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TITLE:

Leukocyte Infiltration of the Cremaster Muscle in Mice Assessed by Intravital Microscopy

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KEYWORDS:

Intravital microscopy, leukocytes, cremaster, adhesion, inflammation, skeletal muscle, mdx

SUMMARY:

Here, we show how to perform intravital microscopy on post-capillary venules of the mouse cremaster muscle. Commonly applied to different models of inflammation and sepsis, particularly those induced by chemokines and cytokines, we highlight its relevance in the study of muscolopathies involving exaggerated muscular leukocyte infiltration.

ABSTRACT:

Intravital microscopy (IVM) is widely used to monitor physiological and pathophysiological processes within the leukocyte recruitment cascade in vivo. The current protocol represents a practical and reproducible method to visualize the leukocyte endothelium interaction leading to leukocyte recruitment in skeletal muscle derived tissue within the intact organism of the mouse. The model is applicable to all fields of research that focus on granulocyte activation and their role in disease.

We provide a step by step protocol to guide through the method and to highlight potential pitfalls and technical difficulties. The protocol covers the following aspects: experimental settings and required material, anesthesia of the mouse, dissection of the cremaster muscle as well as tracheal and carotid cannulation, IVM recordings and offline analysis. Data formats like adherent leukocytes, rolling flux (RF) and rolling flux fraction (RFF) are explained in detail and

appropriate applications are discussed. Representative results from dystrophin deficient mdx mice are provided in the results section.

IVM is a powerful tool to assess leukocyte recruitment in an in vivo setting; however, delineating for example endothelial and leukocyte function may require a combination with ex vivo setups like flow chamber experiments. Furthermore, the genetic background of animals of interest may greatly influence baseline recruitment, requiring individual fine tuning of the protocol provided. Despite its limitations, IVM may serve as a platform to readily translate in vitro findings into a living vertebrate organism.

INTRODUCTION:

Intravital microscopy (IVM) is a commonly applied tool in the field of leukocyte biology. Leukocyte recruitment follows a cascade of well-defined events initiated by leukocyte capture, rolling and adhesion to the endothelial wall, and finally transmigration and extravasation of leukocytes to the actual site of inflammation[1]. Each step is mediated and controlled by various chemokines (e.g., IL-8/CXCL8), receptors (e.g., LFA-1, Mac-1) and corresponding endothelial cell adhesion molecules (e.g., ICAM-1, VCAM-1 and E-Selectin)[2,3]. The interaction of different regulatory sites, controlling factors and mediators of the leukocyte recruitment cascade like RAGE (receptor of advanced glycation end products), ICAM-1 (intercellular adhesion molecule 1), CXCL1/2 (C-X-C motif ligand 1/2) and their receptor CXCR2 were uncovered using IVM[4-9].

The method of IVM has been described for many different organs and tissues such as the intestine[10], skin[11], lymph nodes[12], the embryonic yolk sack[13] and others. However, the most widely studied method of IVM is the cremaster model, first described in rats[14]. Whilst still used in rats[15], the method is nowadays mainly applied in mice due to the high abundance of different transgenic lines. Our group has recently highlighted the potential role of cremaster IVM in the field of inflammatory muscolopathies like Duchenne Muscular Dystrophy (DMD) studying dystrophin-deficient mdx mice[16]. Due to its thin interwoven and easily accessible fiber composition, the cremaster muscle represents the ideal candidate muscle to be studied as a whole mount using light or fluorescent microscopy. Leukocyte recruitment and extravasation mainly take place in post-capillary venules, which can readily be identified on a continuous muscular layer in the cremaster muscle.

The advantage of in vivo imaging compared to other in vitro assays is its biological context in a living organism. At the same time, delineating cell-specific contributions to altered leukocyte recruitment may require additional in vitro models like flow chambers or endothelial assays. The combination of multiple methods will yield most convincing data. Scientists should be aware of the limitations of the cremaster model as any surgical manipulation will lead to increased leukocyte trafficking and recruitment. Hence, baseline recruitment is difficult to estimate with this method. Despite its broad application, IVM of the cremaster can be challenging and a novel setup may take time and resources to establish. We now provide an easy protocol which will help to avoid some of the common mistakes in IVM. Also, limitations will be discussed and complimentary methods will be highlighted where applicable.

IVM of the cremaster represents an ideal approach to be implemented in the field of inflammatory and infectious studies. More specifically, the cremaster model may be of high interest to scientists studying skeletal muscle biology in the context of inflammatory disease.

PROTOCOL:

Animals were housed under controlled and specific pathogen-free conditions at the IBF (Interfakultäre Biomedizinische Forschungseinrichtung), Heidelberg. All the procedures described here were approved by the local IRB and the Regierungspraesidium Karlsruhe, Baden-Wuerttemberg, Germany.

1. Anesthesia administration

1.1. Anesthetize the mouse by intraperitoneal (i.p.) bolus injection of 125 mg/kg ketamine and 12.5 mg/kg xylazine.

1.2. Place and fix the mouse in a dorsal recumbent position on a heating pad to maintain body temperature of the mouse (36.5-38 °C). Use a non-absorbable sterile suture (6/0) to wind a simple loop around the frontal teeth and tape the joining ends of the suture to the heating pad. This fixation aims to keep the mouth open in order to avoid disturbance in breathing.

1.3. Check the mouse for appropriate depth of anesthesia by interdigital toe pinch (withdrawal should not be seen).

1.4. Once sufficient anesthesia is reached proceed to surgical preparation. Repeatedly confirm anesthesia along the ongoing experiment every 30 min. Re-administer drugs as described above if needed.

1.5. Equilibrate physiological salt solution (PSS, composition: 132 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgS0_4$, 2.0 mmol/L $CaCl_2$, 18 mmol/L $NaHCO_3$) with 5% CO_2/95% N_2 for 15 minutes. Continuously bubble PSS with 5% CO_2/95% N_2 throughout the experiment and keep at 37 °C.

2. Surgical preparation of the trachea and carotid artery (optional)

2.1. Dissect the skin from the neck area by gently pulling the skin with tweezers in the midline. Use scissors to apply a circular cut of 1-2 cm in diameter to give enough space for surgical preparation.

2.2. Carefully dissect the surrounding muscle, fat and connective tissues using tweezers.

2.3. Make a transverse cut (~ 1.3 mm) into the trachea using small surgical scissors and introduce a polyethylene tube (I.D x O.D. 0.034” x 0.050”) inside the caudal end of the trachea to secure the upper airways.

2.4. Fix the tube located in the trachea by a single circular knot suture (6/0 USP).

2.5. Locate the carotid artery along the right side of trachea and dissect the surrounding tissue from the carotid artery wall. Alternatively, the jugular or also the tail vein could be used for i.v. access. Try to avoid injury to the vagal nerve, as it is located closely.

2.6. Pass two pieces of suture (6/0) underneath the carotid artery. Place the first cranial suture proximal to the bifurcation of carotid arteries and tie it permanently. The second suture will be located about 2 mm distal from the first one and later be used to secure the tube in the carotid artery. Do not tie it yet.

2.7. Prepare a polyethylene tube (I.D x O.D. 0.011" x 0.024") with a length of 30 cm by bending the end part to a 1 mL syringe needle filled with saline (0.9% NaCl). Rigorous flushing is important to prevent air embolization during preparation.

2.8. Use a 7 mm vessel clip to clamp the carotid artery distally of the second suture.

2.9. Perform a small transverse cut (~0.5 mm) in the carotid artery and introduce the sterile polyethylene tube. Secure the tube in the artery using the second suture prepared before.

2.10. Remove the vessel clip and gently apply a little tension to the syringe connected to the polyethylene tube. This carotid catheter can now be used to administer drugs or take blood samples if required, even monitoring of blood pressure or pulse rate is possible with respective devices.

3. Surgical preparation of the cremaster muscle

3.1. Transfer the mouse on the heating pad (along with the heating pad) to the custom-made plastic frame holding the stage for later microscopy. Orient the mouse with its scrotum facing the microscope stage.

3.2. Localize the scrotum, holding it at its most distal part using tweezers, pull it gently and cut off a circular section of scrotal skin with about 5 mm in diameter. Make sure not to harm the underlying structures such as the cremaster muscle. Make sure to keep the open tissue well hydrated with saline solution.

3.3. Dissect loose connective tissue using two tweezers and localize both testes.

3.4. Hold one testis distally and gently start pulling it out, removing surrounding connective tissue step by step.

3.5. Once the testis is exteriorized, pin its distal end to the rubber ring surrounding the stage. Upon exteriorization, hydrate the tissue with saline solution during the whole process.

3.6. **Critical step:** Carefully remove connective tissue without harming the underlying cremaster muscle. Excess connective tissue may obstruct vision in later microscopy and may produce blurry images.

3.7. Pin the distal part of the testis down and open the cremaster muscle by a small transverse cut (about 1 mm), followed by longitudinal incision from the very distal to the proximal end. As a result the cremaster muscle should open spherically. Macroscopically visible vessel should not be severed, as this may impact hemodynamics.

3.8. Carefully spread the muscle over the glass stage and pin to the rubber ring. Make sure not to touch or harm the central region, as microscopy will be performed here.

Caution: Excess stretch may obstruct blood flow.

3.9. Pin the remaining testis aside to give access to the region of interest. Make sure to moisten with PSS repeatedly to prevent drying. The mounted muscle is now ready for microscopy.

4. Intravital microscopy

4.1. Place the mounted cremaster muscle in the upright microscope and perform microscopy using a 40x objective.

4.2. Acquire data and export using high throughput imaging spectrography.

4.3. Perform recordings under continuous superfusion with preheated (35-37 °C) PSS[17]. Apply superfusion by a small tubing that has been taped to the upright objective of the microscope to allow continuous dripping of PSS alongside the objective down onto the muscle tissue.

4.4. Identify post-capillary venules (confluence of two smaller vessels) and measure the microcirculation on venules with a diameter of 20-40 µm.

4.5. Record high-resolution images of the microcirculation from the cremaster muscle in a time range of 30 s. As there might be slight contraction and relaxation of the muscle tissue during recording, make sure to continuously adjust the focus. Generally, the mouse tends to exhibit stable circulation for about 2 hours. It is possible to acquire several recordings of different vessels from different regions. One or both testis can be used subsequently.

NOTE: As this is a model of inflammation, common ways of induction are: systemic application or local scrotal injection of pro inflammatory mediators as well as superfusion with for example fMLP (*N*-Formylmethionyl-leucyl-phenylalanine). Local TNFα stimulation is commonly used to trigger leukocyte recruitment; LPS induced endotoxemia is a model of SIRS sever systemic

inflammation.

5. **Leukocyte visualization**

NOTE: Adherent and rolling leukocytes can easily be seen without further visualization. To determine the center line velocity of freely moving leukocytes, perform differential staining.

5.1. If eGFP labeled mice are used, directly analyze them by fluorescence microscopy.

5.2. For non-eGFP labeled mouse strains, use rhodamine staining.

5.2.1. Administer rhodamine 6G at 0.2 mg/kg body weight. Repeated doses may be required to achieve sufficient staining intensities. Make sure to keep small volumes, as larger volumes may affect hemodynamic parameters.

5.2.2. For instant staining of leukocytes, administer the stain via the carotid artery. If carotid artery dissection has not been performed, sufficient staining may be achieved by i.p. application using the same dose[18]. As an alternative to carotid artery catheterization, any other venous can be performed.

NOTE: Keep in mind that rhodamine staining, unlike eGFP labeling, shows time decay of fluorescence intensity as well as an overall staining maximum of about 80% of leukocytes at higher concentrations.

5.2.3. Visualize freely moving rhodamine stained leukocytes by fluorescence microscopy.

6. End of experiment

NOTE: The overall estimated time to perform this protocol should be 90 – 150 minutes.

6.1. End the experiment with euthanasia by cervical dislocation.

6.2. For further analyses, such as transmigration, fix the cremaster muscle in PFA whilst still stretched on the stage and stained for histology as required.

7. Offline analysis

7.1. Analyze the following parameters as simple counts during video playback.

7.1.1. Calculate rolling flux [number/min]: number of rolling cells passing an imaginary line/min

7.1.2. Calculate adhesion [number/mm^2]: number of cells adhesive to the vessel wall within the field of view ≥ 30 s/vascular surface [mm^2].

7.2. Measure the following hemodynamic and microvascular parameters are measured using ImageJ.

7.2.1. Calculate vessel diameter [µm] as the inner wall diameter.

7.2.2. Calculate vessel length [µm] as the center line length of the vessel.

7.2.3. Calculate center line velocity $[^{\mu m}/_{s}]$ obtained from frame-to-frame video analysis of freely moving leukocytes in center line.

7.3. Use the following equations as an estimation.

7.3.1. Calculate vascular surface in [mm^2]: vessel length [µm] x vessel diameter [µm] x π x 10^{-6}.

7.3.2. Calculate wall shear rate $[^{1}/_{s}]$: 4.9 × (8 × center line velocity $[^{\mu m}/_{s}]$ × 0.625/vessel diameter [µm]).

7.3.3. Calculate mean blood flow velocity $[^{\mu m}/_{s}]$: center line velocity $[^{\mu m}/_{s}]$ x 0.625.

7.3.4. Calculate total leukocyte flux $\left[^{number}/_{min}\right]$: systemic leukocyte count $\left[^{number}/_{\mu L}\right]$ x (mean blood flow velocity $[^{\mu m}/_{s}]$ x $60/1000$) x π x $\left(^{\text{vascular diameter [µm]}}/_{2 \text{ x } 1000}\right)^2$.

7.3.5. Calculate rolling flux fraction [%]: rolling flux/total leukocyte flux x 100.

REPRESENTATIVE RESULTS:

IVM as per the provided protocol will yield unique insights into the cascade of leukocyte recruitment in skeletal muscle. The results section will focus on typical results obtained by IVM and highlight potential problems that may encounter.

The experimental setup for intravital microscopy is outlined in **Figure 1**. Preparation of the cremaster muscle and removal of connective tissue is crucial to obtain focused microscopic images with a uniform surface. Excess connective tissue eventually leads to blurry microscopic images (**Supplemental Video 1**) when performing IVM. **Figure 2** shows representative microscopic images that can be obtained using IVM. First, post-capillary venules, which should be between 20-40 µm in diameter, are identified by confluence of two smaller vessels. Only adherent and rolling leukocytes can be visualized, circulating leukocytes may only be tracked in slow motion replay of recorded videos. The number of adherent leukocytes is defined as stationary leukocytes over a time of 30 seconds and expressed per mm^2 of vessel surface (**Figure 2**). The number of rolling leukocytes passing a previously defined cross-section of the vessel is counted; rolling velocities may be obtained in offline analysis. Rolling may be expressed as rolling flux (RF = number of rolling cells over a predefined line/min) or as rolling flux fraction (RFF in % = RF/total leukocytes flux passing the vessel). RF is highly dependent on

the number of circulating cells, whereas RFF is less affected by changes in white blood cell counts (WBCs). Note that in many cases leukocyte adherence and rolling are inversely related; a high number of adherent leukocytes may reduce the pool of freely circulating cells and thus dampen leukocyte rolling.

Technical challenges and potential pitfalls

The cremaster muscle encloses the testis and assembles a spherical structure. To mount the muscle onto the recording stage, a longitudinal incision is required to open the sphere. This may affect the overall microvasculature as small vessels might be severed. To avoid bias to altered blood flow, it is advisable to choose the central area for reordering. This area should neither be touched nor manipulated. Additionally, microcautery may prevent bleeding and changes in microvascular hemodynamics. The blood flow of surrounding capillaries generally provides a valuable hint if circulation is affected due to preparation within the field of interest. In general, more proximal vessels (towards the body of the mouse) tend to show better microcirculation, however image capture may be complicated by increased movement and disturbances that result from breathing of the animal.

To prevent the cremaster muscle from drying out during microscopic recording, permanent superfusion of the tissue is eminent. Insufficient or interrupted moistening will result in drying of the tissue and failure of the experiment.

Pinning of the cremaster tissue is necessary to keep it stretched and in place. To flatten the striated muscle, gentle stretching is required. Too little stretch results in uneven tissue, which complicates microscopy, excessive stretching will restrict blood flow and harm the tissue. Again capillary blood flow provides a reliable status of the overall circulatory condition of the tissue. Experience and practice is required to determine the degree of stretch when mounting the tissue.

Long preparation times will affect the overall inflammatory state of the organism and bias data. Therefore, make sure to focus on the question of interest while designing the experiment and keep preparation times as short as necessary. If no drug administration, monitor or blood tests are required a singular preparation of cremaster tissue may be sufficient.

In most settings, different experimental groups will be compared to wild type animals. It is of pivotal importance to make sure that hemodynamic and microvascular parameters are similar between experimental groups (**Table 1**). A multitude of parameters may affect leukocyte recruitment in vivo, including cardiac output, vessel diameter, center line velocity, wall shear rate and the number of circulating leukocytes. **Table 1** displays data from two different transgenic mouse lines, the dystrophin deficient mdx mouse and the lys-eGFP mouse (expressing eGFP under the lys promoter in neutrophils and macrophages). Whereas parameters such as vessel diameter can be controlled by the investigator, centerline velocity and shear rate depend on the hemodynamics of the individual experimental group or the mouse strain. Clearly, the centerline velocity and wall shear rate are significantly different between mdx and lys-eGFP mice (**Table 1**) making meaningful comparison of IVM data

impossible (statistical analysis by t-test with Welch's correction, significance was set at $P < 0.05$). The centerline velocity is influenced by cardiac output, peripheral vascular resistance and intravasal volume. For example, a large volume of rhodamine or any other experimental substance given via the carotid catheter increases post capillary centerline velocity and inhibits leukocyte capture and rolling (**Supplemental Video 2**). On the contrary, cardiac failure, deep anesthesia or hypothermia may decrease post-capillary flow. Before approaching offline analysis of IVM data, requirements of data quality (for example hemodynamic parameters within physiological ranges) should be defined, in order to draw meaningful conclusions and reduce bias.

To determine the centerline velocity, freely circulating leukocytes need to be visualized. Either, fluorescent leukocytes (like in lys-eGFP mice) may be used or leukocytes must be dyed with fluorescent reagents like rhodamine (**Figure 3**). Rhodamine can be applied via the carotid catheter or as an i.p. injection. Since rhodamine is gradually taken up by other cell types as well, a titration of dosage and time is essential. Excess dosing and long intervals will produce significant background during microscopy.

Finally, we like to point out biological diversity of different mouse strains. Besides genetically altered strains, also generally accepted wild type strains may show physiological variance. **Figure 4** compares commonly employed black 6 (C57BL/6) to black 10 (C57BL/10ScSn) mice. Black 6 mice clearly show enhanced rolling, adhesion and transmigration of leucocytes compared to black 10 mice (statistical analysis by one-way ANOVA with Tukey's post-hoc test; significance was set at $P < 0.05$), even though both strain would be referred to as wild type. Therefore, correct genetic background should be considered, especially when utilizing transgenic mice. In our experimental setting, a comparison of black 6 mice and transgenic mdx mice (black 10 background) would largely conceal the enhanced inflammatory state of dystrophin deficient mdx mice over their correct black 10 wild type background (Figure 4).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic experimental setup of IVM on cremaster muscle. After anesthesia, the trachea is cannulated and a catheter is placed in the carotid artery. The cremaster muscle is exposed and prepared for intravital microscopy on an upright microscope using either light microscopy or fluorescent microscopy. Videos are obtained for later offline analysis.

Figure 2: Representative post-capillary venule segment from intravital microscopy with sequential frames. The left column shows light microscopic images, for better visualization the vessel is outlined with dotted lines. The venule is identified by confluent vessels (marked by red asterisks) and a vessel diameter of <40 µm. The right column depicts a schematic representation of the corresponding left image, showing leukocytes in red. The first row indicates adherent leukocytes over a time of 30 seconds. The bottom four rows show sequential images of the same vessel over one second. In the schematic representation, individual leukocytes and their rolling distance are marked. In offline analysis adherence/mm^2 and rolling flux as well as rolling velocity may be calculated.

Figure 3: Rolling leukocytes may be visualized by transgenic expression of fluorescent markers or by secondary staining with rhodamine. Representative microscopic images of leukocyte recruitment of eGFP leukocytes and rhodamine labeled leukocytes from n = 3 animals each. Rolling neutrophils expressing eGFP under the lys promoter from lys-eGFP transgenic mice can be directly detected by immunofluorescent microscopy (left column). Leukocytes without fluorescent makers may be stained by intravenous or intraperitoneal injection of rhodamine (right column). Notably, rhodamine is not exclusively taken up by neutrophils, giving substantially more background.

Figure 4: Comparison of leukocyte recruitment in different transgenic and wild type mouse strains. (**A**) Rolling flux fraction, (**B**) adhesion and (**C**) transmigration of leucocytes is compared between black 6 (C57BL/6J), black 10 (C57BL/10ScSnJ) and mdx mice (C57BL/10ScSn-Dmd^{mdx}J), shown as mean + SEM. Black 6 mice show enhanced leukocyte recruitment compared to black 10 mice. Similarly, mdx mice have greater recruitment compared to black 10, but not compared to black 6 wild type mice. This data demonstrates the importance of allocating correct genetic controls. Data from at least n = 3 animals per group. Statistical analysis by one-way ANOVA with Tukey's post-hoc analysis. Significance was set at $P < 0.05$.

Table 1: Relevant hemodynamic and microvascular parameters from two different transgenic mouse lines. Vessel diameter, center line velocity, wall shear rate and systemic WBCs are displayed for mdx mice lacking dystrophin and lys-eGFP mice. In this example mdx mice (C57BL/10J, black 10 background) and lys-eGFP mice (C57BL/6J, black 6 background) show statistically different centerline velocity and wall shear rate and should not be compared using IVM. Exemplary data from at least n = 3 animals per group are presented as mean ± SEM. Statistical analysis by unpaired t-test with Welch's correction. Significance was set at $P < 0.05$.

Supplemental Video 1: Microscopic effect of insufficient removal of connective tissue on cremaster muscle.

Supplemental Video 2: Altered blood flow by either hypervolemia or hypotension.

DISCUSSION:

IVM as a method has been widely used to study different cell types in different organs and has been extensively described and discussed[19]. The main aim of this study is to provide an efficient approach to set up and perform IVM in the cremaster muscle. Practicing the method will produce reliable and reproducible results. Thus, planning and standardization are key factors to master the technique. Above all, the technique is very dependent on hemodynamic and microvascular parameters, which need to be closely monitored and controlled for. As such, different hemodynamics between groups will yield misleading results.

Despite its role in studying physiological and pathological conditions, IVM alone may not fully unravel individual contribution of specific cell types to leukocyte recruitment in inflammation. To do so, other complementary methods may be combined with IVM. For example, ex vivo methods like flow chamber experiments may delineate between effect of the endothelium or the leukocyte. Also, flow chambers are excellent setups to translate rodent findings to human leukocytes, for example in newborn infants[20]. Further, in vitro methods like FACS or immunohistochemistry should complement IVM experiments. Each method may add specific information acquired from a controlled environment with little confounding factors.

The traumatic cremaster preparation presented here resembles physiological baseline conditions of inflammation as closely as possible. Yet, the mandatory surgical preparation itself is a stimulus of inflammation, which must be considered for interpretation. Pharmacological TNF stimulation of the cremaster tissue may provide an additional mode to boost inflammatory recruitment beyond the traumatic surgical stimulus[16]. Further, different mouse strains may show altered responses towards standardized stimuli. We have demonstrated that even common wild type strains may exhibit different baseline states of leukocyte recruitment. This shows the importance of assigning correct genetic controls, and establishing baseline data for new experimental groups when planning experiments. As transgenic strains are highly abundant these days, it is likely that even same strains may differ over time depending on breeding.

Taken together, IVM is a powerful tool to monitor leukocyte recruitment in the biological context of the mouse and should be accompanied with ex vivo and in vitro techniques. Further, cremaster IVM allows a variety of different modes including specific cell staining, inflammatory stimulation or pharmacological manipulation and pretreatment. As such it may serve as a platform to evaluate different pharmacological mediators and their biological effects in preclinical studies especially in the field of inflammation and more specifically in inflammatory musculopathies.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

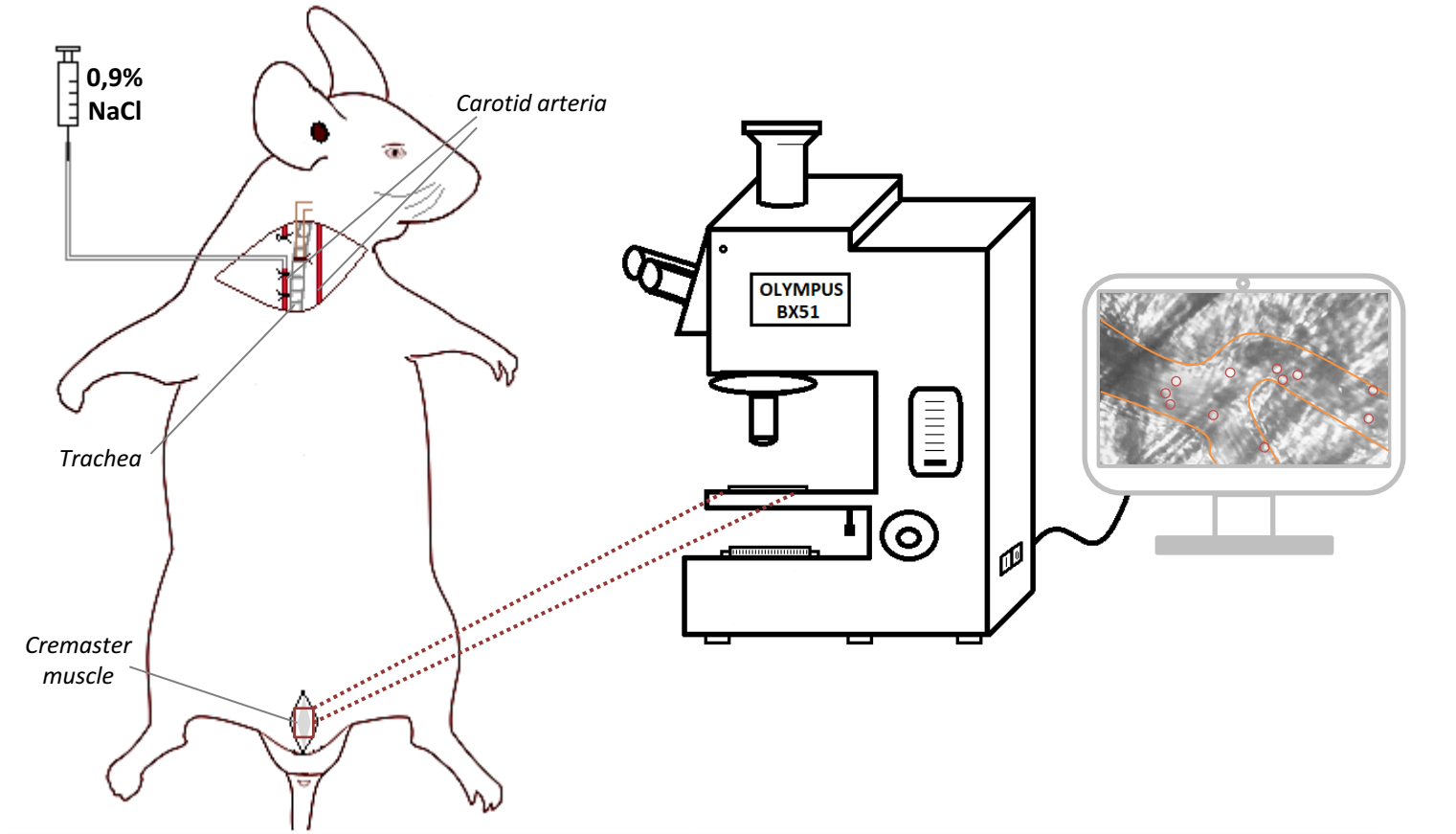

Figure 1

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Figure 2

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Figure 3

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Figure 4

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Supplemental Video 2

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Hannes Hudalla - Supplement Video 2_final.mp4

Mouse strain	**No. of mice**	**No. of venules**	**Vessel diameter**	**Centerline velocity**	**Wall shear rate**	**Systemic WBC**
	N	n	µm	µm/s	s^{-1}	/µl
mdx	3	12	28 ± 4	1150 ± 50	1010 ± 100	5200 ± 300
lys-eGFP	3	15	27 ± 2	2220 ± 90	2030 ± 90	5800 ± 100
P			0.83	0.0005	0.0016	0.13

Name of Material/ Equipment	Generic / IUPAC Name	Company
Material		
Ketanest S	ketamine	Pfizer Pharma GmbH
Xylazine	xylazine (as hidrochloride)	CP-Pharma GmbH
Saline Solution	sodium chloride	B. Braun Melsungen
Syringe needle Omnican F		B. Braun Melsungen
Suture 6/0 USP		Resorba
Polyethylene tube #10		BD GmbH
Polyethylene tube #90		BD GmbH
Rhodamine 6G	ethyl 2-[3-(ethylamino)-6-ethylimino-2,7-dimethylxanthen-9-yl]benzoate	Sigma-Aldrich Chemie GmbH
Setup Equipment		
Upright microscope		Olympus
40-fold objective		Zeiss
ImSpector software		Lavision Biotec GmbH
ImageJ		National Institute of Health, USA

Catalog Number	Comments/Description
PZN: 08509909	anesthesia
Article-nr.: 1205	anesthesia
PZN 02737756	surgical preparation
REF 9161502	surgical preparation
REF 4217	surgical preparation
Supplier No. 427401	surgical preparation
Supplier No. 427421	surgical preparation
CAS Number 989-38-8	leukocyte staining
BX51W1	microscopy
Achroplan 40 × /0.80 W	microscopy
ver. 4.0.469	software
ver. 1.51j8	software

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Title of Article:	**Leukocyte infiltration of cremaster muscle in mice assessed by intravital microscopy**
Author(s):	**Simon Kranig*, Trim Lajqi*, Raphaela Tschada, Maylis Braun, Navina Kuss, Johannes Pöschl, Hannes Hudalla**

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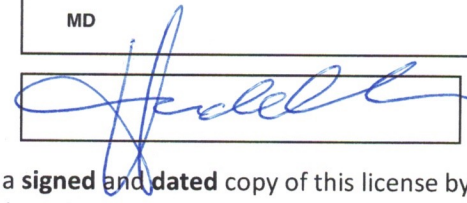
Signature:	
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Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have revisited the manuscript and performed a thorough spell check.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

We have reformatted the manuscript according to the JoVE guidelines. Letter (8.5" x 11" equivalent to 21.59 cm x 27.94 cm) page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs.

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For example: Ketanist, Xylavet, Olympus, Zeiss Achroplan, ImSpector.

All commercial product information has been removed from the manuscript and is only mentioned in the table of materials and reagents.

Protocol:

1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:

1. 4.3: How is superfusion done?

A more detailed description and reference have been added to the protocol and the section on technical challenges.

2. Note between 6 and 7: Which sections, exactly, are you referring to here?

The sections have been merged and can now be found summarized in section 6 "End of Experiment".

3. Please discuss end-of-surgery methods (euthanasia and/or recovery).

Experiments end with euthanasia. The information has been added to the manuscript.

Figures:

1. Please remove 'Figure 1' etc. from the Figures themselves.

The figures were adjusted.

2. Figure 1: '0.9%', not '0,9%'. Please also remove 'Olympus BX51'.

Changes were incorporated.

3. Figures 2 and 3: Please include spaces between numbers and units (e.g., ‘0 s’, ’40 µL’).
The figures were adjusted.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
The table has been revisited.

Reviewers' comments:
Reviewer #1:

This manuscript by Kranig et al. very nice details a complex procedure. The manuscript is well written and will serve as both a nice introductory guide for those starting IVM and a good reference for those already conducting the procedure. I especially like the example of altered flow in addition to the discussion of the importance of flow rate. I did not identify any major deficits. Below I've listed several suggestions and minor edits that could be considered to strengthen the manuscript. One thing that would be good to see in the literature is a bit more discussion of some of the pit falls. They are so rarely discussed but are a huge stumbling block for procedures like this (things like preventing the tissue from drying out on the stage, particularly during long recordings; severing key vessels which can lead to altered/reversed flow; pinching/pinning the tissue in a ways that restricts flow, particularly when pinning the testis; stopping flow from severed small vessels). I do not believe comments on these points are required, but if the researcher has experienced them (and found ways to address them) then this would be an appropriate place to share.
We thank the reviewer for these insightful suggestions. Indeed, we also feel that pitfalls are rarely discussed and make the journal format worthwhile. We have therefore included a paragraph on potential practical and technical challenges in the manuscript.

Major Concerns:
None

Minor Concerns:

Line 90 - it's a bit odd to say that 'Leukocyte recruitment initiates with chemotaxis…' Most of the field would say that it begins with capture, followed by fast rolling, activation, slow rolling, adhesion, etc. This should probably be rephrased accordingly.
We agree and have changed this sentence in the introduction.

Line 139 - ip. should be i.p.
The spelling was changed.

Line 142 - body temp of? please state
Mice show a body temperature ranging from 36.5-38.0°C depending on metabolic rate and activity. We have added the temperature range to the protocol.

Line 178 - I haven't done the catheter method often but are there any precautions taken to prevent bubbles from entering the vasculature?

Yes, the tube should be pre-filled with saline solution and flushed thoroughly to remove bubbles. This aspect has also been added to the protocol.

Line 189 - It sounds like a piece of scrotal tissue is cut off (compared to a simple incision). If this is the case, the rough dimensions and orientation should be stated (i.e. circle with a diameter 3mm, or square 3mm x 3mm, or 3mm2
We remove the scrotal skin in order to get a better visual on the underlying tissue. The section is about 3mm in diameter and we have added this accordingly.

Is anything done to remove hair around the incision or prevent it from getting one the tissue?
This is not a problem in our hands. Occasional hairs are flushed away with the superfusion buffer. In general, hair-removal pastes are now mostly not accepted by IRBs in Germany as they may cause skin irritation.

Line 198 - Where the incision is made matters as care should be taken to avoid severing any of the major vessels. This point should be included
We have added this important information to the respective step in the protocol.

Line 204 - should probably mention that the tissue needs to be well hydrated once exteriorized. Maybe this should be mentioned earlier, before step 5
In our hands, the tissue does not dry out before opening the cremaster muscle. However, we agree that users new to the technique may require earlier superfusion as preparation may take longer and have mentioned it earlier in the manuscript.

Line 234 - i.v. injection would work also (tail vein or retro-orbital)
We agree, and have added this addition to the manuscript.

Line 240 - The tissue is best fixed while still pinned. It helps keep it flat and makes for better mounting.
This has been added to the manuscript as well.

Line 251 - is there any tolerance for how far the leukocyte may move (i.e. 1 cell diameter) before it is considered 'rolling' and not adherent? Would a crawling leukocyte be considered
It should be noted that live images tend to move or lose focus (heart beat, breathing, muscular contractions) and it may be hard to be 100% sure that leukocytes do not crawl. Most publications in the field do not mention exact specifications on what is defined "adherent". We would consider completely stationary cells as adhesive ones, but would also take into account that there might always remain little bias when adjusting focus over time.

Line 261 - the 1x106 factor in the equation confuses me. Is that a conversion factor for going from microns to millimeters? Perhaps it could be clarified
Thank you for pointing this out. Indeed, the equation was wrong as stated. We apologize for this. The correct factor is 10^{-6}. We have also included units for better understanding as well.

Line 312 - Excess (not access)
This has been corrected in the manuscript.

Reviewer #2:

Manuscript Summary:
Overall this paper gives a clear overview of how to go about the mouse cremaster preparation and the potential technical pitfalls that may occur. It is good that the authors have stressed the importance of reproducibility in blood flow in terms of comparing mice and experimental groups. I have the following suggestions:

Major Concerns:

Summary - The prep has been used to study many forms of inflammation most prominently those induced by chemokines and cytokines, i.e. not only sepsis. This should be noted.
The model is indeed studied in many different inflammatory conditions and various agents are used to induce inflammation. We have broadened this statement in the summary.

Introduction, first para - In the interest of recognising other important studies using this model, it may be appropriate to cite some work from Klaus Ley, Paul Kubes and Sussan Nourshargh, in addition to those from their own lab and collaborators.
We have changed the Introduction and now recognize other authors, who have, without doubt, contributed far more significantly to the field.

Protocol:

2. Surgery
It is interesting that these authors choose to cannulate the carotid artery. Most labs I know use the jugular vein or tail vein for i.v. access. Perhaps this could be listed as an alternative, indeed one that is less technically challenging than the carotid artery.
We agree with this point, which is also mentioned by Reviewer #1. The reason we prefer to use the carotid artery is because of its potential use to monitor central blood pressure. We have not mentioned this in the manuscript, as it may complicate the procedure. However, we have added a comment that obviously other i.v. catheters may be used.

3.2. It should be emphasized to hold on to the most distal part of the scrotum
We have included this in the protocol under 3.2 and also reference a similar aspect under 3.8. We agree that this step is crucial in order not to harm vessels, which will be later used for microscopy.

3.7 Our lab uses microcautery to cut open the muscle, as a way of avoiding bleeding. The authors should clarify whether they see that as a necessity.
Collaborators of our lab use microcautery as well, but many labs also seem to be ok without. We believe that the key to success is to prevent bleeding and harming major vessels which is highly dependent on routine and practice. Nevertheless, as we have further elaborated the paragraph on potential pitfalls in the manuscript, we have also mentioned microcautery as a tool to control excess bleeding.

4.6 Perhaps state how long the prep can be imaged for. It is not uncommon to image for 1 or 2 hours, depending on the model in use.
We agree on the time period. This information has been added to the manuscript.
Also in this section it might be useful to list how inflammatory stimuli can be applied to the muscle in order to induce inflammatory cell recruitment, whether systemically, locally via instrascrotal injection, or in the superfusion buffer.

We added paragraph 4.7. to list the different options named to induce inflammation.

7.1.1.2 Please clarify how the total number of cells passing the imaginary line is calculated, in order to determine the RFF.
The correct formula was missing indeed, and has now been added under 3.3.3-4

7.1.1.3 Re the definition of adhesion, do the cells need to be static for the complete 60 seconds? Please define the duration required for a cell to be defined as adherent. Many labs use 30 seconds for this.
Indeed, ≥ 30 s are commonly used in most labs including ours and are generally applied to assess adhesion. We have however reported 60s as well, because in our hands cells that did not move over 30 seconds rarely start moving up to 1 minute. As the protocol prepared here is meant to be a general description, 30 s is the more accepted period to mention. This has been changed.

7.3.3.1 Should this equation be divided by 1x10E6 rather than multiplied. mm2 are smaller than um2
Would it be worthwhile mentioning transmigration as a parameter that can be assessed?
We thank both reviewers for pointing out this mistake in the equation and we have corrected it accordingly. We have also included transmigration after fixation to the potential readouts of the method. In vivo may also be assessed as many other parameters, but we feel this exceeds the scope of this manuscript.

Minor Concerns:

Abstract - I doubt that the method here is "unique." There are many different preps that allow visualisation of the leukocyte recruitment cascade.
You may prefer to stress its practicality, reproducibility, and ease of use relative to other preparations in mice.
We are in full agreement and have changed the wording.

Throughout: take care with the use of 'access' when the intended word is 'excess'
Typos have been corrected.

Discussion - re comparison of wild-type and mdx mice, it would be interesting to note if the leukocyte adhesion parameters also differed between the strains.
They do indeed differ substantially, as has been reported by our lab recently (Kranig et al.). We have analyzed additional animals and assessed adherence per 30 seconds. We also included WT B6 animals to the baseline measurements and now report a comparative Figure 4. To our knowledge this is the first report of baseline strain differences regarding leukocyte recruitment between black 6 and black 10 mice (background of mdx mice). This additional figure was included to highlight the importance of characterizing individual inbred strains and transgenic mice. We feel this is a good example of how wrong conclusions may be drawn if baselines are not characterized well. We have also included this in the discussion of the manuscript.