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**A novel method for segmenting and aligning the pre- and post-
implantation scaffolds of resorbable calcium-phosphate bone
substitutes**

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Abstract

Micro-computed tomography (microCT) is commonly used to characterize the three-dimensional structure of bone graft scaffolds before and after implantation in order to assess changes occurring during implantation. The accurate processing of the microCT datasets of explanted β -tricalcium phosphate (β -TCP) scaffolds poses significant challenges because of (a) the overlap in the grey values distribution of ceramic remnants, bone, and soft tissue, and of (b) the resorption of the bone substitute during the implantation. To address those challenges, this article introduces and rigorously validates a new processing technique to accurately distinguish these three phases found in the explanted β -TCP scaffolds. Specifically, the microCT datasets obtained before and after implantation of β -TCP scaffolds were aligned in 3D, and the characteristic grey value distributions of the three phases were extracted, thus allowing for (i) the accurate differentiation between these three phases (ceramic remnants, bone, soft tissue), and additionally for (ii) the localization of the defect site in the post-implantation microCT dataset. Using the similarity matrix, a $94 \pm 1\%$ agreement was found between algorithmic results and the visual assessment of 556,800 pixels. Moreover, the comparison of the segmentation results of the same microCT and histology section further confirmed the validity of the present segmentation algorithm. This new technique could lead to a more common use of microCT in analyzing the complex 3D processes and to a better understanding of the biological processes occurring after the implantation of ceramic bone graft substitutes.

Keywords: Micro-computed tomography; Bone substitutes; Scaffolds; Image analysis;
Bone growth; Resorption

Introduction

Bone substitutes made of synthetic materials are a viable alternative to autografts and allografts [1,2]. Among these materials, calcium phosphates (CaP) are particularly interesting due to their chemical and physical similarity to bone minerals [3, 4]. Next to their biocompatibility, CaP bone graft substitutes have other desirable properties, including bioactivity and osteoconductivity [4–8]. The CaP materials, such as hydroxyapatite (HA) or β -tricalcium phosphate (β -TCP), are routinely used for filling traumatic and pathological bone defects [9–11]. Compared to HA, β -TCP is gradually resorbed and replaced by neo-bone [12, 13]. The quest continues for the optimum bone scaffold design, in terms of material and pore geometry, and for controlled interactions with the host bone [14,15]. Besides the *in vitro* studies, animal studies have played a significant role in this quest [8, 16–21].

Characterizing the three-dimensional macro-porous bone substitute scaffold before and after implantation requires the use of accurate methods, and microcomputed tomography (microCT) imaging modality is commonly employed [17, 22–27]. Compared to the post-implantation characterization [8, 17, 24–26], the pre-implantation characterization of β -TCP scaffolds, using the microCT technique, has been more accurate and therefore successful [22,23]. Methods to distinguish and isolate the three phases (bone, ceramic, soft tissue) of the explanted ceramic bone substitute samples still do not exist. Furthermore, the spatial and temporal evolution in the mineralization levels, in the form of the newly deposited bone, and of the partially localized resorption

of the β -TCP implant further increase the difficulty in establishing precise and accurate methods for differentiating the grey-intensity levels of the three phases [7, 8, 17, 24]. Specifically, applying the local segmentation technique to the post-implantation microCT dataset requires the use of accurate techniques and new development. In the commonly used global thresholding segmentation technique, two global threshold values must be defined, one separating the grey levels of fibrous tissue and bone, and the second one separating the grey levels of bone and ceramic remnants. Specifically, global thresholding techniques, for example Otsu's method, were applied in several *in vivo* studies to distinguish the bone from the ceramic [7, 17, 24, 26, 28, 29]. These methods often led to inaccurate results. Gauthier et al. [24] reported a significant disagreement between the bone volume fraction measured using microCT and that using scanning electron microscopy. Van Lenthe et al. [17] reported some segmentation errors, and used, for example, dilation-based refinement, in particular for bone being in contact with the scaffold. To take into account the inaccuracy resulting from the global thresholding technique, Komlev et al. [28] classified the microCT data into four phases: bone, scaffold remnants, soft tissue and an uncertain phase. The latter could be bone or partially resorbed scaffold.

An improvement in isolating the different phases after implantation was recently achieved using new segmentation algorithms. Specifically, Jones et al. [30] applied contour-tracking segmentation [31] and watershed algorithms to explanted HA implants, and reported a more accurate segmentation compared to global thresholding. Still, they reported disagreement between the algorithmic results and visual assessment. Polak et al. [25] advantageously combined atlas registration and statistical thresholding

to distinguish bone from ceramic in the explanted samples [25], and reported accurate results. However, this algorithm only applies to scaffolds made of orthogonal rods and the controlled geometry of slow-resorbing ceramic implants. To our knowledge, an accurate method for segmenting the three phases of the *in vivo* resorbable porous bone CaP scaffolds does not yet exist.

Pre- and post-implantation scaffolds datasets were aligned to find the thresholding value to segment the three phases. Komlev et al. [28] who studied Si-TCP scaffolds, used a volume registration method based on 3D correlation and affine linear registration. Their technique is computationally demanding for application to 3D data sets of complete samples. In addition, the Si-TCP material exhibits slow resorption, which means that the pre-implantation scaffold geometry was not greatly changed during implantation [8,17]. The alignment of the pre- and post-implantation structures of geometrically non-uniform and resorbing β -TCP scaffolds poses a great challenge, in particular because of the lack of efficient and accurate techniques for coupling the alignment and the accurate segmentation of the microCT post-implantation.

The present manuscript had two related objectives. The first objective was to develop and implement a novel segmentation method to isolate the three phases present in the explanted ceramic bone substitute scaffolds, i.e. bone, ceramic remnants, and soft tissue. For that purpose, a contour-based method combined with a compaction algorithm specifically treating the fuzzy grey-intensity transitions often found between phases were used to yield more accurate results than those of currently existing global thresholding methods. The second objective was to improve the accuracy of the

segmentation results even more by aligning pre- and post-implantation microCT datasets, hence allowing the extraction of the grey intensity value distribution of each phase. These distributions, once extracted, were subsequently applied to refine the contour-based segmentation method to achieve even more accurate results. Once the post-implantation datasets are accurately segmented in three phases (bone, ceramic remnant, soft tissue), the changes and the biological interaction during the scaffold implantation can be studied more accurately.

Materials and methods

Scaffold fabrication, *in vivo* model, and microCT scanning

Pure β -TCP cylindrical scaffolds of constant porosity were fabricated using the calcium-phosphate emulsions method [32]. Briefly, the calcium-phosphate cement paste (CPC) was mixed with paraffin oil and an emulsifier to obtain a metastable dispersion of oil droplets in the CPC paste. Once set, the oil-filled CPC was incubated at 60°C in a phosphate-buffered solution (0.15 M, pH = 7.4) for 48 hours, cleaned in petroleum ether, and sintered at 1250°C for 1 hour. The resulting blocks were lathed to obtain cylinders ($\varnothing$ = 8mm, L = 13 mm). The samples contained 54% macroporosity (pore diameter greater than 50 μ m) and 21% microporosity (pore diameter smaller than 50 μ m) [32]. The macropores of the sample used in this study had a smooth spherical appearance, with an average pore size close to 1200 μ m, as shown in Fig. 1d of Bohner et al [32]. The β -TCP scaffolds were implanted in the metaphyseal or epiphyseal regions of the sheep for 6, 12 and 24 weeks [8]. Specifically, the scaffolds were implanted in 13-mm deep bone defects/cavities created using an 8-mm diameter drill (KaVo INTrASurg 500s, KaVo Dental AG Biberach, Germany). More details are presented in von Doernberg et al. [8]. Only one randomly selected scaffold, explanted at the 6-week time point was used to demonstrate the utility of the novel processing method presented in this study. The animals were sacrificed, in accordance with the local Ethical Committee and veterinary authorities (application number 176/2003), and bone blocks containing the scaffolds were cut to retrieve the β -TCP *in vivo* scaffolds. The β -TCP scaffolds were microCT-scanned before implantation and after retrieval, using a commercial desktop microCT scanner (μ CT 40, Scanco Medical AG, Bassersdorf, Switzerland) [21–23] at 30- μ m isotropic resolution. The pre-implantation dataset had undergone geometrical and morphometric analyses in recent studies [22,23]. As described in von Doernberg et al. [32], following the microCT scanning, the explanted scaffold was fixed in 40% ethanol, dehydrated in a series of graded ethanol baths (40–100%) and then defatted using xylene. The scaffold was then infiltrated with

polymethylmethacrylate at 4 °C, which was then left to polymerize at room temperature. Once polymerized, the scaffold was then sectioned perpendicular to its longitudinal axis using a precision saw (Leica SP 1600, Leica Microsystems, Nussloch, Germany). Finally, the sections were then stained with toluidine blue dye commonly used in histology staining.

Segmentation and alignment algorithm

The algorithm comprised three consecutive stages, Fig. 1: (I) the initial segmentation of the explanted scaffold; (II) 3D alignment of the pre-implantation and explanted scaffolds; and (III) refined segmentation. The initial segmentation, in stage I, provided a preliminary isolation of three phases found in the explanted scaffold (bone, ceramic and soft tissue). In stage II, the 3D alignment of the scaffold before and after implantation using the microCT dataset isolated the bone deposited in the macropores, and the ceramic remnants of the explanted scaffold. Accordingly, the characteristic grey-value distributions of the ceramic and bone became clearly available. In stage III, these characteristic distributions were used to refine the contour-based segmentation of stage I. Each stage further comprised multiple algorithmic steps.

Initial segmentation of the three phases (Stage I)

In stage I, the initial segmentation algorithm had four successive steps. In step 1, an energy-based active contour algorithm for image segmentation was used to segment/isolate the soft tissue of the explanted scaffold. In step 2, the fuzzy edges resulting from transitions and boundaries between phases present in the explanted scaffold were treated. Step 3 involved diffusing the bone found in the macropores into the background, in order to isolate the ceramic remnants. In step 4, the ceramic remnants were then segmented using an active contour algorithm.

- **Segmentation of soft tissue (Step I.1)**

The microCT image stack/dataset was treated as follows to segment the soft tissue. Firstly, the Sobel edge-detection filter [33] was used to identify strong edge points in the stack, which subsequently were used as seed points to initiate the segmentation process. Secondly, the seed points forming the mask acted as the initial contour in the next step. Using this initial contour, the iterative process inherent to the active contouring algorithm [34] identified contours, gradually isolating the soft tissue by minimizing the total energy of the energy inside and outside the contour curve, $F_1(C)$ and $F_2(C)$ (Eq.1), respectively. Finally, the soft tissue pixels were isolated or identified. The above-mentioned steps were repeated for each 2D image of the microCT image stack.

$$F_1(C) + F_2(C) = \int_{\text{inside}(C)} |I(x, y) - c_1|^2 dx dy + \int_{\text{outside}(C)} |I(x, y) - c_2|^2 dx dy \quad (1)$$

Where C is the variable curve, $I(x, y)$ is the intensity value at (x, y) and c_1, c_2 denote the average energy inside and outside the curve, respectively.

- **Treating the fuzzy transitions between phases (Step I.2)**

For specifically refining the fuzzy transitions between phases in microCT data, two sigmoidal weight functions were used (Fig. 2), a distance relating (W_d) and a grey-level relating value (W_g) (equation 2). Firstly, two windows having a defined number of pixels were used for identifying the transitions in the 2D microCT images in the x and y directions. The sizes of the windows were $1 \times w$ and $w \times 1$ for the x and y directions, respectively, where w was selected based on the fuzziness appearance of the transitions. In the grey-intensity transitions evaluation process, a window was considered to be at the transition from one phase to another if satisfying the following conditions: (a) having at least one pixel of grey level below the threshold of a first phase (TH_1), and (b) having at least one pixel of grey level greater than threshold of second phase (TH_2). Thereafter, the pixels of the window were weighted using the distance followed by the

grey-level weight function. Afterwards, the windows were subjected to a thresholding process in accordance to equation 2.

$$W_g(p) = A + \begin{cases} 1 & \rightarrow f(p) \geq TH_1 \\ 1 - 2(f(p) - TH_1)^2 / (TH_2 - TH_1)^2 & \rightarrow TH_1 \geq f(p) \geq (TH_1 + TH_2) / 2 \\ 2(f(p) - TH_2)^2 / (TH_2 - TH_1)^2 & \rightarrow (TH_1 + TH_2) / 2 \geq f(p) \geq TH_2 \\ 0 & \rightarrow TH_2 \geq f(p) \end{cases} \quad (2)$$

Where A is a weighting constant and $f(p)$ is the grey value of pixel p . The specific numerical values of w, TH1 and TH2 are process-related and inherently depend on the scanning parameters and characteristic histograms of the dataset. As such, these values cannot be a priori assumed and were determined by visually inspecting the microCT datasets. In this study, the values of w, TH1 and TH2 were 6 pixels, 38 and 62 out of 256 grey levels, respectively.

- **Bone/soft tissue interface diffusion** (Step I.3)

This step began by finding the initial seeds for the purpose of diffusing the bone edges into soft tissue. The initial seeds, and more precisely the seed voxels, were identified in the interfaces of the bone and soft tissue, in addition to the ceramic/soft tissue. Using a Gaussian function [35], the seed voxels were then used as initial seed values for an iterative process to diffuse bone edges into the neighboring voxels of the soft tissue. Specifically, once the bone found in the macro-porous space was diffused into the soft tissue, the microCT having two phases (ceramic and soft tissue) remained. Diffusing the bone implied assigning the subset of all voxels of bone/soft tissue interface a grey-intensity value that is similar to the one of bone. This diffusing step eliminated any important grey-intensity gradients between the soft tissue and bone, thereby leaving the microCT image dataset with the grey-intensity gradients mainly pointing towards the ceramic remnant phase.

- **Segmentation of bone and scaffold remnants** (Step I.4)

Step I.4 is identical to Step I.2 used to segment the soft tissue. The initial seeds of the ceramic were found using the Sobel filter, accordingly forming an initial contour. Using an energy-based active contour method, referred to above, the ceramic remnant pixels were iteratively isolated. Once both the ceramic phase (Step 4), and the soft tissue phase (Step 1) were isolated, the remaining unidentified voxels were clearly those of the bone deposited within the macro-porous space.

3D alignment of pre- and post-implantation scaffolds (Stage II)

The 3D alignment process comprised three steps: (1) coarse alignment, (2) machine-based alignment using feature detection, and (3) 3D optimization for aligning the original and explanted scaffolds. The three subsequent steps aimed at finding the 3D rotation angle and displacement matrix, referred to as a transform (Fig. 3), and aligning the scaffold before and after implantation, using the microCT datasets.

- **Coarse alignment (Step II.1)**

In this step, the z-direction projection maps, referred to as the vertical projection, of the microCT image stacks of the pre- and post-implantation scaffolds were used to calculate the initial values of the geometric transform, as explained by the following sequence:

- I. Calculate the z-rotation angle using the volume fraction/projection maps. Once these maps or vertical projection were created as described below, common recognizable features/landmarks in the scaffold before and after implantation became visible.
 1. Add the scaffold's volume fractions in the z direction of the pre-implantation microCT stack.
 2. Add the scaffold's volume fractions in the z direction of the post-implantation microCT stack.

3. Find visually the common and recognizable landmarks in the volume fraction maps/projections.
 4. Calculate the θ'_z angle
 5. Accordingly rotate the pre-implantation microCT image stack
- II. Calculate the x and y rotation angles:
1. Make longitudinal sections at the center, perpendicular to the x axis of the pre-implantation and post-implantation image stacks.
 2. Find common recognizable landmarks within the longitudinal sections (human assisted).
 3. Calculation of θ'_x
 4. Repeat Steps II.1-3 for the y direction and calculate θ'_y
 5. Find the displacement T'_z in the z-direction between the pre- and post-implantation image stacks.

This stage resulted in an initial/coarse calculation of the values of the rotation angles (θ'_x, θ'_y and θ'_z) and the z-direction displacement T'_z

- **Alignment using SURF feature- or landmark-based registration (Step II.2)**

Following the coarse alignment, the Speed Up Robust Features (SURF) algorithm [36], which is an efficient geometric feature detector, was used to register or align the pre- and post-implantation scaffolds more accurately. Specifically, the SURF algorithm found the unique geometric landmarks/forms in both datasets. These features were matched and classified in accordance with the strongest match. A voting criterion for the matching strength was then applied to calculate the 2D geometric transform of the x-y plane. Step II.2 led to refined values of z rotation, x and y displacements, θ'_z, T'_x and T'_y respectively (equation 3).

$$\begin{bmatrix} x'' \\ y'' \\ 1 \end{bmatrix} = \begin{bmatrix} \cos(\theta_z'') & -\sin(\theta_z'') & T_x'' \\ \sin(\theta_z'') & \cos(\theta_z'') & T_y'' \\ 0 & 0 & 1 \end{bmatrix} \begin{bmatrix} x' \\ y' \\ 1 \end{bmatrix} \quad (3)$$

- **3D alignment optimization** (Step II.3)

The much-improved geometric transform of Step II.2 was then used as the input ‘initial values’ for the least-square final 3D alignment optimization using the following procedure:

- I. Initialize the 3D geometric transform with the results of Step II.2
- II. Choose the rotation angle range from $\theta_x'' - \theta_{diff}$ to $\theta_x'' + \theta_{diff}$, $\theta_y'' - \theta_{diff}$ to $\theta_y'' + \theta_{diff}$ and $\theta_z'' - \theta_{diff}$ to $\theta_z'' + \theta_{diff}$.
- III. Choose the displacement range from $T_x'' - T_{diff}$ to $T_x'' + T_{diff}$, $T_y'' - T_{diff}$ to $T_y'' + T_{diff}$ and $T_z'' - T_{diff}$ to $T_z'' + T_{diff}$.
- IV. Calculate the Sum of Squared Difference (SSD) as follows.
 1. For θ_x = all steps between $\theta_x'' - \theta_{diff}$ and $\theta_x'' + \theta_{diff}$ with step $\Delta\theta_x$
 2. For θ_y = all steps between $\theta_y'' - \theta_{diff}$ and $\theta_y'' + \theta_{diff}$ with step $\Delta\theta_y$
 3. For θ_z = all steps between $\theta_z'' - \theta_{diff}$ and $\theta_z'' + \theta_{diff}$ with step $\Delta\theta_z$
 4. For T_x = all steps between $T_x'' - T_{diff}$ and $T_x'' + T_{diff}$ with step ΔT_x
 5. For T_y = all steps between $T_y'' - T_{diff}$ and $T_y'' + T_{diff}$ with step ΔT_y
 6. For T_z = all steps between $T_z'' - T_{diff}$ and $T_z'' + T_{diff}$ with step ΔT_z
 7. $Scaffold_{pretrans}$ = geometrical transformation of scaffold pre-implantation multiplied by transform $(\theta_x, \theta_y, \theta_z, T_x, T_y, T_z)$
 8. Calculate the SSD

$$SSD = \sum \left| (scaffold_{post} - scaffold_{pretrans}) \right|$$
 9. Store SSD value

10. End of all loops
11. $SSD_{min} = \min(SSD)$
12. $Transform_{min} = Transform(SSD_{min})$

The geometric transform ($Transform_{min}$), which is the minimum sum of squared difference (SSD) between the pre- and post-implantation scaffold served for the final refined segmentation (Stage III).

Refined segmentation (Stage III)

Once the alignment of the pre- and post-implantation scaffolds was completed, the three phases present in the explanted scaffold were isolated by comparing with the pre-implantation scaffolds. Specifically, a dense material found in the macro-porous space of the initial scaffold was identified as bone deposited in the macro-porous space. Dense material found in regions previously occupied by ceramics was identified as ceramic or scaffold remnants. Once the bone and ceramic remnants were found, the void phase left was identified as the soft tissue phase. Accordingly, characteristic histograms could be found of the three phases. These characteristic histograms were used, as described below, to segment the dataset of the explanted scaffold, in particular the bone and ceramic remnants:

1. Using its characteristic histogram, the maximum grey value of bone was determined (THBmax).
2. Accordingly, uncertain regions of newly deposited bone (i.e. deposited at space occupied by ceramic in the initial scaffold) of grey value greater than THBmax were identified as scaffold.
3. Similarly, using its characteristic histogram, the minimum grey value of ceramic was determined (THCmin).
4. Uncertain regions of scaffold (i.e. scaffold fragments at macro-porous space of the initial scaffold) of grey level less than THCmin were revised and identified as bone.

Validation of the segmentation algorithm

The segmentation algorithm, stage I-III, was rigorously validated by comparing its results to those from a laborious manual segmentation process done by a trained image-processing scientist, referred to as the operator. In addition, a histology section, which was cut and prepared from the explanted scaffold, was used as a reference for the results obtained from the present segmentation algorithm. Specifically, the histology section, surface-stained with toluidine blue, rendered the three phases (void, bone and ceramic) of the explanted scaffold visible. In addition, the cutting plane of the histology section was manually found within the 3D microCT dataset of the explanted scaffold. A direct comparison of the histology staining results with the algorithmic segmentation results was thus possible.

Five out of the 600 slices, which constituted the post-implantation microCT stack, were randomly selected for the purpose of validation. Each slice/image consisted of 445,440 pixels. Specifically, because of the large number of pixels to process, a quarter of each microCT slice (111,360 pixels per each quarter and a total 556,800 pixels) was manually segmented by the scientist and identified as one of the three phases (bone, ceramic or soft tissue). To manually segment the three phases in the microCT images, the trained scientist utilized both the grey-intensity gradients and the a priori known structural information of the pores and scaffold forms. The similarity matrix was then used to globally compare the algorithmic and operator-based segmentation results. Specifically, the sum of the similarity matrix diagonal elements measures the agreement in the segmentation results of the new algorithm compared with the results of the manual segmentation. In addition, the Dice similarity coefficient (DSC) [37] of the 3 phases, a detailed comparative value (Eq. 4), was used to further compare the manual and algorithmic results:

$$D_p(P_{oper}, P_{alg}) = \frac{2|P_{oper} \cap P_{alg}|}{|P_{oper} + P_{alg}|} \quad (4)$$

Where D_p is the DSC comparing the operator (P_{oper}) and the algorithm-based (P_{alg}) segmentation results of phase P , i.e. bone, scaffold or soft tissue. Moreover, the results of the newly developed method were also compared with the commonly used Otsu's segmentation method [26, 28, 29, 38]. Specifically, the Otsu's method uses statistical techniques to perform global image thresholding. In addition to the validation, the new method was rigorously verified by inspecting the result of every step of the Matlab code (The MathWorks, Inc., Natick, MA, USA).

Statistical Methods

The paired t-test was applied to determine if the results of two segmentation methods, as provided in the similarity matrix summarizing the results of the segmentation methods in the form of the mean and of the standard deviation for the three phases (bone, ceramic remnants, soft tissue), were significantly different from each other. Accordingly, the mean of the surface area fraction of each of the three phases averaged over the five aforementioned quarters provided by the operator-based segmentation was compared to the respective mean provided by one of the two other segmentation groups (i.e. the new segmentation algorithm or Otsu's method). The confidence intervals and indicators used to test for the statistical significance are detailed in the following sections.

Results

The results of the algorithmic segmentation of an example microCT dataset (638x696x600 voxels) are shown in Fig. 4. A raw microCT image in the x-y plane is presented in Fig. 4a. The ceramic, present in the center of the image, is slightly brighter and more radio-dense than both the host bone surrounding the round scaffold, and the bone formed with the scaffold. The ceramic morphology and the round pore geometry can still be seen at 6 weeks in the center of the scaffold. However, the closer to the periphery of the scaffold, the more resorption, the more bone deposition, and the more the initial scaffold morphology changed. Fig. 4b shows an example result for the initial segmentation, stage I, of the microCT dataset. The results of aligning the post- and pre-implantation scaffolds are shown in Fig. 4c and Fig. 4d for the feature-based (step II.2) and optimization alignment (step II.3) algorithms respectively. The reference pre-implantation substitute morphology is shown in blue. Ceramic remnants of the explanted scaffold are shown in red. Ceramic fragments are shown in brown. The SSD optimization refined the alignment, as seen from Fig. 4d versus Fig. 4c. The phases classified in Fig. 4f accordingly provided the characteristic grey-value histograms of both the bone and ceramic remnants (Fig. 6a and Fig. 6b), which were then used to refine the initial segmentation (Fig. 4b). The initial segmentation overlapped with the aligned pre-implantation microCT is depicted in Fig. 4e (red = ceramic remnants, blue = initial scaffold morphology, gold = bone, soft tissue = white, black = new bone locally replacing the ceramics, brown = fragmented ceramic). Small ceramic fragments appeared integrated in bone. Ceramic fragments, found in the host bone, which were outside the defect side, were re-segmented to bone (Fig. 4f).

Figure 5 shows the averaged volume fraction/projection map in the z longitudinal direction of both pre- and post-implantation microCT datasets. Three landmarks, used in the rotation around the z axis for the coarse alignment step, were depicted in both images. The three landmarks, forming a triangle, resulted in three rotation angles that were used for the coarse alignment. The characteristic results from the alignment process are shown in Fig. 6. The characteristic histograms of the ceramic remnants and of the neo-bone deposited in the macro-porous space are shown in Fig. 6a and 6b respectively. The highest grey values of the ceramic remnants (THCmax) and of bone (THBmax) are 92 and 61 respectively. The post-alignment grey-value image of the defect is shown in Fig. 6c. In it, the bone can be distinguished from the ceramic. Using the characteristic histogram of bone and ceramic remnants, the results of the refined segmentation is shown in Fig. 6d.

The histology section, cut perpendicular to the longitudinal axis of the explanted scaffold and then stained, is in Fig. 7a. The optical resolution of the histology section was 0.92 micron. The microCT grey-value section, corresponding to the histology section was manually found in the post-implantation dataset (Fig. 7b). A comparison of the segmentation results of the present new method with those of Otsu's method are shown in Fig. 7c and Fig. 7d, respectively. The Otsu's thresholding method globally overestimated the bone versus the soft tissue, and it overestimated the ceramic remnants versus the bone. The qualitative visual comparison of the histology section and the present segmentation showed they were in a relatively good agreement. For the quantitative validation, 5 microCT slices of the explanted scaffold were randomly selected. The validation results are shown in Fig. 8 for a sample quarter of a randomly

selected grey-value microCT image. The operator- versus the algorithmic-based segmentations of the three phases found is in Fig. 8b and 8c, respectively. Fig. 8d shows the transitions/interfaces among the three phases as detected by the operator. Both the manual segmentation and the present algorithm produced similar results. The similarity matrix of the segmentation results of five randomly selected quarters with a total of 556,800 pixels is summarized in Table 1. Specifically, the total similar surface fraction, which is the sum of the similarity matrix diagonal elements, in the manual and algorithmic segmentation, was $94\pm0.8\%$. The total similar surface fraction in the manual and standard Otsu's segmentation based on these five randomly selected quarters was $80.4\pm2.2\%$ (Table 2). Moreover, the Dice similarity coefficients (the new algorithmic versus manual results) were $92.5\pm1.1\%$, $89.3\pm3\%$ and $96.3\pm0.8\%$ for bone, ceramic remnants and soft tissue, respectively. The Dice similarity coefficients (Otsu's versus manual results) for bone, ceramic and soft tissue were $77.2\pm2.3\%$, $76.1\pm6.4\%$ and $84.8\pm3.7\%$, respectively.

Discussion

In this study, we developed, verified and validated a novel multistage algorithm for the segmentation and alignment of scaffolds of resorbable bone implants, using the pre- and post-implantation microCT datasets. The algorithm comprised three stages: (I) initial segmentation of post-implantation microCT data; (II) alignment and registration of pre- and post-implantation scaffolds; and (III) refinement of the segmentation using the characteristic histograms resulting from stage II. The novel algorithm was rigorously validated by comparing the algorithmic to the operator-based segmentation using the Dice Similarity Coefficient (DSC) [37]. The DSC is a spatial overlap index. The higher the DSC value the greater the similarity of the two subsets or objects. The manual segmentation was very labor intensive and required over five months to complete. Nevertheless, it was required for a proper validation of the new method using an actual microCT image dataset of an explanted scaffold. Specifically, the algorithmic segmentation gave results closely matching the histology section and manual segmentation, when compared to the results of segmentation by the commonly used Otsu's method [26, 28, 29, 38]. Unlike the present algorithm (Fig. 7c), Otsu's method globally under-segmented the bone phase vis-à-vis the soft tissue (Table 2), which led to the loss or merge of structural features as shown in the upper box of Fig. 7d. Except for the transitions/interfaces between two phases such as in the yet unremodeled center of the scaffold, Otsu's method resulted in erroneous segmentation of the interface between ceramic remnants and soft tissue (Fig. 7d). This was partly because of the fuzzy, unclear transitions between phases in microCT data [22], and of the statistical global approach

of Otsu's method [26, 28, 29, 38, 39]. The fuzzy blurred grey-intensity transition resulted from the scanning process and are inherent to the discretization process. Accordingly, a sigmoidal edge segmentation function and the contour-based method markedly enhanced the segmentation results [34, 40, 41].

Prior to segmentation, the microCT dataset was subject to routine image enhancement, which included denoising [35] and image sharpening using an unsharp mask [30, 33]. For added accuracy, anisotropic denoising was used in preference to the isotropic Gaussian denoising process [17, 42]. In addition, the iterative active contouring algorithm combined with the alignment of pre- and post-implantation datasets was crucial to the accurate segmentation algorithm. Specifically, the alignment provided characteristic histograms of bone and ceramic remnants, which were otherwise not accessible. The coarse alignment, which was manually done (Fig. 5), resulted in calculating the initial value of the 3D geometric transform. The 2D SURF feature-based detection led to a more precise geometric transform for the x-y plan, but it was not sufficient because of the large resorption in the explanted scaffold. The 3D SSD minimization alignment, even though computationally demanding, resulted in the accurate alignment, and consequently segmentation.

The novel algorithm is structured and systematic. For example, the initial segmentation (stage I) was essential for the feature detection and, accordingly, the alignment process (stage II), which will otherwise not converge because of the vague features and different scanning parameters of the original and explanted scaffolds. It is the structural alignment that further allowed isolating the scaffold remnants, the new bone formed in

the pore space and the new bone formed by the replacing of the ceramic walls. Moreover, the alignment also allowed for the accurate determination of the boundaries of the defect site, Fig. 4. The characteristic histograms of the new bone and scaffold ceramic remnants became inherently available, the new bone deposited into regions wherein the scaffold material was resorbed was consequently identified, and the segmentation was further enhanced.

A similarity matrix value of $94\pm0.8\%$, based on processing five randomly selected image quarters, with a total of 556,800 pixels indicated the agreement between the novel algorithmic and the operator-based segmentation (Table 1). The $94\pm0.8\%$ value was the sum of the diagonal elements of the similarity matrix (Table 1). The similarity matrix, based on the standard Otsu's method versus the manual segmentation, had a similarity value of $80.4\pm2.2\%$ (Table 2). More specifically, in terms of the surface area fraction of each of the three phases, there was no significant difference between the segmentation result of the present algorithm compared to the manual segmentation at $p < 0.8$, $p < 0.12$ and $p < 0.43$ for the bone, ceramic remnants and soft tissue, respectively. The mean and standard deviation of the surface area fraction of the bone, the ceramic remnants and the soft tissue were $38.3\pm3.1\%$, $12.9\pm2.5\%$ and $48.8\pm4.3\%$ using the present algorithm compared to $38.3\pm2.4\%$, $13.3\pm2.9\%$ and $48.3\pm4.3\%$ using the manual segmentation. The paired t-test was also applied to evaluate the similarity between the segmentation results of Otsu's method compared to the manual segmentation. Specifically, the Otsu's segmentation results of the bone and ceramic remnants in terms of the surface area fractions were $47.3\pm3.8\%$ and $16.8\pm1.5\%$, respectively. These surface area fraction results were significantly higher than those of the manual

segmentation at $p < 0.0003$ and $p < 0.01$, respectively. In terms of the soft tissue fraction, the result of Otsu's method was $35.9 \pm 5.3\%$, which is lower than that, $48.3 \pm 4.3\%$, of the manual segmentation at $p < 0.000046$. Although the validation was very laborious and time consuming, it is essential for establishing confidence in the segmentation results, which will be used for evaluating the complex structures related to bone deposition and scaffold resorption. Furthermore, the comparing of the same microCT and histology section, shown in Fig. 7, confirmed the validity of the present segmentation algorithm. Specifically, Fig. 9, which is an expanded view of the central pore region shown in Fig. 7, shows a good agreement with the histology finding, in particular with respect to the bone formed. The microCT results overestimated the ceramic fraction for the 30-micron resolution compared to the 0.9-micron resolution of the histology section, which makes the ceramic microporosity visible in the histology section photograph. However, a more detailed comparison clearly goes beyond the scope of the present article and will be the subject of future research.

Another advantage of the alignment, besides the higher segmentation accuracy, is the study of the newly deposited bone and the scaffold resorption, in addition to relating them to the initial architectural properties of the pre-implantation scaffold. A priori, the 3D neo-bone and regional resorption geometries and fronts became available, and this is a relevant tool for understanding the biological repair process and its interaction with the scaffold design. The novel segmentation and alignment processes were laborious and required significant image-processing skills. Nonetheless, they are, in our experience, applicable to other scaffolds of different pore shape or of smaller macro pores as long as sufficient geometric features remain unchanged in the explanted

scaffold. A necessary condition of the new segmentation process is that at least three unique non-collinear pore features, not falling on the same straight line, can be found, as shown in Fig. 5. The uniqueness implies variable shape, size, and distribution of the pores in the scaffold. Furthermore, if the scaffold would break or disintegrate largely, it would create a complex case for the applicability not only of this new segmentation protocol but also of other processing protocols because of the structural breakdown of the scaffold. The applicability of the present algorithm was further demonstrated using three additional samples of the β -TCP scaffolds. The first two samples, Fig. 10a-d confirmed the algorithm applicability to two different pores sizes and shapes. Whereas the first sample, Fig. 10a-b, had a large average pore size with a relatively unique pore shapes and with limited number of pores (Fig. 10a-b), the second sample had a smaller average pore size and a more uniform spherical pore shape (Fig. 10c-d). The two samples were explanted at the 6-week time point, whereas the third sample (Fig. 9e-f) was explanted at the 12-week time point. As seen in Fig. 10e-f, except for the ceramic in the center of the defect site, the β -TCP ceramic has been resorbed. Still, the alignment and segmentation algorithm was successfully applied once three non-collinear features (Fig. 10e-f) can be found in the pre- and post-implantation datasets.

Increasing the pore-to-voxel size ratio would further improve the results of the novel method. However, by using a voxel size smaller than 10 microns, the ceramic microporous and grain structure would become visible, thus significantly increasing the computational cost of the novel method. Reducing the pore-to-voxel size ratio renders the alignment imprecise. In our experience, to evaluate the biological process at the macropore porosity level, a pore-to-voxel size ratio of 25 or higher is required.

However, this ratio should not be increased to the limit where the microstructure becomes visible, unless the scientist seeks to study the micropore level effects and interactions. The computational cost of the algorithm depends on a number of factors including the sample size, the code implementation technique and the computing machine. Using a parallelized computing on a 12-core machine (Intel® Xeon® Processor E5-2670 v3 and 64 GB RAM), the required processing time per sample in the study was approximately two days.

The knowledge gained of the regional resorption and bone deposition can be significant. Some blue staining was observed in the histology section within the ceramic remnant, indicating the presence of bone tissue phase within the ceramic walls [25]. Further analysis of the phases present in the ceramic walls requires detailed investigations and new image-processing techniques/modalities, including the high-resolution back-scattered SEM imaging to accurately segment the densified mineral tissue, among others that tissue found in the microporous space, in the histology staining photographs. This will be the focus of future research and future articles.

Conclusion

A novel technique for studying the post-implantation behavior of resorbable CaP bone substitutes using microCT data was developed. In addition to the more accurate segmentation protocol, the alignment of pre- and post-implantation microCT datasets enabled accurate determination of the boundaries of the defect site. This 3D alignment process is unprecedented in terms of localizing the defect site, identifying the global and local resorption of substitutes, classifying the resorption into ceramic zones substituted by bone and others not substituted by bone, and classifying the bone into the host bone, bone formed in the pore space, and finally bone directly replacing the ceramic walls. Recognizing and finding these detailed effects will allow the scientist to study the bone repair process in more details and with higher accuracy. Using the similarity coefficients, the technique was validated and demonstrated a 94-percent accuracy when compared to the operator-based evaluation. Even though the resorption notably altered the geometry of the explanted scaffold, the multi-stage alignment method, using the SSD, converged efficiently. The present novel approach extends the use of the commonly used microCT technique for studying the interactions of the *in vivo* biological processes. The new technique also enhances the accuracy of the morphological and morphometric analyses for the explanted scaffold. Consequently, it could be a step toward a more accurate understanding of the biological processes inside bone implants, and therefore is of interest to the biomaterial scientist.

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Fig. 1. Block diagram of the presented algorithm.

Fig. 2. The sigmoidal weighting functions of the grey-value and distance transitions.

Fig. 3. A schematic of the initial (left) and explanted (right) scaffold. Orthogonal coordinate systems for the 3 rotations and 3 displacements sought in the alignment process

Fig. 4. An example of the alignment and segmentation results: (a) A microCT slice of the explanted β -TCP scaffold; (b) results of the initial segmentation; (c)

alignment result from the feature-based detection (blue = resorbed scaffold not replaced by bone, red = ceramic remnants). The alignment appeared imprecise; (d) the result of 3D alignment using SSD-based optimization; (e) alignment-based segmentation (red = ceramic remnants, blue = resorbed scaffold not replaced by bone, gold = bone, black = ceramic replaced by bone, brown = apparent ceramic fragments in locations where no ceramic was present at the time of implantation); and (f) ceramic fragments outside the defect site were re-segmented to host bone. The arrows indicated two such ceramic fragments. The calibration bars apply to all subfigures.

Fig. 5. Summed Volume fraction (SVF) / z-direction projection maps of ceramic remnants of the explanted (left) and initial (right) scaffold, respectively. Three representative landmarks are common to both vertical maps were used for the course alignment.

Fig. 6. Characteristic histograms of the ceramic remnant (a) and the neo-bone found within the defect site (b), respectively. (c) Characteristic grey-value image of the defect site. (d) Refined segmentation using the characteristic histograms and using the same color scheme of Fig. 4f. The two arrows indicated locations that were over Fig. 4f re-segmented to bone or ceramic fragments displaced in the pore space, respectively (red = ceramic remnants, blue = resorbed scaffold not replaced by bone, gold = bone, black = ceramic replaced by bone, brown = ceramic fragments identified in locations where no ceramic was present at the time of implantation). The calibration bar applies to subfigures (c) and (d).

Fig. 7. Comparing the results of the present method and Otsu's method to the histology section and to the corresponding microCT slice: (a) the histology section; (b) the microCT slice of the same histology section (c) segmentation of the present algorithm; and (d) segmentation of Otsu's method (red = ceramic remnants, gold = bone). The calibration bar in (d) applies to subfigures (c) and (d).

Fig. 8. Validation results of one randomly selected quarter. (a) the gray level microCT slice; (b) the operator-based segmentation; (c) the algorithm-based segmentation; and (d) the inspected interface pixels required judgement by the operator. The calibration bar applies to all subfigures.

Fig. 9. A comparison of the present segmentation result with the histology evaluation of the central region of one and same microCT and the histology section, shown in Fig.7. (a) the toluidine blue histology section; (b) the manual histology section evaluation; (c) the microCT section aligned with the histology section; and (d) the segmentation result of the microCT section using the present algorithm (red = ceramic remnants, gold = bone). All calibration bars are 500 µm.

Fig. 10. The application of the present segmentation algorithm to implants of different average pore sizes and implantation time points: (a) a gray level microCT slice of a 1.22-mm pore size scaffold at 6-week time point; (b) the segmentation result of the micro-CT slice in (a); (c) a gray level microCT slice of a 0.51-mm pore size scaffold at 6-week time point; (d) the segmentation result of the micro-CT slice in (c); (e) a gray level microCT slice of a 1.22-mm pore size scaffold at 12-week time point; (f) the

segmentation result of the micro-CT slice in (e) (red = ceramic remnants, blue = resorbed scaffold not replaced by bone, gold = bone, black = ceramic replaced by bone, brown = ceramic fragments identified in locations where no ceramic was present at the time of implantation).

Table 1. Similarity matrix (manual versus algorithmic segmentation) where BSF = bone surface/total surface, SSF = scaffold remnants surface/total surface, and STSF = soft tissue surface/total surface. All numbers are expressed in the form of mean $\pm$ standard deviation.

		Algorithm-based			Total
		BSF (%)	SSF (%)	STS F (%)	
Operator-based	BSF	35.5 $\pm$	1.0 $\pm$	1.9 $\pm$	38.3
	(%)	2.8	0.3	0.4	$\pm$ 2.4
	SSF	1.4 $\pm$ 0	11.8	0.1 $\pm$	13.3
	(%)	.3	$\pm$ 2.8	0.1	$\pm$ 2.9
	STS	1.4 $\pm$ 0	0.2 $\pm$	46.8	48.3
	F (%)	.9	0.2	$\pm$ 4.2	$\pm$ 4.3
Total		38.3 $\pm$	12.9	48.8	
		3.1	$\pm$ 2.5	$\pm$ 4.3	

Table 2. Similarity matrix (manual versus Otsu's method segmentation) where BSF = bone surface/total surface, SSF = scaffold remnants surface/total surface, and STSF = soft tissue surface/total surface. All numbers are expressed in the form of mean $\pm$ standard deviation.

		Otsu's method			Total
		BSF (%)	SSF (%)	STS F (%)	
Operator-based	BSF	33.1 $\pm$	5.2 $\pm$	0.04	38.3
	(%)	2.6	1.5	$\pm$ 0.07	$\pm$ 2.4
	SSF	1.8 $\pm$ 0	11.6	0.0 $\pm$	13.3
	(%)	.9	$\pm$ 2.4	0.0	$\pm$ 2.9
	STS	12.5 $\pm$	0.0 $\pm$	35.8	48.3
	F (%)	2.0	0.0	2 $\pm$ 5.28	$\pm$ 4.3
Total		47.3 $\pm$	16.8	35.9	

3.8 ± 1.5 ± 5.3

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Calcium-phosphate scaffolds are being increasingly used to repair critical bone defects. Methods for the accurate characterization of the repair process are still lacking. The present study introduced and validated a novel image-processing technique, using micro-computed tomography (mCT) datasets, to investigate material phases present in biopsies. Specifically, the new method combined mCT datasets from the scaffold before and after implantation to access the characteristic data of the ceramic for more accurate analysis of bone biopsies, and as such to better understand the interactions of the scaffold design and the bone repair process.