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Standard Guide for Micro-computed Tomography of Tissue Engineered Scaffolds[1]

This standard is issued under the fixed designation F3259; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide is a resource for conducting micro-computed tomography (microCT) imaging and analysis of porous scaffolds for tissue engineering applications. Considerations are provided for sample preparation, image acquisition parameter selection, post-processing, and data interpretation.

1.2 The information in this guide is intended to be applicable to products that include a porous scaffold component and are designed for tissue engineering repair strategies. The scaffolds may be fabricated from synthetic polymers (e.g., absorbable polyesters) or natural materials (e.g., calcium phosphates), mammalian or human derived materials (e.g., demineralized bone) or combinations of these. While some considerations are provided for imaging of materials that are of moderate to high radiodensity, specific guidelines are not provided for imaging metallic scaffolds.

1.3 Applicability of the guidelines herein will depend on scaffold material type and the user's application (e.g., experimental design, as manufactured characterization) as appropriate.

1.4 The guidelines for microCT discussed herein are most suitable for specimen scanning *in vitro*. Specific guidelines relevant to direct *in vivo* imaging of scaffolds are not included because the imaging parameters will be dependent on the implantation site, animal size, breathing etc. In addition, consensus recommendations for *in vivo* imaging are provided in Bouxsein et al 2010 (**1**).[2] While the specific imaging parameters and processing recommendations discussed in Bouxsein et al are specific to bone imaging, many of the considerations and precautions are also applicable for *in vivo* scaffold imaging.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

F2450 Guide for Assessing Microstructure of Polymeric Scaffolds for Use in Tissue-Engineered Medical Products

F2603 Guide for Interpreting Images of Polymeric Tissue Scaffolds

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *microarchitecture, n*—the set of structural features of an object defined at the microscale.

3.1.2 *volume of interest (VOI), n*—a 3D sub-volume inside an image that contains the features to be analyzed.

4. Significance and Use

4.1 X-ray microcomputed tomography (microCT) is a non-destructive three-dimensional imaging method that can be used to reconstruct the microarchitecture of a tissue engineered medical product (TEMP) scaffold that may or may not contain ingrown tissue. MicroCT was first developed to study ceramics for the auto-industry and adapted for bone morphology at the microscale (Feldkamp et al 1989) (**2**). More recently, the imaging method has been adapted for *in vivo* applications and studies of multiple natural and synthetic materials.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

4.2 Alternate characterization methods for assessing scaffold microarchitecture and tissue ingrowth are limited by their two dimensional nature (e.g., microscopy) and low depth of penetration (e.g., optical coherence tomography), even though their resolution may be increased over microCT. However, microCT is an ideal imaging choice for studying scaffold microarchitecture and tissue ingrowth because it is non-destructive, provides scaffold assessments based on direct measurements rather than stereological methods, offers the ability to perform longitudinal imaging, and can be conducted at length scales relevant to cells and cell attachment (i.e., 1 micron to hundreds of microns).

4.3 The microarchitecture of tissue engineered scaffolds plays a critical role in providing structural support and/or facilitating cell adhesion, proliferation, and phenotype as well as matrix deposition. These parameters are essential elements of the tissue engineering strategy. During scaffold degradation, either *in vitro* or *in vivo*, changes to the microarchitecture continue to influence the eventual tissue repair. Therefore, it is critical to characterize the microarchitecture over time. Such characterization can aid the optimal design of TEMP scaffolds, establishment of manufacturing consistency, and monitoring of scaffold structure and/or tissue response.

4.4 This guide provides a compendium of information related to the use of microCT for the structural assessment of scaffold microarchitecture and tissue ingrowth. While the microarchitecture of tissue engineered scaffolds, as well as changes to it over time, can be assessed using multiple methods, (e.g., such as those described in Guide F2450), this guide focuses on unique considerations for conducting the microCT analyses.

4.5 The user of this guide is provided with considerations for each aspect of a complete microCT study including sample preparation, image acquisition, assessing image quality and artifacts, post-processing, and image interpretation based on the specific application.

4.6 This standard provides imaging and analysis considerations for the following broad types of applications: (a) scaffold microarchitecture analysis *in vitro* either before or after different stages of degradation, (b) *ex vivo* analysis of scaffold microarchitecture following partial degradation in an *in vivo* animal model, (c) deriving microarchitectural information when multiple materials are used in the scaffold, and (d) differentiating between scaffold microarchitectural changes and new tissue ingrowth.

4.7 The information provided in this standard guide is not intended as a test method for microCT characterization because the user's specific application and experimental design will significantly influence the imaging methodology and interpretation.

5. MicroCT Characterization Objectives

5.1 A significant amount of tissue engineering research is focused on developing optimal scaffold microstructure to facilitate tissue ingrowth, modulate cell phenotype, and control the repair response. Due to the non-destructive nature of microCT, many investigators have utilized this imaging technique as a way to measure numerous architectural parameters quantitatively and to track them at progressive time points. Specific indices and types of architectural indices that can be tracked for scaffolds are discussed in Section 10.

5.2 The objective of a microCT assessment of tissue engineered scaffolds is dependent on numerous factors and controlled by the investigators. Some considerations for defining the objective of the study include the need for point-in-time vs longitudinal assessments, quality assurance, and monitoring tissue growth vs scaffold degradation. This guide is suitable for the following experimental objectives when performing microCT assessment of a tissue engineered scaffold:

5.2.1 *Quantification of microstructural features (e.g., strut thickness) in the scaffold.* This type of assessment may be performed as part of a quality assurance characterization, (e.g. to test the degree of agreement between design and production), or to characterize the microstructural features.

5.2.1.1 These analyses are most typically performed on scaffolds that have not been exposed to an *in vivo* environment.

5.2.1.2 The same set of analyses can be applied to scaffolds that have undergone simulated degradation *in vitro*. MicroCT provides a simple and non-destructive way to track microstructural and physical changes to the scaffold during degradation. Since the technique provides a three-dimensional image of the scaffold, it can be used to determine potential areas of non-uniform degradation or other structural features.

5.2.1.3 While assessment of these indices is most commonly completed following all manufacturing processes, similar considerations would apply if assessing the scaffold at interim points in the manufacturing process or after exposure to shelf life aging.

5.2.1.4 Examples of scaffolds imaged with microCT appear in Fig. 1 to illustrate the type of information that may be gathered and the heterogeneity of visual representation for TEMP scaffolds.

5.2.2 *Ex vivo* characterization of changes to the scaffold microarchitecture following degradation and tissue ingrowth *in vivo*. This type of assessment includes removal of the scaffold from the animal model prior to imaging. This characterization would provide the user with information on geometric alterations to the structure of the scaffold over time following implantation in an animal model.

5.2.2.1 Performing microCT of the scaffold after implantation in an animal model has unique challenges as illustrated by the images in Fig. 2.

5.2.2.2 Specific considerations for each aspect of performing a microCT study of a scaffold while implanted in an *in vivo* animal model, however, are beyond the scope of this guide and are covered in Bouxsein et al 2010 (**1**).

5.2.3 *Ex vivo* characterization of tissue ingrowth. In addition, to understanding how the scaffold degrades and is altered following implantation, microCT can be used to quantify the extent of tissue ingrowth and provide some basic information on the type of tissue regenerated.

5.2.3.1 While in theory, using microCT to quantify the amount of tissue ingrowth in the presence of a tissue engineered scaffold is feasible, it is limited by the ability of the microCT to differentiate the radiodensity of scaffold material

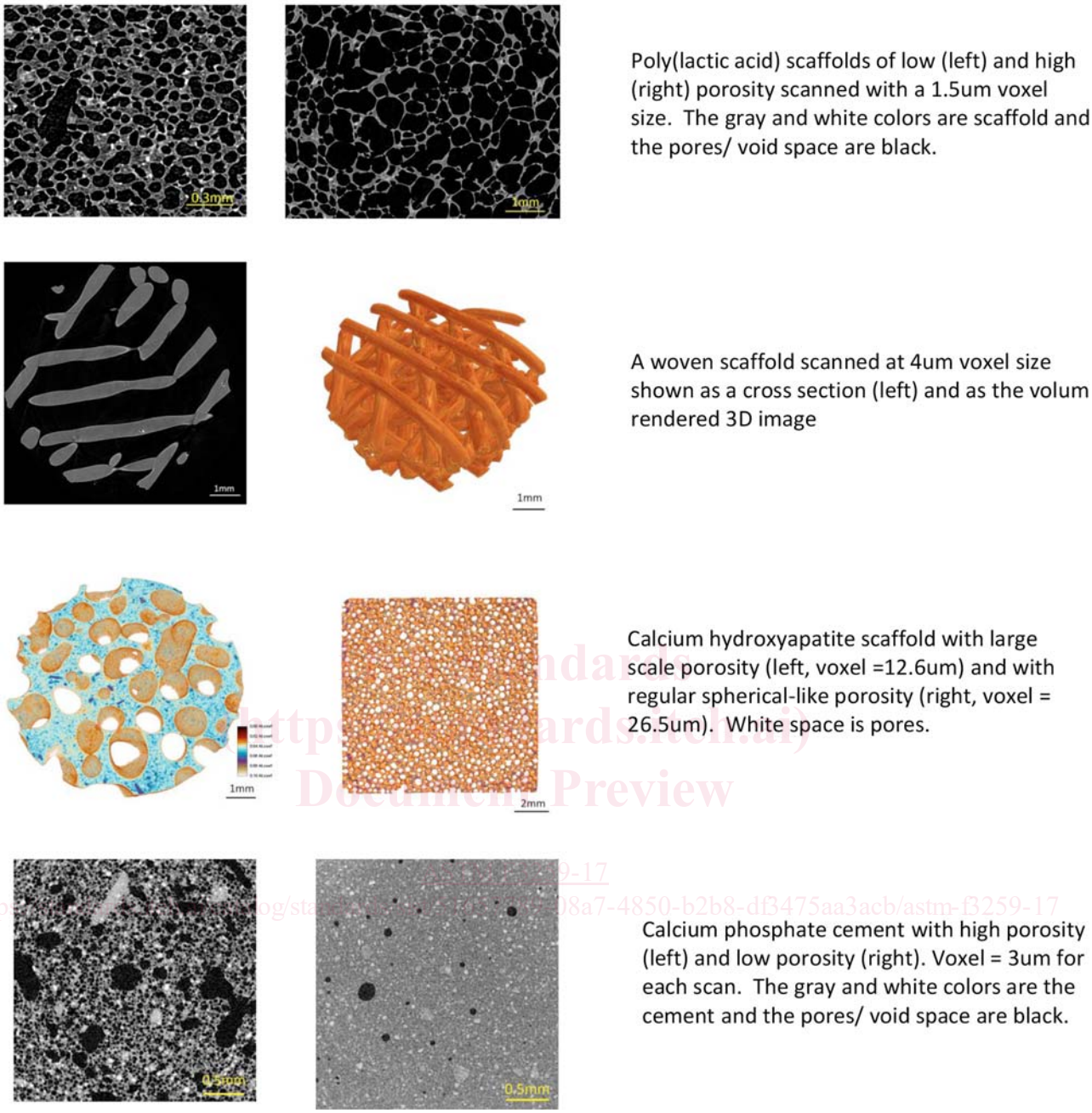


FIG. 1 Examples of TEMP Scaffolds Scanned Alone *in vitro* by X-ray MicroCT

as compared to tissue. In practice, this has been most readily achieved by quantifying the production (Peyrin 2011) (**3**) of bone since this tissue type has a much higher density than that of many synthetic absorbable polymeric scaffolds.

5.3 MicroCT characterizations of tissue engineered scaffolds may be completed on structures that are fabricated from one or multiple materials. The ability to differentiate multiple materials within a scaffold will be dependent on the composition of those materials and their radiodensity.

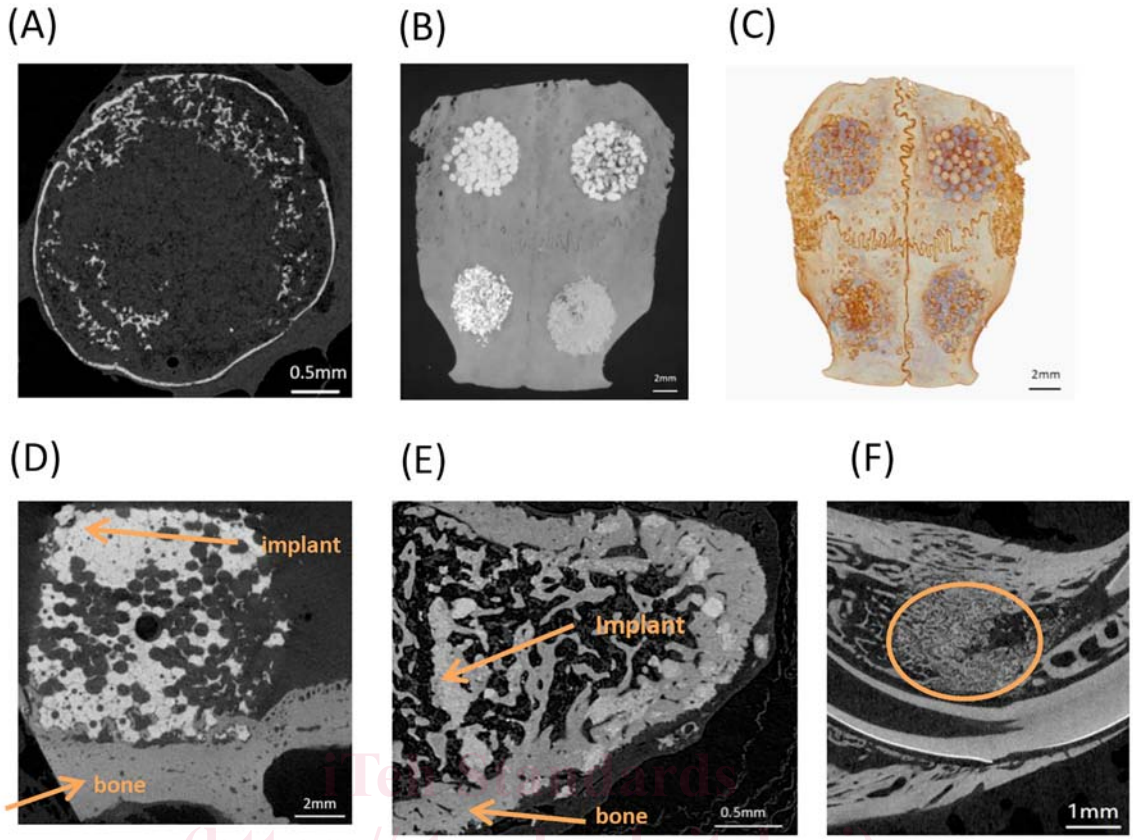
5.4 Some applications may necessitate designing the experiment in order to include various types of controls. Examples of controls which may be used to facilitate microCT image analysis and/or interpretation may include the following:

5.4.1 Blank scaffolds that are stable and do not change their architecture (i.e., without any cells or degradation).

5.4.2 For applications where the tissue engineered scaffold of interest is designed from multiple materials, the microCT experiment may necessitate imaging of different scaffolds, each manufactured with only one of the pure materials. These additional images may be used to aid threshold selection.

6. Sample Preparation

6.1 Scaffold dimensions and/or design are important when preparing TEMPs scaffolds for microCT imaging. During sample preparation, it is recommended that the key features of the scaffold (e.g. pore size, strut thickness, density, etc) which



NOTE 1—These examples illustrate some of the challenges associated with differentiating the scaffold from surrounding tissue if the radiodensity is similar. In (A), a grayscale image of a polycaprolactone (PCL) scaffold impregnated with bone morphogenetic protein (BMP) and implanted in rat muscle tissue. Voxel size 6.8 microns. The PCL scaffold itself is only marginally visible due to weak contrast with the surrounding muscle tissue. In (B), a grayscale image showing calcium phosphate ($CaPO_4$) scaffold material implanted in granular and paste form in four rabbit calvarium defects (4 circles). Voxel size is 24.4 microns. In this case, it is possible to segment the implanted material from surrounding bone due to differences in attenuation because the implanted material has a higher density. White coloring is higher density and black is the lowest density. In (C), A volume rendered image of the rabbit calvarium with the four $CaPO_4$ scaffolds. Blue coloring is higher radiopacity and darker brown is low radiopacity. In (D), A bone-like implant in a granular form has a higher attenuation density than bone (appearing more white), allowing visualisation and segmentation of bone in apposition to the scaffold near the interface. Voxel size is 13.7 microns. In (E), Calcium phosphate cement implanted into the metaphyseal region of an adult rat tibia. The implanted paste has a slightly higher attenuation density than the bone. Voxel size is 2.5 microns. In (F) $CaPO_4$ scaffold implanted *in vivo* into a defect in a rabbit mandible. Voxel size 9.9 microns. This implanted $CaPO_4$ scaffold in a rabbit mandible is not distinguishable from surrounding bone on the basis of attenuation density, but only on the basis of morphometry.

FIG. 2 Examples of TEMP Scaffolds Scanned in Tissue *ex vivo* by X-ray MicroCT

need to be resolved and quantified be identified in order to prepare the sample appropriately. In particular, the desired voxel size for the scaffold should be considered when preparing samples for microCT imaging.

6.1.1 The voxel size is of critical importance to the microCT users and their ability to extract quantitative information. Voxel size is dependent on many aspects of the microCT experiment, including the field of view (see section 6.3), scanning parameters (see Section 7), and reconstruction (see Section 8).

6.1.2 When selecting the size of the specimen holder/ field of view, microCT scan parameters and reconstruction, the voxel size will be calculated and presented to the user. The user should ensure that the voxel size is appropriate for imaging structures of interest. It is recommended to image scaffolds with voxel dimensions that are at least one third and more optimally one tenth of the size of the relevant scaffold features (e.g., strut size).

6.1.3 It should be noted that voxel size is not the same as spatial resolution of the microCT image and microCT manufacturers may report this information differently. A discussion of the difference between voxel size and spatial resolution can be found in Bousxein et al., 2010 (**1**).

6.2 The microCT scan resolution will be determined by the scaffold size and structures of interest within the scaffold. In general, higher resolution microCT scans are obtained when using a smaller sized specimen holder/ field of view (FOV). If very small structures need to be resolved and analyzed, the FOV should be small enough to achieve a resolution sufficient to resolve all the small structures of interest.

6.2.1 Typically, there is no optimal size to address all research questions for a scaffold. In this case, the scaffold may have to be cut into different size specimens to facilitate measurements at different resolutions.

6.2.2 Visual inspection and binary segmentation of structures for analysis provide a good indication of whether sufficient resolution, homogeneity and image quality have been attained in a microCT scan.

6.2.3 Ideally, the scaffold size, specimen holder size, and therefore, the FOV should be consistent for all scaffolds imaged within a study.

6.3 Some small VOIs taken from a larger scaffold with a porous cellular structure may not be a representative selection due to differences in the scale of features. A parameter termed the Representative Volume Element (RVE) has been developed to address this issue (Bachmat et al 1987) (**4**) and has demonstrated some utility in the microCT analysis of snow (Srivastava et al 2010) (**5**). If a material with cellular structure is essentially homogeneous, then the VOI can be selected randomly within the material.

6.3.1 If the VOI is sufficiently large, that is, larger than the RVE, then measured parameters of microarchitecture and porosity will be very similar wherever the VOI is situated.

6.3.2 However if the VOI size falls below the RVE, then stochastic differences in the measured structural parameters increase between VOIs at different locations, since the VOI becomes too small to adequately sample the cellular structures.

6.4 It is critical to secure scaffolds firmly in position to prevent motion artifact (i.e. relative movement between the specimen holder and scaffold) during the scan. Due to the low radiopacity of many TEMPs scaffolds, it is recommended to use low density foam (e.g., polystyrene or polyurethane foams) or another low-attenuating material to secure the scaffold within holder. The securement material should have a lower radiopacity than that of the scaffold itself.

6.4.1 For very low radiodense scaffolds, it may not be feasible to use a lower radiopacity material. In those instances, a higher radiodense material (e.g. spacer) above and/or below the scaffold may provide adequate compression to secure the scaffold and prevent motion artifact.

6.4.2 Other techniques to consider for securing specimens include using 3D printed spacers/cartridges of specific geometries, putty or solvent resistant, double sided tape.

6.5 In general, the voxel intensity of the scanning medium should ideally be significantly lower than that of the scaffold and homogeneous in spatial density distribution. This ensures that scaffold features can be differentiated and resolved.

6.5.1 Scanning in air provides the highest contrast between scanning medium and scaffold.

6.5.2 For scaffolds where hydration is important, other media may be used (e.g., deionized water, saline, phosphate buffered saline, ethanol, and neutral buffered formalin); however it is important to verify whether X-ray attenuation or morphometric properties are significantly affected by the medium.

6.5.3 The same scanning medium should be used for all samples in a microCT study for consistent, quantitative comparisons.

6.6 In situations where contrast agents are needed to enhance images of scaffolds with very low radiodensity, care should be taken to prevent image artifacts. For example, the use of contrast agents or particles of high radiodensity may result in thicker struts due to swelling, accumulation, and/or partial volume artifacts. In these cases, validation of the technique is recommended.

6.6.1 An example of the effect that a contrast agent may have on scaffold visualization with microCT is shown in Appendix X1, Fig. X1.1.

6.7 For longitudinal scans of the same specimen, e.g., imaging a process or changes to the scaffold, maintaining consistent specimen alignment between scans is recommended, particularly if spatial information is compared across multiple scans (e.g. bone ingrowth).

6.7.1 Registration markers may also aid in maintaining consistent alignment.

6.7.2 In addition, software registration can be used to align longitudinal scans.

6.8 Additional considerations may be needed for sample preparation applications where the scaffold has been implanted into an animal model and subsequently explanted for microCT analyses.

6.8.1 If possible, the scaffold with repair tissue should remain hydrated for the microCT scan in order to accurately measure scaffold and tissue features. Hydration of the scaffold during microCT imaging may also be necessary for subsequent analyses (e.g. mechanical testing, histology).

6.8.2 Some of the external tissue may need to be trimmed away from the repair site prior to imaging to aid in determining physical boundaries of the construct and accurately assessing the effects that occur within the scaffold.

6.8.3 If possible, sample orientation should remain the same between samples to allow for ease of segmentation of the VOI during analysis.

6.8.4 Landmarks from radiopaque material (staples, screws, etc) may be used and/or added to help distinguish boundaries between materials with similar radiopacities.

7. Image Acquisition

7.1 MicroCTs are complex imaging systems that require a careful selection of the scanning parameters. These parameters depend on the sample material and on the microCT system used. It is therefore not possible to fix a protocol that is valid for all scaffold types or applications. Solid understanding of the physics behind microCT is essential to acquire the best possible images. However, for a given application and material type, the optimal parameters must be determined only once and then they can be used for all studies using the same scaffold material and application type. In Table 1, the most relevant and critical scanning parameters of tissue engineered scaffolds using X-ray tube based microCT systems are summarized and their effect on scaffold image quality is highlighted.

7.1.1 To set the energy to an appropriate value, it is helpful to know the chemical composition of the scaffold material, as this determines the attenuation of the X-ray beam. In general it can be said that the higher the atomic number, the greater the attenuation and thus, the higher the energy needs to be to penetrate the material.

TABLE 1 General Description of Parameters That Define a Scan[A]

Parameter	Description	Effects
Energy	The energy that can be set in microCT systems refers to the peak voltage applied between the cathode and the anode of the X-ray tube to accelerate the electrons. This peak energy and the anode material determine the photon spectrum and the maximal energy of the converted photons. However, due to the spread of energies emitted from the source, the mean photon energy is typically about 1/3rd of the peak energy.	The higher the energy of the photons, the better the penetration through dense materials. However, materials with similar attenuation coefficients can be separated more effectively at lower energies. Therefore, the energy chosen should be as low as possible but also high enough to ensure that the photons can penetrate all materials in the specimen. When tissue engineered scaffolds are fabricated from low density absorbable polymers, the optimal energies used for imaging scaffolds alone are typically not consistent with those used to image ceramics or metals. However, higher energies may be necessary if calcified tissue, such as bone, has ingrown into the scaffold.
Filter	Filters are typically metal plates that can be inserted in the photon beam to modify the energy spectrum. Filtering narrows the photon energy spectrum and reduces the overall intensity.	Beam hardening artifacts (see Section 8) can be mitigated by narrower photon spectrums. Narrow photon spectrums also aid density resolution. To separate different material types with similar attenuation values within a scaffold it can thus be beneficial to narrow the photon spectrum by using a filter. However, the filters reduce the overall number of photons, which necessitates an increase in measurement time in order to obtain a comparable signal-to-noise ratio (SNR).
Power, Intensity, Current	Intensity or current refers to the current of the accelerated electrons. For a given energy, the power is proportional to the current. Whether power, intensity, or current can be set depends on the microCT model.	The higher the intensity, the more photons are produced and the better the SNR for a given measurement time. For a given energy, an increase in intensity is in general related to an increase in focal spot size.
Focal spot	The size of the emission spot from the X-ray source. This parameter might not be set independent of intensity.	The focal spot size is a critical parameter limiting the resolution for objects measured close to the source. The smaller the focal spot, the better resolution that can be achieved. However, a small focal spot is also related to a low intensity which may necessitate long measurement times. If a scaffold has very thin struts and the goal is to measure the strut thickness, it is essential to work with the smallest possible spot size. However, if contrast differences are of higher importance, a larger spot size may yield better results.
Integration time (Exposure time)	The integration time, sometimes referred to as exposure time, is the time to acquire an individual projection frame. It might be limited to not oversaturate the detector. The maximal integration time depends on the energy, intensity, and filtering.	The longer the integration time, the better the SNR but the longer the measurement time. In order to obtain a reasonable SNR, the integration time may need to be longer for scaffolds with a low attenuation coefficient as compared to scaffolds with high attenuation coefficient. In general, if different materials with comparable attenuation coefficients should be separable in an image (e.g. scaffold in bone), integration time should be selected longer in order to increase SNR.
Frame averaging	Number of times a frame (projection) is acquired at a given position in order to get an averaged frame.	Frame averaging improves SNR and can overcome a potential limitation in integration time. Frame averaging is proportional to the measurement time, but can significantly aid SNR for low density materials and small structures (e.g., scaffold struts with small thickness and width).
Number of projections	The number of projections taken over 180 degrees	The number of projections is a trade-off between image quality and measurement time. For fast overview scans, a relatively low number of projections might be sufficient. However, if the number chosen is too low, aliasing artifacts may distort the image. For standard scans, the values set by the manufacturer should yield reasonable results for most scaffold imaging. Typically, the projections are acquired over 180 degrees, however, some situations may require the user to take projections over 360 degrees. In these situations, the total number of projections and the scan angle should be reported.

[A] For more detailed information on microCT parameters, please refer to Stauber M & Muller R, 2008 (**6**).