The Scar-in-a-Jar: In Vitro Fibrosis Model for Anti-Fibrotic Drug Testing

Simon Stebler [1]

Michael Raghunath [1]✉

Email michael.raghunath@zhaw.ch

[1] Center for Cell Biology and Tissue Engineering, Institute of Chemistry and Biotechnology, Zurich University of Applied Sciences (ZHAW), Wädenswil, 8820, Switzerland

Abstract

Excessive deposition of type I collagen follows in the wake of chronic inflammation processes in dysregulated tissue healing and causes fibrosis that can ultimately lead to organ failure. While the *development* of antifibrotic drugs is targeting various upstream events in collagen matrix formation (synthesis, secretion, deposition, stabilization, remodeling), the *evaluation* of drug effects would use as net read-out of the above effects the presence of a deposited collagen matrix by activated cells, mainly myofibroblasts. Conventional methods comprise lengthy and labor-intensive protocols for the quantification of deposited collagen, some with sensitivity and/or specificity issues. Here we describe the Scar-in-a-Jar assay, an in vitro fibrosis model for anti-fibrotic drug testing that benefits from a substantially accelerated extracellular matrix deposition employing macromolecular crowding and a collagen-producing cell type of choice (e.g., lung fibroblasts like WI-38). The system can be aided by activating compounds such as transforming growth factor-β1, a classical inducer of the myofibroblast phenotype in fibroblasts. Direct image analysis of the well plate not only eliminates the need for matrix extraction or solubilization methods, but also allows for direct imaging and monitoring of phenotypical markers and offers the option for high-content screening applications when adapted to well formats compatible with a screening format.

This is the accepted version of a protocol published in Hinz; B.; Lagares; D. (eds) Myofibroblasts. Methods in Molecular Biology; vol 2299. The final authenticated version is available online at DOI: 10.1007/978-1-0716-1382-5_11

Key words:
Macromolecular crowding
Ultra-flat 3D
Collagen quantitation
Extracellular matrix
Drug discovery
High-content screening
Bioimaging
Immunocytochemistry

1. Introduction

During scarring, an ill-regulated process of wound healing, excessive amounts of extracellular matrix (ECM) (especially type I collagen) are deposited at the site of tissue injury. This process, when locally contained, will form a defined scar, but when perpetuated and spreading through an organ can destroy its microarchitecture, and ultimately its function. Anti-fibrotic drugs therefore aim to disrupt local or organ-wide collagen build-up at a transcriptional, translational, or posttranslational level. In the last decades various methods have been described to quantify the amount of deposited collagen and used in combination with in vitro and ex vivo fibrosis models that allowed researchers to assay for anti-fibrotic properties of drug candidates [1].

A classical method for the colorimetric quantification of collagen is the determination of 4-hydroxyproline, the abundant and unique signature amino acid of collagen (about 14%) [2]. Following total acid hydrolysis of dried and weighed tissue material 4-hydroxyproline is reacted with dimethylaminobenzaldehyde (Ehrlich's reagent) which yields a product that can be determined photometrically at an absorption maximum of $\lambda_{max} = 558 \pm 2$ nm [3]. Several refinements of this hydrolysis method have been introduced aiming at shorter processing times, increased sensitivity, and alternative detection methods such as HPLC or LC-MS [4, 5, 6]. Another popular method to quantify soluble and solubilized collagen is the Sircol Collagen Assay. The assay's central reagent is the anionic dye Sirius red [7]. The original method suffers from specificity issues as the dye binds to basic amino acids regardless of the source and must have led to many overestimations in the literature when performed in serum-containing material [8]. This can be remedied by an additional pepsin digestion and ultrafiltration step followed by the normal Sircol Collagen Assay protocol to yield accurate collagen concentrations [8]. Other quantitative analyses include MS [9], ELISA [10], SDS-PAGE in combination with densitometry [11] or image-

based analyses by confocal microscopy [12, 13, 14], two-photon excitation fluorescence and second and third harmonic generation microscopy [15, 16] that make use of the nonlinear optical property of fibrillar collagen.

The above described collagen quantification methods have been used in combination with various in vitro and ex vivo fibrosis models some of which are highlighted below. Emulating the formation of fibrous-like tissue with excessive ECM build-up potential was achieved in vitro using long-term fibroblast cultures, and allowing them to form multiple cell layers under stimulation with transforming growth factor-β1 (TGFβ1) [17], a known main driver of fibrosis [18]. Another approach for anti-fibrotic drug testing using kidney-derived cells in fact consists of two separate fibrosis models, as under stimulus of TGFβ1, human mesangial cells either formed monolayers (2D) or nodules (3D) solely depending on the growth substrate [19]. More recently, sophisticated examples for in vitro fibrosis models featured lung organoids derived from human embryonic stem cells with engineered gene mutations [20], or from induced pluripotent stem cells (iPSC)-derived human mesenchymal cells cultured in the interstitial spaces between collagen-functionalized hydrogel beads in the presence of TGFβ1 [21]. Biomechanical properties such as stiffening and contraction of progressive fibrotic tissue have been emulated using micropillars or rods that are deflected by contractile forces exerted by contiguous fibroblast layers (after TGFβ1 activation) covering these nano landscapes [22, 23]. Ex vivo precision cut lung slices considerably raise the complexity of the model as the lung tissue architecture of various cell types and ECM is greatly conserved [24]. Airways of healthy or diseased lung of human or animal origin are infused with agarose solution followed by subsequent sectioning into uniformly thick tissue slices (200–1000 μm) and placing in culture. Anti-fibrotic drugs may be tested directly on diseased or healthy lung slices after initiation of fibrosis incubating the tissue in a cocktail containing TGFβ1 [25, 26, 27]. Precision cut lung slices are widely used and considered a bridging element between in vitro and in vivo studies as they help to reduce animal sacrifices while delivering relevant data.

Here we describe the Scar-in-a-Jar, an in vitro fibrosis model that combines (a) a highly accelerated deposition of ECM by fibroblastic cells such as WI-38 lung fibroblasts through macromolecular crowding (MMC) culture conditions and simultaneous fibrotic stimuli such as TGFβ1 and (b) optical high-content screening to quantify deposited collagen circumventing the need to solubilize ECM and losing material along the way [28]. MMC, the addition of preferably

carbohydrate-based macromolecules (50–400 kDa) to culture medium, causes the excluded volume effect. This effect supports protein folding and enzymatic transition complex stabilization by limiting available extracellular space for biomolecule action. Collagen I deposition is boosted on three levels by macromolecular crowding. Firstly, by means of an accelerated conversion rate from procollagen I to collagen I, secondly, an enhanced supramolecular assembly of collagen triple helices to collagen fibers, and thirdly, by an increased stabilization of ECM components by lysyl oxidase- and transglutaminase-mediated cross-linking [29]. In fact, this “crowded state” creates an ECM microenvironment that resembles the in vivo situation more closely than the highly dilute aqueous standard cell culture condition. This is why MMC has been growing in popularity as research discovery tool to unmask processes or functions that may otherwise would have been overlooked [30]. In the Scar-in-a-Jar model, MMC allowed us to deposit significant amounts of ECM (6 day of MMC in a 7 day assay) while keeping the cell density at a subconfluent state [28]. This makes optimal collagen secretion and formation of an ultra-flat 3D model possible, while preventing multilayer formation as in the long-term fibroplasia model [17]. This in turn enabled optical high-content screening by software-assisted determination of immunofluorescent type I collagen area and normalization by a nuclear count [28]. The first adopter of the Scar-in-a-Jar after its publication in 2009 was the pharmaceutical industry, and the assay has since been improved toward medium-throughput (96-well) applications. The Scar-in-a-Jar has stood the test of time as relevant fibrosis model using lung fibroblasts from patients with idiopathic pulmonary fibrosis (IPF) for drug screening (adaptions to the method, *see* **Note 1**) [31]. Recently, an extended (12 day of MMC instead of 6 day) Scar-in-a-Jar assay has been suggested, in combination with clinically validated biochemical markers of ECM synthesis to better monitor long-term effects of anti-fibrotic drugs using ELISA to analyze culture media [32]. These biomarkers are the soluble C-propeptides of collagens I, III, IV–VI and they result from successful cleavage indicating matrix formation. C-propeptides of collagen III and VI have previously been reported to be increased in serum of advanced IPF patients [33]. Of note, α-SMA, a hallmark of myofibroblasts in fibrotic tissue, has recently been detected in serum of patients with IPF [34], and therefore was included in the immunochemical analyses, too. Interestingly, C-propeptides of collagens I, IV-VI were elevated in the first 4 day, while collagen III C-propeptide and α-SMA levels occurred only after 8 days and increased strongly toward day 12 [32]. While this shows that IPF pathology can be emulated in vitro, it also emphasizes the role of the

fourth dimension in assay design.

2. Materials

2.1. Cell Culture and Scar-in-a-Jar Procedure

1. Fibroblastic cells such as normal human lung fibroblasts (WI-38) (*see* **Note 2**).

2. Tissue culture plastic ware: 24-well plates, standard cell culture consumables.

3. Proliferation medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

4. Assay medium: DMEM supplemented with 0.5% FBS, 100 μM L-ascorbic acid 2-phosphate (*see* **Note 3**), a mixture of 37.5 $mg \cdot mL^{-1}$ Ficoll™ (Fc) 70 with 25 $mg \cdot mL^{-1}$ Fc 400 (*see* **Note 4**), 5 $ng \cdot mL^{-1}$ TGFβ1. This medium would contain any drugs used for fibrosis assessment (*see* **Note 1**).

5. Ciclopiroxolamine (CPX) solution (positive control, *see* **Note 5**): Prepare a stock of 0.5 M CPX in methanol, dilute to a working concentration of 1 mM CPX in DMEM (prior to cell culture addition) and add to cell culture at a final concentration of 8 μM.

6. Humidified incubator at 37 °C and 5% CO_2.

7. Potential anti-fibrotic drugs for assessment (*see* **Note 1**).

2.2. Immunocytochemistry

1. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in apyrogenic ultrapure water; pH 7.4.

2. Fixative: Methanol, ice-cold (*see* **Note 6**).

3. Blocking solution: 3% bovine serum albumin in PBS.

4. Antibodies and nuclei staining: Primary type I collagen and compatible secondary detection antibodies. For nuclei staining, 4′,6-diamidino-

2-phenylindoldilactate (DAPI) is suitable (*see* **Note 7**).

2.3. Optical High-Content Screening and Analysis of Data

1. Epifluorescence microscope, microscopic plate reader, or similar device equipped with appropriate filter sets (at least two, preferably one for DAPI) that allows fluorescence image acquisition (*see* **Note 8**).

2. Image analysis software that allows enumeration of nuclei and type I collagen area determination (*see* **Note 9**).

3. Methods

3.1. Cell Culture and Scar-in-a-Jar Procedure

1. Cultivate WI-38 in proliferation medium and passage cells (*see* **Note 10**) when still subconfluent.

2. Plate cells on 24-well plates (*see* **Note 11**) at 50,000 cells per well in 0.5–1 mL of proliferation medium. Plan to have enough experimental wells ready to hold all samples (including desired concentrations and solvent control "vehicle") plus positive control (8 μM CPX in DMEM) and incorporate at least two biological repeats ($n = 2$) per sample and control.

3. Incubate in humidified incubator at 37 °C and 5% CO_2 for 16 h.

4. Change to assay medium (1 mL per well) containing desired drugs (*see* **Note 1**) and controls.

5. Incubate in humidified incubator at 37 °C and 5% CO_2 for 6 day to ensure sufficient ECM deposition.

3.2. Immunocytochemistry

1. Fix cells with ice-cold methanol for 10 min at −20 °C.

2. Remove methanol and wash cells three times with PBS.

3. Add blocking solution and block at room temperature for 30 min.

4. Dilute type I collagen primary antibody in PBS, add to cells, and incubate at room temperature for 90 min.

5. Remove primary antibody and wash cells three times with PBS.

6. From here on work in a dark environment to minimize photobleaching.

7. Prepare a mixture of secondary antibody and DAPI in PBS.

8. Remove primary antibody solution from cells, do **not** wash.

9. Add secondary antibody and DAPI solution to cells and incubate at room temperature for 30 min.

10. Wash cells three times with PBS and leave 0.5 mL of PBS on top of the samples to prevent cells from getting dry.

11. Analyze samples immediately or store at 4 °C in the dark for not longer than 48 h prior to analysis.

3.3. Optical High-Content Screening and Analysis of Data

1. Take multiple images per well (*see* **Note 12**). Look for the strongest signal throughout all experimental samples and controls make sure to adjust exposure times for each channel (nuclei + collagen). Acquire all images using identical settings (*see* **Note 13**).

2. Export and store images in a way that allows analysis of channels individually.

3. Quantify total number of nuclei and corresponding type I collagen content per imaging site (*see* **Note 14**).

4. Normalize type I collagen content by number of nuclei to get area of type I collagen per nuclei per image field in μm^2. Determine mean of each well, calculate fold changes of relevant controls and do statistics on combined biological replicates.

4. Notes

1. GlaxoSmithKline have successfully implemented the Scar-in-a-Jar method to their anti-fibrotic drug discovery and made some updates [31] to our original method [28]: Patient-derived IPF fibroblasts were seeded into black-walled 96-well imaging plates at 10,000 cells per well in DMEM supplemented with 0.4% FBS and 4 mM L-glutamine and incubated for 24 h at 37 °C and 10% CO_2 to reach confluence. Medium was changed to assay medium supplemented with 0.4% FBS, 50 $\mu g \cdot mL^{-1}$ L-ascorbic acid and Ficoll™ crowders (as described above). Anti-fibrotic drugs or vehicle were pre-incubated for 3 h prior to the addition of 1 $ng \cdot mL^{-1}$ TGFβ1 into the medium. Treated fibroblasts were then cultured for 72 h at 37 °C and 10% CO_2. SB-525334 (ALK5 inhibitor) and CZ415 (mTOR inhibitor) were used as positive controls.

2. Initially, IMR-90 and WI-38 fibroblasts were assessed for their ability to deposit substantial amounts of collagen. In comparison, WI-38 showed a slower proliferation rate which was beneficial for our purpose to remain the cell culture subconfluent after 6 day assay duration.

3. The 2-phosphate derivate was previously found to be more stable than ascorbate [35]. We used it as the magnesium salt hexahydrate.

4. In our original publication [28] we further describe the use of a 500 kDa dextran sulfate molecular crowder at 100 $\mu g \cdot mL^{-1}$ as an alternative to Fc 70/400. The use of dextran sulfate even resulted in a quicker method (2 day instead of 6 day); however, type I collagen deposition was rather granular that may result in an under-appreciation of a possible drug effect in image analysis.

5. Since our publication in 2009 [28] the FDA has approved Pirfenidone and Nintedanib for the treatment of IPF in 2014. These drugs may be used as positive controls and benchmark compounds. Further examples of positive controls are described above (*see* **Note 1**).

6. In comparison to 4% paraformaldehyde and live staining, methanol fixation yielded the greatest quantified area of collagen.

7. We used mouse anti-human type I collagen monoclonal primary antibody from Sigma-Aldrich (St. Louis, MO, USA) and Alexa Fluor

594 goat anti-mouse secondary antibody as well as DAPI from Molecular Probes (Eugene, OR, USA).

8. Our data were generated using a Nikon TE600 fluorescence microscope equipped with a Xenon light source, an automated Ludl stage and a Photometrics CoolSNAP high-sensitivity camera.

9. To quantify nuclei and type I collagen content we used Metamorph® Imaging System software.

10. We routinely cultured at low passage (passages 3–8).

11. We found higher well-to-well variations when using 96-well format. Efforts to account for well-to-well variations and port the Scar-in-a-Jar assay to a 96-well plate format for medium-throughput has since been established [31].

12. We acquired nine separate images per well (3×3 grid) covering a total area of 1.35 cm^2 which represents approximately 70% of the 24-well growth area.

13. When using automated image acquisition, it becomes necessary to either turn on autofocus or to define a focus plane by setting focus points in DAPI channel.

14. Using the Metamorph® Imaging System software we accounted for positive DAPI signals when (a) the pixel intensity value of an area was 15 above that of the background and (b) long axis of nucleus measured between 10–15 μm. Suitable fluorescent intensity thresholds for type I collagen were defined according to intensity histograms while fluorescent signals below a defined pixel intensity value were discounted for (background). Corner images of the 3×3 grid were close to the well border. To circumvent illumination variances and interference with auto-fluorescent well borders, triangle-shaped masks (excludes image analysis) were added to these images to account for it. 15. The research group of Ruud Bank recently revisited the Scar-in-a-Jar sucessfully, and found additional value in using PVP40 as an alternative crowder to the Ficoll system that is worth exploring. [36]

References

1. Sundarakrishnan A, Chen Y, Black LD, Aldridge BB, Kaplan DL (2018) Engineered cell and tissue models of pulmonary fibrosis. Adv Drug Deliv Rev 129:78–94. https://doi.org/10.1016/j.addr.2017.12.013

2. Neuman RE, Logan MA (1950) The Determination of Hydroxyproline. J Biol Chem 184:299–306

3. Woessner JF (1961) The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447. https://doi.org/10.1016/0003-9861(61)90291-0

4. Hofman K, Hall B, Cleaver H, Marshall S (2011) High-throughput quantification of hydroxyproline for determination of collagen. Anal Biochem 417:289–291. https://doi.org/10.1016/j.ab.2011.06.019

5. da Silva CML, Spinelli E, Rodrigues SV (2015) Fast and sensitive collagen quantification by alkaline hydrolysis/hydroxyproline assay. Food Chem 173:619–623. https://doi.org/10.1016/j.foodchem.2014.10.073

6. Kindt E, Gueneva-Boucheva K, Rekhter MD, Humphries J, Hallak H (2003) Determination of hydroxyproline in plasma and tissue using electrospray mass spectrometry. J Pharm Biomed Anal 33:1081–1092. https://doi.org/10.1016/S0731-7085(03)00359-5

7. Junqueira LCU, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. Histochem J 11:447–455. https://doi.org/10.1007/BF01002772

8. Lareu RR, Zeugolis DI, Abu-Rub M, Pandit A, Raghunath M (2010) Essential modification of the Sircol Collagen Assay for the accurate quantification of collagen content in complex protein solutions. Acta Biomater 6:3146–3151. https://doi.org/10.1016/j.actbio.2010.02.004

9. Huizen NA, Ijzermans JNM, Burgers PC, Luider TM (2020) Collagen analysis with mass spectrometry. Mass Spectrom Rev 39(4):309–335. https://doi.org/10.1002/mas.21600

10. Seo W-Y, Kim J-H, Baek D-S, Kim S-J, Kang S, Yang WS, Song J-A, Lee M-S, Kim S, Kim Y-S (2017) Production of recombinant human procollagen type I C-terminal propeptide and establishment of a sandwich ELISA for quantification. Sci Rep 7:15946. https://doi.org/10.1038/s41598-017-16290-9

11. Lareu RR, Subramhanya KH, Peng Y, Benny P, Chen C, Wang Z, Rajagopalan R, Raghunath M (2007) Collagen matrix deposition is dramatically enhanced in vitro when crowded with charged macromolecules: the biological relevance of the excluded volume effect. FEBS Lett 581:2709–2714. https://doi.org/10.1016/j.febslet.2007.05.020

12. Clemons TD, Bradshaw M, Toshniwal P, Chaudhari N, Stevenson AW, Lynch J, Fear MW, Wood FM, Iyer KS (2018) Coherency image analysis to quantify collagen architecture: implications in scar assessment. RSC Adv 8:9661–9669. https://doi.org/10.1039/C7RA12693J

13. Bernstein DM, Toth B, Rogers RA, Kling DE, Kunzendorf P, Phillips JI, Ernst H (2020) Evaluation of the dose-response and fate in the lung and pleura of chrysotile-containing brake dust compared to TiO2, chrysotile, crocidolite or amosite asbestos in a 90-day quantitative inhalation toxicology study—Interim results Part 2: histopathological examination, Confocal microscopy and collagen quantification of the lung and pleural cavity. Toxicol Appl Pharmacol 387:114847. https://doi.org/10.1016/j.taap.2019.114847

14. Khorasani H, Zheng Z, Nguyen C, Zara J, Zhang X, Wang J, Ting K, Soo C (2011) A quantitative approach to scar analysis. Am J Pathol 178:621–628. https://doi.org/10.1016/j.ajpath.2010.10.019

15. Stoller P, Celliers PM, Reiser KM, Rubenchik AM (2003) Quantitative second-harmonic generation microscopy in collagen. Appl Opt 42:5209. https://doi.org/10.1364/AO.42.005209

16. Mostaço-Guidolin L, Rosin NL, Hackett T-L (2017) Imaging collagen in scar tissue: developments in second harmonic generation microscopy for biomedical applications. Int J Mol Sci 18:1772. https://doi.org/10.3390/ijms18081772

17. Clark RAF, McCoy GA, Folkvord JM, McPherson JM (1997) TGF-β1

stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasia: a fibronectin matrix-dependent event. J Cell Physiol 170:69–80. https://doi.org/10.1002/(SICI)1097-4652(199701)170:1<69::AID-JCP8>3.0.CO;2-J

18. Pohlers D, Brenmoehl J, Löffler I, Müller CK, Leipner C, Schultze-Mosgau S, Stallmach A, Kinne RW, Wolf G (2009) TGF-β and fibrosis in different organs—molecular pathway imprints. Biochim Biophys Acta BBA Mol Basis Dis 1792:746–756. https://doi.org/10.1016/j.bbadis.2009.06.004

19. Xu Q, Norman JT, Shrivastav S, Lucio-Cazana J, Kopp JB (2007) In vitro models of TGF-β-induced fibrosis suitable for high-throughput screening of antifibrotic agents. Am J Physiol-Ren Physiol 293:F631–F640. https://doi.org/10.1152/ajprenal.00379.2006

20. Strikoudis A, Cieślak A, Loffredo L, Chen Y-W, Patel N, Saqi A, Lederer DJ, Snoeck H-W (2019) Modeling of fibrotic lung disease using 3D organoids derived from human pluripotent stem cells. Cell Rep 27:3709–3723. e5. https://doi.org/10.1016/j.celrep.2019.05.077

21. Wilkinson DC, Alva-Ornelas JA, Sucre JMS, Vijayaraj P, Durra A, Richardson W, Jonas SJ, Paul MK, Karumbayaram S, Dunn B, Gomperts BN (2017) Development of a three-dimensional bioengineering technology to generate lung tissue for personalized disease modeling. Stem Cells Transl Med 6:622–633. https://doi.org/10.5966/sctm.2016-0192

22. Mastikhina O, Moon B-U, Williams K, Hatkar R, Gustafson D, Mourad O, Sun X, Koo M, Lam AYL, Sun Y, Fish JE, Young EWK, Nunes SS (2020) Human cardiac fibrosis-on-a-chip model recapitulates disease hallmarks and can serve as a platform for drug testing. Biomaterials 233:119741. https://doi.org/10.1016/j.biomaterials.2019.119741

23. Asmani M, Velumani S, Li Y, Wawrzyniak N, Hsia I, Chen Z, Hinz B, Zhao R (2018) Fibrotic microtissue array to predict anti-fibrosis drug efficacy. Nat Commun 9:2066. https://doi.org/10.1038/s41467-018-04336-z

24. Neuhaus V, Danov O, Konzok S, Obernolte H, Dehmel S, Braubach P,

Jonigk D, Fieguth H-G, Zardo P, Warnecke G, Martin C, Braun A, Sewald K (2018) Assessment of the cytotoxic and immunomodulatory effects of substances in human precision-cut lung slices. J Vis Exp 135:57042. https://doi.org/10.3791/57042

25. Cedilak M, Banjanac M, Belamarić D, Paravić Radičević A, Faraho I, Ilić K, Čužić S, Glojnarić I, Eraković Haber V, Bosnar M (2019) Precision-cut lung slices from bleomycin treated animals as a model for testing potential therapies for idiopathic pulmonary fibrosis. Pulm Pharmacol Ther 55:75–83. https://doi.org/10.1016/j.pupt.2019.02.005

26. Huang X, Li L, Ammar R, Zhang Y, Wang Y, Ravi K, Thompson J, Jarai G (2018) Molecular characterization of a precision-cut rat lung slice model for the evaluation of antifibrotic drugs. Am J Physiol Lung Cell Mol Physiol 316:L348–L357. https://doi.org/10.1152/ajplung.00339.2018

27. Lehmann M, Buhl L, Alsafadi HN, Klee S, Hermann S, Mutze K, Ota C, Lindner M, Behr J, Hilgendorff A, Wagner DE, Königshoff M (2018) Differential effects of Nintedanib and Pirfenidone on lung alveolar epithelial cell function in ex vivo murine and human lung tissue cultures of pulmonary fibrosis. Respir Res 19:175. https://doi.org/10.1186/s12931-018-0876-y

28. Chen C, Peng Y, Wang Z, Fish P, Kaar J, Koepsel R, Russell A, Lareu R, Raghunath M (2009) The scar-in-a-Jar: studying potential antifibrotic compounds from the epigenetic to extracellular level in a single well: scar-in-a-Jar to assess potential antifibrotics. Br J Pharmacol 158:1196–1209. https://doi.org/10.1111/j.1476-5381.2009.00387.x

29. Chen C, Loe F, Blocki A, Peng Y, Raghunath M (2011) Applying macromolecular crowding to enhance extracellular matrix deposition and its remodeling in vitro for tissue engineering and cell-based therapies. Adv Drug Deliv Rev 63:277–290. https://doi.org/10.1016/j.addr.2011.03.003

30. Benny P, Raghunath M (2017) Making microenvironments: a look into incorporating macromolecular crowding into in vitro experiments, to generate biomimetic microenvironments which are capable of directing cell function for tissue engineering applications. J Tissue Eng 8:2041731417730467. https://doi.org/10.1177/2041731417730467

31. Good RB, Eley JD, Gower E, Butt G, Blanchard AD, Fisher AJ, Nanthakumar CB (2019) A high content, phenotypic ‘scar-in-a-jar’ assay for rapid quantification of collagen fibrillogenesis using disease-derived pulmonary fibroblasts. BMC Biomed Eng 1:14. https://doi.org/10.1186/s42490-019-0014-z

32. Rønnow SR, Dabbagh RQ, Genovese F, Nanthakumar CB, Barrett VJ, Good RB, Brockbank S, Cruwys S, Jessen H, Sorensen GL, Karsdal MA, Leeming DJ, Sand JMB (2020) Prolonged Scar-in-a-Jar: an in vitro screening tool for anti-fibrotic therapies using biomarkers of extracellular matrix synthesis. Respir Res 21:108. https://doi.org/10.1186/s12931-020-01369-1

33. Organ LA, Duggan A-MR, Oballa E, Taggart SC, Simpson JK, Kang’ombe AR, Braybrooke R, Molyneaux PL, North B, Karkera Y, Leeming DJ, Karsdal MA, Nanthakumar CB, Fahy WA, Marshall RP, Jenkins RG, Maher TM (2019) Biomarkers of collagen synthesis predict progression in the PROFILE idiopathic pulmonary fibrosis cohort. Respir Res 20:148. https://doi.org/10.1186/s12931-019-1118-7

34. Holm Nielsen S, Willumsen N, Leeming DJ, Daniels SJ, Brix S, Karsdal MA, Genovese F, Nielsen MJ (2019) Serological assessment of activated fibroblasts by alpha-smooth muscle actin (α-SMA): a noninvasive biomarker of activated fibroblasts in lung disorders. Transl Oncol 12:368–374. https://doi.org/10.1016/j.tranon.2018.11.004

35. Hata R-I, Senoo H (1989) L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol 138:8–16. https://doi.org/10.1002/jcp.1041380103

36. Puerta Cavanzo N, Bigaeva E, Boersema M, Olinga P, Bank RA. Macromolecular Crowding as a Tool to Screen Anti-fibrotic Drugs: The Scar-in-a-Jar System Revisited. Front Med (Lausanne). 2021 Jan 14;7:615774. doi: 10.3389/fmed.2020.615774. PMID: 33521022; PMCID: PMC7841046.