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## Adipose-derived regenerative cells and dermal regenerative biomaterials: An alternative approach for surgical reconstruction --Manuscript Draft--

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<b>Abstract:</b>	Dermal regeneration templates enriched with cultured human adipose-derived stem cells (ASCs) have been developed for regenerative approaches. However, few protocols have used autologous freshly isolated (i.e., uncultured) ASCs on dermal regeneration templates, such as collagen and elastin matrices. Here, a protocol explains a technique for immediate seeding of a collagen and elastin matrix with adipose derived stem cells intra-operatively. This will allow the reconstruction of wounds during a single surgical procedure. Furthermore, all laboratory procedures for cell isolation and seeding can be avoided, by ensuring that all steps are performed inside the operating room. Briefly, the enrichment of the collagen/elastin matrices with adipose-derived regenerative cells can be accomplished with the beginning of fat aspiration procedures to obtain lipoaspirate, which can be processed and purified to

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<b>Author Comments:</b>	<p>Addressed to: Dr. Upponi and Dr. Singh, Addressed to editors of JoVE,</p> <p>Dear Sir or Madam,</p> <p>Thank you very much for the extensive reviews of our article entitled "Adipose-derived regenerative cells and dermal regenerative biomaterials: An alternative approach for surgical reconstruction".</p> <p>We have considered and evaluated the concerns that have been raised by the editor. In the following list we respond to the points:</p> <p>Editorial comments:</p> <ol style="list-style-type: none"> <li>1. All current changes have been now added to the (File name: 52762) as requested by the editor.</li> <li>2. The current abstract has been modified according to the number requested by the editor and also in the editorial manager account.</li> <li>3. The statement is now included: The protocol and the use of human materials follow the guidelines of the human research ethics committee at RWTH Aachen University Hospital (Name in German: Ethik-Kommission des Universitätsklinikums Aachen).</li> <li>4. Figure legends are now included as part of the manuscript text below the results. Titles and detailed description of each figure are now included in the figure legends.</li> <li>5. The term "Modified from.." etc. is now used as requested. A copy will be sent.</li> <li>6. Citation formatting has been modified as requested by the editor.</li> </ol> <p>We thank you for editing and we wish that this manuscript now meets the high standards of JoVE, so a publication of the paper can be accomplished.</p> <p>With kind and warm regards,</p> <p>Ziyad Alharbi, M.D., Ph.D. Department of Plastic Surgery and Hand Surgery - Burns Unit, RWTH - University Hospital Aachen, Pauwelsstr 30 52057 Aachen, Germany Telephone Number: +49-241-80-0 Fax Number: +49-241-80-82634 E-mail address: zalharbi@ukaaachen.de</p>
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**TITLE:**  
**Adipose-derived regenerative cells and dermal regenerative biomaterials: An alternative approach for surgical reconstruction**

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Skin Regeneration; Reconstruction; Wound Healing; Dermal Substitutes; Collagen Matrix; Skin Graft; Fat Graft; Lipoaspirate; Adipose-derived Stem Cells; Stromal Vascular Fraction

**SHORT ABSTRACT:**

This article explains a technique for immediate seeding of a collagen and elastin matrix with adipose derived stem and stromal cells intra-operatively. Thus, tissue engineered construct can be used in the clinical setting to reconstruct wounds in a single surgical procedure, without the need for additional laboratory steps.

**LONG ABSTRACT:**

Dermal regeneration templates enriched with cultured human adipose-derived stem cells (ASCs) have been developed for regenerative approaches. However, few protocols have used autologous freshly isolated (i.e., uncultured) ASCs on dermal regeneration templates, such as collagen and elastin matrices. Here, a protocol explains a technique for immediate seeding of a collagen and elastin matrix with adipose derived stem cells intra-operatively. This will allow the reconstruction of wounds during a single surgical procedure. Furthermore, all laboratory procedures for cell isolation and seeding can be avoided, by ensuring that all steps are performed inside the operating room. Briefly, the enrichment of the collagen/elastin matrices with adipose-derived regenerative cells can be accomplished with the beginning of fat aspiration procedures to obtain lipoaspirate, which can be processed and purified to reach the stromal vascular fraction (SVF) including stem and stromal cells. After enrichment of the above-mentioned scaffolds, they can be used to cover the wound as a neodermis and as a carrier for adipose-derived regenerative cells. Then, skin grafting can be performed to restore the

epidermal integrity for definitive closure in the same operation. These steps favor the reconstruction of the subcutaneous adipose tissue, dermis and epidermis.

## **INTRODUCTION:**

Major burns, acute and chronic wounds, and congenital anomalies can cause severe soft tissue defects that cannot be adequately treated using simple methods of reconstruction. Thus, regenerative methods and tissue engineering has become a promising tool for replacing damaged tissue. This can be exemplified in the formation of biological constructs that can be applied in wound regeneration<sup>1</sup>. However, in terms of tissue engineering approaches, cell seeding on scaffolds is the critical step and the source of stem cells is always of crucial importance. Herein, subcutaneous adipose tissue is a rich and easy-to-access source of adipose-derived stem cells (ASCs), which have a mesenchymal origin, through a minimally invasive procedure (*i.e.*, liposuction). This procedure is a common daily practice in plastic surgery<sup>2, 3</sup>. After liposuction procedure, the surgeon can either inject fat to other region in the body or process fat cells and isolate regenerative potential cells including ASCs for regenerative concepts and tissue engineering approaches.

In the last 10 years, studies designed approaches that seed previously cultured and expanded preadipocytes onto biocompatible 3-D matrices to heal and regenerate wounded tissue<sup>4</sup>. However, less is known about the potential use of autologous, freshly isolated (*i.e.*, uncultured) ASCs with suitable clinically used dermal matrices, such as collagen and elastin matrices. These matrices can be used as a cellular carrier and as a dermal regeneration template in clinical reconstructive approaches even in combination with split thickness skin graft<sup>4</sup>. Our previous work, published in 2013, concentrated on the use of freshly isolated adipose-derived cells and demonstrated that the method of liposuction can influence the viability of the obtained ASCs and the quantity of growth factors present after seeding onto dermal scaffolds<sup>5</sup>. Briefly, it has been found that micro-harvesting techniques (developed by Magalon) result in more quality behavior of isolated adipose-derived stem cells after their attachment to scaffolds<sup>5</sup>. Moreover, these results showed the capability of collagen and elastin matrices to carry freshly isolated cells *ex vivo*.

Thus, direct transfer of ASCs onto available biological matrices, such as dermal regeneration templates, would be possible during a single operation<sup>6,7,8</sup>. Importantly, a short enrichment time that requires no additional lab processing would be ideal for seeding processes in clinical settings. A one-hour ASC enrichment time on collagen and elastin dermal matrices is clinically relevant<sup>7</sup>. Alharbi et al have published these results in 2014.

In the surgical setting, immediate seeding of a collagen and elastin matrix with adipose derived stem cells can take place. Thus, tissue engineered construct can be used in the clinical setting to reconstruct wounds in a single surgical procedure, without the need for additional laboratory steps. Furthermore, adjuvant autologous fat grafting (*i.e.*, direct fat transfer) beneath wound edges can be performed to enhance the quality of wound healing and angiogenesis through the presence of stem cells, growth factors and endothelial progenitor cells. These steps will ensure the construction of the neodermis and fat tissue as well as favoring normal wound healing. The

last step is achieved via the transplantation of a skin graft on top of the tissue-engineered construct to restore the epidermal integrity for definitive closure during the same operation.

This protocol, as illustrated in the video session and Figure 1, shows the main step of the suggested approach for surgical reconstruction. The liposuction procedure is the first step, which provides the fat tissue that can be used as a direct fat graft or processed for the enrichment of a dermal regeneration template with ASCs. In addition, *in vitro* investigations of the adipogenic conversion ability of transplanted cells on dermal templates were performed. Therefore, elective liposuction procedures (*i.e.*, fat harvesting) were performed in our department and consent was obtained from each patient before analytical investigations.

## **PROTOCOL:**

### **Ethical Statement:**

The protocol and the use of human materials follow the guidelines of the human research ethics committee at RWTH Aachen University Hospital (Name in German: Ethik-Kommission des Universitätsklinikums Aachen).

### **1. Enrichment of dermal scaffolds (*i.e.*, collagen and elastin matrices) with adipose-derived regenerative cells**

#### **1.1) Collagen and elastin matrices**

1.1.1) Use acellular dermal substitute sheets (1 mm thick) consisting of a non-cross-linked native bovine collagen matrix, which contains type I, III and V collagen.

#### **1.2) Liposuction and Purification**

1.2.1) Harvest fat<sup>5,7</sup> (Alharbi et al in 2013 and 2014) using a 2 mm diameter cannula with a blunt tip to avoid cellular injury. 20 ml of harvested lipoaspirate is normally sufficient for a wound size of 5 x 5 cm. Harvest another 20 ml for the adjuvant fat injection under and beneath the wound. Note that the amount of purified fat will be notably reduced after centrifugation.

1.2.2) Immediately after the liposuction procedure, place the body of the filled syringe into a centrifuge and centrifuge for 3 min at 1,188 x g.

1.2.3) After centrifugation, decant the oil layer (*i.e.*, the upper level) and drain the aqueous layer (*i.e.*, the lower level) from the syringe. Immediately use the middle layer, which is predominantly composed of fat grafts and represents the purified lipoaspirate, for experiments and for cell isolation.

#### **1.3) Transplantation of purified lipoaspirate to collagen and elastin scaffolds**

1.3.1) Immediately transplant the whole biological matrix containing the adipose-derived stromal vascular fraction on top of the collagen and elastin dermal template.

NOTE: This procedure ensures the presence of the needed cells and growth factors without further manipulation.

1.3.2) Use a micro-lipografting cannula<sup>5</sup>. See Figure 2. Bring the cannula on top of the matrix to gently spread the fat cells over the whole collagen matrix. After placing the purified lipoaspirate on the matrix, ensure that the enriched fat material stays for one hour *ex vivo* without further manipulation.

1.3.3) During this time it is very important to keep the purified fat tissue wet in order to avoid dehydration. Therefore, add ringer solution in calculation of 1 ml for 1 square meter.

1.3.4) During this time debride the wound bed and edges as normally. Depending on wound bed, either use a simple sharp spoon or even water-jet debridement in order to have a bleeding bed with good vascular status. Also fresh the wound edges with a surgical blade until reaching good vascularity. After one hour, take only the enriched material from the remnant fat and begin the next step.

## **2. Placement of the enriched dermal substitute on the wound**

2.1) Transfer the dermal regeneration template by anatomic forceps (2 forceps are preferred) and modify the template carefully to fit onto the wound, ensuring that there are no gaps with the wound bed (Figure 3).

2.2) Optionally modify the size of the matrix by using surgical scissors. This step prevents hematomas and ensures the presence of a normal biological atmosphere for the transplanted cells on the dermal matrix.

## **3. Adjuvant autologous fat transfer beneath and under the wound bed**

NOTE: This step involves the use of the rest of the fat graft that was not used for cellular seeding on matrices.

3.1) Use a 3 ml syringe that includes purified fat graft for injection. Inject fat around the edges of the wound but not on the space of the wound itself. It is crucial that the injection process must be logical without extra injection that may cause more swelling and high risk of fat embolism. One ml of purified fat graft must be enough for 1 – 2 square centimeter. Inject through different points of entry that are separate up to 4 or 5 centimeters according to the length of the filling system.

3.2) After the entrance of the cannula, aspirate first to ensure that there is no contact with a blood vessel and then inject fat while pulling the cannula to the outside.

## **4. Skin grafting on top of the wound for epidermal reconstruction**

4.1) Graft 0.2 mm split thickness skin from a hidden area or an unimportant aesthetic region to restore the epidermal integrity of the wound.

4.2) Use a surgical dermatome to perform this procedure with a specific thickness (Figures 1 and 5). Use 0.2 mm thickness of skin graft that includes the whole epidermis and a tiny part of



the dermis that include only the papillary dermis but not the reticular dermis. Reticular dermis is the deepest layer of the dermis.

4.3) After grafting, use this skin graft to close the wound. Ensure that the skin graft is on top of the dermal regeneration matrix that is applied before on the wound. Make holes on the skin graft using Nr.11 surgical blade every 2 cm in order to let all blood debris and fluids going out to avoid the formation of hematoma and other complications.

4.4) Suture the edges with healthy skin by using non-absorbable sutures (e.g. Prolene 4.0 or 5.0 for small wounds) that can be removed after 10 days.

### **REPRESENTATIVE RESULTS:**

To ensure that this protocol would generate optimal clinical results, several *in vitro* experiments were performed. Last publication demonstrated that freshly isolated uncultured ASCs could be effectively seeded onto collagen and elastin matrices for *ex vivo* cellular enrichment of these constructs after liposuction<sup>9</sup>. We observed an adequate number of ASCs after a 1-h enrichment time. This clinically relevant time is of crucial importance to us, as it makes this approach realistic during surgery without the need for a lab for cellular enrichment. However, it was important to investigate whether these cells are capable of preadipocyte commitment, which is required for the formation an adipose tissue *in vivo* and for adipocyte differentiation. Thus, it was of crucial importance to investigate the adipogenic conversion ability of the cells (Figure 6). Two-photon microscopy was performed using an FV1000MPE microscope attached to a pulsed Ti-Sapphire laser to observe the 3D-structure of the matrix, including the organization of differentiated adipocytes within the matrix. Hoechst 33342 was added for vital staining of the nuclei of the isolated cells on the matrix. The enriched matrix was also stained with BODIPY 493/503 to image the mature fat cells in combination with Hoechst 33342 to stain nuclei. The non-linear optical effect of second harmonic generation (SHG) has been used to identify the enriched collagen elastin matrix. Visualization of the collagen and elastin matrices using two-photon microscopy revealed the differentiation process that occurred on the construct. An *in vitro* BODIPY assay made it possible to detect differentiated adipocytes by assessing lipid droplets and staining intensity on the matrices. Enriched matrices contained differentiated adipocytes. The red structure represents the collagen and elastin construct (*i.e.*, the Matriderm). The green spots are structures that were stained with BODIPY, including fat droplets and the cytoplasm. In addition, the spatial distribution of the cells was investigated using different views to analyze the migration rate of the differentiated cells on the matrix (Videos 1 and 2).

### **Figure Legends:**

#### **Figure 1: Illustration of the methodology described in this paper.**

Fat Aspiration (Liposuction) is first needed to gain the stromal vascular fraction (SVF) after purification and centrifugation containing adipose-derived regenerative cells including ASCs. Seeding of Regenerative Cells on Dermal Regenerative Templates outside the patient takes place as a second step. Third step is the positioning of the tissue-engineered matrix, which is followed by skin closure (skin grafting).

Modified from MedSkin Solutions (<http://www.medskin-suwelack.com/>).

**Figure 2: Sedimentation of purified stromal vascular fraction on matrices (Sterile Technique)**

Transplantation of an autologous fat graft containing stromal vascular fraction, which includes adipose-derived stem and stromal cells onto selected scaffolds for complete enrichment. During this time, surgical debridement of the wound can be accomplished.

**Figure 3: Dermal regenerative templates (collagen and elastin surgical matrices)**

Example of selected collagen and elastin dermal regeneration templates after their placement on top of the wound. These matrices are proofed for dermal reconstruction and can be used as a carrier for isolated cells after their seeding.

**Figure 4: Adjuvant fat grafting**

Preparation of the purified fat graft for autologous fat transfer under the wound and on the sides. This step is a complimentary step which can be performed to enhance the fat volume under the wound. Fat grafting alone has also shown some regenerative effect.

**Figure 5: Skin Grafting**

A surgical dermatome can be used for the transplantation of an autologous split-thickness skin graft on top of the dermal regeneration template after its enrichment with autologous adipose-derived regenerative cells. This figure shows a transplanted skin on the wound several months after the operation.

**Figure 6: In-Vitro results (Differentiation to adipocytes)**

Full differentiation process of committed preadipocytes into adipocytes (*i.e.*, adipose tissue) on dermal matrices (*i.e.*, collagen and elastin scaffolds). This section has been performed in the lab to investigate the ability of preadipocytes toward the differentiation to mature adipocytes; which forms the block of adipose tissue. 2-photon microscopy shows the viable nuclei of adipocytes (blue color) and fat droplets (green color) as well as the structure of the collagen based matrix (red color)

**Video:**

Direct transplantation of adipose-derived cells onto dermal matrices (*i.e.*, collagen and elastin scaffolds) after transplantation.

**DISCUSSION:**

The reconstruction of deep and extensive defects represents a major challenge in plastic and reconstructive surgery. Split thickness skin transfer is an easy approach, but it may lead to unavoidable outcomes, such as scar formation, which occurs due to the absence of dermis, and poor aesthetic results. Furthermore, this approach is not indicated in specific situations (*i.e.*, exposed tendons and bones). The use of surgical flaps outperforms skin grafting because flaps can reconstruct a missing layer of soft tissue, but the shortcomings of this approach include the lack of available large-size harvested tissues and the high rate of high donor site morbidity. Thus, the latter approach is more suitable for defined defects.

Many techniques that utilize autologous sources of adipose tissue for soft tissue replacement have been reported<sup>8, 9</sup>. Direct fat transfer enhances the hypodermal integrity and enhances the signaling process that promotes preadipocyte commitment and adipocyte differentiation. Furthermore, direct autologous fat transfer reduces the volume deficit, which is needed to prevent visible depression of wide scars. In addition, the utilization of collagen scaffolds as a dermal substitute has been attempted<sup>10, 11</sup>. Collagen-based materials promote favorable adipose outcomes and allow the formation of a neodermis<sup>12, 13</sup>. Our lab and other groups demonstrated the ability of 3D collagen sponges to support adipogenesis and to promote the *in vivo* development of new adipose tissue after 12 weeks<sup>14, 15</sup>.

Adipose tissue engineering for soft tissue replacement has attracted scientists and physicians recently. On a cellular level, adipogenic differentiation is even mediated by the 3D structure of the matrix. This 3D environment provides a scaffold structure that functions as a physiologically relevant microenvironment and promotes cell attachment and migration. These results will indeed facilitates cell–matrix interactions<sup>16, 17</sup>. In this context, our lab demonstrated that the transplantation of isolated and cultured preadipocytes within a standardized collagen matrix resulted in well-vascularized adipose-like tissue *in vivo*<sup>18, 19, 20</sup>. In combination with clinical outcomes, these results demonstrated that collagen-based matrices are biocompatible and cytocompatible matrices that are suitable for the cellular environment.

At the beginning many labs tried to use stem cells and recently human ASCs showed more attention for soft tissue reconstruction. ASCs are widely available, abundant, and easily accessible through procedures such as liposuction<sup>21</sup>. Many studies state that the use of this tissue as a stem cell source has potential for regeneration applications<sup>22</sup>.

Rather than using cultured cells, many studies have been conducted toward the using of ASCs directly after their isolation during liposuction procedures. These cells were directly seeded onto collagen and elastin matrices. Pallua et al demonstrated in 2009 that fresh lipoaspirates obtained via liposuction contain many other growth factors. These growth factors have been shown to improve transplantation results<sup>23</sup>. Then studies continued to use freshly isolated, uncultured adipose-derived stem and stromal cells for clinical regenerative approaches. Regardless of the method of liposuction, freshly isolated and uncultured ASCs attached to collagen and elastin matrices exhibited significant viability after 24 h<sup>6</sup>. Very recently, Alharbi et al developed a method for the direct transfer of ASCs onto collagen and elastin matrices to support transplantation. Clinical settings make the direct use of isolated cells more ideal and attractive than the use of additional cultivation steps in the lab. This type of procedure eliminates the need for GMP facilities and will be less expensive. Therefore, this new approach employs a single step in the operating room to avoid delay. For example, a patient could have a single operation in which the enrichment of a selected collagen-based matrix is combined with split thickness skin transfer for soft tissue reconstruction and adjuvant autologous fat transfer for hypodermal reconstruction.

This approach provides a clinical regenerative method using adipose-derived regenerative cells

in combination with dermal regeneration templates with the aim of restoring the dermal and hypodermal structures. It is clear that such approach is still in its infant phase but nowadays tissue-engineering approaches in surgical operating rooms attract many surgeons due to the high potential of stem cells and biomaterials. What is realistic about this concept is that this technique shows the immediate seeding of a collagen and elastin matrix with adipose derived stem and stromal cells intra-operatively. Thus, tissue engineered construct can be used in the clinical setting to reconstruct wounds in a single surgical procedure, without the need for additional laboratory steps.

To recapitulate, in the surgical setting, after successful enrichment of collagen and elastin matrices with autologous fat-regenerative cells, adjuvant autologous fat grafting (*i.e.*, direct fat transfer) beneath wound edges and under the wound bed can be performed to restore the adipose tissue, enhance the quality of wound healing and reduce the depression of scars. These steps will ensure the construction of the neodermis and fat tissue. The last step is completed via the transplantation of a skin graft on top of the wound to restore the epidermal integrity for definitive closure during the same operation.

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#### **DISCLOSURES:**

The authors disclose that the dermal scaffolds were funded by MedSkin Solutions Ag, Germany. The authors also disclose that a clinical observational study will be conducted with ethical approval. No personal financial benefits were provided to any author, as the primary goal was to contribute to the scientific community.

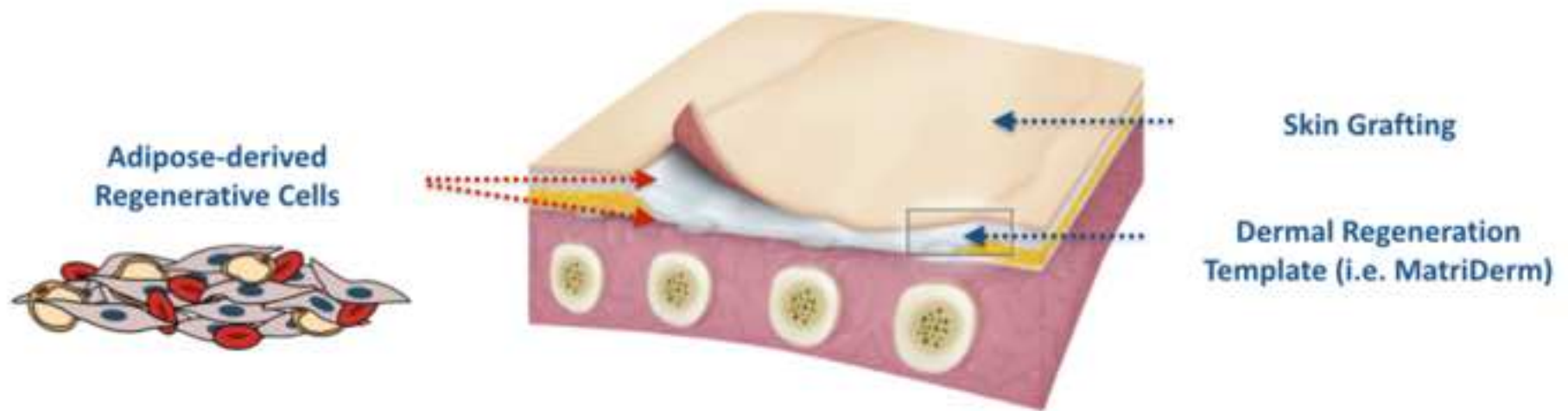
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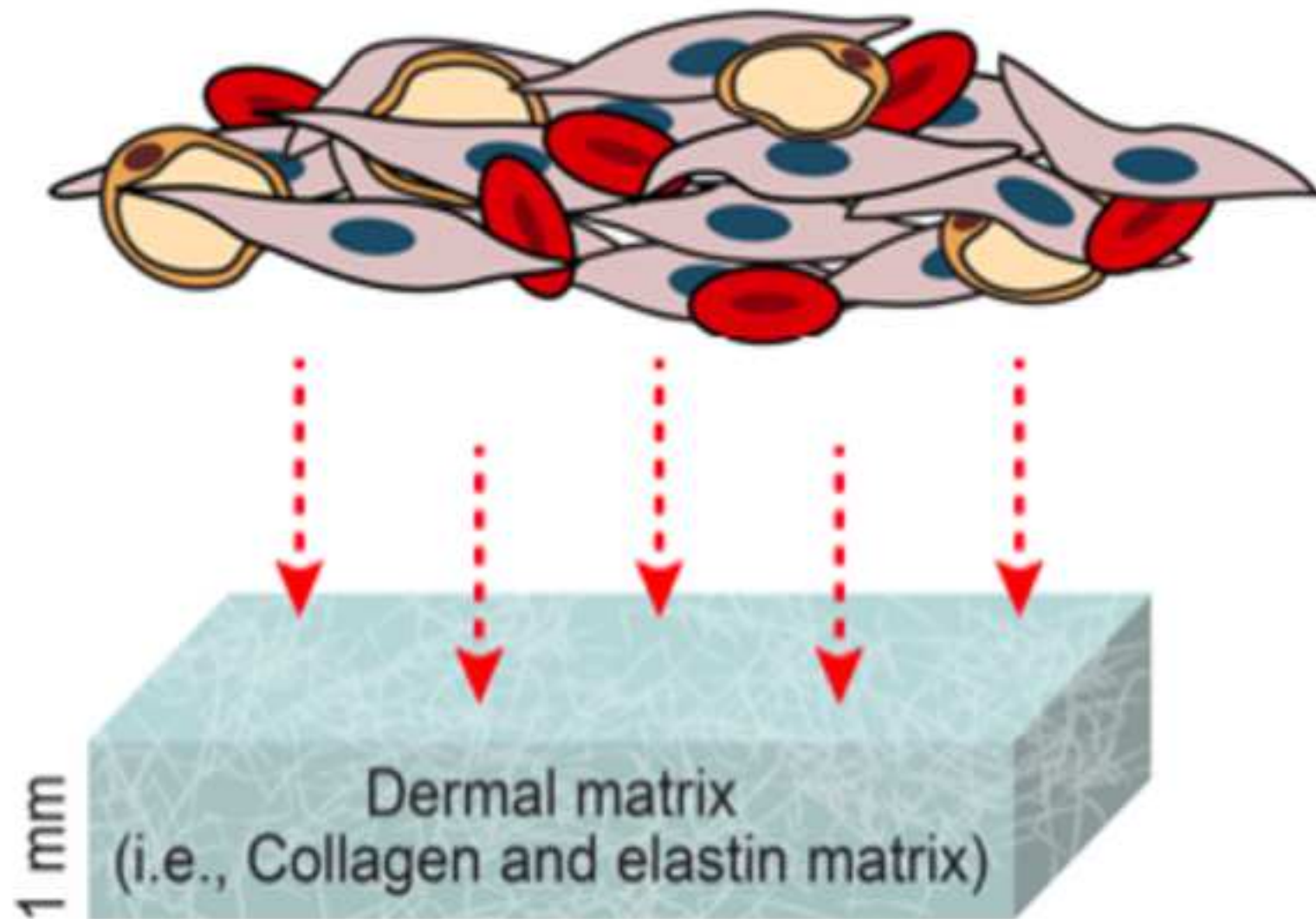
Figure

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Figure

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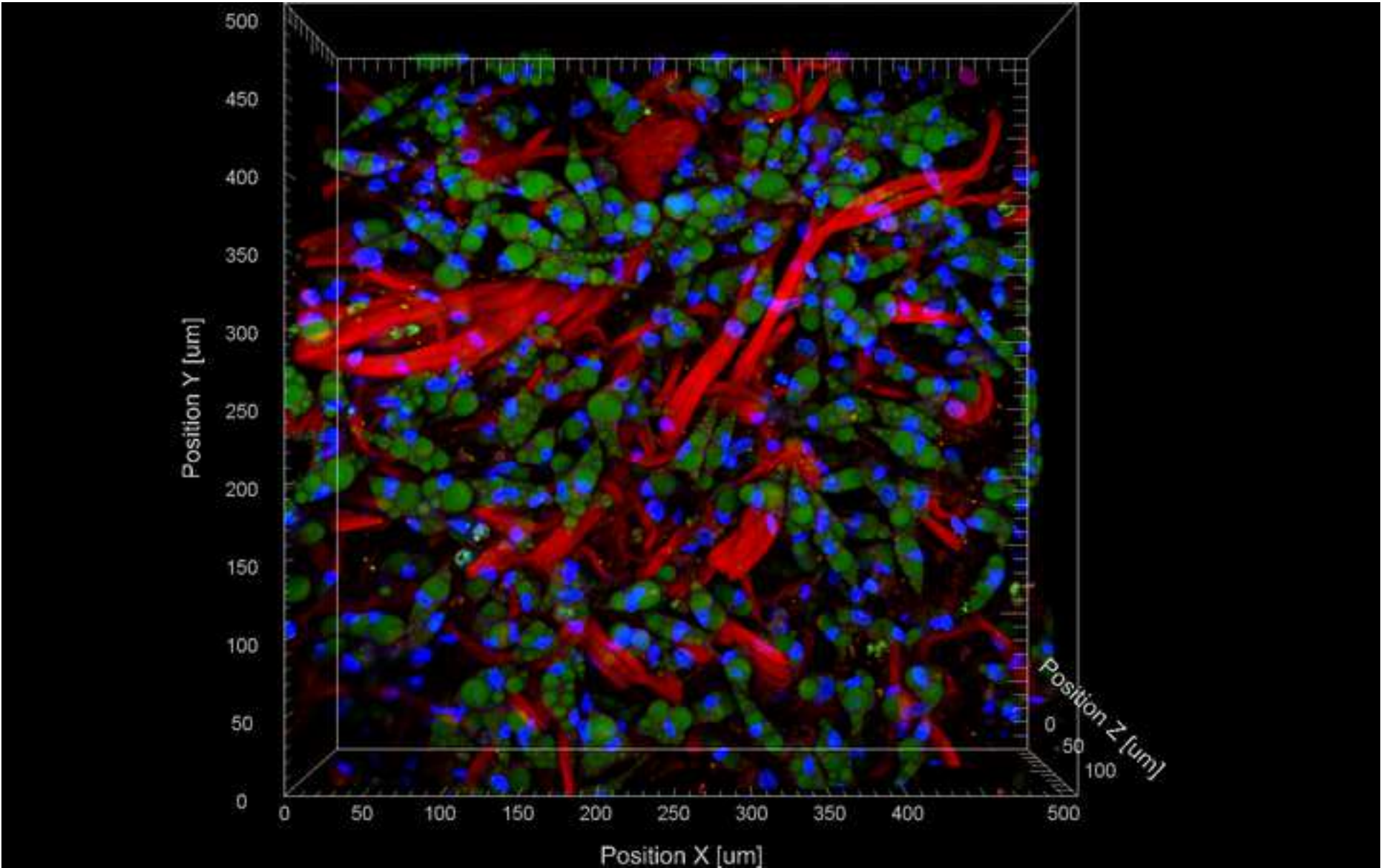


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**DEPARTMENT OF PLASTIC SURGERY AND  
HAND SURGERY – BURN UNIT**

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*Ziyad I. Alharbi, MD, PhD  
Aachen, 02.07.2015*

*Addressed to: Dr. Upponi and Dr. Singh,  
Addressed to editors of JoVE,*

*Dear Sir or Madam,*

Thank you very much for the extensive reviews of our article entitled “**Adipose-derived regenerative cells and dermal regenerative biomaterials: An alternative approach for surgical reconstruction**”.

We have considered and evaluated the concerns that have been raised by the editor. In the following list we respond to the points:

**Editorial comments:**

1. All current changes have been now added to the (File name: 52762) as requested by the editor.
2. The current abstract has been modified according to the number requested by the editor and also in the editorial manager account.
3. The statement is now included: The protocol and the use of human materials follow the guidelines of the human research ethics committee at RWTH Aachen University Hospital (Name in German: Ethik-Kommission des Universitätsklinikums Aachen).
4. Figure legends are now included as part of the manuscript text below the results. Titles and detailed description of each figure are now included in the figure legends.

5. The term “Modified from..” etc. is now used as requested. A copy will be sent.

6. Citation formatting has been modified as requested by the editor.

We thank you for editing and we wish that this manuscript now meets the high standards of JoVE, so a publication of the paper can be accomplished.

With kind and warm regards,

Ziyad Alharbi, M.D., Ph.D.

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