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Paper Title:

Contrast enhanced nanoCT for 3D quantitative and spatial analysis of *in vitro* manufactured extracellular matrix in metallic tissue engineering scaffolds

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Choice of presentation: (highlight the type you prefer)

Oral Presentation

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Poster Presentation

Contrast enhanced nanoCT for 3D quantitative and spatial analysis of in vitro manufactured extracellular matrix in metallic tissue engineering scaffolds

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Introduction and aim

As the field of tissue engineering (TE) matures, the need for novel techniques to characterize engineered constructs (i.e. cells/tissue combined with scaffolds) in a more insightful and quantitative manner becomes crucial. Currently, standard techniques such as histological sectioning show insufficient potential as TE quality control as they only allow assessment of tissue distribution in two dimensions and due to their destructive nature loose information and have a limited depth resolution [1]. Recent advances in 3D imaging techniques and image analysis have demonstrated potential to address some of the above mentioned shortcomings for TE use. In particular X-ray microfocus computed tomography (microCT) holds potential as it has been frequently applied as a 3D quantitative imaging technique for time-lapsed follow-up of mineralization inside scaffolds during *in vitro* static [2] or bioreactor cultures [3]. However, to date when using desktop microCT in standard absorption mode, without the use of a contrast agent, it has not been possible to visualize non-mineralized tissues in 3D scaffolds [4]. Several studies have shown that when using phase contrast imaging, mostly limited to the use of synchrotron radiation, the non-mineralized tissue in 3D constructs can be visualized [5]. However, access to systems allowing phase contrast imaging is restricted and does not allow routine analysis.

In this study, we show that when the high spatial and contrast resolution of nanofocus CT (nanoCT) is combined with two different contrast agents the engineered extracellular matrix (ECM) in TE constructs can be imaged. This contrast-enhanced nanoCT (CE-nanoCT) provides qualitative and quantitative read-outs on the amount of engineered ECM and its spatial distribution throughout the TE construct during static and bioreactor based cell culture.

Materials and methods

TE constructs

3D additive manufactured open porous Ti6Al4V scaffolds ($\varnothing = 6$ mm, $h = 6$ mm),

produced on an in-house developed selective laser melting (SLM) machine [6] were used. Human periosteal derived cells (hPDCs), isolated from periosteal biopsies of different donors were seeded onto pre-wetted 3D Ti6Al4V scaffolds using a static drop-seeding protocol (cell seeding density 30000 cells/cm²) with an average cell seeding efficiency of 60%. TE construct culture ranged from 14 over 21 to 28 days under static or dynamic culture conditions. For bioreactor culture, TE constructs were cultured in an in-house developed perfusion bioreactor equipped with 36 parallel perfusion circuits. Two different perfusion flow rates were used for the bioreactor cultures: a low flow rate at 0.04 ml/min and a high flow rate at 4 ml/min.

Contrast-enhanced nanofocus CT (CE-nanoCT)

After static or dynamic culture the TE constructs were rinsed with 1ml phosphate buffered saline (PBS) and transferred to a 4% paraformaldehyde solution (Sigma) for 2 hours to fixate the ECM. The TE constructs were stored in PBS prior to CE-nanoCT analysis. Two contrast agents were used as received, namely Hexabrix[®] 320 (Guerbet Nederland B.V) and phosphotungstic acid (PTA - VWR International). The latter is known to bind to connective tissues, and more specifically to collagen and fibrin. The former contains a negatively charged ioxaglate, which will be locally repulsed by the negative fixed charge density of the tissue, thus all tissues which do not contain a negative charge will be stained. All samples were first immersed for 30 minutes in a solution of Hexabrix[®] 320 (20% in PBS), then wrapped in parafilm and were stably positioned in the nanoCT system for imaging. Next, the samples were transferred to PBS for 30 minutes, after which they were immersed in a solution of PTA (1.25g/50ml PBS), were again wrapped in parafilm for imaging in the nanoCT system.

The nanoCT system applied in this study was a Phoenix NanoTom S (GE Measurement and Control Solutions). It was equipped with a tungsten target and was operated at a voltage of 90 kV and a current of 170 μ A. A 1 mm filter of aluminum and 1 mm of copper was used to reduce beam hardening and metal artifacts as much as possible. The exposure time was 500 ms and a frame averaging of 1 and image skip of 0 was applied, resulting in a scanning time of 20 minutes. The scanning time was kept low to allow routine screening of the samples and eliminate sample drying during scanning, to enable further use. The reconstructed images had an isotropic voxel size of 3.75 μ m.

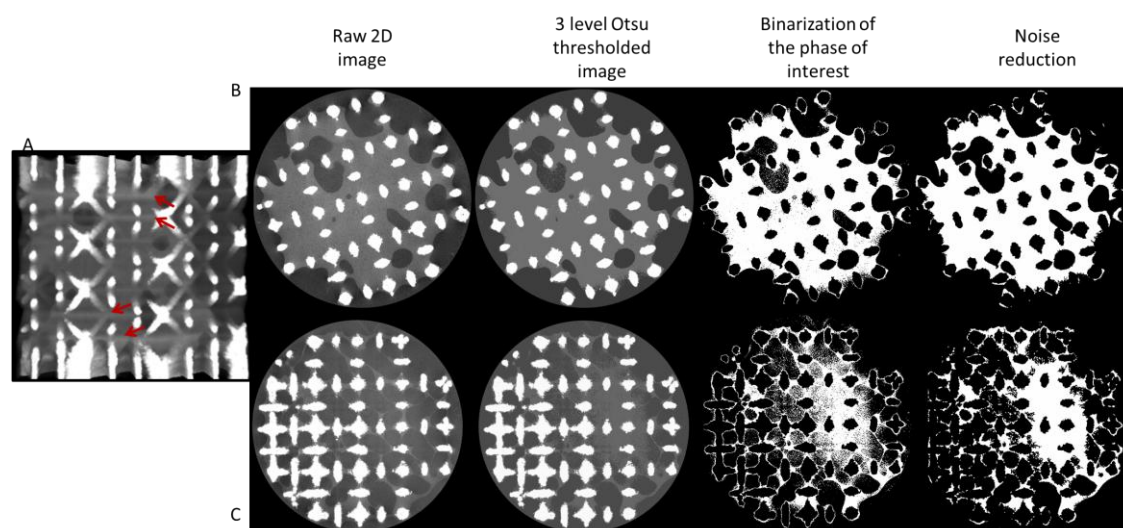


Figure 1: (A) An unprocessed 3D transaxial image of a TE construct. The small red arrows indicate metal artifacts, visible as streaks. Image processing steps for a section (B) without and (C) with metal artifacts at the indicated locations in the TE construct.

For 3D image rendering, CTVox (Bruker micro-CT, Belgium) was applied. For image processing and quantifying the ECM volume in the TE constructs, we used CTAn (Bruker micro-CT, Belgium) according to the scheme in figure 1B and 1C. By using a 3-level automatic Otsu segmentation algorithm [7] on each individual 2D slice, the ECM was separated from both the scaffold and the background, the latter including metallic artifacts. As a result, grey-scale images with distinct grey-scale values for scaffold, ECM and background were generated. A global threshold was chosen manually, but consistently, to select the ECM. In order to reduce the errors introduced by the partial volume effect and by the metallic artifacts prior to analyzing the ECM volume, the binarized images for the Ti6Al4V structure were dilated by 2 voxels and subtracted from the binarized ECM images. The ECM volume was analyzed by performing a 3D analysis on the binarized and processed images.

Even after these image processing steps, metallic streaks were still present in all scanned TE constructs at specific, geometry-related, locations (Fig. 1A and 1C). The effect of these artifacts on the image analysis is apparent when comparing figure 1B, where no artifacts were present, with figure 1C where the artifacts give rise to an overestimation of the ECM volume. To obtain a correct ECM volume, the reconstructed dataset of the full construct was fragmented in different subsets with a comparable amount of metallic artifacts. Sections with a significant amount of metal artifacts present were excluded from the volume analysis and an interpolation in ECM volume was made between the surrounding sections.

Physical characterization of TE constructs

To quantify the cell content of the constructs, the DNA content was determined for each time point ($n = 4$) using a highly quantitative and selective DNA assay (Quant-iT dsDNA HS kit, Invitrogen). After culture, constructs were rinsed with PBS and transferred to the lysis solution containing 350 μ l RLT buffer supplemented with 3.5 μ l β -mercaptoethanol after which they were vortexed for 60s and stored at -80°C . Prior to analysis samples were centrifuged for 1 min at 13 000 rpm and diluted 1:10 in miliQ water. DNA content was determined with a Qubit[®] Fluorometer (Invitrogen) as described by Chen *et al.*, 2012 [8].

The cell viability on the TE constructs was evaluated using Live-Dead viability/cytotoxicity staining (Invitrogen, USA). Constructs were rinsed with 1ml PBS, incubated in the staining solution (0.5 μ l Calcein AM and 2 μ l Ethidium Homodimer in 1ml PBS) for 20 min under normal cell culture conditions and finally imaged using a Leica M165 FC microscope.

Results and discussion

Comparison to physical measurements

Live/dead viability/cytotoxicity staining, a well-accepted technique for the visualization of the cell distribution throughout TE constructs, was employed as a benchmark analysis on the TE constructs cultured under different conditions. The fluorescent images showed that cells were distributed over the entire outer scaffold surface, indicating similar results for all 3 culture conditions (Figure. 2A). However CE-nanoCT images of the same constructs showed that in the case of static culture, ECM was only formed around the periphery of the Ti6Al4V scaffold resulting in partially ECM filled constructs, while in the bioreactor cultured constructs the presence of ECM was seen throughout the entire internal volume of the scaffold (Figure. 2B and 2C). The application of CE-nanoCT thus increased our insight regarding the morphology of the manufactured TE constructs as it provided new information that was impossible to obtain with currently used microscopic techniques. Nevertheless fluorescent or bright-field images still give unique information concerning cell state and should be used in a complementary way to the newly developed technique.

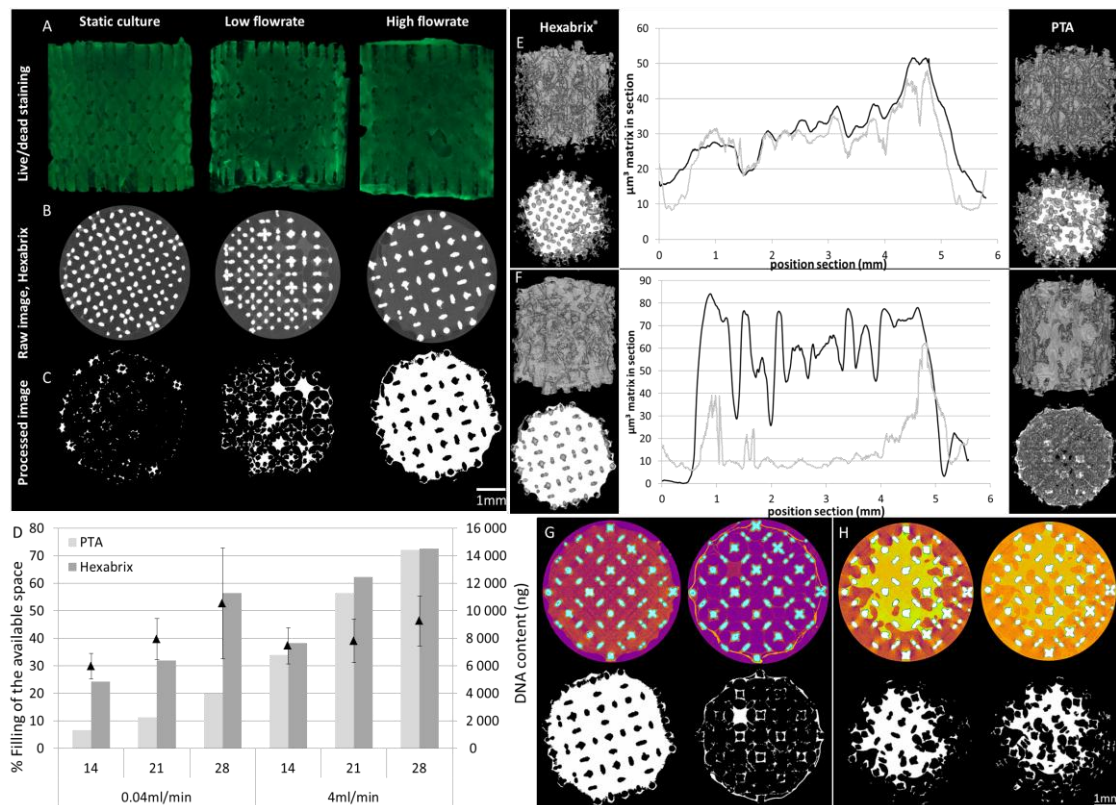


Figure 2: (A) Live/dead staining of TE constructs cultured in different conditions for 21 days, (B) corresponding 2D grey-scale CE-nanoCT cross-sections using Hexabrix®, (C) the corresponding binarized and processed cross-sections. (D) The percentage of ECM filling determined using Hexabrix® and PTA staining (n=2), and the DNA content (ng) of TE constructs cultured at different flow rates for different time points. (E) CE-nanoCT results for a construct cultured at 4ml/min for 14 days (F) CE-nanoCT results for a construct cultured at 0.04ml/min for 28 days; from left to right: Hexabrix 3D reconstruction and 2D cross-section, representative longitudinal ECM distribution throughout the complete TE construct i.e. volume of ECM per slice, with a slice thickness of 3.75 µm, in function of the scaffold height (black: Hexabrix®, grey: PTA), PTA 3D reconstruction and 2D cross-section. (G) Color representation of a typical matching 2D grey-scale CE-nanoCT cross-section for Hexabrix® (left) and PTA (right) staining and the corresponding processed and binarized images (28 days, 0.04ml/min). (H) Color representation of a typical matching 2D grey-scale CE-nanoCT cross-section for Hexabrix® (left) and PTA (right) staining and the corresponding processed and binarized images (14 days, 4ml/min).

Quantification of the ECM volume and spatial distribution

The ECM volume in the TE constructs was determined using CE-nanoCT for both TE constructs cultured statically or using a bioreactor at different flow rates. Although metallic artifacts were reduced by filtering during scanning and subsequent image processing, still metallic artifacts were present in the reconstructed CE-nanoCT images as is visible in figure 1. These artifacts together with the image processing introduced a significant error (i.e. streaks and edges of the scaffold having the same grey-scale as the stained ECM, and a loss in small/thin features respectively) when only a small amount of ECM was present, for example after static culture (Fig. 2B and 2C). Hence 3D quantification was not reliable for statically cultured constructs, and such scans were used only for qualitative, visual inspection of the images. For other, non-metallic scaffold materials, we believe that CE-nanoCT will provide more accurate results, since material-dependent artifacts will be reduced.

For the bioreactor conditions image analysis of the binarized CE-nanoCT slices using the different contrast agents allowed to quantify the internal scaffold filling with ECM

(Fig. 2D). While the DNA content of the different constructs was comparable for the 2 flow rates, figure 2D clearly shows that the matrix formation determined with both Hexabrix[®] and PTA was influenced by the culture conditions. This was confirmed when analyzing the spatial ECM distribution throughout the TE constructs (Figure 2E and F). For the high flow rate, both Hexabrix[®] and PTA staining resulted in similar ECM volumes (Fig. 2H). However, for the low flow rates, Hexabrix[®] staining gave rise to higher ECM volumes compared to PTA staining (Fig. 2H). This could be explained by the collagen-specificity of PTA. Fluid flow is known to enhance matrix synthesis within TE constructs during perfusion culture [3, 9, 10]. Hence, the larger PTA-derived ECM volume for the high flow rate could be explained as an ECM richer in protein components (on which PTA may specifically bind). Furthermore differences in fibrillar collagen organization of the ECM, which are flow dependent [11], could also explain the ECM differences that were observed by PTA staining as the ECM morphology could influence the binding mechanism of the stain to the ECM proteins.

Conclusions

In this study proof-of-concept was given that CE-nanoCT is a non-invasive imaging technique that allows 3D visualization and/or quantification of *in vitro* engineered ECM in TE constructs, even in the worst case scenario of metallic Ti6Al4V scaffolds. Furthermore, there are indications that ECM compositional analysis can be made possible via this method.

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