbitol	Rat and Mouse	Rat: Intraperitoneal injection 30–60 mg/kg Mouse: Intraperitoneal injection 50–90 mg/kg	Effective in procedures of short duration (less than 60 min) in both male and female rats and mice No cumbersome equipment that interferes with the set up on the microscope stage	Not readily available in the US Redosing required in procedures that last longer than 60 min More of a risk of overdose in animals with compromised physiology

to stimuli, it has no analgesic properties. We, therefore, administer Buprenorphine HCl subcutaneously 0.01–0.05 mg/kg prior to skin incision. This provides adequate analgesia once they emerge from general anesthesia. Ketamine cocktails and sodium pentobarbital are also viable options if Isoflurane cannot be used. They have the advantage of analgesic properties, and therefore do not require administration of Buprenorphine HCl prior to skin incision. Again, one drawback to these injectables is a short duration of action requiring multiple doses for sessions that extend beyond 45–60 min. However, what appears to be the critical selection factor is the preference of the individual investigator. For example, three investigators, each performing survival procedures in which similar parameters of an ischemia-reperfusion model were employed to induce AKI in rats, utilized different modalities of survival anesthesia. Collett utilized a ketamine-xylazine cocktail [15], McCurley utilized Isoflurane [23], and Wang utilized sodium pentobarbital [5].

2.2.2. Mouse anesthesia and analgesia in survival studies

Unlike the rat, Isoflurane + Buprenorphine HCl is the preferred choice of some investigators conducting survival procedures in mice [24–28]. This combination is preferred in survival cases because it is easy to titrate to effect and the rapid emergence and recovery from the Isoflurane tends to result in more consistent post-operative course, compared to injectable agents, in the mice. The dose of Buprenorphine HCl in the mouse is 0.05–2 mg/kg subcutaneously (see Table 2).

2.2.3. Post-operative pain management

For both the rat and mouse models, the standard of care in the post-operative period involves the scheduled administration of Buprenorphine HCl for the 1st 48 h following the survival procedure: every 8–12 h in rats, and every 6–12 h in mice. If after 48 h the animals exhibit moderate-severe signs of distress, additional Buprenorphine is provided. The Indiana University School of Medicine IACUC mandates this standard of care. If the animal fails to thrive, then that animal may not tolerate anesthesia during the subsequent imaging sessions. It is, therefore, imperative to maintain daily observations to ensure that the animal's condition is optimal for the next imaging session. We monitor daily weight and skin turgor and observe for signs of distress including greater than 20% weight loss from pre-op measurements, lack of grooming of the coat, decreased activity, porphyrin staining about the eyes, nose and mouth, vocalization, and lack of socialization. In those

animals that fail to thrive, euthanasia should be considered prior to the imaging endpoint.

Non-steroidal anti-inflammatory drugs (NSAIDs) such as Carprofen, 5–10 mg/kg administered subcutaneously in the mouse or rat q 24 h, Ketoprofen, 5–20 mg/kg administered subcutaneously in the rat q 24 h, and Meloxicam 1.0–10 mg/kg in the mouse and 1.0–3.0 mg/kg in the rat, administered subcutaneously q 24 h are also commonly used analgesic agents. Their long-duration of action affords lab personnel a less labor-intensive post-operative period, and the animals respond very well. NSAIDS may be used in combination with opiates such as Buprenorphine. However, given the adverse effects that NSAIDs have on renal blood flow, we do not employ them in our studies of the kidney.

3. Surgical presentation of abdominal organs

All surgical and anesthetic instruments/equipment and reagents recommended for successful surgical presentation and imaging of abdominal organs may be reviewed in Table 3. The overall goal of the surgical procedures is to provide optical access to a stabilized organ while minimizing perturbation of the organ's function and preserving the normal physiological function of the animal. The organ of interest determines the type, and the location of incision as well as the way the animal is presented on the microscope stage. This will also influence whether it is necessary to use an inverted microscope where the imaging objective approaches the organ from underneath the animal, or alternatively an upright microscope, where the imaging objective approaches the organ from above the animal. This may also influence the type of anesthesia utilized. The organs that we most commonly image are the kidney, liver, spleen, and pancreas. Because of anatomical location of these organs and the fact that many of them have long vascular pedicles that allow considerable mobility, two types of incisions are utilized to expose the organs of interest: 1) left flank vertical incision and 2) midline horizontal incision. Many investigators select the inverted microscope as the optimal scope for imaging the intra-abdominal organs that we study [1,5,6,9,10,12–14,16–19,24–29].

What must be considered in these animals prior to induction of anesthesia is their overall health status as a reflection of physiologic integrity. Those animals that are obtained from a commercial vendor are housed in our Laboratory Animal Resource Center (LARC)

Table 3
List of surgical and anesthesia instruments, monitoring equipment and reagents needed to carry out surgical preparation and imaging of abdominal organs in the rat and mouse.

Instruments/Use	Manufacture	Product #
1 cc syringe (inject probes and vehicle solutions	Becton Dickinson	305217
23 gauge inch precision guide needle (serves as the hub for vascular catheters)	Becton Dickinson	305145
Polyethelyne 50 tubing, catheter tubing 100 ft	Braintree Scientific	0.023″ × 0.038″
3–0 silk spool vascular access/ligation in rat	Braintree Scientific	SUT-S 110
5–0 silk spool vascular access/ligation in mouse	Braintree Scientific	SUT-S 106
3–0 PDS incision closure rat	Ethicon	Z316H
3–0 Prolene incision closure rat	Ethicon	8832H
4–0 PDS incision closure mouse	Ethicon	Z773D
4–0 Prolene incision closure mouse	Ethicon	8831H
Vannas-Tubingen Spring scissors for venotomy or arteriotomy	Fine Science Tools	15003-08
Schwartz microserrefine vascular clamps	Fine Science Tools	18052–01 (straight)
		18052–03 (curved
Halsey needle holder	Fine Science Tools	12001-13
Fine Scissors to cut fascia/connective tissue	Fine Science Tools	14058-09
Surgical Scissors to cut skin	Fine Science Tools	14002-12
Kelly Hemostat for rats: muscle clamp to minimize bleeding when cut	Fine Science Tools	13018-14
Kelly Ultrafine Hemostat for mice	Fine Science Tools	13020-12
Dumont #7 forceps to grasp tissues for exposure and dissection (especially when exposing vessels)	Fine Science Tools	11271-30
Graefe Forceps to grasp tissues for exposure and dissection	Fine Science Tools	11050–10 (straight)
		11052–10 (curved)
Adson Forceps with teeth to grasp tissue for exposure and dissection (especially skin and muscle)	Fine Science Tools	11207-12
2 × 2 gauze pads	Fisher Scientific	22-362-178
4 × 4 gauze pads	Fisher Scientific	13-761-52
Cotton-Tipped Applicators	Fisher Scientific	23-400-119
75 mm glass bottom dish for imaging kidney	Willco Wells, Netherlands	GWST-5040
Sterile Saline	Fisher Scientific	NC0336858
Hibiclens Skin Cleanser	Fisher Scientific	19-037-687
Betadine solution aseptic prep	Fisher Scientific	19027132
70% Ethanol solution aseptic prep	Fisher Scientific	HC-1000-1GL
Roboz Germinator 500 bead sterilizer: for instruments between aseptic procedures	Fisher Scientific	DS401
Homeothermic Monitor system: warming pad with thermos-coupling rectal thermometer system	Harvard Apparatus	55-7020
ReptiTherm heating pads for the imaging stage	Zoo Med Laboratories	4″ length × 5″ width
Power Lab 8/35 Data acquisition hardware for monitoring of blood pressure, heart rate	ADInstruments	PL3508
Objective heater with temperature controller	Bioscience	MTHC-HLS-025
Thiobutabarbital	Sigma Aldrich	T133-1G
Ketamine	Sigma Aldrich	1356009-250MG
Xylazine	Sigma Aldrich	1720407-200MG
Acepromazine	Sigma Aldrich	1001502-250MG
Isoflurane	Sigma Aldrich	CDS019936-250MG
Rodent Anesthesia Circuit	Kent Scientific	SOMNO-0307 rats
		SOMNO-0305 mice
Induction chamber	Kent Scientific	SOMNO-0530LG

housing facility for a minimum of 5 days after they are acquired. This allows them to acclimate following the stress of transport and transition to new surroundings. They can establish a norm of light and dark cycle rest and activity in the facility and normalize food and water intake. This acclimation period will also allow the manifestation of any underlying illness that might have existed prior to shipment. The Indiana University School of Medicine IACUC mandates this standard of care. Prior to use, the animals are inspected for overall health: activity, overall appearance, posture, socialization, and grooming are the most common aspects we observe. Once these criteria have been evaluated and the animals are deemed fit, they may be utilized for imaging [1].

3.1. Aseptic surgical technique

Regardless of whether the surgery is a survival or non-survival procedure, the surgical space must be cleaned prior to animal preparation. Traffic in the general area of the preparation room must be at a minimum and air flow should be adequate. The operator should wear a clean gown along with surgical cap/head covering, nitrile or latex gloves (sterile gloves for survival surgery), and in the least, a dust mist face mask. Following proper protective equipment protocol minimizes the incidence of exposure to blood-borne pathogens and laboratory allergens. Instruments should be cleaned and inspected prior to each session for structural integrity and signs of rust. Incisions should be as small as possible,

to minimize heat and fluid loss during the imaging session. And, the tissues must be manipulated gently and as little as possible to minimize the risk of blood loss, tissue ischemia and organ injury.

More than any other category, adherence to aseptic technique, as best as possible, is important in survival imaging. These animals may undergo a series of imaging sessions involving the same organ. It is critical that all materials that contact the animals are sterilized, since this is the only way to optimize valid data acquisition in each imaging session. That includes the imaging stage. Some institutions may not have the capacity to do this. For example, if a group may operate out of a multi-purpose imaging facility in which systematic sterilization of microscopic equipment is not feasible. In such cases, all microscope components that contact the animals are cleaned with 70% alcohol prior to and after each use. It is also important to ensure that the animal's incision is closed with sterile suture utilizing aseptic technique once the imaging session is complete.

The surgical space must be cleaned prior to animal preparation. All surgical instruments should be autoclaved prior to use. The operative site and surrounding area should be shaved and cleansed with a germicidal soap. A 2% betadine application is applied and allowed to stand for a minimum of 1–2 min and then wiped with 70% ethanol solution. This sequence is repeated three times, and then the operative field is covered with a sterile fenestrated drape. A sterile field must be maintained throughout the course of the procedure, and minimal manipulation of the tissues must be prac-

ticed minimizing post-operative adhesions or scarring that could take place. The same set of autoclaved instruments may be utilized in four consecutive surgeries by utilizing a Germinator 500 bead sterilizer to rapidly sterilize the instruments between cases. After the fourth consecutive animal, the instruments must be autoclaved prior to the next use.

A lack of adherence to aseptic technique can lead to formation of excessive adhesions in the region of the operative bed, localized wound infection, and generalized intra-abdominal infection. These complications will have a substantial effect on the results of the multiple-imaging session studies, and may even require cessation of the study prior to the endpoint. For example, in an ischemia-reperfusion model to induce acute kidney injury, the mobility of the vascular pedicle of the kidney can decrease as the result of adhesions from improper implementation of aseptic technique. This, in turn, can lead to an inability to effectively mobilize the kidney for imaging.

3.1.1. Adapting the microscope for intravital microscopy

The microscope stage needs to be big enough to accommodate the animal and allow access to the organs of interest. Imaging of abdominal organs is best accomplished using an inverted microscope, using an approach in which the organ is placed into a customized sterile glass bottom imaging dish, from WillCo Wells, filled with a warm saline solution. The dish is directly mounted into the microscope stage such that the organ can be imaged from below. Oriented in this way, the animal's weight serves to hold the organ in place in the dish, minimizing respiratory and circulatory motion artifacts [1,5,6,9,10,12–14,16–19,24–29]. This approach also requires surgical procedures that are simpler, faster, and less invasive than those needed to immobilize an organ for imaging on an upright system [1].

Once induced under general anesthesia, the animals cannot regulate their core temperature. Therefore, it is necessary to take several steps to maintain the animal's core temperature between 36.5 and 37.5 °C. In preparation for intravital imaging, the animals are placed on a warming pad and a rectal thermometer probe is placed for monitoring core temperature. The thermometer and the warming pad are part of a Homeothermic Monitor from Harvard Apparatus. The thermo-coupling device adjusts the warmth of the blanket based on core temperature measured. The microscope stage also has warming pads so that the animal isn't positioned on a cold surface. The microscope's imaging objective is warmed using an objective heater (Bioscience Tools), which consists of a warming coil connected to a thermo-coupling device. The objective heater is crucial since, the objective will otherwise act as a heat sink, drastically reducing the temperature in the imaged region of the tissue. This is used to reliably achieve temperatures between 36.3 and 37 °C in the imaging dish. Finally, once in position the animal is covered by the warming blanket. Some investigators opt to utilize an environmental climate chamber that encases the microscope stage once the animal is in position for imaging. The temperature within the chamber is adjusted to help maintain optimal core temperature of the animal during imaging [30].

3.2. Surgical presentation of organs via a left flank vertical incision

The left kidney, pancreas, and spleen can all be externalized via a vertical left flank incision. The vascular pedicles of these organs are quite long and allow presentation to the cover glass without undue stress or tension on the vessels (Fig. 3).

3.2.1. Presentation of the left kidney

Our group has made extensive use of intravital microscopy to study the function of the kidney [1,5,6,9,10,12–14,16–19,24–29,31,32]. Please see the video detailing externalization of the left kid-
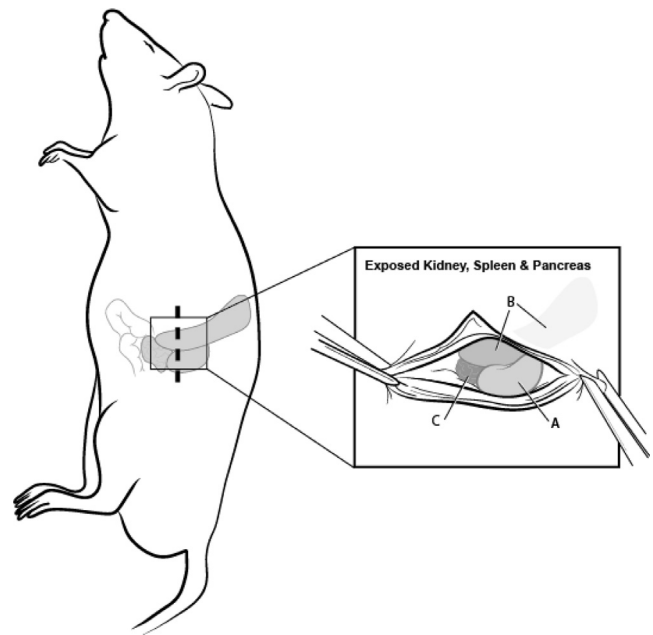
Fig. 3. Externalization of the kidney, spleen and pancreas. A left flank vertical incision provides access to the kidney (A), spleen (B) and pancreas (C). The incision in the figure has been retracted medially and laterally to depict the anatomical relationships of the three organs. The incision length should be no longer than 1.0 cm to prevent retraction of the externalized organ back into the abdominal cavity. Once the organ of interest is externalized, the animal is placed left side down on the microscope stage and organ rests in the glass bottom dish.

ney as demonstrated by Sandoval [9]. The left kidney is utilized for imaging because its vascular pedicle is much longer, and therefore more mobile, than that of the right kidney [9]. The animal is placed on its right side on the surgical table, and a vertical incision is made along the mid anterior axillary line below the level of the costal margin. The position of the incision is critical since, if it is too close to the costal margin, it tends to lead to increased motion artifact. It is easy to palpate the kidney prior to skin incision, and then determine the exact location of the incision based on its position on palpation. We have found that in the rat the initial incision should be no more than 1 cm, while in the mouse it should be no more than 0.5 cm. The length of the incision is also critical; if the incision is too long, the kidney will retract into the abdominal cavity. Should this occur, it can be remedied with a simple interrupted stitch to re-approximate a portion of the wound and reduce the size of the opening.

The kidney is easily externalized for presentation to the cover glass using blunt-tipped forceps to grasp the fat pad along the inferior pole. Once the kidney is externalized via the left flank incision, the animal is placed on the microscope stage on its left side, with the kidney placed in the imaging dish. A small amount of warm sterile saline is placed in the dish to prevent desiccation of the kidney. Once the kidney is positioned in the imaging dish and the animal has been covered with the warming blanket, the microscope objective is raised to the level of the dish in line with the region of kidney contact.

Other investigators have utilized similar surgical approaches to externalize the left kidney, via left flank incision, in mice [33–37] and rats [35,38,40]. Anesthetic agent of choice for mice in some studies was intraperitoneal injection of ketamine cocktail (ketamine 100 mg/kg + Xylazine 8–10 mg/kg) [33–35] and Inactin, 110–130 mg/kg intraperitoneal injection, was utilized for rats that underwent non-survival imaging [35,38,39]. Of note, in Rosivall's study, for visualization of the juxtaglomerular apparatus in the mouse, a 1-mm cortical slice was removed to create a parenchymal

window to expose the most superficial afferent arterioles and glomeruli [35]. Bleeding was minimal and ceased spontaneously within two minutes [35]. It does not appear that such a maneuver physiologically compromised renal function in particular or the animal in general. The Munich Wistar rat strain that was also used in that study has superficial glomeruli so a parenchymal window was not necessary in their cases [35]. Nakano [36], and Kuwahara [37] both utilized a left flank incision to expose the left kidney in mice, however they utilized Isoflurane as an anesthetic agent. This may be because both studies involved mice that were compromised somewhat by exposure to endotoxin [36] or high fat diet-induced kidney disease [37]. Szebenyi utilized ketamine 100 mg/kg + xylazine 100 mg/kg cocktail in a transgenic, GCaMP2 rat strain to externalize the left kidney via a left flank incision for non-survival imaging [39]. Like Rosivall [35] and Shiessl [38], Szebenyi [38] performed non-survival imaging studies in rats that were not compromised physiologically prior to or at the time of imaging.

The weight of the animal generally helps to minimize respiratory and pulsatile motion artifacts [1]. However, motion can be an issue, particularly when the kidney lies close to the diaphragm, and there is an unusually short vascular pedicle. The respiratory motion related to this position can be countered by extending the forelimbs above the head and securing them to the stage with tape. This raises the level of the costal margin somewhat, and therefore decreases the impact of diaphragmatic motion on the kidney. Other situations in which respiratory motion may be a problem are when the depth of the anesthesia is shallow and respirations are accordingly rapid. This tends to occur when an injectable agent has been used. Providing an additional dose of the agent at 25–50% the initial dose results in an improved depth of anesthesia.

3.2.2. Presentation of the spleen, pancreas

The spleen and the pancreas lie along the retroperitoneum and extend left of midline (Fig. 3). They share collateral arterial supply and therefore can be mobilized and externalized together through the same left flank vertical laparotomy incision. Use of blunt-tipped forceps has the potential to damage the spleen or the pancreas. Instead, we utilize a cotton-tipped applicator to scoop underneath the tails of both organs. They will adhere to the applicator as it is withdrawn back through the incision. Imaging of the pancreas and spleen calls for a similar left side down orientation of the animal on the stage, however as the vascular pedicles of these organs are longer they may be externalized further. Again, the weight of the animal aids in minimizing motion artifacts, but as necessary, residual tissue motion can be reduced by placing moistened lens paper on top of the portion of the organ that is in contact with the dish bottom. By having a segment of the lens paper also in contact with the bottom of the dish the organs are fixed in place, and this helps to minimize both respiratory and pulsatile motion artifact. Typically, the spleen lies anterior to the pancreas, so to favor contact on the dish with the spleen predominantly they must be extended toward the anterior side of the animal. To favor contact on the dish with the pancreas primarily, the organs must be extended toward the posterior side of the animal. Our group has conducted relatively few intravital microscopy studies of the spleen and pancreas. The reader is referred to publications by Grayson [40,41] and Ferrer [3,42] for descriptions of studies of the spleen and to publications from the von Herrath laboratory [4,43] for descriptions of studies of the pancreas.

3.3. Surgical presentation of the liver via a midline horizontal incision

Some investigators have found that exposing the liver via a horizontal abdominal approach supports high-resolution imaging with minimal motion artifacts [2,11,18]. With the animal lying
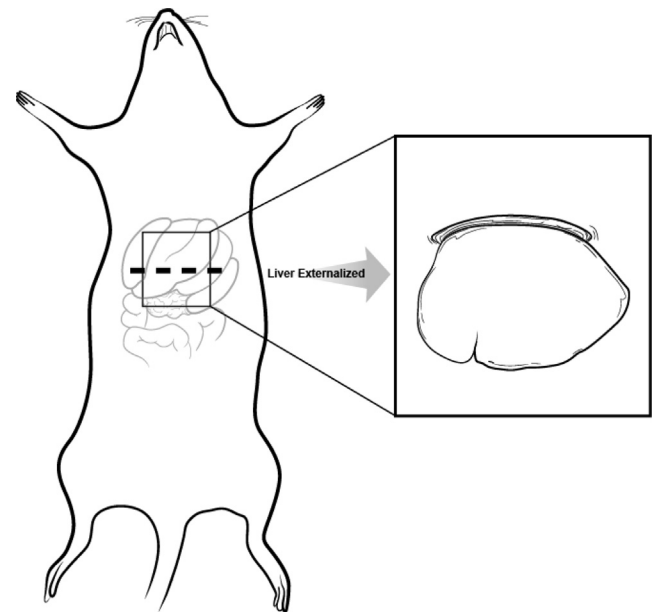
Fig. 4. Externalization of the liver for imaging purposes. A horizontal incision just below the level of the xyphoid process allows for delivery of the liver and placement in a customized glass bottom imaging dish once the animal is placed face down on the microscope stage.

on its back, the liver can be accessed via a midline horizontal incision, placed 1–2 cm below the xyphoid process (Fig. 4). The left lobe of the liver is readily visible once the incision has been made. Mild pressure applied medially on the lateral edges of the incision will externalize the left lobe of the liver, and the animal is placed face down on the stage so that the liver sits in the imaging dish. A saline-moistened gauze is placed below the left lobe. The periphery of the window of the dish is lined with tape, and cyanoacrylate glue is applied to the tape. The dish is then pressed against the liver so that either the gauze or the liver adheres to the glue [18]. Saline is then placed in the dish to avoid desiccation of the liver. While the horizontal approach has also been utilized by investigators outside of our group [7,44], it is not the only approach that has been successfully utilized to image the liver. The midline vertical incision is a common approach employed by investigators [45–49], and the left subcostal incision is yet another approach that has been utilized [50,51].

4. Physiological maintenance and monitoring during imaging

The maintenance of physiological homeostasis is essential for the acquisition of reproducible quantitative data. Significant alterations in core temperature, heart rate, mean arterial pressure, or respiratory rate can have an adverse effect on end organ perfusion, and the reliability of data acquired. See Table 3 for recommended maintenance and monitoring equipment.

4.1. Fluid resuscitation

During the imaging procedure, animals will require fluid resuscitation to compensate for natural losses. Generally, for every hour of imaging we provide 1–3 mL of fluid volume in the rat and 0.2–0.4 mL in the mouse. Fluid can be replaced with saline, and this may include various fluorescent probes to monitor physiological events [1,5,9] (see Section 4.3). Generally, the mouse is more sensitive to fluid volume loss and gains, so we are diligent in our technique to minimize bleeding, and we don't give more than 0.2 mL boluses at a time to minimize placing the animal in a fluid-

overload state. There are several ways in which this fluid resuscitation may be executed. The most common manner is to place an intravenous catheter during the preparation so that the fluid may be given [1,5,9] as a bolus every hour or can be given as a continuous infusion. The most common veins utilized are the jugular and femoral veins. Occasionally we access the tail vein via butterfly catheter set up. Whether we provide an infusion or a simple bolus is dictated by length of procedure and scope of the project. Those procedures in which multiple fluorescent probes or compounds are given at various time points during the procedure and/or last longer than 2 h generally require placement of an intravenous catheter.

4.2. Hemodynamic monitoring

While we observe the animal periodically for signs of adequate perfusion as it relates to color of the mucous membranes and the extremities, there are some projects that require accurate monitoring of blood pressure, and heart rate. These studies involve assessment of vascular flow rates within the organs of interest. For example, our studies of glomerular sieving require an arterial catheter whose transducer provides a real-time readout of blood pressure and heart rate [9,12,16,23]. The most common arteries utilized are the common carotid or femoral arteries. Customized blood pressure cuffs for the rat and mouse tail may be used as an alternative to invasive arterial catheters. While they are non-invasive, they do require that the temperature of the tail is maintained above room temperature to adequately determine pressure and pulse. Finally, consideration of the animal's airway is extremely important. Typically, when an animal is under general anesthesia, the tongue is externalized to one side so that it does not retract into the mouth and potentially obstruct the upper airway [1]. For this reason, we strive to ensure that our survival imaging procedures last less than 2 h. Still some investigators choose to place a tracheostomy tube to control the airway throughout the session [32–36].

4.3. Organ perfusion

It can be difficult to monitor perfusion of the imaging field in an organ during the imaging process. However, as the organs are being prepared for intravital imaging, careful inspection will provide evidence of adequate perfusion. In general, if the organ's color grows darker, then venous constriction or congestion must be considered. If the organ develops a pale color then arterial ischemia must be considered. Both phenomena are primarily characteristic of over-rotation of the vascular pedicle, which is easily remedied by reducing the over-rotation, or abnormally high tension because of the vascular pedicle being overstretched, which may require freeing up any connective tissue that may be restricting mobilization of the organ. In certain organs, blood flow can be evaluated through the microscope eyepiece epifluorescence, using the contrast of the dark red blood cells against the auto fluorescence of the surrounding parenchyma [1,5,9].

In general, a fluorophore that marks the vasculature is necessary to determine perfusion in the region of interest. Poor perfusion may also result from an excessive dose of the anesthetic agent. In the cases of inhalational agent excess, it may be corrected by decreasing the percentage of vapor delivered. Given the standard dose ranges for ketamine cocktails overdose is quite rare. The same is true for Thiobutabarbital. If it does occur, additional 1 mL bolus of saline in the rat and 0.2 mL in the mouse can help support low blood pressure while the ketamine cocktail dose diminishes. However, animals prepared using Thiobutabarbital are at risk of a fatal overdose because of the agent's long duration of action.

5. Imaging abdominal organs using an upright microscope

Investigators tend to utilize inverted microscopes to study abdominal organs of interest [1,5,9,32–34,45,46,50,51]. However, approaches have been developed for imaging on upright microscope stands, which may be useful to investigators without access to an inverted microscope system. For studies of the kidney, a technique utilizes custom-designed cups placed in the body cavity to immobilize the kidney. The kidney is placed in the cup facing up and covered with a glass coverslip, so that the kidney is imaged from above [1].

The surgical approach used to image the kidney on an upright microscope differs from that used for an inverted microscope. The animal is still placed on its right side, but the incision is carried out horizontally and extends from just left of midline to the level of the mid-axillary line of the left flank [1]. It is positioned equidistant between the costal margin and the superior iliac crest. Self-retained retractors are placed to expose the kidney, and it is mobilized and placed into the cup. Given that each kidney has variability in size and shape, moistened cotton helps remove dead space in the cup. In addition, we attach a glass coverslip to the cup to contact the kidney surface. This provides further reduction of motion artifacts. The advantages are that the kidney is easier to inspect and access, and the images acquired are of high quality due to minimal motion artifact. A heating chamber that covers the stage maintains core temperature of the animal.

This approach has several disadvantages. First, the preparation time can be up to 30 min longer compared to the inverted microscope. Second, the length of the incision is significantly longer, and it increases the likelihood of fluid and heat loss and tissue desiccation. This also presents a more difficult post-operative recovery in cases of survival imaging procedures. Third, the variation in size and shape of the kidney between animals can lead to increased time to ensure proper fit into the cup. Masedunkas [52] described the creation of several custom holders for various organs in the rat and mouse, for imaging on the upright microscope. They have achieved quality images with minimal motion artifacts [52]. Other investigators have modified the microscope stage to image a specifically isolated organ on an upright microscope. Cao developed a special microstage device to hold either the liver or pancreas in place for upright imaging and greatly reduced motion artifact [53]. Klinger developed an imaging chamber that makes it possible to image small intestinal mucosa, with minimal motion artifact on an upright microscope [54].

An alternative approach for investigators limited to using an upright microscope is to use an objective inverter system (LSM Tech, www.lsmtech.com). This system utilizes a pair of mirrors such that, when introduced between the turret and the objective lens, reverses the orientation of the objective lens and converts an upright microscope into an inverted microscope. Studies from the laboratory of Weigert demonstrate that this system supports imaging with little or no deterioration in image quality [52].

6. Approaches for longitudinal studies of abdominal organs

Methods supporting serial imaging of the same animal can be used to study processes occurring over days or weeks. Points to consider are maintenance of viable tissue for repeat imaging as it relates to proper aseptic surgical technique, adequate post-operative management to maintain physiologic parameters, general health of the animal, and the ability to serially follow the same regions of the organ during the serial imaging sessions. Hackl et al. have described a technique for sequential imaging in the mouse kidney to track the migration of glomerular epithelial cells [32]. In this study, the investigators surgically exposed the same mouse

kidney 3 times within a 48-h period. In this study, a cocktail of ketamine (100 mg/kg) and xylazine (10 mg/kg) induced a surgical plane of anesthesia. They utilized a left flank vertical incision to expose the left kidney for imaging [32]. Upon completion of the first imaging session, the left kidney was placed back in the abdominal cavity. This can be done carefully by utilizing a slightly moistened cotton-tipped applicator to gently push the kidney back, through the incision, into the abdominal cavity. They then closed the incision in 2 layers (fascial and skin) with sutures. Again at 24 and 48 h after the initial imaging procedure, the same ketamine cocktail was used for anesthesia [32]. The sutures were removed and the kidney was exteriorized for imaging [32]. In the same fashion as described in Section 2.2.3, post-operative recovery and management of the animal is the same in multiple survival imaging sessions that involve reopening a previous laparotomy to externalize organs of interest. Within that time span they observed no gross evidence of adhesions or fibrosis associated with the kidney because of repeated opening and closing of the left flank incision. Nor, did repeat opening and closure interfere with their ability to obtain quality images.

The question of whether reopening a laparotomy incision for serial imaging of an organ elicits an immune response or activates healing pathways that may interfere with the data obtained from imaging has not been fully addressed in the literature. Ritsma describes a less invasive approach for long term serial imaging of abdominal organs by utilizing an indwelling imaging window surgically implanted adjacent to the organ of interest [30,55]. White blood cell counts before and after implantation of the window did not indicate a systemic inflammatory response [55]. In addition, there was no evidence of local inflammatory response based on immunohistochemistry of the tissue fixed to the ring [55]. The use of imaging windows is well-established in the field of tumor biology, where dorsal skinfold chambers [56] and mammary imaging windows [57] have been used to track the behavior of tumor cells in mice. The reader is referred to the chapter by Vrisekoop et al. in this volume for a description of an abdominal imaging window that has been used for sequential imaging of the liver, intestines, kidney, spleen and pancreas. This system remains functional for a minimum of 4 weeks [30], and with various modifications can study liver metastasis [55], pancreatic islets transplanted under the capsule of the kidney [58] and ovarian physiology [59].

7. Summary

In conclusion, intravital microscopy places unique and exacting requirements on methods of animal anesthesia, surgery and maintenance. While selection of the optimal anesthetic regimen, that maintains a surgical plane of anesthesia, depends upon the species and sex of the animal, survival versus non-survival procedure, and physiological stability of the animal, personal preference, based on experience, of the individual investigator performing or supervising the procedure plays a vital role as well. The type of incision and mobilization of the organ of interest is influenced by the way the organ is presented to either an inverted or upright imaging objective. So, great care is taken to minimize trauma to the tissues and organs of interest, especially in survival procedures. Finally, maintaining homeostasis throughout the preparation and imaging period is vital. Core temperature, as well as temperature at the level of the organ, near the objective, must be maintained within an acceptable range to ensure that organ perfusion and physiology are not compromised. The importance of adhering to these principles and techniques for obtaining meaningful, reproducible data in intravital microscopy studies cannot be overstated. Nor, can the positive impact that the implementation of imaging windows has

had on the potential for successful serial imaging studies of abdominal organs for weeks at a time.

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