Ex vivo method to visualize and quantify vascular networks in native and tissue engineered skin

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Abstract
Background and aims Neovascularization plays a pivotal role in tissue engineering and tissue regeneration. However, reliable technologies to visualize and quantify blood vessel networks in target tissue areas are still pending. In this work, we introduce a new method which allows comparing vascularization levels in normal and tissue-engineered skin.
Materials and methods Normal skin was isolated, and vascular dermal regeneration was analyzed based on tissue transillumination and computerized digital segmentation. For tissue-engineered skin, a bilateral full skin defect was created in a nude mouse model and then covered with a commercially available scaffold for dermal regeneration. After 3 weeks, the whole skin (including scaffold for dermal regeneration) was harvested, and vascularization levels were analyzed.
Results The blood vessel network in the skin was better visualized by transillumination than by radio-angiographic studies, the gold standard for angiographies. After visualization, the whole vascular network was digitally segmented showing an excellent overlapping with the original pictures. Quantification over the digitally segmented picture was performed, and an index of vascularization area (VAI) and length (VLI) of the vessel network was obtained in target tissues. VAI/VLI ratio was calculated to obtain the vessel size index.
Conclusions We present a new technique which has several advantages compared to others, as animals do not require intravascular perfusions, total areas of interest can be quantitatively analyzed at once, and the same target tissue can be processed for further experimental analysis.

Keywords Vascularization · Dermal regeneration · Skin · Tissue engineering

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Introduction

Skin-related clinical problems could occur due to many different etiologies including disease-related processes, trauma, or aging. Classical surgical treatments include autologous or heterologous split or full skin transplantation, which presents several complications including morbidity of the donor site and disease transmission. An alternative approach is the use of scaffolds for dermal regeneration

which are placed over the wound bed, serving as three-dimensional matrix for cell infiltration and further dermal regeneration [1]. Only an adequate vascular blood supply can ensure the presence of oxygen, nutrients, and immune cells during tissue regeneration [2]. Thus, establishment of vascular networks is of crucial importance for clinical success in regenerative medicine and represent a major challenge in tissue engineering technologies [3, 4] including scaffold-based dermal regeneration [4]. To study and compare different scaffolds and new therapeutic approaches, methods and technologies for blood vessel visualization and quantification in experimental models are indispensable. Light, fluorescence, confocal, and electron microscopy have been used for ex vivo blood vessel visualization. These techniques have adequate resolution, but they require special labeling, are time-consuming, and expensive. Moreover, several stains (e.g., antibodies against endothelial cells) do not distinguish between functional vessels and non-perfused vessels [5]. Non-microscopical methods such as computerized tomography, magnetic resonance imaging, and positron electron tomography are also commonly used in visualization of blood vessels, but they require sophisticated equipment, and due to the low resolution power, ranging between 100 to 500 μm, these techniques do not permit to study microvascular processes [6]. Other approaches to visualize entire vessels is the use of intravascular perfusion with different tracers such as contrast phase liquid followed by a radiographic analysis of the target tissue or perfusion of the animal with latex or plastic material followed by tissue clearance or degradation [7]. Perfusion of the animals with tracers excludes the use of the same target tissue for other studies such as protein or RNA analysis. Moreover, results strongly depend on the levels of artificial perfusion and distribution of the tracers in the animal.

Taken together, no reliable technology for vascular network visualization and quantification in native tissues and bioartificial substitutes (e.g., dermal scaffolds) has been published so far. In this work, we hypothesized that based on the thin structure of the skin and the intrinsic blood perfusion of the vessels, the entire blood vessel network of the target tissue can be visualized by light contrast enhancement and further digitally segmented to obtain quantitative data. In this work, we quantified the vascularization levels of a commercially available scaffold for dermal regeneration describing a new ex vivo technique which allows visualization and quantification of area and length of the blood vessel networks in native and tissue-engineered skin.

Materials and methods

Target tissue analysis was performed in nine female nu/nu mice (Taconic, Copenhagen, Denmark) 6 to 8 weeks of age (body weight 20–25 g). Animals were divided in three groups (three animals each) consisting in transillumination of normal skin, tissue perfusion followed by radioangiography and a group for scaffold-based dermal regeneration. Animals were anesthetized with ketamine (Ketanest®, Pfizer. Kalsruhe. Germany; 10 mg/kg) and xylazine (Rompun®, Bayer Leverkusen, Germany; 2.4 mg/kg) via intraperitoneal injection. The University of Luebeck review committee for experimental work on laboratory animals approved all experiments.

Blood vessel visualization

Under general anesthesia, skin from the back of the animals was removed and quickly placed over a transilluminator (Hama, LP 5000K, Germany). Digital pictures were obtained in TIF format (Olympus camera, C-5060, 5.1 megapixels) and stored for further digital analysis.

Specimen preparation and X-ray settings

Under general anesthesia, animals were perfused with 1.5 ml of $BaS0_4$ (Barilux, Sanochemia, Germany) by intracardiac injection. To follow the perfusion in the animal, green ink was added and mixed with the $BaS0_4$ solution. After perfusion, animals were sacrificed, and skin was removed. An X-ray analysis was performed using mammography technique (9 mAs, 24 kV, Senographe DMR+, GE Medical Systems, Milwauke, WI, USA).

Scaffold-based dermal regeneration in vivo

Under general anesthesia, a bilateral full skin defect was created in the back of three animals (15 mm diameter), and the skin was replaced by a commercially available scaffold for dermal regeneration. Integra matrix (Integra®DRT, Integra Life Science Corporation, Plainsboro, NJ, USA). To avoid possible artefacts during tissue harvesting, a titanized mesh (TIMESH, GfE Medizintechnik GmbH, Germany) was placed between the wound bed and the scaffold for dermal regeneration. After 3 weeks, the whole skin from the back of the animals, including the scaffolds and mesh, was removed, and digital pictures were obtained as described above.

Computerized vessel segmentation and quantification

The basics of the vessel segmentation algorithms used here have been previously described [7, 8]. Briefly, vessel segmentation takes place in two steps: first, vessel enhancement, resulting in a vessel map where vessel structures appear with improved contrast, followed by the final vessel segmentation. The segmentation process is semiautomatic.

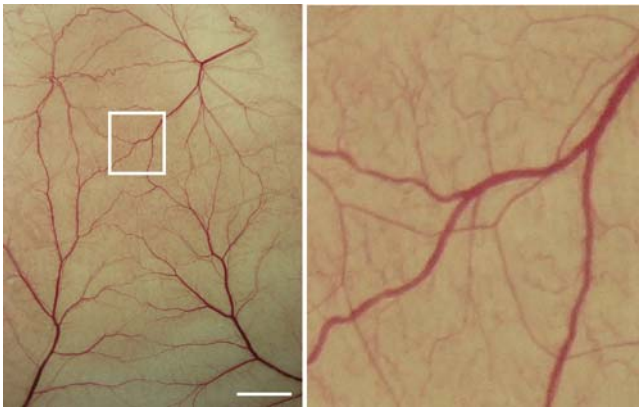

Fig. 1 Blood vessel network visualization. After tissue transillumination, blood vessels from different sizes were visualized. *Left panel* shows the general structure of the vascular network in the back of the animal. *Right panel* shows a close-up of the tissue where even very small vessels were visualized. *Scale bar* represents 5 mm

Initially, an automatic segmentation proposition is presented, and if the result is not satisfactory, the image can be manually edited to eliminate false-positive segmentations as well as to add new vessel structures, which have either not been segmented at all or have been only partially segmented. After segmentation, area and length of the image covered by vessels (white pixels) can be easily obtained. To compute the length, the result of the vessel segmentation needs to be thinned [9] obtaining only the vessel center lines with a width of one pixel (see Fig. 3). Results can be expressed in metric or pixel units. The vessel-analysis software described here can be freely downloaded at: http://www.isip.uni-luebeck.de/index.php?id=150

Results

Direct blood vessel visualization and digital segmentation is a novel technique which allows visualizing and quantifying blood vessels in native or engineered tissues. Enhancement of native contrast between blood vessels and surrounding tissue by transillumination allowed easy visualization of vessels in fresh isolated tissues. Broad areas of tissues were analyzed (Fig. 1, left panel) giving us an idea about the structure of the whole vascular network in the target and surrounding tissues. With this technique, very small vessels (>20 μm) were detected (Fig. 1, right panel). To compare this visualization method with radio-angiographies, one of the gold standards in the field, animals were perfused as described in the "Materials and methods" section, and

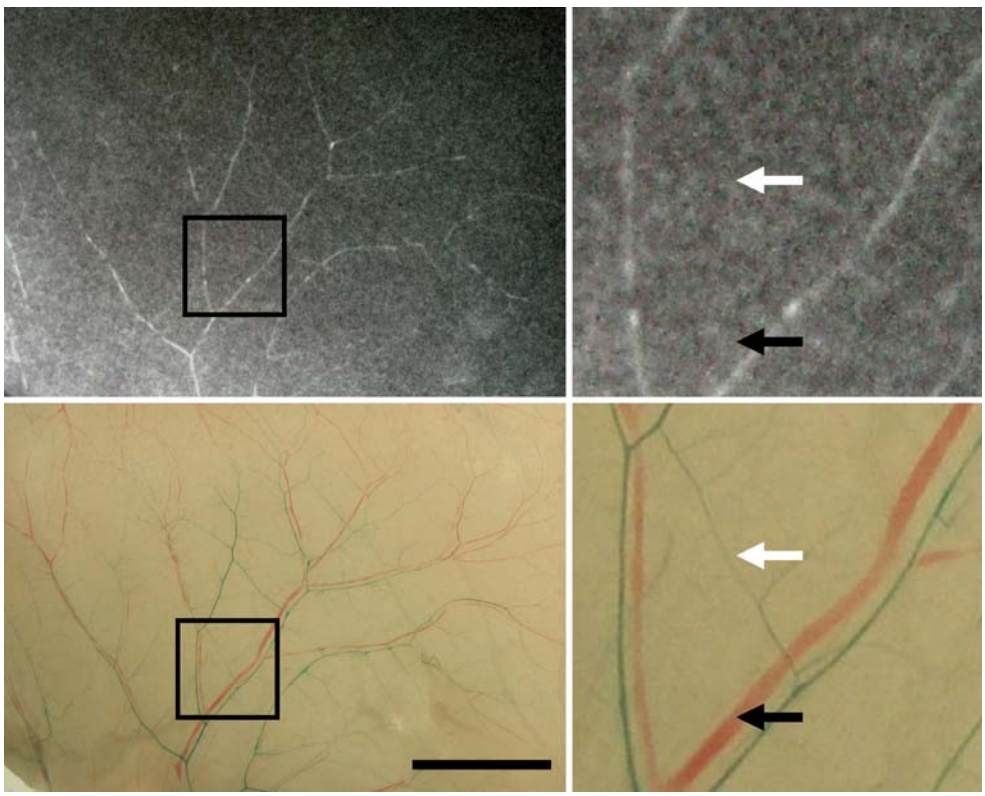

Fig. 2 Validation of transillumination for vessel visualization. Animals were perfused with a mix of $BaSO_4$/green ink. After that, skin was removed and tissue transillumination (*upper*) was compared with radio-angiographic analysis (*lower*). Results showed that several small perfused (*green vessels*/*white arrow*) and not perfused (*red vessels*/*black arrow*) vessels seen by transillumination are not present in the radio-angiogram. *Scale bar* represents 5 mm

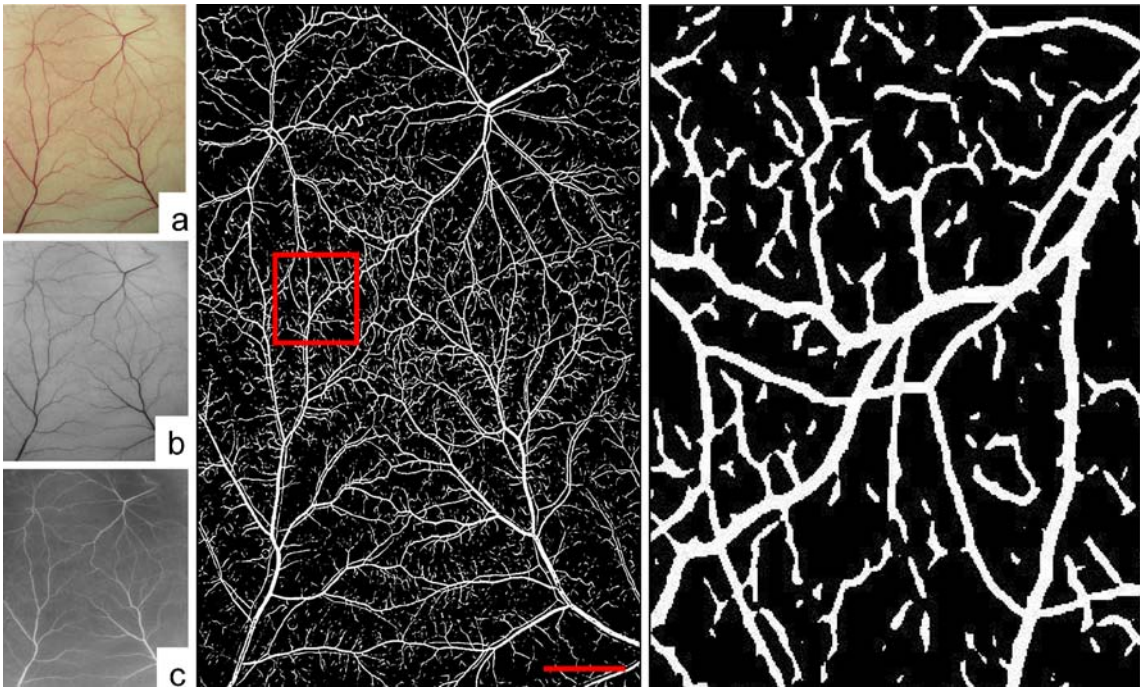

Fig. 3 Digital segmentation of the blood vessel network. *Left panels* show the image processing before segmentation. The steps were: **a** normal picture, **b** picture transformation to gray scale, and **c** inversion of the gray scale. Then, pictures were transformed into a black/white image where white structures correspond to blood vessels. *Middle panels* show the segmentation of the Fig. 1 with a close-up (*right panel*) showing that even small vessels were segmented. *Scale bar* represents 5 mm

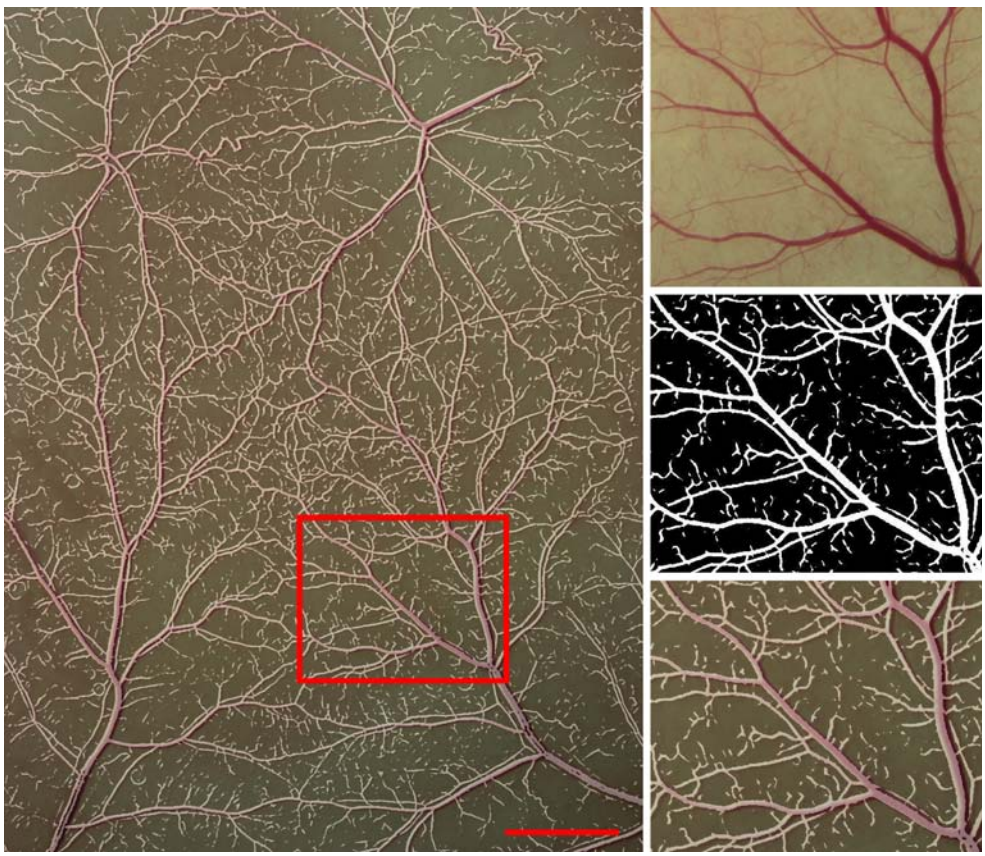

Fig. 4 Segmentation overlapping. To validate the results obtained by digital segmentation, original and segmented pictures were overlapped. *Left picture* shows an excellent correlation between both pictures where *pale pink vessels* represent the overlapping between original (*red*) and segmented (*white*) vessels. A close-up (*left panel*) shows that vessels observed by transillumination were all segmented and vice versa

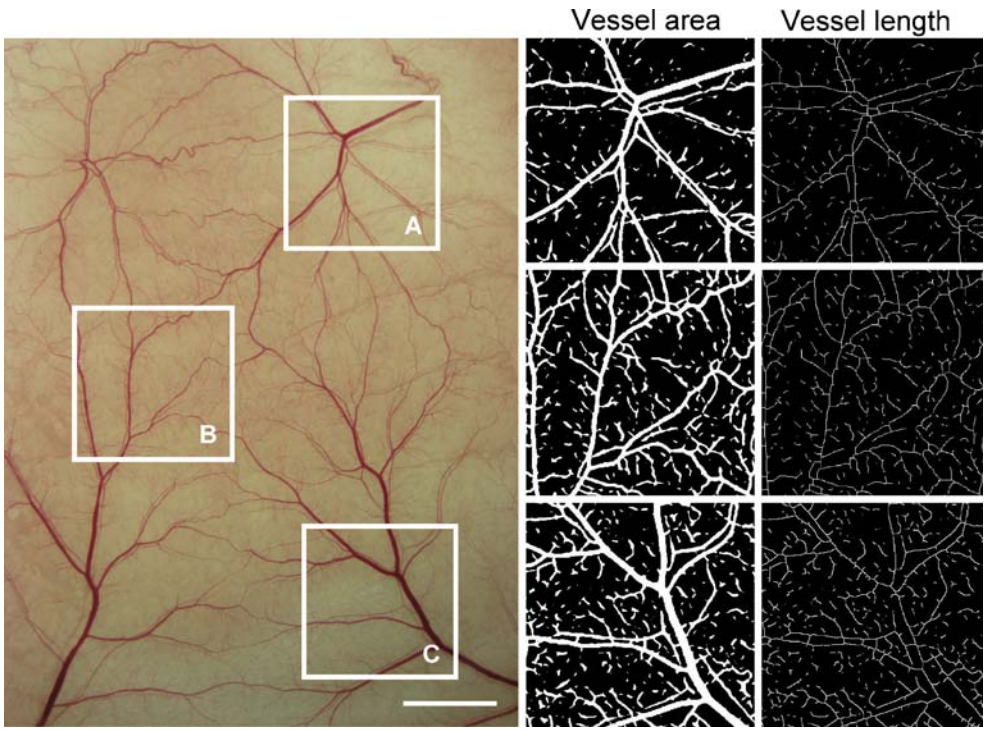

Fig. 5 Quantification of the vascular networks. Different areas of the original picture (*left*) were chosen and segmented in terms of vessel area and length

tissues were isolated. Then, radio-angiographic analysis and transillumination were compared in the same tissue area. Results showed that radio-angiographic studies are strongly influenced by the tracer perfusion level of the vessels and their size. Figure 2 shows that some vessels cannot be detected either because they are too small or because they are not well perfused, resulting in an underestimation of the vascular network. Moreover, the resolution of the visualized vessels by radio-angiographies was much lower than the resolution obtained by transillumination.

After blood vessel visualization by transillumination, a digital image was taken, and computerized analysis was performed as described in the "Materials and methods" section. For segmentation, the original images were converted into gray scale (Fig. 3b) and then inverted such that vessels appear brighter than the background according to the conventions used by the vessel enhancement algorithms

Table 1 Vascular area index (VAI), vascular length index (VLI), and vascular size index (VAI/VSI) were quantified in the three different regions selected in Fig. 5

Region	VAI (%)	VLI (%)	VSI
A	17.08	3.80	4.49
B	21.56	5.00	4.31
C	23.23	6.39	3.64

Results show that, with this method, small differences between different target areas can be detected.

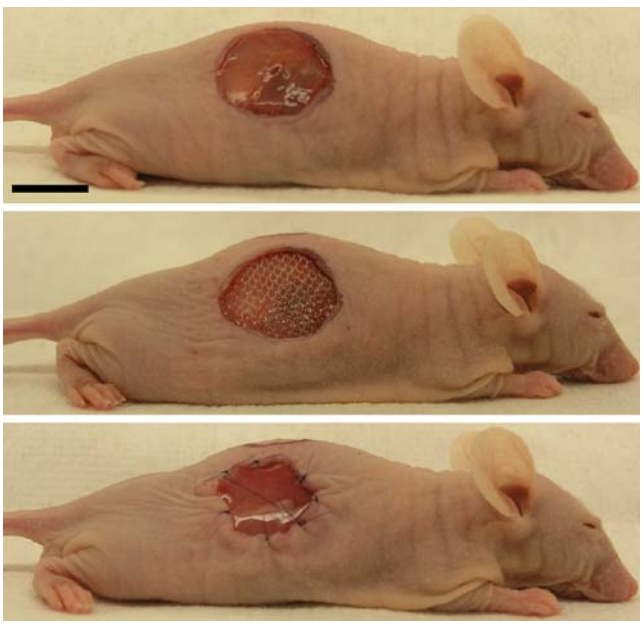

Fig. 6 Full skin dermal regeneration model. A 15-mm diameter bilateral full skin defect was created in the back of the animal and replaced by a scaffold for dermal regeneration. To avoid possible artefacts during the tissue harvesting, a titanized mesh was placed between the wound bed and the scaffold (*middle picture*). *Scale bar* represents 1 cm

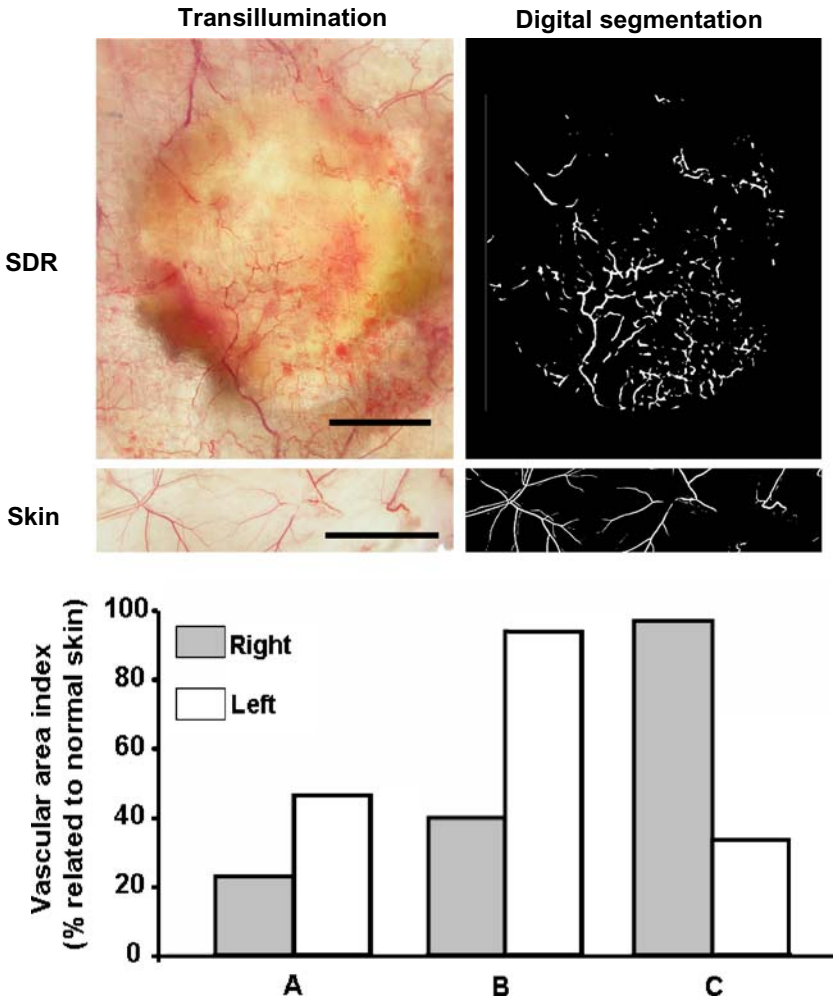

Fig. 7 Assessment of blood vessel network in a scaffold-based dermal regeneration model. Three weeks post-scaffold transplantation, the whole skin of the back of the animal (including scaffolds and mesh) was removed, and transillumination and digital segmentation were performed. Vascular structures in normal skin and in the scaffold were clearly visualized and segmented (*upper panels*). Quantification of three different animals (**a**, **b**, and **c**) are shown in the graph where *left and right* represent the side of the defect. *Scale bar* represent 0.5 cm in scaffold for dermal regeneration and 1 cm in normal skin

(Fig. 3c). Finally, segmentation was automatically performed by the software. Results show that even the smallest vessels can be segmented by this program (Fig. 3, right). To evaluate the reliability of the segmentation, both images were overlapped, resulting in an excellent correlation between the original picture and the digital segmentation (Fig. 4).

To quantify the vascular area index (VAI), vascular length index (VLI), and vascular size index (VSI; VAI/VLI), different target areas were selected (Fig. 5, left), and digital segmentations were performed in terms of the area and length of the vessels (Fig. 5, right panels). After that, the percentage of white pixels (blood vessels) from the total number of pixels in the picture was automatically calculated by the program, thus obtaining the VAI, VLI, and VSI. Results showed that small differences in vascularization can be detected by the combination of tissue transillumination and digital segmentation (Table 1).

As shown in Fig. 6, a bilateral full skin defect was created in the back of the animals, a titanized mesh was placed over the wound bed, and the defect was covered by a scaffold for dermal regeneration. After 3 weeks of regeneration, animals were anesthetized, and the whole skin of the back (including mesh and scaffolds) was removed, and transillumination and digital segmentation were performed. For quantification of the vascularization levels in the scaffold for dermal regeneration, normal skin and tissues in regeneration were analyzed in the same sample and compared among them. Control healthy tissue from the same animal was used for normalization. One hundred percent of vascularization was assigned to the normal skin, and vascularization of the scaffold was quantified related to this areas. Figure 7 (upper panel) shows that after transillumination, vascular networks in the scaffold for dermal regeneration can be clearly visualized,

digitally segmented, and further quantified. Results obtained for the analysis of six scaffold for dermal regeneration (three animals) shows broad differences in vascularization levels with percentages ranging from 22.8 to 97.1 (average±SD: 55.6±31.9).

Discussion

Like in other fields of tissue engineering, scaffold-based dermal regeneration requires the development of reliable tools for visualization and quantification of vascular networks. In this work, we developed a novel method which allows quantifying blood vessels and presents several advantages compared to other established techniques. The operative process takes approximately 10 min, non-sophisticated equipment or technical expertise is required, and fresh target tissue can be frozen or treated for further histological or molecular analysis. As no chemical additives are needed to visualize blood vessels, other samples (e.g., serum) can be obtained to be analyzed in parallel to the target tissue, saving animals and enabling individual comparisons. Moreover, this new technique allows distinguishing leaky vessels, and the quality and resolution of the image give insight into vessel dimensions, intervascular distances, number of branching points, vascular area, and total length of the vascular network. Detection of small vessels in both native and tissue-engineered skin equivalents is possible without further treatments in this model. The method described here depends on the excision of the area of interest which allows the analysis of only one single time point. Due to that, dynamic parameters such as vessel infiltration in scaffold cannot be determined. However, the combined use of this new method with dorsal skinfold chambers could allow daily quantification of the vascularization of scaffold for dermal regeneration [10]. A drawback of the technique is the fact that it is restricted only to scaffolds or tissues which can be transilluminated. In this context, normal mouse skin and scaffold for dermal regeneration are ideal, while other scaffolds or tissues may not allow the penetration of light. To avoid possible artefacts due to the inclusion of prevascularized subcutaneous tissue in the sample, in this model, a titanized mesh was initially placed between the wound bed and the scaffold for dermal regeneration serving as a physical border during the tissue harvesting. After transillumination and digital segmentation, the blood vessel area and blood vessel length can be calculated as percentages of white pixels from the total number of pixels in the picture. To obtain comparable data between different animals or treatments, an area of reference was used for normalization, and results were expressed as a percentage of vascularization related to control areas, thus avoiding possible artefacts due to differences in the hematocrit level or vascular dilatation between animals. With the method presented here, a vascular area index (VAI) and vascular length index (VLI) was obtained. As only the vessel length counts, VLI is independent from the vessel calibre, allowing to obtain data about vascular density, in contrast with VAI where a higher percentage of vascularization could be obtained for the presence of few bigger vessels. A ratio between both represent an index of vessel size (VSI=VAI/VLI). High levels of vascularization due to few big vessels will have a big VSI, whereas tissues vascularized by small capillaries will have a smaller VSI with tendency to one.

In this work, we also propose that mouse skin could serve as a new general model for testing scaffold vascularization in different fields of tissue engineering.

Conclusion

In this work, we showed that digital segmentation of the blood vessel network allows quantifying vascularization of tissue target areas independent of the size. The combined use of tissue transillumination and digital segmentation of the vascular network provide a simple, reliable, and quantitative tool for studying vascularization of scaffolds in laboratory animals. This tool may also serve as a screening model to study vascularization of different biomaterials or the angiogenic or antiangiogenic effects of therapeutic substances after systemic application to the animal or directly into the scaffold.

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Conflict of interest statement None disclosed.

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