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Quantification of collagen matrix deposition in 2D cell cultures: a comparative study of existing assays

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ABSTRACT

Collagen matrix deposition is an important biomarker to predict the regenerative capacity of new biomaterials or the therapeutic potential of new drugs in collagen-associated diseases. Several methods for the quantification of matrix collagen in tissue samples are established, e.g., Picro-Sirius red assay, hydroxyproline assay, antibody-based assays, or the 3,4-DHPAA-based assay. These methods have been extended to quantify deposited collagen in *in vitro* cell culture models, although their applicability has been questioned due to the much lower concentration and eventually lower relative abundance of deposited collagen in cell cultures than in tissue. Here we compare the performance of the above-mentioned methods for the quantification of deposited matrix collagen in 2D cell cultures under different conditions: culture time, addition of collagen deposition-stimulating molecules, and post-culture processing step (decellularization). We show that the available methods can deliver accurate results within different experimental windows. We provide a comprehensive analysis of the relevant experimental parameters that influence the assay, and the sensitivity limits for the different methods, as well as the involved effort. In a comparative table, we provide guidance for the selection of the most appropriate collagen quantification assay for different culture conditions.

1. Introduction

Collagen is the major component of the extracellular matrix in vertebrates and plays an important role in numerous biological processes [1]. Collagen matrix deposition is a biomarker for tissue healing and regeneration and for the severity of diseases like cancer, fibrosis [2,3], and collagenopathies [4–6]. Quantifying the level of collagen matrix deposition is essential for the comprehensive investigation of these biological processes.

Several methods allow quantification of collagen in tissue samples, where collagen is a major component. These methods have also been extended to the quantification of deposited collagen in *in vitro* cell cultures, although the collagen amount (absolute and relative) in cell cultures can be significantly lower than in tissue. The Picro-Sirius red colorimetric assay uses a strong anionic dye (Sirius red F3B) to interact with the positively charged side groups of basic amino acids present in the collagen molecules in acidic conditions. The dye is released from the collagen with a basic elution solution and is detected by colorimetry. This assay has been used to quantify total collagen in tissue specimens and in cell culture lysates [7,8]. It has also been applied to quantify soluble collagen in the culture medium and to quantify deposited collagen in cell layers [9]. Researchers have drawn attention to the low selectivity and specificity of the Picro-Sirius red staining in the presence of non-collagenous proteins [10,11] and the consequent overestimation of the collagen content [12,13]. Modifications of the assay to increase selectivity and accuracy in solubilized tissue samples, cell lysates and culture medium have been proposed. One study introduced a pepsin digestion step followed by column ultrafiltration before Picro-Sirius red staining [13]. Others show the benefit of loading the tissue homogenate onto a PVDF membrane prior to Picro-Sirius red staining followed by image-based analyses to enhance the assay's accuracy [14].

The 3,4-dihydroxyphenylacetic acid (3,4-DHPAA) based fluorometric assay detects collagen in solubilized collagen samples after collagenase degradation by reaction of 3,4-DHPAA in the presence of sodium borate buffer (pH 8.0) and $NaIO_4$ with N-terminal glycine-containing peptide fragments [15,16]. The authors indicate that proteins like bovine serum albumin, casein, lysozyme, myoglobin, thrombin, and amyloid β protein do not interfere with the assay, but potential interference of other proteins such as elastin, fibronectin or laminin, was not assessed. The assay has been applied to quantify

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collagen in cell culture lysates, non-decellularized and decellularized tissue specimens [15,17,18]. It has also been applied to quantify the activity of enzymes that generate N-terminal glycine-containing peptides [19–21].

Hydroxyproline (Hyp) is found within the [Gly-X-Y] triplet repeat sequence characteristic of collagens. In the Hyp assay, the total amount of Hyp in the sample is quantified after collagenase degradation (optional) and acid or alkaline hydrolysis steps. The released Hyp is then oxidized via chloramine-T and reacted with Ehrlich's reagent to form a chromophore. According to reported literature, the Hyp assay provides a relatively accurate estimation of collagen content in tissue samples [12,22–24].

Antibody-based assays utilize collagen-specific antibodies for the quantification of specific collagen types. When combined with fluorescently labeled antibodies, it allows quantification of deposited collagen by immunofluorescence (IF) microscopy [25,26]. When using enzyme-conjugated antibodies, it enables quantification by enzyme-linked immunosorbent assay (ELISA) in cell cultures and tissue samples [15,27].

A recent review article compared the different methods for quantification of collagen in tissue samples [28]. The authors concluded that the Hyp assay was suitable for estimating overall collagen content. ELISA-based quantification was considered highly sensitive and can differentiate collagen types. The Picro-Sirius red staining was declared not accurate due to non-specific interactions. A different study compared the results of three of these assays in cell culture and tissue lysates and came to a different assessment [15]. The 3,4-DHPAA-based assay was found to be 10 and 5 times more sensitive than ELISA and Picro-Sirius red assays, respectively. The Picro-Sirius red assay was considered to overestimate the collagen content. The Hyp assay was not compared.

Considering the strong differences in the working principle (from collagen-type specific to non-specific interactions), in the involved reporter molecules (small molecules or large antibodies), in the sample processing conditions (no processing, digestion or decellularization) and in the sensitivity of the detection methods associated with the four methodologies for quantification of collagen deposition, differences in assay performance are not surprising. A systematic study that compares the performance of the different assays in the quantification of deposited collagen in cell cultures appears necessary to unequivocally clarify the range of applicability and sensitivity of the methods. We note that the quantification of deposited collagen matrix is complementary to the quantification of the expression levels of collagen genes [29], of the soluble collagen in culture medium [13], or the total collagen present in cell lysates which also includes intracellular collagen [30]. While these measurements can be taken as an indirect estimation of the potential level of deposited matrix collagen in cell cultures, they do not provide a direct quantification of it.

In this article, we systematically compare the performance of the above-mentioned methods for the quantification of deposited collagen in model 2D cultures of fibroblasts and evaluate their reliability and sensitivity as a function of deposited collagen concentration, cell density and post-culture processing conditions (decellularization). Our results are relevant to inform the selection of the appropriate assay for collagen quantification, which is important for the prediction of the regenerative capacity of new biomaterials or the therapeutic potential of new drugs in collagen-associated diseases.

2. Experimental

2.1. Cell culture and stimulation of collagen production

Normal human neonatal dermal fibroblasts (NHDFs-Neo) were purchased from Lonza (CC-2509). The Fibroblast Growth Medium-2 (FGM-2) BulletKit containing 2 % fetal bovine serum (FBS) ("FGM-2 + 2% FBS", Lonza, CC-3132) was used as recommended medium by Lonza for the growth of human dermal fibroblasts. Cells were cultured in FGM-2 + 2 % FBS medium with 1 % penicillin/streptomycin (Gibco, 15140–122) at 37 °C in a humidified atmosphere with 5 % CO_2. Cell passages 4–14 were used for the experiments. 96-well plates (Greiner bio-one, 655180) were used for the biochemical assays. 18-well μ-slides with a polymer bottom (Ibidi, 81816) were used for experiments requiring microscopic imaging. Both plates are tissue-culture treated and are expected to show comparable affinity for collagen. Cells were seeded at a density of 5×10^4 cells per well and cultured for 24 h with 100 μL medium. After 24 h, the medium was refreshed ("Ctrl", which stands for "control"). In the experiments with collagen deposition-stimulating conditions, the medium was supplemented with 0.2 mM Asc (L-ascorbic acid sesquimagnesium-2-phosphate hydrate, Sigma Aldrich, A8960) or with Asc in combination of 10 ng mL^{-1} TGF-β1 (Transforming growth factor beta-1, "Asc + TGF-β1", R&D Systems, 240-B- 002). After the medium change, cells were cultured for an additional 2, 4, and 6 days in all experimental conditions, resulting in cells being in culture for a total of 3, 5, and 7 days for the collagen quantification experiments. Within this time, the medium was exchanged every 48 h with respective stimulants.

We note that Asc is an essential cofactor for prolyl hydroxylases and lysyl hydroxylase enzymes involved in post-translational modification of collagen. Hydroxylation of proline stabilizes the collagen triple helix by additional hydrogen bonds, while lysine hydroxylation stabilizes the collagen fibers by covalent crosslinks [31]. TGF-β1 is a profibrotic factor that can promote cell proliferation, differentiation, and collagen biosynthesis. It also induces the inhibition of MMPs activity, which leads to the accumulation of collagen [32,33]. We found that the typical concentration range used to accelerate collagen deposition in 2D cell cultures with Asc is 0.1–0.2 mM [8,29,34–36] and with TGF-β1 is 1–15 ng mL^{-1} [8,37].

2.2. Decellularization of 2D cell cultures

Cell layers were rinsed once with prewarmed PBS (Phosphate Buffered Saline) followed by incubation with 100 μL of decellularization solution (20 mM NH_4OH with 1 % Triton X-100 in PBS) for 7 min at room temperature (RT) [38]. Afterward, the samples were rinsed with PBS 6 times. In all steps the solutions were slowly added along the wall of the well and carefully removed to minimize the damage to the matrix. During the washing steps, 50 μL of the washing solution was left in the well to minimize peeling off the exposed matrix from the edges and loss of matrix in the subsequent washing steps [39].

After decellularization, the remaining matrix was fixed with 4 % paraformaldehyde (PFA, Thermo Fisher Scientific, J61899) at RT for 20 min. To confirm the decellularization, samples were stained with phalloidin (Alexa Fluor 488 label, Invitrogen, A12379) and DAPI. For this purpose, the fixed samples were washed 3 times with PBS followed by the incubation with 0.5 % Triton X-100 in PBS for 10 min at RT and blocking step with 1 % bovine serum albumin (BSA, Sigma Aldrich, A7906-100G) in PBS (PBSA) at RT for 45 min. The samples were incubated with 50 μL phalloidin solution at a 1:50 dilution in 1 % PBSA at 37 °C for 45 min. For nuclear staining, the samples were incubated with 1 μg mL^{-1} of DAPI in PBS for 5 min at RT. Samples were rinsed 3 times with PBS and stored at 4 °C until imaging.

To visualize the matrix after the decellularization procedure, the samples were stained with collagen I antibody (Human pro-collagen I alpha 1 antibody, R&D Systems, MAB6220–100), or with Sirius red dye following the protocols described in the following sections. The protocols used for non-decellularized samples were also applied to decellularized samples.

2.3. Estimation of cell density via crystal violet (CV) staining

Cells in the wells were rinsed once with prewarmed PBS and fixed with 100 μL of precooled (4 °C) methanol-ethanol mixture (2:1) for 20 min at RT, followed by washing 3 times with PBS. The methanol-ethanol

fixation permeabilizes cells and facilitates the penetration of the crystal violet dye through cellular multilayers. Then, 100 μL of 0.1 % crystal violet (Sigma, C0775-25G) solution in Milli-Q H_2O was added at RT and incubated with gentle shaking at 150 rpm for 20 min. Wells were rinsed with deionized H_2O multiple times until washing water appeared clear and colorless by eye. The wells were filled with deionized H_2O and shaken for 30 min. This step was repeated 3 times. The plates were air-dried for 30 min. To elute the bound dye, cells were incubated with 120 μL of 33 % acetic acid with shaking at 250 rpm for 20 min. 100 μL of the elution solution was transferred to another 96-well plate and the absorbance at 590 nm was measured using a Tecan Infinite 200 PRO microplate reader. Wells without fibroblasts were treated in the same way and used as controls for background subtraction. For the standard curve, cell seeding densities ranging from 0.5 to 12 × 10^5 cells mL^{-1} were utilized.

2.4. Immunofluorescence (IF) staining of extracellular collagen I in cell cultures

Cells cultured in an 18-well μ-slides as described above were washed with prewarmed (37 °C) PBS and fixed with 100 μL of PFA for 20 min at RT. Samples were rinsed 3 times with PBS and blocked with 100 μL of 1 % PBSA at RT for 45 min. Samples were incubated with 50 μL of 10 μg mL^{-1} of mouse anti-human pro-collagen I alpha 1 monoclonal antibody (R&D Systems, MAB6220–100) solution in 1 % PBSA for 45 min at 37 °C. After this, samples were rinsed 3 times with PBS and incubated with 50 μL of 4 μg mL^{-1} of donkey anti-mouse secondary antibody (Alexa Fluor 647 label, Fisher Scientific, 10226162) solution in 1 % PBSA at 37 °C for 45 min. The incubation solution of the secondary antibodies also contained 1 μg mL^{-1} of DAPI. Samples were rinsed 3 times with PBS and stored at 4 °C until imaging. Zeiss Axio Observer Z1 microscope and LSM 880 confocal microscopes were used for imaging the samples at different magnifications.

2.5. Quantification of extracellular collagen I via immunofluorescence imaging

Images of IF stained samples were captured using a Zeiss Celldiscoverer 7 microscope with 20×/NA 0.7 objective. Imaging parameters were set based on the control sample showing the highest collagen fluorescence intensity, typically those with longer culture durations or those treated with collagen deposition-stimulating factors. These settings were then used consistently across all samples. The image tiling function was used to define 20 specific positions across the entire well, which were kept the same for all other wells and conditions. Images taken from those positions were used to measure the mean fluorescence intensity generated by the anti-collagen I antibody staining. The ImageJ/Fiji software's (version 2.9.0/1.53 t) "Measure" macro was utilized to analyze the mean fluorescence intensity for each image. A control well stained only with secondary antibody was used to measure the mean fluorescence intensity value of background, which was then subtracted from the sample values. Measurements were taken from 2 independent experiments, each with 2 technical replicates.

2.6. Quantification of extracellular collagen I by cell-based indirect ELISA

Cells cultured in a 96-well plate were fixed with 4 % PFA, rinsed 3 times with PBS and blocked with 100 μL of 1 % PBSA for 45 min. The deposited collagen was stained using 50 μL of mouse anti-human pro-collagen I alpha 1 monoclonal antibody (R&D Systems, MAB6220–100) at a concentration of 5 μg mL^{-1} diluted with 1 % PBSA for 45 min at 37 °C. Samples were washed 3 times with PBS and 50 μL of HRP-conjugated goat anti-mouse IgG polyclonal antibody (GeneTex, GTX213111–01) at a concentration of 0.625 μg mL^{-1} in 1 % PBSA was added to the wells. After incubation for 45 min at 37 °C, cells were rinsed 6 times with PBS and incubated with 120 μL substrate solution in the dark for 15 min at 37 °C. The substrate solution was previously prepared by dissolving 1 tablet of *o*-phenylenediamine dihydrochloride (OPD, Sigma, P8287) in 25 mL of 0.05 M phosphate-citrate-buffer in deionized H_2O, pH 5.0 (Sigma, P4809) to a final OPD concentration of 0.4 μg mL^{-1}. Immediately before use, 10 μL of 30 % H_2O_2 (EMD Millipore, 386790-100ML) was added to the substrate solution. After 15 min of incubation, 100 μL of the reaction solution was transferred to a 96-well plate containing 30 μL of 3.0 M HCl (stop solution). The absorbance of the stopped reaction was measured at 492 nm using a Tecan Infinite 200 PRO microplate reader. A secondary antibody control (without primary antibody) was carried out and subtracted from the sample values. In the same experiment, CV staining was performed to assess cell density, and the resulting mean absorbance values were used to normalize the ELISA data.

2.7. Quantification of extracellular collagen by Picro-Sirius red assay

A reported protocol was used for our experiments [36,40,41]. Cell cultures in 96 well plates were rinsed with prewarmed PBS and fixed. Two different fixation methods were tested: (1) fixation with 100 μL Bouin's solution (Carl Roth Gmbh + Co. KG, 6482.2) for 1 h at RT, or (2) fixation with 100 μL of 4 % PFA for 20 min at RT. The fixed samples with Bouin's solution were washed with PBS until the yellow colour was no longer visible on the plate. The PFA fixed samples were rinsed 3 times with PBS. Inspection of the samples by optical microscopy before and after fixation was performed to check for possible cell loss during the fixation process. Fixed samples were incubated with 100 μL Picro-Sirius red dye solution (Abcam, ab246832) at RT with gentle shaking for 1 h. The dye solution was removed, and samples were washed 4 times with 200 μL of 0.01 N of HCl to remove unbound dye. Microscopic analysis was performed to assess the specificity of the staining (Fig. S4). The excitation and emission filters were at wavelengths of 545 ± 25 nm and 605 ± 70 nm, respectively. The bound dye was eluted by incubation with 120 μL of 0.1 N NaOH and shaking for 30 min. 100 μL of the elution solution was transferred to a different 96-well plate and absorbance was measured at 550 nm using a Tecan Infinite 200 PRO microplate reader. Wells without cells were treated identically and the absorbance values were used for background subtraction. In the same experiment, CV staining was done to get an estimate of the cell density and the resulting mean absorbance values were utilized for normalizing the Picro-Sirius red assay readings. The standard curve was constructed with human collagen I (StemCell Technologies, 07005) solution in PBS within the concentration range 0–500 μg mL^{-1}.

2.8. Quantification of extracellular collagen by 3,4-DHPAA-based assay

The assay was conducted based on the reported protocol, with slight modifications [15]. *Clostridium histolyticum* type I collagenase (ColG, Sigma, C0130-100MG) dissolved in 50 mM Tris-HCl buffer, pH 7.5 with 5 mM $CaCl_2$ (1.0 mg mL^{-1}, stored at −20 °C), was diluted in PBS to a concentration of 48 μg mL^{-1} before use. We used PBS buffer for the collagenase treatment instead of sodium borate buffer (pH 7.5) used in the original protocol since sodium borate buffer caused cell lysis already at incubation times >30 min. The cell layers were washed once with prewarmed PBS and incubated with 50 μL of the enzyme solution at 37 °C for 5 h. The following reagents were added to the well: (i) 50 μL of 125 mM sodium borate buffer (pH 8.0), (ii) 50 μL of 0.75 mM 3,4-DHPAA in Milli-Q H_2O (Sigma, 850217-1G), and (iii) 50 μL of 1.25 mM $NaIO_4$ in Milli-Q H_2O. The sequence in which reagents are added is important for the successful completion of the fluorogenic reaction. The reaction mixture was incubated for 10 min at 37 °C before fluorescence detection. Afterwards, the fluorescence intensity of the reaction mixture was measured immediately using a Tecan Infinite 200 PRO microplate reader (excitation: 375 nm; emission 465 nm). Fluorescence measurements were performed in the presence of cells, in the same wells where the enzymatic digestion and the fluorogenic reaction were performed.

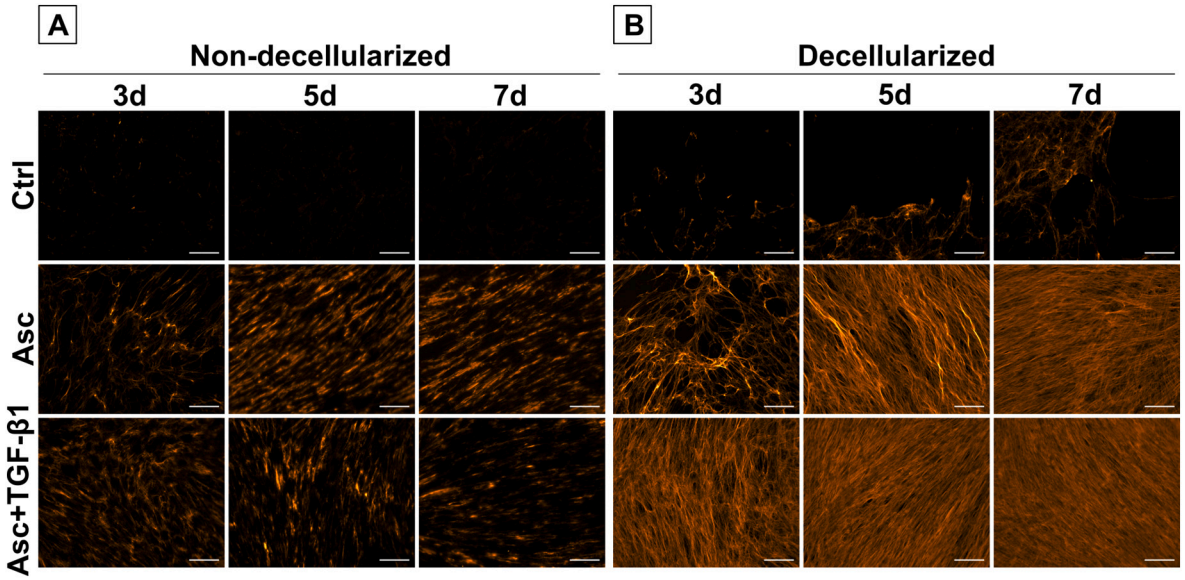

Fig. 1. Fluorescence images of NHDF cultures stained with collagen I antibody in (A) non-decellularized and (B) decellularized samples. Cells were cultured for 3, 5 and 7 days at three different culture conditions: Ctrl, Asc, Asc + TGF-β1. Images were captured using a 20×/NA 0.75 objective (Zeiss Axio Observer Z1 microscope). Scale bar = 100 μm.

Replicates without collagenase treatment were used for background subtraction. In the same experiment, CV staining was performed to estimate the cell density. The mean absorbance values from the CV staining were used to normalize the fluorescence signal from 3,4-DHPAA-based fluorometric assay. For standard curve, a human collagen I solution in PBS at the concentration range of 0–80 μg mL^{-1} was used.

2.9. Quantification of extracellular collagen by Hyp assay

The Hyp assay was performed using a commercial Hyp assay kit (Sigma, MAK008-1KT). Decellularized samples in 96 cell plates were digested with 50 μL of ColG solution at a concentration of 48 μg mL^{-1} overnight at 37 °C. Digestion with ColG was carried out to detach the matrix from the 96-well plates. The homogenization step was performed by shaking the plates in an orbital shaker (Heidolph Instruments, Titramax 100) at maximum speed for 30 s. The supernatant was transferred into a pressure-tight polypropylene screw cap tube (Corning, 430915) and mixed with 50 μL of 12 M HCl. Samples were hydrolyzed at 120 °C for 3 h followed by centrifugation at 16,000 rcf for 5 min. The entire liquid phase was transferred to a 96-well plate and evaporated to complete dryness in an oven at 60 °C. 100 μL of chloramine T/oxidation buffer mixture was added to the dry samples and incubated at RT for 5 min. Then, 100 μL of DMAB reagent diluted in a perchloric acid/isopropanol solution at 1:1 ratio was added and incubated at 60 °C for 90 min. The absorbance of the samples was measured at 560 nm using a Tecan Infinite 200 PRO microplate reader. A ColG solution without decellularized matrix was treated identically and used for background subtraction. To assess the cell density, CV staining was conducted, and the mean absorbance values were used for data normalization in the Hyp assay. Human collagen I diluted in PBS was used to generate a standard curve over a concentration range of 0 to 100 μg mL^{-1}.

2.10. Quantification of extracellular collagen I in mouse embryonic fibroblast (MEF) cultures by IF staining and cell-based indirect ELISA

Wild-type mouse embryonic fibroblasts (MEFs WT #18) and Hsp47 gene knockout mouse embryonic fibroblasts (MEFs KO #13) were purchased from RIKEN BRC cell bank (RCB5291 and RCB5293) and cultured in DMEM medium (Gibco, 21969–035) supplemented with 1 % penicillin/streptomycin, 1 % GlutaMax (Gibco, 35050–061) and 10 % FBS at 37 °C in a humidified atmosphere with 5 % CO_2. The cell seeding and culturing conditions were the same as those applied to NHDFs.

The IF staining and ELISA protocols used for NHDFs were also applied for collagen quantification in MEFs cultures, but different primary and secondary antibodies were used: 5 μg mL^{-1} anti-collagen I primary antibody (Abcam, ab21286) and 4 μg mL^{-1} of goat anti-rabbit secondary antibody (Alexa Fluor 647 label, Invitrogen, A21245) were used for IF staining. 2,5 μg mL^{-1} anti-collagen I primary antibody (Abcam, ab21286) and 1.775 μg mL^{-1} HRP-conjugated goat anti-rabbit IgG polyclonal antibody (GeneTex, GTX213110–01) were used for ELISA. In the same experiment, CV staining was performed to assess cell density, and the resulting mean absorbance values were used to normalize the ELISA data.

2.11. Statistical analysis

Biochemical assays for collagen quantification were performed in 3 independent experiments with 3 technical replicates. GraphPad Prism 10 was used for plotting graphs and statistical analysis. The data is presented as mean ± standard deviation (SD). Comparisons among 3 groups (1-factorial experiments) were performed using 1-way ANOVA followed by Tukey multiple comparison test. A two-way ANOVA followed by Tukey multiple comparison test was used for 2-factorial experiments. The outliers were identified using the Grubbs' method at an α-level of 0.0001. *p*-values under 0.05 were considered significant. The annotations on graphs are: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

3. Results and discussion

3.1. Establishment of NHDFs culture conditions

Initial experiments were performed to establish cell culture conditions that result in different levels of collagen deposition and cell density. We used fibroblasts (NHDF) as matrix producing cells over a 7-day period (Ctrl samples). To stimulate collagen deposition, Asc or Asc + TGF-β1 were added to the medium. Under our experimental conditions, NHDF cultures reached confluency on day 3 and formed areas with heterogenous cell distribution (later visualized as mono and multilayer

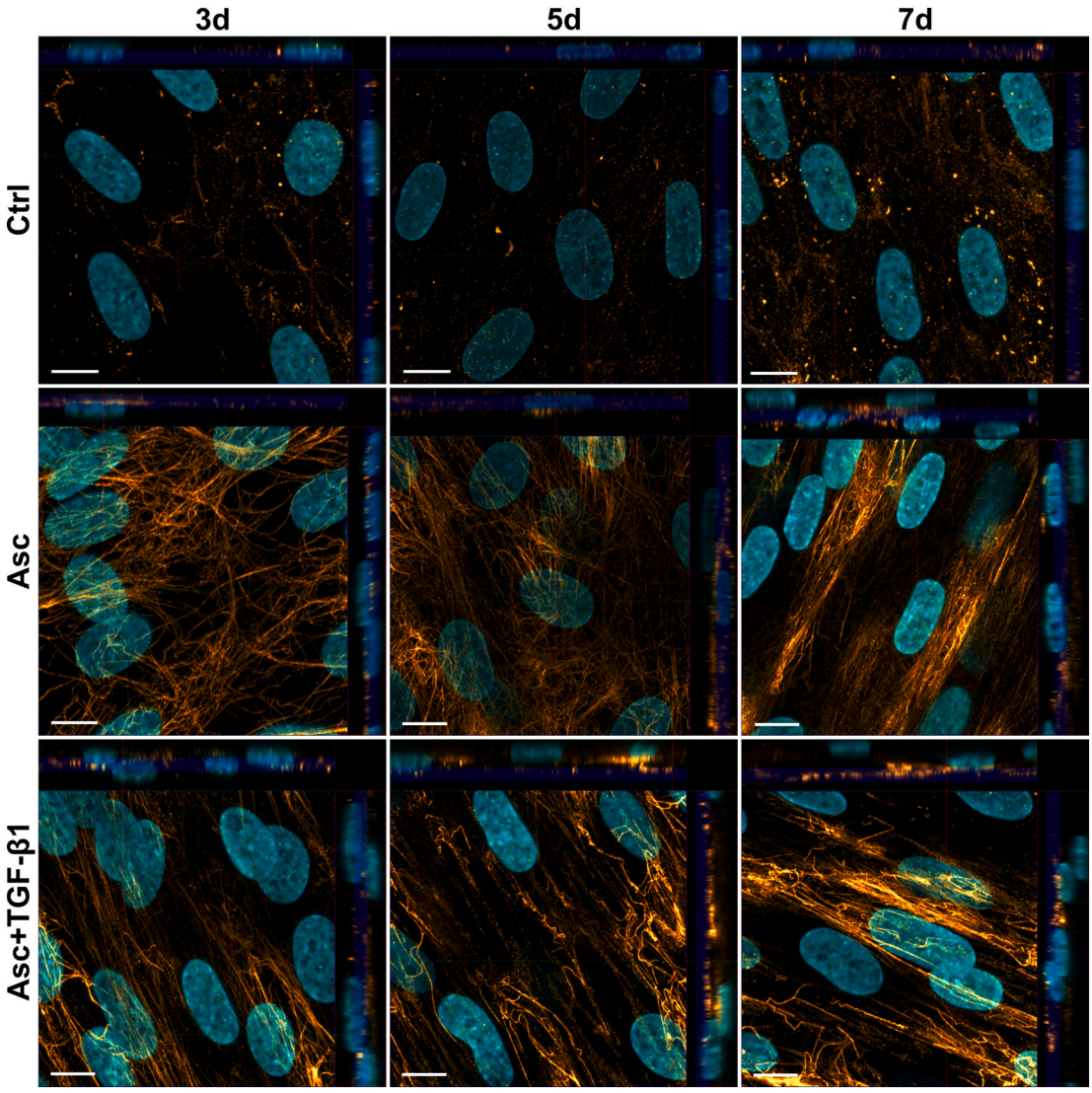

Fig. 2. Orthogonal projection of fluorescence microscopy images showing deposited collagen I in NHDF cultures at different culture conditions. Collagen I staining is shown in gold and DAPI staining in turquoise. Images were captured using a 63×/NA 1.4 oil objective (LSM 880 confocal microscope with Airyscan detector in SR-mode). Scale bar = 10 μm.

areas) particularly when stimulated with Asc or Asc + TGF-β1 (Fig. S1 A). According to CV staining, cell density increased from day 3 to day 7 at all experimental conditions and it was significantly higher in samples stimulated with Asc + TGF-β1 on days 5 and 7 and with Asc on day 7 (Fig. S1 B).

3.2. Establishment of collagen I imaging method via IF

IF staining of NHDF cultures allowed visualization of deposited collagen I and its morphological features. Collagen I signal was observed in all culture conditions (Fig. 1), with a higher intensity observed in Asc and Asc + TGF-β1 conditions vs Ctrl samples (Fig. 1 A). In high-resolution images, collagen I appeared as thin fibrils in samples treated with Asc or Asc + TGF-β1. In samples with higher cell densities, fibrils were oriented preferentially along the cell body. In contrast, NHDFs not exposed to stimulating factors showed a dotted collagen pattern and fewer fibrils (Fig. 2). In decellularized samples, a higher collagen I signal was detected, and a denser network of collagen fibrils was observed compared to non-decellularized samples (Fig. 1 A-B). This indicates a better accessibility of the staining antibodies to deposited collagen I in the absence of cells.

Although conditions for decellularization were optimized, some damage to the deposited matrix was revealed in the images of the Ctrl samples, which had overall lower collagen density (Fig. S2 B). This can lead to underestimated values of deposited collagen in the following collagen quantification experiments with decellularized Ctrl samples. DAPI and Phalloidin staining of decellularized samples indicated that residual DNA and F-actin remained after the decellularization process, particularly in samples with higher cell density (Fig. S3 A-B).

3.3. Quantification of deposited collagen I by image-based analysis and cell-based indirect ELISA

To quantify the deposited collagen I across the well by image analysis, stitched images of the well were obtained after IF staining (Fig. S2 A-B). At first sight, a higher mean intensity in the collagen I signal was observed in areas of the well with cell monolayers (Fig. 3 A1, A2) compared to areas with cell multilayers (Fig. 3 B1, B2) in non-decellularized samples at culture times ≥5 days in the presence of collagen deposition-stimulating factors. Maximum intensity projection images of areas with cell multilayers revealed that the major contribution to the collagen I signal was from the top part of the multilayer

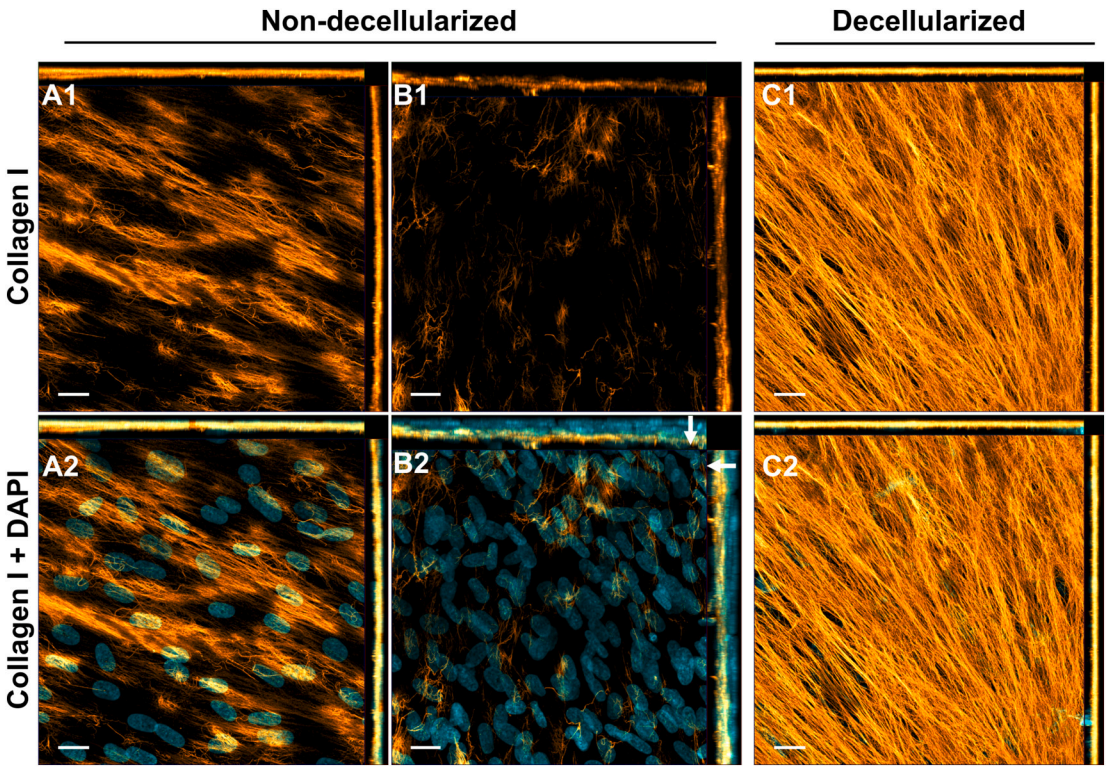

Fig. 3. Maximum Intensity Projection (MIP) of fluorescence images depicting collagen I deposition in areas with varied cell densities within a 7-day NHDF culture treated with Asc. (A, B) Images from non-decellularized samples. (A1, A2) Image corresponds to an area with lower cell density, while B1, B2 presents an area with higher cell density. (C1, C2) Image from decellularized samples. Collagen I staining is shown in gold, while DAPI staining in turquoise. Arrows in the image indicate the orientation from the bottom to the top plane in z-direction. Images were captured using a 20×/NA 0.8 objective (LSM 880 confocal microscope). Scale bar = 20 μm.

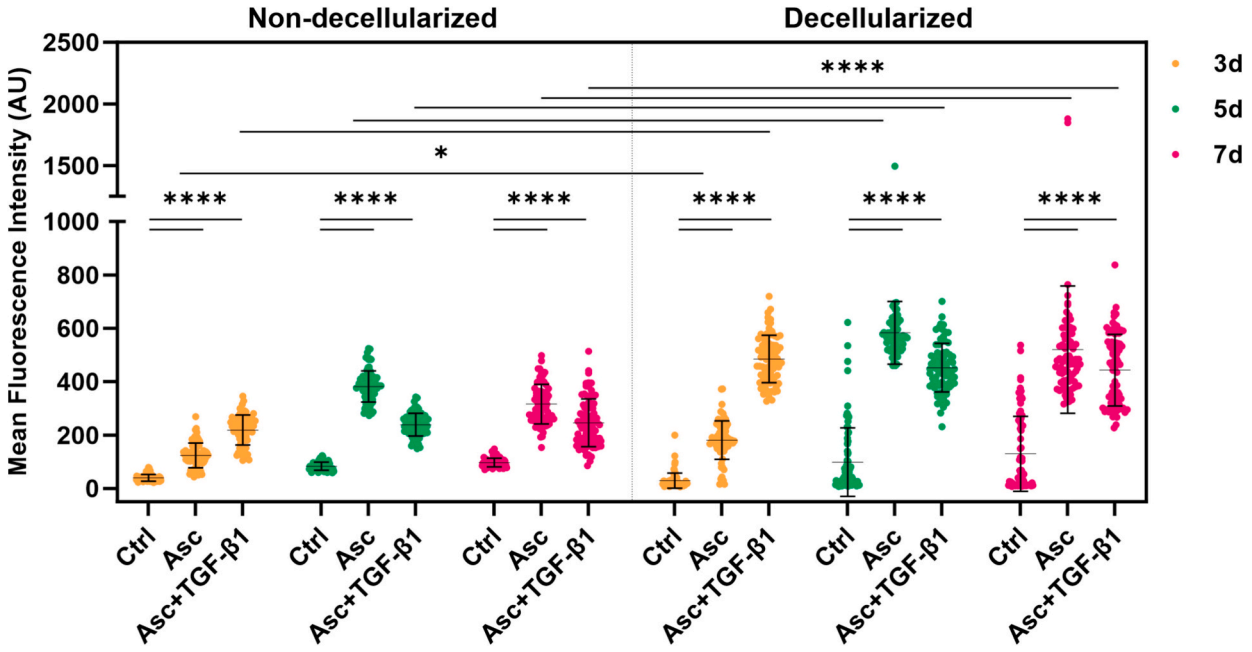

Fig. 4. Image-based quantification of mean fluorescence intensity after staining with collagen I antibody in non-decellularized and decellularized samples. Data were quantified from 2 independent experiments, each performed in duplicate. Data are represented as mean ± SD. AU: arbitrary unit. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test (* = $p < 0.05$, **** = $p < 0.0001$).

(Fig. 3 B2). This could be due to a hindered diffusion of the staining antibodies to underlying cell layers since no permeabilization of the cell membrane was performed to avoid staining of intracellular collagen. A similar analysis with decellularized samples showed a uniform distribution of collagen I (Fig. 3 C1, C2) throughout the entire well (Fig. S2 B, 7d Asc) and confirmed that decellularization facilitates access of

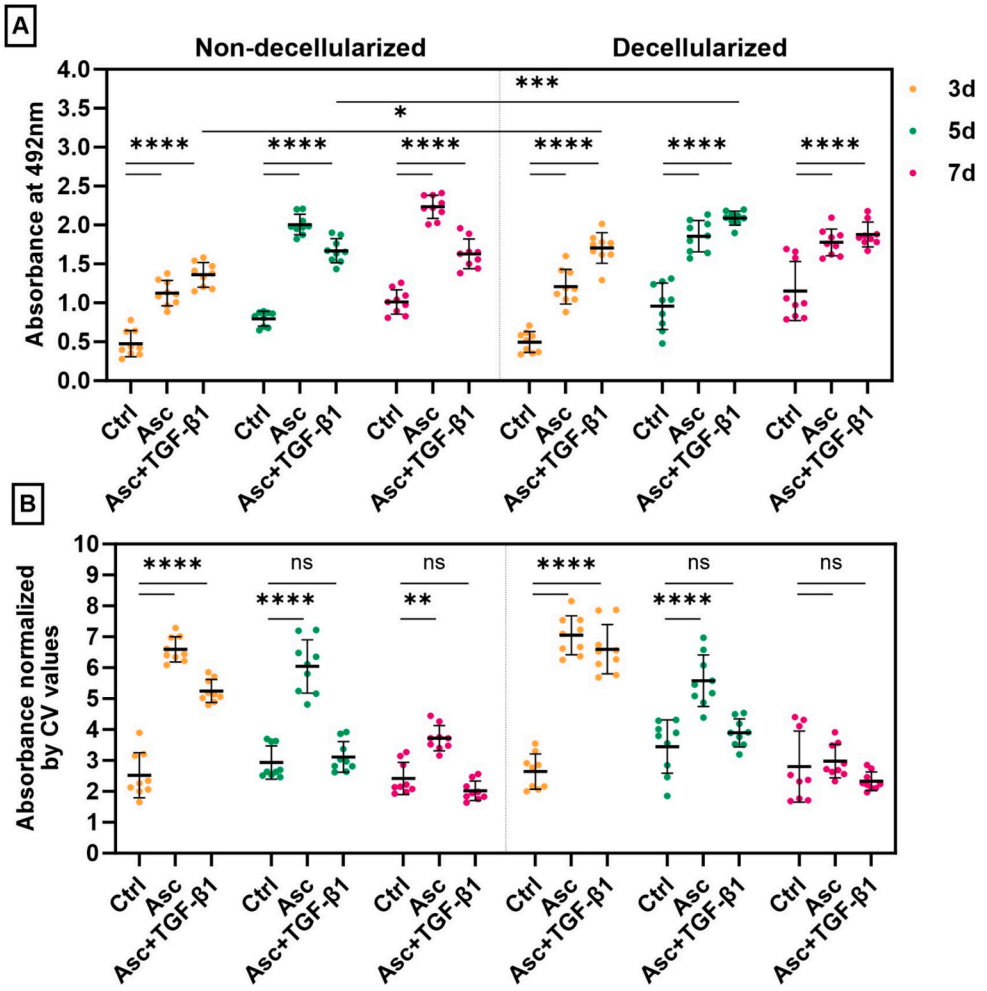

Fig. 5. Quantification of deposited collagen I in non-decellularized and decellularized samples using cell-based indirect ELISA. Data are presented before (A) and after normalization by CV absorbance values (B). Data were obtained in 3 independent experiments, each performed in triplicate. Data are represented as mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test (ns = not significant, *p < 0.05, ** = $p < 0.01$, *** = $p < 0.001$, **** = p < 0.0001).

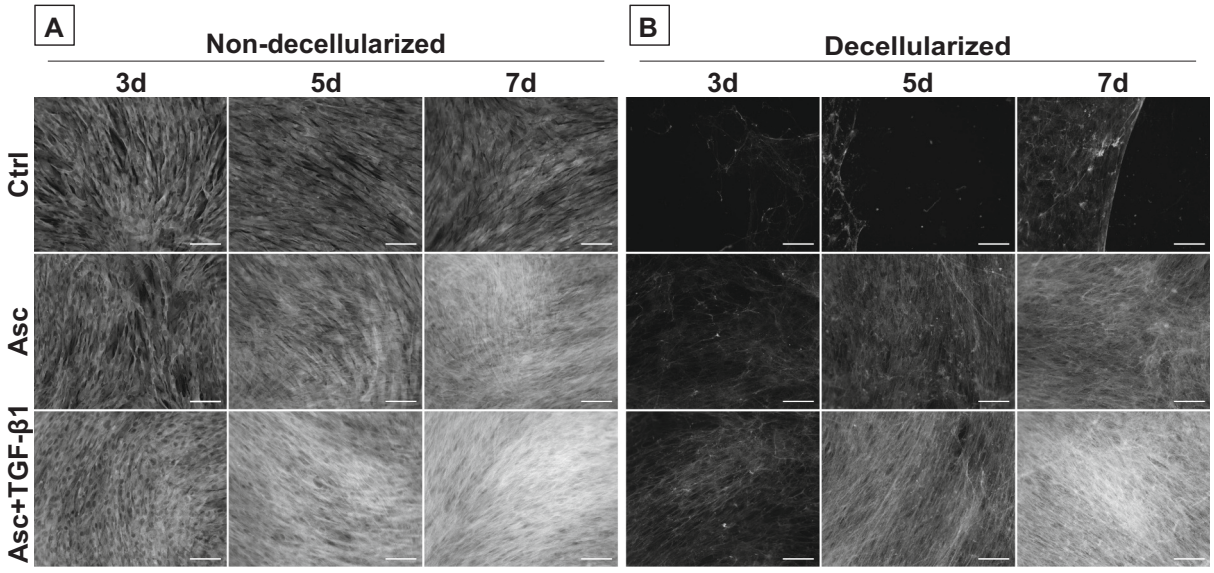

Fig. 6. Picro-Sirius red staining (A) of non-decellularized and (B) decellularized samples after PFA fixation. Images were captured using a 20×/NA 0.75 objective (Zeiss Axio Observer Z1 microscope). Scale bar = 100 μm.

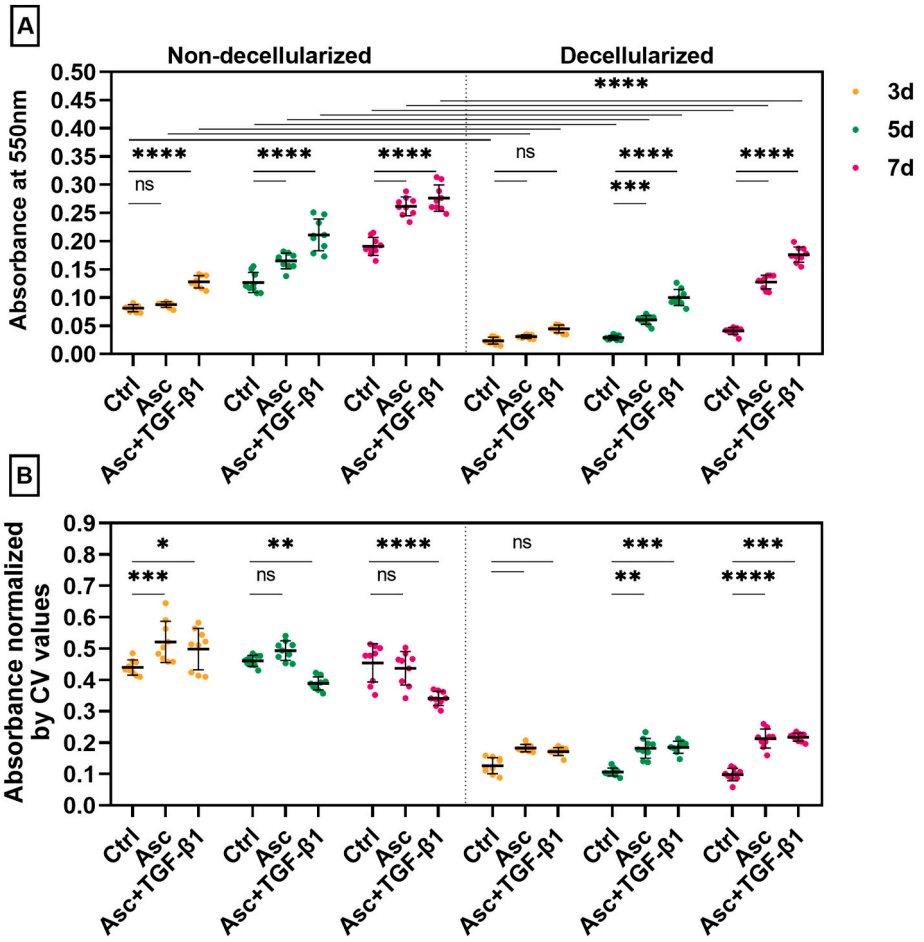

Fig. 7. Quantification of collagen deposition in non-decellularized and decellularized samples fixed with PFA using Picro-Sirius red colorimetric assay. Data are presented before (A) and after normalization by CV absorbance values (B). Data were obtained in 3 independent experiments, each performed in triplicate. Data are represented as mean ± SD. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test (ns = not significant, *p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

staining antibodies to collagen I structures, most probably by exposing the collagen matrix beneath the cell layers. This result indicates that quantification of deposited collagen I in non-decellularized samples by IF image analysis could underestimate the real collagen amount in 2D cell cultures at longer culture times, where multilayers are formed.

Fig. 4 shows the quantification of the mean fluorescence intensity by image analysis for the different culture conditions. Higher collagen I deposition values were detected in stimulated samples regardless of the samples' post-culture processing state. An increase in collagen I deposition was observed from day 3 to day 5 in Asc stimulated samples, but not in Asc + TGF-β1, and no difference was observed between day 5 and day 7 in both samples. Non-decellularized samples exhibited a lower mean fluorescence intensity compared to decellularized samples. Since image-based nucleus quantification was not reliable in areas with cell multilayers, the results could not be normalized by the number of cells.

Deposited collagen I was also quantified using cell-based ELISA (Fig. 5). A similar trend as in the IF quantification was observed in the samples treated with stimulating factors (non-decellularized samples) (Fig. 5A). The ELISA data were normalized by the cell density in the cultures estimated based on CV assay. Although statistical differences in the collagen deposition per cell were observed in Crtl vs stimulated samples at 3 days cultures, these differences diminished or vanished at longer culture times (Fig. 5B).

3.4. Quantification of deposited collagen by Picro-Sirius red assay

Microscopy evaluation of NHDF cultures stained with Picro-Sirius red dye after Bouin's solution fixation showed extracellular structures indicative of collagen deposition (Fig. S4). However, the dye also stained the intracellular space and, in some cases, also the nuclei of the cells. Optimization of the staining protocol through shorter incubation times with the dye, or changes in the cell fixation method (using PFA instead of Bouin's solution) did not eliminate the intracellular signal (Fig. S4). In addition, we performed decellularization of samples to enhance the specificity of the staining toward extracellular matrix. The microscopic inspection of the samples revealed fibrillar structures under all experimental condition (Fig. 6).

To further evaluate collagen deposition, we quantified the eluted dye from samples cultured under different conditions using absorbance measurements (Fig. 7). In non-decellularized samples, an increase in the absorbance was observed with culture time and the addition of collagen deposition-stimulating factors compared to Ctrl, except in the Asc-treated condition on day 3 (Fig. 7 A). However, this increase was no longer observable after normalization of the data by the CV absorbance values for most conditions (Fig. 7 B). In decellularized samples, the eluted dye concentration was significantly lower than from non-decellularized samples (Fig. 7 A) and the treatment with collagen deposition-stimulating factors led to a significant increase in the signal on days 5 and 7, both pre- and post-normalization (Fig. 7 A-B). These

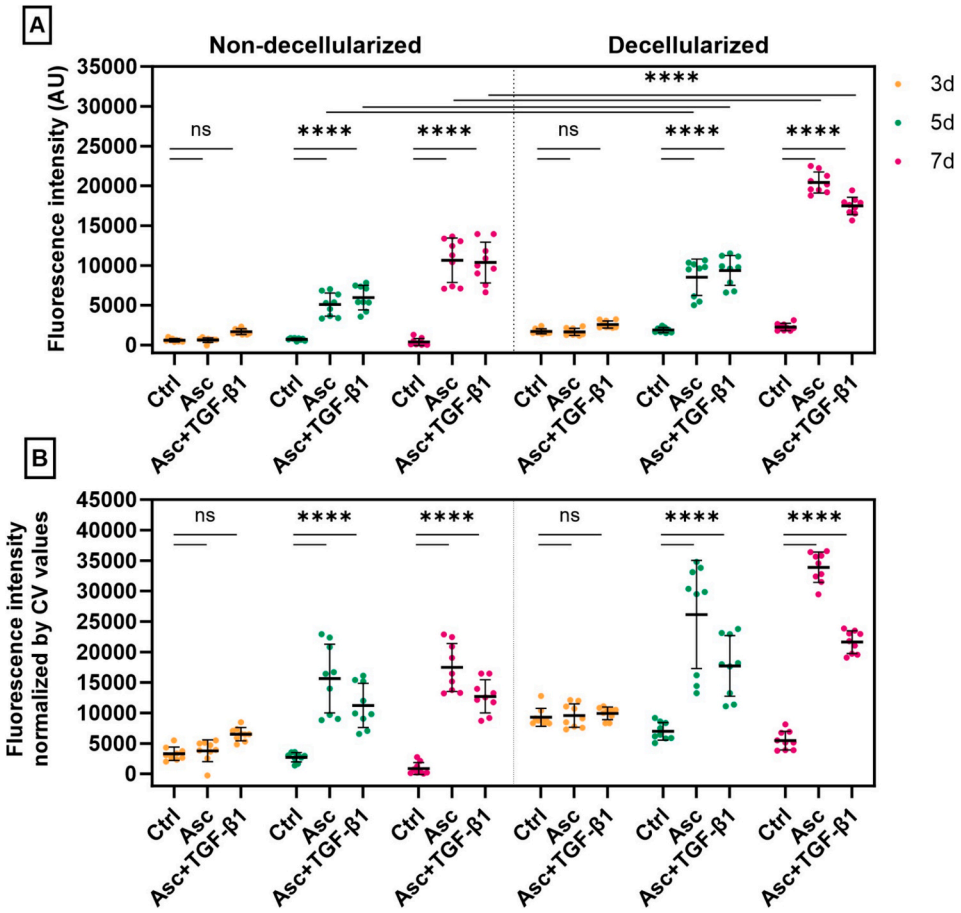

Fig. 8. Quantification of digested deposited collagen in non-decellularized and decellularized samples using 3,4-DHPAA-based fluorometric assay. Data are presented before (A) and after normalization by CV absorbance values (B). Data were obtained in 3 independent experiments, each performed in triplicate. Data are represented as mean ± SD. AU: arbitrary unit. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparison test (ns = not significant, **** = $p < 0.0001$).

results from Picro-Sirius red assay suggest that the contribution of the non-specific staining precludes the detection of the differences in collagen deposition in 2D fibroblast cultures between control and collagen deposition-stimulating conditions, however the extracellular collagen can still be estimated in decellularized samples under collagen deposition-stimulating conditions and in culture times of ≥5 days.

3.5. Quantification of digested collagen by 3,4-DHPAA-based assay

The 3,4-DHPAA-based fluorometric assay was used to quantify the collagen derived degradation peptides after enzymatic treatment of the cell layers with bacterial type I collagenase. In non-decellularized samples, we observed a significant difference in digested collagen signal between Asc and Asc + TGF-β1 stimulated conditions compared to Ctrl on day 5 and 7 (Fig. 8 A). Similar trends were also observed in decellularized samples, with a higher overall fluorescence intensity for all collagen deposition-stimulating conditions on day 5 and 7 (Fig. 8 A). Two different reasons could explain this observation: (I) the absence of cells facilitated the access of collagenase to collagen underneath the cells, or (II) the decellularization reagents weakened intermolecular interactions in collagen fibrils [42] and facilitated the digestion of collagen. A similar trend was seen after normalization by the CV absorbance values in non-decellularized and decellularized samples (Fig. 8 B). No differences between samples were observed on day 3, indicating that the assay is not sensitive at low culture times.

3.6. Quantification of hydrolyzed deposited collagen in decellularized samples by Hyp assay

The Hyp assay was applied to decellularized samples after collagenase digestion and acid hydrolysis at high temperature. The assay was not used on non-decellularized samples since the acid hydrolysis instantly lyses the cells and the intracellular collagen would interfere with the quantification. Hyp was detected in cultures treated with Asc and Asc + TGF-β1 incubated for 5 and 7 days. In the Ctrl samples, the Hyp assay rendered negligible values, consistent with the understanding that Asc is an essential cofactor for hydroxylation of proline during collagen biosynthesis (Fig. 9 A). In day 3 samples, no significant differences were observed with or without stimulating conditions, indicating that the assay is not sensitive enough to detect low amounts of deposited collagen. After normalization, an increase in Hyp under collagen deposition-stimulating conditions was observed at longer culture times compared to Ctrl samples (Fig. 9 B).

3.7. Quantification of collagen I deposition in MEFs cultures

We tested if IF staining and cell-based ELISA were sensitive enough to differentiate collagen deposition levels in cell cultures with a reduced collagen synthesis capacity. We used MEF WT and MEF KO cells where the latter cell type shows slowed secretion and reduced deposition of collagen due to the lack of Hsp47 collagen-specific chaperon [43,44]. At 5-day cultures, KO cells displayed a significantly higher cell density than WT cells under both Ctrl and Asc treatment conditions (Fig. S5 A-B). No

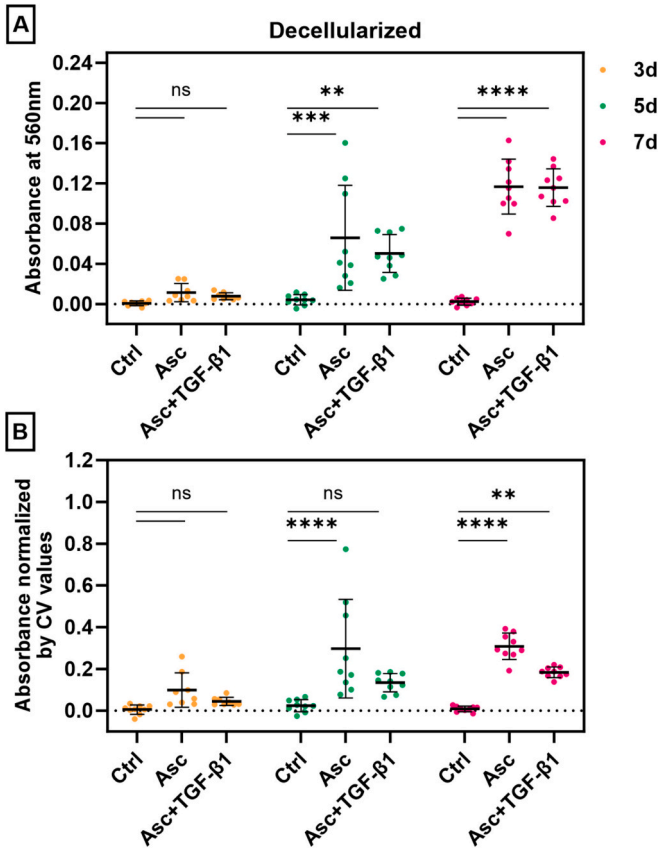

Fig. 9. Quantification of digested deposited collagen in decellularized samples using Hydroxyproline assay. Data are presented before (A) and after normalization by CV absorbance values (B). One outlier was identified and removed from each of the Asc and Asc + TGF-β1 conditions on day 3. These outliers are not presented in the graph and were excluded from the statistical analysis. Data were obtained in 3 independent experiments, each performed in triplicate. Data are represented as mean ± SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparison test (ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$).

significant differences in cell density were observed between the Ctrl and Asc treated conditions in both cell types (Fig. S5 B).

IF staining showed extracellular fibrils in Asc stimulated cultures of MEF WT at days of 5 and 7, but not in MEF KO cultures (Fig. S6). The predominant staining was extracellular, though a weak intracellular signal was also observed (Fig. S6). We believe the reason for the intracellular staining was due to fixation-induced cell blebbing [45], which was observable during fixation with 4 % PFA and was no longer observed after fixative removal and sample washing (data not shown). Collagen I quantification by cell-based ELISA confirmed the observations from IF staining (Fig. S7 A-B). Experiments in decellularized samples were performed to avoid possible interference from intracellular staining, which showed similar trends as in non-decellularized ones with the more variability (Fig. S7 A-B). These results prove the applicability of these assays to other cell types.

3.8. Comparative discussion of the different collagen quantification methods

A number of experimental details are to be considered when selecting a methodology to quantify collagen in *in vitro* 2D cell cultures [46]. (i) Which form of collagen is the focus of quantification: intracellular, secreted, solubilized or deposited? (ii) Which collagen type needs to be quantified? (iii) At which stage of cell proliferation in culture is collagen quantification needed: during the early phases, at the point of confluence, or at advanced stages when cells form multilayers? (iv) Is the deposited collagen stable enough to withstand a decellularization step? (v) What are the time and cost constraints for the assay? The answers to these questions narrow down the number of applicable methods. Table 1 compares the characteristics of the assays tested in this article to quantify deposited collagen in 2D cultures, and guides method selection.

Collagen types produced by neonatal human dermal fibroblasts (NHDFs) in this study are type I, III and V, with a predominance of type I [47]. The collagen quantification methods assessed in this study respond to different collagen types. The Picro-Sirius red and the Hyp assay do not differentiate between collagen types, whereas the 3,4-DHPAA-based assay, due to the use of collagenase type I for digestion, primarily quantifies collagen types I to III [48]. The antibody-based assays are specific for collagen type I. The collagen specificity of the assay is expected to influence the amount of deposited collagen quantified by the different methods (Table 2). Similar collagen amount was detected by Hyp and 3,4-DHPAA-based assays, which is consistent with the expected predominant secretion of collagen I and III by fibroblasts. The much higher collagen amount detected by the Picro Sirius assay is associated with non-specific binding to other matrix proteins, and to cellular structures in non- decellularized samples (Table 2). The cell-based ELISA does not allow quantification of collagen amount since an appropriate standard curve cannot be constructed from soluble collagen.

Quantification of deposited collagen at early time points (i.e., 3-day NHDF cultures) requires a methodology with high sensitivity. Cell-based ELISA was the only method able to show the differences in collagen deposition between Ctrl and stimulated conditions at 3-day cultures, which indicates that this assay has the highest sensitivity. However, this method seems to provide underestimated values of the total collagen I deposition levels in overconfluent cultures, where the staining antibody might have restricted access to underlying collagen in multicellular areas of the culture. A potential way to circumvent this limitation could be to do ELISA on digested samples. Other collagen quantification methods like High Performance Liquid Chromatography coupled to Mass Spectrometry could also be applied on digested samples. However, this methodology requires specialized equipment and might be less practical for routine collagen quantification. The Picro-Sirius red colorimetric assay exhibits low sensitivity and requires long culture times and previous decellularization of the sample to provide reliable data. The non-specific interaction of the dye with other cellular structures precludes quantification in the presence of cells. The alternative 3,4-DHPAA-based fluorometric assay provides more reliable results, also at intermediate time scales, and does not require decellularization. It can detect collagen deposition at day 5 and under collagen stimulation conditions.

The findings from the Hyp assay after normalization by CV staining at all culture times are similar to those from the 3,4-DHPAA-based assay. However, the Hyp assay necessitates a prior decellularization step, and demands over a day for data collection. Conversely, the 3,4-DHPAA-based assay requires a shorter sample preparation time and offers applicability to non-decellularized samples. According to our results, the 3,4-DHPAA-based assay is an appealing alternative to the commercial Hyp assay.

4. Conclusions

The comparison of collagen quantification by different methods evidence that the available methods can deliver accurate results within narrow experimental conditions. The selection of the most appropriate assay can save time and avoid misleading results as a consequence of insufficient selectivity, sensitivity or time investment. Combinations of different methods might be needed to reliably monitor and quantify collagen matrix deposition in studies across different culture times and conditions. The cell-based ELISA was the only method able to detect differences in the collagen deposition on day 3. 3.4-DHPAA-based assay

Table 1
Comparative features of collagen quantification assays.

Collagen quantification method	3,4 – DHPAA-based assay	Picro-Sirius red assay	Hydroxyproline assay	Cell-based ELISA*	IF imaging*
Specific molecular motif recognized in collagen structure	Glycine or N- terminal glycine containing peptides	Basic amino acids or basic amino acids containing sequences	Hydroxyproline	Sequences in collagen I (Gln23-Lys277, Gly1094-Leu1464)	
Molecular interactions involved	Complexation	Electrostatic interactions	Oxidation and complexation	Molecular recognition	
Specificity for collagen type	Specificity relies on the collagenase used. Semi-specific for collagen I-III	Semi-specific for collagen types, staining can also be interfered by non-collagenous proteins	Semi-specific for collagen types	Specific for collagen I	
Imaging-derived morphological insights	No	Yes	No	No	Yes
Minimum days of culture studied for reliable results with NHDFs	5d	5d	7d	3d	3d
Sample pretreatment requirements	Collagenase treatment	Decellularization	Decellularization and hydrolysis	–	–
Detection method	Fluorometric	Colorimetric	Colorimetric	Colorimetric	Fluorescence imaging
Price** of kits	No significant differences				–
Price** of self-made assay solutions	1×	2×	2×	50×	–
Time/Effort required**	<1 h	1.5 h	2 h	2.5 h	2 h
Duration of the experiment **	5.5 h	3 h	27.5 h	4 h	3.5 h***
Presence of commercial kits	Yes	Yes	Yes	Yes****	No
Device required	Fluorimeter	Spectrophotometer	Spectrophotometer	Spectrophotometer	Fluorescence microscope

* Antibody used: MAB6220–100, R&D Systems.
** The values shown are approximate estimates calculated for 100 assays performed on a single plate.
*** The value does not include the time required to acquire images of antibody-stained samples. It usually takes several hours to capture and process high-resolution Z-stack images or visualize the entire well.
**** The cell-based ELISA kits are available with different antibodies.

Table 2
Comparison of collagen concentration (μg mL^{-1}) in non-decellularized and decellularized samples using Picro-Sirius red, 3,4-DHPAA-based, and Hydroxyproline assays.

		Collagen concentration (μg mL^{-1})					
		Non-decellularized			Decellularized		
		3d	5d	7d	3d	5d	7d
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Picro-Sirius red assay	Ctrl	119 ± 8	179 ± 23	263 ± 21	43 ± 9	50 ± 5	66 ± 8
	Asc	127 ± 7	229 ± 17	357 ± 21	53 ± 5	92 ± 11	180 ± 16
	Asc + TGF-β1	180 ± 14	290 ± 36	376 ± 30	71 ± 9	144 ± 18	244 ± 17
3,4-DHPAA-based assay	Ctrl	2.8 ± 1	3 ± 1	5 ± 1	1 ± 1	2 ± 1	3 ± 1
	Asc	2.1 ± 1	17 ± 4	36 ± 10.	1 ± 1	23 ± 7	62 ± 4
	Asc + TGF-β1	6.8 ± 2	20 ± 5	36 ± 9	4 ± 1	26 ± 6	52 ± 4
Hydroxyproline assay	Ctrl	ND	ND	ND	3 ± 2	6 ± 4	5 ± 2
	Asc	ND	ND	ND	11 ± 6	50 ± 37	86 ± 19
	Asc + TGF-β1	ND	ND	ND	9 ± 2	39 ± 12	85 ± 12

ND = Not Determined.

was able to detect differences in collagen deposition after 5 days, and Hyp and Picro-Sirius red assays provided coherent results after decellularization. Collectively, the three last assays demonstrate comparable sensitivity in measuring collagen deposition. Our results can help researchers to more accurate quantification of collagen matrix deposition in the design and in vitro testing of biomaterials for tissue regeneration and the study of collagen-associated diseases.

CRediT authorship contribution statement

Syuzanna Hambardzumyan: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Jennifer Y. Kasper:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Aránzazu del Campo:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioadv.2025.214436.

Data availability

Data will be made available on request.

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