THE DORSAL SKINFOLD CHAMBER: WINDOW INTO THE DYNAMIC INTERACTION OF BIOMATERIALS WITH THEIR SURROUNDING HOST TISSUE

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Abstract

The implantation of biomaterials into the human body has become an indispensable part of almost all fields of modern medicine. Accordingly, there is an increasing need for appropriate approaches, which can be used to evaluate the suitability of different biomaterials for distinct clinical indications. The dorsal skinfold chamber is a sophisticated experimental model, which has been proven to be extremely valuable for the systematic *in vivo* analysis of the dynamic interaction of small biomaterial implants with the surrounding host tissue in rats, hamsters and mice. By means of intravital fluorescence microscopy, this chronic model allows for repeated analyses of various cellular, molecular and microvascular mechanisms, which are involved in the early inflammatory and angiogenic host tissue response to biomaterials during the initial 2-3 weeks after implantation. Therefore, the dorsal skinfold chamber has been broadly used during the last two decades to assess the *in vivo* performance of prosthetic vascular grafts, metallic implants, surgical meshes, bone substitutes, scaffolds for tissue engineering, as well as for locally or systemically applied drug delivery systems. These studies have contributed to identify basic material properties determining the biocompatibility of the implants and vascular ingrowth into their surface or internal structures. Thus, the dorsal skinfold chamber model does not only provide deep insights into the complex interactions of biomaterials with the surrounding soft tissues of the host but also represents an important tool for the future development of novel biomaterials aiming at an optimisation of their biofunctionality in clinical practice.

Keywords: Dorsal skinfold chamber, biomaterials, inflammation, angiogenesis, intravital fluorescence microscopy, *in vivo*, implantation, tissue engineering.

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Introduction

Recent progress in the fields of material science, biotechnology and tissue engineering has resulted in a tremendous increase in the introduction of different biomaterial devices in pharmacy and medicine. These include drug delivery systems (Svirskis *et al.*, 2010), metallic implants (Ryan *et al.*, 2006), prosthetic vascular grafts (Roll *et al.*, 2008), bone and cartilage substitutes (Busenlechner *et al.*, 2008; Berghaus *et al.*, 2010), synthetic surgical meshes (Shankaran *et al.*, 2011) and scaffolds for the engineering of artificial tissues (Kim *et al.*, 2010). However, a riskless and successful use of such devices in clinical practice is only possible, if they exhibit an adequate biocompatibility. This means that they should not induce a severe local or systemic inflammatory reaction. On the other hand, they should exhibit an optimal biofunctionality, i.e., the ability to perform the specific task for which they are intended (Morais *et al.*, 2010). For the development of novel devices fulfilling these preconditions there is an increasing need for appropriate technologies and assays, which allow for the systematic evaluation of the interaction of implanted biomaterials with the host tissue.

This interaction is a highly dynamic process, which is subsumed under the term 'foreign body reaction' and comprises distinct steps (Anderson *et al.*, 2008). Immediately after implantation into the body, a layer of adsorbed host proteins forms onto the surface of a biomaterial, which critically influences the subsequent cellular reaction to the implant (Gray, 2004; Thevenot *et al.*, 2008). In the acute phase, this reaction is characterised by the activation of leukocytes, which infiltrate the implantation site and produce various cytokines and growth factors (Keselowsky *et al.*, 2007; Velard *et al.*, 2010). Moreover, monocytes are recruited from the blood stream and start to differentiate into macrophages (Kou and Babensee, 2011). Persistent inflammatory stimuli may result in a chronic foreign body reaction. This is associated with the accumulation of lymphocytes and fusion of individual macrophages to multinucleated giant cells, which are typically found at the interface between the implanted biomaterial and the surrounding host tissue (Xia and Triffitt, 2006). In addition, fibroblast proliferation and migration as well as development of new blood vessels results in the formation of a vascularised granulation tissue or a fibrous capsule around the implant, determining its final incorporation at the implantation site (Morais *et al.*, 2010).

Because a foreign body reaction involves a broad range of different cell types, cytokines and growth factors, it is

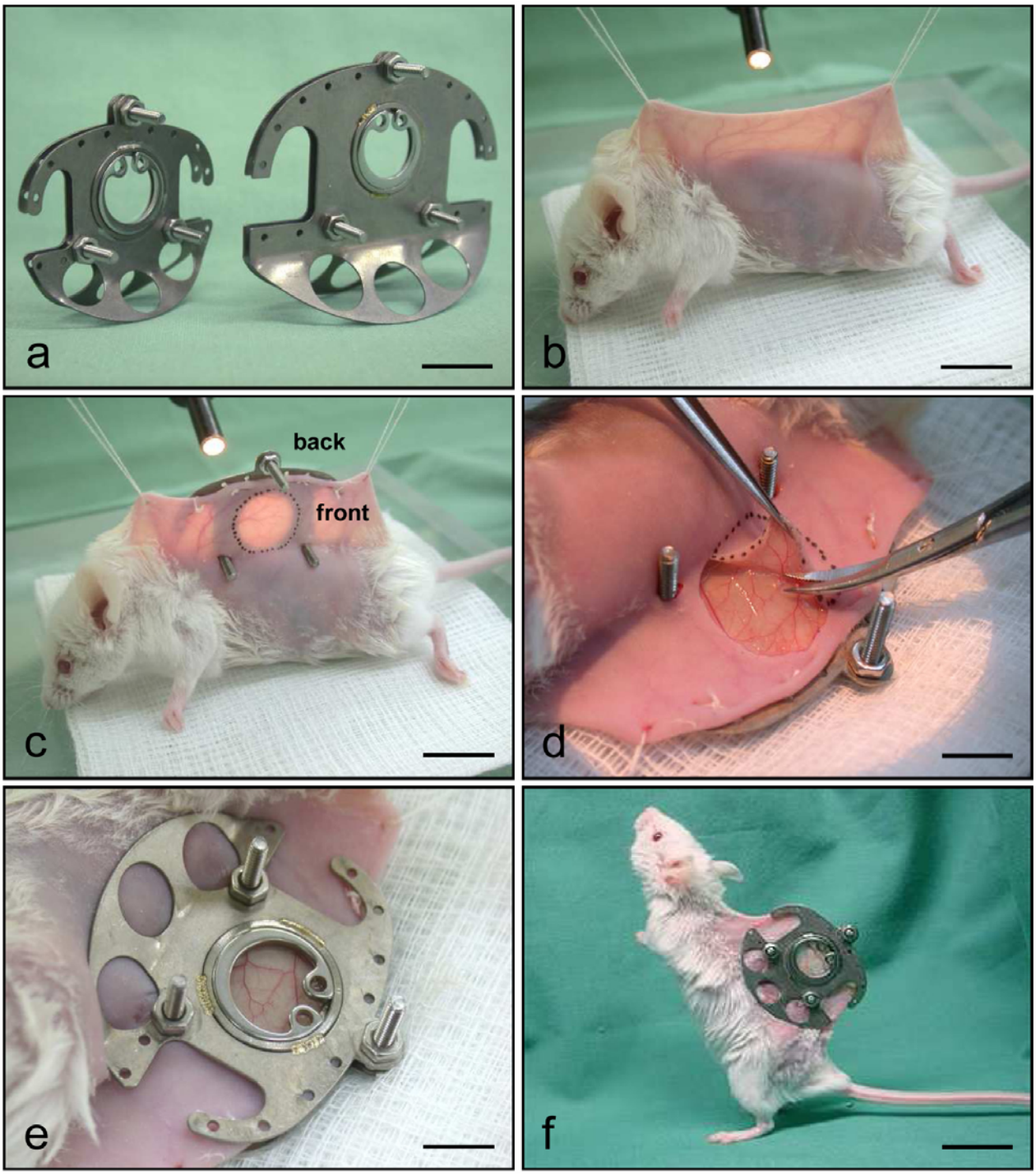

Fig. 1. (a) Window chambers consisting of two symmetrical titanium frames for insertion into the dorsal skinfold of mice (chamber on the left side, weight: ~2 g) and Syrian hamsters or rats (chamber on the right side, weight: ~4 g). **(b-e)** Stepwise preparation of a dorsal skinfold chamber in an anaesthetised balb/c mouse. **(f)** The animal tolerates the chamber well, as indicated by normal orientation behaviour. Scale bars: **(a)** = 14 mm; **(b, c)** = 16 mm; **(d, e)** = 8 mm; **(f)** = 19 mm.

not possible to simulate adequately this complex process under *in vitro* conditions or via computer. Accordingly, the *in vivo* analysis of biomaterials by means of sophisticated animal models represents an essential prerequisite for the successful development of novel biomedical devices and their establishment into clinical practice. In the following, this review will focus on the dorsal skinfold chamber model. By means of intravital fluorescence microscopy, this chronic model has been broadly used during the last two decades for the repetitive *in vivo* analysis of various biomaterials and, thus, has provided deep insights into the mechanisms regulating the angiogenic and inflammatory host tissue response to implanted biomaterials (Menger *et al.*, 1990a; Kraft *et al.*, 2000; Laschke *et al.*, 2005a; Rücker *et al.*, 2006; Laschke *et al.*, 2007; Wieghaus *et al.*, 2008).

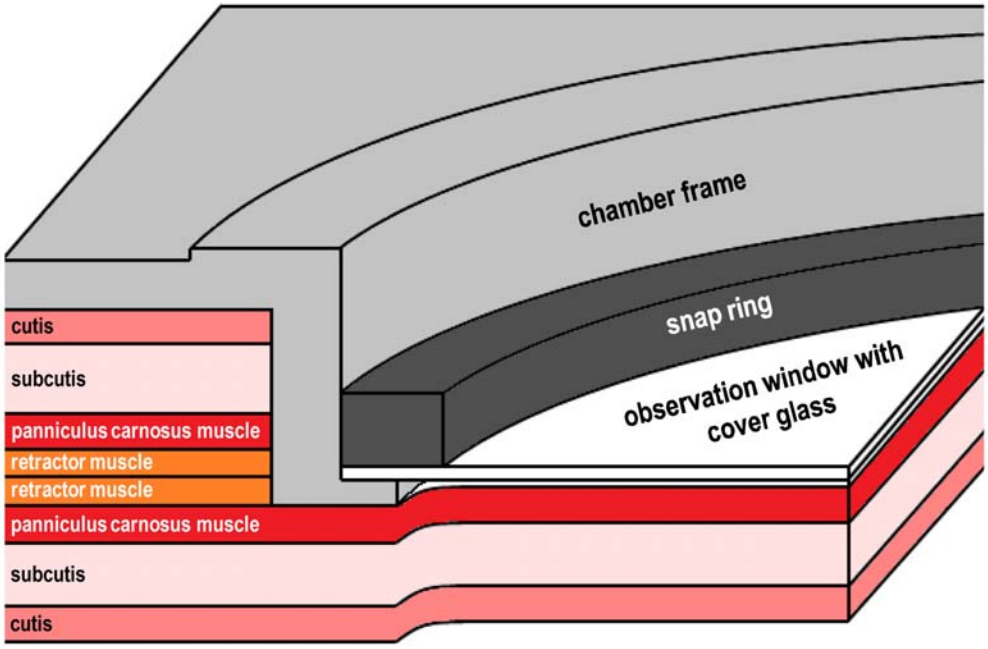

Fig. 2. Schematic drawing showing the different tissue layers of the skinfold, which are included in the observation window of the chamber, i.e. panniculus carnosus muscle, subcutis and cutis. These layers are covered with a removable cover glass incorporated into one of the chamber frames.

Preparation of the dorsal skinfold chamber and biomaterial implantation

For the preparation of the dorsal skinfold chamber, which consists of two symmetrical frames (Fig. 1a), animals should have a body weight ranging between 22-25 g in mice, 60-80 g in hamsters and 150-200 g in rats. The back of the anaesthetised animals is carefully shaven and chemically depilated avoiding micro-injuries to the skin. Subsequently, the hair-free back is cleaned under 37 °C warm water ensuring a complete removal of the depilatory cream, which may otherwise induce inflammatory irritations. Then, the animals are put in prone position and the back is exposed to medical disinfectant spray. The skinfold of the animals is extended and examined under transillumination in order to position the major feeding and draining blood vessels congruently. The sandwiched skinfold is then cranially and caudally affixed at midline with two 5-0 silk sutures (Fig. 1b). The first chamber frame is fixed by 5-0 silk sutures on its superior edge to the back side of the skinfold and two openings are carefully prepared at the base of the skinfold that is close to the body, through which the connecting screws are passed from the back to the front side (Fig. 1c). After marking the circular area of the later observation window, the animals are placed in lateral position under a stereo-microscope. By means of microsurgical instruments, one layer of skin and subcutis with the panniculus carnosus muscle as well as the two layers of the retractor muscle are completely removed within this area (Figs. 1d and 2). Thereby it is of major importance that the size of the removed area (~15 mm in diameter) exceeds the diameter of the observation window (~11 mm) to avoid tissue compression after positioning the second chamber frame. Moreover, special care should be taken during the removal of the second layer of retractor muscle, because it is in direct contact to the underlying panniculus carnosus muscle, which serves for later microscopic analyses and, thus, should not be damaged. Subsequently, the second frame of the chamber is put on the connecting screws with a frame-to-frame distance of 400-500 µm using stainless steel nuts as spacers to prevent compression of both the supplying arterioles and the draining venules of the chamber. During the entire chamber implantation procedure, the operation field is kept moist with 37 °C physiological saline to avoid drying of the tissue. Finally, the chamber preparation is hermetically closed by placing a cover glass in the observation window of the second frame, which is fixed by means of a snap ring and provides direct microscopic access to the microcirculation of the chamber (Fig. 1e). After the preparation, which takes not longer than 20-30 min for a trained person, the animals should recover for at least 48 h before implantation of biomaterials in order to exclude deterioration of the microcirculation due to the anaesthesia and the surgical trauma of the preparation procedure. The animals tolerate the chambers well, as indicated by normal daily feeding, cleaning and sleeping habits, which do not differ from those of animals without chambers (Fig. 1f).

For the *in vivo* analysis in the dorsal skinfold chamber, the implanted biomaterials should not be thicker than 1 mm to guarantee the closure of the chamber free from air. Moreover, the biomaterials should not substantially exceed a size of ~3 x 3 mm to enable analyses of the border zones in direct vicinity to the implants as well as of distant areas within the chamber, which may serve as control

tissue that is not affected by the implanted biomaterials. Thus, biomaterial implants, of which many are quite big in size for clinical applications, cannot be tested in their original size. However, this does not necessarily represent a disadvantage for the analysis of the angiogenic and inflammatory host tissue response at the implantation site. In fact, the testing of small biomaterial implants of identical size bears the advantage that the biocompatibility of these implants is directly comparable under the standardised conditions of the dorsal skinfold chamber model.

For implantation of a biomaterial, the cover glass of the chamber is temporarily removed and the biomaterial is freely positioned onto the panniculus carnosus muscle taking care to avoid contamination, mechanical irritation or damage of the prepared tissue. Due to this mode of implantation, there is no mechanical loading of the biomaterial. However, mechanical stress has been shown to be a crucial determinant for the angiogenic activation and differentiation of stem cells (Kasper *et al.*, 2007; Glaeser *et al.*, 2010) and, thus, may in particular affect the vascularisation of stem cell-seeded biomaterials, such as scaffolds for tissue engineering (Sandino *et al.*, 2010).

To overcome the disadvantage that the mechanical environment is completely excluded in the chamber model, static mechanical load can be applied to biomaterial implants by pressing a silicone pad, which is fixed with an adjustable screw to one of the chamber frames, on the cutis of the back side of the observation window (Becker *et al.*, 1994; Schäfer *et al.*, 2005; Contaldo *et al.*, 2007). The combination of this technical modification with a micromotor may in future studies even allow exposing biomaterial implants to dynamic mechanical stresses over prolonged time periods.

Characteristics of the dorsal skinfold chamber model

The classical experimental approach to study biocompatibility, vascularisation and tissue incorporation of biomaterials is their implantation into a subcutaneous pocket. In this case, data are normally based on histological and immunohistochemical analyses of isolated biomaterial samples at a single observation time point. In contrast, the dorsal skinfold chamber is a chronic *in vivo* model, which allows for the non-invasive and repetitive analysis of the angiogenic and inflammatory host tissue response to implanted biomaterials in individual animals over time by means of intravital microscopy, as discussed in detail below. This maximises the amount of data obtained from each animal and, by this, limits the statistical variability and reduces the number of animals required for a study. Moreover, in contrast to histological and immunohistochemical analyses, intravital microscopic measurements in the dorsal skinfold chamber are performed in living animals, which enables the qualitative and quantitative assessment of functional parameters such as vessel growth, microvascular perfusion, vascular permeability and cellular interactions with a high spatial and temporal resolution. For this purpose, the microscopic images are stored on videotape, DVD or hard disc for the subsequent analysis by means of a computer-assisted off-line analysis system.

The dorsal skinfold chamber model is based on different chamber techniques, which have been introduced in rabbits and mice during the last century to study the growth and behaviour of living cells and tumour implants by intravital microscopy (Sandison, 1928; Algire, 1943; Arfors *et al.*, 1970). The basic principle of this model is to provide a chronic access to exposed tissues in an implanted chamber for microscopic imaging through an observation window. In case of the dorsal skinfold these tissues comprise striated muscle, i.e., the panniculus carnosus, subcutis and skin.

To enable analyses under physiological conditions, animals are usually allowed to recover for 2-3 days from the surgical trauma of the chamber implantation before starting the experiments. Moreover, it is necessary that the tissues inside the chamber are not affected by the implanted chamber material itself. Therefore, chambers originally consisted of aluminium, which was covered with Teflon S to guarantee low weight, low thermal conductivity and biological inertness (Endrich *et al.*, 1980). Nowadays, the chambers are usually made of titanium, which also exhibits these material properties and additionally provides an improved stability (Menger *et al.*, 2002). Besides, non-metallic chambers composed of polymer materials have been fabricated, which can be applied to studies employing ionising or non-ionising radiation in animal exposure experiments without inducing thermal effects (Ushiyama *et al.*, 2004). Moreover, they are compatible for magnetic resonance imaging (MRI) (Gaustad *et al.*, 2008; Erten *et al.*, 2010).

The dorsal skinfold chamber model has been established in rats (Papenfuss *et al.*, 1979), immuno-competent mice (Cardon *et al.*, 1970), nude and severe combined immuno-deficient mice (Leunig *et al.*, 1992; Lehr *et al.*, 1993) as well as in hamsters (Endrich *et al.*, 1980). In contrast to rats and mice, preparation of the dorsal skinfold chamber in hamsters bears the major advantage that the retractor muscle is only loosely attached to the underlying panniculus carnosus muscle without many vascular interconnections between the two muscle layers. Accordingly, the retractor muscle can easily be removed without surgical trauma to the tissue, which serves for later microscopic analyses. Moreover, the hamster dorsal skinfold preparation is characterised by a better translucency due to a thinner panniculus carnosus muscle, resulting in an improved microscopic image quality when compared to the mouse or rat chamber. On the other hand, the mouse as experimental animal is less expensive and genetically better defined than the hamster. In addition, there is a multitude of knock-out and transgenic strains, which allow for the analysis of physiological and pathological processes on a molecular basis. The availability of a broad range of monoclonal antibodies directed against distinct cell surface molecules further enables for detailed immunohistochemical analyses of isolated tissue samples. For these reasons, biomaterial studies in the dorsal skinfold chamber model are increasingly performed in mice.

The dorsal skinfold chamber can easily be positioned under an upright microscope for intravital microscopic analyses of the microcirculation (Figs. 3a-d). For this purpose, the animal has to be fixed on a stage, which allows for horizontal positioning of the observation window under

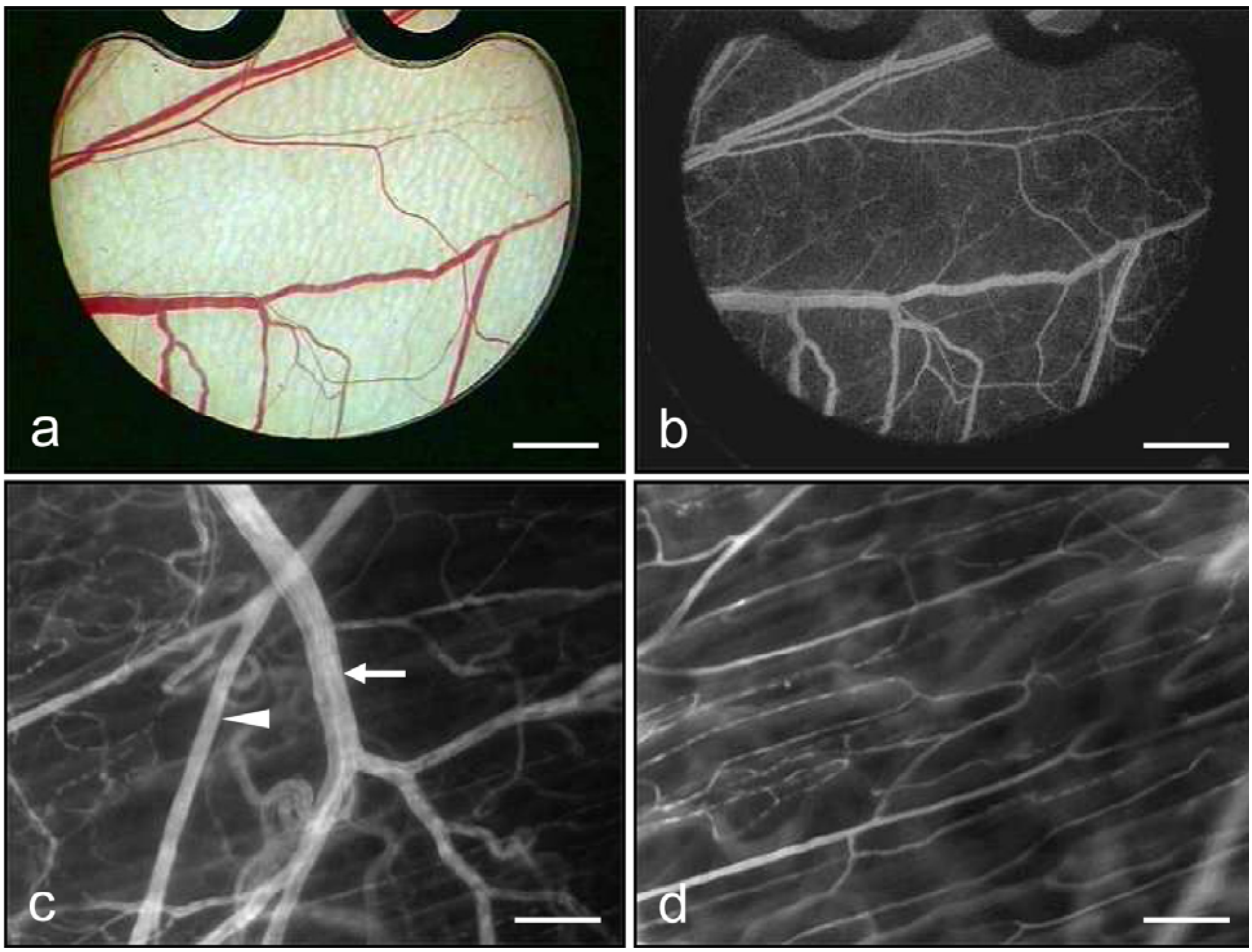

Fig. 3. **(a, b)** Intravital overview of the observation window of a mouse dorsal skinfold chamber as documented **(a)** by transillumination and **(b)** by fluorescence epi-illumination microscopy (blue light with intravascular plasma contrast enhancement by 5 % FITC-labelled dextran (150 kDa) i.v.). **(c, d)** Fluorescence microscopic view of the micro-angioarchitecture in the chamber showing an arteriole (arrowhead in **c**), a collecting venule (arrow in **c**) and a parallel arrangement of muscle capillaries **(d)**. Scale bars: **(a, b)** = 1.7 mm; **(c)** = 170 μm; **(d)** = 85 μm.

the microscope objectives. In principle, microscopy of the chamber is possible by means of long distance objectives or water and oil immersion objectives, because the snap ring and surrounding frame form a convenient space for fluids. However, ideal objectives should exhibit a narrow, tapered shape and a long working distance. This allows the microscopic examination of the entire observation window. When using large objectives with a small working distance, microscopic examination of the margins of the observation window may not be possible due to collision with the snap ring or the connecting screws of the chamber frames.

Intravital microscopic analyses are repetitively performed over an observation period that is not longer than 2-3 weeks, because the elasticity of the dorsal skinfold decreases over time. This can lead to tilting of the chamber, which may affect the perfusion of the prepared tissue. Accordingly, the dorsal skinfold chamber model is typically used to analyse the early inflammatory and angiogenic host tissue response to implanted biomaterials rather than the chronic foreign body reaction. Although this may represent a disadvantage of this model, it has to be considered that this early observation period may be of particular interest in biomaterial testing, because especially the initial vascularisation of implants has been proposed to be a crucial determinant for adequate biomaterial incorporation into the host tissue, minimising the risk of extrusion, migration and infection (Sclafani *et al.*, 1997; Naik *et al.*, 2007).

Using the dorsal skinfold chamber model in combination with transillumination microscopy techniques, it is possible to assess parameters of vascularisation such as microvessel diameter, density and blood flow. Even more versatile analyses of cellular and molecular aspects can be done by means of epi-illumination fluorescence microscopy. For this purpose, different fluorescent dyes are injected intravenously via a jugular catheter or directly into the tail vein or retrobulbary space. For instance, fluorescein isothiocyanate (FITC)-labelled dextran with a molecular weight of 150 kDa or albumin is applied for contrast enhancement of individual microvessels by intravascular staining of plasma. Because of their high molecular weight, these fluorescence markers only extravasate in case of disturbed endothelial integrity and, thus, can be used for the analysis of vascular leakage during inflammatory or

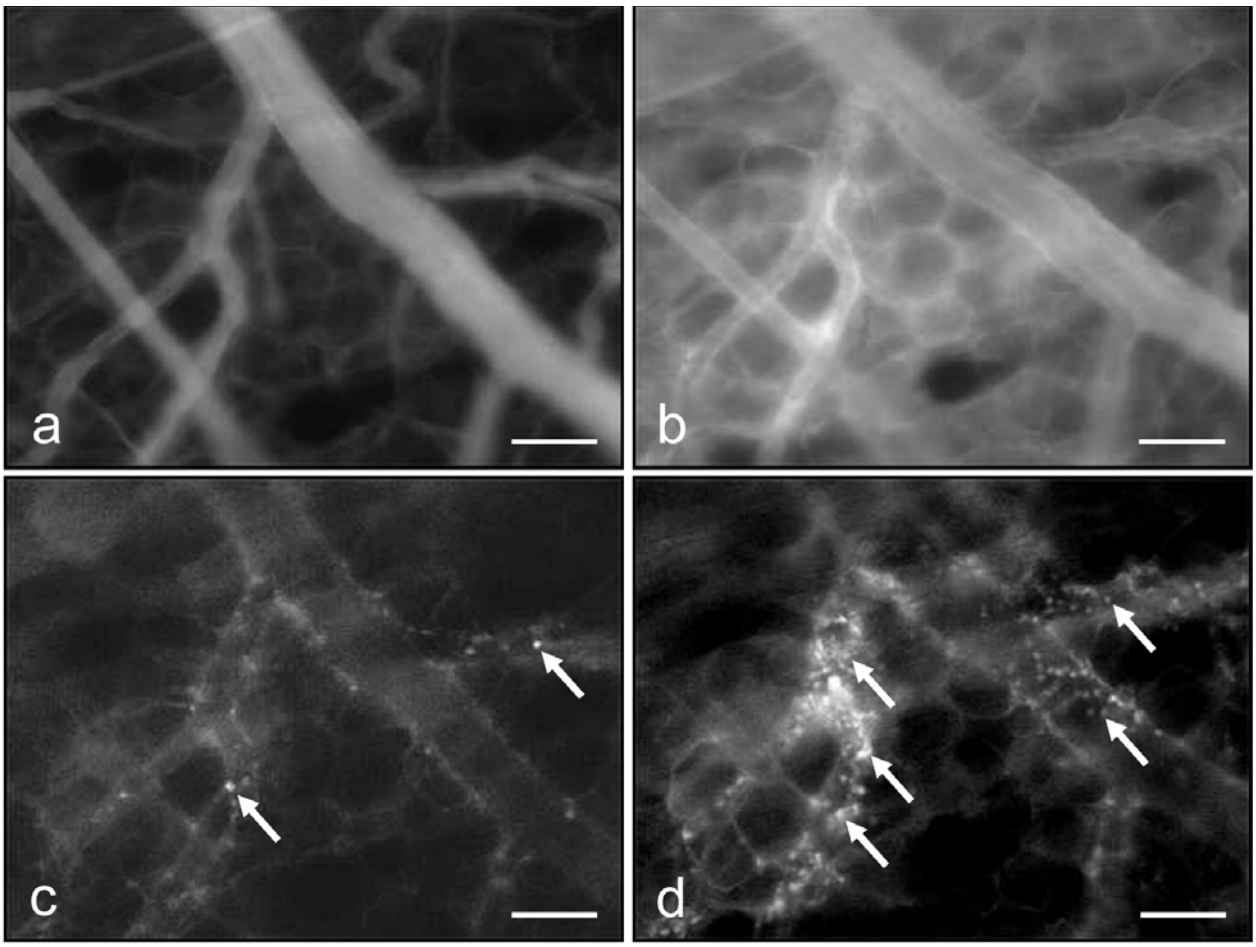

Fig. 4. Intravital fluorescence microscopy of postcapillary and collecting venules of a dorsal skinfold chamber **(a, b)** Blue light epi-illumination with intravascular plasma contrast enhancement by 5 % FITC-labelled dextran (150 kDa) i.v.; **(c, d)** Green light epi-illumination for visualisation of rhodamine 6G-stained leukocytes) under physiological conditions **(a, c)** and 2 h after induction of inflammation by topical administration of tumour necrosis factor (TNF)-α **(b, d)**. **(a, b)** Inflammation is typically associated with extravasation of the contrast medium, as indicated by increased brightening of the tissue of panel b when compared to that of panel **a**. **(c, d)** TNF-α-treatment also causes massive accumulation of rhodamine 6G-stained leukocytes (**c**, **d**, arrows), apparently interacting with the endothelium. Scale bars: 85 µm.

angiogenic processes (Menger *et al.*, 1992a) (Figs. 4a and b). To study the interaction of leukocytes and platelets with the microvascular endothelium, the cells can be stained *in situ* with rhodamine 6G (Baatz *et al.*, 1995) (Figs. 4c and d). In addition, individual necrotic cell death can be detected by the use of propidium-iodide (Harris *et al.*, 1997), whereas apoptotic cells can be identified by nuclear condensation, margination and fragmentation following local bisbenzimide staining (Vollmar *et al.*, 2001).

Besides conventional epi-illumination fluorescence microscopy, imaging in the dorsal skinfold chamber model has increasingly been performed in recent years by means of confocal microscopy techniques (Isaka *et al.*, 2004; Makale, 2007; Strieth *et al.*, 2008). Using a pinhole aperture for point illumination, single-photon laser-scanning microscopy allows the detection of light from a specific volume within the plane of focus, so that the resultant image is comparatively free of scattered light and attendant blurring (Makale, 2007). Accordingly, the obtained microscopic images exhibit an improved optical resolution and contrast. Moreover, it is possible to concatenate individual optical sections to create a three-dimensional reconstruction of the object of interest. As a variation of this technique, multiphoton microscopy uses near-infrared lasers for two-photon excitation, which allows the generation of bright, high-resolution images in sample depths of 500-1000 µm, while photobleaching and phototoxicity-induced tissue damage is markedly reduced (Makale, 2007; Ishii and Ishii, 2011).

In addition to microscopy, several other non-invasive techniques have been used in the past for the analysis of microcirculatory parameters within the dorsal skinfold chamber, including Laser Doppler flowmetry for the assessment of microvascular tissue perfusion (Menger *et al.*, 1992b) or phosphorescence quenching for the measurement of tissue oxygenation (Kerger *et al.*, 1996). However, these techniques are indirect in nature and, thus, do not allow for the direct visualisation of distinct cellular

mechanisms, such as the interaction of leukocytes with the microvascular endothelium or the ingrowth of new blood vessels into implanted biomaterials.

Biomaterial research in the dorsal skinfold chamber model

During the last two decades, many experimental studies have been performed using the dorsal skinfold chamber model to study the inflammatory and angiogenic host tissue response to various biomaterials. These include prosthetic vascular grafts, metallic implants, surgical meshes, bone substitutes, scaffolds for tissue engineering as well as locally or systemically applied drug delivery systems (Figs. 5a-c). The following chapters provide an overview of these studies and highlight the most interesting conclusions, which can be drawn from their findings.

Prosthetic vascular grafts

Implantation of prosthetic vascular grafts for both replacement and bypass procedures represents a central therapeutic approach in the field of cardiovascular surgery. However, this approach bears the risk of early graft failure due to infection (Chiesa *et al.*, 2002; Zetrenne *et al.*, 2007; Herscu and Wilson, 2009) or thrombotic occlusion (Kraiss and Johansen, 1995; Bonhomme *et al.*, 2010). A major prerequisite for the prevention of these serious complications is a rapid and adequate vascularisation of the vascular prostheses during the early healing process. In fact, insufficient graft vascularisation contributes to a poor incorporation of the implant into the surrounding host tissue, which may result in perigraft seroma formation and subsequent infection (Borrero and Doscher, 1988). Moreover, deficient transmural ingrowth of newly formed microvessels may cause thrombotic graft occlusion due to an impaired development of neointimal coverage (Rahlf *et al.*, 1986). For these reasons, it is of major importance to identify material properties, which promote the early vascularisation of the implanted prosthetic vascular graft material.

The most frequently used materials for the fabrication of prosthetic vascular grafts are polytetrafluorethylene (PTFE) and polyethylenterephtalate (Dacron). After implantation of these materials into the dorsal skinfold chamber of Syrian golden hamsters it could be demonstrated that Dacron grafts, which exhibit a high porosity with an internodal distance of 212 µm, induce a strong angiogenic host tissue response with the development of dense microvascular networks and a large number of capillaries piercing into the interstices of the implants (Menger *et al.*, 1990a). In contrast, transmural capillary ingrowth cannot be observed in the less porous PTFE grafts with an internodal distance ranging between 1-30 µm (Menger *et al.*, 1990a,b). By measuring the dynamic breaking strength, i.e. the force necessary to remove the grafts from the host tissue within the chamber, it could further be shown that the extent of graft vascularisation positively correlates with the integration of the materials into the perigraft tissue (Menger *et al.*, 1990a,b). Taken together, these findings clearly indicate that synthetic implants can be optimised for different applications by modification of their porosity. Porous implants should be preferred for vascular prostheses, whereas dense materials with a low porosity, such as PTFE with an internodal distance of 1 µm, may be more useful for the replacement of biological membranes, such as pericardium or peritoneum (Menger *et al.*, 1990b).

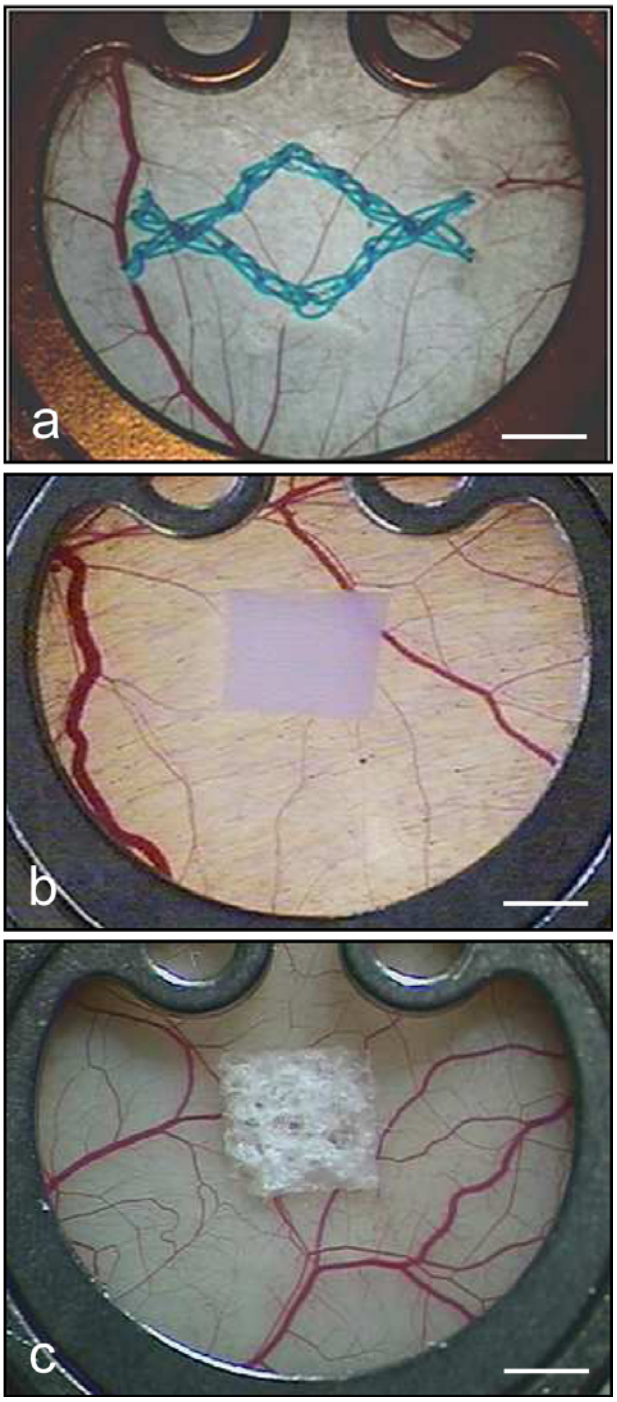

Fig. 5. Overview of the observation window of a hamster **(a, b)** and a mouse dorsal skinfold chamber **(c)** directly after implantation of the surgical mesh Ultrapro® **(a)**, the synthetic bone substitute Ostim® **(b)** and a porous polyurethane scaffold for tissue engineering **(c)**. Scale bars: 1.6 mm.

Besides fully synthetic materials, biosynthetic and biological materials are used for the fabrication of vascular prostheses, because they are supposed to offer improved surgical handling characteristics and healing conditions. Biosynthetic composite grafts consisting of ovine collagen and polyester (Omniflow®) have shown an early vascularisation and tight incorporation into the surrounding chamber tissue (Menger *et al.*, 1992c). Of interest, this has not been the case for biolised bovine artery grafts (SolcoP®), which may be due to the formation of a fibrous collagen capsule around the implants (Menger *et al.*, 1992c). In a recent study, Esguerra *et al.* (2010) used the dorsal skinfold chamber model to analyse the *in vivo* vascularisation and biocompatibility of bacterial cellulose as a potential novel biomaterial for the reconstruction of small-diameter blood vessels. Bacterial cellulose is a three-dimensional asymmetric hydrogel-like structure configured as a microfibril network, which is produced by the bacterium *Acetobacter xylinum* (Brown *et al.*, 1976). Although bacterial cellulose lacks a defined pore size, it exhibits a porosity of >90 % (Esguerra *et al.*, 2010). Moreover, it is possible to generate tubes of bacterial cellulose with mechanical properties that are adequate for vascular grafts (Bodin *et al.*, 2007). Of interest, it was found that bacterial cellulose exhibits a biocompatibility, which is comparable to that of PTFE and polyglycolid acid (PGA) implants (Esguerra *et al.*, 2010). Nonetheless, because bacterial cellulose only induces a poor angiogenic host tissue response during the first 14 days after implantation into the dorsal skinfold chamber, further long-term studies have to clarify the suitability of this biological material for the fabrication of vascular prostheses, which are adequately incorporated into the surrounding host tissue.

Metallic implants

Various metallic implants are commercially available for osteosynthesis in traumatology and orthopaedic surgery. Their majority is based on titanium and stainless steel due to the mechanical properties of these materials. Because metallic implants are in direct contact with the soft tissue at the implantation site, the dorsal skinfold chamber consisting of striated muscle tissue, subcutis and cutis is an ideal model to study their effects on the local microcirculation under *in vivo* conditions. Using this model, Kraft *et al.* (2000) demonstrated that titanium only induces a transient up-regulation of leukocyte-endothelial cell interactions in venules of the host tissue during the first 24 h after implantation, indicating a high degree of biocompatibility. This is also the case for commonly used titanium alloys, such as titanium-aluminium-vanadium (Ti-6Al-4V), titanium-aluminium-niobium (Ti-6Al-7Nb) and titanium-molybdenum (Ti-15Mo) (Kraft *et al.*, 2005; Pennekamp *et al.*, 2006, 2007). Furthermore, modification of titanium implants by plasma pre-treatment and collagen I coating contributes not only to a reduced foreign body reaction but also enhances angiogenesis at the implantation site (Hauser *et al.*, 2009). In contrast, implants made of silver induce a permanent breakdown of the microcirculation with a massive increase of vascular leakage and extravasation of leukocytes 3 days after implantation into the dorsal skinfold chamber (Kraft *et al.*, 2000). This should be considered in situations, where silver-coated implants are recommended due to their antimicrobial properties (Monteiro *et al.*, 2009). Stainless steel implants also provoke an inflammatory host tissue reaction, which, however, is only moderate and of temporary nature. Studies could show that this reaction can be reduced by using stainless steel alloys containing less amounts of nickel (Kraft *et al.*, 2001a) or by sol-gel calcium phosphate coating of the material (Kraft *et al.*, 2002).

After implantation of metallic devices, wear particles may detach from their surface and accumulate in the surrounding tissues. The biocompatibility of these fretting particles may differ significantly from that of the bulk implants, because their composition can deviate due to tribochemical reactions or corrosion (Jacobs *et al.*, 1998). Based on these considerations, wear particles produced from stainless steel or titanium were analysed in the hamster dorsal skinfold chamber and compared to the corresponding bulk implants (Kraft *et al.*, 2001b; Kraft *et al.*, 2003). By this, no marked differences could be found between titanium bulk and debris. However, stainless steel wear debris induces a massively increased inflammatory reaction with oedema formation and breakdown of the microcirculation within 24 h after implantation when compared to bulk stainless steel. This may be explained by the increased release of nickel and chromium from the wear particles, indicating that not only the bulk properties of metallic devices but also the microcirculatory implications of inevitable wear debris play a crucial role in determining the implants' biocompatibility (Kraft *et al.*, 2001b; Kraft *et al.*, 2003).

Surgical meshes

Hernia repair represents one of the most frequent operations in general surgery (Rutkow, 2003). Meanwhile, implantation of surgical meshes has become the golden standard in hernia surgery. The major advantage of this procedure is a tension-free repair of the abdominal wall defect, resulting in reduced post-operative pain and significantly decreased recurrence rates of less than 20 % when compared to conventional suture closures (Luijendijk *et al.*, 2000; Burger *et al.*, 2004). However, implantation of surgical meshes also bears the risk of infection, adhesions, and seroma or fistula formation (Robinson *et al.*, 2005; Jezupovs and Mihelsons, 2006). Accordingly, one major challenge in modern hernia repair is the development of novel surgical mesh materials, which exhibit an optimal biocompatibility and which are adequately integrated into the surrounding host tissue. For these reasons, the dorsal skinfold chamber has been introduced as a model, which allows for the first time the repetitive *in vivo* analysis of inflammatory effects, vascularisation and incorporation of different surgical mesh materials (Laschke *et al.*, 2005a).

Prolene®, Ultrapro® and Vicryl® meshes are commonly used meshes, which differ in polymer composition, mesh architecture and resorbability. After their implantation into the hamster dorsal skinfold chamber, it was found that the multifilament Vicryl® mesh induces a more pronounced angiogenic and inflammatory host tissue response when compared to the monofilament Prolene®

and Ultrapro® mesh (Laschke *et al.*, 2009a). However, this is not associated with an improved material incorporation. In fact, the granulation tissue surrounding the Vicryl® mesh is instable due to low collagen content and massive infiltration of histiocytes, multinucleated giant cells and polymorphonuclear granulocytes (Laschke *et al.*, 2009a). These results indicate that a stronger angiogenic and inflammatory response to an implanted surgical mesh does not necessarily result in a better incorporation into the host tissue.

Based on the finding that immunosuppressive therapy affects angiogenesis and collagen synthesis during wound healing (Kelley *et al.*, 1990; Schäffer *et al.*, 2007), a recent study analysed, how the immunosuppressive drugs rapamycin and cyclosporine A influence the incorporation of Ultrapro® meshes (Laschke *et al.*, 2009b). This is an issue of major clinical relevance, considering the fact that the incidence of incisional hernias following orthotopic liver transplantation has markedly increased during the last ten years (Kahn *et al.*, 2007). Interestingly, rapamycin dose-dependently inhibits the ingrowth of new microvessels from the host striated muscle tissue of the chamber into the meshes, resulting in a significantly decreased microvessel density at the implantation site when compared to cyclosporine A-treated and non-treated control animals (Laschke *et al.*, 2009b). This is associated with a reduced collagen content of the newly formed granulation tissue around the implants. Accordingly, immunosuppressed patients should not receive rapamycin in case of incisional hernia repair to guarantee optimal mesh incorporation.

A severe complication of surgical mesh implantation is bacterial infection. Using the dorsal skinfold chamber model in combination with intravital fluorescence microscopy it is possible to study *in vivo* the interaction of bacteria with the microvascular endothelium (Laschke *et al.*, 2005b; Kerdudou *et al.*, 2006). Roller *et al.* (2008) injected FITC-labelled *Staphylococcus aureus* into Syrian golden hamsters, which were equipped with dorsal skinfold chambers containing Ultrapro® meshes and analysed the adherence of the circulating bacteria in microvessels surrounding the implants. This study could demonstrate that Ultrapro® meshes do not increase the adherence of bacteria in microvessels at the implantation site under physiological conditions as well as under tumour necrosis factor (TNF)-α-induced local inflammation. Therefore, Ultrapro® meshes cannot be considered as an important trigger for an increased bacteria-endothelial cell interaction, which leads to the conclusion that this mechanism seems not to play a major role in the development of late-onset Ultrapro® mesh infections.

Bone substitutes

For the reconstruction of bone defects due to fractures, non-unions or resection of benign tumours and bone cysts, transplantation of autologous bone is still considered the golden standard in bone repair, because it guarantees optimal osteogenesis, osteoinduction and osteoconduction (Giannoudis *et al.*, 2005). However, this approach bears the disadvantage of a prolonged overall surgical procedure and anaesthesia time with all its risks. Moreover, harvesting of bone autografts from the iliac crest can be associated with local complications, such as nerve injury or infection (Seiler and Johnson, 2000). Alternatively, thermodisinfected and cryopreserved allogenic bone graft substitutes may be used. Recently, Ring *et al.* (2011a) implanted chips consisting of dehydrated human femoral head into dorsal skinfold chambers of balb/c mice and found that surface activation with glow discharge gas plasma accelerates and improves their vascularisation without inducing adverse effects in the host microcirculation. These promising findings indicate that this novel technology may be used in the future to increase the incorporation rates of clinically applied allogeneic bone implants.

Most of the commercially available synthetic bone substitutes are chemically based on calcium phosphate compounds. These include the granulated ceramics Algipore®, BioOss®, ChronOs® and Endobon®, as well as the absorbable bone cements Calcibon®, Biobon® and Norian SRS®. Of interest, Roetman *et al.* (2010) showed that all of these biomaterials induce a comparable host tissue response in the chamber model, excluding material-specific reasons for the reported sporadic failure of these bone substitutes in clinical practice. NanoBone® and Ostim® are two synthetic bone substitutes, which contain nanocrystalline hydroxyapatite (ncHA). In case of NanoBone®, ncHA is embedded in a highly porous matrix of silica gel (Götz *et al.*, 2008), whereas Ostim® is a suspension of ncHA in water, making it a highly viscous, injectable bone substitute (Huber *et al.*, 2008). After implantation into the dorsal skinfold chamber, both materials have been shown to exhibit an excellent biocompatibility comparable to that of cancellous bone, as indicated by a lack of venular leukocyte activation in the host striated muscle tissue (Laschke *et al.*, 2007; Abshagen *et al.*, 2009). In contrast to NanoBone®, Ostim® is rapidly degraded, which allows for a guided neovascularisation directed towards areas of degradation (Laschke *et al.*, 2007) (Figs. 6a-d). This may facilitate the early invasion of osteoblasts into the biomaterial and, thus, the formation of new bone after the injection of Ostim® into bone defects.

Medpor® is a porous polyethylene, which is commonly used in plastic and reconstructive surgery for the restoration of contour in the craniofacial skeleton (Xu *et al.*, 2009; Berghaus *et al.*, 2010). For such facial surgical procedures, patients often undergo perioperative steroid therapy to reduce postoperative oedema formation and to shorten recovery time (Kara and Gökalan, 1999; Kargi *et al.*, 2003). However, it was found that Medpor® implanted into the dorsal skinfold chamber of prednisolone-treated balb/c mice exhibits a significantly reduced vascularisation when compared to vehicle-treated controls (Ehrmantraut *et al.*, 2010). These findings indicate that perioperative steroid therapy may be avoided in case of Medpor® implantation to achieve a rapid incorporation of the material at the implantation site. In addition, Strieth *et al.* (2010) could show that the biocompatibility of porous polyethylene can be improved by incorporation of the extracellular matrix Matrigel™ enriched with recombinant vascular endothelial growth factor (VEGF). In fact, this modification efficiently reduced the transient initial inflammatory response to

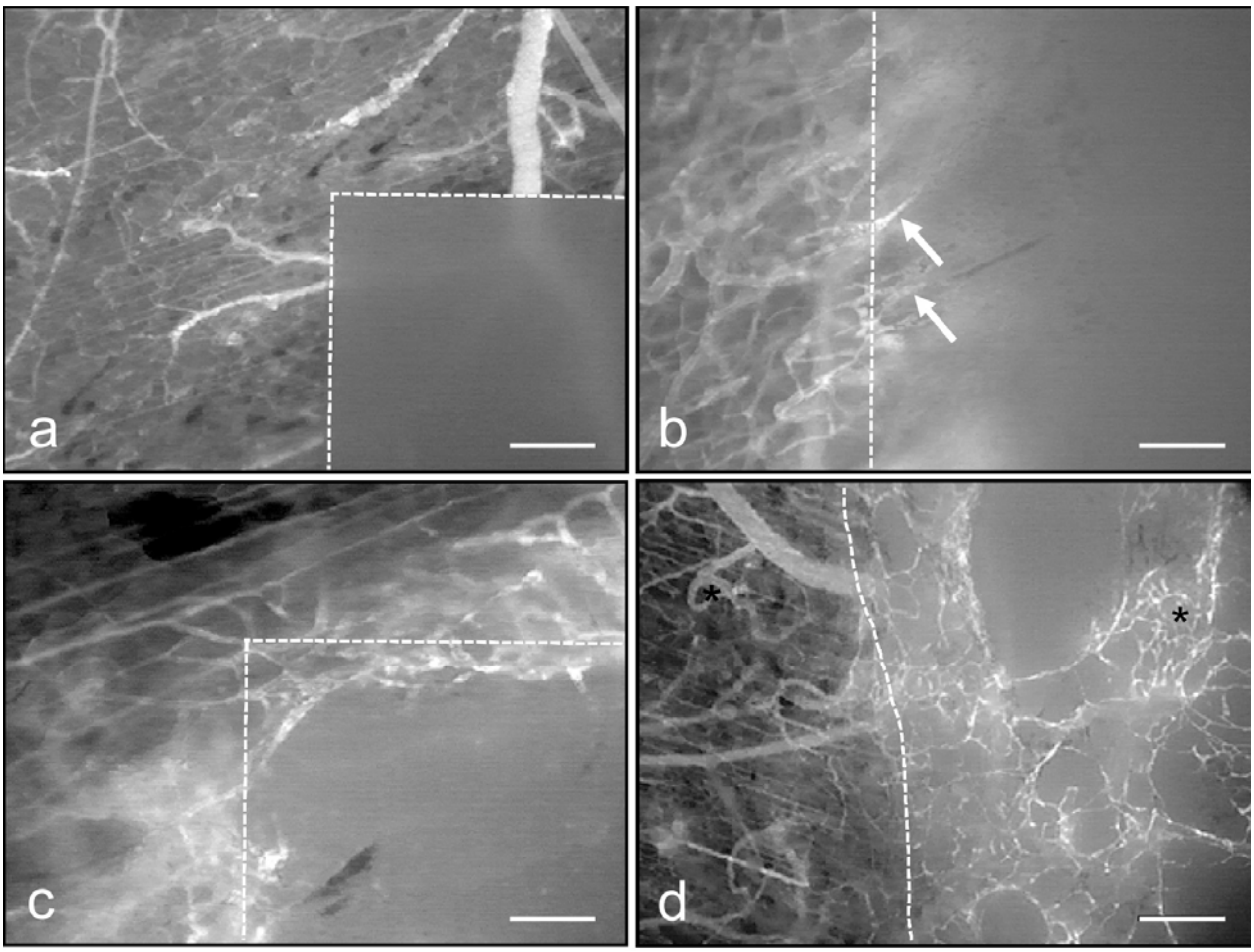

Fig. 6. Intravital fluorescence microscopy (blue light epi-illumination microscopy with intravascular plasma contrast enhancement by 5 % FITC-labelled dextran (150 kDa) i.v. of the synthetic bone substitute Ostim® (border marked by dotted line) at 20 min **(a)** as well as at day 3 **(b)**, 6 **(c)**, and 14 **(d)** after implantation into the dorsal skinfold chamber of a Syrian golden hamster. At day 3, first signs of angiogenesis can be observed in the border zone of the implant, characterised by capillary sprouts (**b**, arrows). During the following days, these sprouts interconnect with each other to form new microvascular networks around the implant **(c)**. At day 14, newly formed microvessels can also be detected in central surface areas of the implant (**d**, asterisk). Scale bars: **(a, d)** = 260 μm; **(b, c)** = 100 μm.

the implants with a significantly decreased leukocyte-endothelial cell interaction in venules of the chamber preparation.

Taken together, these studies demonstrate that the dorsal skinfold chamber allows for the comparative *in vivo* study of the angiogenic and inflammatory reaction to various bone substitutes under standardised conditions by means of intravital fluorescence microscopy, although this model cannot directly predict bone ingrowth into the implants. Accordingly, a comprehensive characterisation of bone substitutes should be performed by combining this model with other sophisticated *in vivo* approaches, which enable the study of osteoconductivity and osseointegration, such as the bone chamber (Winet and Albrektsson, 1988; Albrektsson *et al.*, 1989) or non-union models (Garcia *et al.*, 2008).

Scaffolds for tissue engineering

In tissue engineering, scaffolds serve as three-dimensional matrices for cells to attach, proliferate and finally form a functional tissue substitute (Gloria *et al.*, 2010; Carletti *et al.*, 2011). During the last few years, various scaffold types have been evaluated in terms of biocompatibility and vascularisation in dorsal skinfold chambers of mice. These scaffolds consisted of collagen (Ichioka *et al.*, 2005), collagen-elastin (Ring *et al.*, 2010a), collagen-chitosan-hydroxyapatite hydrogel (Rücker *et al.*, 2006), acellular dentin (Rücker *et al.*, 2008), hydroxyapatite (Rücker *et al.*, 2008), β-tricalcium-phosphate (Lindhorst *et al.*, 2010), poly(ether ester) (Druecke *et al.*, 2004), poly(L-lactide-co-glycolide) (PLGA) (Rücker *et al.*, 2006), polyethylene glycol terephthalate/polybutylene terephthalate (PEGT/PBT) (Ring *et al.*, 2006a,b), lactocapromer terpolymer (Ring *et al.*, 2010b, 2011b), polyurethane (Laschke *et al.*, 2009c, 2010a) and supramolecular nanofibres (Ghanaati *et al.*, 2009). Notably, it was found that the initial vascularisation of the scaffolds is crucially dependent on their biocompatibility. For instance, polyurethane scaffolds, which do not induce a leukocytic inflammatory host tissue response, are only poorly vascularised throughout the first 14 days after implantation (Laschke *et al.*, 2009c, 2010a). In contrast, PLGA scaffolds, which induce a

slight inflammation, show a strong angiogenic reaction (Rücker *et al.*, 2006). On the other hand, collagen-chitosan-hydroxyapatite hydrogel scaffolds, promoting a massive inflammation of the chamber tissue with an increased number of apoptotic cells, demonstrate a complete lack of angiogenesis at the implantation site (Rücker *et al.*, 2006). Finally, hydroxyapatite and dentin scaffolds, which exhibit a biocompatibility comparable to that of isogeneic bone, promote vascularisation to a similar extent as observed in syngeneically implanted bone tissues (Rücker *et al.*, 2008).

The vascularisation of scaffolds is an essential determinant for the success of clinical tissue engineering applications. In fact, long-term survival of three-dimensionally constructed tissues, which cannot survive by diffusion alone, crucially depends on a rapid ingrowth of new blood vessels, which provide nutrients and oxygen not only to the cells of the margin but also of the centre of the tissue substitutes (Laschke *et al.*, 2006). By means of the dorsal skinfold chamber model, several approaches have been tested, which may contribute to an improved scaffold vascularisation. These include the modification of pore sizes. In fact, scaffolds with large pores of 250-300 μm in diameter significantly improve the ingrowth of new microvessels when compared to scaffolds with pore sizes <75 μm (Druecke *et al.*, 2004, Ring *et al.*, 2006a,b). Moreover, the surface of scaffolds can be activated by biomimetic coating with gas plasma, calcium-phosphate and collagen, resulting in an increased microvessel density of the materials after implantation (Ring *et al.*, 2007, 2010a,c). In addition, scaffolds are loaded with antimicrobial peptides, such as cathelicicin LL37 (Steinstraesser *et al.*, 2006), angiogenic growth factors, such as VEGF (Lindhorst *et al.*, 2010), or growth factor-containing extracellular matrices, such as Matrigel™ (Laschke *et al.*, 2008a), to stimulate the angiogenic host tissue reaction. Finally, vascularisation of scaffolds can be accelerated by vitalisation with osteoblast-like cells (Schumann *et al.*, 2009; Tavassol *et al.*, 2010) or bone marrow cells (Ichioka *et al.*, 2005; Schumann *et al.*, 2009).

However, all of these approaches face the common problem that angiogenesis is a complex time-consuming process. Previous studies have shown that the physiological growth of blood vessels is not faster than ~5 μm/h (Zarem, 1969; Orr *et al.*, 2003). Accordingly, the ingrowth of microvessels into implanted scaffolds can only be accelerated to a limited extent. This problem may be overcome by the generation of preformed microvascular networks within scaffolds prior to their implantation. In this case, the preformed networks simply have to anastomose to the existing blood vessels of the host tissue after scaffold implantation, representing the process of inosculation (Laschke *et al.*, 2009d) (Figs. 7a-e). Of interest, recent studies indicate that preformed microvessels actively contribute to the process of scaffold vascularisation. After implantation of *in situ* prevascularised PLGA scaffolds from green fluorescent protein (GFP)-transgenic mice into dorsal skinfold chambers of wild-type recipient animals, intravital fluorescence microscopy demonstrates that GFP-positive vessels grow out from the scaffolds into the surrounding host tissue, where external inosculation occurs (Laschke *et al.*, 2008b). Furthermore, the GFP-positive preformed vessels contribute to sprouting angiogenesis in the centre of the scaffolds, further increasing the microvessel density of the implants (Laschke *et al.*, 2008b). However, even this approach does not result in adequate blood perfusion of the scaffolds during the very first days after implantation. This is most probably due to the fact that inosculation can only take place, when the preformed blood vessels within a scaffold and the preformed blood vessels of the host tissue have grown towards each other to finally form interconnections.

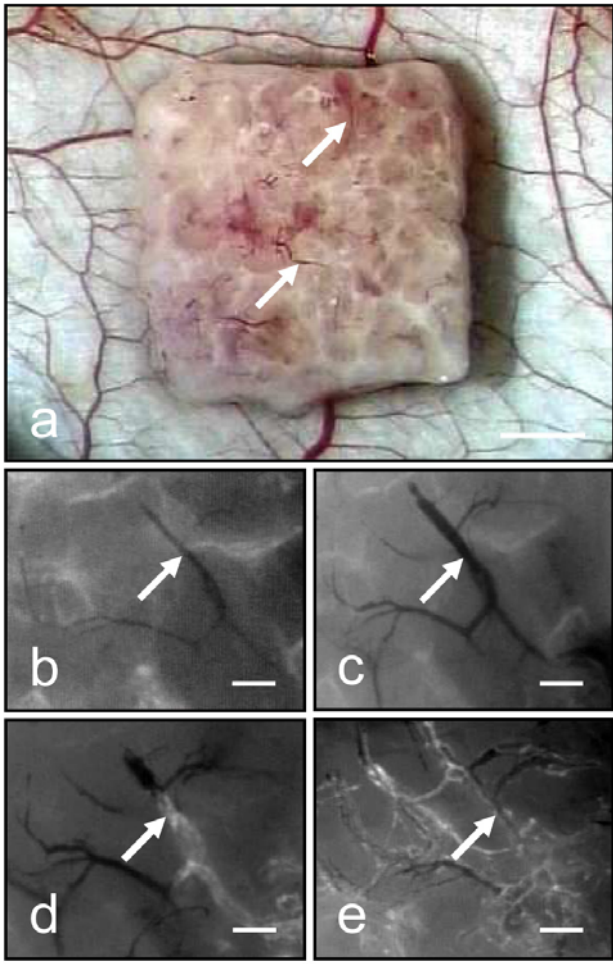

Fig. 7. **(a)** Porous polyurethane scaffold, which was prevascularised for 20 days in the flank of a donor mouse and subsequently transferred into the dorsal skinfold chamber of a recipient animal. The prevascularised scaffold exhibits already a complete microvascular network of newly formed blood vessels (arrows). **(b-e)** Intravital fluorescence microscopy of a preformed blood vessel (arrow) within a tissue portion of the prevascularised scaffold directly after implantation into the dorsal skinfold chamber **(b)** as well as at day 3 **(c)**, 6 **(d)** and 14 **(e)** (blue light with intravascular plasma contrast enhancement by 5 % FITC-labelled dextran (150 kDa) i.v.). Note that until day 3 the preformed microvessel appears dark, indicating lack of FITC-dextran-labelled blood perfusion **(b, c)**. At day 6 and 14 the microvessel is reperfused, as indicated by FITC-dextran staining **(d, e)**. Scale bars: **(a)** = 900 μm; **(b-e)** = 100 μm.

Therefore, novel strategies are needed, which further accelerate the process of inosculation. First studies provide evidence that this may be achieved by *in vitro* short-term cultivation of *in situ* prevascularised scaffolds. This short-term cultivation results in the destabilisation of their preformed microvascular networks (Laschke *et al.*, 2011), which represents an essential step in the process of angiogenesis. Moreover, the outgrowth of vessel sprouts from prevascularised scaffolds may be promoted by embedding them in an extracellular pro-angiogenic matrix, resulting in an improved external inosculation in the surrounding host tissue after implantation (Laschke *et al.*, 2010b).

Drug delivery systems

During the past decade, there has been a tremendous increase in the number of drug delivery systems being developed for the local or systemic treatment of pathologic conditions. Many of these systems are based on controlled-release biodegradable polymers, such as PLGA, which permit the sustained and targeted delivery of therapeutic agents. Nickerson *et al.* (2009) have recently analysed how the degradation of PLGA affects the local microcirculation in dorsal skinfold chambers of mice. They found that the influence of PLGA degradation is limited to luminal arteriolar expansion without apparent effects on the total length of smooth muscle-coated microvessels, indicating that the polymer itself does not induce a relevant arteriogenic remodelling after implantation. In contrast, incorporation of phthalimide neovascular factor (PNF1) into PLGA films resulted in a strong angiogenic host tissue response, which is characterised by the formation of new microvascular networks in direct vicinity of the implants (Wieghaus *et al.*, 2008). Moreover, PNF1 treatment significantly increased the diameters of local arterioles and venules. Similar effects can be observed by incorporation of sphingosine-1-phosphate (S1P) or S1P receptor-selective agonists into the PLGA (Sefcik *et al.*, 2008, 2011).

Ibuprofen is a non-selective cyclooxygenase (COX) inhibitor, which is increasingly used as a pain-reducing component in modern wound dressings. However, because inhibition of COX may also suppress angiogenesis during wound healing, Ring *et al.* (2008) recently studied the vascularisation of ibuprofen-containing polyurethane foams implanted into dorsal skinfold chambers of mice. Surprisingly, they could demonstrate that the ibuprofen-releasing implants even induced a slightly stronger angiogenic host tissue response when compared to ibuprofen-free foams. Thus, local release of small-dose ibuprofen seems not to have a negative effect on the development of new blood vessels at the implantation site. This finding should be taken into account during the development of novel devices for the local application on wounds.

The dorsal skinfold chamber is not only a suitable *in vivo* model for biomaterial implantation experiments but is also very useful to gain new insights into the transport and tissue-specific uptake of systemically applied drug delivery systems. In particular in the field of oncology, a broad spectrum of nanoparticulate carriers is currently under investigation for the development of novel anti-neoplastic agents. By means of sophisticated real-time imaging technologies, it is possible to analyse the processes involved in their delivery to different tumour types established in the dorsal skinfold chamber (Monsky *et al.*, 1999; Jiang *et al.*, 2009; Erten *et al.*, 2010; Hak *et al.*, 2010). These include the interaction of the particles with the microvascular endothelium, their extravasation and intracellular uptake into individual tumour cells (Tada *et al.*, 2007). Of interest, these processes have been shown to be particularly dependent on the size of the used particles (Kawai *et al.*, 2009), their charge (Krasnici *et al.*, 2003) and the local tissue temperature (Gaber *et al.*, 1996; Liu *et al.*, 2004), which should be considered for the development of novel drug delivery systems exhibiting improved pharmacokinetics and anti-tumour activity.

Conclusions

The dorsal skinfold chamber in combination with sophisticated imaging technologies offers the unique opportunity to analyse *in vivo* the biocompatibility and vascularisation of small biomaterial implants, which should not exceed a size of 3 x 3 x 1 mm. Typically, these analyses are repetitively performed over a limited observation period of 2-3 weeks after biomaterial implantation. Thus, this model is not suitable for long-term studies focusing on the chronic foreign body reaction, but rather for the investigation of the early inflammatory and angiogenic host tissue response to implanted biomaterials, which is crucial for their adequate incorporation into the host tissue. Accordingly, this model has been increasingly used during the last two decades to study the *in vivo* performance of a broad spectrum of medical devices, including prosthetic vascular grafts, metallic implants, surgical meshes, bone substitutes, scaffolds for tissue engineering and drug delivery systems. These studies have shown that chemical composition, material architecture and surface characteristics are important determinants for the angiogenic and inflammatory host tissue response to the implants. Thus, the dorsal skinfold chamber model does not only provide deep insights into the complex interactions of biomaterials with the surrounding host tissue but also represents an important tool for the future development of novel biomaterials aiming at an optimisation of their biofunctionality in clinical practice. For this purpose, sophisticated analyses focusing on completely new aspects of the *in vivo* behaviour of implanted biomaterials may be performed by means of the dorsal skinfold chamber model, which cannot easily be done in other animal models. For instance, the *in vivo* visualisation of the lymphatic vascular system within the chamber (Menger *et al.*, 2003; Schacht *et al.*, 2004) may make it possible to study the lymphatic removal of biomaterial wear particles at the implantation site. Moreover, selective heating (Harder *et al.*, 2005) or cooling (Thorlacius *et al.*, 1998; Westermann *et al.*, 1999) of the chamber may offer the unique opportunity to analyse the temperature-dependent biofunctionality of thermosensitive biomaterials (Vermonden *et al.*, 2010;

Wang *et al.*, 2010) without affecting the general condition of the experimental animal.

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Discussion with Reviewers

Reviewer I: Please discuss the advantages and the limitations of this experimental model in more detail, regarding (1) advantages compared to subcutaneous implantation, (2) methods used for the following of the newly formed tissue, (3) the absence of mechanical strain.

Authors: (1) The classical experimental approach to study biocompatibility, vascularisation and tissue incorporation of biomaterials is their implantation into a subcutaneous pocket. In this case, data are normally based on histological and immunohistochemical analyses of isolated biomaterial samples at a single observation time point. In contrast, the dorsal skinfold chamber is a chronic *in vivo* model, which allows for the non-invasive and repetitive analysis of the angiogenic and inflammatory host tissue response to implanted biomaterials in individual animals over time. This maximises the amount of data obtained from each animal and, by this, limits the statistical variability and reduces the number of animals required for a study. Moreover, in contrast to histological and immunohistochemical analyses, measurements in the dorsal skinfold chamber are performed in living animals, which enables the detailed assessment of functional parameters such as vessel growth, microvascular perfusion, vascular permeability and cellular interactions.

(2) Apart from conventional epi-illumination fluorescence microscopy, imaging in the dorsal skinfold chamber model has increasingly been performed in recent years by means of confocal microscopy techniques (Isaka *et al*., 2004; Makale, 2007; Strieth *et al*., 2008, text references). Using a pinhole aperture for point illumination, single-photon laser-scanning microscopy allows the detection of light from a specific volume within the plane of focus, so that the resultant image is comparatively free of scattered light and attendant blurring (Makale, 2007). Accordingly, the obtained microscopic images exhibit an improved optical resolution and contrast. Moreover, it is possible to concatenate individual optical sections to create a three-dimensional reconstruction of the object of interest. As a variation of this technique, multiphoton microscopy uses near-infrared lasers for two-photon excitation, which allows the generation of bright, high-resolution images in sample depths of 500-1000 µm, while photobleaching and phototoxicity-induced tissue damage is markedly reduced (Makale, 2007; Ishii and Ishii, 2011, text references).

In addition to microscopy, several other non-invasive techniques have been used in the past for the analysis of microcirculatory parameters within the dorsal skinfold chamber, including Laser Doppler flowmetry for the assessment of microvascular tissue perfusion (Menger *et al*., 1992b, text reference) or phosphorescence quenching for the measurement of tissue oxygenation (Kerger *et al*., 1996, text reference). However, these techniques are indirect in nature and, thus, do not allow for the direct visualisation of distinct cellular mechanisms, such as the interaction of leukocytes with the microvascular endothelium or the ingrowth of new blood vessels into implanted biomaterials.

(3) For implantation of a biomaterial, the cover glass of the chamber is temporarily removed and the biomaterial is freely positioned onto the panniculus carnosus muscle taking care to avoid contamination, mechanical irritation or damage of the prepared tissue. Due to this mode of implantation, there is no mechanical loading of the biomaterial. However, mechanical stress has been shown to be a crucial determinant for the angiogenic activation and differentiation of stem cells (Kasper *et al*., 2007; Glaeser *et al*., 2010, text references) and, thus, may in particular affect the vascularisation of stem cell-seeded biomaterials, such as scaffolds for tissue engineering (Sandino *et al*., 2010, text reference).

To overcome the disadvantage that the mechanical environment is completely excluded in the chamber model, static mechanical load can be applied to biomaterial implants by pressing a silicone pad, which is fixed with an

adjustable screw to one of the chamber frames, on the cutis of the back side of the observation window (Contaldo *et al*., 2007, text reference). The combination of this technical modification with a micromotor may in future studies even allow exposing biomaterial implants to dynamic mechanical stresses over prolonged time periods.

Reviewer I: What is the infection rate of the animals after the implantation of the dorsal skinfold chamber?

Authors: The infection rate of the animals is rather low. In well trained hands, less than 10 % of dorsal skinfold chambers show local signs of infection, i.e. vasodilation and oedema formation, after implantation. To guarantee this low infection rate, it is important to disinfect thoroughly the back of the animals and all instruments during the implantation procedure of the chamber. Moreover, the whole procedure should not exceed 20-30 min to reduce the time to a minimum, during which the prepared tissue layers within the observation window are exposed to the ambient air.

Reviewer I: Are there any cases where the animals felt much discomfort and then eventually the operation failed?

Authors: The authors are not aware of such cases. As already stated in the manuscript, the animals tolerate the chambers well, as indicated by normal daily feeding, cleaning and sleeping habits, which do not differ from those of animals without chambers.

Reviewer I: Is it possible that the chamber can be used for the growth of embryonic tissues, stem cells or cancer cells?

Authors: Because the cover glass of the observation window can temporarily be removed, the dorsal skinfold chamber suits ideally for transplantation and implantation experiments. Accordingly, this model has been extensively used to study the growth and vascularisation of various tumour types (Vajkoczy *et al*., 2000; Bingle *et al*., 2006; Al-Jamal *et al*., 2010, additional references), ovarian follicles (Laschke *et al*., 2002, additional reference), endometrial tissue (Laschke *et al*., 2005c, additional reference), and transplanted stem cell spheroids (Moosmann *et al*., 2005, additional reference).

Reviewer II: Mice, rats and hamsters have been used as the animals for the implantation of the dorsal skinfold chamber. How are these three types of animals compared when this technique is used?

Authors: The basic principle of this model, i.e. to provide a chronic access to exposed tissues in an implanted chamber for microscopic imaging through an observation window, is identical in mice, rats and hamsters. However, as already described in our article, there are minor differences between these species when applying this model. In contrast to rats and mice, preparation of the dorsal skinfold chamber in hamsters bears the major advantage that the retractor muscle is only loosely attached to the underlying panniculus carnosus muscle without many vascular interconnections between the two muscle layers. Accordingly, the retractor muscle can easily be removed without surgical trauma to the tissue, which serves for later microscopic analyses. Moreover, the hamster dorsal skinfold preparation is characterised by a better translucency due to a thinner panniculus carnosus muscle, resulting in an improved microscopic image quality when compared to the mouse or rat chamber. On the other hand, the mouse as experimental animal is less expensive and genetically better defined than the hamster. In addition, there is a multitude of knock-out and transgenic strains, which allow for the analysis of physiological and pathological processes on a molecular basis. The availability of a broad range of monoclonal antibodies directed against distinct cell surface molecules further enables for detailed immunohistochemical analyses of isolated tissue samples. For these reasons, biomaterial studies in the dorsal skinfold chamber model are increasingly performed in mice.

Reviewer II: How are the findings derived from the dorsal skinfold chamber compared with the actual clinical outcome when the biomaterials tested in animals are used in humans?

Authors: The dorsal skinfold chamber model is typically used to analyse the early angiogenic and inflammatory host tissue response to biomaterials during the first 2-3 weeks after implantation. Because biomaterials in humans are usually not analysed during the first 2 weeks after implantation, a direct comparison of the findings derived from the dorsal skinfold chamber model with the actual clinical outcome is difficult. Moreover, because in clinics materials are normally analysed only functionally, there are no systematic angiogenesis data available during the first 2 weeks. However, it has to be considered that this early observation period is of particular interest in biomaterial testing, because especially the initial vascularisation of implants has been proposed to be a crucial determinant for adequate biomaterial incorporation into the host tissue, minimising the risk of extrusion, migration and infection.

Reviewer II: Are the daily activities of the mouse with an implanted chamber affected as the metal frames look big as compared with the size of the animal?

Authors: The chamber frames are made of titanium and, thus, the entire chamber for mice exhibits a low weight of ~2 g, representing less than 10 % of the body weight of the animals. Accordingly, the animals tolerate the chambers well, as indicated by normal daily activities, feeding and cleaning habits, which do not differ from those of their litter mates.

Reviewer II: In this review, this chamber is described as a chronic *in vivo* model. What does "chronic model" mean?

Authors: In acute animal models analyses are typically performed only at a single observation time point. In contrast, the term "chronic model" means that the dorsal skinfold chamber allows for the non-invasive and repetitive analysis of the angiogenic and inflammatory host tissue response to implanted biomaterials in individual animals over an observation period of 2-3 weeks.

Reviewer II: The intravital microscopy is described as a sophisticated technique in the review, so how does it work?

Authors: Intravital fluorescence microscopy can be used

to study distinct cellular and molecular processes in the living animal with a good temporal and spatial resolution by the application of different fluorescent dyes (examples are listed in our article). Upon excitation of these dyes by light of defined wavelength, they emit photons, which are then focused to a camera detector. The camera images are recorded on DVD or hard disk for subsequent off-line analysis by means of a computer-assisted image analysis system.

Reviewer II: How do transgenic mice help with this chamber in various types of biomedical research?
Authors: During the last years, transgenic or knock-out mouse strains have gained major importance in biomedical research with the chamber model, because they provide completely new insights into the regulation of inflammatory and angiogenic processes on a molecular basis. For instance, the analysis of venular leukocyte-endothelial cell interaction in tumour necrosis factor (TNF)-α-treated dorsal skinfold chamber preparations of mice deficient in angiopoietin (Ang)-2 could identify Ang-2 as an important autocrine regulator of endothelial cell inflammatory responses (Fiedler *et al*., 2006, additional reference). Moreover, intravital fluorescence microscopy of *in situ* prevascularised scaffolds from green fluorescent protein (GFP)-transgenic mice implanted into dorsal skinfold chambers of wild-type recipient animals has been used to study the interaction of the implants' preformed blood vessels with the host microvasculature at the implantation site (Laschke *et al*., 2010, 2011, text references). Based on the findings of such studies it may be possible in the future to develop novel biomaterial implants with an improved biocompatibility and vascularisation potential.

Reviewer II: In orthopaedic or traumatic studies, some bone substitutes are quite big in size. Can this chamber be used for testing the biocompatibility of these big substitutes?
Authors: For the *in vivo* analysis of bone substitutes in the dorsal skinfold chamber, the implants should not be thicker than 1 mm to guarantee the closure of the chamber free from air. Moreover, the bone substitutes should not substantially exceed a size of ~3 x 3 mm to enable analyses of the border zones in direct vicinity to the implants as well as of distant areas within the chamber, which may serve as control tissue that is not affected by the implants. Thus, bone substitutes, which may be quite big in size for clinical applications, cannot be tested in their original size. This, however, does not necessarily represent a disadvantage for the analysis of the angiogenic and inflammatory host tissue response at the implantation site. In fact, the testing of small biomaterial samples of identical sizes bears the advantage that the biocompatibility of these samples is directly comparable under the standardised conditions of the dorsal skinfold chamber model.

Reviewer II: Are there any possibilities where the tested biomaterials induce severe immune responses or inflammation that jeopardise the animal's survival or daily activities?
Authors: Implanted biomaterials may only induce a local but not systemic inflammatory host response. Accordingly, testing of biomaterials in the dorsal skinfold chamber model does not jeopardise the animal's survival or daily activities.

Reviewer III: To what extent has confocal microscopy been applied in the skinfold model?
Authors: In the past, most studies in the dorsal skinfold chamber model have been performed by means of epi-illumination fluorescence microscopy. However, in recent years confocal microscopy techniques have increasingly gained in importance when using this model (Isaka *et al*., 2004; Makale, 2007; Strieth *et al*., 2008, text references), because the obtained microscopic images exhibit an improved optical resolution and contrast. Moreover, it is possible to concatenate individual optical sections to create a three-dimensional reconstruction of the object of interest. Finally, multiphoton laser-scanning microscopy allows the generation of bright, high-resolution images in sample depths of 500-1000 µm, while photobleaching and phototoxicity-induced tissue damage is markedly reduced (Makale, 2007; Ishii and Ishii, 2011, text references).

Reviewer III: There is an emerging field of biomaterial immunology. What unique contributions may the skinfold implant make to studies in this area?
Authors: The dorsal skinfold chamber model in combination with intravital microscopy techniques offers the unique opportunity to study repetitively *in vivo* the dynamic inflammatory host tissue response to implanted biomaterials throughout an observation period of 2-3 weeks. For this purpose, leukocytes are stained *in situ* with the fluorescent dye rhodamine 6G to analyse their interaction with the microvascular endothelium of blood vessels at the implantation site. Moreover, activation and accumulation of mast cells around biomaterial implants can be studied by topical ruthenium red staining of the chamber tissue. Finally, distinct subsets of immune cells may be isolated and reinjected after *ex vivo* labelling to investigate their recruitment at the implantation site. This approach may allow an even more detailed analysis of the time-dependent contribution of different immune cell types to the foreign body reaction of the host tissue within the dorsal skinfold chamber.

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