

Supplementary information

Porous yet Dense (PyD) matrices: using ice to shape collagen 3D
cell culture systems with increased physiological relevance

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MATERIALS AND METHODS

Ice templating and topotactic fibrillogenesis

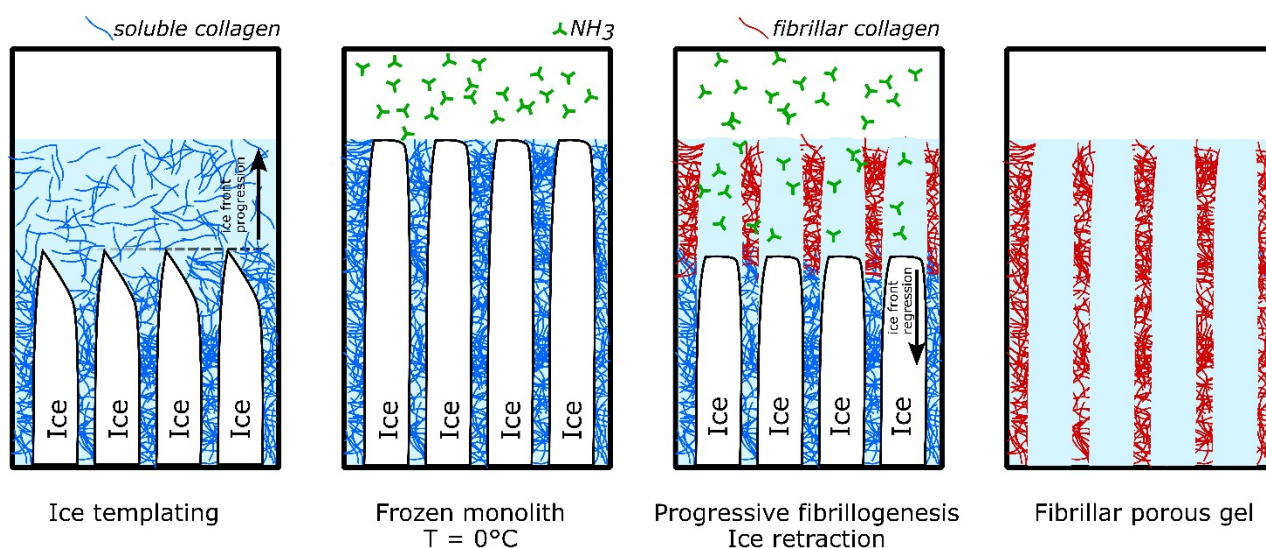


Figure S1. Sequential steps of ice templating and topotactic fibrillogenesis are required for the preparation of PyD matrices. After freezing, samples are exposed to ammonia vapours at 0°C until complete thawing of ice crystals. Simultaneously with the ice thawing due to cryoscopic depression effect of ammonia, the local pH increases in the areas where the ice has melted, leading to the fibrillogenesis of collagen walls.

Hydroxyproline titration

Collagen solutions concentration was estimated using a modified protocol of the previously described hydroxyproline assay.¹ Briefly, $50\ \mu\text{L}$ of the extracted collagen solution or $100\ \mu\text{L}$ of the supernatants of the collagenase degradation solutions at each time point were hydrolysed overnight with an equal volume of 6 M hydrochloric acid at 106°C . The samples were then dried at 106°C and the residues were resuspended with 1 mL of water. 0.2 mL of freshly prepared chloramine T in citrate buffer solution were added and samples were left at room temperature for 20 min. 0.2 mL of perchloric acid were subsequently added and hydroxyproline was oxidised at room temperature for 5 min. Next, 0.2 mL of freshly prepared 4-(dimethylamino)benzaldehyde (DMBA) solution in acetone were added and the samples were incubated in a water bath at 60°C for 20 min. Following the incubation, the samples were left at room temperature for 20 min, 0.2 mL of each sample were transferred to a 96-well plate and the absorbance was read at 557 nm (Varioskan LUX, Thermo Scientific). A reference curve was also prepared for each experiment using hydroxyproline standards.

Image analysis

For all acquired z-stacks and tile scans, the channels were split and appropriate regions of interest (ROI) were defined for the subsequent analyses. Standard processing and noise reduction treatment (Despeckle, Remove outliers, Subtract background, Thresholding) and filtering were used based on the intended analysis.

Pore size measurements (Analyze Particles)

SEM images of porous scaffolds surfaces were binarized after being processed with threshold and noise removal. Particle analysis was used to measure Feret's diameter of the pores in each image. Pores size was determined through MinFeret values, the minimum calliper diameter.

Nuclei position (TO-PRO™-3 staining) – 2D (StarDist plugin)

Before applying a Z-projection on the TO-PRO™-3 channel, the z-stack was pre-processed for noise reduction followed by Thresholding. The StarDist plugin² was applied on this Z-projection, set on Versatile (fluorescent nuclei) model and on default parameters to normalise the image. Several trials were performed adjusting the NMS (non-maximum suppression) settings so to obtain the best possible segmentation for the objects (nuclei). The output images were cross-checked with the z-stacks for nuclei and F-actin labelling to exclude artefacts. The selected produced image was further treated with the 3D manager e.g., to erase objects that corresponded to artefacts. The ROI set produced by the StarDist plugin was corrected based on the final image and measurements of the nuclei were performed.

Minimum distance: cells-scaffold's surface – 2D (Macro Reference Distances)

Using this macro³ the minimum (and maximum) distances for all objects compared to a reference file of XY coordinates can be determined. A number of new columns depending on the selected settings (e.g. minimum and maximum distances to the reference set, angle of minimum distance direction etc.) is added to the results table produced by "Measure" on ROI Manager. This analysis was performed on the final StarDist images for PyD and NP-col scaffolds only.

A freehand line on the sample surface was drawn on an adjusted for brightness and contrast Z-projected image of the F-actin channel. XY coordinates of this ROI were saved according to the step-by-step instructions of the macro, and served as the reference file. After obtaining the Results table with the measurements from the ROI manager, the macro was run and on the final StarDist image the lines showing the calculated minimum distances from the nuclei centroids to the sample surface were drawn. The calculated minimum distances were further corrected if needed based on the position of the nuclei

regarding the samples surface. For instance, for a calculated distance of 3.67 μm for a nucleus that was above the sample's surface (the "0" reference), the corrected distance was -3.67 μm .

Objects counting – 3D

In this analysis the objective was to obtain measurements for isolated 3D objects of interest, namely the labelled nuclei and actin cytoskeletons, retaining as much as possible their original structure after image segmentation.

Nuclei (TO-PRO™-3 staining) – 3D (TrackMate plugin with StarDist detector)

The TO-PRO™-3 channel z-stack was processed for noise reduction, thresholded, and filtered with Gaussian Blur 3D. The TrackMate plugin⁴ was applied on the processed z-stack using the StarDist detector² and LAP tracker for image segmentation following the corresponding instructions.⁵ The obtained segmented Z-stack was added in 3D Manager excluding objects on XYZ edges. The labelled objects were exhaustively verified and treated with Delete, Split, and Merge options as needed to correct discrepancies between the original and the segmented z-stack. In case an object, even after extensive efforts for corrections, was not corresponding to the original z-stack, it was excluded from the analysis. "Measure 3D" was applied to the final objects list.

F-actin (phalloidin staining) – 3D (TrackMate plugin with Thresholding detector)

The F-actin channel z-stack was processed for noise reduction. The TrackMate plugin was applied on the processed z-stack defining an appropriate threshold value for the Thresholding detector, and using LAP Tracker for image segmentation. The obtained segmented Z-stack was added in 3D Manager excluding objects on XYZ edges. As for the labelled nuclei, exhaustive verification and corrections took place on the labelled cytoskeletons and all objects not corresponding to the original z-stack, were excluded from the analysis. "Measure 3D" was applied to the final objects list.

Proliferating cells position (Ki67 staining) – 2D (StarDist plugin)

The same procedure as described above for the TO-PRO™-3 channel was followed for the Ki67 z-stack. The StarDist plugin was applied on a processed Z-projection of the Ki67 channel and the final segmented image was obtained after the corrections needed. The Results table was also produced as before.

Minimum distance: proliferating cells-scaffold's surface – 2D (Macro Reference Distances)

The same procedure as described above for the TO-PRO™-3 channel was followed for the Ki67 z-stack. The Reference Distances macro was applied on the corrected segmented image from StarDist

plugin based on the Ki67 labelling. A final image showing the minimum distances was obtained along with the extended Results table. Again, the calculated minimum distances were further corrected if needed based on the position of the nuclei regarding the samples surface.

Colocalization (TO-PRO™-3 – Ki67 staining) – 2D

Before applying Z-projections on the TO-PRO™-3 and Ki67 channels, z-stacks were pre-processed for noise reduction. Z-projections were subjected to Enhance Contrast (saturated pixels 0,3% - Normalize). The final ROI set obtained from the StarDist plugin for the nuclei labelling was applied on the enhanced projections and these areas only were taken into account for the colocalization analysis.

Colocalization Colormap

The Colocalization Colormap plugin⁶ was used to obtain a quantitative visualisation of colocalization between processed Z-projections of the TO-PRO™-3 and Ki67 channels.. The Z-projection for the nuclei labelling was always set as “Channel 1” while the Ki67 projection as “Channel 2”. A manual threshold was set on the two images to include all nuclei pixels and the analysis resulted to the corresponding colormap and a correlation index for each examined sample.

RESULTS

Materials for 3D cell culture

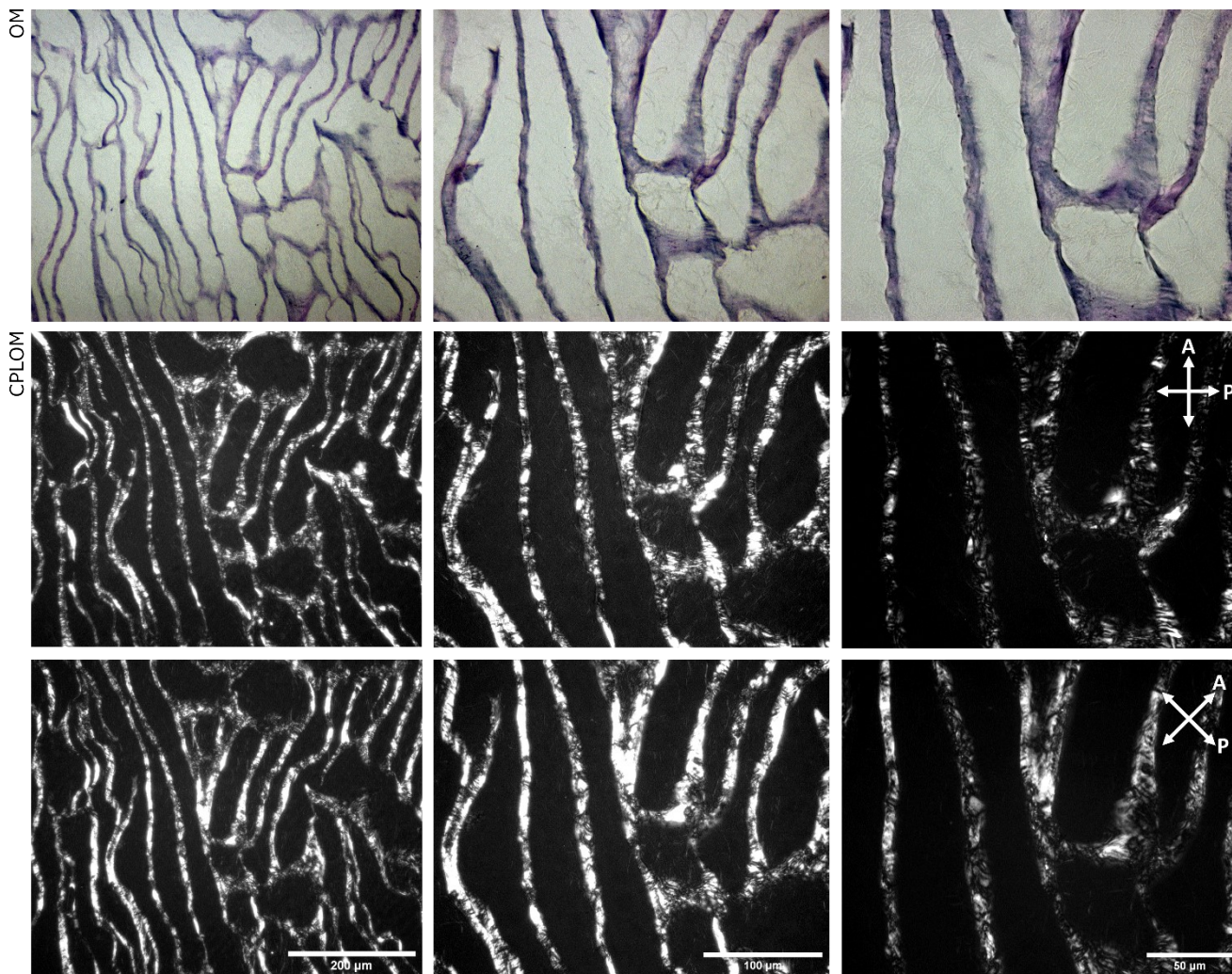


Figure S2. Microscopic characterisation of the PyD collagen matrices at different magnifications. Top row: Optical microscopy images (haematoxylin staining). Middle and bottom rows CPLM images of the same sections at 0 and 45° between polarizer and horizontal axis, respectively.

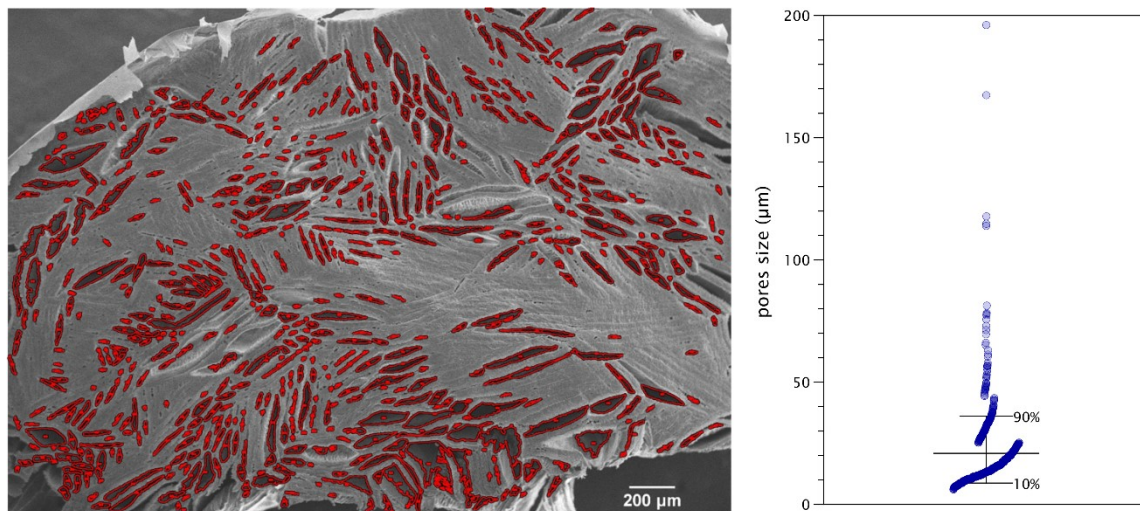


Figure S3. Pore size quantification by Feret minimum diameter.

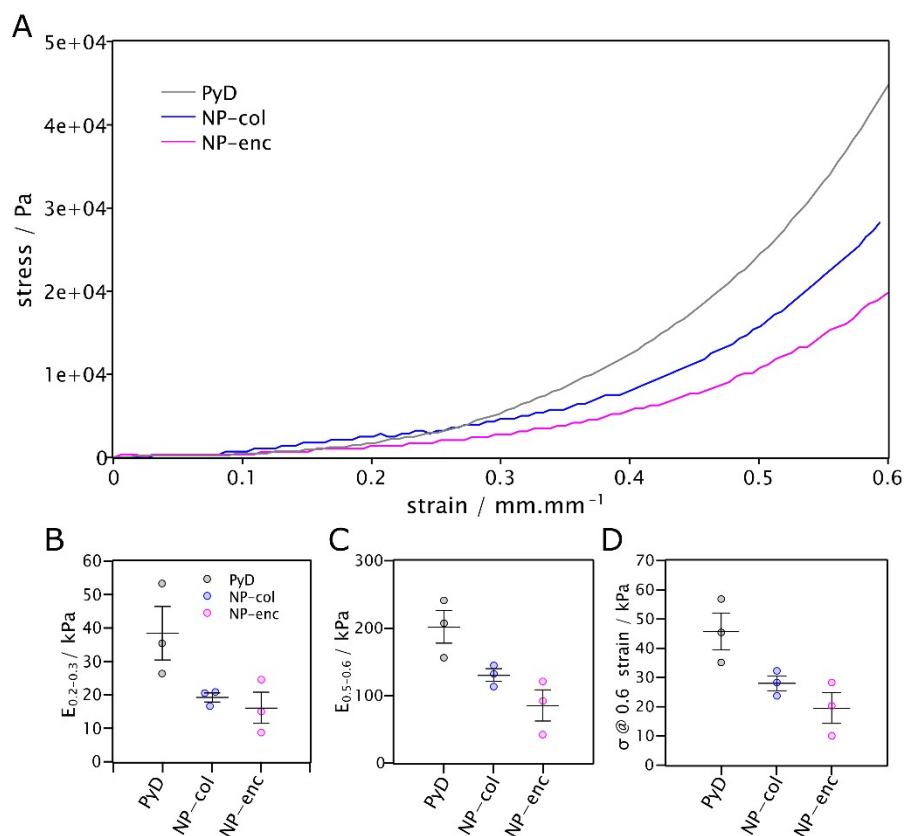


Figure S4. Mechanical properties of type I collagen materials. A) Stress-strain profiles of PyD, NP-col and NP-enc samples obtained in compression. B) Scatter plot of elastic moduli at strain levels $0.2 < \epsilon < 0.3$. C) Scatter plot of elastic moduli at strain levels $0.5 < \epsilon < 0.6$. D) Scatter plot of maximum stress at $\epsilon = 0.6$.

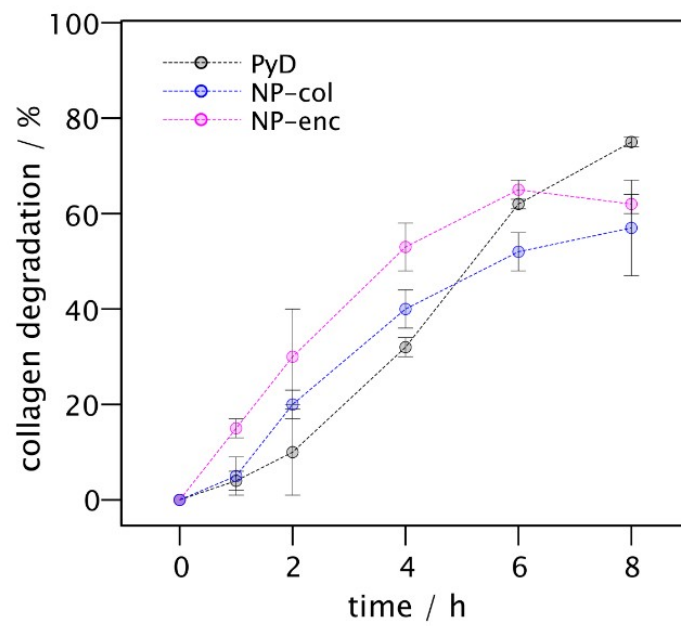


Figure S5. Type I collagen materials degradation kinetics in presence of collagenase. The mean degradation percentage \pm SD is shown for each time point for the 3 types of materials.

Proliferative status of seeded/encapsulated fibroblasts and colocalization analysis

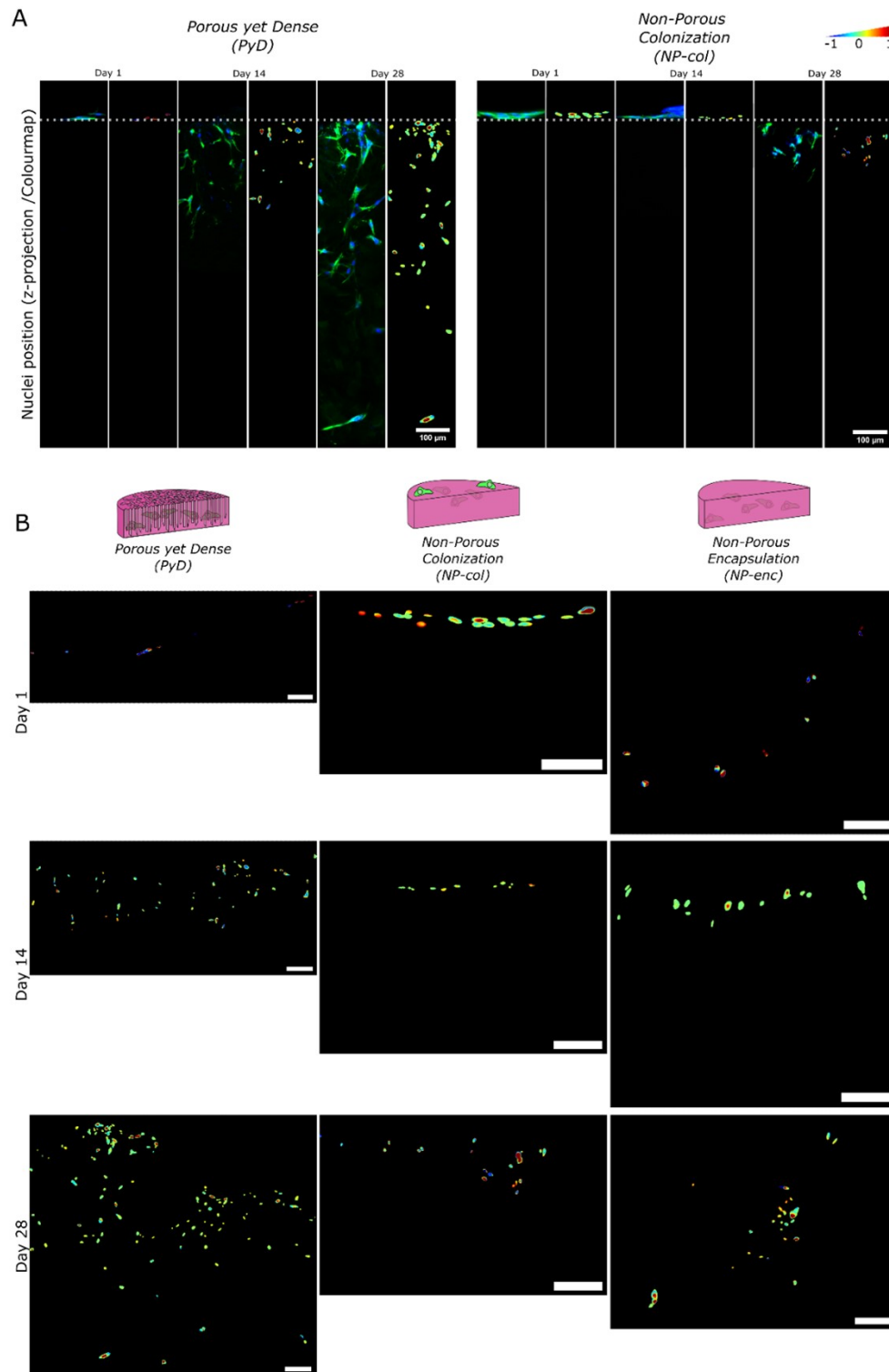


Figure S6. A) ROI of Z projections at days 1, 14, and 28 of confocal microscopy images (left column for each day. Nuclei: blue, TO-PRO™-3 / F-actin: green, phalloidin) and their corresponding colormaps with colocalization analysis of TO-PRO™-3 and Ki67 labelling as shown in Fig. 4 (right side). B) Colocalization colormaps of the processed Z-projections of confocal microscopy images for the TO-PRO™-3 and Ki67 channels. Scale bars: 100 μ m.

Table S1. Colocalization index values for the colormaps presented in Fig. S5.

| | PyD | NP-col | NP-enc |
|--------|--------|--------|--------|
| Day 1 | 0.4778 | 0.6790 | 0.6195 |
| Day 14 | 0.5735 | 0.8065 | 0.6987 |
| Day 28 | 0.6919 | 0.5935 | 0.6741 |

Morphometric analysis of the seeded/encapsulated fibroblasts

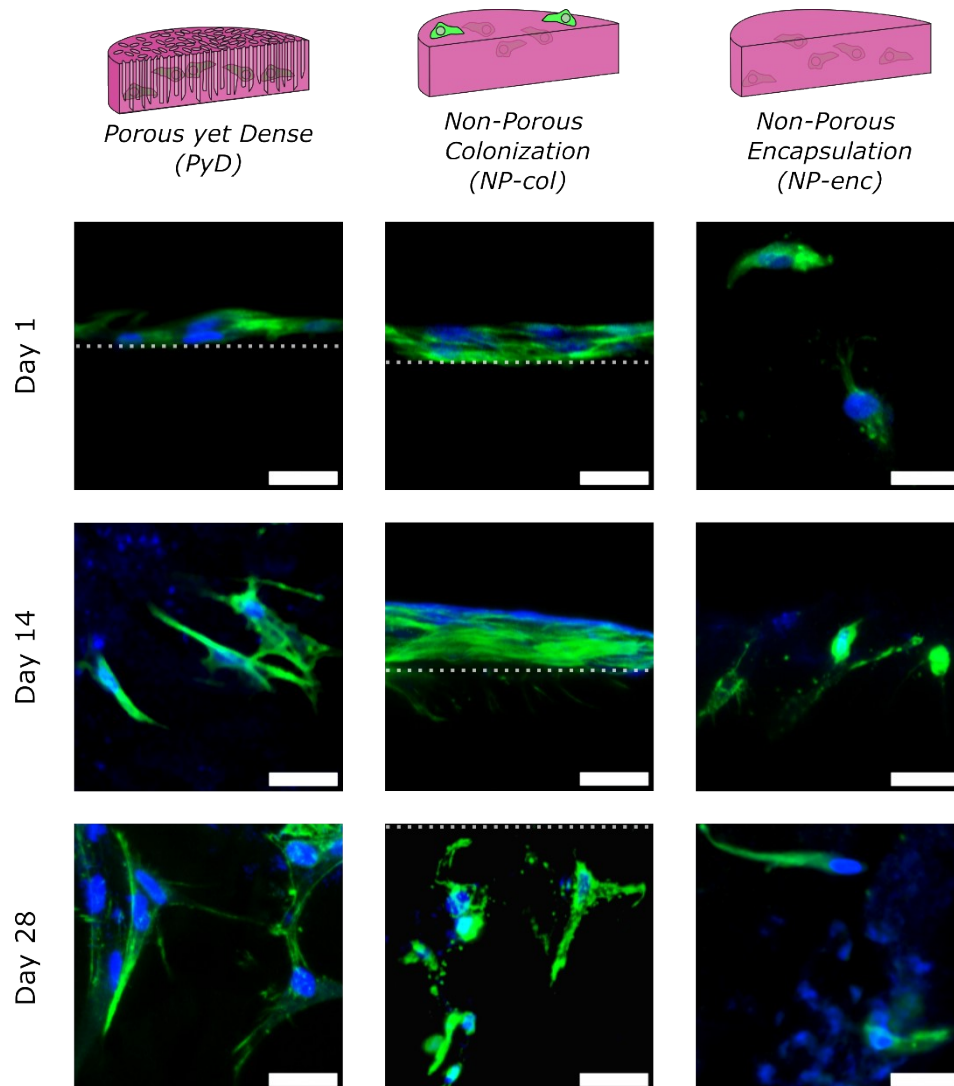


Figure S7. Z-projections of composite confocal microscopy images showing representative examples of NHDFs in the examined samples at the different time points. The dotted lines represent schematically the top surface of the materials. Nuclei: blue (TO-PRO™-3), F-actin: green (phalloidin) Scale bars: 30 μm .

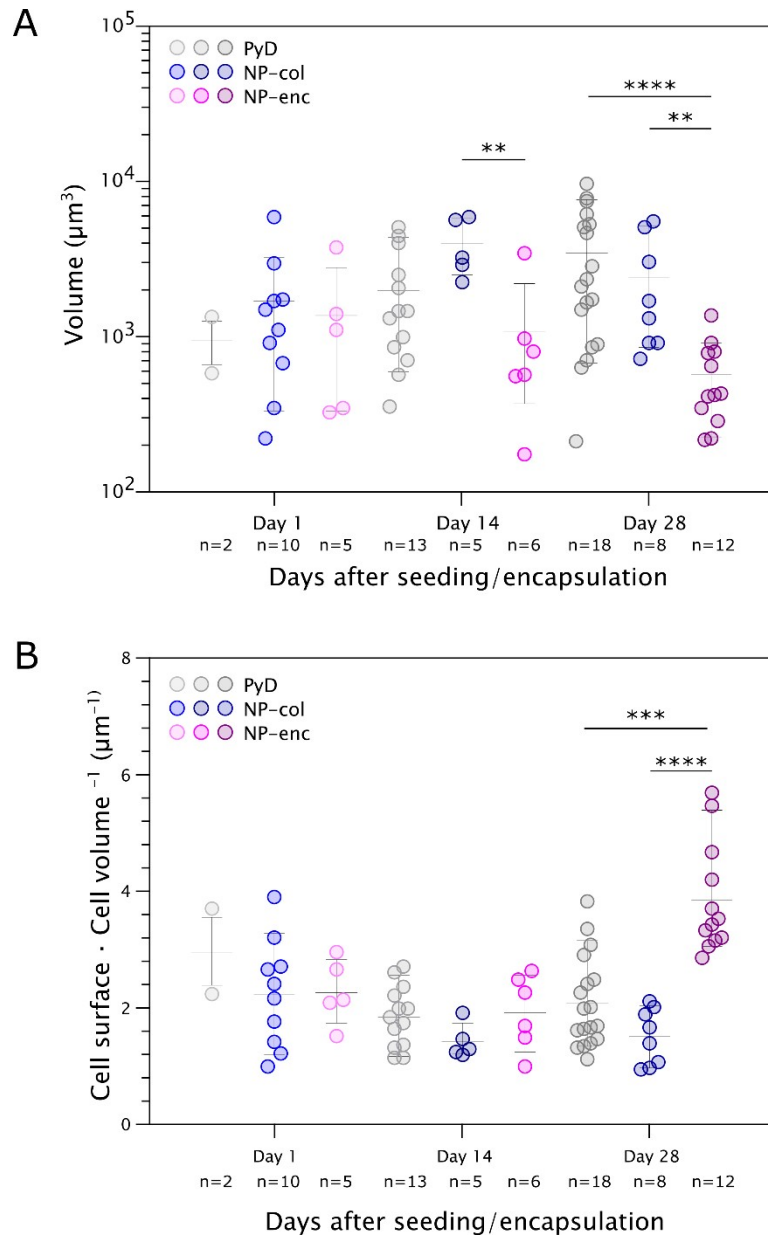


Figure S8. 3D morphometric analysis at the cytoskeletal level of fibroblasts colonising PyD and NP collagen materials, based on actin labelling. Scatter plots (mean \pm interdecile range plotted) of A) the cellular volume and B) cell surface / cell volume ratio measurements of NHDFs at days 1, 14, and 28 after seeding (n = the number of identified cells based on actin labelling using TrackMate plugin with Thresholding detector). Statistically significant differences between different materials for the same time points are shown (**p < 0.0021, ***p < 0.0002, ****p < 0.0001).

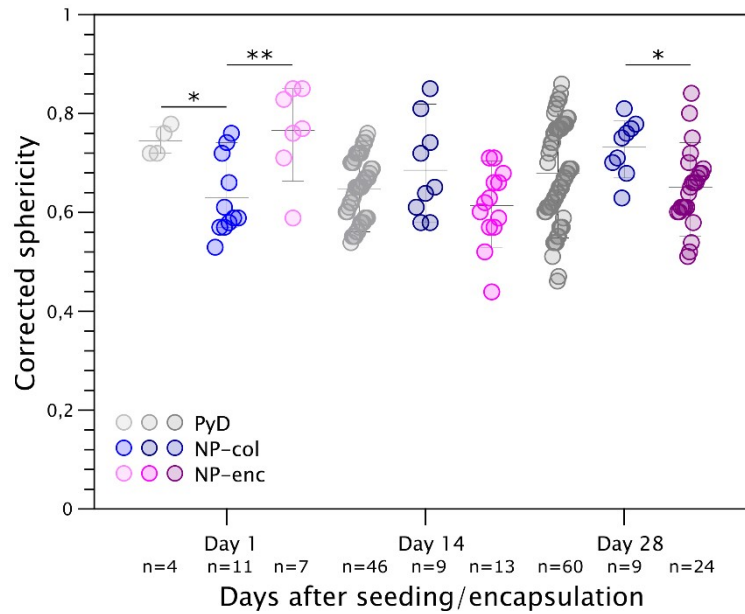


Figure S9. 3D morphometric analysis at the nuclear level of fibroblasts colonising PyD and NP collagen materials, based on TO-PRO™-3 labelling. Scatter plot (mean \pm interdecile range plotted) of the corrected sphericity measurements of NHDFs nuclei at days 1, 14, and 28 after seeding (n = the number of identified nuclei based on TO-PRO™-3 staining using TrackMate plugin with StarDist detector). Statistically significant differences between different materials for the same time points are shown (* $p < 0.0332$, ** $p < 0.0021$).

NOTES AND REFERENCES

- 1 J. F. Woessner, *Arch. Biochem. Biophys.*, 1961, **93**, 440–447.
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- 4 D. Ershov, M.-S. Phan, J. W. Pylvänäinen, S. U. Rigaud, L. Le Blanc, A. Charles-Orszag, J. R. W. Conway, R. F. Laine, N. H. Roy, D. Bonazzi, G. Duménil, G. Jacquemet and J.-Y. Tinevez, *bioRxiv*, 2021, 2021.09.03.458852.
- 5 J.-Y. Tinevez, <https://imagej.net/plugins/trackmate/trackmate-stardist>.
- 6 A. Gorlewicz, <https://github.com/AdamGorlewicz/Colocalization-colormap>.