Contrast enhanced nanoCT parameter optimisation for volumetric analyses of *in vitro* manufactured tissue-engineered bone constructs

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Aims

As the field of tissue engineering (TE) matures, there is a growing need for novel imaging techniques for the characterisation of engineered constructs (i.e. cells/tissue combined with scaffolds) in a more insightful and quantitative manner. In a proof of concept study, we recently showed that combining the high spatial and contrast resolution of nanofocus computed tomography (nanoCT) with two different contrast agents (i.e. contrast-enhanced nanoCT or CE-nanoCT) allowed the visualization and quantification of *in vitro* formed neo-tissue (both cellular and extracellular matrix) in bioreactor-cultured TE constructs (1). Although the contrast difference between the neo-tissue fraction of the construct and the background was increased using the contrast agents, the image quality was compromised due to metal-related image artefacts thus hindering accurate, and automated quantification in the entire dataset.
Therefore, a range of image processing settings was evaluated to optimise image quality and enable automation of the analysis procedure. Additionally, using a design of experiments (DoE) approach we improved/enhanced the quality of the obtained datasets allowing the determination of the optimum staining time, contrast agent concentration combinations.

Method

TE constructs

Selective laser melted porous, 3D Ti6Al4V scaffolds were used (2). Human periosteal derived cells (hPDCs) (3) were seeded on the scaffolds as described before using a static drop-seeding protocol (1). The constructs were subsequently cultured in an in-house developed perfusion bioreactor system for 7 to 21 days or in a static setup for 21 days to allow cell proliferation and neo-tissue formation. For all conditions the constructs were cultured in normal growth medium which was refreshed every two days. For the perfusion bioreactor system a flow rate of 1 ml/min was used.

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 neo-tissue. 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) (1). Different concentrations and staining times of both contrast agents were used for the DoE according to a 3-level, 3-parameter fractional factorial design (Table 1). Constructs cultured for 3 weeks in the static system represented the low neo-tissue level, 1 week in the perfusion bioreactor system the mid-level and 3 weeks in the perfusion bioreactor the high level. Constructs were initially stained and scanned with Hexabrix after which they were rinsed in PBS overnight at least 3 times to remove all traces of the first contrast agent. The same constructs were subsequently stained with PTA and scanned again.

Neo-tissue volume	staining time	Concentration
1 week perfusion	120 min	40% Hex -- 5% PTA
1 week perfusion	240 min	20% Hex -- 2.5% PTA
1 week perfusion	30 min	60% Hex -- 7.5% PTA
3 weeks perfusion	120 min	20% Hex -- 2.5% PTA
3 weeks perfusion	240 min	60% Hex -- 7.5% PTA
3 weeks perfusion	30 min	40% Hex -- 5% PTA
3 weeks static	120 min	60% Hex -- 7.5% PTA
3 weeks static	240 min	40% Hex -- 5% PTA
3 weeks static	30 min	20% Hex -- 2.5% PTA

Table 1: Conditions for 3-level, 3-parameter fractional factorial design (DoE): amount of neo-tissue, staining time, and concentration of the consecutively used staining solutions.

The nanoCT system applied 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 µA. 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 and eliminate sample drying during scanning, to enable further use. The reconstructed images had an isotropic voxel size of 3.75 µm.

Image processing and analysis

CTvox (Bruker micro-CT, Belgium) was used for 3D visualization of the constructs. For image processing and quantifying the neo-tissue formed in the TE constructs, CTAn (Bruker micro-CT, Belgium) was applied. A 2 to 5 level automatic Otsu segmentation (4) based on the class-id was used on each individual 2D slice, and for each grey-scale fraction, the neo-tissue and scaffold surface area were determined per image (2D analysis) and plotted over the height of the construct. Based on the surface area histograms generated for each greyscale fraction, optimal thresholding levels were determined for all conditions of the subsequent DoE analysis.

In order to reduce the errors introduced by the partial volume effect and by the metallic artifacts, the binarized images for the Ti6Al4V structure were dilatated by 2 voxels and subtracted from the binarized neo-tissue images. Subsequently, both black and white speckles smaller than 200 voxels were removed and a 2D closing operation with 2 voxels was applied. The neo-tissue volume was analyzed by performing a 2D and 3D analysis.

DoE analysis

DoE was applied to obtain consistent and objective conclusions on the impact of the investigated parameters (i.e. volume of neo-tissue, staining concentration and incubation time) on the on quality of the CT datasets (i.e. amount of wrongly segmented images). In this study, the latter is determined both by the accuracy of the 2D and 3D quantification as well as the level of automation in the image analysis. All images were processed as described before and histograms showing the distribution of the neo-tissue along the axial direction of the constructs were generated, thus allowing quantification of the number of wrongly segmented images which were subsequently used as an input for the DoE analysis.

Results and discussion

Optimization of the image processing and analysis

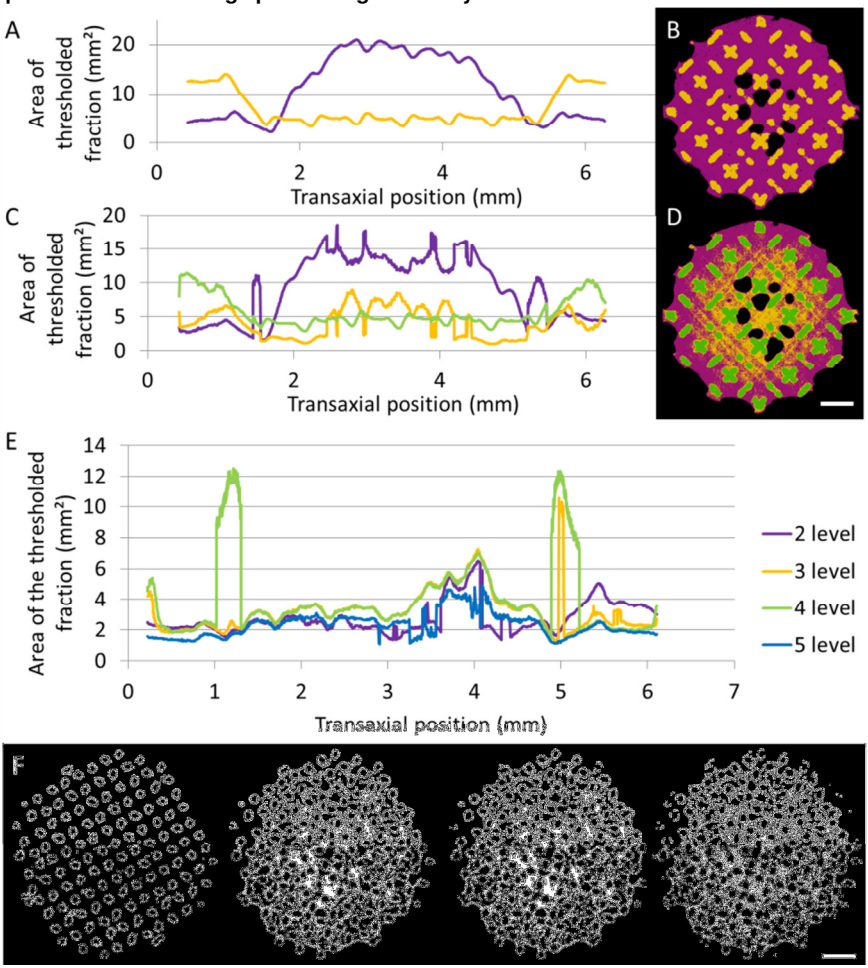

Figure Y: (A): Surface area histogram of the two greyscale fractions containing neo-tissue (purple) and scaffold material (yellow) for a 2D, 2-level thresholded construct. (B) Representative segmented image of the histogram shown in A for a transaxial position of 3mm. (C) Surface area histogram of the three greyscale fractions containing neo-tissue (purple), a combination of fractions of the neo-tissue, edge effects and scaffold material (yellow) and scaffold material (green) with a 2D, 3-level thresholding algorithm. (D) Representative segmented image of the histogram shown in C for a trans-axial position of 3mm. (A-D) were generated based on the same construct cultured for 3 weeks in the perfusion bioreactor system and stained for 240 min with 60% Hex. (E) Surface area histogram of the PTA stained neo-tissue containing greyscale fraction for a construct stained with 7,5% PTA for 240 min and processed with a 2 to 5 level 2D Otsu segmentation (left to right respectively). (F) Binarized images (for the neo-tissue containing greyscale fraction) for the 2 to 5 level (left to right respectively) segmented trans-axial sections at 4.1 mm in E. Scale bars are 1mm

To optimize the image processing and analysis settings, CE-nanoCT datasets of the constructs with a hypothesized maximal contrast (maximal staining time and concentration) were segmented using a 2 to 5 level Otsu algorithm. Figures 1.A and 1.B show for a Hexabrix-stained sample a clear separation between the scaffold material and neo-tissue using a 2-level segmentation. In figure 1.C and 1.D, it can be seen that the additional segmentation level does not increase the quality of the segmentation. Figures 1.C and 1.D represent the results for the 3-level segmentation. In the middle of the construct, where there was a lower presence of metal artifacts as compared to the top and bottom, the sum of the yellow and purple grey-scale fractions correspond well to the 2-level segmented neo-tissue fraction. However, at the top and bottom of the construct, metal artifacts were incorporated in the grey-scale fraction of the neo-tissue (yellow). Therefore a 2-level Ostu segmentation was selected for the Hexabrix stained constructs.
Also for the PTA-stained constructs, the amount of thesholding levels had a strong influence on the 2D and 3D analysis (Fig. 1.E). Both the 2- and the 5-level segmentation resulted in a lower PTA positive signal compared to the 3 and 4-level segmentation as could also be observed in Figure 1.E and 1.F. While for a 2-level segmentation the neo-tissue could not be distinguished from the noise (Fig. 1.F - left), a 5-level segmentation resulted in an additional segmentation of the neo-tissue in different greyscale fractions. Both the 3- and 4- level segmentation resulted in a similar neo-tissue distribution profile, except near the top and bottom of the construct. There the 4-level segmentation strongly overestimated the neo-tissue volume because the metal artifacts were included in this greyscale fraction. Hence a 3-level segmentation was selected for further processing of all PTA stained datasets.

Influence of the concentration and incubation time of the contrast agent, and the neo-tissue volume on the image quality – DoE

Despite the optimized image processing strategy, there were significant differences regarding the accuracy and quality of the analysis. For the high concentration and incubation time of Hexabrix, a good segmentation between the scaffold, neo-tissue and background was found (Fig. 2.A). For a lower concentration and incubation time of Hexabrix, and for certain zones in the construct the segmentation was not successful in distinguishing between the background signal (noise and metal artifacts) and the neo-tissue (Fig. 2.C - insets), as is also apparent in the streaks visible in the 3D representations (Fig. 2.B). This resulted in discontinuities in the surface area histogram (Fig. 2.C).
The DoE analysis indicated that for the Hexabrix stained constructs only the concentration had a significant effect on the quality of the datasets (Figure 2.D). A higher concentration resulted in a significant reduction of the number of incorrectly quantified sections, thereby increasing the accuracy of the analysis. As Hexabrix is an equilibrium contrast agent (1) with a small molecular weight, it can easily infiltrate in the neo-tissue. Thus, the lowest incubation time assessed in this study was probably already sufficient for a complete diffusion of Hexabrix

throughout the neo-tissue. For the 60% Hex stained constructs, independent of the staining time and matrix quantity, no discontinuities were present in the histogram (Fig. 1.A), thereby resulting in a quantitative analysis of the neo-tissue volume. For these constructs, automated image processing and analysis is possible, thus being user-independent.
For the PTA stained constructs an increase of the staining parameters, as well as a higher amount of neo-tissue present resulted in a significant decrease in incorrectly thresholded sections. Although again concentration had the strongest effect, an increase in staining time also resulted in a significant increase of the image quality. The molecular weight of PTA is higher compared to that of Hexabrix. Additionally, PTA binds to the collagen- and fibrin-rich part of the neo-tissue (1). As a result, the diffusion of PTA through the neo-tissue is slower, causing a significant effect of the incubation time on the image quality. This was also linked to the amount of neo-tissue present had a significant effect on the image quality. For the low amount of neo-tissue, only thin strands of PTA-stained neo-tissue were present. As the thickness of these strands is close to the spatial resolution of the images (~ 7.5 – 10 µm), the partial volume effect plays an important role and will reduce image quality.

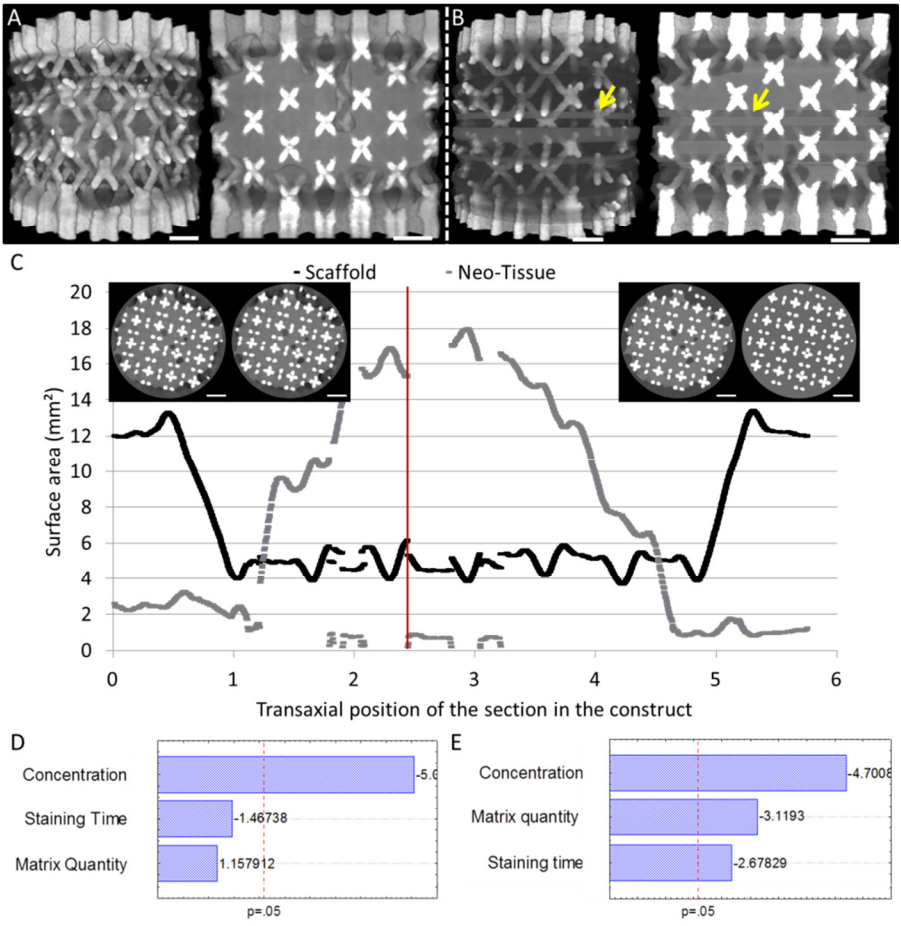

Figure 2: (A) 3D visualizations of a 3 week bioreactor cultured construct stained (A) with 60% Hexabrix for 240 min and (B) with 20% Hexabrix for 120 min. Yellow arrows indicate background signal, (C) The surface area histogram of scaffold (black) and neo-tissue (grey) in a construct cultured for 3 weeks in the perfusion bioreactor and stained with 20% hexabrix for 120 min. Insets show each time a raw image (left) and the corresponding segmented image (right) for the slice left and right of the red line. (D, E) Pareto chart of standardized effects for the fraction of incorrectly quantified slices for respectively Hexabrix and PTA stained constructs. Scale bars are 1 mm

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