Fiber-based fluorescence lifetime imaging of recellularization processes on vascular tissue constructs

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Supporting Information (jbio.201700391)

1. Instrument design and FLIm data pre-processing

The instrument used to acquire fluorescence lifetime images (figure S1a) has a wavelength selection module that separates the sample's autofluorescence in four spectral bands (SB). The bands were chosen to approximately match the spectral emission properties of collagen, with emission peak at $\sim$ 390 nm [1–5], nicotinamide adenine dinucleotide (NADH), in its free and protein-bound state, with emission peaks at $\sim$ 440 and 450 nm, respectively, and flavin adenine dinucleotide (FAD), with an emission peak at $\sim$ 540 nm [1, 5]. Figure S1b shows the normalized emission spectra of the pure fluorophore solutions acquired with a time resolved fluorescence spectrometer, together with color-coded spectral bands of the wavelength selection module in the FLIm setup. The preparation of the fluorophore solution was done following [6]. Briefly, free NADH solution consisted of 250 μM NADH in 0.1 M MOPS buffer. Bound NADH solution was made with 250 μM NADH mixed 1:1 in volume with 1000 unit/ml lactate dehydrogenase (LDH, Sigma no. L3916). FAD (Sigma n.F6625) was diluted at 2 mg/ml in DI water at pH 7.4.

2. Image post-processing

EGFP labeled cells for both hAEC and hMSC were used to validate the FLIm results, in terms of cell presence and progression on the scaffolds. Steady-state fluorescence microscopy was used to image the entire scaffolds on each imaging day. The images were further processed to quantify the cellular area on the scaffold, Figure 2Sa shows a representative image of hAEC on the fibrous side of AR-BP on day 1 after seeding on its raw form (i), as a 16-bit converted gray scale image (ii), and after applying Otsu segmentation to threshold the cells out of the scaffold (iii). the cell area was quantified as the number of white pixels time the pixel size (14.3 μm^2). Figure S2-vi and -v show the cellular area over time for both cell types seeded on both sides on the scaffold.

Antigen removed bovine pericardium changes its fluorescence properties when cultured in cell media, or PBS. Therefore the fluorescence lifetime of the scaffold, which constitutes the background for cellular detection, shortens over time. To overrule possible effects of the changing background, and enhance the effect of the cellular presence, fluorescence lifetime images were normalized to the mean fluorescence lifetime on the area of cell seeding. Figure S2b-i shows the fluorescence lifetime map in SB1 of a representative piece of AR-BP with seeded hAEC. The fluorescence lifetime of the scaffold decreases over days in culture (from 1 to 7), and because it is the dominant contributor to the signal, the lifetime values of the area with seeded cells also shortens. A circular region of interest of $<$ 4 mm in diameter (seeding side had a diameter of 4 mm) was selected within the seeding area (white dashed circles in figure S2b-i), and its mean fluorescence lifetime was measured. The image was then divided by that mean fluorescence lifetime to obtain the normalized images shown in figure S2b-ii, which have a relative lifetime color scale. Relative lifetime equal 1.00 indicates regions on the scaffold with affected by the presence of cells. On these images, the cell progression area was estimated by contouring an ellipse around the area of relative lifetime equal to 1.00 $\pm$ 1% (black circles in figure S2b-ii).

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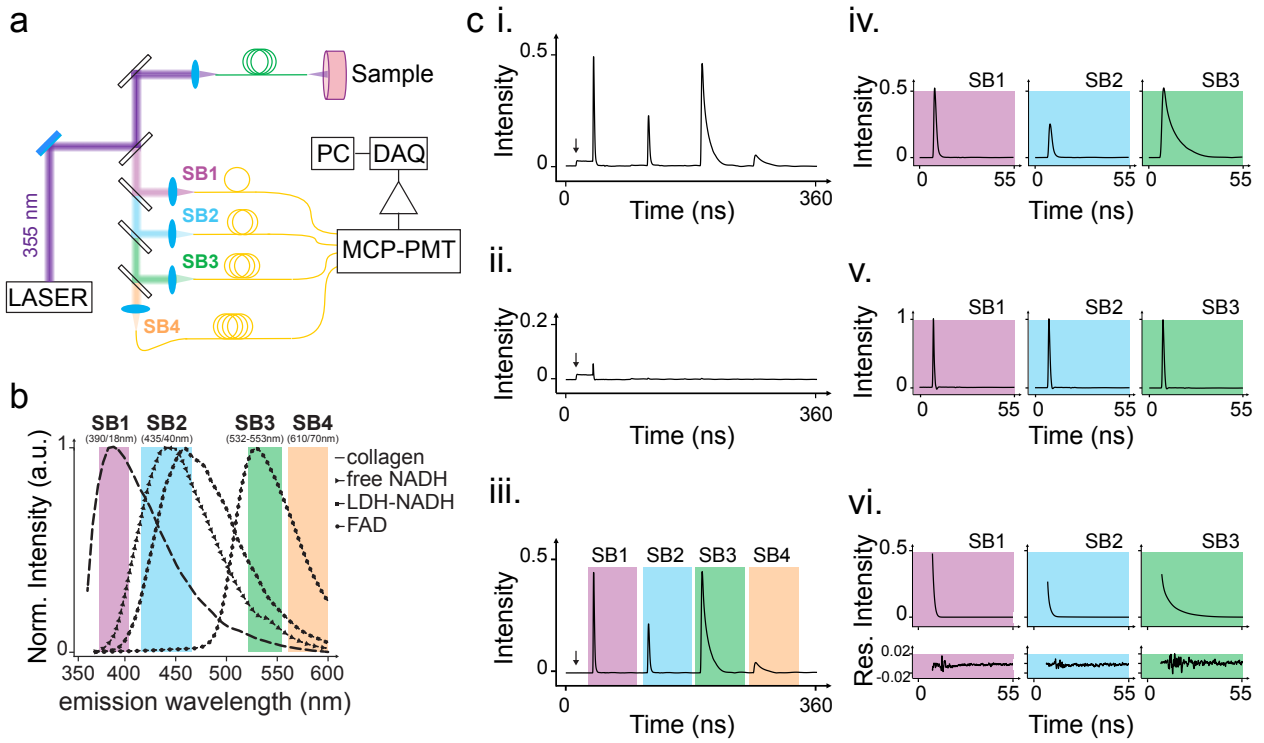


Figure 1: **S1** a) Fiber optic based fluorescence lifetime imaging setup, adapted from [7]. b) Spectral bands (SB) on the fluorescence lifetime imaging instrument: SB1 = 390/18 nm, SB2 = 435/40 nm, SB3 = 532-553 nm, and SB4 = 610/70 nm, and emission spectra of the main contributors to the autofluorescence of the samples used in this study: collagen, free nicotinamide adenine dinucleotide, NADH bound to lactate dehydrogenase (LDH-NADH), and flavin adenine dinucleotide (FAD). c) Schematic representation of data processing pipeline using fluorescence signal from a phantom consisting of 4 plastic fluorescent (chroma) slides in air. i) The raw signal is a waveform that reports the decay of fluorescence intensity in all 4 spectral bands. ii) The raw background is acquired by pointing the fiber distal tip away from the sample. iii) Background is subtracted from the signal. The four spectral bands are here highlighted with different colors (SB1-4). Arrows in i-ii point at the most prominent background feature, which has been subtracted in iii. iv) The background subtracted signal is segmented into four individual portions, one per each spectral band. SB4 is not shown for simplicity in this and the next steps. v) The instrument response function (IRF) is also acquired at each spectral band, by measuring the fluorescence decay of 2-DASPI.f) After deconvolving the IRF from the background subtracted signal using least square deconvolution with Laguerre expansion we obtain the fluorescence intensity decays, at each spectral band. The residuals of the expansion are also shown.

3. Fluorescence lifetime drift of AR-BP over time

Figure S3 shows representative pieces of acelllular AR-BP cultured in PBS (figure S3a,c) and D20 (hMSC media; figure s3b,d) during 9 days. Fluorescence lifetime changes are detected with different degree in the three spectral bands used in this study (SB1, SB2, and SB3), and for both sides of BP, the serous and the fibrous side. The changes in SB1 and SB2 are the most pronounced, as those are the bands where the ECM fluorophores are more prominent. SB3 shows smaller intensity contributions (the intensity for day 0, serous side, is not strong enough to retrieve a lifetime measurement) and shorter, almost constant fluorescence lifetime readouts over time, which strengthens the importance of SB on cellular detection. The mean and standard deviation of the fluorescence lifetime of two pieces per condition (side and culture media) were calculated in each spectral band, and are shown in figure S3c-d. The fluorescence lifetime of AR-BP pieces cut from adjacent regions of BP (like in the subset of images shown in figure S3) are very close, and thus the standard deviations are difficult to appreciate in the plot. Standard deviation fluctuates between 0 and 0.14 ns.

4. Histology and immunohistochemistry

Histological analysis with H&E stained samples provided a verification of cellular presence at the end of the 7-day experiments (figure S4), and showed the formation of endothelial monolayers (figure S4b-c) and how MSCs preferentially

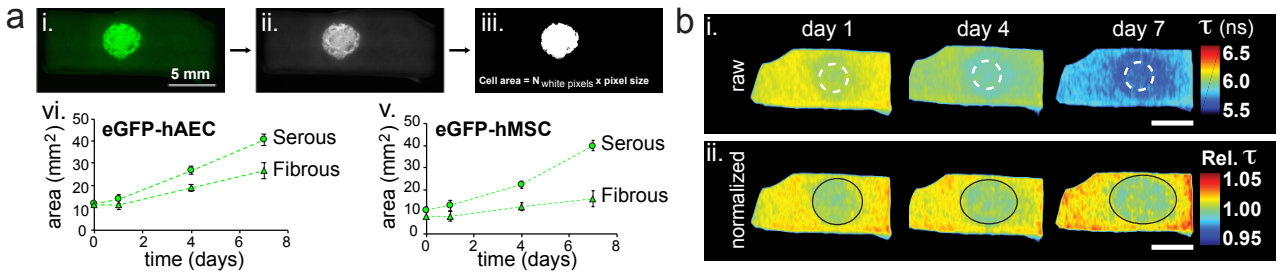


Figure 2: **S2** a) Image post-processing: fluorescence microscope images. i) Steady-state fluorescence microscope image of hAEC on the fibrous side of AR-BP, ii) corresponding gray scale image, and iii) Otsu segmented image. iv) eGFP-hAEC and v) eGFP-hMSC area on either side of AR-BP over time. b) Image post-processing: relative lifetime maps. i) Fluorescence lifetime maps in SB1 of an AR-BP scaffold seeded with hAEC exhibited an average lifetime drift over 7 days in cell culture media. White dashed circle indicates the selected region for further image processing. ii) Normalized lifetime images to the mean lifetime obtained from the selected region in *a*. Black circle indicates area of cellular contribution. Scale bars = 5 mm.

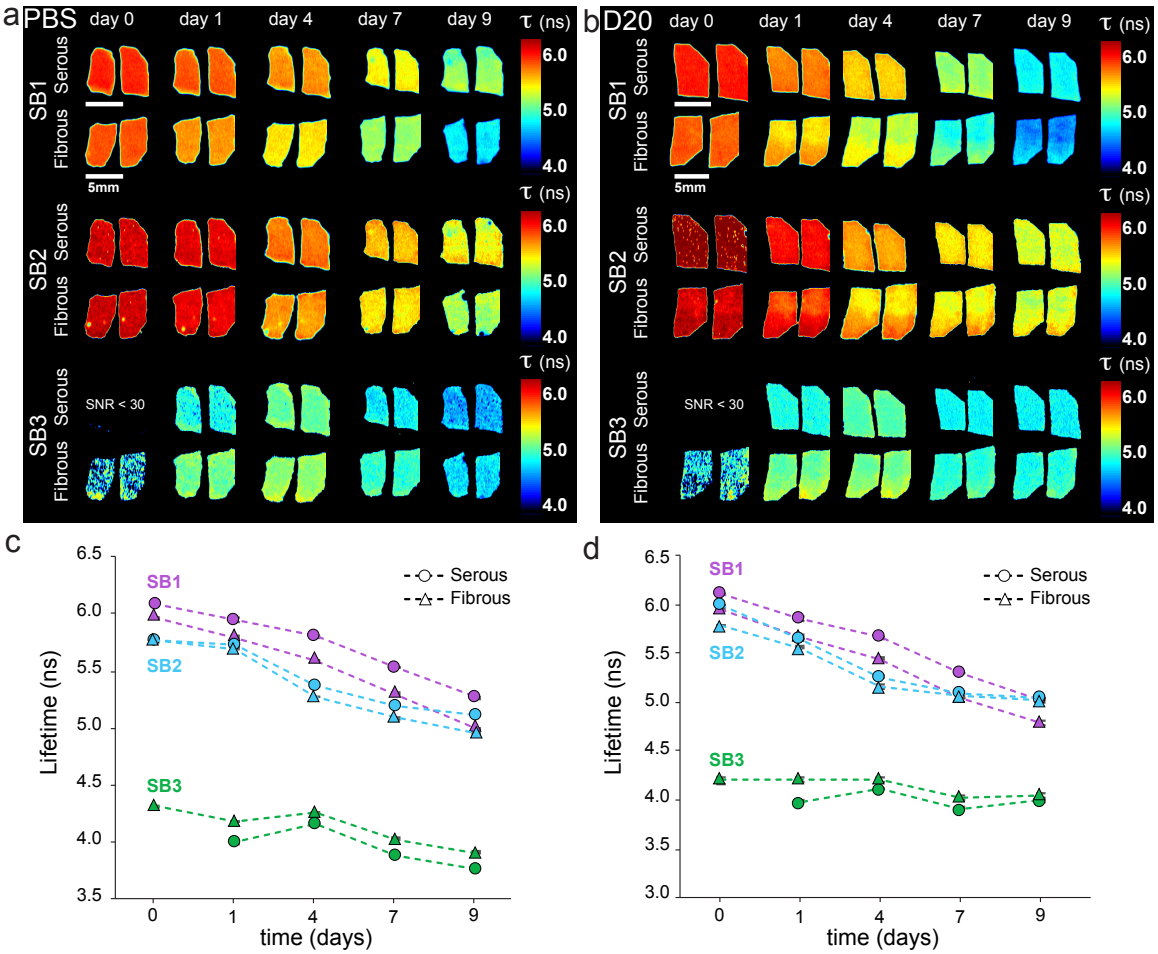


Figure 3: **S3** Representative fluorescence lifetime maps of AR-BP pieces cultured over 9 days in (a) PBS, and (b) D20. Scale bar = 5 mm. Mean fluorescence lifetime value for n = 2 pieces per side and culture media (C) PBS, and (d) D20 over 9 days, for SB1, SB2, and SB3. Error bars indicate standard deviation.

penetrate the scaffold when seeded on the fibrous side (figure S4d-e). Additional immunofluorescence staining was performed to confirm the cells were alive and displayed characteristic morphology (figure S4f).

For immunofluorescence staining, scaffolds with cells were fixed with 4% formaldehyde, incubated with 0.1% Triton x-100 in 1% BSA for 10 min and stained with Hoechst 33342 (blue, 1:10,000), and Actin-stain-594 Phalloidin (red, 1:40). Scaffolds were immersed in ProLong Gold antifade reagent and placed on a slide with Gene Frame around as spacer.

For histological analysis, the samples were fixed in formalin, embedded in paraffin and hematoxylin an eosin (H&E) staining was performed according to standard procedure. Images were acquired using Aperio ScanScop and processed using ImageScope software 12.3.2.5030 (Leica).

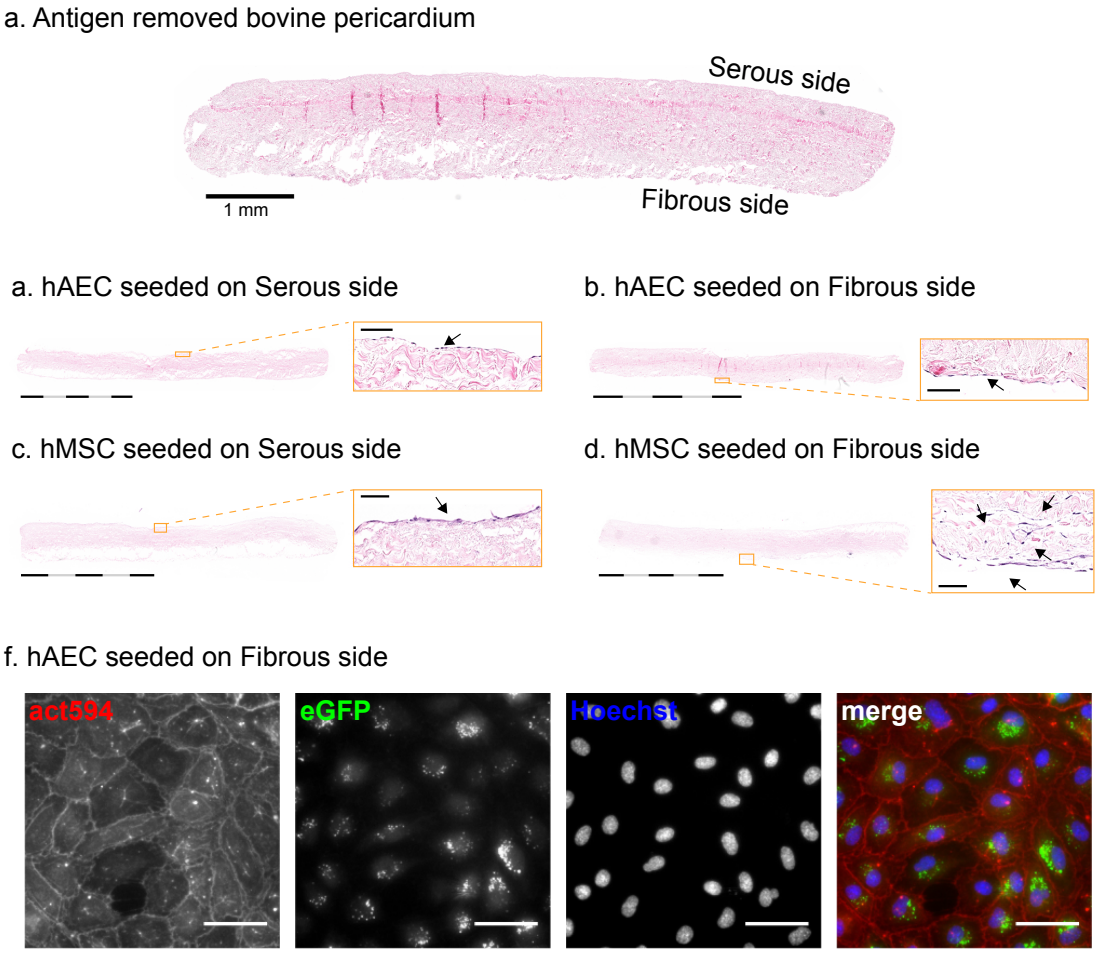
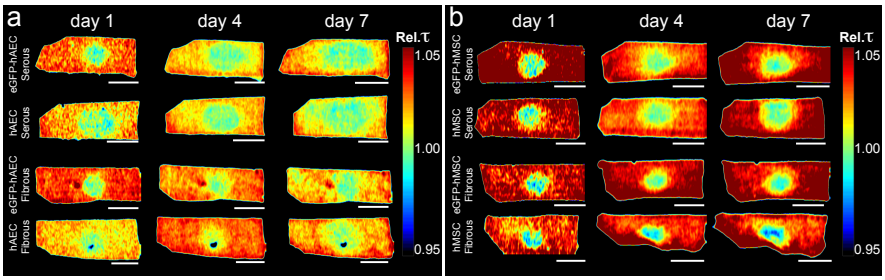


Figure 4: **S4.** Histological cross sections stained with H&E of a) acellular AR-BP, hAEC seeded on the b) serous and c) the fibrous side of AR-BP, and hMSC seeded on the d) serous and the e) fibrous side AR-BP. Scale bars = 5 mm. Scale bars in boxes = 100 μm. Arrows point at cells. f) Actin-stain-594 Phalloidin, unspecific eGFP, and Hoechst 33342 stain of hAEC seeded on the fibrous side of AR-BP. Scale bar = 50μm.

5. eGFP-labeled and unlabeled cells

To verify the success of cell seeding and cellular presence on AR-BP, eGFP-labeled cells were used for both cell types, hAEC and hMSC. To validate the observed fluorescence lifetime changes where not due to the fluorescence label, unlabeled cells were also utilized. Figure S5 shows representative relative lifetime maps of labeled (n = 9) and unlabeled (n = 11) (a) hAEC and (b) hMSC seeded on both sides of AR-BP over 7 days in culture, for SB3. The resulting relative lifetime images were equivalent, there wasn't a significant difference from labeled and unlabeled cell progression rates as reported by one-way ANOVA analysis (figure S5c).



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day	SB	P-value
1	1	0.186
	2	0.156
	3	0.057
4	1	0.243
	2	0.099
	3	0.732
7	1	0.054
	2	0.055
	3	0.291

Figure 5: **S5.** Fluorescence relative lifetime maps of AR-BP with eGFP-labeled and unlabeled (a) hAEC and (b) hMSC, in the fibrous and serous sides, over 7 days in culture with respective cell culture media. Scale bars = 5 mm. c) Comparison between cell area of eGFP labeled (n = 9) and unlabeled (n = 11) cells for each imaging day and spectral band. P-value from one-way ANOVA analysis.

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