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Verification of cell viability in bioengineered tissues and organs before clinical transplantation

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ABSTRACT

The clinical outcome of transplantations of bioartificial tissues and organs depends on the presence of living cells. There are still no standard operative protocols that are simple, fast and reliable for confirming the presence of viable cells on bioartificial scaffolds prior to transplantation. By using mathematical modeling, we have developed a colorimetric-based system (colorimetric scale bar) to predict the cell viability and density for sufficient surface coverage. First, we refined a method which can provide information about cell viability and numbers in an *in vitro* setting: *i)* immunohistological staining by Phalloidin/DAPI and *ii)* a modified colorimetric cell viability assay. These laboratory-based methods and the developed colorimetric-based system were then validated in rat transplantation studies of unseeded and seeded tracheal grafts. This was done to provide critical information on whether the graft would be suitable for transplantation or if additional cell seeding was necessary. The potential clinical impact of the colorimetric scale bar was confirmed using patient samples. In conclusion, we have developed a robust, fast and reproducible colorimetric tool that can verify and warrant viability and integrity of an engineered tissue/organ prior to transplantation. This should facilitate a successful transplantation outcome and ensure patient safety.

1. Introduction

Bioengineered tissues and organs with simple architectures have recently been successfully transplanted in patients with end-stage organ failure or disease [1,2]. Among them are tracheal scaffolds, either as decellularized donor tissue [2] or as nanotechnology-based artificial materials [3]. For a positive outcome, it seems that interactions between natural or artificial scaffolds and autologous bone marrow stromal cells, using bioreactors, play pivotal roles for clinical tissue and whole organ regeneration [4,5]. In the near future, this tissue engineering (TE) strategy could also be transferred to more complex structures, such as the heart or lung, and promising experimental findings in these areas have been reported [6–8].

In a clinical transplantation, scaffolds are usually reseeded with cells in bioreactors and implanted into humans within a few hours [2,3]. During this period, timing is important for obtaining vital information about the cells on the graft prior to transplantation. There is a wide range of techniques available to image and quantify the number of cells that are viable and proliferating, such as flow cytometry, scanning electron microscopy, confocal microscopy, *etc.* Unfortunately, the time- and labor-intensive protocols and the need for trained staff to reliably evaluate data can delay results. This severely increases the risk of implanting re-seeded scaffolds with non-functional cells. Cell labeling prior to seeding on the scaffold can be an alternative useful tool for cell tracking, but ethical guidelines would limit this technology in patients as the incorporated cell marker may produce unpredictable side effects.

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In this study, we aimed to produce a reliable method to evaluate the pre-implantation state of cell-seeded bioartificial scaffolds in real time, *i.e.* maximum 2–3 h. To deliver a safe, reproducible and non-laborious method to detect and validate the viability and proliferation rate of attached cells on bioartificial scaffolds, we modified the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) overnight protocol to be effective within less than 3 h [9], and stained cells with Phalloidin to label intracellular F-actin Refs. [10], and 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei by a fluorescent microscope. This structural analytical panel was validated in rat transplantation studies of unseeded and seeded tracheal grafts. On the basis of these data, we could further use mathematical models to develop a colorimetric-based system to predict graft/scaffold cell density with a corresponding surface coverage. This method can provide critical information to decide whether a graft is suitable for transplantation or if additional intervention, e.g. additional cell seeding, is necessary.

2. Materials and methods

Male Sprague Dawley rats ($n = 16$) were used as donors for mesenchymal stromal cell (MSC) isolation and as recipients in the *in vivso* transplantation model. All animals were treated in compliance with the "Principles of laboratory animal care" formulated by the National Society for Medical Research and the "Guide for the care and use of laboratory animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Ethical permission was approved by the Stockholm South Ethical Committee (Sweden) (registration number S74-12).

2.1. Rat mesenchymal stromal cell isolation

Eight animals were used for the *in vitro* study. MSCs were isolated and processed as previously described [11]. Briefly, animals were sacrificed and the bone marrow was flushed out gently from both the femur and tibia with phosphate buffered saline (PBS, Invitrogen, Sweden). The obtained cells were centrifuged and the pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Sweden) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen, Sweden) and 1% antibiotic-antimycotic (Invitrogen, Sweden). The cell suspension was seeded in culture flasks (Corning, USA) and cultured for 24 h (37 °C, 5% CO_2). Non-adherent cells were removed and culture medium was changed every three days. Rat MSCs from passages 2 to 5 were used in the study.

2.2. In vivo animal model

Animals ($n = 8$) were anesthetized with a mixture of ketamine and xylazine [ketamine: 100 mg/kg intramuscular (i.m.; Intervet, Boxmeer, Netherlands); xylazine 10 mg/kg (i.m.; Intervet)] injection as a bolus. Under sterile conditions, the tracheae of the recipient animals (200 g–300 g) were exposed *via* an anterior midline cervical incision. Thereafter, we divided the sternohyoid muscles and dissected the cervical fat lobe. The pre-tracheal fascia was opened, the cervical trachea mobilized and traction sutures were placed to retract trachea superiorly.

After that the animals' cervical tracheae were resected and 1 cm replaced by the synthetic based tracheal graft. We utilized a continuous 6-0 polyproyplene (Prolene; Ethicon, Inc, Somerville, NJ) suture to anastomose the posterior trachea and 6-0 absorbable polygalactin (Vicryl, Ethicon) interrupted sutures for the anterior trachea. The anastomotic suture knots were then tied outside the lumen. During the entire surgery, the animals were maintained on spontaneous ventilation. After hemostasis, we closed the tissue and skin in a usual fashion and animals were allowed to recover on a heating pad. All animals were observed on a daily basis. Euthanasia was induced at the endpoint of the study (30 days), implanted tracheae were harvested and analyzed both macroscopically and microscopically.

2.3. Colorimetric cell activity assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-assay (Roche, Sweden) is a colorimetric assay that was used to detect viable cells and evaluate the metabolic activity of cells. All samples were analyzed in triplicates. Media and scaffold only were used as negative controls. The MTT substrate (40 μl) was added to each well and were either incubated: 4 h (Protocol I and II) or 1 h (Protocol III) at 37 °C with 5% CO_2. Next, 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl (400 μl) was added across all protocols (I-III) and further incubated overnight (Protocol I) or 1 h (Protocol II and III) at 37 °C, 5% CO_2. The samples were read on a spectrophotometer (SpectraMax 250, Molecular Devices, USA), the absorbance was measured at 570 nm.

2.4. Phalloidin and DAPI staining on seeded synthetic scaffolds

Pieces from the seeded scaffold were cut to an appropriate size with a 6 mm biopsy punch. Samples were fixed in formaldehyde 4% (Histolab, Sweden) for 5–10 min, washed and stained with Phalloidin (Molecular Probes, Sweden) diluted in PBS/0,1% Triton X-100 (2 U/ml) (Sigma–Aldrich, Sweden) and incubated for 30 min. Samples were washed and counterstained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma–Aldrich, Sweden). Stained cells were either visualized with a fluorescent microscope (Olympus BX-60, Japan) using a 4× (dry, numerical aperture 0,13; Olympus, Japan), 10× objective (dry, numerical aperture 0,3; Olympus, Japan) and 20× objective (dry, numerical aperture 0,5; Olympus, Japan) or on a confocal microscope (Nikon A1+, Japan) using a 10× objective (dry, numerical aperture 0,45; Nikon, Japan).

2.5. Color change evaluation

2.5.1. Cell coverage quantification

Using the DAPI stained samples, cell numbers were either manually counted with ImageJ 1.46R (NIH, Maryland, USA) or an automated software CellProfiler 2.0 (BROAD Institute, Massachusets, USA). The readouts were converted to surface densities, denoted σ having units cm^{-2}, by dividing by the area of the samples (circular pieces with diameter 6 mm). The counted cell densities on samples were plotted against the seeding density, where the error bars indicate the mean and standard deviation of triplicate samples. The cell densities were then converted into values indicating the degree of cell coverage of the surface, using $\theta = A_{cell}\,\sigma \times 100\%$ i.e. multiplying σ by the average area of a single cell, A_{cell}. The images of phalloidin-stained cells [11] were analyzed using the CellProfiler software package to determine the value $A_{cell} = 280\ \mu m^2$.

2.5.2. Color change analysis of laboratory samples

After applying the MTT test to the samples, but prior to solubilizing the formazan crystals, the media was temporarily removed from the wells. A single color digital picture, showing a uniformly illuminated top view of the well plate and the samples, was obtained and imported into MATLAB. The image was converted into a grayscale image by applying the formula $Y = 0.2989 \times R + 0.5870 \times G + 0.1140 \times B$ to the RGB values in the image (using the MATLAB function rgb2gray.m provided with the Image Processing Toolbox). This allowed a single grayscale value, Y, to be obtained for each pixel in the image. The regions of the image corresponding to the upper surface of each sample were selected manually using mouse and cursor input for further analyses. The numerical value of the color change of a seeded sample was calculated using Equation (1):

$$\Delta C = \frac{Y_c - Y_s}{Y_c - Y_m} \times 100\% \tag{1}$$

where Y_s is the average of the grayscale values of the pixels in the sample, Y_c is the average of the grayscale values of the pixels in control samples (unseeded), and Y_m is the average of the grayscale values of pixels of regions where there was maximum purple staining (seeded samples). Hence $\Delta C = 0\%$ corresponds to no color change in the sample relative to the control, and $\Delta C = 100\%$ corresponds to a sample that is completely stained dark purple. The color change values of the samples were calculated using equation (1). The average and standard deviations were calculated for all triplicate sets for the different seeding densities. In order to extrapolate higher values of cell coverage and density, equation (2) was formulated:

$$\theta = \exp\left(\alpha\left(\left(\frac{\Delta C}{100\%}\right)^{\beta} - 1\right)\right) \times 100\% \tag{2}$$

The parameters α and β were fitted using least squares (using the MATLAB function fminsearch.m) to the data of the cell coverage with respect to the color change, yielding $\alpha = 4.25$ and $\beta = 0.58$. The functional form of $\theta(\Delta C)$ was chosen based on the assumption that maximum color change i.e. $\Delta C = 100\%$ corresponds to full coverage of cells on the sample. Equation (2) was used to relate a linear scale of color change, with values in the range of $\Delta C = 0$–100%, to the corresponding cell coverage and cell density values.

2.6. Synthetic scaffolds

The scaffold was designed based on the patient's CT-scan performed 4 weeks prior to the transplantation. The scan was analyzed and then used to create an electrospun, nanofiber-based composite made from polyethylene terephthalate (PET)/polyurethane (PU) supplied by Nanofiber Solutions® (Columbus, OH). The utilized FDA-approved materials, PET and PU, (Regulations. U.S. FDA, 1998) are non-biodegradable polymers that are non-cytotoxic and retain mechanical properties [12].

2.7. Fiber alignment evaluation

Fiber alignment has been evaluated by a custom-made software. Scanning electron microscopy (SEM) images were firstly binarized and then rotated around its center in step of 2°. For each direction the fraction of bright pixels was computed and the resulting standard deviation calculated. Results were represented by means

of a unitary polar plot that is specific for each analyzed electrospun mat. As measurement index, the eccentricity of the fitting ellipse of the polar plot was computed as follows:

$$E = \frac{A_M - A_m}{A_M + A_m} \quad (3)$$

where A_M is the major axis and A_m the minor axis. According to this formulation; $E = 1$ indicates a straight line (unidirectional fiber alignment) and $E = 0$ indicates a perfect circle (randomly arranged fibers). Moreover, the orientation of the fitting ellipse is indicative of the average fiber alignment [11].

2.8. Patient mononuclear cell isolation and cultures

Under aseptic conditions, bone marrow (200 ml) was obtained by punctation of the right and left crista iliaca. Mononuclear cells were isolated though density gradient separation. White blood cells, mononuclear cells, CD34+ cells, colony forming unit-fibroblast, flow cytometric characterisation, viability and sterility analyses were performed. MNCs were resuspended in low-glucose DMEM (Invitrogen, Stockholm, Sweden), transferred at room temperature to Good Manufacturing Practice (GMP) facility (VECURA, Karolinska University Hospital, Huddinge, Sweden). Cells were seeded under sterile conditions with a specially designed bioreactor for 72 h at 37 °C/5%CO_2. Patient's consent was given for all analytical evaluations and publication.

2.9. Scanning electron microscopy

To evaluate cell adhesion and integration of the seeded synthetic tracheal scaffold from the transplanted patient, small pieces from the external and internal part were fixed with 2.5% glutaraldehyde (Merck, Germany) in 0.1 M cacodylate buffer (Prolabo, France) for 2 h at room temperature, rinsed in cacodylate buffer, and dehydrated though an ethanol gradient. Samples were dried overnight and gold sputtered. The samples were used for analysis by SEM (JSM6490, JEOL, Japan).

2.10. Histological analysis

Samples of synthetic trachea from rats or brushing sample from patient were fixed in 10% neutral buffered formalin solution in PBS (pH 7.4) at room temperature. They were washed in distilled water, dehydrated in graded alcohol, embedded in paraffin (Merck, Darmstadt, Germany), and sectioned at 5 mm thickness. Sections were stained with Hematoxylin and Eosin stain (H&E) (Merck, Darmstadt, Germany) and imaged with a microscope at 4× or 20× magnification (Olympus BX-60, Japan).

2.11. Statistical analysis

Results were expressed as mean ± standard deviation. GraphPad Prism 5 (GraphPad Software, California, USA) was used for all statistical analysis, with significance levels of $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$ and $^{****}p \leq .0001$. All data were compared using either an unpaired *t*-test or two-way ANOVA analysis.

3. Results

3.1. Qualitative and quantitative evaluation of attached cells on scaffold

We designed an analytical method that provides rapid information about cell viability and/or cell proliferation on engineered tissues or organs prior to clinical transplantation. We first analyzed cells on scaffold with a commercially available MTT-assay using its standard protocol (4 h MTT and overnight incubation with SDS; Protocol I), and then applied two modified protocols with reduced incubation time: 5 h (4 h with MTT and 1 h SDS; Protocol II) and 2 h (1 h with MTT and 1 h SDS; Protocol III). This was to ensure that the processing time modifications did not alter the qualitative information as compared to the standard protocol. Rat MSCs from passages 2 to 5 were seeded at a density of 7500 cells/cm^2 on PET/PU fiber coated 24 well plates (2 cm^2 surface-area/well; Nanofiber solutions®) as previously described [11]. All samples were measured by absorbance and we obtained significantly differences for all three protocols as compared to unseeded scaffolds (control) (control *versus* protocols I: $p \leq .01$; II: $p \leq .001$ and III: $p \leq .0001$) (Fig. 1A). This showed that the modification in processing time did not alter the qualitative information as compared to the standard method. Therefore, we further evaluated Protocol III (2 h) for its quantitative data. We seeded different cell numbers (2500; 5000; 10,000; 50,000 or 100,000) on 96-well plates with PET/PU nanofiber inserts for 48 h to investigate if cell numbers corresponded to different absorbancy measurements. The higher absorbancy measurements corresponded with higher cell numbers (Fig. 1B,C).

3.2. Quantification of cell density and coverage on scaffolds using mathematical modeling

In order to simplify the readout of the MTT method, we wanted to investigate whether the formazan crystal color change could predict the approximate cell number and surface coverage. Thus, seeded scaffold samples were analyzed after 1 h with MTT without using SDS to solubilize the formazan crystals from the cells (Fig. 2A). Images were imported into MATLAB and RGB color triplets of each pixel in the imported image were converted to grayscale values. An empirical mathematical model was then used to relate cell density, which was calculated on confocal images (Fig. 2B) using Cellprofiler™ 2.0 software package (BROAD Institute, Massachusetts, USA), to the measured color change. The color change values of the samples were computed using equation (1). In Fig. 2C, cell coverage and cell density was plotted against the color change for five different cell numbers used (2500; 5000; 10,000; 50,000 or 100,000). The graph showed that the greater the color change, the greater the coverage and density of cells on sample. We used the obtained data to develop a colorimetric scale bar that can provide the essential information about cell viability, density and surface coverage for engineered tissues and organs.

3.3. Bioengineered graft evaluation in an animal model

Rat tracheal nanofibrous scaffolds made from electrospinning (with an average fiber dimension of internal 0.61 ± 0.25 μm and external 0.52 ± 0.37 μm) were seeded with rat MSCs for 48 h and then orthotopically implanted into male Sprague Dawley rats. Prior to transplantation, we used our optimized MTT method and applied the developed colorimetric scale bar to estimate the surface coverage of the scaffold (Fig. 3A,B). Based on the color changes we found that the scaffold surface coverage was 63.8 ± 9.2%. Throughout the observational period of 30 days post implantation, animals ($n = 5$) showed no signs of health impairment or breathing difficulties. The implanted tracheae were then harvested, and subsequent histological analyses revealed respiratory epithelialization on the internal surface of the implants (Fig. 3C). There were no signs of bacterial or fungal contamination. The external surfaces displayed no marks that would indicate ongoing inflammatory processes, and seemed to have integrated well into the surrounding connective tissue. However, when unseeded rat tracheal scaffolds were transplanted ($n = 3$), after 3 ± 1 days the animals had to be sacrificed due to dyspnea. Histological analyses of these grafts showed near-total luminal occlusions and signs of severe inflammatory responses (Fig. 3D).

3.4. A translational approach, development of a clinical tracheal graft

A 21-year-old female patient suffered from an iatrogenic induced severe tracheal damage that affected the entire organ. An immediate transplantation was necessary to replace the entire trachea with a synthetic based TE tracheal graft. This surgery took place in August 2012 at the Department of Cardiothoracic Surgery and Anesthesiology at the Karolinska University Hospital, Stockholm (Sweden).

From the patient's CT scan, a scaffold was customized using electrospun PET/PU nanofibers (Fig. 4A). The average diameter of the fibers was 1.97 ± 0.32 μm on the internal and 2.15 ± 0.41 μm on

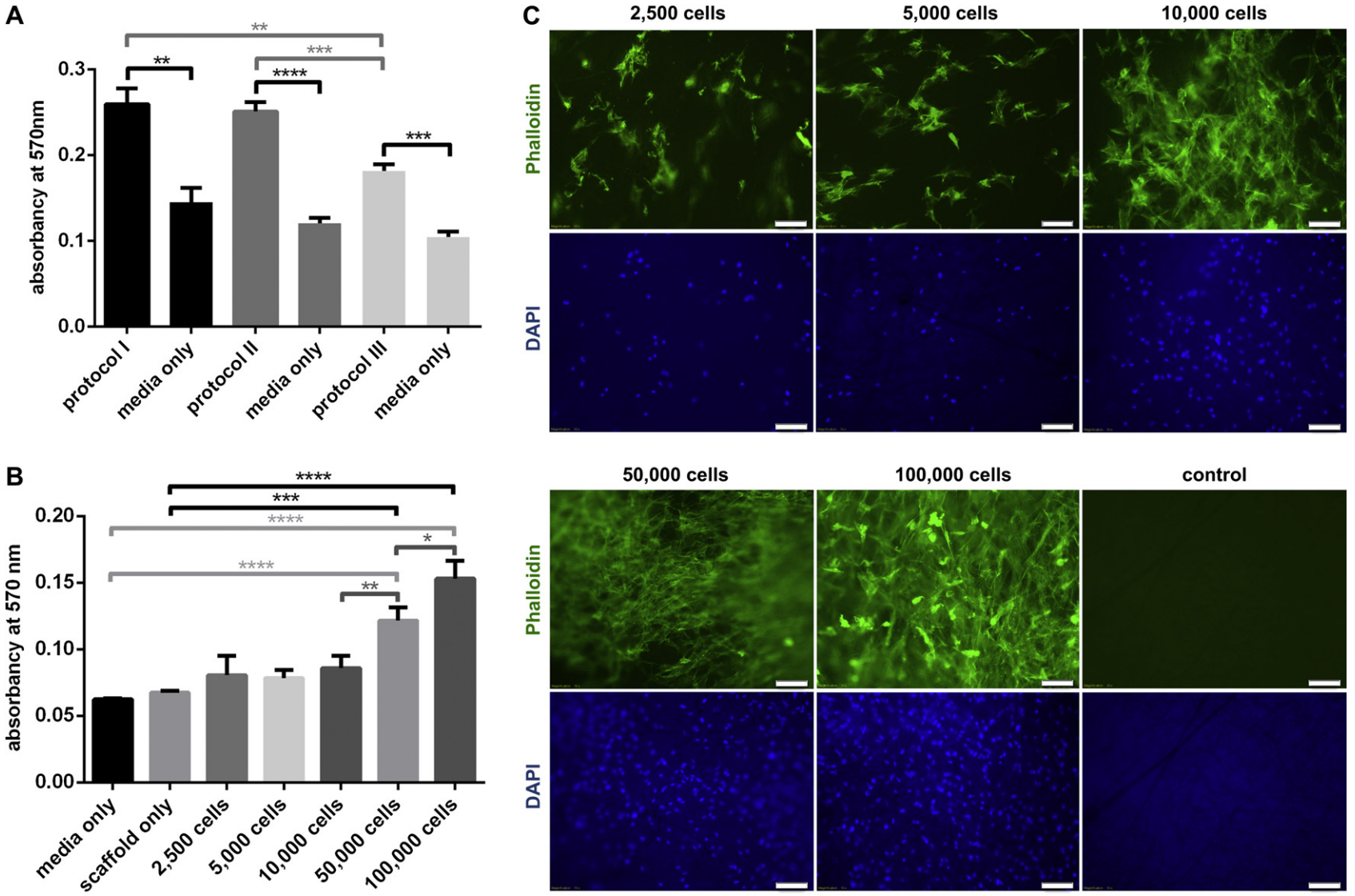

Fig. 1. Absorbance at 570 nm after having performed the colorimetric MTT-assay with the different protocols I-III on seeded PET/PU fiber coated plates after 48 h of culture compared to negative control i.e. media only (A). Absorbance at 570 nm after performing the colorimetric MTT-assay on reseeded pieces of the synthetic scaffold made of PET/PU with either 2500 cells; 5000 cells; 10,000 cells; 50,000 cells or 100,000 cells compared to negative controls i.e. media and scaffold only ($n = 3$) (B). Fluorescent images of seeded synthetic scaffolds with different cell numbers after 48 h culture stained with Phalloidin (green) and DAPI (blue). Magnification 10× scale bar representing 200 μm (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

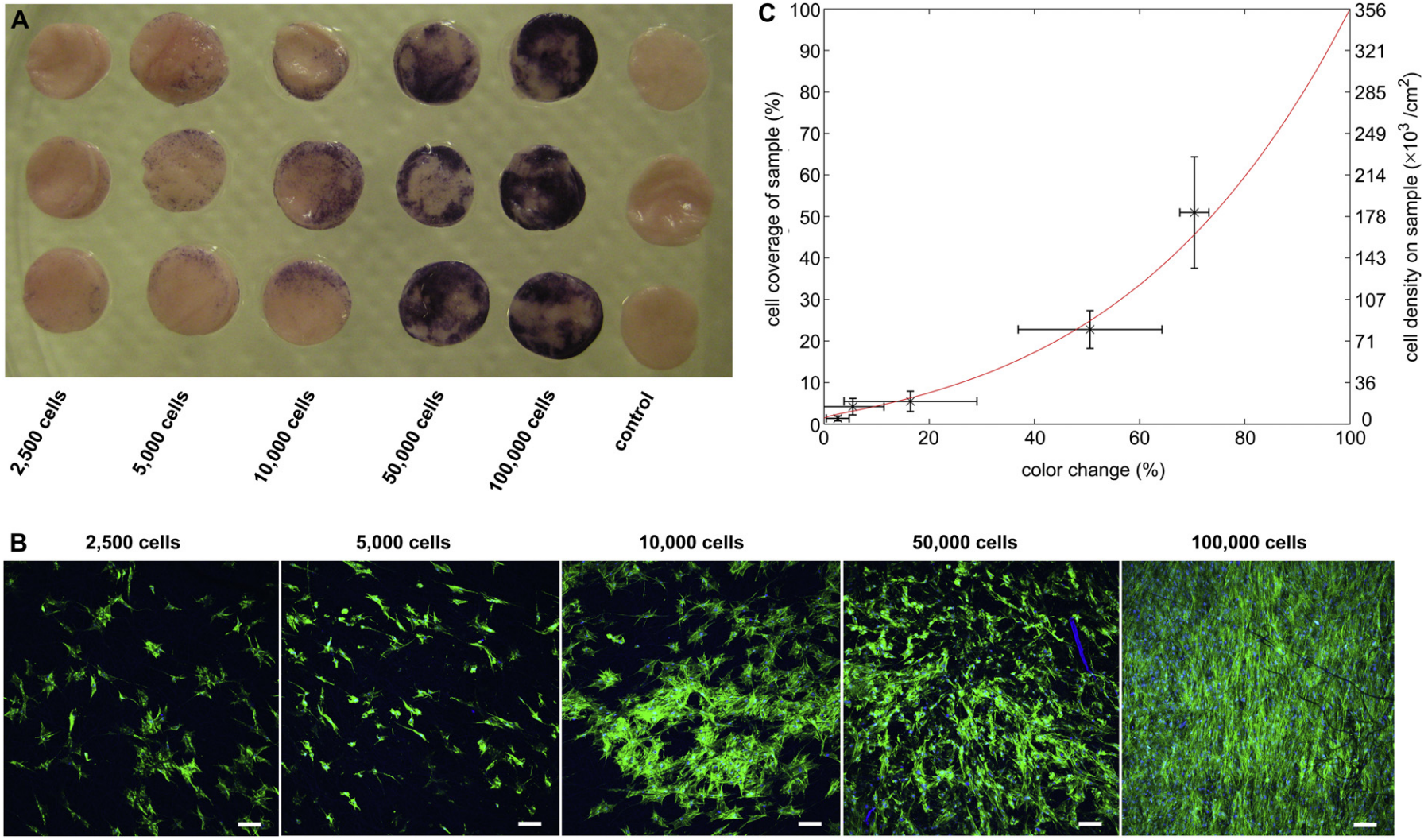

Fig. 2. Macroscopic pictures of seeded synthetic scaffolds with different cell numbers after 1 h incubation with the MTT-reagent (A). Confocal microscopy images on seeded pieces of the synthetic scaffold made of PET/PU with either 2500 cells; 5000 cells; 10,000 cells; 50,000 cells or 100,000 cells, stained with Phalloidin (green) and DAPI (blue). Maximum intensity images of the seeded scaffolds. Magnification 10×; scale bar representing 100 μm (B). Graph is showing cell coverage and cell density on the samples with respect to the color change (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

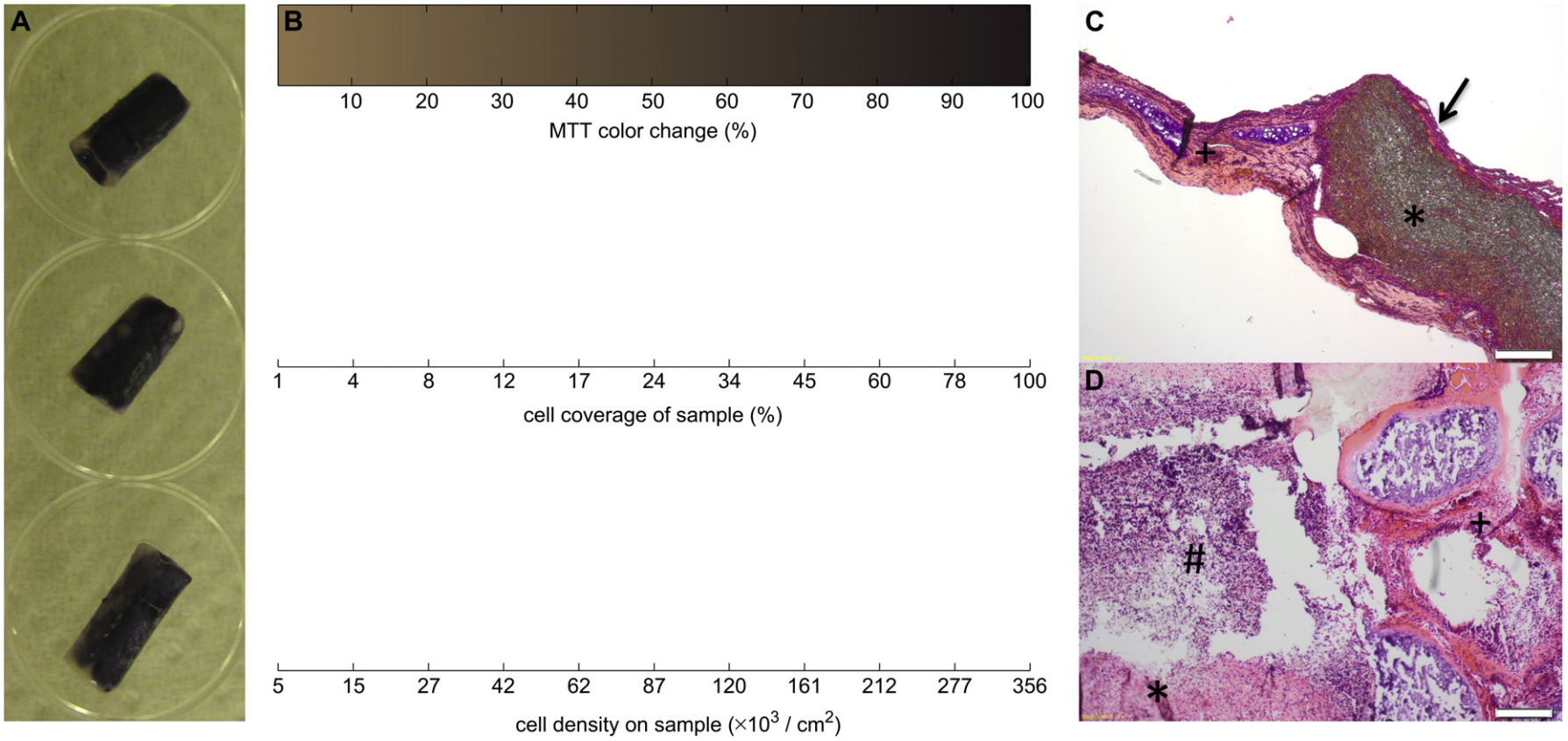

Fig. 3. Macroscopic images of MSCs seeded on synthetic grafts, analyzed with MTT by protocol III (A). Color scale bar is showing the cell coverage and cell density on a seeded sample corresponding to a given color change (B). Images of paraffin sections stained with H&E. Image shows synthetic graft seeded with rat MSCs at day 30 (post transplantation); asterisk indicates synthetic scaffold; + indicates the native trachea; arrow indicates epithelial layer. Magnification 4×; scale bar representing 500 μm (C). Image shows synthetic graft without MSCs on day 3 (post transplantation); asterisk indicates synthetic scaffold; + indicates the native trachea; # indicates increased number of granulocytes. Magnification 4×; scale bar representing 500 μm (D).

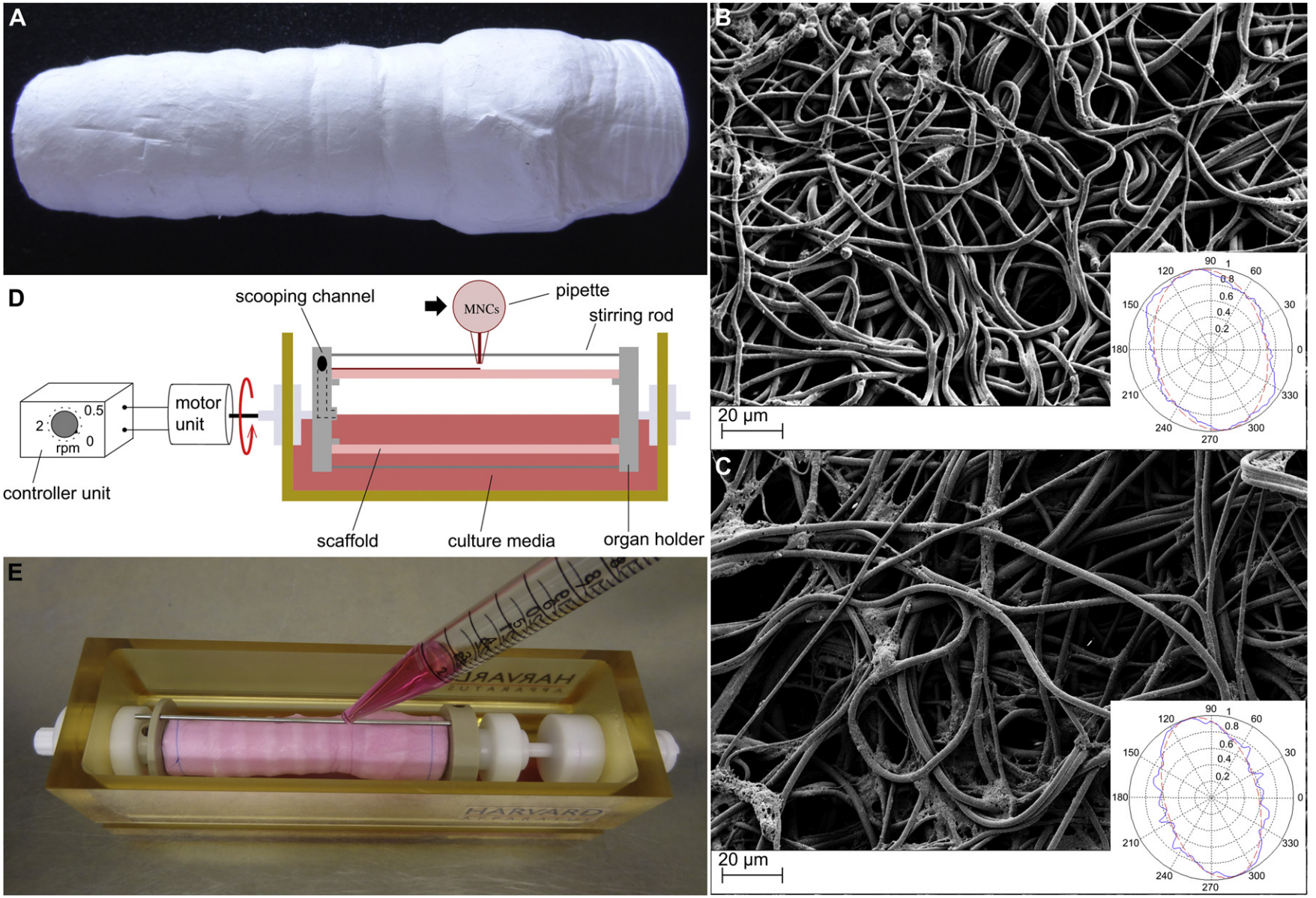

Fig. 4. Picture of a synthetic tracheal scaffold made of PET/PU-based nanofibers (A). SEM images of a synthetic scaffold showing the fiber network made of PET/PU. Insets report the computed fiber alignment pattern. Magnification 1,500×; scale bar representing 20 μm (internal B; external C). Schematic drawing of the bioreactor culturing system composed of the bioreactor, motor unit and controller unit with a synthetic tracheal scaffold mounted inside with an organ holder (D). Image of the seeding process on the tracheal scaffold inside the bioreactor prior to transplantation (E).

the external side. The organization of the electrospun fibers mimics the fiber network of a native trachea. To determine the alignment of the transplanted scaffold, SEM images were evaluated on a custom-made image analysis software. The alignment of the fibers was measured at 0.29 ± 0.09 μm on the internal (Fig. 4B) and 0.27 ± 0.08 μm on the external side (Fig. 4C). The scaffold was seeded and cultured for 72 h at 37 °C/5%CO_2 with the patient's own MNCs isolated from bone marrow in a bioreactor as previously described (Fig. 4D,E) [3].

3.5. Evaluation of the clinical samples, analytical panel with mathematical modeling

Based on our experimental *in vitro* and *in vivo* results described above, we could develop a colorimetric scale bar and translate it to a clinical scenario of tissue engineered tracheal transplantation. Samples were collected from the tissue engineered tracheal graft prior to transplantation into the patient at two different time points: a) in the morning of the transplantation (samples 1) and b) during the operation when the graft was trimmed to the anatomical needs of the patient (sample 2). Autologous human MNCs stained with Phalloidin/DAPI were counted from three images taken from two clinical samples that yielded cell densities of 115 × 10^3/cm^2 and 148 × 10^3/cm^2. Simultaneous absorbancy readout using the 2h MTT protocol (Protocol III) showed significant differences between the seeded and unseeded control scaffolds (Figs. 5A and 6A). This suggested that the seeded graft contained cells that had attached and were viable and proliferating on both internal and external surfaces.

To investigate the value of our experimentally developed color scale bar, we analyzed the color changes on clinical samples and

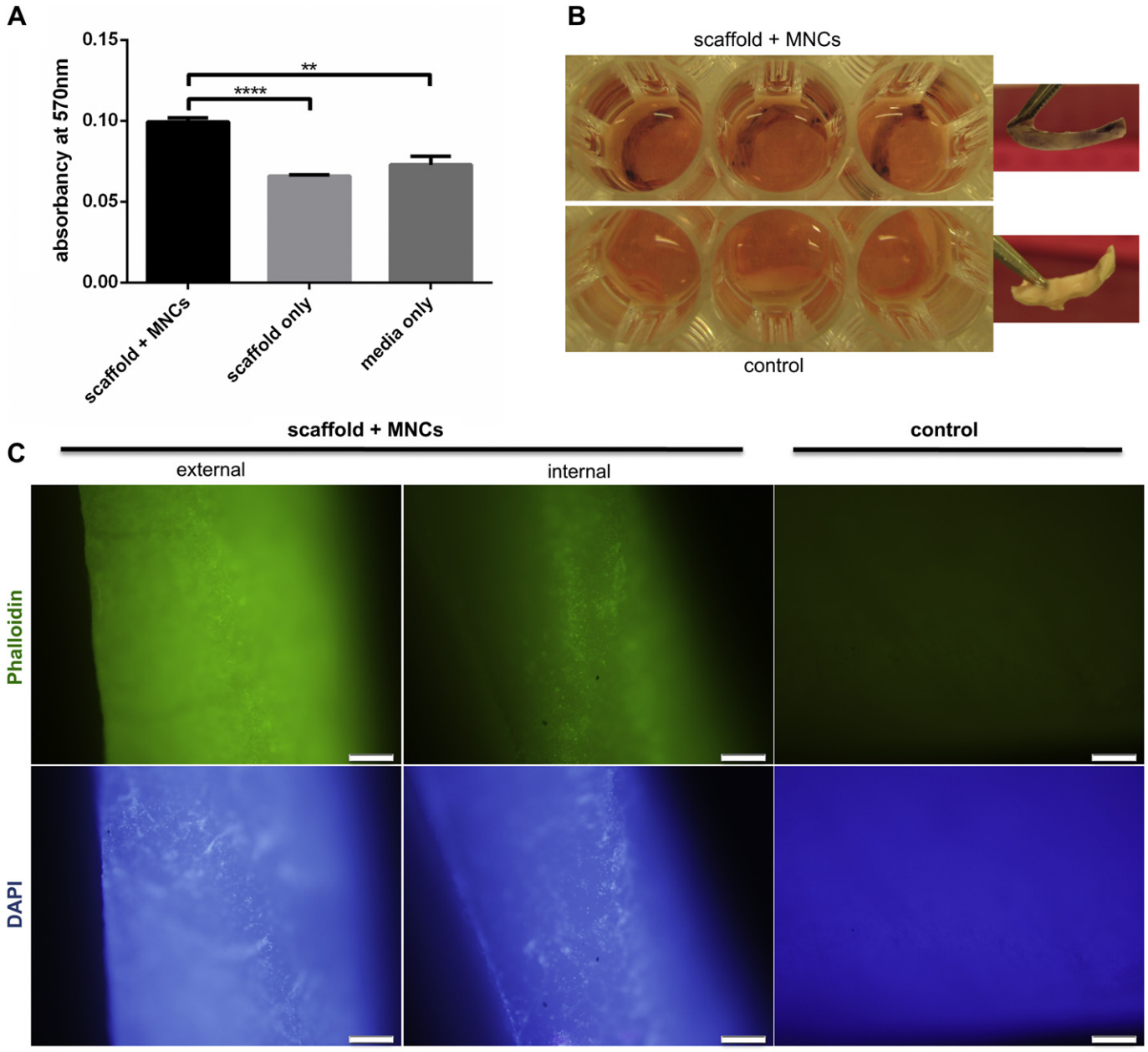

Fig. 5. Absorbance at 570 nm after having performed the colorimetric MTT-assay on sample I of the transplanted synthetic tracheal scaffold into patient (A). Macroscopic pictures of sample I reseeded with MNCs (upper part; $n = 3$) and control scaffolds without cells (lower part; $n = 3$), thin rings obtained from the distal part (graft-bioreactor fixation area) of the synthetic trachea (B). Fluorescent images of the internal and external part of the tracheal scaffold (sample I) stained with Phalloidin (green) or DAPI (blue). Unseeded pieces served as controls ($n = 3$). Magnification 10×; scale bar representing 200 μm (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

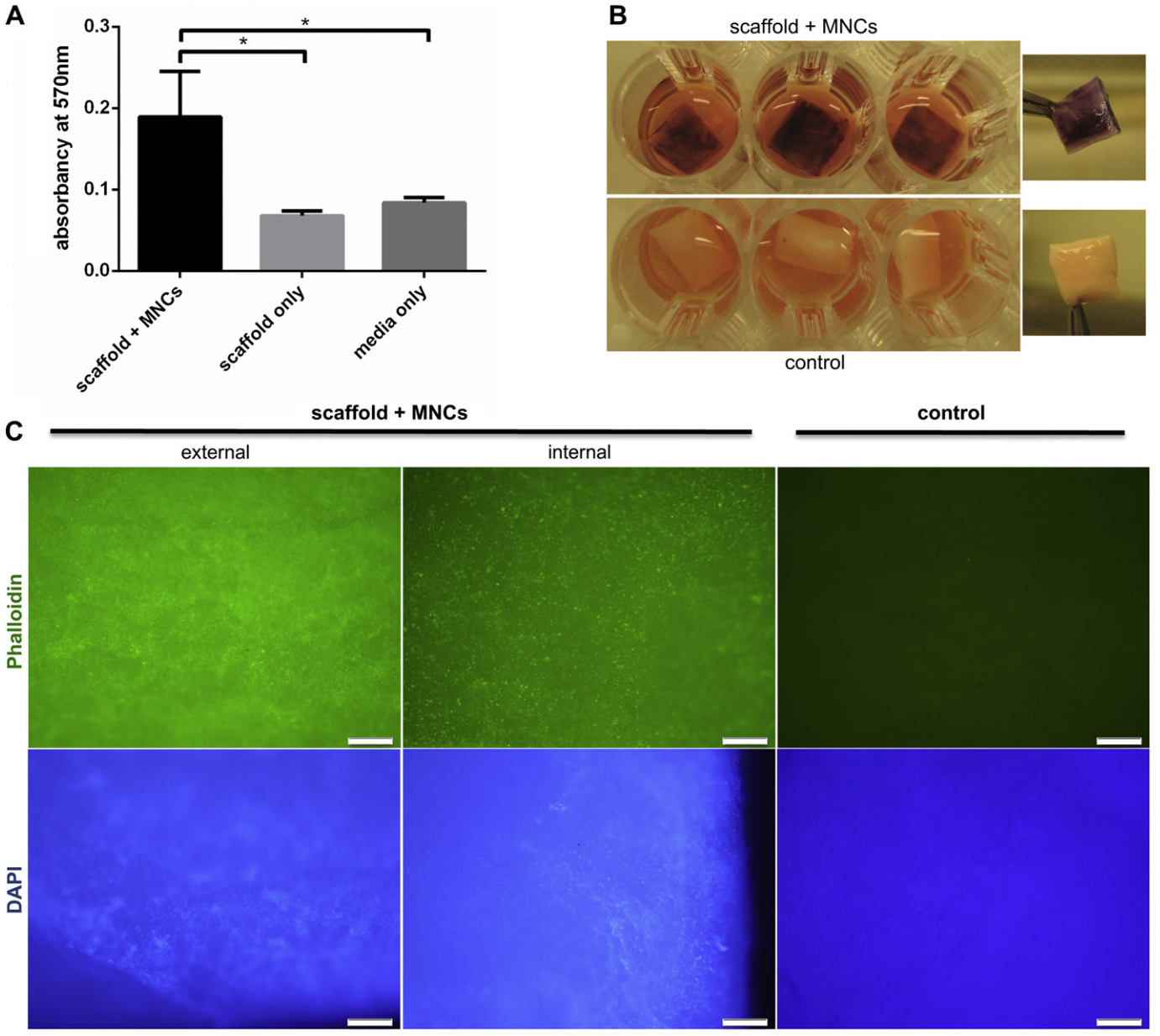

Fig. 6. Absorbance at 570 nm after having performed the colorimetric MTT-assay on sample II of the transplanted synthetic tracheal scaffold into patient (A). Macroscopic pictures of sample II reseeded with MNCs (upper part; $n = 3$) and control scaffolds without cells (lower part; $n = 3$), pieces from the distal part (obtained intraoperatively) of the synthetic trachea (B). Fluorescent images of the internal and external part of the tracheal scaffold (sample II) stained with Phalloidin (green) or DAPI (blue). Unseeded pieces served as controls ($n = 3$). Magnification 10×; scale bar representing 200 μm (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

their corresponding cell numbers. Digital images of clinical samples stained with formazan crystals (Figs. 5B and 6B) were quantified for the color changes as described above. When applying equation 1 to the grayscale values of the pixels in the digital image, the values Y_c and Y_m were the same as those used in the laboratory samples. The result was $\Delta C = 67\%$, which was consistent with the measured cell density (Figs. 5C and 6C). The calibration curve (Fig. 2C) also showed that this corresponded to an average cell coverage on the entire samples of approximately 40%. However, when equations (1) and (2) were applied to each pixel in the image and the resulting histogram of the θ values was analyzed, it was found that 20% of the surface area of the sample had less than 25% cell coverage, whereas 20% of the area of the sample had greater than 75% coverage (Fig. 3B). This was confirmed by observations made from SEM images, which showed a wide variation in cell coverage ranging from an almost complete absence of cells (Fig. 7A*)* (the area where the scaffold was fixed to the bioreactor) to a full confluence (Fig. 7B). The early clinical evaluation revealed an initial graft epithelialization as judged from the 1-week post-operative brushing (Fig. 7C). The intermediate post-operative outcome (5 months) has shown a patent and non-contaminated graft without any signs of inflammation.

4. Discussion

The recent clinical successes of transplanting bioengineered tissues or organs suggest TE can potentially be routinely used in the very near future [1–3]. Hence, it is important to formulate ethical and clinical guidelines by establishing standard operating procedures that are both reproducible and reliable for TE. It is even

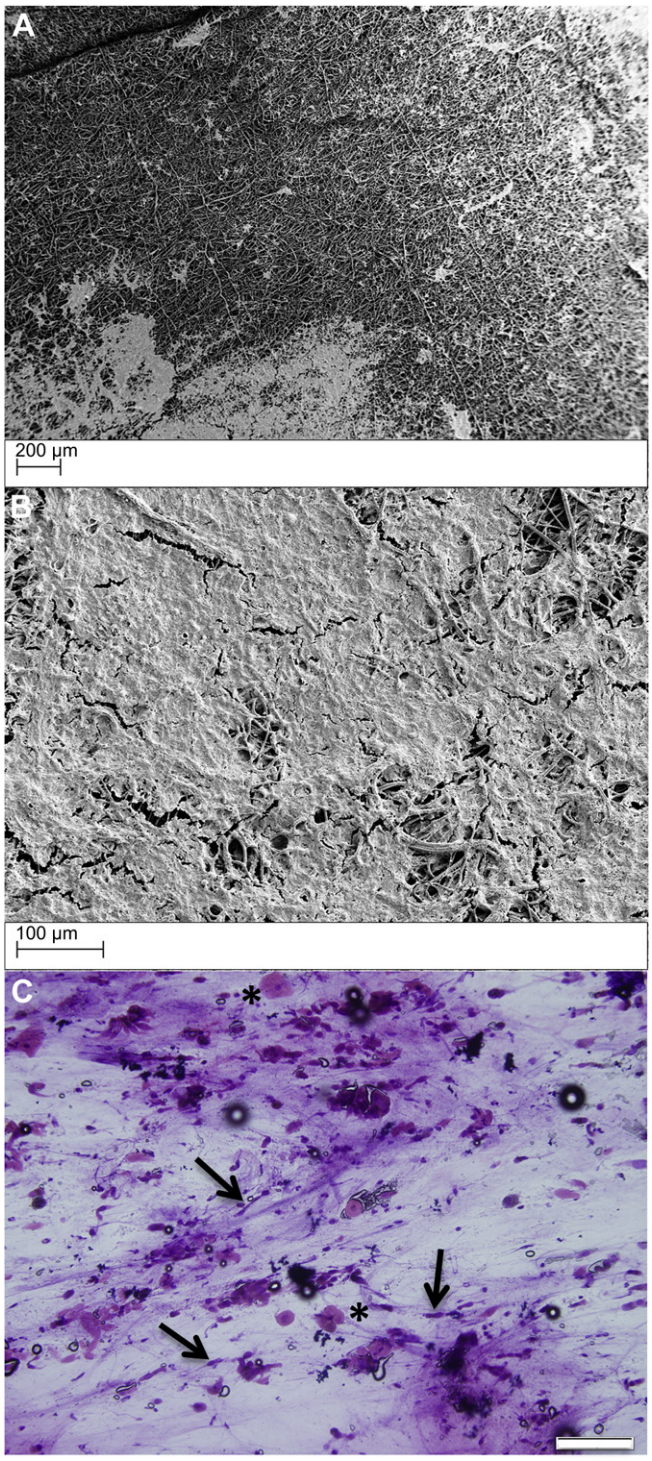

Fig. 7. Scanning electron microscopy images of the tracheal scaffold used in the clinical transplantation either showing the graft-bioreactor fixation area (Magnification 100×; scale bar representing 200 µm) (A) Or aside from this area (Magnification 400×; scale bar representing 100 µm) (B) Image shows a clinically obtained brushing sample of the patient's graft (H&E stained) at day 7 (post transplantation). Magnification 20×; scale bar representing 100 µm; arrows indicate epithelial cells, asterisks indicate basal cells (C).

more important to have qualitative and quantitative validations of the graft prior to transplantation, specifically concerning *i)* cell attachment, *ii)* cell viability, *iii)* proliferation rate, and *iv)* cell distribution while minimizing staff labor and laboratory equipment for performing the analyses. However, there is as yet no standardized analytical protocol that is applicable routinely in a clinical setting. Therefore, we set out to establish a reliable and simple evaluation panel that can give the most relevant information on graft features to the responsible clinicians and scientists involved. In order to meet the clinical requirements, analytical methods need to be time- and cost-effective, uncomplicated and simple with high reproducibility. We optimized an analytical panel, which includes a modified processing protocol (protocol III) from a commercially available MTT assay and fluorescence staining with Phalloidin/DAPI. We successfully proved the validity and reproducibility of this evaluation panel with both *in vitro* and *in vivo* studies. This was also transferred to a clinical setting during a recent trachea TE organ transplantation. The colorimetric MTT assay was first described by Mosman [9]. It is a well-accepted method to measure viability and proliferation of cultured cells. It is based on two substrates: the yellow tetrazolium salt MTT and SDS in 0.01 M HCl. MTT is cleaved to metabolically active cells to form purple formazan crystals, which provides a macroscopic indicator. The purple color change can be macroscopically recognized which makes this assay superior to many other marketed cell viability/proliferation assays such as Cell Titer Glo®, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) [13]. In the next step, the crystals are solubilized with SDS. The resulting color change is finally detected with a simple (ELISA-) spectrophotometer by measuring the absorbancy, which correlates with viability, proliferation and cell numbers [14]. Our data have demonstrated both experimentally (Figs. 1A and 2A) and clinically (Figs. 5A,B and 6A,B) that the reduced processing time to 2 h in the MTT assay could yield dual validation: i) macroscopic color change and ii) absorbancy readouts which correlated well with number of attached cells on the synthetic scaffolds.

We also show here that this simple and fast analytical panel can provide qualitative and quantitative data (cell viability, cell distribution) that are essential for clinical decision-making. We found that graft areas distal from the graft-bioreactor holder fixture had around 70% of the surface covered with cells, while nearly no cell adhesion was found in the holder fixture region. Similar observations were also found in transplantation studies carried out in animals here. Although the graft was not 100% seeded with autologous cells, the early clinical examination of the patient's graft did not show any bacterial or fungi contamination. Hence, we can assume that it may not necessarily be important to have a scaffold with confluent cell covering of the surface: around 70% coverage seems to be sufficient. However, although we have data on cell densities, we still do not know the patterns of proliferation and distribution of seeded cells on grafts *in situ.* Cell recruitment such as local resident and circulating stem and progenitor cells may also play a key role in regeneration [15]. Further studies could be performed to elucidate the different regulatory pathways or mechanisms involved during an *in situ* tracheal regeneration.

The complexity of TE and regeneration would remain a challenge because the restoration or creation of three-dimensional tissues and organs require a combination of stem cells, scaffolds and signaling molecules. Mathematical modeling is another approach, which we could make predictions of cliniscal outcomes from the evaluations. Here, we have successfully developed a mathematical model and converted it to a colorimetric scale bar that can predict cell coverage and different cell densities. This easy-to-use device can potentially be widely used and be well-accepted in a medical field to confirm the viability and integrity of the engineered tissue/organ directly prior to transplantation. The ability to acquire immediate visual information can certainly assist with the decisions about whether the tissue or organ is ready for implantation into a patient or a second bone marrow isolation and

reseeding is necessary with the postponement of transplantation. From an ethical point of view, it is most important to guarantee that the implanted tissue or organ graft is safe and does not jeopardize the patient's health. Within the fast growing field of TE and its clinical application, this analytical panel is an important and highly desired tool that can be implemented routinely to facilitate a successful and safe transplantation outcome.

5. Conclusion

We have developed a robust, fast and reproducible colorimetric tool that can verify and warrant viability and integrity of an engineered tissue/organ prior to transplantation. This should facilitate a successful transplantation outcome and ensure patient safety.

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