**In vivo visualization of uterine mast cells by two-photon microscopy**

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**Abstract**

Transgenic mice expressing fluorescent proteins in specific cell populations are widely used for the study of *in vivo* behavior of these cells. We have recently reported that uterine mast cells (uMCs) are important for implantation and placentation. However, their *in vivo* localization in uterus before and during pregnancy is unknown. Herein, we report the direct observation of uMCs *in vivo* using double-transgenic C57BL/6j Mcpt5-Cre ROSA26-EYFP mice with high expression of enhanced yellow fluorescent protein in MC protease 5 (Cma1 (Mcpt5))-expressing cells by intravital two-photon microscopy. We were able to monitor MCs live *in utero* during the murine estrous cycle and at different days of pregnancy. We demonstrated that uMCs accumulated during the receptive phase of the female (estrum) and persisted in large numbers at early pregnancy stages and around mid-gestation and declined in number in non-pregnant animals at diestrus. This intravital microscopy technique, including a custom-made microscope stage and the adaption of the surgical procedure, allowed the access of the uterus and implantations for imaging. The introduced application of intravital microscopy to C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice offers a novel and powerful *in vivo* approach to further address the evident relevance of uMCs to reproductive processes with obvious clinical implications.

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**Introduction**

Besides the well-documented role of mast cells (MCs) in IgE-mediated allergic responses (Galli & Tsai 2012), MCs appear now as key effector cells influencing a variety of physiological as well as pathophysiological processes (Bischoff 2009, Anand et al. 2012). Not only do they contribute to innate and adaptive immune responses (Galli et al. 2005, St John & Abraham 2013), but also they are directly involved in non-immunological processes including tissue remodeling and angiogenesis (Varayoud et al. 2004, Bosquiazzo et al. 2007, Theoharides et al. 2010). Their effects are mainly mediated by granule-stored mediators such as metalloproteases, MC-specific proteases, tissue plasminogen activator (tPA), vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) (Galli et al. 2005) and galectin 1 (Gal1, Woidacki et al. 2013) that are released upon activation. We have recently reported that MCs migrate from the periphery to the uterus responding to hormonal fluctuations (Jensen et al. 2010). This has already suggested their importance for pregnancy, a period characterized by great hormonal changes. In a mouse model, Kit (c-Kit) deficiency, which is associated with a lack of MCs including uterine MCs (uMCs), was related to impaired implantation and placentation that negatively influenced fetal growth (Woidacki et al. 2013). We found these effects to be mediated by Gal1 as bone marrow-derived MCs (BMMCs) from WT mice but not from Lgals1 (Gal1)-deficient mice could rescue the impaired reproductive phenotype (Woidacki et al. 2013). The *in vivo* behavior of MCs at the fetal–maternal interface is, however, largely unknown. As there were some controversial reports on the role of MCs in pregnancy (Salamonsen et al. 1996, Varayoud et al. 2004, Menzies et al. 2012, Woidacki et al. 2013), it is of vital importance to localize uMCs at the fetal–maternal interface and to later dissect their behavior and interaction with other cells live.

Transgenically modified mice with fluorescent-labeled MCs using MC-specific Cre recombinase/loxP-mediated recombination are very useful for the
visualization of MCs in vitro but particularly in vivo (Luche et al. 2007, Scholten et al. 2008). In 2001, the production of Cre reporter alleles that express enhanced yellow fluorescent protein (EYFP), a color variant of the green fluorescent protein, was described (Srinivas et al. 2001). In this mouse model, EYFP was inserted into the ubiquitously expressed ROSA26 locus, framed by a loxP-flanked stop sequence. Crossing these ROSA26-EYFP strains with transgenic strains expressing Cre either in a ubiquitous or in a cell-specific pattern would now allow monitoring Cre activity in living tissues (Srinivas et al. 2001). Scholten et al. (2008) were the first to describe the combination of the Mcpt5-Cre transgenic mouse line crossed with the ROSA26-EYFP Cre excision reporter strain. Due to the lack of Mcpt5-Cre transgene expression in MCs of the mucosal type, EYFP signal was detected exclusively in connective tissue-type MCs (Dudeck et al. 2011) and the serine protease Mcpt5 showed unique tissue specificity for connective tissue-like MCs (Lützelschwab et al. 1998).

Two types of MCs are known: mucosal and connective tissue-type MCs. This classification is based on phenotypic differences rather than on their tissue localization (Metcalfe et al. 1997, Schwartz & Huff 1998). Additionally, for rodents, a third population is described. These cells show characteristics of both mucosal and connective tissue-type MCs (Schwartz & Huff 1998, Michaloudi & Papadopoulos 1999, Woidacki et al. 2013). Unfortunately, to the best of our knowledge, no model is currently available in which both types of MCs can be visualized in vivo. Although the percentage of Mcpt5-expressing MCs from isolated uterine cells ranged between 5 and 20% (Woidacki et al. 2013), double-transgenic C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice with high expression of EYFP in Mcpt5-expressing cells emerge as a powerful tool for the visualization of uMCs live and in vivo by two-photon microscopy. Here, the simultaneous absorption of two photons, which precedes the emission of the fluorescence, is harmless to the tissue, avoids photo-bleaching and enables a better tissue penetration (Paoli et al. 2009). Based on these methodological advantages, two-photon microscopy became a preferred technique for intravital imaging (Zipfel et al. 2003, Olivieri & Tadokoro 2013). Until recently, the visualization of cells in vivo at the fetomaternal interface was hampered by the fact that in vivo microscopy was not established for the uterus or the placenta. Recently, we have succeeded in visualizing the fetomaternal interface in vivo by modifying the technique for intravital imaging (Zenclussen et al. 2012, 2013).

In this study, the application of this intravital technique to C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice provided direct observations of MC presence and localization within the virgin and pregnant mouse uteri live and in vivo. This information opens up vast new possibilities to understand the behavior and functional relevance of uMCs in health and disease.

Materials and methods

Animals

Mcpt5-Cre mice (Scholten et al. 2008) were crossed with R26 StopFLOX-EYFP reporter mice (Srinivas et al. 2001). Cre reporter strains were produced by targeted insertion of EYFP and ECFP into the ROSA26 locus, which express EYFP in cells with a deleted loxP-flanked stop element. These mice were then housed at the Central Animal Facility at the Otto-von-Guericke University Magdeburg, Medical Faculty, with a 12 h light:12 h darkness cycle. In all experiments, mice at the age of 10–16 weeks were used to avoid age-dependent influences on their reproductive phenotype (Talbott 1971, Parkening et al. 1978, Holinka et al. 1979). All procedures were in accordance with the institutional guidelines of Magdeburg, and the study was previously approved by the Landesdirektion Dessau (42502-2-978 Magdeburg). This is in agreement with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, USA.

A vaginal lavage was performed to determine the exact state of the estrous cycle by analyzing the content and type of cells in the vaginal smear. For this, the vagina was gently washed with 20 μl 0.9% sodium chloride solution (Berlin-Chemie, Berlin, Germany). For identification of the state of the estrous cycle, the cells were distributed on an object slide and analyzed by light microscopy with a total magnification of 200× (ECLIPSE-S, Nikon, Düsseldorf, Germany). For in vivo imaging of the virgin uterus, we focused on diestrus and estrus as we previously found these two phases to be the time points with major differences in the number of MCs between each other (Woidacki et al. 2013). For in vivo imaging of the pregnant uterus, C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice were mated allogeneically with BALB/c males. The day of vaginal plug was set as day 0 of pregnancy. Pregnant females were separated from the males upon plug detection. In vivo imaging of the uterus was performed on gestational days 0, 2, 5, 8, 10 and 14.

Processing of the uterus/implantations for the two-photon microscopy technique

Animals were anesthetized by s.c. injection of ketamine (CP-Pharma, Burgdorf, Germany) (125 μg/g body weight of mouse) and Rompun (Bayer Health Care, Bayer Vital GmbH) (50 μg/g body weight of mouse). After s.c. anesthetization, mice were ventilated through a tracheal tube with a mechanical small animal respirator (Mini-Vent, Hugo Sachs Elektronik, March-Hugstetten, Germany) receiving a mixture of 0.6 vol% isoflurane (Baxter, Unterscheiβheim, Germany) and oxygen for narcosis using a Univentor 400 Anaesthesia Unit (Univentor, Zejtun, Malta).

Animals were kept on top of a heating pad at 37 °C and hairs at the abdomen were removed using depilatory cream (Veet, Mannheim, Germany). Fluorescence labeling of blood vessels was done by retro-orbital injection of 100 μl Rhodamine B (10 mg/ml, Sigma–Aldrich) or 12.5 μl QTracker 655 nontargeted QDots (Invitrogen) dissolved in 80 μl aqua ad injectabilia (Berlin-Chemie). The uterus could be accessed by a lateral section at the abdomen. The female mouse and its uterine horns were stabilized on a microscope stage (custom-made design,
was maintained throughout the experiment. Caffeine was provided continuously in dissolved form using caffeine-soaked cotton. We have shown previously that the addition of caffeine can prevent uterine muscle contraction without any effect on blood circulation (Zencussen et al. 2013). Low-melting agarose (2% in PBS, Peqlab, Erlangen, Germany) was carefully placed around and above the whole uterus to keep the organ at physiological temperatures. After preparation, a heater with a glass-covered opening was placed on top. The temperature probe connected to this heater was placed in very close proximity to the organ where a temperature of 37 °C was maintained throughout the experiment.

**Imaging of uMCs by two-photon microscopy**

For imaging of the uterus, two-photon microscopy was performed using a Zeiss LSM-710 microscope (Carl Zeiss, Jena, Germany) with simultaneous detection via three external non-descanned detectors. The microscope was equipped with a MaiTai TiSa laser (Newport/Spectra-Physics, Darmstadt, Germany) on an Axio Examiner upright stage and a water-dipping lens (NA 1.0, magnification ×20). Illumination was performed at a wavelength of 850 or 950 nm. EYFP-positive MCs were detected at 500–550 nm emission. Blood vessels were labeled either with Rhodamine B and detected with a bandpass filter of 565–610 nm or with QTracker 655 nontargeted QDots detected at 640–710 nm. Tissue structure was detected by its second-harmonic generation (SHG) signal below 480 nm based on the non-centrosymmetric structure of collagen (Williams et al. 2005). Images were recorded as z-stacks overarching the whole uterine horn with 3 μm Z-spacing using Zen software 2009 (Carl Zeiss). For 3D reconstruction, Volocity software (Perkin Elmer/Improvision, Waltham, MA, USA) was used. Images are representative for three observations in total.

**Quantification of uMCs**

The number of MCs was determined in representative z-stacks of uteri from virgin females either at diestrus or estrus of the estrous cycle (n = 5 each) or during pregnancy (days 0, 2, 5, 8, 10 and 14) (n = 3 per stage) using Zeiss LSM Image Browser software (Carl Zeiss MicroImaging GmbH). The mean number of MCs was calculated as the total number of MCs per recorded z-layer of one z-stack. Statistical analysis was performed by ANOVA test, followed by post hoc Tukey's multiple comparison test.

**Results**

**Two-photon microscopy represents a powerful tool for the visualization of uMCs live and in vivo**

We first visualize uMCs intravitaly in surgically exposed virgin and pregnant murine uteri. Application of two-photon laser scanning microscopy allowed the live imaging of uMCs up to depths of ~200 μm in double-transgenic C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice, depending on biological divergences such as pregnancy stage and technical implementation. Compared with the methods applied to fixed tissue, the intravital microscopy technique allowed us to detect uMCs live.

Caffeine was used for reducing the intensive uterine contractions at a concentration of 20 mg/ml. Its application allowed high-quality recording of images, 3D renderings and videos. However, day 14 of pregnancy represented the maximum limit for imaging because at later pregnancy stages not only the uterine contractions but also fetal movement are present, and it becomes impossible to reduce fetal and uterine movements without a surgical manipulation of the uterus.

For imaging of the highly blood-perfused, sensitive and contracting uterus, tissue immobilization was evaluated by a new surgical technique. This was performed in accordance with the procedures described previously (Zencussen et al. 2013). One horn of the murine uterus bicornis was carefully exposed (Fig. 1A), fixed by a self-made aluminum foil fixator and permanently embedded in caffeine-soaked cotton rolls (Fig. 1B) to reduce uterine contractions. As MCs are mainly located in the uterus rather than in placental tissue (Woidacki K, Zencussen AC, 2010; unpublished observations), we avoided damage to the uterine wall, a procedure shown to be beneficial for imaging of the placenta (Zencussen et al. 2012). Therefore, the focus was on the mesometrial side of the intact uterus (Fig. 1C) and one implantation (Fig. 1D), with special emphasis on the zone between the uterus and the decidua basalis (Fig. 1D). The permanent moistening of uterine tissue was assured by the addition of low-melting agarose. The implantation to be analyzed was covered by the heating plate containing an opening that allowed the access to the zone of interest (Fig. 1E).

Monitoring of vitality after surgery and during intravital imaging was assured by visualization of the blood flow. In our first experiments, different laser wavelengths were tested to excite simultaneously both EYFP and blood flow. In further preliminary works, Rhodamine B, a red fluorescent blood dye, was used. Implementation of both criteria – simultaneous excitation of EYFP and Rhodamine B at the maximum with one certain wavelength and usage of higher and therefore more tissue-gentle and harmless wavelength – revealed that Rhodamine B was not optimal for our setup (data not shown). Therefore, QTracker 655 nontargeted QDots were administrated by retro-orbital injection which allowed the visualization of both blood flow and MCs during the whole imaging process using deep red filter with an absorption wavelength between 640 and 760 nm for detection of emission of QDots. Visualization of uMCs, blood flow and SHG signal at once was then facilitated by excitation at a wavelength of 950 nm. These fine-modulated settings allowed us to monitor MCs live and in vivo within the virgin as well as...
prenant uteri of C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice in which Mcpt-5-expressing MCs are positive for EYFP. This is the first report not only in visualizing the pregnant decidua live at different days but also in reporting the presence of MCs in the uterus of a live mouse, confirming the presence of these cells at the fetal–maternal interface.

**MCs are present in the virgin uterus and accumulate during the receptive phase of the estrous cycle, the estrus**

By using intravital microscopy together with two-photon microscopy, we could confirm in vivo that uMCs are mainly located in close proximity to blood vessels (Figs 2A, B and 3A, B, C, D, E and F). Furthermore, this method allowed the assessment of the quantitative distribution of uMCs along the murine estrous cycle (Fig. 2A and B). By using flow cytometry and histology, we have recently reported that the number of MCs peaked during the receptive phase of the female (estrus, Figs 2A and 4) and their number decline at diestrus (Figs 2B and 4), but also show their density and localization. In previous studies, we could detect uMCs in longitudinally cut uterine sections distributed inter-implantational and in close proximity to the developing fetus (Woidacki et al. 2013). By using intravital microscopy for detection of EYFP-positive uMCs in C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice, we could not only confirm that uMCs accumulate during the receptive phase of the female (estrus, Figs 2A and 4) and their number decline at diestrus (Figs 2B and 4), but also show their density and localization. In previous studies, we could detect uMCs in longitudinally cut uterine sections distributed inter-implantational and in close proximity to the developing fetus (Woidacki et al. 2013). In this study, we observed that uMCs were additionally distributed in large numbers at the mesometrial site of the uterus that is in close proximity to the decidua basalis (Fig. 1D).

**uMCs are abundant during early pregnancy and their frequency declines as gestation advances**

To analyze the presence, distribution and density of uMCs during pregnancy, we concentrated first on days 0, 2 and 5 of the pre- and peri-implantation phase and later on days 8, 10 and 14 of the post-implantation phase. Uterine tissues at all analyzed pregnancy stages are characterized by remarkably large numbers of Mcpt-5-expressing MCs (Figs 3A, B, C, D, E, F and 4). During the pre-implantation phase (days 0 and 2) when the uterus is preparing for the nidation of the blastocyst (Wang & Dey 2006), an accumulation of uMCs at the mesometrial uterine site was evident (Figs 3A, B and 4). During the implantation process at gestational day 5, when the blastocyst is in close physical and physiological contact...
to the uterine endometrium (Dey et al. 2004), uMCs were present in comparable numbers as in non-pregnant uteri at estrus albeit this did not reach significance levels when compared with diestrus (Figs 3C and 4). Around day 8 of gestation when placentation starts (Rugh 1990) and spiral arteries appear histologically within the decidua basalis (Burke et al. 2010), uMCs are present (Fig. 3D) in slightly lower numbers than at early pregnancy (Fig. 4). At gestational day 10 when the placenta and the fetus are simultaneously growing, uMCs could be still detected mesometrially but their number was significantly lower than at estrus (Figs 3E and 4). At mid-gestation (day 14 of pregnancy) when the main focus is on fetal rather than on placental development (Suzuki et al. 1997), uMCs are present but in significantly lower numbers when compared with estrus (Figs 3F and 4).

**Discussion**

The *in vivo* adaptation of two-photon microscopy for monitoring MC distribution at the fetal–maternal interface offers a novel, impressive and powerful approach to address the evident relevance of uMCs to reproductive processes. The application of two-photon microscopy to transgenic mouse models increases exponentially (Zipfel et al. 2003). Of interest for the reproduction field, it has been recently reported that this methodology can be used for the tracking of dendritic cells (DCs; Zenclussen et al. 2013) and regulatory T cells (Teles et al. 2013) as well as for the imaging of the mouse placenta under physiological (Zenclussen et al. 2012) and pathophysiological conditions (De Moraes et al. 2013).

The generation of new mouse models whose MC deficiency is independent of restricted Kit gene expression (Musch et al. 2008, Dudeck et al. 2011, Feyerabend et al. 2011) initiated a continuing controversy about the scientific significance of the Kit-dependent models (Katz & Austen 2011, Brown et al. 2012) which were used for the investigation of MC biology *in vivo* during the last decades. The application of intravital microscopy to C57BL/6J-Mcpt5-Cre ROSA26-EYFP mice in which Mcpt5-producing MCs are detectable by the expression of EYFP (Scholten et al. 2008) represents a useful tool to further address the Kit-independent action of MCs and is of special interest for us.
We adapted the technique introduced by Zenclussen et al. (2013) to the specific area of the uterus in which MCs are mainly distributed. Besides, we also investigated pregnant uteri that constituted a special challenge because of the increasing contractions as pregnancy advances. Hence, we report that imaging of the uterus was possible using a custom-made holder without any further manipulation of the tissue. MCs accumulated at the receptive phase of the female, namely in estrus when the uterine tissue is prepared for the nidation of the blastocyst. A similar distribution pattern could be observed live for DCs which additionally showed a clustering in estrus (Zenclussen et al. 2013) and are known to be crucial for the implantation process (Plaks et al. 2008). The accumulation of MCs at estrus could affect the physical as well as physiological contacts between the blastocyst and the uterine epithelium. In this study, a remarkably large number of uMCs at the mesometrial site of the virgin and pregnant uteri at all pregnancy stages were located in close proximity to blood vessels. To date, MC distribution pattern in uterus was exclusively referred to histological dying techniques applied to fixed tissue (Gaytan et al. 1991, Karaca et al. 2008). In this study, we demonstrate that the number of MCs in the uterus is much higher as it was suspected. We can further confirm their localization close to vessels; this may be beneficial for the release of MC-specific mediators into the vasculature. Moreover, the immediate vicinity of MCs located at the mesometrial region to the feto-placental unit suggests an interaction with other immune cells such as uterine NK cells, which are located within the mesometrial lymphoid aggregate of pregnancy (MLap) as well as within the decidua basalis (Croy et al. 2006). This together with the observation that uMCs are additionally located inter-implantational (Woidacki et al. 2013) suggests a significant role of uMCs and MC-specific mediators in reproductive processes such as implantation, placentation and later spiral artery remodeling. Recently published data point out that their absence is associated with abnormal implantation, aberrant placentation and inadequate spiral artery remodeling (Woidacki et al. 2013).

During the pre-implantation phase, the uterine tissue undergoes tremendous remodeling processes as a reaction to the invasion of fetal-derived trophoblasts (Rugh 1990). MCs are best known for their broad array of granule-stored mediators. The majority of these mediators are directly or indirectly involved in tissue remodeling and angiogenic processes including matrix metalloproteases, tryptases, chymases, tPA, TGFβ, fibroblast growth factor and VEGF (Galli et al. 2005). During the implantation process when the trophoblast cells penetrate the uterine epithelium and decidualization starts, we found uMCs to be present and abundant at the mesometrial area of the uterus. In mice, the inner cell mass of the blastocysts is oriented mesometrially (Dey et al. 2004). The remarkably large amount of uMCs at the mesometrial site of the uterus before and during implantation strongly suggests that they exert a positive influence on the establishment of pregnancy. However, and in contrast to this concept, in 1996 a significant role for MCs in the process of implantation was excluded. This conclusion was based on a negligible amount of MCs found in uterine tissue (Salamonsen et al. 1996). Back then, the quantification of MCs was only possible ex vivo in fixed tissue by means of immunohistochemical techniques. The introduced visualization of numerous uMCs live and in vivo using intravital microscopy suggests that inadequate fixation procedures of the tissue could cause false results as suggested by El Sayed & Dyson (1993). We did not perform confocal microscopy of fixed or unfixed tissue of C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice to state whether this alternative method yields similar results as ours in in vivo observations. It is, however, evident from this study that MCs are present in significant numbers and at strategic sites at the fetal–maternal interface, which supports recent data on their important role for pregnancy establishment (Woidacki et al. 2013).

During the post-implantation phase, when placentation starts (day 8) (Rugh 1990) and spiral arteries appear within the decidua basalis (Burke et al. 2010), uMCs remained present in the uterus. Recently, we demonstrated that mice deficient in uMCs showed decreased placental surface areas and an inadequate remodeling of spiral arteries compared with WT C57BL/6j mice (Woidacki et al. 2013), whose uterine tissue is characterized by the presence of MCs at days 8 and 10 of pregnancy as shown in the present paper. Defective placental development as well as spiral artery formation cause fetal growth retardation (Ashkar et al. 2000, Song et al. 2002, Watson & Cross 2005) as both structures are crucial for maternal and fetal blood exchange, and therefore, for supplying the fetus with oxygen and nutrients. Even at day 14 of pregnancy when the fetus but not the placenta needs to grow (Suzuki et al. 1997), the amount of uMCs is still considerable although significantly lower when compared with that at estrus. These observations are indicative of the involvement of uMCs in pregnancy establishment rather than its maintenance. This may be due to the secretion of soluble factors contained in their granules. One important mediator was found to be Gal1. MCs deficient in Lgals1 were not efficient in restoring placental growth as MCs from WT animals did when transferred into Kit-deficient mice (Woidacki et al. 2013). It is, however, possible that other uMC mediators are involved in this late process, too. Moreover, based on our data (Woidacki et al. 2013), we speculated that the mucosal MC phenotype that is also present in uterine tissue plays a role in mediating pre-, peri- and post-implantation events. Thus, the unique uterine micromilieu during pregnancy might influence the distribution/expression balance between both the MC phenotypes. The detection of uMCs that undergo a
transdifferentiation process (Woidacki et al. 2013) might support this assumption.

Although the detected EYFP-positive cells represent connective tissue-type MCs based on the expression of the serine protease Mcpt-5 (Dudeck et al. 2011) and uMCs display a unique population composed of mucosal, connective tissue-type and a third transdifferentiation cell-type MCs (Spicer 1960, Woidacki et al. 2013), C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice are a valuable and reliable model for studying uMC behavior live and in vivo. To the best of our knowledge, except one alternative model that is based on a cross of a ROSA26-tdRFP reporter strain (Luche et al. 2007) with the Mcpt5-Cre transgenic mouse strain (C57BL/6j-Mcpt5-Cre Rosa26-tdRFP) (Roers A 2012, personal communication), no other MC-specific reporter strains are available.

During the imaging process, uterine contractions, which would have negatively influenced the quality of pictures and movies, were reduced to a minimum by applying caffeine-soaked cotton rolls that ensured a permanent drenching of the uterus. Former problems of intravital technology concerning the amenability of the organ could be resolved by performing handling modifications of the animal and uterus respectively. According to the applied imaging procedures and a custom-made microscope stage, intravital images of placenta (Zenclussen et al. 2012) and uterus (Zenclussen et al. 2013) emerged as an adequate appliance for the basic fixation of the uterus for analyzing uMCs. A self-made fixator composed of tear-resistant aluminum foil served as a fine fixation for the uterus. The addition of low-melting agarose allowed a constant and long-lasting moistening of the focal area.

The application of two-photon microscopy to C57BL/6j-Mcpt5-Cre ROSA26-EYFP mice represents a novel and exciting approach to track MC behavior in vivo. It could be a powerful tool to bring to light the so-far controversial discussed influence of MCs on reproductive processes and pregnancy. We do hope that the introduced visualization of uMCs might help in the future for addressing elementary questions regarding MCs and their participation in physiological and pathophysiological processes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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