

A method for direct correlation of 2D-micro-computed tomography datasets onto histomorphometric slides to achieve quantification of 3D osteoid volume in bone substitutes.

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Aims

Micro-Computed Tomography (mCT) belongs to the most powerful tools for the three-dimensional (3D) assessment of bone and scaffold architecture [1]. While it is possible to refer to clear landmarks in mCT scans of actual bone structure, the assessment of calcified osteoid within scaffolds is problematic, due to the missing obvious morphological correlates and landmarks [2].

Therefore, bone formation within scaffolds is mostly analyzed using indirect parameters such as changes in volume or surface alteration, preserving histomorphometry the gold standard in the direct analysis of bone formation: Newly formed bone tissue, that is mostly calcified extracellular matrix (osteoid) within scaffold structures can be quantified directly, for example by calculating the percentage area of osteoid within the total 2D-size of the respective 2D-scaffold region [2, 3]. However, mCT provides the possibility to quantify the 3D volume of the scaffold without further effort, whilst 3D quantification in histomorphometry is extensively time consuming [4].

Crucial for reliable mCT-analysis in scaffold research is the segmentation of the obtained and reconstructed datasets [2]. Therefore, certain grey-value ranges are excluded from the dataset as described previously [2]. Hypothetically, osteoid structures within scaffolds could be assessed by finding the grey-value-range that is representing calcified tissue by setting a defined threshold. This would subsequently allow excluding soft tissue or the scaffold structure, as well as artifacts and could lead to a direct evaluation of the amount of calcified osteoid within the volume of interest (VOI).

However, the respective osteoid-threshold cannot be clearly identified by analyzing the mCT-scans, because of the missing clear correlates. By creating an overlay of mCT-slices with the corresponding histomorphometric section the calcified osteoid could clearly be identified by setting corresponding ROIs in the mCT and histomorphometric slice. From the ROI in the mCT-dataset grey values can be extracted and applied to the VOI to determine the volume of the osteoid. This approach would combine the major advantages of mCT and histomorphometry.

Method

General

Osteoid formation of β -Tricalciumphosphate (β -TCP) scaffolds seeded with human mesenchymal stem cells (MSC) was assessed using standardized *in-vivo* tissue engineering protocols obtaining ectopic osteoid formation. MSC were harvested from five donor patients. The patients underwent surgical treatment for atrophic non-unions at Heidelberg University Hospital and gave informed consent to harvest cells during surgery according to the declaration of Helsinki in its present form prior to the operation. The procedure was approved by the ethics committee of the Ruprecht-Karls-University of Heidelberg (S-355/2010). Isolation, cultivation, and the expansion of the MSC followed established protocols [3].

Scaffold preparation and mCT-procedure

Scaffolds were seeded with MSC and underwent implantation into severe combined immunodeficiency (SCID) mice for eight weeks. After explantation, scaffolds were embedded in paraffin and underwent mCT-scanning using a SkyScan 1076 scanner and the following specifications: tube current 200 μ A, voltage 50kVp, pixel size 9 μ m. mCT-datasets were reconstructed using NRecon.

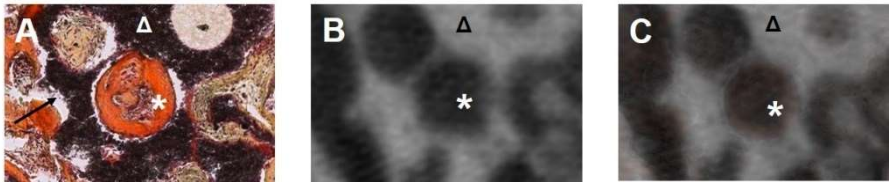


Figure 1: Registration process, showing representative steps. Prominent structures (A, \rightarrow) alongside the osteoid tissue (A, *) are detected in histomorphometry (A) and in the corresponding region in the corresponding mCT-slice (B). Dataviewer is used to create an overlay (C) of the two modalities. Subsequently, the osteoid (+) can be clearly identified and differentiated from the surrounding structures such as the β -TCP (Δ). The ROI representing the osteoid (B, *) is set and transferred to CTAn to identify the respective grey-value-range. Soft-tissue-thresholding not shown.

Histomorphometric processing

After the mCT-scanning-procedure, the paraffin-embedded scaffolds underwent histomorphometric processing. Slides with a thickness of 4.5 μ m each were prepared. Prior to cutting, the reconstructed mCT-datasets were analyzed in detail to determine the planes of interest within the implant. An imaginary z-axis was placed through the implant, beginning orthogonally to the first cutting plane and going from there through the whole VOI. Several regions on the z-axis of the implant were defined and the cutting and staining processes were subsequently adapted, thus the number of 4.5 μ m cuts from the beginning of the implant were counted and related to the position on the z-axis, selected in mCT. The slides were stained using a hematoxylin-eosin-saffron (HES) staining to provide clear visual distinction of osteoid from surrounding tissue and β -TCP (Fig. 1A). Care was taken to ensure the blocks were processed in exactly the same orientation they had during the mCT-scan.

Registration process

The histomorphometric slices and mCT scans were screened to find the best possible matches. The first slice of the mCT-dataset was defined showing scaffold structures. This slice was matched with the histomorphometric section and adapted if the matching was not yet suitable: with a pixel size of 9 μ m in the mCT scan, two histomorphometric slides (each cut at 4.5 μ m thickness) corresponded to one mCT-slice (Fig. 2B).

The corresponding slices were registered to each other using the 2D registration function of DataViewer (Fig. 2C) aiming to create an exact overlay to define the ROI (calcified osteoid in this case) in the mCT slice.

Thresholding process

The ROI defined from the overlay image was transferred to the mCT-dataset, opened in CTAn. From the defined ROI, the grayscale values were extracted and defined as the “osteoid threshold”. Furthermore, an upper and a lower threshold was defined to create a global threshold using the same method. For the upper threshold, a ROI was marked in the β -TCP parts of the scaffold, the lower threshold was defined by assigning a ROI in the soft tissue at the outer boundaries of the scaffold.

Calculation of percentage osteoid area and 3D osteoid volume in mCT

The area of the osteoid in the respective 2D-slice and the volume of osteoid in the whole scaffold were calculated by CTAn. The “osteoid threshold” was applied to the mCT dataset and divided by the values obtained after global thresholding.

Calculation of percentage osteoid area in histomorphometry

Aperio ImageScope (Leica Biosystems, Germany) was used to calculate the percentage amount of osteoid in the 2D histomorphometric section by applying the positive pixel count algorithm supplied by the manufacturer with some modifications. Pixels revealing the reddish-colored osteoid were referred as “positive”, the other pixels as “negative”. Percentage osteoid area was calculated by dividing “positive” by “negative” pixel counts.

Troubleshooting

The osteoid tissue appears to be of significantly lesser density compared to the surrounding β -TCP parts of the scaffold, leading to halo artifacts between the osteoid and the high-density β -TCP (Fig. 2). For reduction, the CTAn-specification “morphological operations” was used following recommendations of the Bruker micro-CT Academy newsletter 08/2015 [5]. By modification of the dataset using the “opening”-operation binarized objects with thickness equal or less than twice the original opening pixel radius were removed leading to a significant reduction of the halo artifacts.



Figure 2: Halo artifact reduction: the raw 2D mCT-slice showing osteoid formation (A, *). After applying the osteoid threshold to the dataset, halo artifacts (B, →) occur alongside the osteoid (B, *). After application of the morphological operations-specification as described above, the halo artifacts are significantly reduced (C, →) while the osteoid tissue is still imaged (C, *).

Statistics

Results obtained by both methods were compared using the Mann Whitney-test, correlation of the results were analyzed by Spearman’s rank correlation coefficient. Statistical analyses and graphs were made using GraphPad Prism (Version 5.01, GraphPad Software, La Jolla, USA). Results were described as statistically significant for $p < 0.05$.

Results

Percentage osteoid area detected in histomorphometry and mCT

Osteoid formation was 8.65% in histomorphometry, 8.51% in 2D mCT and 7.45% in the VOI analyzed by application of the thresholds on the 3D mCT dataset (Fig. 3). The differences were not significant ($p=0.762$ for histomorphometry vs. 2D mCT; $p=0.353$ for histomorphometry vs. 3D mCT; $p=0.529$ for 2D mCT vs. 3D mCT).

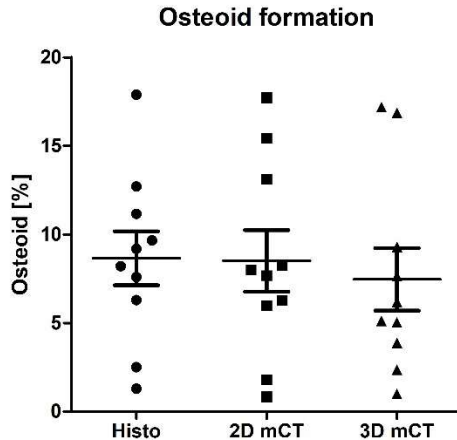


Figure 3: Osteoid formation depending on the methods. Data shown as mean and standard error of the mean. No significant differences between the methods was found. Histo: histomorphometry.

Correlation between the methods

To assess whether the results obtained by the different methods are comparable, correlation was tested for all combinations (Fig. 4, A-C). The inter-methodological comparison revealed strong correlations (>0.8), making the results obtained by the different methods comparable.

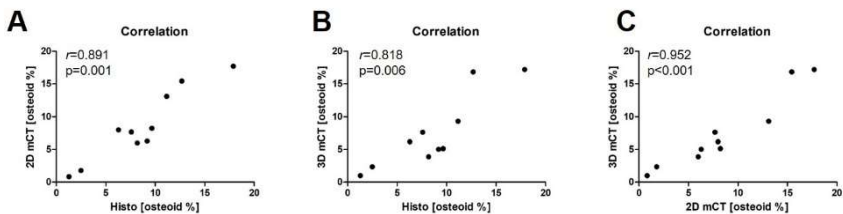


Figure 4: Correlation analysis between the methods.

Conclusion

The method presented allows for clear identification of osteoid in histomorphometry and accurate transfer to mCT datasets. Results obtained by the different approaches show strong correlations, making the quality of the methods comparable. The presented approach therefore combines the advantages of histomorphometry and mCT in the research field concerning bone substitutes. By application of the obtained osteoid-defining threshold to the whole 3D-mCT-dataset, time-saving quantification of osteoid-tissue within the whole VOI is possible.

References:

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