Video Article

Intravital Microscopy of the Microcirculation in the Mouse Cremaster Muscle for the Analysis of Peripheral Stem Cell Migration

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URL: http://www.jove.com/video/50485
DOI: doi:10.3791/50485

Keywords: Stem Cell Biology, Issue 81, migration, intravital microscopy, cremaster muscle, bone marrow, endothelium, microsurgery

Date Published: 11/5/2013

Citation: Donndorf, P., Ludwig, M., Wildschütz, F., Useini, D., Kaminski, A., Vollmar, B., Steinhoff, G. Intravital Microscopy of the Microcirculation in the Mouse Cremaster Muscle for the Analysis of Peripheral Stem Cell Migration. *J. Vis. Exp.* (81), e50485, doi:10.3791/50485 (2013).

Abstract

In the era of intravascular cell application protocols in the context of regenerative cell therapy, the underlying mechanisms of stem cell migration to nonmarrow tissue have not been completely clarified. We describe here the technique of intravital microscopy applied to the mouse cremaster microcirculation for analysis of peripheral bone marrow stem cell migration *in vivo*. Intravital microscopy of the M. cremaster has been previously introduced in the field of inflammatory research for direct observation of leucocyte interaction with the vascular endothelium. Since sufficient peripheral stem and progenitor cell migration includes similar initial steps of rolling along and firm adhesion at the endothelial lining it is conceivable to apply the M. cremaster model for the observation and quantification of the interaction of intravascular administered stem cells with the endothelium. As various chemical components can be selectively applied to the target tissue by simple superfusion techniques, it is possible to establish essential microenvironmental preconditions, for initial stem cell recruitment to take place in a living organism outside the bone marrow.

Video Link

The video component of this article can be found at http://www.jove.com/video/50485/

Introduction

The purpose of the present article is to describe the technique of intravital microscopy (IVM) applied on the mouse cremaster microcirculation for direct observation and analysis of peripheral bone marrow stem cell migration.

The current concept of stem cell based tissue and organ regeneration involves the homing of bone marrow derived stem cells to the injured tissue[1]. A crucial step for successful stem cell migration includes stem cell interaction with the local endothelium within the injured organ followed by transendothelial migration and eventually organ engraftment[2]. Intravital analysis of these stem cell - endothelial cell interactions forms an ideal parameter for the quantification of a stem cell based regenerative response in different pathophysiological settings *in vivo*. Furthermore, it seems conceivable, that, in the future, intravital analysis of the migratory capacity of specific stem cell populations within standardized microcirculatory environments, might be applied prior advancing these populations to further clinical testing.

Intravital microscopy has been initially developed for the observation and quantification of leucocyte-endothelial cell interaction *in vivo* in the field of inflammatory research[3]. First successful recordings of intravital microscopy studies were reported by Cohnheim already in the 19th century, studying frogs' tongues and mesenteries under a light microscope[4]. Since its first use IVM has undergone a tremendous technical development and today certain essential components form prerequisites in order to perform quantitative IVM: (i) tissue preparations that permit optical access, (ii) molecular probes that can be detected by a microscope, (iii) a microscope connected to a detection system and (iv) computer based analysis systems that can extract parameters of interest from the image data set[4].

A variety of tissue preparations has been introduced for IVM studies including the mesenteries and liver of the mouse and rat[5], the dorsal skinfold chambers of mouse [6] and hamster, the rabbit ear and the hamster cheek pouch to name a few.

However, in the following we will focus on the mouse cremaster muscle, representing an ideal tissue for intravital observations, as preparation and visualization are possible by a well-standardized surgical procedure, and in general no problems of movement artifacts occur. The open cremaster muscle preparation was carried out for the first time in the 1970s by Baez and colleagues [7]. Originally described for rats, it has been adopted successfully also to the mouse[8]. After previous studies had mainly focused on leucocyte interactions with the vessel wall, our own group recently introduced the mouse cremaster muscle preparation as a valuable tool for direct visualization and quantitative analysis of stem cell-endothelium interactions within a defined microenvironment[9]. Various stem cell subpopulations have been studied utilizing this model, including murine c-kit$^+$ bone marrow stem cell and mesenchymal stem cells, as well as human CD 133$^+$ bone marrow stem cells[10-12]. Following cell isolation from donor bone marrow and fluorescent labeling for visualization, the stem cells are selectively applied into the cremaster microcirculation utilizing an arterial injection via the femoral artery, thereby avoiding any cell entrapment within remote organs. Furthermore,

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the cremaster muscle model is particularly useful since various chemokines potentially mediating local stem cell migration within the respective settings, can be topically applied to the target tissue by simple superfusion technique.

Protocol

The entire protocol after cell isolation takes approximately 2 hr.

1. Microsurgical Preparation

1. Perform stem cell isolation from the donor bone marrow and fluorescent labeling of the isolated subpopulation according to standardized protocols[9,12] Allow cells to rest during the time the animal operation is performed. For fluorescent labeling we recommend CFDA (Carboxyfluorescein diacetate succinimidyl ester).
2. Anesthetize a male mouse weighing 20-25 g with ketamine (75 mg/kg) and xylazine (2.5 mg/kg). Throughout the surgical procedures and experimental protocols, anesthesia should be maintained by supplements (10% of initial injection, i.p.) as needed (usually every 30-60 min).
3. Shave gently the anterior aspect of the right scrotum as well as the right thigh and groin. Collect all lose hair with a moistened cotton swab. Place the mouse ventral side up on a plexiglass viewing stage and fix the feet using elastic drape. The stage is custom-made, consisting of a base plate and a second plate at the caudal edge of the base plate for elevation of both legs and the scrotum. Place the stage on a heating pad to maintain body temperature at 37 °C. After fixation on the stage, place the animal under an operation microscope and perform the following operation steps using 10- to 16-fold magnification.
4. Make the initial incision using skin scissors at the right thigh, just anterior of the femoral vessels from the knee until to the groin.
5. Identify the femoral artery and follow it proximally, mobilizing the artery from surrounding soft tissue. Identify and cut a large branch to the left testicle using thermocautery. Thereafter place a stay suture superficially at the left testicle. Gentle traction will facilitate further proximal mobilization of the femoral artery.
6. After completion of the proximal mobilization, identify and cut a large branch running medially at the thigh by thermocautery. Thereafter ligate the femoral artery in the distal part of the right thigh. Gentle traction on the ligature will aid further preparation.
7. Turn attention now to the proximal femoral artery again and place a suture around the artery as proximally as possible. Prepare a knot but do not tie it down yet. Apply gentle traction to the suture and create an arterial kinking.
8. After establishing the kinking, incise the artery is carefully using a microscissor. Take special care not to damage the femoral vein.
9. Insert a prepared microcatheter (inner diameter 0.28 mm) via the incision. The catheter must be deaired (0.9% sodium chloride, NaCl) and connected to a 1 ml syringe prior insertion.
10. After successful insertion of the catheter, carefully loosen the kinking in order to facilitate passage of the catheter. Arterial blood flow should be immediately visible within the catheter. Gently advance the catheter a few millimeters beyond the previous kinking. Thereafter, tie down the prepared ligature for fixation of the catheter.
11. Gently flush the catheter (0.9% NaCl) and aspirate to confirm correct position. Use a piece of tape to further secure the catheter to the operation stage.
12. After adequate fixation, put the catheter aside for the following preparation steps of the cremaster muscle. Apply regular flushing and aspiration of the catheter every 5-10 min in order to prevent clot formation.
13. Turn attention now to the right scrotum and make an incision by cutting the skin and fascia above the ventral aspect of the right scrotum. Take care not to touch the underlying tissue with any instruments.
14. Extend the incision up to the inguinal fold and caudally to the distal end of the scrotum.
15. Carefully separate underlying soft and connective tissue above the cremaster sack, without touching it. Identify the distal end of the cremaster, and place a stay suture here to slightly extend the muscle. Resect now remaining soft tissue beneath the muscle sack by gently pulling the muscle caudally and anteriorly.
16. After complete separation from connective tissue, open the cremaster sack at one point on the cranial edge using scissors. Extend the incision from this opening down to distal end of the muscle.
17. Seal the small vessel connecting the testicle and the cremaster using thermocautery, thereafter cut the remaining connections between the muscle and the testicle using scissors.
18. Lay aside the testicle, out of the microscopy field.
19. The opened cremaster now lays flat on the stage. Place four 6-0 Prolene stay sutures at the edges of the tissue and fix them with elastic drape in order to spread the cremaster muscle tissue. From now on continuously moisten the tissue with Phosphate-buffered saline (PBS) using a 1 ml syringe (**Figure 1**).
20. Allow now the preparation to rest for 15 min. Thereafter inject 0.05-0.1 ml of 1% Rhodamine-Dextran via the left femoral artery for contrast enhancement of the microvasculature. If any chemokines or inflammatory agents are planned for application, you should now topically moisten the muscle with the respective reagent using a 1 ml syringe. In the case of a control experiment, continue moistening with PBS.

2. Intravital Microscopy

1. Keep the mouse fixed on the operation stage and move under a fluorescence microscope modified for epi-illumination and connected to a CCD (charge-coupled device) video camera. Take special care not to dislocate the femoral artery catheter.
2. Conduct experiments using a water immersion objective.
3. After adjusting the objective for sufficient visualization of the cremaster microcirculation and before cell injection, stochastically define six postcapillary venules for later analysis of firm stem cell adherence.
4. Administer fluorescent-labeled stem cells, dissolved in 200 µl PBS, at 0.4 x 10^6 cells per injection, with a total of five consecutive injections utilizing the femoral artery catheter and a 1 ml syringe. Carefully shake the cell sample prior to the first injection.
5. Record stem cell rolling immediately after cell injection, thereby defining "rolling" as a more than 50% reduction of cell velocity along the endothelial lining in combination with typical cellular "stick and release" movements. Perform counting of rolling stem cells and all stem cells passing a predefined venular distance during 1 min of observation following every single injection. The amount of stem cell rolling is expressed as percentage of all passing cells. Do not count cells exhibiting random brief tethering phenomena as rolling cells.

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6. Following the last cell injection determine the number of firmly adherent stem cells by counting and express the amount in relation to the calculated endothelial surface of the six predefined venules (diameter x length x π) as adherent cells/mm^2 . Firm adhesion is considered when no cell movement is detectable for 30 sec.
7. After completion of the intravital microscopy carefully remove the cremaster muscle and store it in correspondence to later analysis (immunohistochemistry or molecular biology). Sacrifice the animal by cervical dislocation. The analysis of intravital microscopy recordings for quantification of stem cell rolling and firm endothelial adhesion, as well as microvascular hemodynamics, such as red blood cell velocity (mm/sec), vessel length (μm), vessel diameter (μm), wall share rate (sec^{-1}); should be performed offline by an investigator blinded to the respective experimental tissue treatment utilizing an image processing software.

Representative Results

In general, interaction of directly injected stem or progenitor cells with the vascular endothelium within the cremaster muscle microcirculation is a rare event and occurs exclusively in postcapillary venules (diameter: 30-80 μm). Due to fluorescence labeling rolling and firmly adherent stem cells can be clearly quantified in separation from circulating endogenous leucocytes in the observed venules (**Figure 2**).

The microcirculatory conditions represented by the blood flow velocity and wall shear rate usually do not differ significantly between the respective experimental groups with different chemokine treatment (**Table 1**).

Local treatment of the cremaster muscle tissue with different chemokines or mediators of inflammatory response, *e.g.* SDF-1α (stromal cell-derived factor-1 alpha) or TNF-α (tumor necrosis factor-alpha) is capable to mimic specific microenvironmental conditions. Such conditions enhance stem cell-endothelial cell interactions in a different amount, depending on the specific chemokine/mediator applied and the stem cell population injected (**Figure 3**)[12]. The possibility to clearly quantify these differing patterns of stem cell-endothelial cell interactions enables for evaluation of the migratory potential of specific stem cell populations within defined microenvironments *in vivo* prior advancing these populations to further clinical testing.

Furthermore, the impact of certain pathophysiological conditions affecting the endothelial function, such as nitric-oxide deficiency, has been shown by the use of knockout animals[9].

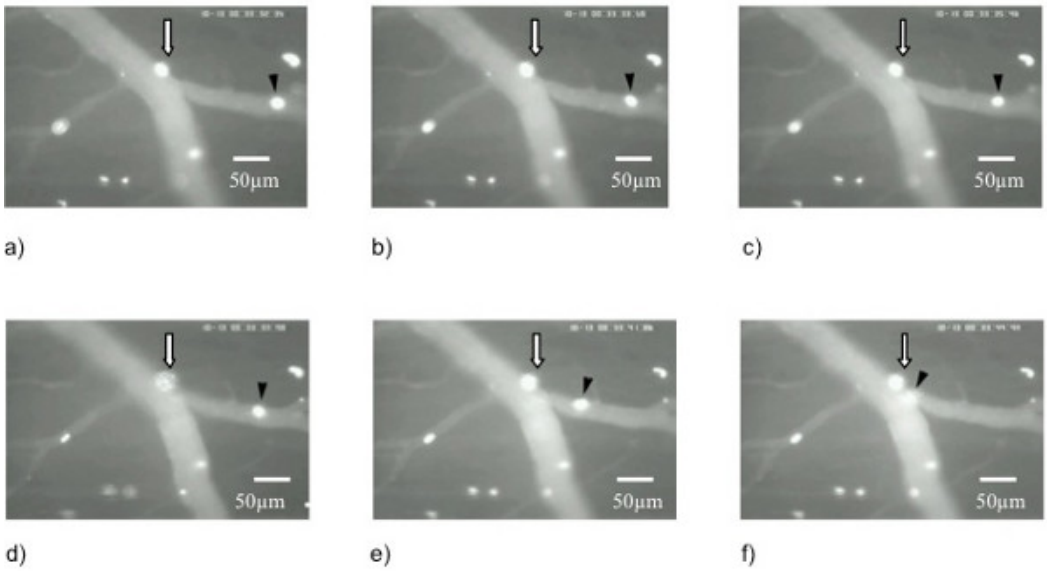


Figure 1. Illustration of the key microsurgical steps (a-f) in mouse cremaster muscle preparation for subsequent intravital microscopy.

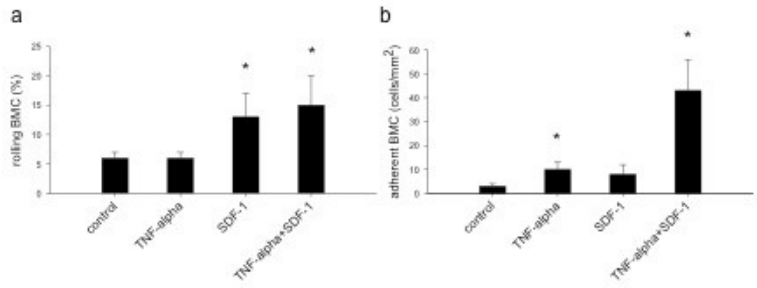
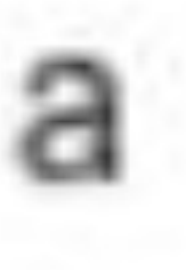
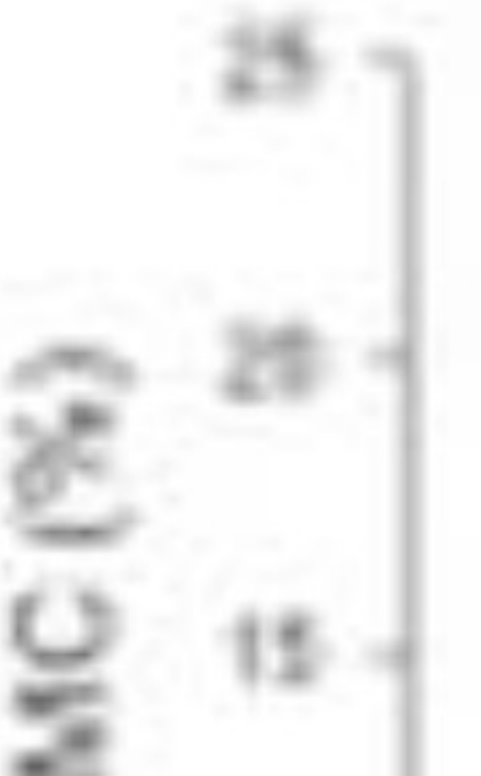


Figure 2. Intravital microscopy of c-kit$^+$ cells in a murine cremaster muscle preparation. Intravascular background is provided by rhodamin-dextran. (**a-f**) A series of six consecutive images with rolling (black arrows) and firmly adherent (white arrow) carboxy-fluorescein d-acetate succinimidylester- labeled c-kit$^+$ cells in the venular vasculature. *Lab Invest*, 2008, Vol 88. Kaminski, A., Ma, N., Donndorf, P. *et al*.





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Mouse Type	Experimental Group	red blood cell velocity (mm/sec)	wall shear rate (sec^{-1})
Wild type	Control	0.7±0.2	43±32
Wild type	SDF-1α	0.5±0.2	51±31
Wild type	TNF	0.4±0.2	45±24
Wild type	SDF-1 α +TNF α	1.0±0.5	117±26

Table 1. Red blood cell velocities and wall shear rates in venules of murine cremaster muscles at baseline. Analysis was performed using CapImage Software (Zeintl, Heidelberg, Germany) in muscle preparations of wild-type mice after treatment with SDF-1α, TNF-α. Data are expressed as mean ± s.d. Differences between the individual groups were not found being significant. *Lab Invest,* 2008, Vol 88. Kaminski, A., Ma, N., Donndorf, P. *et al*.

Discussion

Intravital fluorescence microscopy allows for direct visualization and quantitative analysis of stem cell-endothelium interactions. The cremaster muscle model is particularly useful, since microsurgical exposure and techniques intravital visualization have been well established during the research on leucocyte-endothelium interactions and various chemical components can be selectively applied to the target tissue by simple superfusion technique. Due to the absence of movement artifacts and severe image noise, this particular model, in comparison to other intravital-microscopy settings, provides optimal conditions for visualization of cell-cell interactions.

The model allows revealing essential microenvironmental preconditions, for initial stem cell recruitment to take place in a living organism. If required, endogenous leucocytes can serve as positive controls for validation and prove of principle of stem cell behavior.

Representing an acute animal model and utilizing direct arterial injection instead of venous application, the potential limitations of chronic cell migration models and/or systemic cell delivery - *e.g.* cell loss due to entrapment within remote organs - can be avoided. On the other hand, acute surgical tissue trauma is likely to induce a certain degree of tissue activation itself. Therefore, an atraumatic surgical preparation is mandatory and stable microcirculatory conditions following cremaster muscle preparation need to be achieved in a reproducible fashion prior starting with experimental groups.

The presence of extensive leucocyte accumulation within the postcapillary venules and significant leakage of the fluorescent dye to the surrounding tissue at the beginning of the intravital microscopy recordings in control animals indicate major surgical tissue trauma.

However, even when applying meticulous preparation techniques, results derived from different investigators are not necessarily completely comparable due to slightly differing baseline tissue activation. This is why subsequent experiments should be performed by the same investigator whenever possible.

Designed for the analysis and quantification of the initial steps of stem cell recruitment to target tissues, the current model does not allow statements on definite stem cell organ-engraftment.

Disclosures

All animal experiments, conducted for this manuscript have been approved by the local animal care and use committee.

The authors declare that they have no competing financial interests.

References

1. Madlambayan, G.J. *et al.* Bone marrow stem and progenitor cell contribution to neovasculogenesis is dependent on model system with SDF-1 as a permissive trigger. *Blood.* **114**, 4310-4319, doi:10.1182/blood-2009-03-211342 (2009).
2. Lapidot, T., Dar, A., & Kollet, O. How do stem cells find their way home? *Blood.* **106**, 1901-1910 (2005).
3. Lehr, H.A., Vollmar, B., Vajkoczy, P., & Menger, M.D. Intravital fluorescence microscopy for the study of leukocyte interaction with platelets and endothelial cells. *Methods Enzymol.* **300**, 462-481 (1999).
4. Gavins, F.N. & Chatterjee, B.E. Intravital microscopy for the study of mouse microcirculation in anti-inflammatory drug research: focus on the mesentery and cremaster preparations. *J. Pharmacol. Toxicol. Methods.* **49**, 1-14, doi:10.1016/S1056-8719(03)00057-1 (2004).
5. Leister, I. *et al.* A peritoneal cavity chamber for intravital microscopy of the liver under conditions of pneumoperitoneum. *Surg. Endosc.* **17**, 939-942, doi:10.1007/s00464-002-9082-5 (2003).
6. Sriramarao, P., Anderson, W., Wolitzky, B.A., & Broide, D.H. Mouse bone marrow-derived mast cells roll on P-selectin under conditions of flow *in vivo*. *Lab Invest.* **74**, 634-643 (1996).
7. Baez, S. An open cremaster muscle preparation for the study of blood vessels by *in vivo* microscopy. *Microvasc. Res.* **5**, 384-394 (1973).
8. Bagher, P. & Segal, S.S. The mouse cremaster muscle preparation for intravital imaging of the microcirculation. *J. Vis. Exp.* doi:10.3791/2874 (2011).
9. Kaminski, A. *et al.* Endothelial NOS is required for SDF-1alpha/CXCR4-mediated peripheral endothelial adhesion of c-kit+ bone marrow stem cells. *Lab Invest.* **88**, 58-69, doi:10.1038/labinvest.3700693 (2008).
10. Furlani, D. *et al.* HMGB-1 induces c-kit+ cell microvascular rolling and adhesion via both toll-like receptor-2 and toll-like receptor-4 of endothelial cells. *J. Cell Mol. Med.* **16**, 1094-1105, doi:10.1111/j.1582-4934.2011.01381.x (2012).

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11. Furlani, D. *et al.* Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc. Res.* **77**, 370-376, doi:10.1016/j.mvr.2009.02.001 (2009).
12. Donndorf, P. *et al.* Analysing migratory properties of human CD 133(+) stem cells *in vivo* after intraoperative sternal bone marrow isolation. *Cell Transplant.* doi:10.3727/096368912X657729 (2012).

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