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Running Head:

Abstract

Tissue engineering by self-assembly allows for the fabrication of living tissue surrogates by taking advantage of the cell's inherent ability to produce and deposit tissue-specific extracellular matrix. However, the long culture periods required to build a tissue substitute in conducive to phenotypic drift *in vitro* microenvironments result in phenotype and function losses. Although several biophysical microenvironmental modulators (e.g. surface topography, substrate stiffness, mechanical stimulation) have been used to address these issues, slow extracellular matrix deposition remains a limiting factor in clinical translation and commercialisation of such therapies. Macromolecular crowding is an alternative *in vitro* microenvironment modulator that has been shown to accelerate extracellular matrix deposition by several orders of magnitude, thereby decreasing culture periods required for the development of an implantable device, whilst maintaining cell phenotype and function. Herein, we provide protocols for the production of rich in extracellular matrix tissue surrogates from human dermal fibroblasts, equine tenocytes and equine adipose derived stem cells using the principles of macromolecular crowding and the subsequent characterisation thereof by means of immunofluorescent staining and complementary fluorescence intensity analysis.

Keywords

Macromolecular crowding; Excluding volume effect; Cell therapies; Immunocytochemistry; Extracellular matrix

1. Introduction

Modern tissue engineering capitalises the inherent capacity of cells to create native supramolecular assemblies. However, during *in vitro* expansion, deprived of their optimal tissue context, cells lose their phenotype and function. To overcome such issues, numerous *in vitro* microenvironments modulators, including surface topography, substrate elasticity, mechanical loading, are at the forefront of scientific research and technological innovation [1]. The fabrication of native supramolecular assemblies, or tissue surrogates, is highly dependent on the rate of deposition of extracellular matrix (ECM) which, under traditional cell culture conditions, is extremely slow due to the very dilute culture conditions [2]. Macromolecular crowding (MMC) has been proposed as an efficient method to not only enhance ECM deposition, but to also maintain their phenotype during *in vitro* culture [3]. MMC is a biophysical phenomenon that enhances thermodynamic activities and biological processes by several orders of magnitude [4, 5]. It is based on the addition of inert macromolecules to the culture media and it acts by recreating the highly confined or crowded *in vivo* environment [2]. Several molecules have been tested as macromolecular crowders, including dextran sulphate, Ficoll™, polyethylene glycol, polyvinylpyrrolidone and carrageenan [6-10]. Among them, carrageenan, a highly sulphated polysaccharide, has been shown to induce maximum ECM deposition in permanently differentiated and stem cell cultures due to its higher polydispersity / more effective excluding volume effect [3, 6, 11-16].

Immunofluorescent labelling techniques are a very valuable tool for *in situ* identification of a wide variety of biological molecules. This identification can be performed directly, by means of the use of a primary antibody against the molecule of interest labelled with a fluorochrome [17, 18], or indirectly, by utilising a primary antibody against the molecule of interest and a fluorescently-labelled secondary antibody that targets specifically the primary antibody [19]. By using different combinations of primary and secondary antibodies conjugated with different fluorochromes, the spatial relations of two or three different molecules can be assessed simultaneously in one sample

[20]. In addition, with the aid of a fluorescence microscope connected to a camera and publicly available software, a semi-quantitative analysis can be performed in order to estimate the relative amounts of antigen that are present in different samples, thereby increasing the value of such techniques. Herein, we describe protocols for the fabrication of rich in ECM tissue surrogates from human dermal fibroblasts, equine tenocytes and equine adipose derived stem cells using the principles of MMC and the subsequent characterisation thereof by means of immunofluorescent staining and complementary fluorescence intensity measurements.

2. Materials

All cell culture materials should be handled following aseptic technique. Human primary cells should be handled using Biosafety Level 2 practices and containment. Diligently follow all the waste disposal regulations of your country / institution when disposing any kind of waste material.

2.1 Cell Culture

1. Human normal adult dermal fibroblasts (hDFs), cryopreserved (ATCC).
2. Equine tenocytes (eTCs), cryopreserved.
3. Equine adipose derived stem cells (eADSCs), cryopreserved.
4. Dulbecco’s modified Eagle’s medium, high glucose (DMEM).
5. Foetal bovine serum (FBS).
6. Penicillin-streptomycin solution 100× (PS).
7. Growth medium: Prepare growth medium by adding 10 % FBS and 1 % PS solution to the appropriated volume of DMEM.
8. Trypsin-EDTA solution.
9. Hank’s balanced salts solution 10× (HBSS) without Ca^{2+} and Mg^{2+}.
10. Double distilled water, sterile.
11. Carrageenan powder, suitable for gel preparation.

12. 100 mM L-ascorbic acid 2-phosphate solution. Prepare a stock solution of 100 mM L-ascorbic acid 2-phosphate in ddH2O and sterile-filter with a syringe and a 0.2 µm syringe filter. Store it in frozen aliquots at -20 ºC and protect it from the light.
13. 0.2 μm surfactant free cellulose acetate sterile syringe filters.
14. 10 mL luer lock sterile syringes.
15. 75 cm^2 tissue culture flasks.
16. 48-well tissue culture plates.
17. MMC growth medium: weigh the appropriated amount of carrageenan powder in a 1.5 mL microcentrifuge tube in order to prepare 0.5 mL of medium per well to be treated, considering a concentration of carrageenan of 75 µg/mL (the concentration may need to be optimised for the cell population of interest). Always prepare 2 mL extra in order to compensate for pipetting errors. In order to disinfect the carrageenan, irradiate it with UV-C light for 15 minutes (see **Note 1**). Supplement growth medium with 1 µL of the 100 mM ascorbic acid solution per mL of medium in order to reach a concentration of 100 µM. This will be used as the control medium and will also be used to prepare MMC growth medium by adding carrageenan. For the preparation of MMC growth medium, recover the disinfected carrageenan from the 1.5 mL microcentrifuge tube by suspending it in 1 mL of growth medium supplemented with ascorbic acid and transfer the volume to a tube with the remaining medium. Repeat this step at least twice in order to ensure the complete recovery of the carrageenan from the tube. For non-crowded control wells, keep aside the appropriated volume of growth medium supplemented with L-ascorbic acid 2-phosphate for this purpose (0.5 mL per well + 2 mL extra for compensating pipetting errors). For the solubilisation of the carrageenan in the growth medium supplemented with ascorbic acid, incubate the tube in a thermostatic bath at 37 ºC for at least 30 minutes (see **Note 2**).

2.2 Immunofluorescent characterisation of the ECM

1. Phosphate buffered saline (PBS): 137 mM sodium chloride, 2.7 mM potassium chloride, and 10 mM phosphate buffer. Add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 to 800 mL of dH_2O. Adjust the pH to 7.4 with HCl before adding dH_2O to 1L.
2. Fixing solution: paraformaldehyde (PFA) 2% (w/v) in PBS. Weight 0.2 g of PFA 95% powder and dissolve it by heating and stirring in a glass beaker containing 10 mL of 1x PBS (*see* **Note 3**). Allow dissolution for 1 hour (*see* **Note 4**). Once the PFA has been completely dissolved, transfer the resulting fixation solution to a 50 mL centrifuge tube. Store at 4°C. Filter the solution through a 0.2 μm sterile syringe filter before use.
3. Blocking solution: 3% bovine serum albumin (BSA) in PBS. Weight 0.3 g of bovine serum albumin and dissolve it in 10 mL of 1x PBS in a 15 mL centrifuge tube by vortexing. Keep it at 4°C for short term or -20°C for long-term storage.
4. Primary antibodies (Abcam): mouse monoclonal anti-collagen type I (ab90395), rabbit polyclonal anti-collagen type III (ab7778), rabbit polyclonal anti-collagen type IV (ab6586), rabbit polyclonal anti-collagen type V (ab7046), rabbit polyclonal anti-collagen type VI (ab6588), rabbit polyclonal anti-fibronectin (ab2413) (*see* **Note 5**). Prepare the dilutions of the primary antibodies in 1× PBS as indicated: mouse monoclonal anti-collagen type I (1:200), rabbit polyclonal anti-collagen type III (1:200), rabbit polyclonal anti-collagen type IV (1:200), rabbit polyclonal anti-collagen type V (1:200), rabbit polyclonal anti-collagen type VI (1:200), rabbit polyclonal anti-fibronectin (1:200).
5. Secondary antibodies (ThermoFisher): goat anti-rabbit conjugated to AlexaFluor®488 (A-32731), goat anti-mouse conjugated to AlexaFluor®555 (A-32727) (*see* **Note 6**). Prepare the dilutions of the secondary antibodies in 1× PBS as indicated: goat anti-mouse AlexaFluor®555 (1:500) for collagen type I immunofluorescent staining; goat anti-rabbit AlexaFluor®488 (1:500) for collagen types III, IV, V, VI and fibronectin immunofluorescent staining. Protect them from exposure to light (**see Note 7**).

6. DAPI solution: weight 1 mg/mL of DAPI and dissolve it in ddH_2O to produce a 2000× solution. Dilute with methanol to obtain the 1x solution.
7. Mounting medium (Vectashield).
8. 8mm Ø round glass cover slips.

2.3 Fluorescence intensity measurements

1. Inverted fluorescence microscope, equipped with 20× objective, filters suitable for DAPI (Ex/Em: 358⁄ 461 nm), AlexaFluor®488 (Ex/Em: 495/519 nm), AlexaFluor®555 (Ex/Em: 555/565 nm) and a connected camera.
2. Q-Capture Pro 7 software for image acquisition or similar.
3. ImageJ (version: 1.51j8) software.

3. Methods

For the preparation of the rich in ECM tissue surrogates, routine cell culture practices should be applied.

3.1 Preparation of rich in ECM tissue surrogates

1. Fill the appropriated number of 75 cm^2 tissue culture flasks (T75) with 12 mL of growth medium. Equilibrate the flasks for 30 minutes in a cell culture incubator at 37 ºC and 5 % CO_2.
2. Thaw a vial of cryopreserved hDFs, eTCs, eADSCs, low passage preferred (*see* **Note 8**) in a thermostatic bath at 37 ºC and seed the pre-equilibrated tissue culture flasks at 2,500 cells per cm^2 for the hDFs or 5,000 cells per cm^2 for the eTCs and eADSCs. Incubate the cells overnight at 37 ºC and 5 % CO_2 under humidified atmosphere and replace the medium with 12 mL of fresh growth medium per T75 flask. Replace the growth medium every 3 - 4 days.

3. Once the cell cultures have reached approximately 85 % confluency, discard the growth medium and wash the cell layers twice with 5 mL of 1× HBSS per T75 flask. Discard the HBSS and add 2 mL of trypsin-EDTA solution to each flask and incubate 5 minutes at 37 ºC. Assess cell detachment by microscopical examination and once all cells are detached from the flasks, add 4 mL of growth media, collect the cell suspension and centrifuge it for 5 minutes at 400*g*, 700*g* or 1200*g* for the hDFs, eADSCs, and eTCs, respectively. Discard the supernatant and resuspend the cell pellet in 1 mL of growth medium per every cultured T75. Determine the cell concentration with the aid of a haematocytometer or similar.
4. To plate the desired number of wells in 48-well tissue culture plates, adjust cell concentration in order to seed 25,000 cells per cm^2 for the hDFs and eTCs and 15,000 cells per cm^2 for the eADSCs and ensure that each well has 0.5 mL of growth medium and incubate the plates at 37 ºC and 5 % of CO_2. In order to appreciate the effects of MMC on ECM deposition, at least three wells are used per immunofluorescence marker to be analysed, and three wells without macromolecular crowder are used as control group. Plate the necessary number of wells per immunofluorescence assay in duplicate in order to have non-primary antibody controls in triplicate per condition.
5. After 24 hours of culture, examine the cells by microscopy and ensure that they are healthy and properly attached to the cell culture substrate before replacing the medium.
6. Replace the medium of the wells to be treated with MMC medium with 0.5 mL of the growth medium containing ascorbic acid and carrageenan or only ascorbic acid for control wells. Incubate the plates at 37 ºC and 5 % CO_2.
7. After 3 days of culture, the plates can be examined by microscopy and processed for immunofluorescent staining.

3.2 Immunofluorescent characterisation of the ECM

All the following steps must be performed at room temperature (unless otherwise stated).

1. At the end of every time point, aspirate the cell culture media of the corresponding plate and wash every well three times during 5 minutes with 500 μL of 1× sterile HBSS. Fix the samples with 150 μL/well of the filtered fixation solution pre-cooled at 4°C for 15 minutes (*see* **Note 9**).
2. Remove the fixation solution and wash the wells three times during 5 minutes with 250μL of 1× PBS. Samples can be kept at 4 ºC in 1x PBS if immunofluorescent staining is not to be performed right away.
3. Add 150 μL per well of BSA blocking solution for 30 minutes to block unspecific binding sites for the primary antibody.
4. After the aforementioned 30 minutes, drain the blocking solution and incubate the samples with 75 μL per well of the corresponding primary antibody solution for 90 minutes. For negative control wells, add 75 μL of 1× PBS per well (*see* **Note 10**).
5. Remove the primary antibody solution and wash the wells three times during 5 minutes with 250μL of 1× PBS (*see* **Note 11**). Incubate with 75 μL per well of the corresponding secondary antibody solution for 30 minutes. In this case, all the samples including the negative controls must be incubated with the corresponding secondary antibody solution (*see* **Note 12**). Protect the samples from light from here on.
6. Remove the secondary antibody solution and wash three times during 5 minutes with 250μL of 1x PBS.
7. Remove the PBS and incubate the samples 5 minutes with 75μL per well of 1× DAPI solution in order to stain nuclei.
8. Remove the DAPI solution and wash three times during 5 minutes with 250μL of 1× PBS.
9. Add 10 μL per well of VECTASHIELD® mounting media and place a cover slip in each well (*see* **Note 13**).
10. Analyse the samples by using ultraviolet excitation filter (e.g. Ex: 360-370 nm / Em: 420-460 nm) to visualize cell nuclei stained with DAPI, a blue excitation filter (e.g. Ex: 460-495 nm /

Em: 510-550 nm) to visualize collagen types III, IV, V, VI and fibronectin stained with AlexaFluor® 488 and finally, a green excitation filter (e.g. Ex: 535-555 nm / Em: 570-625 nm) to visualise collagen type I stained with AlexaFluor®555 (**Figure 1**).

3.3 Imaging and quantification of immunofluorescent staining

Perform the steps relative to the image acquisition process in dark, in order to prevent the loss of fluorescence intensity from the fluorophores as a consequence of light excitation.

1. Once the software has been launched, position the plate on the sample stage of the microscope and visualize a live preview from the camera input: be sure that the light path selector of the microscope is set on "camera" to view the image on screen.
2. It is pivotal to keep the same exposure time for all the samples examined for the same antigen (*see* **Note 14**). Adjust the exposure time for DAPI, and carefully adjust the focus on the cell layer. Select the correct filter to excite the fluorophore bound to the secondary antibody of interest, regulate the exposure time in order to avoid saturation, and take note of the longest exposure time void of saturation. Repeat these actions for all the samples, excluding the negative controls, then set the lowest obtained exposure time value to examine all of the samples and controls.
3. Position the plate on the sample stage exposing the first sample to the light path, select the violet filter and acquire a 20× magnified picture of the nuclei stained with DAPI. Without moving the sample, turn the filter block turret to the appropriated filter for the fluorophore bound to the secondary antibody of interest, regulate the focus and acquire the image. Repeat this step five times in randomly selected fields of the well for each replicate of each condition.
4. Using the emission filter for the secondary antibody of interest, acquire five pictures from five randomly selected fields of the non-primary antibody negative controls (*see* **Note 15**). Save all the acquired pictures in TIFF format before proceeding with the analysis of fluorescence intensity, which can be performed with the ImageJ software.

5. To proceed with the analysis, open the desired picture with the ImageJ software, open the "Analyze" menu, click on "Set measurement", tick "Mean grey value" option, then "OK". Open again the "Analyze" menu and this time click on "Measure": a results window will appear. Repeat this step for all the pictures of the replicate in examination and transfer the data to an Excel worksheet to further process them. Repeat this step for all the replicates of all the examined conditions, including the non-primary antibody negative controls.
6. By using Excel average function, obtain the average of the mean grey values for each replicate, including negative controls, and then subtract the negative control mean value to each corresponding replicate to obtain their specific fluorescence intensity (*see* **Note 16**).
7. Calculate the average and standard deviation of each condition from the values of the corresponding replicates for further statistical analysis and graphical representations (**Figure 2**).
8. To create a representative picture of the assay, open the desired image in ImageJ software together with the corresponding DAPI field. For both of the pictures, open the 'Image' menu, select 'Type' and click on'32-bit'. Once all the images that need to be merged in a single picture have been turned to 32-bit format, open the 'Image' menu, select 'Colour', then 'Merge channels': match each picture with the desired channel, tick the 'create composite' box and click 'Ok' to obtain the composite picture (*see* **Note 17**). Once the picture has been saved in the preferred format, it is possible to add a scale bar using the dedicated Image J tool (*see* **Note 18**).

4. Notes

1. The efficacy of UV-C disinfection depends on many parameters, as are the intensity and wavelength of the UV radiation, the time of exposure, the distance from the source of irradiation, the presence of particles that can protect the microorganisms from UV, and a microorganism's ability to withstand UV during its exposure. We currently disinfect the

carrageenan with success inside of an open 1.5 mL microcentrifuge tube in vertical position with the UV lamps typically included in the biological safety cabinets used for cell culture, but more dedicated equipment can be also used with the same efficacy if similar conditions are met.

2. Once the carrageenan is suspended into the medium, if the tube is vortexed and observed through a light source, particles in suspension can be appreciated. Once the carrageenan is completely dissolved into the medium, no particles can be appreciated following the same observation procedure. Is very important to ensure the completely dissolution of the carrageenan into the medium as non-dissolved particles can deposit on the bottom of the plates and negatively affect the cell viability.
3. Avoid temperatures over 60 °C (optimal are between 55 – 57 ºC), which result in methanol formation and the reduction of the effective concentration of PFA. This can damage the cytoskeleton of the cells and increase the auto-fluorescence of the samples.
4. For safety reasons, make the fixation solution under a chemical hood and cover the glass beaker, with aluminium foil for example, to prevent the release of PFA toxic vapours and the evaporation of the PFA. Likewise, all the steps that involve the use of the fixation solution must be executed under a chemical hood due to the PFA toxicity.
5. To perform the localisation of several molecules in the same sample by indirect immunofluorescent staining, it is required for the primary antibodies against the antigens of interest to be raised in different species in order to be able to be recognised by different specific secondary antibodies (e.g. the primary antibody anti-collagen type I has been raised in mouse and can be combined with any of the other primary antibodies raised in rabbit for their use in multicolour immunostaining).
6. For the selection of the secondary antibodies, several guidelines must be followed. First, the secondary antibody must be specific for the target species corresponding to the primary antibody (e.g. goat anti-mouse secondary antibodies for primary antibodies derived from mice

and goat anti-rabbit secondary antibodies for primary antibodies derived from rabbit). Moreover, to avoid cross-reactivity with the non-intended primary antibody targets in multicolour immunostaining and increase specificity, secondary antibodies cross-adsorbed against IgGs from the non-target species and sera from different species can be used (e.g. for the present study, we used goat anti-mouse secondary antibody cross-adsorbed against human serum and rabbit and goat IgGs, as well as goat anti-rabbit secondary antibody cross-adsorbed against mouse and goat IgGs). The selection of the fluorochromes conjugated with the secondary antibodies and the appropriated filter set it is crucial to have the minimal signal overlapping and maximal specificity in case of detecting several molecules at the same time in multicolour immunofluorescent stainings. For this, it is very important to excite each fluorochrome with wavelengths as close as possible to its maximal excitation, avoiding at the same time to excite the other fluorochromes present in the sample. Also, for collecting the fluorescence of each fluorochrome specifically, the emission filter should match as close as possible the maximal emission wavelength of the corresponding fluorochrome, avoiding at the same time the emission of the other fluorochromes present in the sample. As an example, the filter set distribution and fluorochromes selected for the present study is shown in **Figure 3**.

7. Protect the samples from the light to avoid the loss of fluorescent signal due to photobleaching. The photobleaching effect (also termed as fading) causes a permanent photochemical alteration, by cleaving covalent bonds in the fluorochromes. These irreversible modifications make the fluorochrome unable to emit fluorescence and thereby decrease the signal in the samples.
8. (a) The hDFs that can be acquired from ATCC are generally in early passages (p0 - p1). In order to expand them, before the preparation of the tissue surrogate, a cell density of 2,500 cells per cm^2 in growth medium was used. The subculture routine included 2 × 5 min washes with 5 mL of 1× HBSS, 2 mL of trypsin-EDTA solution for 5 minutes at 37 ºC, cell recovery

with 4 mL of growth medium per each T75 flask, centrifugation for 5 minutes at 400 *g*, resuspension, counting and plating at 2500 cells per cm^2. Normally, the cells reach confluency after a week of culture, and medium is changed twice per week, using 12 mL per T75. For cell cryopreservation, a cell density of 500,000 cells / mL of 10 % DMSO in FBS was used. (b) The eTCs used for this experiment were isolated by migration method from superficial digital flexor tendon of equine donors after surgical removal of the paratenon. Expansion in culture was performed using a seeding density of 5,000 cells per cm^2. Subculture was performed as for the case of the hDFs, except for the centrifugation of the cell suspension, which was performed at 1,200 *g*, and for a centrifugation step which was added in the thawing procedure in order to remove DMSO traces before plating. For cell cryopreservation, a cell density of 1,000,000 cells per ml of 10 % DMSO in FBS was used. (c) eADSCs were extracted by collagenase type I digestion from fat samples of the mane of 4 horses. eADSC subcultures were performed at 5,000 cells per cm^2 following the same procedure as for the hDFs, except for the centrifugation step at 700 *g* for 6 minutes. For cell cryopreservation, a cell density of 1.8-2 x 10^6 cells per mL of 10 % DMSO in FBS was used.

9. Be extremely careful during fixation and washing steps to prevent the detachment of the cell layer.
10. Alternatively, incubation with the primary antibody solutions can be performed overnight at 4 °C.
11. When working with a high number of experimental groups or samples, always proceed with the washes in a fractional way in order to avoid drying the samples. Drying may cause a background increase due to unspecific binding of the primary antibody to the sample.
12. In order to demonstrate the non-specific binding of the secondary antibodies to the sample, the non-primary antibody control wells should be incubated with the corresponding secondary antibody solution. For multicolour immunostaining, these wells should be incubated with a solution containing the mixed secondary antibodies as applied to the sample wells.

13. Be extremely careful when placing the cover slips. Introduce them vertically in one edge of the well and leave them fall slowly in order not to form bubbles and not damage the cell layer. Do not press them forcefully once properly positioned, as this can cause the loss of the sample.
14. The selection of an optimal exposure time suitable for all the examined samples, defined as the maximal exposure time at which the sample with the highest fluorescence intensity does not show signs of saturation, will allow for the comparison of the collected data between the different samples. The acquisition of an excessively saturated image would result in an underestimation of fluorescence intensity.
15. Background fluorescence can result from several factors, such as non-specific binding of the secondary antibodies, or auto-fluorescence from the culture plate, the cultured cells and from collagen itself. The quantification of non-primary antibody controls fluorescence is therefore performed in order to estimate the amount of this non-specific fluorescence, which will eventually be subtracted from the mean grey value measurements of the corresponding samples.
16. In order to proceed with further statistical analysis, values from each of the single replicates are needed. Therefore, subtract to the value of each replicate of the condition in analysis, the mean value obtained from all the three replicates of the corresponding non-primary antibody control.
17. It is possible to merge more than two pictures, or channels, in this step, in order to appreciate the relative localisation of the examined antigens. To reduce the background interference that can derive from non-specific binding of the antibodies as well as from autofluorescence of several biological molecules, or even from the plastic substrate, the ‘Subtract background’ Image J tool can be useful. To use it, right after the images have been turned to 32-bit format, open the ‘Process’ menu, click on ‘Subtract background’, select the same rolling ball radius for every picture in the assay and confirm with ‘OK’, then proceed with the merging of the

channels. Choose a larger rolling ball radius to maximise the preservation of positive pixels intensity, or a smaller one to maximise background subtraction.

18. There are several options to add a scale bar with ImageJ. If the pixel/length ratio is known, it is possible to open the 'Analyze' menu, select 'Set scale', type '1' in the 'Pixels' box and type the corresponding distance in the 'Known distance' box, then specify the unit of length and confirm with 'OK'. If the pixel/length ratio is not known, it is possible to use the 'Straight' tool from Image J toolbar in order to trace a segmented line covering a known distance in the picture, then open the 'Set scale' window and fill the 'Known distance' and 'unit of length' boxes, then confirm with 'OK'. A third option provides that a second picture, with the same pixel/length ratio of the former and already containing its scale bar is open and selected in Image J: in such case, it is only needed to tick the 'Global' box in the 'Set scale' window before clicking 'OK' to apply the same scale to all the open pictures. To finally add the scale bar to the desired picture, all the listed options provide that the 'Analyze' menu is open, in order to select 'Tools > Scale bar', personalize the scale bar and confirm with 'OK'.

5. Acknowledgements

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6. Figures

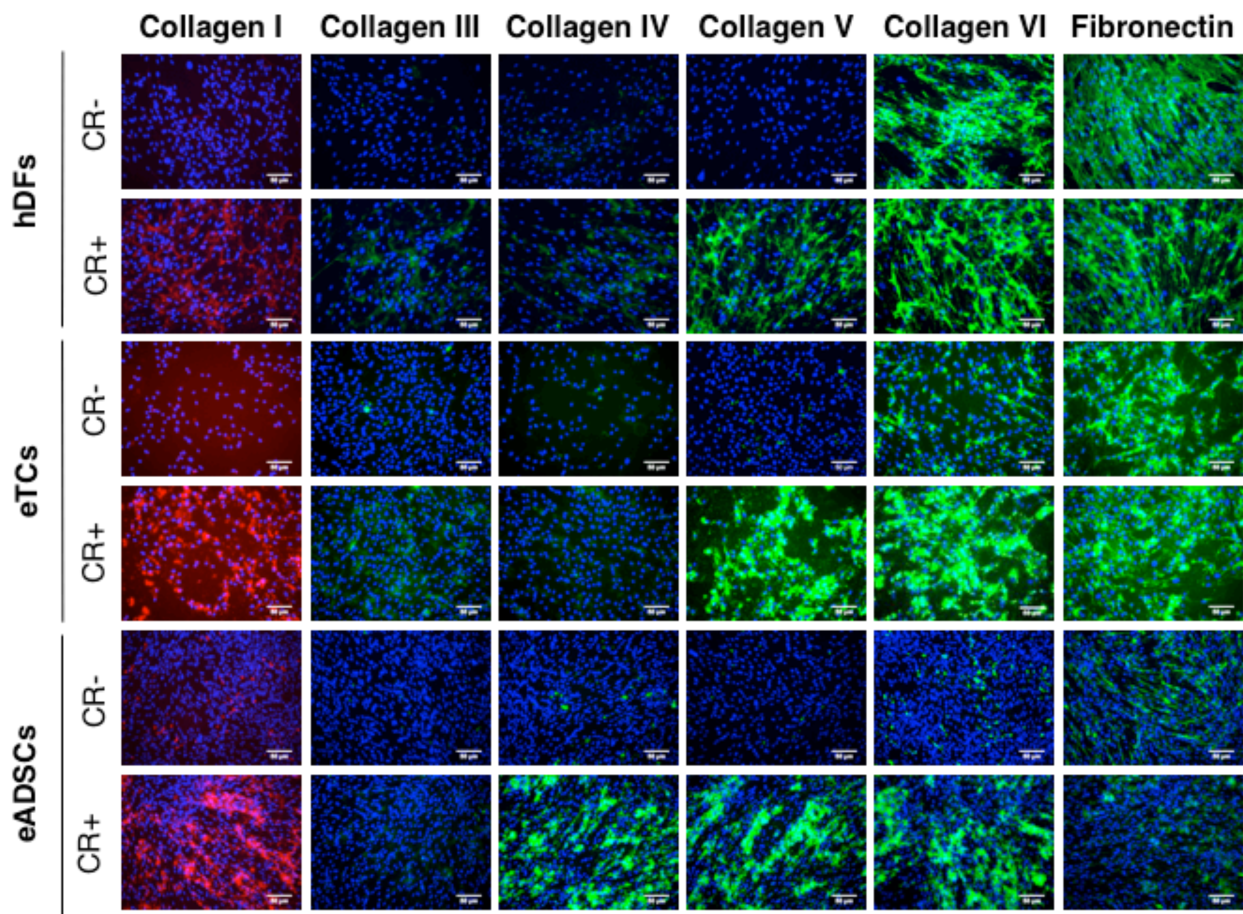

Figure 1: Immunofluorescent labelling of different extracellular matrix proteins in hDFs, eTCs and eADSCs after 3 days in the presence (CR+) or absence (CR-) of macromolecular crowding (carrageenan). Macromolecular crowding increases deposition of matrix proteins in a cell type-dependent fashion.

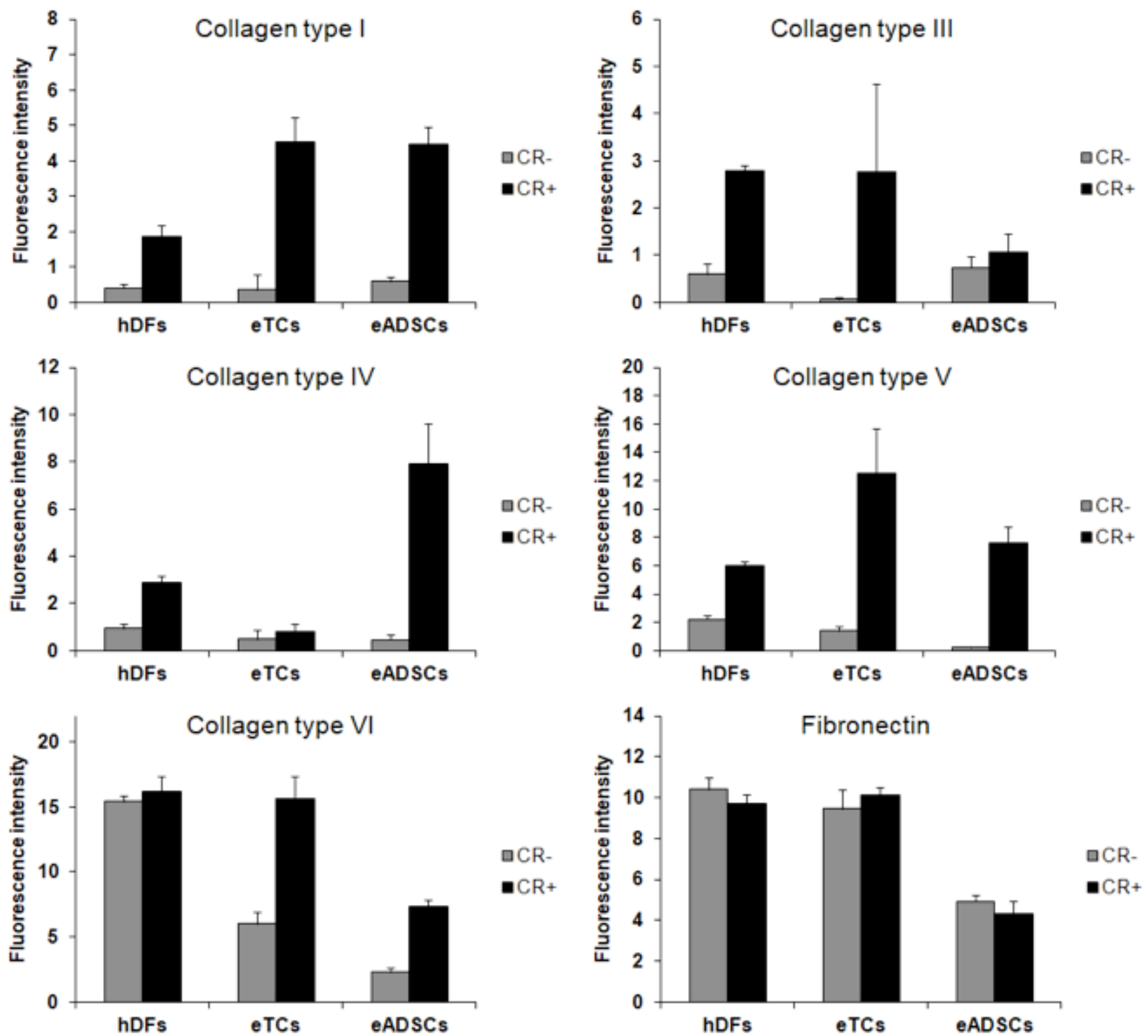

Figure 2: Fluorescence intensity analysis of different extracellular matrix proteins in hDFs, eTCs and eADSCs after 3 days in the presence (CR+) or absence (CR-) of macromolecular crowding (carrageenan). Fluorescence intensity provides an estimation of the relative amounts of the different extracellular matrix proteins produced by the different cell types in the presence or absence of macromolecular crowding.

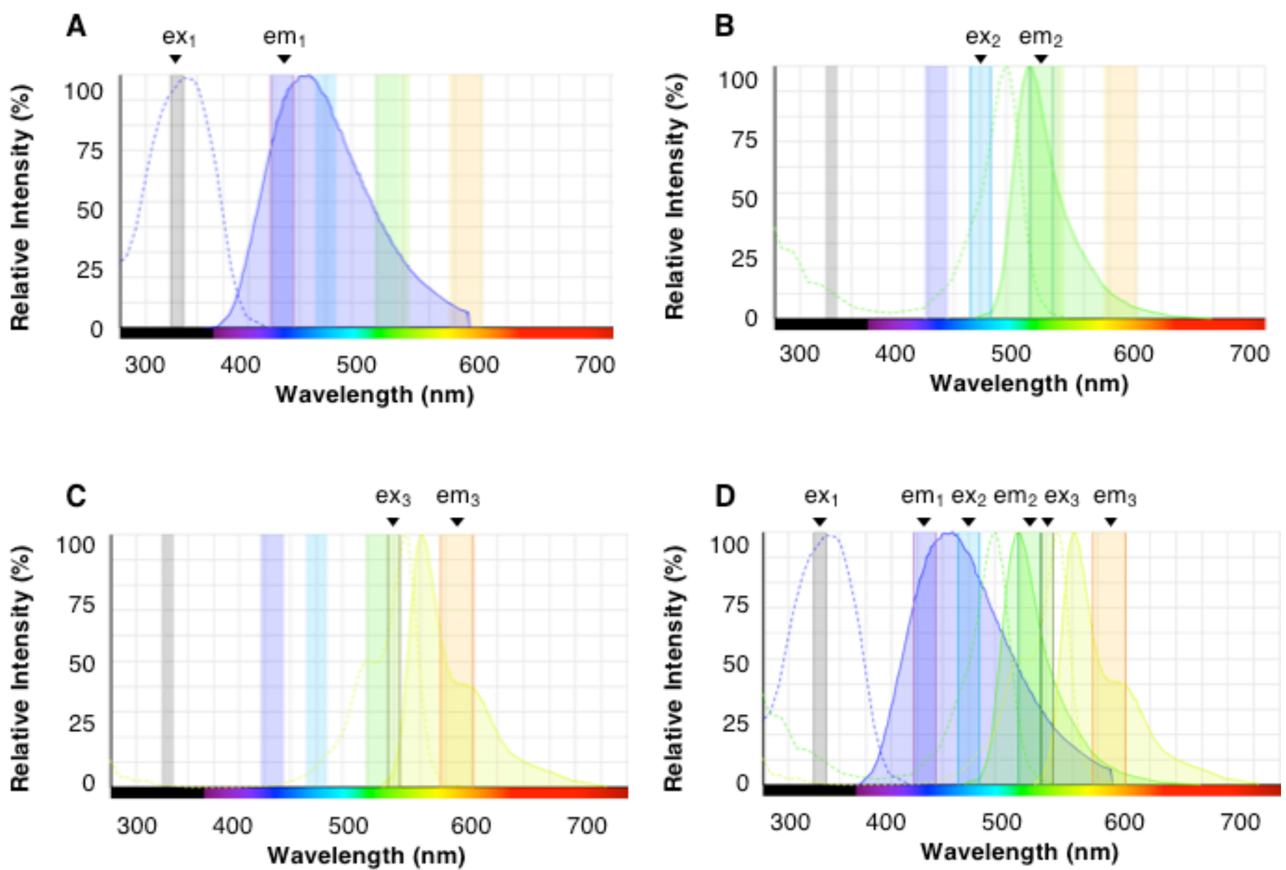

Figure 3: Fluorescence spectra of the different fluorochromes used and filter set distribution. Excitation (dashed line) and emission (continuous line) spectra and filter set used for DAPI (ex1: 350/10; em1: 440/20) (A), Alexa® 488 (ex2: 478/18; em2: 530/20) (B), Alexa® 555 (ex3: 545/10 and em3: 598/28) (C) and all the fluorochromes together (D). Minimal spectral overlapping can be appreciated when using the present filter set and fluorochromes selection.

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