Development of algorithms and methods for three-dimensional image analysis and biomedical applications

SSD ING-INF/06 Bioingegneria Elettronica ed Informatica

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In loving memory of Alessandra
ABSTRACT

Tomographic imaging is both the science and the tool to explore the internal structure of objects. The mission is to use images to characterize the static and/or dynamic properties of the imaged object in order to further integrate these properties into principles, laws or theories. Among the recent trends in tomographic imaging, three-dimensional (3D) methods are gaining preference and there is the quest for overcoming the bare qualitative observation towards the extraction of quantitative parameters directly from the acquired images. To this aim, Computed Tomography (CT) and Magnetic Resonance Imaging (MRI), as well as the related micro-scale techniques (\(\mu\)-CT and \(\mu\)-MRI), are promising tools for all the fields of science in which non-destructive tests are required. In order to support the interpretation of the images produced by these techniques, there is a growing demand of reliable image analysis methods for the specific 3D domain. The aim of this thesis is to present approaches for effective and efficient three-dimensional image analysis with special emphasis on porous media analysis. State-of-the art as well as innovative tools are included in a special software and hardware solution named *Pore3D*, developed in a collaboration with the Italian 3rd generation synchrotron laboratory Elettra (Basovizza - Trieste, Italy). Algorithms and methods for the characterization of different kinds of porous media are described. The key steps of image segmentation and skeletonization of the segmented pore space are also discussed in depth. Three different clinical and biomedical applications of quantitative analysis of tomographic images are presented. The reported applications have in common the characterization of the micro-architecture of trabecular bone. The trabecular (or cancellous) bone is a 3D meshwork of bony trabeculae and void spaces containing the bone marrow. It can then be thought of as a porous medium with an interconnected porous space. To be more specific, the first application aims at characterizing a structure (a tissue engineering scaffold) that has to mimic the architecture of trabecular bone. The relevant features of porosity, pore- and throat-size distributions, connectivity and structural anisotropy indexes are automatically extracted from \(\mu\)-CT images. The second application is based on *ex vivo* experiments carried out on femurs and lumbar spines of mice affected by microgravity conditions. Wild type and transgenic mice were hosted in the International Space Station (ISS) for 3 months and the observed bone loss due to the near-zero gravity was quantified by means of synchrotron radiation \(\mu\)-CT image analysis. Finally, the results of an *in vivo* study on the risk of fracture in osteoporotic subjects is reported. The study is based on texture analysis of high resolution clinical magnetic resonance (MR) images.
L’imaging tomografico è l’insieme di scienza e tecnica che consente la visualizzazione della struttura interna di oggetti. Ha come obiettivo la produzione di immagini in grado di caratterizzare le proprietà statiche e/o dinamiche di un oggetto al fine di desumere da tali proprietà principi, leggi o teorie legate all’oggetto stesso e/o alle sue condizioni. Tra le recenti tendenze nell’imaging tomografico, vi è un notevole interesse verso le tecniche in grado di produrre immagini tridimensionali (3D) e vi è il crescente desiderio di andare oltre la mera osservazione qualitativa delle immagini a favore dell’estrazione di parametri quantitativi direttamente da esse. A questo scopo, rivestono particolare interesse per i molti campi della scienza in cui si richiedono test non distruttivi la Tomografia Computerizzata (TC) e la Risonanza Magnetica (RM) come anche le relative tecniche microscopiche (μ-TC e μ-RM). Al fine di supportare l’interpretazione delle immagini prodotte, vi è un’accresciuta richiesta di metodi per l’analisi di immagini concepiti per lo specifico caso 3D. Scopo del presente lavoro di tesi è presentare approcci per un’efficace ed efficiente analisi di immagini tridimensionali con particolare enfasi verso l’analisi di mezzi porosi. Gli approcci presentati sono stati implementati in una speciale soluzione hardware e software dal nome Pore3D, sviluppata all’interno di una collaborazione con il laboratorio di luce di sincrotrone Elettra (Basovizza, Trieste). Verranno esposti algoritmi e metodi che consentono la caratterizzazione di diversi tipi di mezzi porosi. Le fasi cruciali di segmentazione e scheletizzazione dello spazio dei pori saranno discusse in dettaglio. Verranno inoltre presentate tre diverse applicazioni di analisi quantitativa di immagini tomografiche in ambito biomedico. Comune denominatore di queste applicazioni è la caratterizzazione della micro-architettura dell’osso trabecolare. L’osso trabecolare (o spugnoso) è formato da una rete di trabecole ossee intrecciate tra loro in modo da formare delle cavità in cui è contenuto midollo osseo. L’osso trabecolare è quindi un particolare mezzo poroso con spazio dei pori interconnesso. Più in dettaglio, la prima applicazione ha come obiettivo la caratterizzazione di una struttura (uno scaffold per ingegneria dei tessuti) che deve simulare la micro-architettura dell’osso trabecolare. In questa applicazione, parametri caratteristici come la porosità, la distribuzione delle dimensioni dei pori e delle gole come anche indici per descrivere il grado di interconnettività e di anisotropia della struttura sono calcolati in modo automatico a partire da immagini di microtomografia computerizzata (μ-TC). La seconda applicazione è basata su esperimenti ex vivo condotti su femori e spine lombari di topi affetti da condizioni di microgravità. Topi wild type e transgenici sono stati ospitati per tre mesi nella Stazione Spaziale Internazionale (SSI) e la riduzione ossea osservata è stata quantificata a partire da immagini μ-TC con
luce di sincrotrone. Infine, vengono riportati i risultati di uno studio
in vivo sul rischio di frattura in soggetti osteoporotici. Lo studio è ba-
sato sull’analisi della tessitura di immagini cliniche ottenute con una
speciale Risonanza Magnetica ad alta risoluzione.
## CONTENTS

1. **INTRODUCTION**  
   1.1 The science of imaging  
   1.2 Trends in clinical and biomedical imaging  
      1.2.1 Quantitative analysis of images  
      1.2.2 2D vs. 3D imaging  
   1.3 Framework of the thesis  
   1.4 Outline of the thesis  

2. **TOMOGRAPHIC THREE-DIMENSIONAL IMAGING**  
   2.1 Principles of X-ray computed microtomography  
   2.2 Absorption and phase contrast micro-CT  
   2.3 Micro-CT setups  
      2.3.1 Synchrotron radiation micro-CT at SYRMEP  
      2.3.2 Conventional X-ray micro-CT at TomoLab  
      2.3.3 Comparison between SYRMEP and TomoLab  
   2.4 Principles of Magnetic Resonance Imaging (MRI)  
   2.5 Comparison between micro-CT and MRI  

3. **THE PORE3D PROJECT**  
   3.1 Motivations of the project  
   3.2 History of the project  
   3.3 The current release  
      3.3.1 The SaaS paradigm  
      3.3.2 Software architecture  

4. **THREE-DIMENSIONAL IMAGE PROCESSING**  
   4.1 Image quality  
      4.1.1 Spatial resolution  
      4.1.2 Contrast resolution  
      4.1.3 Noise  
      4.1.4 Artifacts in micro-CT and MRI  
   4.2 Segmentation  
      4.2.1 Threshing  
      4.2.2 Region-based segmentation  
      4.2.3 Clustering and multi-phase segmentation  
      4.2.4 Other approaches  
      4.2.5 Pre- and post-segmentation filters  
   4.3 Volume of Interest (VOI) selection  

5. **THREE-DIMENSIONAL IMAGE ANALYSIS**  
   5.1 Basic analysis  
   5.2 Structural anisotropy analysis  
   5.3 Model based morphometric analysis  
   5.4 Blob analysis of closed cell structures  
      5.4.1 Blob separation via watershed segmentation
<table>
<thead>
<tr>
<th>5.5</th>
<th>Skeleton analysis of open cell structures</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5.1</td>
<td>Skeletonization</td>
<td>56</td>
</tr>
<tr>
<td>5.5.2</td>
<td>Nodes and branches analysis</td>
<td>59</td>
</tr>
<tr>
<td>5.6</td>
<td>Texture analysis</td>
<td>62</td>
</tr>
<tr>
<td>5.7</td>
<td>Representative Elementary Volume (REV)</td>
<td>66</td>
</tr>
<tr>
<td>6</td>
<td>BIOMEDICAL APPLICATIONS</td>
<td>69</td>
</tr>
<tr>
<td>6.1</td>
<td>Characterization of bone tissue engineering scaffolds</td>
<td>69</td>
</tr>
<tr>
<td>6.1.1</td>
<td>Materials and Methods</td>
<td>74</td>
</tr>
<tr>
<td>6.1.2</td>
<td>Results and Discussion</td>
<td>79</td>
</tr>
<tr>
<td>6.1.3</td>
<td>Conclusion</td>
<td>86</td>
</tr>
<tr>
<td>6.2</td>
<td>Characterization of bone alterations after microgravity</td>
<td>86</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Materials and Methods</td>
<td>89</td>
</tr>
<tr>
<td>6.2.2</td>
<td>Results and Discussion</td>
<td>91</td>
</tr>
<tr>
<td>6.2.3</td>
<td>Conclusion</td>
<td>96</td>
</tr>
<tr>
<td>6.3</td>
<td>Characterization of the risk of fracture in osteoporotic subjects</td>
<td>96</td>
</tr>
<tr>
<td>6.3.1</td>
<td>Materials and Methods</td>
<td>98</td>
</tr>
<tr>
<td>6.3.2</td>
<td>Results and Discussion</td>
<td>99</td>
</tr>
<tr>
<td>6.3.3</td>
<td>Conclusion</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>CONCLUSION</td>
<td>101</td>
</tr>
<tr>
<td>AUTHOR'S PUBLICATIONS</td>
<td>103</td>
<td></td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>105</td>
<td></td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Absorption and phase contrast modes</td>
<td>12</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Sketch of the SYRMEP beamline</td>
<td>14</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Main μ-CT setups</td>
<td>15</td>
</tr>
<tr>
<td>Figure 4</td>
<td>The TomoLab station</td>
<td>16</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Basic principles behind MRI</td>
<td>18</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Comparison of a μ-CT and μ-MRI image</td>
<td>21</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Full 3D vs. “slice-by-slice” approach</td>
<td>24</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Approaches for intense computation</td>
<td>26</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Software and hardware architecture of Pore3D</td>
<td>27</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Artifacts in μ-CT: Beam hardening</td>
<td>34</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Example of a slice of a 4D μ-CT time series</td>
<td>35</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Artifacts in μ-CT: Ring artifacts</td>
<td>36</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Polar transformation for ring removal</td>
<td>37</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Artificial image for testing ring removal filter</td>
<td>38</td>
</tr>
<tr>
<td>Figure 15</td>
<td>VOI selection issues</td>
<td>46</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Mean Intercept Length (MIL) method</td>
<td>49</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Concepts used for blob analysis</td>
<td>53</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Blob separation example</td>
<td>55</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Removal of “incomplete” blobs</td>
<td>56</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Skeleton analysis example (Part I - Processing)</td>
<td>57</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Skeleton analysis example (Part II - Analysis)</td>
<td>58</td>
</tr>
<tr>
<td>Figure 22</td>
<td>Skeleton pruning</td>
<td>59</td>
</tr>
<tr>
<td>Figure 23</td>
<td>Comparison of skeletonization algorithms</td>
<td>59</td>
</tr>
<tr>
<td>Figure 24</td>
<td>Example of pores and throats</td>
<td>60</td>
</tr>
<tr>
<td>Figure 25</td>
<td>Node-pore correction method</td>
<td>61</td>
</tr>
<tr>
<td>Figure 26</td>
<td>Connectivity by skeleton analysis</td>
<td>62</td>
</tr>
<tr>
<td>Figure 27</td>
<td>The concept of Tissue Engineering</td>
<td>70</td>
</tr>
<tr>
<td>Figure 28</td>
<td>Slice of each considered dataset</td>
<td>73</td>
</tr>
<tr>
<td>Figure 29</td>
<td>Comparison of automatic segmentation techniques</td>
<td>77</td>
</tr>
<tr>
<td>Figure 30</td>
<td>Effects of the post-thresholding cleaning</td>
<td>78</td>
</tr>
<tr>
<td>Figure 31</td>
<td>Results with manual segmentation</td>
<td>83</td>
</tr>
<tr>
<td>Figure 32</td>
<td>Results with automatic segmentation</td>
<td>84</td>
</tr>
<tr>
<td>Figure 33</td>
<td>Analysis of the representativeness of the VOIs</td>
<td>85</td>
</tr>
<tr>
<td>Figure 34</td>
<td>Mice Drawer System (MDS)</td>
<td>90</td>
</tr>
<tr>
<td>Figure 35</td>
<td>Color map of trabecular thickness in the femur</td>
<td>93</td>
</tr>
<tr>
<td>Figure 36</td>
<td>Color map of trabecular thickness in the lumbar spine</td>
<td>94</td>
</tr>
<tr>
<td>Figure 37</td>
<td>MRI of a healthy and osteoporotic calcaneus</td>
<td>98</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Sources and properties employed for imaging</td>
<td>2</td>
</tr>
<tr>
<td>Table 2</td>
<td>Trends in clinical and biomedical imaging</td>
<td>4</td>
</tr>
<tr>
<td>Table 3</td>
<td>Fields of computer science related to images</td>
<td>5</td>
</tr>
<tr>
<td>Table 4</td>
<td>Main tomographic imaging techniques</td>
<td>10</td>
</tr>
<tr>
<td>Table 5</td>
<td>Comparison between SYRMEP and TomoLab</td>
<td>16</td>
</tr>
<tr>
<td>Table 6</td>
<td>Comparison between CT and MRI</td>
<td>20</td>
</tr>
<tr>
<td>Table 7</td>
<td>Proposed automatic thresholds for each VOI</td>
<td>79</td>
</tr>
<tr>
<td>Table 8</td>
<td>Misclassification error for each considered method</td>
<td>80</td>
</tr>
<tr>
<td>Table 9</td>
<td>Results of quantitative analysis</td>
<td>81</td>
</tr>
<tr>
<td>Table 10</td>
<td>Results of quantitative analysis in the femur of Wt mice</td>
<td>92</td>
</tr>
<tr>
<td>Table 11</td>
<td>Results of quantitative analysis in the femur of Tg mice</td>
<td>92</td>
</tr>
<tr>
<td>Table 12</td>
<td>Quantitative analysis in the lumbar spine of Wt mice</td>
<td>95</td>
</tr>
<tr>
<td>Table 13</td>
<td>Quantitative analysis in the lumbar spine of Tg mice</td>
<td>95</td>
</tr>
<tr>
<td>Table 14</td>
<td>Texture analysis of the MR images of the calcaneus</td>
<td>99</td>
</tr>
</tbody>
</table>
## ACRONYMS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
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<tr>
<td>CMOS</td>
<td>Complementary Metal Oxide Semiconductor</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>DART</td>
<td>Discrete Algebraic Reconstruction Technique</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual-energy X-ray Absorptiometry</td>
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<tr>
<td>FOV</td>
<td>Field Of View</td>
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<tr>
<td>HAp</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>IDL</td>
<td>Interactive Data Language</td>
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<tr>
<td>MIL</td>
<td>Mean Intercept Length</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>pQCT</td>
<td>peripheral Quantitative Computed Tomography</td>
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<tr>
<td>PTN</td>
<td>Pleiotrophin</td>
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<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>SART</td>
<td>Simultaneous Algebraic Reconstruction Technique</td>
</tr>
<tr>
<td>SR</td>
<td>Synchrotron Radiation</td>
</tr>
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<td>TE</td>
<td>Echo Time</td>
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<td>Tg</td>
<td>Transgenic</td>
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<tr>
<td>TR</td>
<td>Repetition Time</td>
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<tr>
<td>VOI</td>
<td>Volume of Interest</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
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Light is what drives life. It is hard to imagine the world and life without it. The sensing of light by living things is almost universal. Plants use light through photosynthesis to grow. Animals use light to hunt their prey or to sense and escape from predators. Light is at once both obvious and mysterious. It continues to surprise scientists. For instance, scientists have always taken for granted that light travels faster than anything else in the universe. A recent finding seems to state that neutrinos are even faster, thus making the need to shed more light on how light exactly behaves.

Light is also what makes vision possible. Vision depends on how light affects the eye and this depends on the condition of the eye itself. Human eye and vision mechanisms are as fascinating as the light. Objects are visible because they modify the light that reaches them. Transparent objects transmit most of the incident light, thus making it possible to see through them, while opaque objects absorb and reflect light. Sight is probably the most important of the human senses since it furnishes most of the information we have about our surroundings and it affects our understanding like any other sense.

One of the common and, at the same time, major challenges to researchers is the question of how to acquire and/or represent the information about something so that the information can be assimilated, interpreted, and utilized. Being vision so important and effective for our understanding, the search for a visual representation (i.e. an image) is probably the first and most frequently pursued approach to address this challenge. Without any doubt, humans learn new things mainly by “seeing” them.

Humans want to see also what cannot be seen with the unaided eye. The desire to look the wonders of the universe sharpened the development of magnifying tools like the telescope that allowed men to improve the knowledge of remote objects. The need of inspecting the surface of very small objects has lead to optical, electron and scanning microscopy which is an essential tool for several fields of
1 INTRODUCTION

Table 1. Main energy sources and object properties employed for imaging

<table>
<thead>
<tr>
<th>Energy Sources</th>
<th>X-rays, γ-rays, visible light, ultraviolet light, annihilation radiation, electric fields, magnetic fields, infrared light, applied voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Object Properties</td>
<td>mass density, electron density, proton density, atomic number, velocity, current flow, relaxation, temperature, chemical state</td>
</tr>
<tr>
<td>Image Properties</td>
<td>transmissivity, opacity, emissivity, reflectivity, conductivity, magnetizability, resonance, absorption</td>
</tr>
</tbody>
</table>

science. Devices like microscopes and telescopes allow to see objects that are not within the resolution range of the naked eye just because they are too small or too far. A different need is to inspect the internal structure of an opaque object regardless its dimension. In this case, there is the will to address the question of how to look inside an object without dissecting it. This latter desire has lead to the science of tomographic imaging.

1.1 THE SCIENCE OF IMAGING

Within the purposes of this thesis, imaging refers to both the science and the tool to explore the internal structure of objects. It employs a variety of energy sources and material properties to produce useful images (see Table 1). The mission of imaging is to use images to characterize the static and/or dynamic properties of the imaged object, preferably in quantitative terms, in order to further integrate these properties into principles, laws or theories.

Medical imaging

Medical imaging is the main field of application as well as the main driving force in the development of imaging methods. Human medicine is the quest for understanding one particular object, the human body, and its structure and function under all conditions of health. This quest has yielded models of human health and illness that are immensely useful e.g. in preventing disease and disability, detecting and diagnosing illness and injury, and designing therapies to alleviate pain. With the same approach, imaging has become a powerful tool for several fields of science, e.g. material science, geosciences, food science just to cite a few. Imaging yields in any practical application additional information about the underlying properties of the imaged object.

Images of complex objects reveal characteristics of the object such as its transmissivity, opacity, emissivity, reflectivity, conductivity, and magnetizability as well as changes in these characteristics with time (see Table 1). For example, images created by X-rays transmitted
Trends in clinical and biomedical imaging through a region of the imaged object can reveal intrinsic properties of the region such as effective atomic number $Z$, mass density and electron density. In ultrasonography, images are produced by capturing energy reflected from interfaces in the object that separate phases with different acoustic impedances, where the acoustic impedance is the product of the physical density and the velocity of ultrasound in the phase. Magnetic Resonance Imaging (MRI) of relaxation characteristics following magnetization of object phases is influenced by the concentration, mobility, and chemical bonding of hydrogen and, less frequently, other elements present in the object. For the specific case of human body, maps of the electrical field (electroencephalography) and the magnetic field (magnetoencephalography) at the surface of the skull can be analyzed to identify areas of intense neurological activity in the brain. Nuclear medicine images, including emission computed tomography (ECT) with pharmaceuticals releasing positrons (positron emission tomography - PET) and single photons (single-photon emission computed tomography - SPECT), reveal the spatial and temporal distribution of target-specific pharmaceuticals in the human body. Depending on the application, these data can be interpreted to yield information about physiological processes such as glucose metabolism, blood volume, flow and perfusion, tissue and organ uptake, receptor binding, and oxygen utilization.

All the aforementioned as well as other techniques provide an array of imaging methods that are immensely useful for displaying structural and functional information about the human body as well as any other object investigated exploiting one of these techniques.

1.2 TRENDS IN CLINICAL AND BIOMEDICAL IMAGING

Clinical and biomedical imaging is improving more and more. A few recent trends in clinical and biomedical imaging can be identified and they are summarized in Table 2. These trends include the combination of different imaging modalities and a gaining interest towards functional imaging and molecular methods. While analog imaging still survives for some applications, images are directly acquired and stored in digital form in the vast majority of the applications. Moreover, images can be easily digitalized and therefore, from now on, when referring to images it is implicit that they are digital images and they will be digitally processed.

More interestingly, three-dimensional (3D) methods are gaining preference over 2D imaging and there is the will to overcome the bare qualitative observation of images. In fact, the extraction of quantitative information about the imaged object leads to a more comprehensive and objective understanding of its structure and it allows to better establish further interrelationships with the properties of the object itself. The present thesis focuses on the combination of these latter major challenges, i.e. quantitative image analysis methods suitable for the specific case of 3D images.
Table 2. Trends in clinical and biomedical imaging

<table>
<thead>
<tr>
<th>From</th>
<th>To</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analog</td>
<td>Digital</td>
</tr>
<tr>
<td>2D</td>
<td>3D</td>
</tr>
<tr>
<td>Static</td>
<td>Dynamic</td>
</tr>
<tr>
<td>Anatomical/Structural</td>
<td>Functional/Physiobiochemical</td>
</tr>
<tr>
<td>Qualitative</td>
<td>Quantitative</td>
</tr>
</tbody>
</table>

1.2.1 Quantitative analysis of images

The use of the word *quantitative* might be misleading when combined to imaging and in particular when related to X-ray imaging since it can be related to the techniques of Dual-energy X-ray Absorptiometry (DXA) and peripheral Quantitative Computed Tomography (pQCT) both used for making measurements of the bone mineral density. Also in microtomography, the word *quantitative* might lead to the concept of holotomography \[1\] \[2\]. Throughout this thesis, the word *quantitative* refers to morphological (or structural) and textural analysis of 3D images.

It is customary to subdivide the broad domain of techniques related to the digital handling of images into image processing, image analysis, and computer vision (see Table 3). There is a significant overlap in the range of techniques and applications that these cover. This implies that the basic methods that are used and developed in these fields are more or less identical, something which can be interpreted as there is only one field with different names. However, the following characterization based on the input/output of the techniques appear relevant but should not be taken as universally accepted.

Roughly, image processing consists in transforming one image into another image, often with the same support. The purpose is typically to enhance its features by e.g. compensating artifacts and reducing noise. Image analysis goes one step further by extracting numerical values, i.e. parameters and/or indexes that quantify or attempt at quantifying some features of the imaged scene. Finally, computer vision is a field of study which generally aims at designing systems that mimic the human sense of sight. It implies providing a symbolic description of an image or scene with the ultimate aim of an automatic decision about the scene itself. While the expression “computer vision” is sometimes improperly used as a broader term including “image analysis” and “image processing”, it will not be used throughout the thesis.

The kind of information obtainable by the acquired images is related to the acquisition technique as well as the related acquisition parameters. For instance, in the clinical practice, X-ray based imaging is very effective for showing doctors a broken bone, but if they want a look at patient’s soft tissues they will likely use an MRI. After
this choice, additional acquisition parameters have to be specified depending on the anatomical site to image and other factors. Provided that the “best” image has been acquired, part of the art of interpreting clinical and biomedical images is to bridge among image characteristics, object properties, biology and chemistry, as well as to determine how all of these aspects are affected by the condition of the investigated object. Roughly, the art of interpreting images is a matter of “seeing” and “sensing” with, of course, an additional stock of knowledge.

Imaging devices are improving more and more. As a consequence, the quality of images in terms of e.g. spatial and contrast resolution, lack of noise and artifacts is also increasing more and more. While an increased quality reduces the risk of misinterpreting the images, any additional support for minimizing this risk is for sure of great help in every practical applications. In fact, in order to support the interpretation of the images, there is a growing demand of image analysis approaches for the automatic, accurate, reliable and reproducible extraction of relevant image features. This is the role of quantitative analysis of images: supporting the human sense of sight, not necessarily mimicking or substituting it.

1.2.2 2D vs. 3D imaging

The acquisition of 3D data on natural and experimental samples via e.g. CT or MRI techniques may be time consuming and expensive to obtain for some scientific applications. Often only two dimensional (2D) data is measured on an outcrop at the macroscale and/or on a thin section at the microscale. Analytical techniques commonly applied to the study of microstructure are restricted to 2D because of the equipment used, e.g. optical and/or scanning electron microscopy. The advantage of these techniques is that they provide low-cost and rapid quantitative inspection of sample textures, still providing some parameters that cannot be easily obtained by using 3D techniques. The main drawback is that sample preparation for such techniques is often destructive and no direct information along the third dimension is given. Therefore, the obtained 2D data may not fully represent the true 3D structures.

The three-dimensional imaging techniques of CT and MRI, as well as the related micro-scale μ-CT and μ-MRI, are therefore very attrac-
tive as they are non destructive, no special sample preparation is, in general, required and a more comprehensive characterization of the sample is achievable. When combined with effective direct 3D image analysis approaches, three dimensional techniques are indeed powerful tools for the biomedical field.

1.3 FRAMEWORK OF THE THESIS

Author’s PhD activities were carried out in a collaboration between the University of Trieste and the Elettra laboratory of the Sincrotrone Trieste S.C.p.A. who funded the PhD scholarship entitled “Development of algorithms and systems for quantitative analysis of 3D images”.

Being Elettra a multidisciplinary environment, the Author had the opportunity to be involved in different research projects mainly in the field of biomedical science [3] [4] [5] [6]. Some research activities have been developed in collaboration with external research groups affording different topics. In particular, the fields of marine biology and Earth sciences were marginally explored during the three-years PhD program [7] [8] [9]. In all the cases, the main effort usually consisted in providing support for the image analysis of the acquired data. In most of the cases, this support had also the aim to test the effectiveness of the newly developed image analysis methods in practical applications.

When the experiments were out of the main topic of interest of the Author, as in marine biology and Earth sciences applications, the additional challenges of a better understanding of the motivations of the study as well as an effective communication with the team members (e.g. geoscientists and marine biologists) were faced by the Author. This has lead to a more “open-minded” stock of knowledge, thus allowing in some cases to transfer the acquired skills to the biomedical science with interesting results.

1.4 OUTLINE OF THE THESIS

The manuscript is divided in two parts coherently with the dual characteristic of the activities (development of methods and applications of the methods) carried out by the Author during his PhD program.

The first part starts describing the basic principles of 3D tomographic techniques (CT and MRI), with special emphasis on X-ray microtomography (µ-CT) with both conventional sources and synchrotron radiation. A description and comparison of the two µ-CT facilities used during some of the experiments performed during the PhD activities is also reported in chapter 2.

The main Author’s effort was directed to the improvement of the Pore3D project [10], which is now a software and hardware tool for the processing and the analysis of 3D images. Pore3D is delivered
1.4 Outline of the thesis

Software as a Service (SaaS) paradigm. The reasons why this paradigm is an effective and efficient solution for addressing the challenge of 3D image analysis are reported in chapter 3. The image processing and analysis algorithms and methods implemented into the the *Pore3D* project are presented in chapter 4 and chapter 5. To be more specific, after imaging and prior to the actual analysis, image processing plays a fundamental role. Three-dimensional image enhancement as well as image quality aspects are discussed in chapter 4. The key step between the acquired images and the analysis process is named segmentation. A review of the main implemented approaches for the segmentation of 3D images is also part of the chapter. Approaches for quantitative analysis of 3D images are then reviewed in chapter 5. The review follows an “application-oriented” presentation, i.e. the analysis methods are presented within a description of the most frequently faced situations in porous media characterization, which is one of the most recurrent applications of 3D imaging.

The second part of the thesis reports three biomedical applications in which image analysis methods were applied by the Author. These applications have in common the characterization of the microarchitecture of trabecular bone, which can be thought of as a “special” porous medium with an interconnected porous space containing bone marrow. The first application (section 6.1) is based on conventional X-ray μ-CT imaging and it aims at developing an automated protocol for the characterization of the microarchitecture of a bone tissue engineering scaffold (a structure that has to mimic the architecture of trabecular bone) directly from the acquired images [3] [4]. Relevant features like porosity, pore-size distribution, throat-size distribution, connectivity and structural anisotropy indexes are extracted.

In the second application (section 6.2), bone alterations occurring in mice exposed to a near-zero gravity are investigated. Mice were hosted in the International Space Station (ISS) for 3 months during the Mouse Drawer System mission (up until now the longest permanence in space of mice). Wild type (Wt) mice as well as transgenic mice over-expressing Pleiotrophin (PTN-Tg) under the control of the human bone specific osteocalcin promoter were selected. PTN-Tg mice were used to investigate whether these mice are protected from space related osteoporosis and whether the PTN over-expression could be considered a countermeasure for the bone loss observed in microgravity as preliminary studies have shown. It is easy to understand the these specimens are particularly precious and therefore non destructive tests are highly desirable. Quantitative analysis of synchrotron radiation X-ray μ-CT images was then performed prior to histomorphometric analysis in order to get a 3D characterization of the turnover in femur and lumbar spine bones [5].

The last application (section 6.3) aims at evaluating the risk of fracture in osteoporotic subjects by means of *in vivo* MRI images of the calcaneus. The spatial resolution of clinical MRI is comparable to the thickness of human trabeculae (approximately 0.1 ÷ 0.2 mm), there-
Therefore specific methods for this low resolution regime has to be developed. Texture analysis of the images is performed in this application and the computed descriptors are used for the evaluation and prediction of the risk of fracture.
Tomography refers to imaging by sections (or sectioning) through the use of any kind of penetrating wave. The word derives from the Greek word *tomas* which means “part” or “section”. A tomographic image represents the idea of “a section”, “a slice” or “a cutting”. The expressions 3D imaging as well as volume imaging can be used when referring to this kind of imaging method.

Common tomography involves gathering projection data from multiple directions and feeding the data into a tomographic reconstruction algorithm in order to get the “slices”. Tomographic images can be obtained by using several different physical sources or phenomena (see Table 4) leading to the widely used techniques of Computed Tomography (CT), Positron Emission Tomography (PET), Single-Photon Emission CT (SPECT), Magnetic Resonance Imaging (MRI) and Electron Tomography (ET). In this thesis only X-ray CT (in particular X-ray μ-CT) and MRI techniques are taken into account.

X-ray CT imaging employs the principle of reconstructing images from measurements of X-ray transmission through the imaged object. MRI imaging exploits powerful magnetic field to align the magnetization of some atoms in the imaged object (hydrogen nuclei of water molecules) and radio frequency fields to systematically alter the alignment of this magnetization. Both techniques allow to get volume renderings Volume rendering and also to get virtual sections, i.e. the display of a 2D image extracted from an arbitrary oriented plane intersecting the 3D volume.

In this chapter, the basic principles of X-ray Computed Tomography (CT) imaging with special emphasis on the related micro-scale technique X-ray microtomography (μ-CT) and Magnetic Resonance Imaging (MRI) are described. The applications reported in the second part of the thesis are based on quantitative analysis of μ-CT and MRI images.
Table 4. Main tomographic imaging techniques

<table>
<thead>
<tr>
<th>Source/Phenomenon</th>
<th>Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-rays</td>
<td>Computed Tomography (CT)</td>
</tr>
<tr>
<td>γ-rays</td>
<td>Single-Photon Emission CT (SPECT)</td>
</tr>
<tr>
<td>positron annihilation</td>
<td>Positron Emission Tomography (PET)</td>
</tr>
<tr>
<td>radio-frequency waves</td>
<td>Magnetic Resonance Imaging (MRI)</td>
</tr>
<tr>
<td>electrons</td>
<td>Electron Tomography (ET)</td>
</tr>
</tbody>
</table>

2.1 PRINCIPLES OF X-RAY COMPUTED MICROCTOMOGRAPHY

X-rays are basically the same thing as visible light rays. Both are wavelike forms of electromagnetic energy carried by particles called photons. The difference between X-rays and visible light rays is the wavelength of the individual photons. A larger atom is more likely to absorb an X-ray photon while smaller atoms, where the electron orbitals are separated by relatively low jumps in energy, are less likely to absorb X-ray photons. This principle is exploited in radiography.

The expression “radiography” refers to procedures for recording, displaying, and using information carried by an X-ray beam to a radiographic film or a detector system. Radiography is the basis for X-ray computed tomography (CT) and microtomography (μ-CT).

During a μ-CT scan the sample is placed on a high-resolution rotation stage and illuminated with X-rays in transmission geometry. Radiographs (or projections) are usually collected at fixed angular increments (the sample can be either rotated continuously or step by step). The total rotational angle depends upon the geometry of the beam and on the sample, but typically is 180° when a monochromatic nearly parallel beam is used (typically at a synchrotron) or 360° when a cone-beam geometry is used (typically in a laboratory apparatus). After passing through the sample the X-rays are then imaged with a 2D detector. For μ-CT the X-rays are normally converted into visible light by a scintillator screen. The visible light rays are then detected by either a cooled CCD camera or a CMOS-based detector. The scintillator screen can be coupled to the chip of the camera by direct deposition on a fiber optic taper or by magnifying lenses. For larger samples, flat-panel detectors based on an amorphous silicon scintillator with no magnification lens are often used. In clinical CT the sample (the patient) slides through the “donut” (the gantry) while the system source-detector rotates around the patient.

Depending on the sample size and the employed detector configuration, a large number of projections are collected for each tomographic scan. Theoretically the minimum number of projections is \( \pi /2 \) times the largest number of sample pixels in the direction perpendicular to the rotation axis [14]. In practice, a smaller number of projections is often used since a trade-off among total scan duration,
image accuracy and delivered dose is required. The 3D tomographic image is reconstructed starting from the sample projections.

The transmission (or absorption) X-ray µ-CT technique is based on the mapping of the linear attenuation coefficient of X-rays traversing the investigated sample \[15]\). The attenuation depends upon the composition and density of the scanned sample. Mathematically, if different regions \(i = 1, ..., n\) occur along the path of X-rays, the transmission through the imaged object is (Beer-Lambert law):

\[ I = I_0 e^{-\sum_{i=1}^{n} \mu_i x_i}. \]

Each region contributes “reducing” the initial intensity of the beam \(I_0\) to the final recorded attenuation and the contribution is proportional to the segment \(x_i\) of the path that presents the linear attenuation coefficient \(\mu_i\). The attenuation is considered along a line of pixels in the radiographic image perpendicular to the incoming X-ray beam at a specific height, and the solution of the function \(\mu(x, \theta)\) for all the angles \(\theta\) is found by applying the Radon transform. This solution is repeated for each line of pixels at all angles to create the tomographic reconstruction\(^1\). For parallel beam geometry (i.e. for a synchrotron source) the filtered backprojection or a Fourier transform-based technique is typically used. For cone-beam geometry (e.g. typically a laboratory tube source, but also for synchrotron sources with focusing optics) a Feldkamp reconstruction is normally used \[16\], though it is less accurate in particular when the cone angle is larger than about 20 degrees. For cone-beam geometry, or in the case of limited projections covering a rotation range less than 180 degrees, novel approaches based on the use of iterative methods, such as the Simultaneous Algebraic Reconstruction Technique, SART \[17\] and the Discrete Algebraic Reconstruction Technique, DART \[18\], provide images with high spatial resolution from a limited number of projections.

After the reconstruction of multiple slices, a complete tomographic dataset is obtained, which is composed of 3D “pixels”, better named voxels. The distribution of regions with different density and/or chemical composition inside the investigated sample can be visualized by means of virtual slicing or volume rendering procedures.

For a given X-ray source and X-ray energy, the choice of the best experimental parameters needed to obtain high-resolution images, such as the total number of projections or the spatial resolution of the detector, is mainly affected by X-ray dose delivery restrictions (clinical applications or possible damage of the sample) or scanning times (i.e. clinical or fast-tomography applications). In some cases, an alternative method is to zoom-in on a given region of interest of the sample increasing the spatial resolution of the detector only in that region leading to the so-called local area tomography \[19\]. This approach, however, has the drawback of projection truncation, leading to cupping artifacts in the reconstructed slices \[20\].

\(^1\) For mathematical details about the Radon transform and the computation of the tomographic reconstruction from the recorded projections see \[14\].
Two main different modes (absorption and phase contrast) used in μ-CT are here described. In absorption mode the contrast is given by the difference between the linear attenuation coefficients (essentially the atomic numbers of the elements and the density). Larger differences lead to better contrast resolution and better contrast makes easier the subsequent image analysis. This is the conventional mode and, in this case, the detector is placed close to the sample to avoid phase effects due to propagation from sample to detector (Figure 1). Too low transmission results in bad photon statistics whereas too high transmission results in too low contrast between elements. When the beam is monochromatic, some artifacts are avoided (see further subsection 4.1.4) and it is possible to perform quantitative analysis (with the meaning mentioned at the beginning of subsection 1.2.1) since the gray level is directly linked to the absorption coefficient, related to the atomic number and density (absorptiometry).
2.3 Micro-CT setups

The phase contrast mode [21] is mainly observed when the beam is partially coherent and when the distance between the sample and the detector is appropriately increased in comparison to the absorption mode (Figure 1). However it has been shown [22] that also polychromatic sources may be used for phase-contrast imaging. The contrast is due to interference after propagation between parts of wave at either side of an interface that have suffered different phase retardation. This contrast is superimposed to the conventional absorption contrast and it is efficient for edge detection especially when absorption only leads to weak contrast but also where small differences in mass density is present [6]. Increasing the distance between the sample and the detector results initially to stronger and broader signature of edges in the material. At larger distance, broad Fresnel fringes cover the image, turning it into a hologram with less and less direct resemblance to the object. In the so-called edge detection regime, the reconstructed quantity using conventional reconstruction algorithm is proportional to the Laplacian of the refractive index [2]. This gives signature of interfaces in the material but in practice, segmentation of such images for further image analysis may be sometimes difficult.

Phase (sensitive) radiography and tomography applies not only to light materials (e.g. polymers, plants) but also to heavier ones, such as alloys or geomaterials. The mean attenuation in the latter samples is appreciable but in some cases the difference in attenuation coefficient between different phases could be small to be detected in the absorption mode. Fresnel fringes can also signal the presence and the position of isolated features with a transverse dimension small compared to the spatial resolution such as cracks with submicron opening. The possibility to obtain 2D and 3D information about the internal structure of nontransparent samples with a sensitivity much greater than classical X-ray absorption imaging is of great interest for materials science samples.

2.3 MICRO-CT SETUPS

Two different setups to perform X-ray microtomography experiments were exploited in the applications described in this thesis (chapter 6). The first one is the µ-CT apparatus of the SYRMEP beamline of the 3rd generation Italian synchrotron radiation facility (Elettra). The second one is a laboratory µ-CT instrument named TomoLab equipped with a micro-focus X-ray tube and based on a cone-beam geometry.

2.3.1 Synchrotron radiation micro-CT at SYRMEP

Elettra operates at two electron energies, namely 2.0 GeV and 2.4 GeV respectively. Some of the X-ray experiments of this thesis have been carried out at the SYRMEP² (SYnchrotron Radiation for MEdical

² Website: http://www.elettra.trieste.it/experiments/beamlines/syrmep/
Tomographic three-dimensional imaging

Physics) beamline. The source is one of the Elettra bending magnets (Figure 2a). Depending on the experimental setup, a white beam can be used or a monochromator may be placed between the source and the sample in order to get a monochromatic beam. In the applications of this thesis only the monochromatic configuration depicted in Figure 2b was used without additional tools for magnification. A system of in-vacuum tungsten slits shapes the beam in the desired rectangular dimension. A double silicon crystals monochromator follows the slits and it allows to select a specific X-ray energy between 8.3 and 35 keV. Downstream the monochromator, a second system of in-air slits shapes further the monochromatic beam right before the experimental hutch. The X-ray beam is thus practically parallel as depicted in Figure 3. Thanks to the parallel and monochromatic beam setup, an “exact” and quantitative reconstruction free of geometrical artifacts is possible.

2.3.2 Conventional X-ray micro-CT at TomoLab

At Elettra a conventional μ-CT system named TomoLab is available. It has been designed as a complementary instrument to the SYRMEP beamline both for the energy range and the beam

Figure 2. Sketch of the SYRMEP beamline: a) X-ray beam coming out from a bending magnet of Elettra; b) Schematic view of the SYRMEP beamline in monochromatic beam configuration (the size of the elements in the sketch does not correspond to the real size).
2.3 Micro-CT setups

![Diagram of Micro-CT setups]

**Figure 3.** Main $\mu$-CT setups. a) Cone-beam: micro-focus X-ray source with 2D detector (the sample results magnified in the projection image); b) Nearly parallel-beam: synchrotron radiation X-ray source with 2D detector (the sample does not result magnified).

size at sample. The TomoLab$^3$ is a cone-beam desktop $\mu$-CT station equipped with a sealed micro-focus X-ray tube. The system operates at a voltage range from 40 up to 130 kV, and a maximum current of 300 $\mu$A. The detector is a water cooled CCD camera offering a good combination between a large field of view (49.9 mm $\times$ 33.2 mm) and a small pixel size (12.5 $\mu$m $\times$ 12.5 $\mu$m). Due to the cone-beam geometry (see Figure 3) it is possible to achieve a spatial resolution close to the focal spot size (5÷8 $\mu$m). It is also possible to perform phase-contrast measurements, although the achieved spatial coherence is limited with respect to the one achievable with synchrotron sources.

2.3.3 Comparison between SYRMEP and TomoLab

Some of the relevant differences between SYRMEP and TomoLab are summarized in Table 5. The TomoLab station features a polychromatic cone beam while the monochromatic configuration of the SYRMEP beamline was exploited in the experiments of this thesis. Regarding the energy range (or voltage in the case of TomoLab), SYRMEP operates in the 8.3÷35 keV range while the TomoLab in the 40÷130 kV $\approx$ 20÷65 keV range. The spatial resolution and subsequently the field of view (FOV) at the TomoLab station can be tuned by changing the ratio between the source-to-detector and source-to-sample distances (magnification). On the other hand, no magnification was possible at the beamline with the setup adopted in the experiments of this thesis and therefore spatial resolution and FOV were governed only by the imaging detector. The main limitation of the synchrotron source is its vertical beam size that is limited to 4÷6 mm depending on the X-ray beam energy. As a consequence, plane images of larger samples have to be obtained by a vertical scan of

$^3$ Website: [http://www.elettra.trieste.it/Labs/TOMOLAB/](http://www.elettra.trieste.it/Labs/TOMOLAB/)
2 Tomographic Three-Dimensional Imaging

Figure 4. The TomoLab station: a) external cabinet; b) internal view where the micro-focus X-ray source (in the background with a red case), the rotational stage and the detector (black tube in the foreground) are visible.

Table 5. Comparison between the SYRMEP beamline and the TomoLab

<table>
<thead>
<tr>
<th></th>
<th>SYRMEP</th>
<th>TomoLab</th>
</tr>
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<tbody>
<tr>
<td>Beam</td>
<td>monochromatic</td>
<td>polychromatic</td>
</tr>
<tr>
<td>Energy range</td>
<td>8.3–35 keV</td>
<td>40–130 kV</td>
</tr>
<tr>
<td>Beam shape</td>
<td>nearly-parallel</td>
<td>cone</td>
</tr>
<tr>
<td>Spatial resolution</td>
<td>detector-limited</td>
<td>source-limited</td>
</tr>
</tbody>
</table>

the sample through the stationary X-ray beam. SYRMEP provides a monochromatic parallel beam that permits to tune the most suited energy to the specific sample improving image quality (see subsection 4.1.4).

2.4 PRINCIPLES OF MAGNETIC RESONANCE IMAGING (MRI)

The biggest and most important component of an MRI system is the magnet. There is a horizontal tube, known as the bore – the same one the patient enters in clinical MRI systems – running through the magnet from front to back. This magnet is capable of producing a large, stable magnetic field.

Most MRI systems use a superconducting magnet, which consists of many coils or windings of wire through which a current of electricity is passed, creating a magnetic field of up to 1.5 or 3.0 tesla. Maintaining such a large magnetic field requires a good deal of energy, which is accomplished by superconductivity, or reducing the

---

4 The Earth’s magnetic field measures 0.5 gauss (1 tesla = 10,000 gauss). MRI systems uses really powerful magnets.
resistance in the wires to almost zero. To do this, the wires are continually bathed in liquid helium.

There are also three gradient magnets inside the MRI machine. These magnets are much lower strength compared to the main magnetic field. They may range in strength from 180 gauss to 270 gauss. While the main magnet creates an intense and stable magnetic field around the patient, the gradient magnets create a variable field, which allows different parts of the patient to be scanned.

Another part of the MRI system is a set of coils that transmit radiofrequency waves into the patient’s body. There are different coils for different parts of the body (knees, shoulders, wrists, heads, necks and so on). These coils usually conform to the contour of the body part being imaged, or at least reside very close to it during the exam.

When patients slide into an MRI machine, they take with them the billions of atoms that make up the human body. For the purposes of an MRI scan, only hydrogen atoms are useful but they are abundant since the body is mostly made up of water and fat. These atoms are randomly spinning, or precessing, on their axis (Figure 5). All of the atoms are going in various directions, but when placed in a magnetic field, the atoms line up in the direction of the field.

These hydrogen atoms have a strong magnetic moment, which means that in a magnetic field, they line up in the direction of the field. Since the magnetic field runs straight down the center of the machine, the hydrogen protons line up so that they’re pointing to either the patient’s feet or the head. About half go each way, so that the vast majority of the protons cancel each other out – that is, for each atom lined up toward the feet, one is lined up toward the head. Only a couple of protons out of every million are not canceled out (Figure 5b). There is a slight preference for hydrogen protons to align along the direction of the magnetic field (a slightly lower and therefore preferred energy state) rather than opposite to it (a slightly higher energy state). This imbalance depends on the strength of the magnetic field. At a magnetic field strength of 1.5 T, approximately 6 magnetic hydrogen protons per million hydrogen atoms are aligned along the magnetic field that are not canceled by oppositely oriented protons. This does not sound like much, but the sheer number of hydrogen atoms in the body is enough to create extremely detailed images. Summing over the magnetic protons in a object element gives a net tissue magnetization due to the preferential alignment and imbalance of hydrogen protons in tissues when placed in a static magnetic field. However, because the magnetic field due to this imbalance is so much smaller than the magnetic field applied by the MRI system, a more refined and subtle approach is then needed to measure this small tissue magnetization.

Next, the MRI machine applies a radio frequency (RF) pulse that is specific only to hydrogen. The system directs the pulse toward the area of the body one wants to examine. When the pulse is applied, the unmatched protons absorb the energy and spin again in a different direction. This is the “resonance” part of the acronym MRI. The
Figure 5. Basic principles behind MRI. a) Atoms normally spin in random directions; b) In magnetic field produced by MRI, atoms line up either north or south but there are a few unmatched atoms; c) When radio frequency pulse is applied, the unmatched atoms spin the other way; d) When radio frequency is turned off the extra atoms return to normal position emitting energy. This energy is the signal used for the creation of the image.

RF pulse forces them to spin at a particular frequency, in a particular direction. The specific frequency of resonance is called the Larmour frequency and is calculated based on the particular tissue being imaged and the strength of the main magnetic field (Figure 5c). The strength of the RF wave and its duration determine the tip angle of tissue magnetization.

At approximately the same time, the three gradient magnets jump into the act. They are arranged in such a manner inside the main magnet that when they are turned on and off rapidly in a specific manner, they alter the main magnetic field on a local level. What this means is that it is possible to pick exactly a desired area. This area is referred to as the “slice”. More precisely, these three magnetic gradients are used to localize the sources of signals along each of the three coordinates. In planar (or two-dimensional Fourier transform)
Principles of Magnetic Resonance Imaging (MRI)

MR imaging, one gradient is turned on during tissue excitation (as RF signal is sent into the patient) to provide slice selection, another gradient is turned on during signal measurement, and a third gradient is turned on between signal excitation and signal measurement to provide phase encoding.

When the RF pulse is turned off, the hydrogen protons slowly return to their natural alignment within the magnetic field and release the energy absorbed from the RF pulses. This is called “relaxation”. When they do this, they give off a signal that the coils pick up and send to the computer system (Figure 3d). The rate at which this relaxation occurs is different for different tissues and is the fundamental source of contrast in the so-called T1-weighted MRI images. The system goes through the patient’s body point by point, building up a map of tissue types. It then integrates all of this information to create 2D images or 3D models with a mathematical formula known as the Fourier transform. The computer receives the signal from the spinning protons as mathematical data and the data is converted into a picture. That’s the “imaging” part of the acronym MRI.

In addition to T1-weighted images, one can take advantage of other principles to produce MR images. The net magnetization is made up of contributions from many protons, which are all precessing. During the RF pulse, the protons begin to precess together (they become “in phase”). Immediately after the RF pulse, the protons are still in phase but begin to dephase due to four possible effects, i.e. spin-spin interactions, magnetic field inhomogeneities magnetic susceptibility and chemical shift effects. When dephasing occurs due to all four effects, it is called T2* (T2 star) decay or T2* relaxation. When dephasing is due only to the effect called spin-spin interactions, the dephasing it is called T2 decay or T2 relaxation. T2 is a parameter that is characteristic of specific tissue and characterizes the rate of dephasing for the protons associated with that tissue. It can be measured and used as source of contrast for MR images.

In addition to T1 and T2 images, several different pulse sequence acquisition strategies exist (e.g. spin echo, multiecho spin echo, inversion recovery, gradient recalled echo). A very simplified pulse sequence is a combination of RF pulses, signals and intervening periods of recovery. It consists of several components, the main ones are the repetition time (TR) and the echo time (TE). The repetition time (TR) is the time from the application of one RF pulse to the application of the next RF pulse and is measured in milliseconds (ms). The TR determines the amount of relaxation that is allowed to occur between the end of one RF pulse and the application of the next. Therefore the TR determines the amount of T1 relaxation that has occurred. The echo time (TE) is the time from the application of the RF pulse to the peak of the signal induced in the coil and is also measured in ms. The TE determines how much decay of transverse magnetization is allowed to occur before the signal is read. Therefore, the TE controls the amount of T2 relaxation that has occurred. The application of
Table 6. Quick comparison between CT and MRI. (Where not specified the comparison applies to both the clinical and the micro-scale techniques)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CT</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanned object</td>
<td>Almost any</td>
<td>Paramagnetic atoms should be in the object (e.g. water or contrast agents)</td>
</tr>
<tr>
<td>Spatial resolution</td>
<td>Comparable to MRI in clinical CT.</td>
<td>Comparable to CT in clinical MRI.</td>
</tr>
<tr>
<td></td>
<td>Even below 1 µm in µ-CT</td>
<td>Up to 20 µm (in plane) for µ-MRI</td>
</tr>
<tr>
<td>Voxel type</td>
<td>Usually isotropic in µ-CT</td>
<td>Usually anisotropic in µ-MRI</td>
</tr>
<tr>
<td>Scanning time</td>
<td>Fast</td>
<td>Slow</td>
</tr>
<tr>
<td>In vivo concerns</td>
<td>Ionizing radiation</td>
<td>Non-invasive</td>
</tr>
</tbody>
</table>

RF pulses at certain repetition times and the receiving of signals at pre-defined echo times produces contrast in MRI images⁵.

For the sake of completeness, the micro-scale MRI technique is also briefly mentioned, even though µ-MRI experiments were not performed by the Author during his PhD program. Micro-MRI is mainly used for pre-clinical investigations on small animal models. A µ-MRI image has a good spatial resolution (up to 100 µm and even 25 µm in very high strength magnetic fields) and an excellent contrast resolution, in particular for soft tissues. The biggest drawbacks of a µ-MRI experiment is its cost, due to the high cost of the device (a few millions of euros). Furthermore, the image acquisition time is extremely long, spanning into minutes and even hours. This negatively affects animals that should be anesthetized for long periods of time, thus making hard to perform some kind of in vivo studies.

2.5 COMPARISON BETWEEN MICRO-CT AND MRI

An accurate and detailed comparison of the two tomographic techniques introduced in this chapter should require a severe distinguish between the clinical systems and the related micro-scale techniques as the fields of application are, of course, usually different. Only a very rough comparison is mentioned in this section and some significant aspects are summarized in Table 6.

When focusing to the clinical use, X-ray based imaging is usually very effective for hard tissues (e.g. bone, teeth), while MRI is usually

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⁵ For more details about contrast formation in the most commonly used pulse sequences see [24].
Comparison between micro-CT and MRI

Figure 6. Comparison of a μ-CT and μ-MRI image. a) X-ray μ-CT slice of a mouse acquired in vivo; b) Analogous post mortem μ-MRI slice. The scale bar is 5 mm. (Image courtesy of Dr. Alberto Astolfo)

better suited for patient’s soft tissues, including organs, ligaments and the circulatory system. The patient can not move very much during an MRI scan. MRI scans require patients to hold still for 20 to 90 minutes or more. Even very slight movement of the part being scanned can cause distorted images (motion artifacts) that will have to be repeated. MRI systems are also very expensive to purchase, and therefore the exams are also very expensive. A CT scan is faster (few seconds, thus the risk of motion artifacts is reduced) and, in general, cheaper. The fact that MRI systems do not use ionizing radiation, as X-ray based imaging devices do, is a comfort to many patients.

The micro-scale techniques μ-MRI and μ-CT are mainly used for research purposes. While the spatial resolution of clinical systems is somewhat comparable, the microscale techniques present significant differences in the spatial resolution achievable. With μ-CT, it is possible to reconstruct images having isotropic voxel size even below 1 μm. This is sometimes called nano-CT. Voxel size is rarely isotropic in μ-MRI because the slice thickness is usually greater than the in-plane resolution. Isotropic voxel size is very useful for further quantitative analysis of images.

In order to visually appreciate some differences between an X-ray based technique and MRI, Figure 6 reports an example of a mouse imaged in vivo by means of a suitable μ-CT setup at SYRMEP and post mortem by means of μ-MRI. The μ-CT experiment was performed with energy $E = 24$ keV, 4 mm Aluminum filter, distance sample-to-detector = 40 cm and resulting voxel size = 56 μm, the total scan time was about 5 minutes. Being an in vivo study, the radiation dose was a concern and therefore the resulting voxel size was far from the minimum usually achievable at SYRMEP. The post mortem sample was imaged by means of a Bruker 9.4 T MRI scanner using a 3D Flash (gradient echo) scanning sequence with echo time $TE = 10$ ms, repetition time $TR = 34$ ms, flip angle $\alpha = 30$ deg and resulting pixel size (in-plane) = 60 μm. The acquisition took about 9 hours. It can be noticed from Figure 6 that bony structures appear brighter in the
X-ray $\mu$-CT while they appear darker in the analogous $\mu$-MRI. Soft tissues are more detailed in the $\mu$-MRI.
The handling of tomographic 3D images requires a great amount of hardware resources and this is particularly true for datasets produced by the \( \mu \)-CT technique introduced in the previous chapter. For instance, a \( 2048 \times 2048 \) pixels detector may produce a 8GB dataset of 8-bit images or a 32GB dataset in the case of 32-bit images. In some cases, different areas of a big sample are scanned and the analysis has to take into account multiple datasets in order to characterize the whole scanned object. Moreover, when ultra-fast tomography is performed, time resolved experiments are possible (see also subsection 4.1.4) and there is the need to consider several datasets in order to characterize the dynamic changes through time of the imaged sample. Therefore, software and hardware tools able to manipulate huge amount of data are necessary.

In this chapter the \textit{Pore3D} project is described starting from the motivations behind it to the current release. It will be shown that \textit{Pore3D} is an effective software and hardware solution for addressing the challenges of tomographic image processing and analysis. The image processing and analysis algorithms and methods implemented in the current release of the \textit{Pore3D} project will be presented in chapter 4 and chapter 5.

### 3.1 Motivations of the project

In some cases 3D processing or analysis can be performed with multiple 2D operations (as represented in Figure 7) and therefore a sequential application of 2D procedures produce effective results. In fact, 3D image processing can be decomposed in a sequence of 2D image processing steps for e.g. ring artifacts removal in tomographic images or, with little effort, decomposable filtering such as the gaussian smoothing. The volume fraction (or porosity) assessment is also a “decomposable” problem as it can be computed by taking into account the contribution of several 2D segmented images (“slices”). In this case, the 3D domain is seen as a “special” case to which “ex-
3D analysis with the “slice-by-slice” approach

```
3D Volume (MxNxP)

slice 1 (MxN) 2D processing slice 1 (MxN)

slice 2 (MxN) 2D processing slice 2 (MxN)

... 2D processing ...

... 2D processing ...

slice P (MxN) 2D processing slice P (MxN)

3D Volume (MxNxP)
```

3D analysis with the “full 3D” approach

```
3D Volume (MxNxP)

3D processing 3D Volume (MxNxP)
```

Figure 7. Full 3D vs. “slice-by-slice” approach. In the “slice-by-slice” approach the 3D domain is thought as a sort of “multiple 2D” domain and the 3D volume is sliced into a stack of 2D images in order to apply easily available 2D techniques. The full 3D approach operates directly in the three-dimensional domain.

tend” established 2D approaches and therefore well known software tools may be exploited for this purpose. Also parallel implementation of algorithms and methods comes straightforward in this case and therefore fast processing is possible with this “slice-by-slice” approach (a so-called “embarrassingly parallel” approach). However, this strategy is not correct when direct 3D information is needed. This is the case for the assessment of e.g. the number of objects in the considered volume. The number of objects per 2D “slice” cannot be linked to the true 3D number of objects per unit volume since on a 2D section objects might appear separated whereas they are connected along the third dimension. Moreover, the connectivity of phases as well as the structural anisotropy cannot be obtained from 2D measurements since these are direct 3D parameters. An interesting analysis approach based on the skeletonization of the pore space (see section 5.5) also requires 3D tools. Parallel implementations do not come straightforward in this case and therefore attention must be paid when trying to offer an efficient solution.

Several commercial software (e.g. MAVI1, Avizo2) and public available libraries (e.g. DIPlib3, ITK4) as well as research codes (e.g. the 3DMA packages [25], Blob3D [26], Quant3D [27]) have been developed in recent years for 3D image analysis. Limited analysis are

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1 Website: http://www.mavi-3d.de
2 Website: http://www.vsg3d.com
3 Website: http://www.diplib.org
4 Website: http://www.itk.org
available in some cases and “black box” tools are sometimes offered, especially in commercial software, thus making hard to understand if assumptions are required before attempting the analysis process. However, an effective solution that takes into account also the hardware parameter was yet to be available as all the aforementioned software is conceived for a stand-alone application on a local machine. Due to local hardware limitations, most of these software tools allow to process only relatively small datasets. Moreover, some of these software tools, in particular research codes, are not able to take advantage of multi-core and/or multi-processor machines.

3.2 History of the project

Several years ago the SYRMEP research group of the Elettra Synchrotron Light Laboratory started to develop the Pore3D project\[10\]. The main aim was to merge many of the features implemented in the above-mentioned software, customizing in some cases their characteristics or adding new tools, on the basis of the specific know-how acquired inside the SYRMEP group. Moreover, an in-house developed software assures a complete control of the algorithm implementation, allowing also different strategies of analysis as a function of the specific scientific application. This avoids “black box” approaches. Although any kind of 3D data set is a valid input, the Pore3D project was conceived and optimized for the handling of µ-CT images and it offers tools for performing all the approaches for image processing and analysis that will be presented in the next chapters.

Originally prototyped in MATLAB by K. Parnian (ICTP, Trieste, Italy) with the collaboration of S. Favretto (University of Trieste, Trieste, Italy), the software was completely rewritten by the Author in C language with parallel programming extensions in order to improve the performance of the product (both in terms of computational time and memory requirements) and, most significantly, in order to migrate to an innovative Software-as-a-Service solution.

3.3 The current release

The current release of Pore3D is based on an interesting paradigm, here named Scientific Software-as-a-Service (SaaS), that allows to exploit special hardware resources, thus offering performances not available with local machines.

3.3.1 The SaaS paradigm

Universities, research institutions as well as R&D (Research and Development) departments of industries may require the computation of large amount of data. The classical way to face this issue is by acquiring the necessary hardware and software tools. However, this ap-

Website: http://ulisse.elettra.trieste.it/uos/pore3d
Figure 8. Approaches for intense computation. In the traditional way to solve the problem of intense computation, each industry builds its dedicated computing center with software and hardware sized for its specific application. In the SaaS-based solution, state-of-the-art software and up-to-date hardware are offered as a service by the SaaS provider. Each industry pays these resources on demand (usually according “pay as you go” policies).

A cheapest and better way to face the problem of intense computation is by contacting a scientific Software as a Service (SaaS) provider that is able to offer a “pay as you go” solution. In this way, universities and industries are not required to invest large amount of money to acquire high performance stand-alone machines and/or expensive software tools to install on the local machines, but they take advantage of the tools offered by the scientific SaaS provider for only the necessary time period. Moreover, by using the internal specialized hardware tools, the solution offered by the scientific SaaS is typically faster than any solution that exploits common hardware. In addition, in a scenario that involves geographically distributed collaborations among universities and/or R&D departments of industries, there is the need to take into account ways for permitting an effective commu-
3.3 The current release

Figure 9. Software and hardware architecture of Pore3D. A specialized middleware (cloud and storage computing) available at Elettra is exploited.

communication among the involved departments. The SaaS based solution relieves the involved departments of the responsibility to implement communication methods offering a common point easily reachable by all the distributed members via common network technologies (i.e. a web browser). Therefore, the SaaS solution greatly accelerates research activities both in terms of computational cost and communication. Universities and industries may also decide to deliver their own custom software solutions through a scientific SaaS provider, resulting in a better protection of the intellectual properties. The SaaS-based approach as well as the classical one are represented in Figure 8.

The Pore3D SaaS presents all the aforementioned properties allowing universities and industries to solve the specific problem of an effective and efficient analysis of large 3D images. The Pore3D SaaS overcomes the classical approach to intense computation exploiting the concept of cloud computing. Huge datasets of µ-CT images can be efficiently processed and analyzed without being limited by the local hardware resources.

3.3.2 Software architecture

Pore3D consists of a software library (the “engine”) and a “bridge” (or wrapper) for the high-level scripting environment IDL. Users exploit the features of Pore3D by preparing a script in IDL language. The engine is then responsible to reach the cloud computing and storage resources of Elettra, thus allowing to manipulate large amount of datasets.
data in a reasonable amount of time. This scenario is represented in Figure 9.

The choice of IDL as user interface has been conceived taking into account that users may be interested in further data manipulation (e.g., computing image histogram) and/or representing data in custom graphical form (e.g., plotting the computed pore size distribution). Simple tasks in image processing and visualization can be easily and effectively performed via IDL. In fact, the philosophy behind the Pore3D (at least at this stage) is a strict focus onto innovative and state-of-the-art 3D image processing and analysis with visualization of images and results via IDL (or with external tools).
The whole process that starts from image acquisition and ends with the actual analysis usually involves several persons with different skills of expertise. Although the produced images are of course related to the imaging technique, it is not uncommon that the image analysis step is not carried out by the same team members that performed the acquisition. This is the common scenario of e.g. the clinical practice in which imaging is performed by technical personnel while the image evaluation (monitoring or diagnosing) requires the skills of a medical doctor. Doctors start their evaluations assuming that the “best” image has been provided them. However, they are still required to recognize the artifacts that the imaging technique may produce in order to avoid misinterpretation of the final images. This philosophy holds also in every scientific applications that involves imaging. Since every imaging techniques has its own prerogative in terms of quality, analysts are required to recognize the artifacts introduced by the acquisition techniques in order to correctly apply image analysis approaches.

Image analysis aims at extracting numerical values, i.e. parameters and/or indexes that quantify or attempt at quantifying some features of the imaged object. An image analysis process includes one or more image processing steps consisting in transforming the original image into an enhanced image better suited for further analysis. In an image enhancement system, there is no conscious effort to improve the fidelity of a reproduced image with regard to some ideal form of the image (as is done in image restoration). Image enhancement is generally subjectively performed and, when included into an image analysis workflow, it usually aims at ease or improve the key step named...
Moreover, there is no general unifying theory of image enhancement because there is no general standard of image quality that can serve as a design criterion for an image enhancement processor [28]. Consideration is given in this chapter to the techniques that have proved useful for human observation and image analysis improvement.

This chapter presents methods and techniques for performing the image enhancement required for the subsequent analysis process. In particular, the final output of what is called image processing throughout this chapter is a segmented Volume of Interest (VOI). The segmented VOI is the input for almost all the approaches for the actual analysis that will be presented in chapter 5. Techniques for performing the segmentation step and the selection of the VOI as well as the effects that these steps will have on the final quantitative analysis are discussed in this chapter. Being the segmentation strongly affected by the quality of the acquired images, the chapter starts defining the parameters for evaluating the quality of an image with focus on µ-CT and MRI images. It is hereafter assumed that the “best” image was obtained with accurate image acquisition and that the analysis is based on the images only with the voxel size as the unique additional information required to appropriately express the computed measures. The only small exception to this assumption is related to the ring artifacts removal as described in subsection 4.1.4. In general, software-based approaches for the compensation of the most recurrent imaging artifacts are logically part of the imaging process and they also usually have to be included into the imaging process as they might require intermediate data (e.g., the projections or the sinograms during CT reconstruction). In this chapter, a ring removal algorithm that operates directly on the final images (i.e., without the need of additional data from the imaging process) will be presented. While this kind of ring removal breaks the assumption that the “best” image was produced in the imaging step, the assumption that all the analysis presented in this thesis is based only on the acquired images still holds.

4.1 IMAGE QUALITY

The degree to which an image achieves its purpose is described by the vague term image quality. In part, image quality connotes how clearly the image displays information about the morphology and features of the imaged sample. Image quality depends on other aspects as well, including whether the proper area of the sample is examined, whether the correct images are obtained, and last but not least whether the desired characteristic of the sample is detectable by imaging.

Image quality is influenced by four fundamental characteristics of the image: spatial resolution, contrast resolution, noise, and distortion or artifacts. In any image, the clarity of information is affected
by these aspects and how they interact with each other. When imaging an object, researchers and scientists have to find the best trade off among all these aspects. These aspects are also strongly related to the imaging device and the conditions of the imaged sample.

4.1.1 Spatial resolution

Resolution is perhaps a confusing term in describing the characteristics of a visual image since it has a large number of competing terms and definitions. In its simplest form, image resolution is defined as the smallest discernible or measurable detail in a visual presentation. Researchers in digital image processing and analysis use the term resolution mainly for spatial resolution. For the digital image processing, spatial resolution is related to the spacing of pixels or voxels in an image, usually expressed in pixels per unit (millimeters or micron) or, equivalently and more commonly, in pixel or voxel size, i.e. the number of millimeters or microns for one pixel or voxel.

Every digital image presents an element of blurring to well-defined (sharp) boundaries (edges) in the object. An increased size of the pixels or voxels composing the images leads to blurry images in which details below this size are not recognizable. Also, when the size of a detail is comparable with the voxel size, the detail can be easily misinterpreted as noise. When measuring physical features of the imaged object (e.g. the thickness or length of a part of the object), higher voxel size will lead to less precise measures.

Users are generally interested in achieving the smallest voxel size while imaging the largest area of the considered object. However, as stated above, this usually implies that the other image quality parameters worsen. Radiation dose might be also an additional concern. If the final contrast is too poor or the amount of noise is too high, the desire of an extreme spatial resolution and the largest area of interest can potentially degrade images sufficiently to make them unusable for the characterization of the imaged object.

4.1.2 Contrast resolution

Contrast is the second major feature of an image. This characteristic describes how well the image distinguishes different features in the object. The contrast between adjacent regions representing different phases of the imaged object can be described as the difference in brightness between the two regions. In X-ray imaging, the contrast is a reflection primarily of atomic number and physical density differences among the different phases of the sample. Sometimes a substance can be introduced into the sample to enhance the contrast. This substance, termed a contrast agent (or dye), is selected to provide a signal different from that of the surrounding phases.

Every imaging application reflects a choice of a specific imaging technique among many alternatives to yield images of greatest poten-
tial to provide the desired information. For a specific imaging application, image contrast can be influenced by careful trade-off of the technique factors used to produce the image. In radiographic imaging of the breast, for example, subtle differences among tissues can be accentuated in the image by use of low peak kilovoltage and small amounts of beam filtration. These choices enhance the differential transmission of X-rays through tissues that vary slightly in atomic composition and physical density. The choice of specific pulse sequences and other variables in MRI strongly influences the resulting contrast among structures in the image. Choices in the \(\mu\)-CT acquisition part (e.g. the number of projections) as well as variations in the parameters of the reconstruction process also affect the contrast resolution of the final images. The properties of the detector used to acquire the image are also crucial for the resulting contrast.

4.1.3 Noise

Every image contains information that is not useful for the characterization of the object’s condition. This information not only is of little interest to the observer, but often also interferes with the representation of image features crucial to the analysis process. Irrelevant information in the image is defined as image noise. Image noise has different sources, but mainly it arises from a noisy sensor or channel transmission errors. Noise usually appears as discrete isolated pixel or voxel variations that are not spatially correlated. These pixels or voxels often appear visually to be markedly different from their neighbors.

Noise added to an image generally has a higher-spatial-frequency spectrum than the normal image components because of its spatial decorrelatedness. Digital image processing low-pass filters (e.g. mean, gaussian, median) are able to successfully average out noisy pixels or voxels in most of the cases. However, tomographic images are the result of a reconstruction process that already includes some “averaging” step, e.g. the “filtered” part of the filtered back projection algorithm for CT images. Additional de-noising steps can be also introduced within the reconstruction process, since processing the tomographic projections is generally a better choice than processing the final slices. Therefore, by correctly “tuning” the reconstruction steps, the final images usually present a negligible amount of noise. Hence, there is no need to further post-processing. Moreover, if the number of acquired projections can be increased, the final CT images will result with higher quality also in terms of noise.

4.1.4 Artifacts in micro-CT and MRI

Artifacts are unrealistic features of the image, i.e. the image may present an edge or a variation in brightness that the imaged sample does not present. Artifacts can seriously degrade the quality of tomo-
graphic images, sometimes to the point of making them unusable or with the risk of leading to totally wrong interpretations. To optimize image quality, it is necessary to understand why artifacts occur and how they can be prevented or suppressed. A complete description of all the artifacts that can occur in tomographic imaging goes beyond the goals of this paragraph. Only the most recurrent artifacts and, in particular, those that seriously complicate the image analysis process are mentioned.

**Artifacts in micro-CT**

It is possible to group the origins of the most recurrent artifacts in $\mu$-CT into three categories\,[29]: (i) physics-based artifacts, which result from the physical processes involved in the acquisition of CT data; (ii) sample-based artifacts, which are caused by such factors as sample movement (when trying to image a sample whose morphology is dynamically changing) or the presence of metallic materials in or on the sample; (iii) scanner-based artifacts, which result from imperfections in some elements (usually the detector) composing the scanning device.

When using a polychromatic beam, the slices reconstructed from $\mu$-CT experiments commonly display the so-called beam hardening artifacts\,[20]. These artifacts, due to the differential absorption of the X-ray spectrum by the sample, lead to a misleading recovery of the linear absorption coefficients, mainly appearing as bright sample borders in the reconstructed slices as in Figure 10. For regular objects the effect is easily detected. In irregular objects it is commonly difficult to differentiate between beam hardening artifacts and actual material variations because of the additional appearance of dark stripes in the images.

Beam hardening can be compensated by using filtration, calibration correction, as well as correction software. A flat piece of attenuating material, usually aluminum can be used to “pre-harden” the beam by filtering out the lower-energy components before it passes through the object. A $\mu$-CT device can be calibrated by using phantoms in a range of sizes. This allows the detectors to be calibrated with compensation tailored for the beam hardening effects of different setups. Iterative correction algorithms exist and may be applied when the tomographic projections are being reconstructed. However, in $\mu$-CT the beam hardening effect does not occur with monochromatic sources. In fact, if the beam hardening effect is a serious concern for a $\mu$-CT experiment, the simplest way to get rid of it is by taking advantage (if possible) of monochromatic sources. Although software and hardware procedures for beam hardening compensation exist\,[15], it has to be taken into account these procedures might affect other image quality aspects (e.g. additional of artifacts or a reduction in spatial resolution).

Quite often image processing is limited to a sub-volume of interest totally included in the imaged object. The whole tomographic
dataset that includes the background as well as uninteresting areas of the sample are rarely used as direct input of an analysis workflow. Therefore, if a polychromatic source has to be used and the final images present beam hardening, the artifact can be bypassed by selecting a region of interest far from object boundaries where the effects of the beam hardening are usually stronger. This volume of interest may be then used for the characterization of the sample. However, this “trick” makes sense only in the case of single phase materials (as in Figure 10).

Figure 10. Artifacts in μ-CT: Beam hardening. On the left: Slice of a human skull bone fragment in which beam hardening effects (unrealistic brightening of the exterior parts of the imaged object even if the material is homogeneous) are noticeable (μ-CT parameters: $V = 80$ kV, $I = 100$ μA). The intensity profile of the highlighted red line shows the so-called cupping trend. (The scale bar is 5 mm)

The object-based artifacts here considered are the metal/streak artifacts and the motion artifacts. Metallic materials in or on the sample lead to streak artifacts. They occur because the density of the metal is beyond the normal range that can be handled by the detector or the reconstruction software, resulting in incomplete attenuation profiles. The streaking caused by overranging can be greatly reduced by means of special software corrections. Reconstruction software usually offers interpolation techniques to substitute the overrange values in attenuation profiles. A few streak artifacts due to high absorbing elements within the imaged object are visible in Figure 12.

Sample motion may occur in in vivo studies but also when temperature changes and/or other variations of the conditions of the imaged object alter its structure. This movements occurring during the scan can cause misregistration artifacts. Motion artifacts usually appear as shading or streaking in the reconstructed image. Of course, steps have to be taken to prevent voluntary motion in e.g. in vivo experiments. In general, when trying to get a 3D “snapshot” of a sample that dynamically changes, researchers have to privilege the setup that results in the fastest scan possible.
Figure 11. Example of a slice (the same through time) of a 4D μ-CT time series. The sample is imaged from the beginning of bubble growth through the point of failure when the bubbles “pop”. Only the most significant time steps are shown. It can be noticed that motion artifacts strongly affect the early stages of the growing process, thus making quantitative analysis almost impracticable. From time \( t = 12 \) to the last μ-CT dataset (\( t = 17 \), not reported) the sample remains in a steady (motion artifacts-free) condition. (The scale bar is 0.5 mm)
As the scanning time of a \(\mu\)-CT scan using synchrotron radiation sources can be very fast, multiple consecutive scans might be considered. This is sometimes called 4D or time-resolved tomography and it results of particular interest when trying to image a sample whose conditions change through time. Figure 11 shows the results of a geological application in which 4D tomography was exploited with the aim to perform a bubble tracking through time in order to characterize the growing ratio of the expansion process\(^1\). The motion artifacts are a serious concern for 4D tomography. It can be noticed from Figure 11 that motion degrades images too much, thus making the analysis impracticable for the early and more interesting stages of the growing process.

The main concern before considering an analysis approach of \(\mu\)-CT images is probably related to scanner-based artifacts, and in particular to the so-called ring artifacts. In fact, the most recurrent artifact in tomographic images is the presence of concentric rings that arise from inhomogeneities in the individual pixel response of detector elements. Ring artifacts seriously complicate the segmentation of the image. Therefore, a significant reduction of these artifacts is essential prior to any quantitative analysis process.

![Image showing ring artifacts in \(\mu\)-CT images](image)

**Figure 12.** Artifacts in a \(\mu\)-CT slice (\(\mu\)-CT parameters: \(V = 50\) kV, \(I = 160\) \(\mu\)A) of a DELRIN\textsuperscript{®} phantom (diameter 20.0 mm): a) original image in which ring artifacts and a few streak artifacts due to high absorbing elements within the pores are visible; b) de-ringed imaged with the algorithm proposed in [30]. (A zoomed crop of a small area in the vicinity of rings center is also reported)

Image processing correction algorithms are used for the compensation of ring artifacts. Rings in \(\mu\)-CT images result from the back-

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\(^1\) Images were acquired in a collaboration among the Earth and Planetary Sciences department of the McGill University (Montreal, Canada), Sincrotrone Trieste S.C.p.A. (Basovizza - Trieste, Italy) and the Swiss Light Source (Villigen, Switzerland). Quantitative analysis of the produced 4D datasets was performed by the Author during a two months stage period at the Earth and Planetary Sciences department of McGill University (Montreal, Canada).
projection of stripe artifacts in the sinogram image, so de-striping the sinogram is a classical way to face the problem. However, if a reconstructed slice is transformed into polar coordinates assuming the center of rings as the center of the cartesian to polar conversion, the problem of ring artifacts compensation can be still brought back to a de-striping issue \( \text{Figure 13} \). Doing so, de-striping filters may be applied directly to transformed reconstructed slices avoiding the need of additional imaging data (i.e. flat fielding and sinogram images). Four of the most common de-striping approaches have been revised by the Author in \( [31] \). Two approaches, namely the Sijbers and Postnov \( [32] \) and Münch et al. \( [33] \), resulted very effective for the processing of \( \mu \)-CT images.

\[ \text{Figure 13.} \] Polar transformation for ring removal of an experimental X-ray \( \mu \)-CT slice of a human skull bone fragment (\( \mu \)-CT parameters: \( V = 80 \) kV, \( I = 100 \) \( \mu \)A): a) original image; b) polar transformed image. (The scale bar is 5 mm).

In the Sijbers and Postnov approach \( [32] \), within a sliding window of user defined size \( W \), a set of homogeneous rows is detected. The homogeneity criterion is based on an user specified threshold value \( T \). Working on this set, an artifact template is generated and used for the correction of each column of the image. A preliminary segmentation process is proposed in the original version \( [32] \), however it has been shown by the Author in \( [30] \) that this should be considered as an optional step. The idea of the Münch et al. filter \( [33] \) is based on wavelet and Fourier transform. At first, the original image is wavelet decomposed into \( L \) levels in order to separate the structural information into horizontal, vertical and diagonal bands at different resolution scales. Subsequently, the bands containing the stripe information are FFT transformed to further tighten the stripe information into narrow bands. This stripe information is then removed by using a Gaussian function with damping factor \( \sigma \). Finally, the de-striped image is reconstructed from the filtered coefficients.
Artifacts in MRI

Artifacts in MRI are produced by interaction of several factors, however, most are fundamentally classifiable into one of the following four basic categories [34]: (i) inhomogeneity and temporal instability of the static magnetic field; (ii) inhomogeneity of the radio-frequency magnetic field, (iii) non-linearity and eddy currents of gradient magnetic field and (iv) motion artifacts. In this section, attention is given to radio-frequency related and motion artifacts only as they present analogies with some CT artifacts.

The radio-frequency (RF) pulses used in MRI are also in the frequency range of common extraneous RF sources, such as radio broadcasts, electric motors and other electrical machinery. When RF noise enters an MRI system, it may manifest itself in several ways. If the noise contains multiple frequencies, the entire image may be diffusely degraded. If, however, the noise contains only a fixed frequency, it may be projected through the image as a vertical stripe perpendicular to the frequency encoding direction. The exact position of this line on the image depends upon the frequency of the interference relative to the central frequency of resonance for the imaged and the frequency encoding gradient strength. The resulting stripe artifacts
are therefore similar to the stripe artifacts of the sinogram image created during the CT reconstruction process.

The most recurrent artifacts in \textit{in vivo} MRI are those related to motion. MRI is sensitive to all forms of macroscopic and microscopic motion. Spin-echo sequences, particularly those with long echo times TE, are especially sensitive to motion. Cardiac or respiratory motion are the most common motion related causes of image degradation.

\textbf{4.2 Segmentation}

In image enhancement the desired output is an improved version of the input picture. In image analysis the input is still pictorial but the desired output is a description of the given image through several parameters. Although the whole analysis workflow can include several image enhancement steps in the early phases of the process as well as several manipulations of some intermediately computed descriptors prior to the final response, at some point the vast majority of image analysis approaches requires a segmentation step. Segmentation is the “bridge” between the image processing and image analysis fields.

It should be emphasized that there is no single standard approach to segmentation. Many different types of image or scene parts can serve as the segments on which descriptions are based, and there are many different ways in which one can attempt to extract these parts from the picture. The perceptual processes involved in segmentation of a scene by the human visual system are not yet well understood. For this reason, no attempt will be made here to define criteria for successful segmentation. As mentioned also for the evaluation of the enhancing of an image, a successful segmentation must be judged by the utility of the description that is obtained using the resulting objects. As image enhancement is generally subjectively performed, a “good” segmentation is also subjectively assessed.

Segmentation is basically a process of pixel or voxel classification; the picture is segmented into subsets by assigning the individual pixels to classes. For example, segmenting a picture by thresholding its gray level means classifying the pixels into “dark” and “light” classes, in an attempt to distinguish e.g. dark objects from their light background. A more formal definition based on set theory is that, given a definition of “uniformity,” a segmentation is a partition of the picture into disjoint subsets, each of which is uniform, but such that no union of adjacent subsets is uniform. The goal of a segmentation technique is to define the “uniformity” criterion such that the result is a “good” segmentation.

The most commonly used approaches for the segmentation of three-dimensional images are now exposed. It is almost impossible to enumerate all the approaches for segmentation published in the literature. Moreover, some of them are difficult to extend to the three-dimensional case and therefore will not be taken into account in the
following subsections. Computational aspects have also to be taken into account as the memory requirement is a serious concern for volumetric image processing. Focus will be given only to efficient approaches.

4.2.1 Thresholding

Thresholding is probably the simplest method of image segmentation. During the thresholding process, individual voxels in an image are marked as “object” voxels if their value is greater than some threshold value (assuming the object to be brighter than the background) and as “background” voxels otherwise. The opposite convention can be adopted. Typically, an object voxel is given a value of 1 while a background voxel is given a value of 0. However, object voxels can be labeled as 255 (the maximum value in an 8-bit range) as usually the minimum access unit of a digital processor is actually a byte (1 byte = 8-bit). After thresholding, a binary image is usually created by representing each voxel white or black, depending on the voxel’s label.

The key parameter in the thresholding process is the choice of the threshold value. Several different methods for choosing a threshold exist. The simplest way is to let users manually choose a threshold value. This value is usually chosen via visual evaluation of the results produced by a few trials with different threshold values. The starting point for this trial and error assessment is usually performed by observing the image intensity histogram. In the case of an image intensity histogram that is clearly bimodal, with two relatively narrow peaks corresponding to the object and to the background, the threshold may be easily determined as the histogram minimum lying between the two peaks. However, images may present an image histogram that does not consist of two separate peaks. In the presence of such a monomodal histogram, more efforts have to be performed. The result of this kind of thresholding is obviously subjective as the threshold is manually assessed. Moreover, factors like room lighting, monitor brightness/contrast settings, operator fatigue and limited gray-scale shade perception can affect the reproducibility of this segmentation approach [35].

Automatic thresholding algorithm exist. They overcome the subjectivity of manual thresholding and they can also speed up the analysis process as the manual assessment of the threshold might be a time-consuming task for some applications, e.g. where a great number of images have to be analyzed. A few automatic thresholding methods are hereafter briefly reviewed. Kittler and Illingworth [36] proposed a method that consists in arbitrarily dividing the histogram into two parts, modeling each part with a normal distribution, comparing the model with the histogram and assuming as optimal the threshold the minimizes a minimum error criterion function. Ridler and Calvard [37] advanced an iterative scheme in which at step $n$, a
new threshold $T_n$ is established using the average of the foreground and background class means. Iterations terminate when the changes $|T_n - T_{n+1}|$ become sufficiently small. Otsu \[38\] suggested minimizing the weighted sum of intra-class variances of the foreground and background voxels to establish an optimum threshold. The Tsai’s method \[39\] determines the threshold imposing that the first three moments of the input image are preserved in the output image. The Pun’s method \[40\] is based on entropic thresholding: an entropy-thresholded image is the one that preserves (as much as possible) the information contained in the original unthresholded image in terms of entropy. Kapur et al. \[41\] improved the Pun’s approach considering the image foreground and background as two different classes of events. When the sum of the two class entropies reaches its maximum, the image is said to be optimally thresholded. All of these approaches are applied in the application reported in section 6.1. It will be there shown (Figure 29) the effects of an application of a different threshold value in an experimental $\mu$-CT dataset.

Adaptive thresholding (sometimes called local or dynamic thresholding) techniques have been also proposed. Whereas the conventional thresholding operator uses a global threshold for all voxels, adaptive thresholding changes the threshold dynamically over the image. This more sophisticated version of thresholding can accommodate changing lighting conditions in the image, e.g. those occurring as a result of a strong illumination gradient or shadows. This situation rarely occurs in tomographic imaging, therefore adaptive thresholding are seldom used. An interesting approach for adaptive thresholding is the Niblack’s algorithm \[42\]. The concept of this algorithm is to build a threshold surface based on local mean $m$ and local standard deviation $s$ computed in a $N \times N \times N$ neighborhood of each voxel. For each voxel, the threshold $T$ is computed as $T = m - k \cdot s$ where $k$ is a constant user-defined “tuning” parameter (the other parameter is the “window” size $N$). The algorithm produces a large amount of binarization noise. Post-segmentation filters (see subsection 4.2.5) need usually to be applied. Adaptive thresholding of 3D images might be computationally very intensive. Moreover, since some “tuning” parameters have to be assessed by means of a “trial and error” approach, the total time of the segmentation might result excessive for some applications.

For porous media applications, other techniques might be considered. For instance, Moore et al. \[43\] suggest to use the threshold that produces a binary image in which the resulting porosity (percentage of background voxels in comparison with the total) is similar to either the theoretical porosity (based on some \textit{a priori} knowledge) or the measured porosity (based on complementary techniques, e.g. mercury intrusion porosimetry). However, with this approach an external data (the theoretical or measured porosity) is needed for the segmentation and in the introduction of this chapter it was stated that focus is here given to approaches based only on the contents of the images.
4.2.2 Region-based segmentation

The thresholding methods described in the previous section are based on the intensity properties of an image. The logical extension is to exploit also spatial properties of the image for segmentation.

Region growing is one of the conceptually simplest approaches to image segmentation. Neighboring pixels of similar amplitude are grouped together to form a segmented region. However, in practice, constraints, some of which are reasonably complex, must be placed on the growth pattern to achieve acceptable results. Some algorithms have to be considered semi-automatic segmentation methods as at first user intervention is required (seed selection).

Brice and Fenema [44] have developed a region-growing method based on a set of simple growth rules. In the first stage of the process, pairs of pixels are combined together in groups called atomic regions if they are of the same amplitude and are spatially connected. Two heuristic rules are next invoked to dissolve weak boundaries between atomic regions.

Adams and Bischof [45] have proposed a seeded region growing algorithm in which the user manually selects a set of seeds that are placed in areas of visual homogeneity. The seeds can be single voxels for nearly noise-free images, or they can be small clusters of voxels to provide some degree of noise tolerance for noisy images. Then, conventional region growing proceeds with one new pixel added to each of the $N$ seeded regions. The process proceeds until adjacent regions meet at a common boundary.

Most region growing techniques have an inherent dependence upon the location of seeds for each region. As a consequence, the segmented result is sensitive to the location and ordering of seeds. The region growing process is also iterative and therefore it can be computationally intensive for high resolution volume images as $\mu$-CT datasets.

4.2.3 Clustering and multi-phase segmentation

The previously introduced thresholding approach usually aims at classifying voxels into two classes (named also phases or clusters) by defining a threshold value. Some applications may require a classification into more than two classes (see for instance [6]). If the $N$ classes correspond to subpopulations of the voxels having different intensities, it is intuitively possible to segment the images by defining $N-1$ thresholds. In this case, the classes should show up as peaks on the histogram of intensity values. These peaks should be at least some minimum distance apart, to insure that they represent distinctive subpopulations. One might also require that there be relatively deep valleys between them. Under these circumstances, the picture is usually segmented by choosing more than one threshold (multiple thresholding) so as to separate the peaks, e.g., at the bottoms of the
valleys between the peaks.

However, sometimes the classes should show up as dense regions on the image histogram and therefore multiple thresholding might be ineffective. A classification (or clustering) algorithm might be more effective, in particular when the number of phases is more than two (multi-phase segmentation)\[^46\]. All classification algorithms are based on the assumption that the image in question depicts one or more features and that each of these features belongs to one of several distinct and exclusive classes. The classes may be specified \textit{a priori} by the human supervisor (as in \textit{supervised classification}) or automatically clustered (i.e. as in \textit{unsupervised classification}) into sets of prototype classes, where the supervisor merely specifies the number of desired categories. Image classification analyzes the numerical properties of various image features and organizes data into categories. Classification algorithms typically employ two phases of processing: \textit{training} and \textit{testing}. In the initial training phase, characteristic properties of typical image features are isolated and, based on these, a unique description of each classification category, i.e. training class, is created. In the subsequent testing phase, these feature-space partitions are used to classify image features. The description of training classes is an extremely important component of the classification process. In supervised classification, statistical processes (i.e. based on an \textit{a priori} knowledge of probability distribution functions) or distribution-free processes can be used to extract class descriptors. Unsupervised classification relies on clustering algorithms to automatically segment the training data into prototype classes.

The most commonly used clustering method is the \textit{K}-Means algorithm. It is an unsupervised clustering algorithm that classifies the input data points into multiple classes based on their inherent distance from each other. The algorithm starts selecting \textit{K} cluster centers. The selection can be performed manually, randomly, or by a heuristic. Each voxel in the image is then assigned to the cluster that minimizes the distance between the voxel and the cluster center. This difference is based on gray-level intensity but it can combine also location measures. Cluster centers are then updated by averaging all of the voxels in the cluster. These steps are repeated until no voxels change clusters.

\[4.2.4\text{ Other approaches}\]

It is possible to segment an image into regions of common attribute by detecting the boundary of each region for which there is a significant change in attribute across the boundary. Boundary detection can be accomplished by means of \textit{edge detection} operators\[^47\]\[^48\]. However, some of these operators are not easy to extend to the 3D case. A three-dimensional modeling of the concept of \textit{edge} is required. Moreover, a detected boundary may often be broken. Edge linking techniques are usually essential in order to bridge short gaps in such
a region boundary. These linking techniques require special handling of the 3D case as well. Therefore, edge detection is rarely exploited for the segmentation of volume images.

Active contours (or snakes) [49] [50] is a method of molding a closed contour to the boundary of an object in an image. The model is a controlled continuity closed contour that deforms under the influence of internal forces, image forces and external constraint forces. The internal contour forces provide a piecewise smoothness constraint. The image forces manipulate the contour toward image edges. The external forces are the result of the initial positioning of the contour by some a priori means. Roughly, it can be viewed as a special edge detection technique that ensures that at the end at least two connected components are created, the internal and the one external to the final contour. As for common edge detection, extensions to the 3D case usually do not come straightforward, as the concept of “active surface” has to be defined and implemented.

4.2.5 Pre- and post-segmentation filters

Additional steps might be part of a segmentation process. Smoothing filters might be applied on the gray-scale input image in order to ease the application of e.g. a thresholding or a region growing approach. Binary filters, i.e. algorithms that process the “black and white” representation of the output image are also effective for some applications.

An interesting class of pre-segmentation filters are the so-called edge-preserving smoothing filters. They aims at smoothing regions without affecting object edges. Interesting approaches are the Perona and Malik [51] anisotropic diffusion model and the Tomasi and Manduchi bilateral filter [52].

Post-segmentation techniques include the application of morphological operators [53] but also more refined techniques based on the labeling of connected components (sometimes called blobs). A connected component is an isolated cluster of object voxels in a binary image (see also section 5.4). A simple post-segmentation technique removes connected components having volume (i.e. number of voxels) below a specified threshold value, as small connected components can usually be considered as “noise”. Yanowitz and Bruckstein [54] proposed a different criterion for removing undesired connected components based on the relation of a connected component with the original gray scale image. If for a particular blob the average of its edge values does not exceed a specific input value, the process eliminates it. The edge values are computed according to an edge detection operator applied to the original image. Other post-segmentation techniques are based on topological observations. For instance, if image background appears in the segmented result, a simple way for get rid of it is by removing the connected components “in touch” with the borders of the image. An example of this will be shown in Figure 19.
4.3 Volume of Interest (VOI) selection

While the selection of the Volume of Interest (VOI) to use as input for the image analysis process is usually performed before the actual segmentation step, the criteria for its selection can also be determined by the segmentation step. In other words, a different VOI selection can suggest a different segmentation approach and difficulties in the segmentation might suggest the need to select a different VOI. These steps have to be considered somehow interconnected.

Roughly, the selected VOI should be small enough to be easily handled by the available computer hardware but at the same time it should be large enough to comprehend all the representative features of the sample. A common rule suggests to select VOIs having size of about one order of magnitude larger than the characteristic size of the underlying structure. This concept is sometimes referred to as macroscopic homogenization but it requires some a priori knowledge about the underlying structure.

When imaging a porous media, the final images present usually at least three phases, i.e. the material composing the media, the porous phase and the background. Sometimes additional elements may be present in the final images, e.g., the sample holder or the thin film used for embedding the sample. Therefore, when trying to segment the whole dataset, the segmentation process becomes a three-phases issue. While the object can be easily separated as it appears different from the porous phase and the background, it is usually hard to separate the background from the porous phase as they cover the same gray level intensity range. If the analysis is limited to a VOI totally included within the sample, the segmentation issue can be brought back to a two-phase classification process: The voxels that do not belong to the object phase form by consequence the porous phase. Therefore it is usually worthwhile to perform an accurate selection of the VOI prior to the segmentation step in order to simplify the actual segmentation.

The accurate selection of the VOI is also essential for further correct analysis. “Is 10 people a lot?” - one might ask. It is probably a lot for an elevator, while one would feel pretty lonely in a stadium among the audience waiting for the final lap of the Olympic marathon. The concept of “a lot” has to be contextualized otherwise it does not have its full meaning. To “contextualize” may be probably translated in image analysis with the concept of normalization with respect of the selected VOI. The following simple example might help in understanding why numerical results are strongly affect by the selection of the VOI. Consider two glasses where one has double capacity than the other one. If one fills with water the smaller glass to its maximum, the result is that the glass is full. By filling with the same amount of water the big glass, the result is that the glass looks half empty (or half full). Anyway, the absolute water volume does not change, however, it also does not have much meaning. In fact, generally, in image analysis the absolute values are of rare interest. Values have to be

Normalization

45
Figure 15. VOI selection issues: a) original µ-CT slice ($V = 40$ kV; $I = 200$ µA) of an alginate-based bone tissue engineering scaffold; b) segmented image where two VOIs are highlighted but only the blue VOI leads to a correct characterization of the porosity. (The scale bar is 2 mm)

In conclusion, the results of an image analysis process are strongly related to the VOI selection (both in terms of size and position) and before complaining about anomalous results or being excited for attractive values, it is important to have a good rationale for the choice of the VOI. The rationale used for VOI selection is as important as the one used for the segmentation. A strategy for checking the representativeness of the VOI will be presented in the application reported in section 6.1.
The goal of the chapter is to present the main approaches for three-dimensional image analysis with special emphasis on the case of porous media, as they represent the most recurrent application of tomographic imaging. With the exception of texture analysis (discussed in the final part of the chapter), every image analysis workflow starts from a segmented Volume of Interest (VOI). Approaches for image segmentation were exposed in the previous chapter.

Provided that the “best” segmentation is obtained, the analysts are still required to supervise the analysis process. In fact, there is no unique methodology for the extraction of quantitative information from 3D images as this chapter will clarify. It is very dissimilar to analyze porous media made up of bubble-like pores (e.g. some volcanic rocks, artificial metallic and polymeric foams, food products like bread and cheese), fibrous materials (e.g. felts, paper, artificial composite materials), trabecular structure (e.g. trabecular bone, bone tissue engineering scaffolds) or randomly distributed materials (e.g. most of the reservoir rocks and many other industrial products). While it is possible to assess basic parameters without additional modeling, assumptions for the pore space are sometimes required to get a more effective quantitative characterization.

The main assumption required when performing an image analysis process of a porous medium is based on the quest whether the imaged object presents either a set of closed cells (or pores) or, instead, a globally interconnected pore space is observable and pore bodies as well as connecting channels have to be identified and characterized. However, sometimes the imaged medium physically presents a closed cells/pore space but the imaging technique and/or the segmentation process is not able to recognize this. This is due because e.g. the closed cells present thin walls having size below the sp-
tial resolution of the adopted imaging technique. Image processing in order to move from an open cells to a closed cells space is then required.

5.1 BASIC ANALYSIS

A first group of descriptors is named basic (or first-order) characteristics (also called Minkowski functionals or quermass integrals or intrinsic volumes). They are: volume fraction or density ($V$), specific surface area or surface density ($S$), integral of mean curvature ($M$) and Euler number ($\chi$). These parameters can be computed using the algorithms proposed in [56].

The volume density simply represents the density of the sample, i.e. the number of voxels belonging to the object phase with respect to the total number of voxels in the considered VOI. The measure $1 - V$ represents the density of the complementary phase and it is usually denoted as porosity if the complementary phase is the pore space or with specific names depending on the specific applications (e.g. vesicularity in vulcanology). The specific surface area is the ratio of the interface between the phases (the solid phase and the porous one) to the total VOI volume. It is usually expressed in mm$^{-1}$.

Even though they are expressed with a physical unit (mm$^{-2}$ and mm$^{-3}$, respectively), integral of mean curvature and the Euler number are used to characterize the kind of spatial coverage of the 3D pore space in terms of presence of concave vs. convex structures and connectivity. They have to be intended more as a descriptor indexes and their absolute values do not have a physical meaning. A positive value for $M$ implies the dominance of convex structures, while $M < 0$ occurs in the case of predominance of concave structures. The $M$ value computed on the object phase is the opposite of the $M$ value computed on the porous phase, which is coherent with the duality character of convexity and concavity for a two-phase material. Provided that the considered sample presents one connected structure with no closed void cavities, Euler number $\chi$ is an indicator of the connectedness of the 3D complex pore space. It provides a measure of connectivity density indicating the number of redundant connections between void structures per unit volume: the breaking of a single connection will leave the network less connected increasing the value of $\chi$, while the addition of a redundant connection will decrease it [57]. Therefore, $\chi$ is positive when the number of isolated pores that are not connected to each other exceeds the number of multiple connections between the pores. On the other hand, $\chi$ is negative (up to several hundreds in a volume of few mm$^3$) for a completely connected network of pores. In this case it represents the number of multiple connections. Being the Euler number a topological quantity, it does not carry information about positions or size of connections but it is a simple global measure of connectivity which gives higher values for poorly connected structures and lower val-
5.2 Structural anisotropy analysis

Figure 16. Mean Intercept Length (MIL) method. The number of intersections (represented in green) along randomly generated lines (as the ones with origin in point $P_1$ and $P_2$ respectively) is counted. Random orientations are used. This approach is usually used also for the assessment of the standard morphometric parameters $Tb.Th$, $Tb.Sp$ and $Tb.N$.

Information about the anisotropy of the microstructure is often necessary. The anisotropy corresponds to the preferential orientation(s) of the structure and it allows to establish resistance to the strengths in a given preferential direction. In general, a structure is isotropic if it has no preferred orientation or, more rigorously, if the perpendicular to any element of surface has an equal probability of lying within any element of solid angle. Cowin and Laborde \[58\] introduced the term fabric as a description of the local anisotropy of a material’s microstructure and a fabric tensor was defined as any positive definite second rank tensor, which quantitatively describes fabric. Based on an assumption of orthotropy, the elastic mechanical properties may be formulated as a function of fabric and density \[59\].

While a few methods have been proposed in the literature \[27\], the most commonly used method for the characterization of anisotropy is based on the Mean Intercept Length (MIL) concept. The basic principle of the MIL method is to count the number of intersections between randomly oriented lines and the pore/material interface as a function of the line orientation $\omega$ \[60\]. The mean intercept length (an intercept is the linear segment between two intesections) is calculated as the ratio between the total length $L$ of the line and the number of intersections. This idea is represented in Figure 16.
A common way to analyze MIL measurements is by plotting the MIL values as a function of the angles (two angles are necessary to describe a solid angle). A “flat” profile suggests isotropy, while the variations in this profile allow to check the presence of preferential direction(s). Three dimensional MIL measurements may also be fitted to an ellipsoid which can be expressed as the quadratic form of a second rank tensor $M$. Cowin defined a MIL fabric tensor $H$ as the inverse square root of $M$. Since the eigenvectors $(u_1, u_2, u_3)$ of the fabric tensor $H$ give information about the direction of the axes of the ellipsoid, and the eigenvalues $(t_1, t_2, t_3)$ express the radii of the ellipsoid, the latter can be used to define the degree of anisotropy, which denotes the ratio between the maximal and minimal radii of the MIL. The eigenvalues can be summarized using the isotropy index $I = t_3/t_1$ and the elongation index $E = 1 - t_2/t_1$.

### 5.3 Model Based Morphometric Analysis

Thickness measures are of particular interest in several scientific applications but, in most cases, they require a model assumption and verification that is in general a nontrivial task. In the past, a few descriptors have been proposed to this aim. They were initially developed for bone histomorphometric analysis but they can be easily extended to the 3D case. These parameters are trabecular thickness ($Tb.Th$), trabecular separation ($Tb.Sp$) and trabecular number ($Tb.N$). Since this nomenclature was standardized, it is quite common to see these names in the characterization of other objects than the trabecular bone.

While other models exist (e.g. the so-called rod-like model), the computation of these parameters is usually based on the parallel plate model. According to this model, the considered sample is thought of as a meshwork of intersecting parallel plates and $Tb.Th$ should represent the mean plate thickness, $Tb.Sp$ is a measure of the mean plate separation (a measure of the thickness of the void phase) and $Tb.N$ is the mean number of plates traversed by a line of unit length perpendicular to the plates. A common way for computing these parameters in the 3D domain is based on the aforementioned mean intercept length (MIL) concept. The $Tb.N$ (usually expressed in $\text{mm}^{-1}$) is the number of test line intersections with the pore/material interface per unit test line length and the other parameters are determined according to the model assumption as $Tb.Th = (1 - V_f) / Tb.N$ and $Tb.Th = V_f / Tb.N$ being $V_f$ the first Minkowski functional determined as reported in section 5.1 and sometimes denoted as $BV/TV$. 
These parameters have become a sort of “standard” in porous media analysis as several commercial as well as free software offer simple tools for their computation. Unfortunately, the fact that these parameters are based on model assumptions is sometimes omitted. An application of this kind of analysis to porous media that present an architecture far from being similar to the one of trabecular bone may produce totally wrong results. The Structure Model Index (SMI) has been proposed in the literature \cite{67} (see also \cite{68}) in order to check if the parallel-plate model could be assumed. However, in general, model-independent measures have to be privileged. It will be shown that skeleton analysis may provide a more accurate assessment of structure thickness as well as structure separation.

### 5.4 Blob analysis of closed cell structures

If the pore space is formed by an isolated set of “blobs”, a series of descriptors for size and shape of each “blob” can be computed. The analysis is based on the already mentioned concept of connected components (see subsection 4.2.5) and their labeling. Labeling connected components (or blobs) has a recursive nature. The first object voxel in the binary image is labeled. Then an object voxel in the neighborhood of this voxel belongs to the component as well, and therefore it is marked with the same label. Then, in turn, the neighboring voxels of this last labeled voxel are checked and labeled again. This procedure continues until all the voxels of this component are labeled. The procedure stops because no more neighboring object voxels are connected to the blob. The 3D neighborhood can be composed by 6, 18 or 26 voxels and the choice of the kind of neighborhood affects the final number of labeled blobs and therefore the whole analysis process. Usually, the components of the object phase are considered connected according to a 26-connectivity rule, which means that the blobs of the complementary phase are considered connected with a 6-connectivity rule.

For each labeled blob, commonly computed descriptors are:

- **Volume (V):** computed taking into account the actual number of voxels belonging to the connected component and the voxel size of the acquired images. It is usually expressed in mm$^3$ or μm$^3$;

- **Diameter of the maximum inscribed sphere ($d_{max}$):** computed as two times the maximum value of the Euclidean distance transform;

- **Equivalent diameter ($d_{eq}$):** specifies the diameter of a sphere with the same volume as the blob, computed exploiting the inverse formula of the volume of a sphere, i.e. $d_{eq} = 2 \sqrt[3]{(3V/4\pi)}$;

- **Minor axis length ($l_{min}$):** length of the shortest segment among all the segments passing through the center of mass and fully
three-dimensional image analysis

included into the connected component. The so-called “star” is generated using random orientations and it is represented in Figure 17.

- Major axis length (l_max): length of the longest segment among all the segments determined as above;
- Sphericity (c): ratio between d_max and d_eq;
- Aspect ratio (r): ratio between l_min and l_max;
- Extent (e): ratio between V and the volume of the minimum bounding box, i.e. the smallest parallelepiped oriented according to image axis containing the blob (see Figure 17);
- Solidity (s): ratio between V and the volume of the convex hull, i.e. the smallest convex solid shape that can contain the blob (see Figure 17).

It should be noticed that theoretically d_max and l_min measure the same property, even though different algorithms are used for this assessment and therefore small numerical differences might occur. Moreover, the meaning of d_eq is mainly related to the shape of the blob, as it will be discussed later. Therefore, when limiting the analysis to a characterization of the size of the blob, V, l_min and l_max are probably a sufficient set of descriptors.

The other proposed parameters might help in understanding shape and relative position of the blob. Value for c and r far from 1 indicate an anisotropic (generally “elongated”) shape due to strong differences between the d_eq and d_max, and l_min and l_max respectively. Because of such an anisotropic shape, the value of e for this blob becomes meaningless being affected by the orientation of the shape with respect to its bounding box. The value of e can be used for inferring information about the relative position of the blob. An elongated shape perfectly aligned with image x-, y- or z-axis implies a value for e very close to 1. This value decreases as the misalignment or the “elongation” of the shape increase. While presenting different absolute values, the descriptors c and r behave coherently. Therefore the proposed set of descriptors for blob shape characterization can be reduced to the e, s and r descriptors, promoting r instead of c, the former being related to the already preferred l_min and l_max. The descriptors e, c, s and r are dimensionless.

Figure 17 shows a 2D version of some of the aforementioned concepts used for the characterization of the blobs. For all the represented blobs, the convex hull is very close to the perimeter (or surface in 3D) of the objects as none of the represented blobs present concavities. So the values for the solidity s would result close to 1 for all the blobs represented in Figure 17.
5.4 Blob analysis of closed cell structures

![Image of blob analysis concepts](a)

![Image of blob analysis concepts](b)

![Image of blob analysis concepts](c)

Figure 17. Illustrations of some of the concepts used in blob analysis: a) original image; b) image axis-aligned minimum bounding box (dotted line), convex hull (solid line) and center of mass of each blob; c) “star” composed by segments passing through the center of mass and fully included into the connected component (only a few of the randomly generated segments are reported). For the sake of clarity concepts are represented in 2D but they can be easily extended to the 3D domain.

5.4.1 Blob separation via watershed segmentation

For porous media having isolated pores, the segmentation should be able to produce a binary image having each pore represented by a set of contiguous voxels (connected component). However, in some cases this could be hard to obtain due to a limited spatial and contrast resolution or due to an imperfect segmentation process. Moreover, some volumes feature pores physically interconnected which however have to be considered as separated or they are better characterized if they would be separated. Therefore, it is sometimes necessary to isolate each pore in order to achieve an accurate characterization.

Approaches for pore separation remind in some way those for segmentation presented in section 4.2 as this issue is actually a voxel classification process. Supervised or semi-automatic methods exist. They require to manually specify two or more seeds inside the wrongly
segmented blob and then a region growing procedure starts. While this approach is widely exploited for 2D images, appropriate visualization tools for the 3D case are needed as it might be difficult to correctly place the seeds in a 3D volume. Moreover, this approach is time consuming when a huge number of blobs have to be processed. Therefore automatic methods are usually preferred for the 3D case.

The morphological dilation and erosion operators might help for the pore separation issue. They might be effective when limiting the analysis to the counting of blobs, however they could affect the accuracy of a more comprehensive characterization of the pore space that includes size and shape descriptors.

The pore separation issue can be automatically faced by means of a combination of distance transform and watershed segmentation with subsequent masking of the original binary image and the watershed segmented image. Sometimes, it could be necessary to preprocess the original binary image with morphological operators (dilation and erosion) or more refined operations based on morphological reconstruction such as the so-called H-minima filter. Watershed segmentation usually produces over-segmented results where too many small regions are identified. The H-minima filter is an effective way to avoid over-segmentation. Figure 18 shows an application of the automatic separation process performed exploiting this approach.

After pore isolation, the characterization of the blobs come straightforward and the set of parameters already introduced can be automatically computed. However, since VOIs usually are extracted from a region totally included into the imaged sample, an additional step is required in order to obtain unbiased results. The characterization of the size and shape of each blob needs to take into account only the ones fully included into the VOI, otherwise the resulting distribution would be affected by “incomplete” pores truncated by VOI margins. An additional “border cleaning” is required. A volume rendering of the blobs before and after the removal of “incomplete” pores is reported in Figure 19.

5.5 Skeleton analysis of open cell structures

When the imaged porous medium features an interconnected pore space, one of the goals of the analysis process might be a quantification of the degree of connectivity. While it was shown in the previous sections that an interconnected pore space can be transformed into a “disconnected” set of blobs, approaches for the characterization of the connectivity were not introduced so far. When this measure is of particular interest, skeleton analysis might be a more effective and more general approach as it allows also to quantify size and shape of the pores composing the pore space. Figure 20 and Figure 21 show an application of this approach.
Figure 18. Blob separation example on a milk aerated chocolate sample imaged by SR µ-CT (Energy = 13 keV. The scale bar is 0.5 mm): a) original image; b) edge-preserving smoothed image; c) thresholded image; d) connected components labeling of image (393 blobs found); e) distance transform of the pore space (intensity inverted and rescaled for representation purposes); f) watershed segmentation of image; g) superposition of image and image h) connected components labeling of image (583 blobs found). (For the sake of clarity only one slice of the considered VOI is represented, however the processing is performed in the 3D domain.)
Figure 19. Removal of “incomplete” blobs. Volume rendering of the VOI considered in Figure 18 in which each pore is represented with a different color: a) whole VOI (583 blobs); b) VOI without partial pores (339 blobs remaining). (Rendering performed with the commercial software VG Studio MAX)

Skeleton analysis is based on the extraction of the skeleton of a binary 3D image, i.e. a description of the binary object in terms of nodes and branches [71]. Ways for performing an effective skeletonization and characterization of the resulting network of nodes and branches are now discussed.

5.5.1 Skeletonization

The curve-skeleton (or, simply, skeleton) is a set of idealized connected thin lines that preserve the original topology and captures both boundary and region information forming what can be intuitively thought as the “spine” (or medial axis) of the object. Several automatic methods for curve-skeleton extraction have been proposed in the literature and they can be categorized into the following classes: (i) thinning and boundary propagation [72] [73]; (ii) distance transform based [74] and (iii) general-field functions based [75] [76] [77] methods (see also [78] for a review of curve-skeleton algorithms).

However, it is hard to perform an accurate comparison among different classes and techniques since there is no generally adopted definition of a curve-skeleton object as well as no clear criteria for the evaluation of results [78]. Practically, the skeleton is defined in terms of these desirable properties: homotopy, i.e. the skeleton is topologically equivalent to the original image (the skeleton and the original image have the same number of connected components, tunnels and cavities); thinness, i.e. the skeleton is one voxel wide; and medialness, i.e. skeleton is centrally located within the foreground objects (a skeleton should grant this property to be called medial axis). Different skeletonization algorithms behave differently in terms of these properties.
Two classes of skeletonization algorithms are here briefly reviewed. The first class includes algorithms that operate in the discrete (voxels) domain and they are mainly thinning methods. They produce a curve-skeleton by iteratively removing voxels from the boundary of an object until the required thinness is obtained. Thinning methods rely on the concept of simple point. A simple point is an object point (voxel) that can be removed without changing the topology of the object. An important property of simple points is that they can be locally characterized, i.e. one can determine whether a voxel is simple or not by only inspecting its local neighborhood, thus making thinning algorithms much more efficient. The thinning process starts from the object’s boundary and continues inward until no more simple points can be removed. At every iteration, each boundary voxel is tested against a set of topology preserving conditions and possibly removed. Two algorithms belonging to this class are the Lee et al.
Figure 21. Skeleton analysis example (Part II - Image analysis): a) Volume rendering of the segmented VOI; b) Skeleton of the pore space superimposed to image (a); c) Pore space modeling. By inflating the maximal spheres on skeleton nodes it is possible to move from the nodes/branches model to the pore/channels model. (Rendering performed with VG Studio MAX)

These two algorithms are fully automatic, i.e. without any tuning parameter. Users can not modify the “branchness” of the output skeleton. The usual way to reduce undesired branches is by further applying a pruning method as shown in Figure 22. The Palágyi and Kuba algorithm does not ensure medialness as Figure 23 shows.

The second class of skeletonization algorithms includes methods that build a continuous model from the original binary image and therefore they are able to produce sub-voxel and “tunable” skeletons. An effective skeletonization algorithm based on the concept of gradient vector flow was introduced by the Author in. It is able to produce a thin and centered curve-skeleton also in presence of noisy objects, a situation quite common in intensity volumes produced by biomedical imaging. The proposed method can be summarized in three steps as followed: (i) computing a force field at each object voxel; (ii) detecting the critical points of the force field; (iii) connecting the critical points by following the force field.
5.5 Skeleton analysis of open cell structures

**Figure 22.** Pruning effects on an artificial volume: a) skeleton extracted with [72]; b) same skeleton of (a) after pruning. (Rendering performed with the commercial software Amira - Visage Imaging)

**Figure 23.** Comparison of skeletonization algorithms: a) skeleton extracted with [73] with subsequent pruning; b) skeleton extracted with [81]. (Rendering performed with Amira - Visage Imaging)

In **Figure 23** a comparison among the mentioned skeletonization algorithms is shown. Differences are visible and, for further quantitative analysis, the most significant aspect that has to be considered is the resulting number of nodes and branches.

5.5.2 *Nodes and branches analysis*

By scanning the skeleton it is possible to extract the number of nodes and branches as well as pore and throat thickness measurements based on the concept of maximal inscribed sphere [82]. The
idea consists in inflating a sphere centered at a skeleton point. The inflating process stops when the sphere “.touches” the walls of the pore space. The diameter of this maximal sphere is an estimation of the local thickness. In principle, the spheres centered on skeleton nodes are used for the assessment of pore thickness, while the minimum thickness along a skeleton branch is usually denoted as throat (see Figure 24).

However, there is no unambiguous geometrical definition of where a pore ends and a connecting channel begins, making a geometrical determination of pore and throat difficult. Conceptually, the nodes correspond to pore bodies and the branches of the pore space skeleton correspond to the channels (or paths) connecting the pores. Unfortunately, without further modification, this 1-to-1 correspondence does not exist. Imperfect skeletonization may produce a very short branch between two skeleton nodes. Therefore, while every channel

---

Figure 24. Example of pores and throats: a) Volume rendering of a fragment of the considered VOI with skeleton of the porous phase; b) Superposition of pore bodies determined as cluster of maximal spheres on skeleton nodes; c) Superposition of throats determined as minimum maximal sphere along a branch. (Rendering performed with VG Studio MAX)
5.5 Skeleton analysis of open cell structures

**Figure 25.** Node-pore correction method. a) Several skeleton nodes (labeled in red) occur where a single pore has to be assumed (for instance in the large pore in the top left area). b) The inflated spheres on each skeleton nodes overlap as the nodes are at a close range forming a cluster of spheres. The counting of the resulting clusters of inflated spheres leads to a more accurate assessment of the number of pores. (Rendering performed with VG Studio MAX)

has its corresponding branch in the skeleton, not every branch corresponds to a channel. Similarly, while each pore body is represented by some nodes of the medial axis, several nodes may occur in the same pore body (see Figure 25) [83]. Therefore a merging (or correction) criterion should be adopted: two or more nodes are merged if there is an overlap among the maximal sphere centered at the nodes. The node having the largest maximal sphere is chosen to be the one that should be assumed as the center of a pore. This criterion successfully improve the assessment of the pore diameter distribution, assuming that each skeleton node is the center of a pore. However, some concerns may still remain for the throats size determination. In fact, it is more difficult to develop a criterion able to discard branches that do not correspond to channels.

Provided that the pore space is one connected structure with no closed void cavities, a simple indicator of the connectedness of the 3D complex pore space is the Euler number $\chi_V$. It can be computed as the fourth Minkowski functional as already introduced, but the Euler number can be assessed also from the number of nodes $n$ and the number of branches $b$ as $\chi_V = n - b$. It provides a measure of connectivity indicating the number of redundant connections between void structures. The breaking of a single connection will leave the network less connected increasing the value of $\chi_V$, while the addition of a redundant connection will decrease it (see Figure 26). In order to normalize the Euler number with respect to the size of the considered volume $V$, the parameter connectivity density $\beta$ computed as $\beta = (1 - \chi_V)/V$ is commonly adopted.
three-dimensional image analysis

Figure 26. Connectivity by skeleton analysis. On the left: network with no redundant connection, i.e. there is only one path possible for connecting node P to node Q. On the right: same network with a redundant connection in red allowing multiple path definition.

One more interesting parameter obtainable by skeleton analysis is the pore coordination number, i.e. the number of channels (skeleton branches) connected to a pore (skeleton node). The mean value of all the coordination numbers is an additional parameter for characterizing pore space connectivity.

5.6 TEXTURE ANALYSIS

All the previously introduced approaches require a segmented volume of interest as starting point. However, in some cases, it is interesting to analyze the gray-scale distribution of a particular region of interest in an image. A common case is when a segmentation is impracticable because the voxel size of the final images is comparable to the size of the structures of the imaged object. So-called sub-voxels segmentation and analysis methods have been proposed but their effectiveness is arguable as they sometimes consist of nothing more than an upsampling with an interpolation technique. A reliable way to proceed in this case is to focus the analysis on the texture of the selected region of interest. Texture analysis can be used also for binary images.

The concept of texture is strictly related to the human vision behaviour and it appears to depend upon three ingredients: (i) some local order repeated over a region which is large in comparison to the order’s size, consisting of (ii) nonrandom arrangement of elementary parts that are (iii) roughly uniform entities having approximately the same dimensions everywhere within the textured region. Although this description of texture seems perceptually reasonable, it does not immediately lead to a quantitative textural measure. Various approaches have been proposed in the literature to attain this aim. A preliminary distinction can be made among three different approaches: a basic one in which the image histogram is analyzed,
a more refined one based on the analysis of a suitably created matrix, named Gray Level Co-occurrence Matrix (GLCM) and the fractal approach.

The normalized intensity histogram of an image is the discrete function:

\[ p(i) = \frac{n_i}{n} \]  \hspace{1cm} (5.1)

where \( n_i \) represents the number of pixels with intensity \( i = 0, 1, \ldots, L - 1 \), with \( L \) the number of possible intensity levels and \( n \) is the total number of pixels in the image. Starting from \( p(i) \) the following parameters are commonly adopted as simple descriptors of texture:

- **Mean (a measure of average intensity):**
  \[ m = \sum_{i=0}^{L-1} i p(i) \]  \hspace{1cm} (5.2)

- **Standard deviation (a measure of average contrast):**
  \[ \sigma = \sqrt{\sum_{i=0}^{L-1} (i - m)^2 p(i)} \]  \hspace{1cm} (5.3)

- **Smoothness:**
  \[ S = 1 - \frac{1}{n + \sigma^2} \]  \hspace{1cm} (5.4)
  where
  \[ \sigma_n = \frac{\sigma}{L - 1} \]

  \( S \) measures the relative smoothness of the intensity in the image. It is equal to 0 for an image of constant intensity and approaches to 1 for images with large excursions in the values of their intensity levels.

- **Uniformity:**
  \[ U = \sum_{i=0}^{L-1} p^2(i) \]  \hspace{1cm} (5.5)
  which has a maximum if all gray levels have the same value (maximally uniform) and it decreases from there.

- **Entropy (a measure of randomness):**
  \[ e = - \sum_{i=0}^{L-1} p(i) \log_2 p(i) \]  \hspace{1cm} (5.6)
Haralick et al. [57] have proposed a number of more refined textural features based on the Gray Level Co-occurrence Matrix (GLCM) concept. While originally introduced for 2D images, the GLCM method can be easily extended to the 3D case. The GLCM of an image is an estimate of the second-order joint probability \( q(i,j) \) of the intensity values of two voxels \((i \text{ and } j)\) a distance \(d\) apart along a given direction \(\theta\), i.e. the probability that \(i\), called reference voxel, has the same intensity value of \(j\), called neighbor voxel. This joint probability takes the form of a square matrix with row and column dimensions equal to the number of discrete gray levels (256 levels for 8-bit images) in the image being examined. If an intensity image is entirely flat (i.e. contained no texture), the resulting GLCM should be completely diagonal. As the image texture increases (i.e. as the local pixel intensity variations increase), the off-diagonal values in the GLCM become larger.

In presence of a quite isotropic texture, the analysis is usually limited to a single displacement \(d\) (usually \(d = 1\)). Starting from \(q(i,j)\) the following parameters are commonly used as textural description:

- **Contrast:**

  \[
  C = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} |i-j|^2 p(i,j) .
  \]  

  It represents a measure of average difference between the reference pixel and the neighbor pixel in the whole image. The contrast is \(C \in [0, (L - 1)^2]\) where the case \(C = 0\) occurs in the presence of a constant image.

- **Correlation:**

  \[
  R = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} \frac{(i - \mu_r)(j - \mu_c)q(i,j)}{\sigma_r \sigma_c} .
  \]

  It is a measure of how the reference pixel is correlated to its neighbor pixel, where \(\mu_r, \mu_c, \sigma_r, \text{ and } \sigma_c\) are the mean and standard deviation values computed separately on rows and columns of the matrix as:

  \[
  \mu_r = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} i q(i,j) ,
  \]

  \[
  \mu_c = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} j q(i,j) ,
  \]

  \[
  \sigma_r = \sqrt{\sum_{i=0}^{L-1} \sum_{j=0}^{L-1} (i - \mu_r)^2 q(i,j)} ,
  \]

  \[
  \sigma_c = \sqrt{\sum_{i=0}^{L-1} \sum_{j=0}^{L-1} (j - \mu_c)^2 q(i,j)} .
  \]
5.6 Texture analysis

\[
\sigma_c = \sqrt{\sum_{i=0}^{L-1} \sum_{j=0}^{L-1} (j - \mu_c)^2 q(i,j)}.
\]

The correlation \( R \in [-1,1] \) is equal to 0 if no correlation is present while \( R = \pm 1 \) means perfect correlation. The correlation cannot be computed for constant images (a division by 0 occurs).

- Energy (or uniformity or second angular moment):

\[
E = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} p(i,j)^2.
\]  \hspace{1cm} (5.9)

The energy \( E \in [0,1] \) is equal to 1 for a constant image.

- Homogeneity:

\[
H = \sum_{i=0}^{L-1} \sum_{j=0}^{L-1} \frac{p(i,j)}{1 + |i-j|}.
\]  \hspace{1cm} (5.10)

It is a measure of how much elements of the matrix are close to the diagonal of the GLCM. The homogeneity \( H \in [0,1] \) is equal to 1 for a diagonal GLCM.

Since the notion of texture relies on the existence of some “order”, it is possible to resort to the fractal theory, proposed by B. Mandelbrot in [88] for the study of fractal objects. In fact, a fundamental property of this mathematical object concerns the auto-similarity, for which the structure of the object is replicated in the same way at different scale. This property can be characterized in terms of fractal dimension that represents a measure of the “roughness” of an object. When it is used as textural descriptor, it can be interpreted as an index of “coverage”: high values for fractal dimension imply the presence of a texture that covers the image leaving a few and small “void” areas. Different methods have been proposed for the estimation of the fractal dimension (see for instance: [89] and [90]).

For binary images, the fractal dimension \( D \) can be computed by using the box counting method [88]. In the box-counting method, a grid consisting of a box size \( e \) is superimposed on the boundary of the object to be quantified. The number of boxes of a given size \( e \) that contain the boundary point \( N(e) \) is computed. This procedure is repeated for different box dimensions. Then \( D \) is the absolute value of the slope in the linear portion of the plot of the log number of boxes versus the log box size \( e \) and for 3D objects it results \( 2 < D < 3 \). The fractal dimension describes how an object occupies space and it is related to the complexity of its structures. When it is used as textural descriptor it can be interpreted as an index of “space coverage”: high values for fractal dimension imply the presence of a diffuse texture that covers the space leaving few and small void areas.
5.7 REPRESENTATIVE ELEMENTARY VOLUME (REV)

As already mentioned in section 4.3, the selection of the Volume of Interest (VOI) both in terms of size and position affects the results of the quantitative analysis process. The representativeness of the chosen VOI is an important issue for the accuracy of the results. The adoption of the term Representative Elementary Volume (REV) was also advanced when a VOI is really representative [9]. However, checking the representativeness of an adopted Volume of Interest is a non trivial task.

In all the cases in which this is practicable, it is a good and simple idea to extract several VOIs (without overlapping areas, if possible) from different regions of the 3D volume and to apply the analysis protocol to all these VOIs. The consideration of different VOIs allows to search for a dependence from the size of the VOI itself or from the area of the original dataset from which the VOI is extracted, i.e. how the investigated parameters behave when the size of the VOI is kept constant but its position is altered. The standard deviation (SD) of the set of these multiple measures may preliminary suggest a “good” VOI selection. However, more accurate considerations that combine both size and position of the VOI are required. In fact, a low SD may imply that a too small VOI was chosen and an insufficient number of elements was taken into account. An oversized volume would also result in minimal changes of the computed values when altering the VOI position as the differences would simply average out.

A simple but automatic method was proposed by the Author in [3] for checking VOI representativeness when more than one VOIs can be extracted from a single 3D dataset. The analyst starts from the VOI size that ensures to select a certain number (e.g. three or four) of VOIs without no overlapped areas. Selecting a significant number of non-overlapped VOIs might be difficult for increased sizes. This trade-off between the number of VOIs and the VOI size has to be assessed by trial and error and it is related to the size and the structure of the imaged sample. Then, each VOI is reduced in turn by cropping an equal amount of voxels from each side and the cropped VOI is re-subjected to the analysis. The adoption of cropped versions from the original VOI instead of simply considering other resized VOIs simplify the computational requirements of this validation process and does not require user’s involvement for the selection of the smaller VOIs.

The dispersion bar of all the considered VOIs and for each VOI size can then be plotted. The sensitivity of each computed quantitative parameter with respect to the size of the VOI and, consequently, the representativeness of the considered VOI is suggested by the trend line among the different sizes and the dispersion bars. In general, a steady behaviour for both the trend line and the width of the dispersion bars suggests a proper VOI size. While, of course, this proper VOI size may differ among the parameters, generally the best trade-off is identified in order to assess all the parameters from the same
VOIs. An application of this method for the check of VOI representativeness was adopted in the application reported in section 6.1.
Three applications of quantitative analysis of tomographic images are now presented. All of these applications have in common the characterization of the micro-architecture of trabecular bone. More precisely, the first application aims at characterizing a structure (a tissue engineering scaffold) that has to mimic the architecture of trabecular bone. The second application is based on ex-vivo experiments carried out on femur and lumbar spine of mice affected by microgravity conditions. Finally, the results of an in vivo study on osteoporotic subjects by means of MRI are reported.

The trabecular (or cancellous) bone is a 3D meshwork of bony trabeculae and void spaces containing the bone marrow. It can then be thought of as a porous medium with an interconnected porous space. Therefore, the approaches exposed in the previous chapters for image analysis of this kind of porous medium are then applicable as the following applications will show.

### 6.1 CHARACTERIZATION OF BONE TISSUE ENGINEERING SCAFFOLDS

Tissue engineering is widely recognized as one of the most promising approaches for tissue repair and reconstruction [92]. The basic elements of the tissue engineering approach are illustrated in Figure 27. In its simplest form, tissue engineering starts from cells - including stem cells, genes and/or proteins (not necessarily extracted from the destination patient) - transplanted within a porous degradable material known as a scaffold. The scaffold is then placed into a
Figure 27. The concept of Tissue Engineering. Cells are extracted from the patient (i, ii) and then transplanted within a porous scaffold. The whole system is then placed into a bio-reactor (iii). After the evolution of cells, a newly formed tissue is available (iv) and ready to replace the damaged tissue of the patient (v).

Bio-reactor in order to stimulate tissue regeneration. At the end, the newly formed tissue is ready to replace the damaged tissue of the patient. A key step of the whole process is the micro-architecture of the scaffold as it affects several properties of the final tissue.

Being the widest tissue in human body, skin has been the first application of tissue engineering with attractive results. The more recent trends in this field include investigations on bone regeneration. The application reported in this section focuses on the characterization of alginate-based bone tissue engineering scaffolds by means of $\mu$-CT image analysis.

It is mandatory for any optimal scaffold material to act as a temporary three-dimensional support for cell adhesion, growth and mineral matrix deposition. Moreover, ideal scaffolds should be able to integrate into surrounding tissue and mimic the morphology of the natural bone tissue. Strict requirements for scaffolds are biocompatibility, a design closely resembling the natural extracellular structure, an appropriate surface chemistry to promote cellular attachment, differentiation and proliferation, and a sufficient mechanical strength to withstand $in\ vivo$ stresses and physiological loading. Finally, the degradation of the ideal scaffold should proceed in a controlled way, still keeping a sufficient structural integrity until the newly grown tissue has replaced the scaffold’s supporting functions.

Approaches in scaffold design must be able to satisfy the aforementioned principles. Also, material chemistry and the micro-architecture
determine the functional properties of a scaffold as well as how cells interact with it [93]. In particular, for bone tissue engineering, osteoconductivity largely depends upon the geometry of the scaffold. The degree of porosity and interconnection of the pores are also crucial for the in vivo bone tissue ingrowth in terms of blood vessels invasion, migration and proliferation of osteoblasts and matrix deposition in the void spaces. Therefore, critical issues for the design of a scaffold concern pore size, pore geometry, spatial distribution of pores and their interconnections in order to correctly derive mechanical and mass-transport properties and improve the effectiveness of biomaterials for bone tissue engineering [94]. However, assessing these structural properties is a challenging task. While it seems obvious that a scaffold needs to have pores and channels as cells need to grow within it and these need to be supplied with nutrients, it is not so obvious to determine a priori what their ideal shape, dimensions and interconnections should be as well as how these parameters can be effectively assessed [95].

Both bioactive ceramics and polymers have been developed for use as bone composite scaffolds [96] [97] [98] [99]. Polymers have some advantages over ceramic materials. Their biodegradation rates and mechanical properties can be tailored for specific applications. They are particularly amenable for implantation and can be easily manufactured into desired shapes. The major concern associated with polymer scaffolds deals with their low mechanical strength and shape retention failure. In addition, synthetic polymers demonstrate insufficient cell adhesion and their hydrophobic surfaces hinder cell growth [100] [101]. They also lack functional groups available for further surface modifications [102]. When implanted in vivo, some synthetic polymers release acidic degradation products and invoke a chronic immune reaction [103].

Composite scaffolds made of biodegradable natural polymers are very promising constructs: they are endowed with excellent biocompatibility and suitable mechanical properties, and they can be loaded with growth factors involved in bone formation. Natural polymers offer the advantage of being very similar, often identical, to the natural macromolecular environment of cells. This similarity introduces the interesting capability of designing biomaterials with a true molecular biological functionality, rather than a mere morphological similarity. Among the natural polymers, polysaccharides are very versatile, enabling to be decorated with signal molecules (oligosaccharides, peptides) and to interact with inorganic components. Alginate is the name of a family of linear copolymers produced by brown algae and bacteria. Alginate has the ability to form stable gels in the presence of millimolar concentrations of calcium or other divalent cations [103]. Cell encapsulation in calcium alginate beads represents a well established method for cell protection from the host immune system, [105] [106] but the biological inertness of alginate has largely hampered its use in all those applications where cell adhesion is mandatory for survival and proliferation. Moreover, while calcium cross-linked
gels make use of a simple chemistry and can be introduced into the body in a minimally invasive surgery, they are generally associated over longer time intervals with poor shape definition and volume instability in vivo \[107\] \[108\]. One possible approach to overcome the biological and mechanical limits of alginate is the use of HAp as inorganic reinforcing and osteoconductive component of alginate HAp composite scaffolds \[109\] \[110\]. Hydroxyapatite reinforced polymer biocomposites offer a robust system to engineer synthetic bone substitutes with tailored mechanical, biological, and surgical functions \[111\] \[112\] \[113\] \[114\] \[115\] \[116\]. Several studies have consistently shown that HAp typically exhibits excellent biocompatibility, bioactivity, and if porous, osteoconduction \[94\] \[4 \]. Therefore, the basic design rationale for preparing HAp-reinforced polymer composites is to further reinforce a solid biocompatible polymer matrix with a bioactive inorganic filler, mimicking the role of HAp in bone. In recent years, various alginate-based constructs for the use in bone engineering have been proposed \[114\] \[118\] \[119\] \[120\] \[121\]. A reliable characterization of the microarchitecture of the produced scaffold is necessary.

Commonly, two-dimensional (2D) imaging systems such as scanning electron microscopy (SEM) are used for the characterization of microstructures \[122\], although 2D systems show limits for the evaluation of some characteristics such as pore interconnectivity and anisotropy. To this aim, the most promising technique seems to be the computed microtomography (µ-CT) \[123\] widely used for the characterization of scaffolds \[124\] \[125\] \[126\] \[94\] \[4 \]. However, accurate image analysis protocols able to extract quantitative measures and indices directly from µ-CT images are yet to be well defined.

The challenging part of the quantitative analysis of µ-CT images lies on the segmentation, i.e. a voxel classification process in which an image is separated into subsets by assigning individual voxels to classes \[127\]. Micro-CT images are usually segmented by thresholding gray levels and the preferred choice of selecting the threshold value is mainly based on manual visual assessment \[128\] \[129\] \[130\] \[131\] \[125\]. However, such a process is subjective, time-consuming and factors like room lighting, monitor brightness settings, operator fatigue and limited gray-scale shade perception can affect the reproducibility of visual thresholding \[132\]. Therefore it is worthy to investigate alternative objective and automatic thresholding techniques in order to overcome the subjectivity of manual thresholding.

Jones et al. \[133\] proposed an edge-based kriging segmentation algorithm \[134\] which however involves the manual choice of two cutoff values. Furthermore, a curve integration method was proposed in \[135\] \[136\] but a manual assignment is first required to train the process. Moore et al. \[43\] suggest to use the threshold that produces a binary image in which the resulting porosity (percentage of background voxels in comparison with the total) is similar to either the theoretical porosity (based on known porogen concentration) or the measured porosity (based on mercury intrusion porosimetry). How-
ever, with this technique an external data (the theoretical or measured porosity) is needed for the segmentation.

In this application, an effective image analysis protocol with a fully automatic segmentation is presented. Different automatic thresholding techniques are examined and quantitatively compared against a manual thresholding performed onto three different \( \mu \)-CT datasets of alginate/hydroxyapatite (Alg/HAp) composite scaffolds (Figure 28). After the comparison, the thresholding technique resulting as the best one was selected to be part of the proposed automated protocol. Several quantitative descriptors are then selected as relevant and applied to both the manually and automatically segmented images in order to evaluate the differences between the proposed method and the results of a manual segmentation. Different Volumes Of Interest (VOIs) with variable size and position are also considered in order to assess the representativeness of the chosen VOIs, which is an important issue for the accuracy of the results.

Figure 28. A 2.5\( \times \)2.5 mm\(^2\) (400\(^2\) pixels) slice of each considered \( \mu \)-CT dataset: a) Alg/CaCO\(_3\); b) Alg/nHAp; c) Alg/HapF. (The scale bar is 0.5 mm in all the images)
6 BIOMEDICAL APPLICATIONS

6.1.1 Materials and Methods

Materials preparation

Sodium alginate (Alg) samples isolated from *Laminaria hyperborea* stipe were provided by FMC Biopolymer (Norway). Hydroxyapatite powder (HApF) was from Fluka (USA) while the preparation of nano-Hydroxyapatite (nHAp) was achieved following the indications reported in [137]. Briefly, nHAp was prepared by wet chemical method using CaCl$_2$ (Sigma, USA) and (NH$_4$)$_2$HPO$_4$ (Sigma, USA) as Ca and P precursors, respectively. To precipitate stoichiometric nHAp, 0.3 M aqueous solution of (NH$_4$)$_2$HPO$_4$ was slowly added drop by drop to 0.5 M aqueous solution of CaCl$_2$. The rotation speed of stirrer was adjusted to 1000 rpm and the reaction temperature was maintained at 60°C. The minimum pH was adjusted to 10 by adding concentrated NH$_4$OH using an injectable syringe. The resultant precipitate was aged for 24 h under stirring at the same speed. After aging, the obtained white precipitate was filtered, washed four to five times with distilled water until complete removal of ammonium chloride. Final precipitate was centrifuged at 10000 rpm for 10 min and dried in oven for 24 hours at 120°C. The nHAp powder was finally pounded in a mortar several times in order to obtain an homogeneous powder. As reported in [4], TEM images were used to identify the average dimensions of the commercial HApF granules leading to an average value of about 150 nm. The average dimension of the particles for the nHAp powder resulted to be about 120 nm.

As the preparation of the samples with HApF and nHAp is the same, for simplicity in this paragraph the acronym HAp is used for both the formulations. Alg/HAp composite scaffolds were prepared by mixing alginate 2% (w/v) and HAp at different concentrations in water using calcium release method. HAp powder was homogenously dispersed into a stirred solution of alginate in water, followed by the addition of 60 mM of D-gluconic acid δ-lactone (GDL) to release calcium ions from HAp. Aliquots of this gelling solution were then cured in 24-well tissue culture plates (h=18 mm, φ=16 mm, Costar, Cambridge, MA) for 24 h at room temperature to allow complete gelification. The hydrogels in the tissue-culture plate were then step-wise cooled by immersion in a liquid cryostat. Ethylene Glycol in water (3:1) was used as refrigerant fluid. Temperature was decreased step-wise from 20°C to -20°C by 5°C steps with 30 min intervals for equilibration. The samples were then freeze-dried for 24 hours to obtain porous scaffolds. For control experiments, pure alginate gels (HAp-free) were prepared by replacing HAp with CaCO$_3$ (corresponding to 30 mM of Ca$^{2+}$). Pure alginate gels were then processed as HAp composite gels.

Three composite scaffolds were prepared for μ-CT image analysis: pure alginate gels (hydroxyapatite-free) prepared by replacing the hydroxyapatite with CaCO$_3$ (corresponding to 30 mM of Ca$^{2+}$) hereafter referred as Alg/CaCO$_3$, an alginate with hydroxyapatite powder
6.1 Characterization of bone tissue engineering scaffolds

from Fluka (hereafter Alg/HApF) sample and a alginate with nano-hydroxyapatite (Alg/ nHAp) sample.

X-ray micro-CT imaging

Micro-CT images of the samples were acquired using the TomoLab station (see subsection 2.3.2). All the scans were performed with the following parameters: distance source-sample = 100 mm; distance source-detector = 400 mm; 1440 tomographic projections over a 360 degree scan angle; tube voltage = 40 kV; tube current = 200 μA; exposure time = 2.6 sec; focal spot size = 5 μm; resulting voxel size = 6.25 μm. While the μ-CT setup is the same for all the samples, independent conversion of the images to 8-bit format was performed according to an automatic min/max normalization procedure. Doing so, the three μ-CT datasets have to be considered as independent and therefore the three gray-level scales are not comparable.

VOI selection

In this application, four VOIs of 2.5×2.5×2.5 mm$^3$ (400$^3$ voxels) were extracted from each considered dataset. This particular VOI size was chosen because it ensures to select four VOIs without no overlapped areas. Selecting an equal number of non-overlapped VOIs was observed to be difficult for increased sizes. Each VOI was reduced in turn to 350$^3$, 300$^3$, 250$^3$, 200$^3$, 150$^3$ and 100$^3$ voxels (i.e. 2.23, 1.93, 1.63, 1.33, 0.93, 0.63 mm$^3$) in order to perform an a posteriori test for the representativeness of the adopted VOIs as suggested in section 5.7.

Automatic image segmentation

After VOI extraction, a segmentation process is required. In this application, seven automatic fixed-threshold techniques are taken into account. In principle, a global threshold for the whole 3D dataset is preferable and this is coherent with the typical behavior of a human operator inspecting all the reconstructed stack of images. However, in this study each VOI was subjected to the automatic segmentation techniques. This allows to check for the robustness of the automatic thresholding methods to be checked, i.e. how much the methods are affected by small variations in the histogram such as those occurring when considering different VOIs extracted from different areas of the CT dataset.

The automatic thresholding techniques considered in the present study are: the Kittler and Illingworth [36] the Ridler and Calvard [37] iterative scheme; the well-known Otsu [38] method; the approaches proposed by Tsai [39] and Pun [40] as well as an improvement proposed by Kapur et al. [41] were also considered; finally the method suggested by Rajagopalan et al. [42] was also applied. In this application, the segmentation process includes a parameter-free post-thresholding step as an attempt to reduce misclassified voxels. Since a
scaffold possesses an interconnected porous network, an easy way to perform an effective post-thresholding “cleaning” procedure is based on the connected components labeling algorithm \[138\]. In fact, the interconnectivity implies that a correctly segmented image should in principle possess only one connected component for the object phase (representing the scaffold itself) and also only one connected component with very high volume for the background (representing the porous network). Few connected components with small volume could appear after the thresholding if a non-optimal threshold is selected and they have to be considered as noise. Nevertheless, the binary object representing the scaffold has the prerogative to include voxels of the faces of the VOI, while the binary noise is “disconnected” from the VOI faces. Therefore, an effective “cleaning” filter could be obtained by simply removing internal connected components, i.e. connected components that do not “touch” VOI margins. This filter is included into the proposed segmentation process as a post-thresholding step (see Figure 30). An example of the results of the whole segmentation process (thresholding and post-processing) is reported in Figure 29.

In order to test the goodness (or discrepancy) of an automatic thresholding, several comparison methods have been proposed in the literature (see [139] for a review). In this application the automatically segmented images obtained according to the automatic methods are compared against a manually segmented image. Of course, the manually assessed threshold may not be the “best” one - which still remains unknown - but practically it makes sense to consider the manually thresholded image as a reference for the aim of this study. The automatic segmentation of each image is obtained by thresholding with the mean value of all the thresholds determined on each of the four VOI extracted from the original image. On the other hand, the manual binarization is realized by using a threshold proposed by an expert human supervisor. In both cases the post-processing step follows. The comparison is a simple strategy that allows to evaluate which one of the automatic thresholding techniques could be reasonably considered as the most effective at producing an image that is the most similar to the one proposed by an expert human supervisor. In the present paper the Misclassification Error (ME) \[140\] between the manually and the automatically segmented image is adopted. The ME is calculated as:

\[
ME = 1 - \frac{TP + TN}{N}
\]  

where \(TP\) is the number of object voxels correctly detected (True Positives), \(TN\) is the number of background voxels correctly detected (True Negatives) and \(N\) is the total number of voxels. ME varies from 0, for a perfectly classified image, to 1 for a totally wrongly binarized image.
Figure 29. Comparison of the different segmentation results (thresholding plus post-processing in all the cases): a) original slice (the scale bar is 0.5 mm); b) Kittler’s method (threshold = 50); c) Ridler’s method (threshold = 58); d) Otsu’s method (threshold = 59); e) Tsai’s method (threshold = 61); f) Pun’s method (threshold = 42); g) Rajagopalan’s method (threshold = 41); h) Kapur’s method (threshold = 81). (For the sake of clarity only one central slice of the considered VOI of the Alg/CaCO₃ sample is shown, however 3D processing has been performed on the whole stack)
Figure 30. Effects of the proposed post-processing on a slice of a VOI extracted from the CT dataset of the Alg/CaCO$_3$ sample: a) original image; b) simple thresholding according to the Kittler and Illingworth method; c) elimination of all the connected components in touch with VOI margins resulting in an image with only binary noise; d) difference of (b) and (c) images, resulting in a correctly segmented image. (For the sake of clarity only one slice of the VOI is shown though the processing is performed in 3D)

Quantitative analysis

Basic characteristics were extracted directly from the segmented images. Focus was posed onto the following parameters: porosity ($1 - V_V$) and specific surface area ($S_V$) as well as indexes for the characterization of the anisotropy (isotropy index $I$ and elongation index $E$). A model-independent approach was preferred when attempting to characterize pore thickness. Pore and throat size distributions were assessed after skeletonization of the pore space. The skeletonization algorithm reported in [81] was adopted. Information about the connectivity of the pore space was extracted from the connectivity index $\beta$ and the mean coordination number $Z$. 

78
Table 7. Thresholds proposed by automatic methods for each VOI.
(Mean and standard deviation (SD) of the VOIs are included.)

<table>
<thead>
<tr>
<th>Kit</th>
<th>Rid</th>
<th>Ots</th>
<th>Tsa</th>
<th>Pun</th>
<th>Raj</th>
<th>Kap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg/CaCO₃ (manual threshold determination: 51)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOI₁</td>
<td>50</td>
<td>38</td>
<td>60</td>
<td>62</td>
<td>42</td>
<td>43</td>
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<td>VOI₂</td>
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<td>57</td>
<td>58</td>
<td>60</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>VOI₃</td>
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<td>57</td>
<td>58</td>
<td>60</td>
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<td>40</td>
</tr>
<tr>
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<td>58</td>
<td>59</td>
<td>61</td>
<td>42</td>
<td>41</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>0.500</td>
<td>1.000</td>
<td>1.000</td>
<td>0.000</td>
<td>1.299</td>
</tr>
<tr>
<td>Alg/nHAp (manual threshold determination: 53)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOI₁</td>
<td>52</td>
<td>64</td>
<td>66</td>
<td>72</td>
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<td>46</td>
</tr>
<tr>
<td>VOI₂</td>
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<td>67</td>
<td>68</td>
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<td>65</td>
<td>66</td>
<td>73</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
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<td>64</td>
<td>65</td>
<td>71</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>mean</td>
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<td>65</td>
<td>66</td>
<td>73</td>
<td>46</td>
<td>46</td>
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<tr>
<td>SD</td>
<td>0.000</td>
<td>1.225</td>
<td>1.090</td>
<td>1.479</td>
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<td>0.000</td>
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<tr>
<td>Alg/HApF (manual threshold determination: 40)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOI₁</td>
<td>38</td>
<td>56</td>
<td>57</td>
<td>64</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>VOI₂</td>
<td>38</td>
<td>56</td>
<td>58</td>
<td>65</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>VOI₃</td>
<td>38</td>
<td>58</td>
<td>59</td>
<td>66</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>VOI₄</td>
<td>38</td>
<td>58</td>
<td>60</td>
<td>67</td>
<td>30</td>
<td>28</td>
</tr>
<tr>
<td>mean</td>
<td>38</td>
<td>57</td>
<td>59</td>
<td>66</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>SD</td>
<td>0.000</td>
<td>1.000</td>
<td>1.118</td>
<td>1.118</td>
<td>0.000</td>
<td>0.500</td>
</tr>
</tbody>
</table>

6.1.2 Results and Discussion

Table 7 reports threshold values for the manual assessment and for each automatic determination method considered in this study. Mean values and standard deviation (SD) of the threshold values were computed for all of the VOIs separately for each scaffold. The very low SD values observed in the Pun’s method and in the one proposed by Kittler and Illingworth indicate that they are insensitive to VOI selection. On the other hand, other methods (e.g. Kapur) exhibit high SD suggesting a dependence from the region of interest to which the VOIs belong. The latter methods have to be discarded because fluctuations in the threshold value affect further quantitative analysis.

Figure 29 shows that the threshold proposed by the Pun’s method is too far from the manual one and also the post-processing is not able to remove the binary noise introduced by the thresholding. This is confirmed in Table 8 in which ME values for each automatic thresholding method are reported. Moreover, since an automatic min-/max normalization procedure was performed on each of the three
Table 8. Misclassification error for each considered automatic method and for each considered VOI with respect to the manual thresholding. (Mean value of all the considered VOIs for all the samples is also reported.)

<table>
<thead>
<tr>
<th></th>
<th>Kit</th>
<th>Rid</th>
<th>Ots</th>
<th>Tsa</th>
<th>Pun</th>
<th>Raj</th>
<th>Kap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg/CaCO₃</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOI₁</td>
<td>0.014</td>
<td>0.070</td>
<td>0.076</td>
<td>0.096</td>
<td>0.396</td>
<td>0.583</td>
<td>0.132</td>
</tr>
<tr>
<td>VOI₂</td>
<td>0.014</td>
<td>0.072</td>
<td>0.078</td>
<td>0.099</td>
<td>0.400</td>
<td>0.580</td>
<td>0.136</td>
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<tr>
<td>VOI₃</td>
<td>0.014</td>
<td>0.067</td>
<td>0.073</td>
<td>0.092</td>
<td>0.419</td>
<td>0.601</td>
<td>0.127</td>
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<tr>
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<td>0.064</td>
<td>0.070</td>
<td>0.089</td>
<td>0.413</td>
<td>0.597</td>
<td>0.123</td>
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<tr>
<td>Alg/nHAp</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VOI₁</td>
<td>0.011</td>
<td>0.088</td>
<td>0.093</td>
<td>0.124</td>
<td>0.463</td>
<td>0.463</td>
<td>0.187</td>
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<tr>
<td>VOI₂</td>
<td>0.010</td>
<td>0.082</td>
<td>0.087</td>
<td>0.117</td>
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<td>0.474</td>
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<td>VOI₃</td>
<td>0.011</td>
<td>0.092</td>
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<td>0.130</td>
<td>0.466</td>
<td>0.466</td>
<td>0.197</td>
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<td>VOI₄</td>
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<td>0.088</td>
<td>0.093</td>
<td>0.126</td>
<td>0.467</td>
<td>0.467</td>
<td>0.190</td>
</tr>
<tr>
<td>Alg/HApF</td>
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<tr>
<td>VOI₁</td>
<td>0.008</td>
<td>0.045</td>
<td>0.051</td>
<td>0.062</td>
<td>0.415</td>
<td>0.515</td>
<td>0.132</td>
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<tr>
<td>VOI₂</td>
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<td>0.047</td>
<td>0.053</td>
<td>0.065</td>
<td>0.404</td>
<td>0.502</td>
<td>0.141</td>
</tr>
<tr>
<td>VOI₃</td>
<td>0.007</td>
<td>0.044</td>
<td>0.050</td>
<td>0.061</td>
<td>0.397</td>
<td>0.496</td>
<td>0.137</td>
</tr>
<tr>
<td>VOI₄</td>
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<td>0.043</td>
<td>0.049</td>
<td>0.059</td>
<td>0.409</td>
<td>0.506</td>
<td>0.130</td>
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<tr>
<td>mean</td>
<td>0.011</td>
<td>0.067</td>
<td>0.073</td>
<td>0.093</td>
<td>0.427</td>
<td>0.521</td>
<td>0.151</td>
</tr>
</tbody>
</table>

CT dataset, the three gray-level histograms are not comparable and it is worthy to notice that, for instance, the Otsu’s method proposes the same threshold (59) for both the Alg and Alg/HApF samples while the Kittler and Illingworth method better adapts to the shifts in the image histogram suggesting a value closer to the manually selected one. Therefore, among the considered, the method proposed by Kittler and Illingworth is the most effective because it presents the lowest mean value of ME (about 1%) together with a very low dependence on the selected VOI.

Table 9 reports the quantitative results computed after the manual and the automatic segmentation with the Kittler and Illingworth method, respectively. It can be noticed that the differences in the segmentation affect the computation of parameters in a negligible way and this is confirmed by the statistical analysis (Wilcoxon test) that revealed no p-value below the 0.05 significance level. With more details, in all the cases automatic segmentation provides threshold values lower than the manual ones. This means that the scaffold results slightly thicker in the case of the automatic segmentation producing lower values of porosity as well as higher values for specific surface area. Moreover, the differences observed for the basic characteristics in the case of the Alg/HApF sample are slightly greater than the ones observed for the other two samples which is consistent with the fact
6.1 Characterization of bone tissue engineering scaffolds

Table 9. Comparison between the characterization of the considered samples adopting a manual segmentation (manual thresholding and post-processing) and the proposed automated segmentation (Kittler-Illingworth method and post-processing).

Mean value ± standard deviation among the considered Volumes of Interest (VOI) is reported except for pore size, throat size and coordination number. Mean value ± standard deviation of the total distribution of the considered pores is reported for pore size, throat size and coordination number.

<table>
<thead>
<tr>
<th></th>
<th>Alg/CaCO₃</th>
<th>Alg/nHAp</th>
<th>Alg/HApF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Manual segmentation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 - V_V$</td>
<td>0.833±0.003</td>
<td>0.808±0.004</td>
<td>0.825±0.004</td>
</tr>
<tr>
<td>$S_V$ [mm⁻¹]</td>
<td>12.3±0.4</td>
<td>10.5±0.4</td>
<td>9.2±0.3</td>
</tr>
<tr>
<td>$I$</td>
<td>0.71±0.02</td>
<td>0.62±0.02</td>
<td>0.67±0.03</td>
</tr>
<tr>
<td>$E$</td>
<td>0.22±0.01</td>
<td>0.33±0.02</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>$\beta$ [mm³⁻¹]</td>
<td>15.5±0.4</td>
<td>12.7±1.8</td>
<td>15.0±1.5</td>
</tr>
<tr>
<td>$Z$</td>
<td>4.8±3.1</td>
<td>4.7±3.4</td>
<td>6.5±7.0</td>
</tr>
<tr>
<td>Pore size [mm]</td>
<td>0.28±0.12</td>
<td>0.31±0.13</td>
<td>0.32±0.15</td>
</tr>
<tr>
<td>Throat size [mm]</td>
<td>0.08±0.05</td>
<td>0.08±0.06</td>
<td>0.09±0.06</td>
</tr>
<tr>
<td><strong>Automated segmentation</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>$1 - V_V$</td>
<td>0.826±0.003</td>
<td>0.797±0.004</td>
<td>0.811±0.004</td>
</tr>
<tr>
<td>$S_V$ [mm⁻¹]</td>
<td>12.4±0.4</td>
<td>10.8±0.4</td>
<td>9.6±0.3</td>
</tr>
<tr>
<td>$I$</td>
<td>0.71±0.02</td>
<td>0.67±0.02</td>
<td>0.68±0.03</td>
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<tr>
<td>$E$</td>
<td>0.23±0.01</td>
<td>0.28±0.02</td>
<td>0.27±0.03</td>
</tr>
<tr>
<td>$\beta$ [mm³⁻¹]</td>
<td>14.4±0.7</td>
<td>11.7±1.5</td>
<td>14.6±1.4</td>
</tr>
<tr>
<td>$Z$</td>
<td>4.5±2.7</td>
<td>4.7±4.0</td>
<td>6.0±5.2</td>
</tr>
<tr>
<td>Pore size [mm]</td>
<td>0.27±0.11</td>
<td>0.30±0.12</td>
<td>0.30±0.13</td>
</tr>
<tr>
<td>Throat size [mm]</td>
<td>0.08±0.05</td>
<td>0.08±0.05</td>
<td>0.09±0.06</td>
</tr>
</tbody>
</table>

that the automatic proposed threshold is two gray-levels lower than the manual one (38 vs. 40) instead of just one gray-level found in the other cases (50 vs. 51 for the alginate sample and 52 vs. 53 for the Alg/nHAp sample).

On the other hand, the differences in the descriptors based on anisotropy and skeleton analysis are not affected by the slightly thicker scaffold obtained in the automatic segmentation case. In fact, the MIL method for the computation of the isotropy and elongation indexes is based on the number of intersections between a linear grid and the pore/material interface. Although slight variations in the threshold values influence the structure thickness, this fact does not directly imply variations in the pore/material interface. It is important to underline that, since the adopted implementation uses random orientations, minimal variations in the results are observed each time the code is executed. However, a simple test performed by re-computing
100 times the isotropy index $I$ and the elongation index $E$ on a single $2.5 \times 2.5 \times 2.5$ mm$^3$ (400$^3$ voxels) VOI revealed a coefficient of variation of about 0.007 for $I$ and about 0.020 for $E$ demonstrating that the random component in the computation of these parameters is not a concern. In the case of the Alg sample the isotropy index $I$ is identical in both segmentations ($0.71 \pm 0.02$ for the manual segmentation and $0.71 \pm 0.01$ for the automatic segmentation) while just slightly increasing values can be noticed for the Alg/nHAp sample (from $0.62 \pm 0.02$ for the manual segmentation to $0.67 \pm 0.04$ for the automatic segmentation) as well as for the Alg/HApF sample (from $0.67 \pm 0.03$ for the manual segmentation to $0.68 \pm 0.03$ for the automatic segmentation). In a similar way, the elongation index $E$ increases for the Alg and Alg/nHAp samples while a minimal decrease may be observed for the Alg/HApF sample. It is reasonable to conclude that all samples present closely the same degree of anisotropy and that the slight variations in the segmentation threshold do not significantly affect the estimation of $I$ and $E$.

In the case of the automatic segmentation, the differences in the results of skeleton analysis are related to the increasing thickness of structures, which reduces the number of cavities in the scaffold’s walls compared to the manually segmented case. A reduced number of branches and consequently a reduced number of nodes in the porous network are then identified by the skeleton analysis. However, due to the high number of nodes and branches considered, the pore and throat size distribution as well as the connectivity density and the coordination number are only marginally affected by these variations. Figure 31 and 32 present an almost identical behavior and, in addition, mean values (and standard deviation) for the pore and throat size distribution for all the considered samples present negligible differences.

The arbitrary selection of the $\mu$-CT Volume of Interest (VOI) seems to not significantly affect the results since generally low SD are observed. However, more accurate considerations that combine both size and position of the VOI are required. In fact, a low SD may imply that a too small VOI was chosen and an insufficient number of elements was taken into account. An oversized volume would also result in minimal changes of the computed values when altering the VOI position as the differences would simply average out. Figure 33 reports the results for the VOI representativeness check. For each parameter and for each sample, the mean value and the dispersion bar ($\pm$ SD) among the four VOIs cropped to the considered resize are reported. The sensitivity of each computed parameter with respect to the size of the VOI and, consequently, the representativeness of the considered VOI is suggested by the trend line among the different sizes and the dispersion bars. In general, a steady behavior for both the trend line and the width of the dispersion bars suggests a proper VOI size. While, of course, this proper VOI size may differ among the parameters, generally the best trade-off is identified in order to assess all the parameters from the same VOIs. For the basic characteristics
6.1 Characterization of bone tissue engineering scaffolds

![Histograms of pore and throat size distribution for Alg/CaCO₃, Alg/nHAp, and Alg/HApF.](image)

**Figure 31.** Distribution of pore and throat size for the considered samples in the case of the manual segmentation: a) Alg/CaCO₃; b) Alg/nHAp; c) Alg/HApF. Each histogram is computed taking into account all the four VOIs and the size of the bins is equal to the voxel size of the images (6.25 µm).

It can be noticed that even small VOIs may be used for the estimation of these parameters if the mean value of different VOIs is computed. A similar behavior can be observed for the isotropy indexes suggesting that the proposed 2.53 mm³ VOI allows to get reliable results for the considered samples with respect to the adopted imaging resolution. On the other hand, the skeleton analysis-based parameters (pore and throat diameter, connectivity density, and coordination number) need an adequate VOI size. In fact, a more fluctuating trend for the small sizes can be noticed. This is because an insufficient number of nodes and branches are determined if small VOIs are considered. Reminding that the mean pore size is around 300 µm and that only spheres totally incorporated into VOI margins are included in the computation of results, in a 0.6×0.6×0.6 mm³ (100³ voxels) VOI a very low number of pores can be identified. Reliable results require an enlarged VOI and the 2.5×2.5×2.5 mm³ (400³ voxels) VOI seems to be a good choice for the considered samples and the adopted imaging resolution. From a theoretical point of view, the adopted test for representativeness supposes that the investigated volume presents a micro-structural pattern repeated in some way throughout the sample and the test aims at identifying a VOI size that surely includes the pattern. Although such a perfectly repeated pattern cannot be
Figure 32. Distribution of pore and throat size for the considered samples in the case of the automatic proposed segmentation: a) Alg/CaCO$_3$; b) Alg/nHAp; c) Alg/HApF. Each histogram is computed taking into account all the four VOIs and the size of the bins is equal to the voxel size of the images (6.25 µm)

theoretically supposed, the considered samples do present a regular architecture and therefore this test do supply useful information.

Focusing on the specific sample characteristics, it is allowed to conclude that the addition of HAp (independently of average granule dimensions, at least in range from 120 to 150 nm) practically does not affect the overall characteristics of the freeze-casted alginate gels. Consequently, it appears that it is probably the combination of the 3D architecture of alginate gels together with the given procedure of freezing and sublimation which prevail in producing the final dry scaffold architecture.

In bone biomaterial engineering, early studies showed that a minimum pore size of 100 µm was required to allow bone tissue ingrowth in ceramic scaffolds [141]. Further investigations were carried out and, although the optimal pore size vary with scaffold material, the general consensus is that an ideal scaffold should possess a global porosity in the range of 80-90% with interconnects of at least 50 µm in diameter between its macropores in the 100-400 µm pore size range [142] [143] [144] [145] [146].
Figure 33. Analysis of the representativeness of the proposed VOI size (only the case of automated segmentation is considered) for each quantitative descriptor: a) porosity; b) specific surface area; c) elongation index; d) isotropy index; e) connectivity density; f) coordination number; g) pore diameter; h) throat diameter. The average value with the dispersion bar (±standard deviation) of the four VOIs are reported for each cropped VOI size and for each considered sample.
The results of the $\mu$-CT analysis show that the Alg/HAp scaffolds analyzed in the present study have a suitable architecture for tissue engineering applications in terms of pore and throat size. Moreover, a quantification of the degree of anisotropy and interconnectivity confirm that Alg/HAp biocomposite scaffolds for bone ingrowth are indeed trabecular structures with high and isotropic connectivity [4].

6.1.3 Conclusion

In this application, a parameter-free and model-independent methodology for the characterization of bone tissue engineering scaffolds directly from $\mu$-CT images is presented. An automatic segmentation method composed by the Kittler and Illingworth thresholding and a parameter-free post processing cleaning step able to reduce misclassified voxels is proposed. After segmentation, the porosity as well as more refined descriptors such as pore and throat size, degree of interconnectivity and isotropy indices can then be computed. By analyzing $\mu$-CT images of three different alginate/hydroxyapatite scaffolds it was here investigated how variations in the segmentation affect these numerical quantitative descriptors. Since the results showed negligible differences, it is here suggested that an automatic and objective segmentation has to be preferred. The proposed descriptors are also influenced by the selection of the Volume of Interest (VOI) both in terms of the area of the CT dataset which the VOI belongs and its dimensions. In the present study, it is suggested also how to correctly investigate on these crucial aspects. The resulting framework is an automatic tool for the characterization of bone tissue engineering scaffolds by means of $\mu$-CT image analysis.

6.2 CHARACTERIZATION OF BONE ALTERATIONS AFTER MICRO-GRAVITY

Bone is a complex dynamic tissue undergoing a continuous remodeling process throughout a lifetime. It adjusts to changes in mechanical demands, to prevent accumulation of fatigue damage, to repair microfractures, to ensure the viability of the osteocytes and to allow the skeleton to participate in calcium homeostasis. The remodeling process is characterized by a rapid resorption and a slower formation phase. Two different cell types play a major role in the process: osteoblasts (bone forming) and osteoclasts (bone resorbing). Osteoblasts are differentiated cells derived from lining cells, the immediate precursors residing on the bone surface, that synthesize a membrane associated alkaline phosphatase and regulate the deposition of the bone matrix molecules, including type I collagen and a variety of other non-collagenous proteins. Osteoblasts become osteocytes as soon as they are surrounded by a mineralized matrix. Instead, osteoclasts are multinucleated giant cells, responsible for the mineralized
6.2 Characterization of bone alterations after microgravity

Bone matrix resorption, that are formed by the fusion of mononuclear progenitors of the monocyte/macrophage family.

Bone cells act to increase or to decrease skeletal mass on the basis of its degree of utilization. The skeleton main role is locomotion that has to be performed counteracting the Earth gravity. When skeleton has not to stand against gravity, as it happens during space flights, exercise and movements are reduced leading to a decrease of whole bone mass and density and making bones brittle [147]. Early in the space program, studies performed on the crew members of the Gemini and Apollo missions showed a severe bone demineralization associated with an increase of calcium and phosphorus excretion. Skylab missions allowed further studies that highlighted how the serum levels of bone formation markers decreased whereas urinary markers of bone loss increased over 30% in the austronauts [148]. Analysis on cosmonauts aboard the Russian MIR space station provided irrefutable data derived from longer missions. In fact, the members of the Russian crew experienced a significant bone loss in the tibia already after 1 month, that continued throughout the flight period up to 6 months [149]. This bone loss was observed in both trabecular and cortical bone compartments. Interestingly, space flight did not alter bone mass in the non-weight-bearing radius. The post-flight follow-up revealed that a recovery time in normal gravity of the same length of the flight duration was insufficient to restore the bone mineral density at a level corresponding to the pre-flight status [150]. Consistently with results from humans, also the bone loss observed in rats during flight periods was not recovered by reambulation in normal gravity for a time corresponding to the flight duration after return to Earth [150]. Most recent Dual-emission X-ray Absorptiometry (DXA) measurements of 14 crew members which were aboard the International Space Station (ISS) for 4-6 months revealed bone loss at an average rate of 0.8% and 1.5% per space/month in the lumbar spine and in the femur, respectively. The determination of the bone loss rates provided also further evidence that space permanence more severely affects the trabecular than the cortical bone compartment [147] [149].

Because of the paucity of the involved subjects and the limits in the possibility to study microgravity effects on human bone, several studies regarding bone loss in space have been performed taking advantage of rat or mouse models. Most of the data collected from these animals were in agreement with those obtained from human studies. Nonetheless, investigators have to take into account that results have to be interpreted considering the specific experimental conditions, such as animal age, sex, body weight, pregnancy and variable delay in post-flight sample collection (i.e. sacrifice of the animals), that could affect the final results. Early studies conducted in Wistar rats during a 26-days space flight indicated a reduction in periosteal bone formation and longitudinal bone growth evidenced by the appearance of an extensive arrest line in the periostium of cortical bone and a decrease in the primary spongiosa width. Interestingly, the bone formation rate was not uniformly depressed in the cross section
of the tibia and was less severe at the level of the crest of the anterior tibia, where muscles are inserted \textsuperscript{151}. This evidence suggests that the deleterious effects of mechanical unloading in microgravity can be partially avoided by muscular contractions \textsuperscript{152}. A more recent study showed that bone resorption rates in rats exposed to 20-days space flight remained comparable with controls \textsuperscript{153} whereas several other studies confirmed a reduced presence and activity of osteoblasts and demonstrated the existence of site-specific changes in bone resorption \textsuperscript{154} \textsuperscript{155} \textsuperscript{156} \textsuperscript{157}.

Space flights lead to a decrease in osteoblast number and activity \textsuperscript{154} \textsuperscript{158} \textsuperscript{159}, most likely as result of an altered differentiation of osteoblast precursors. In rats exposed for 1-week to the space environment during the Spacelab 3 mission, tibial osteoblasts had a smaller cytoplasmic area, probably leading to reduced collagen secretion \textsuperscript{154}. In the same animal model, osteoclast number and activity remained unchanged in most studies of bones from space flight animals. An increase in the osteoclast population was only observed in space permanence of less than one-week duration, in different bone sites in pregnant rats \textsuperscript{160}. A 14-days period in space provoked a decrease in gene expression of bone matrix proteins, including osteocalcin, osteonectin, and type I collagen in Sprague-Dawley rat weight-bearing bones together with a reduction of osteoid surface and trabecular bone volume \textsuperscript{161}. When the differentiation of the cells of the osteogenic lineage to more mature forms is inhibited the expression level of two bone formation markers, such as alkaline phosphatase and osteocalcin, change in an opposite direction \textsuperscript{162}.

In this application skeleton alterations occurring in mice exposed to a near-zero gravity are investigated. Mice were hosted in the International Space Station (ISS) for 3 months during the Mouse Drawer System (MDS) mission (Shuttle Discovery Flight 17A/STS-128 on August 28th, 2009. MDS re-entered on November 27th, 2009 with Shuttle Atlantis Flight ULF3/STS-129 after 91 days), the longest permanence in space of mice. For the MDS experiment in addition to wild type (Wt) mice also transgenic mice over-expressing Pleiotrophin under the control of the human bone specific osteocalcin promoter (PTN-Tg) were selected. Pleiotrophin is an extracellular matrix-associated growth/differentiation factor widely expressed in embryonic development \textsuperscript{163} \textsuperscript{164}. However in postnatal life PTN is found mainly in bone and brain \textsuperscript{164} \textsuperscript{165} \textsuperscript{166}. PTN is considered an osteotrophic agent because mice overexpressing the human PTN gene had a higher mineral content respect to wild type control. Moreover PTN-transgenic mice compensate for the bone loss observed after ovariectomy \textsuperscript{167}. At cellular level, PTN was chemotactic to a variety of osteoblastic cell line \textsuperscript{168} and osteoprogenitors from human bone marrow \textsuperscript{169} suggesting that pleiotrophin might play a role during bone remodeling attracting osteogenic cells to sites of new bone formation. Indeed, PTN was found to be expressed in sites of new bone formation \textsuperscript{168} \textsuperscript{170}. PTN was synthesized by osteoblasts at an early stage of development and enhanced the osteogenic differentiation of mouse and
6.2 Characterization of bone alterations after microgravity

human bone marrow derived cells \[169\] [170]. For the PTN positive effects on new bone formation and its protective effects on ovariectomized females in bone loss \[167\], we selected PTN-Tg mice to investigate whether these mice were protected from space related osteoporosis and whether the PTN over-expression could be considered a countermeasure for the bone loss observed in microgravity.

6.2.1 Materials and Methods

Animals

Bone alterations were investigated onto 3 Wt and 3 PTN-Tg male mice (2 months old at the time of launch) after 3 month permanence in the ISS\textsuperscript{1}. Wild-type C57BL/J10 male mice were purchased from Jackson Laboratories and delivered to Charles River Laboratories and then to NASA Kennedy Space Center (KSC) Science Lab Specific Pathogen Free (SPF) animal facility. The mice not utilized for the MDS flight experiment were delivered to the animal facility of IST and kept in SPF Individually Ventilated Cages (IVC) conditions until the ground and vivarium control experiments started. PTN-Tg male mice were sent from the animal facility of IST to Charles River Laboratories for rederivation, breeding and husbandry activities. Mice that participated to the MDS experimentation were sent by Charles River Laboratories to the NASA KSC Science Lab SPF animal facility whereas the remaining animals were delivered to the animal facility of IST to be housed in the same conditions of the wild-type mice. Under vivarium conditions, mice were kept in rooms characterized by 20-24°C temperature, 40-60% relative humidity and 12 hours of light/dark cycle. Food (Mucedola srl, Milan, Italy) and water were provided \textit{ad libitum} to all mice but the animals housed in the MDS modules during flight and ground control experiment. In these last cases only water was provided \textit{ad libitum} while 5 g/die food was automatically provided by the MDS module. IVC system cages had a size of $300 \times 160 \times 140$ mm while cages of $330 \times 150 \times 120$ mm were used for the vivarium control experiment.

Unfortunately, only three mice (Wt2, PTN-Tg1 and PTN-Tg2) returned to Earth alive at the end of the mission. Three mice died during the flight (Wt3 after 16 days, PTN-Tg3 after 24 days and Wt1 after 44 days). Dead mice were immediately frozen by astronauts for subsequent analysis of their bones by \(\mu\)-CT. To check MDS habitat impact, a ground replica of the flight experiment was conducted housing six mice in the MDS spare model for three months. During the

\textsuperscript{1} The MDS experiment was approved by the American Institutional Animal Care and Use Committee (IACUC) with protocol nr. FLT-09-070(KSC) as well as by the Ethics Committee of the Animal Facility of the National Institute for Cancer Research (IST, Genova, Italy) and by the Public Veterinary Health Department of the Italian Ministry of Health with protocol nr. 4347-09/03/2009-DGSA.P and performed in accordance with the principles expressed in the “Guide for the care and the use of laboratory animals” (Office of Science and Health Reports of the USA National Institute of Health, Bethesda, USA).
ground replica of the experiment, the mice Wt₁, Wt₃ and PTN-Tg₃ were sacrificed exactly at the same experiment day of the death of the corresponding mice on the ISS. Moreover three Wt and three PTN-Tg mice of the same age, but raised in a vivarium in standard cages and conditions, were considered as further controls. Values derived from the vivarium samples were averaged in all types of analysis, thus always considering only one value per experiment data.

Animal habitat

The MDS model has been designed by Thales Alenia (Milano, Italy) and consists of an external $421 \times 480 \times 516$ mm container [171] in which various subsystems are integrated². All the subsystems are studied in order to support animal survival for a period much longer than the scheduled experimental time of 100 days. The subsystems integrated in the MDS module are principally the following: Mice Chamber (MC), Liquid Handling Subsystem (LHS), Food Delivery Subsystem (FDS), Air Conditioning Subsystem (ACS), Illumination Subsystem (ILS), Observation Subsystem (OSS).

Synchrotron radiation X-ray micro-CT imaging

Synchrotron radiation X-ray μ-CT imaging was performed at the SYRMEP Beamline of the ELETTRA Synchrotron Radiation Facility (Trieste, Italy). The 1200 radiographic projections were acquired with a beam energy of 19 keV over $180^\circ$ with a resulting voxel size of 9 μm.

VOI selection and segmentation

Investigations were focused on the lower third of the left femurs from the patella towards the shaft of the femur. The analysis of the trabecular structure was limited to a restricted subvolume, which corresponded to the maximum rectangular prism (about 5 mm high)

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² Further details as well as additional pictures about the MDS payload can be found at [http://www.nasa.gov/mission_pages/station/research/experiments/MDS.html](http://www.nasa.gov/mission_pages/station/research/experiments/MDS.html)
Characterization of bone alterations after microgravity registrable inside the inner cortical wall. In the case of cortical analysis, a 540 µm thick portion 4 mm far away from the patella was considered. In the lumbar spine district the analysis was focused onto the Vertebral Body in the VII Lumbar ring and the maximum rectangular prism (about 2 mm high) registrable inside the inner cortical wall was selected and subjected to quantitative analysis. Images were segmented with manual thresholding.

Image analysis

The following quantitative descriptors for trabecular bone architecture were extracted: total volume ($TV$ - expressed in $\mu m^3$), bone volume ($BV$ - expressed in $\mu m^3$), bone volume to total volume ratio ($BV/TV$ - expressed as a percentage), bone surface to bone volume ratio ($BS/BV$ - per millimeter), trabecular thickness ($Tb.Th$ - expressed in micrometers), trabecular number ($Tb.N$ - per millimeter), and trabecular separation ($Tb.Sp$ - expressed in micrometers). Information about the anisotropy, i.e. the presence of preferential orientation(s) of the structure was extracted according to the mean intercept length (MIL) method. MIL measurements were then summarized adopting the isotropy index $I$ and the elongation index $E$. Skeleton analysis was also applied in order to extract the connectivity index $\beta$.

6.2.2 Results and Discussion

Table [10] and [11] report the quantitative results for the femur bone of both Wt and Tg strains. Among all the vivarium mice examined, Wt mice showed a higher mean trabecular number ($Tb.N$) respect to PTN-Tg mice and as a consequence a wider mean trabecular separation ($Tb.Sp$). This confirms preliminary lab observations. Although an effective statistical analysis was not performed due to the reduced number of considered samples, in all the Wt and PTN-Tg samples affected by microgravity condition a significant decrease in bone volume to total volume ratio ($BV/TV$) values as well as connectivity index ($\beta$) with respect to the ground and vivarium controls was observed. This means that when a more dense trabecular structure is revealed the structure is also more interconnected, i.e. the additional trabeculae are in part redundant and not only essential to the structure itself. The computed trabecular number ($Tb.N$) value confirmed the tendency of the $BV/TV$ parameter, resulting decreased in the flight conditioned samples. No significant changes in the mean trabecular thickness ($Tb.Th$) were observed in the animal femurs regardless of the three different experimental conditions. The specific surface of the trabecular structure ($BS/BV$) was observed to be a quite stable parameter: the determined values were comparable in both Wt and PTN-Tg and did not dramatically increase after flight. Coherently with the previously considered parameters, the $Tb.Sp$ increased in all the samples after flight, whereas the $Tb.N$ parameter decreased when compared to ground and vivarium controls. Moreover, when
Table 10. Results of quantitative analysis in the femur of Wt mice. Tb.Th and Tb.Sp are expressed in mm, Tb.N in mm³, BS/BV in μm³, β in mm³ and BV/TV is a percentage value.

<table>
<thead>
<tr>
<th></th>
<th>Vivarium</th>
<th>Ground</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Wt1</td>
<td>Wt2</td>
</tr>
<tr>
<td>BV/TV</td>
<td>3.3±0.8</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>BS/BV</td>
<td>0.10±0.005</td>
<td>0.099</td>
<td>0.114</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>0.020±0.001</td>
<td>0.020</td>
<td>0.018</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>0.595±0.123</td>
<td>0.616</td>
<td>0.564</td>
</tr>
<tr>
<td>Tb.N</td>
<td>0.911±0.237</td>
<td>1.410</td>
<td>0.490</td>
</tr>
<tr>
<td>I</td>
<td>0.89±0.03</td>
<td>0.92</td>
<td>0.87</td>
</tr>
<tr>
<td>E</td>
<td>0.06±0.01</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>β</td>
<td>30.3±18.0</td>
<td>38.0</td>
<td>38.0</td>
</tr>
</tbody>
</table>

Table 11. Results of quantitative analysis in the femur of Tg mice. Tb.Th and Tb.Sp are expressed in mm, Tb.N in mm³, BS/BV in μm³, β in mm³ and BV/TV is a percentage value.

<table>
<thead>
<tr>
<th></th>
<th>Vivarium</th>
<th>Ground</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD</td>
<td>Tg1</td>
<td>Tg2</td>
</tr>
<tr>
<td>BV/TV</td>
<td>1.6±0.4</td>
<td>2.4</td>
<td>0.9</td>
</tr>
<tr>
<td>BS/BV</td>
<td>0.117±0.003</td>
<td>0.116</td>
<td>0.110</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>0.017±0.001</td>
<td>0.017</td>
<td>0.018</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>1.136±0.295</td>
<td>0.709</td>
<td>2.040</td>
</tr>
<tr>
<td>Tb.N</td>
<td>0.911±0.237</td>
<td>1.410</td>
<td>0.490</td>
</tr>
<tr>
<td>I</td>
<td>0.85±0.02</td>
<td>0.88</td>
<td>0.78</td>
</tr>
<tr>
<td>E</td>
<td>0.09±0.03</td>
<td>0.05</td>
<td>0.15</td>
</tr>
<tr>
<td>β</td>
<td>9.4±1.3</td>
<td>24.0</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Comparing vivarium, ground and flight femurs, the analysis of the trabecular thickness distributions confirmed a significant decrease of the Tb.N values in both Wt and PTN-Tg mice that experienced microgravity conditioning. After the three months exposure to microgravity, in PTN-Tg1 and PTN-Tg2 the trabeculae were in proportion less reduced than in Wt2.

In order to investigate with more details these findings, the trabecular thickness distribution was also assessed. Color maps of the distribution of the trabecular thickness in mice after flight conditioning as well as ground and vivarium controls are reported in Figure 35. Since, the Wt3 mouse died after 16 days of microgravity exposure, its trabecular thickness distribution was already affected. The alteration of the trabecular distribution in the Wt1 mouse (died after 44 days) was found to be even more severe and comparable to the one observed in the Wt2 mouse survived to the flight. On the contrary, the PTN-Tg3
that died after 24 days presented a trabecular thickness distribution similar to the one of PTN-Tg ground and vivarium controls.

At variance with observations made in femurs, no significant differences were noticed for all the investigated morphometric parameters between lumbar spines of vivarium Wt and PTN-Tg mice (Table 12 and 13). Instead, coherently with the femur observation, an important reduction of the BV/TV ratio and connectivity index in the Wt lumbar spines was observed after the flight. Moreover, after the microgravity conditioning, there were evidences of alteration also in the mean number (Tb.N) and separation (Tb.Sp) in both Wt and in PTN-Tg mice. In particular, these were more pronounced in Wt mice suggesting a possible protection of the transgene expression against
flight bone loss. The color maps of the distribution of trabecular thickness in lumbar spines are reported in Figure 36.

The combination of anisotropy index (I) and elongation index (E) did not allow to observe significant changes in the degree of anisotropy of the trabecular architecture of all the considered weight bearing bones after flight conditioning. The differences in the numerical results seem to be negligible for both strains and for all the samples. However, the method adopted for the structural anisotropy analysis considers subvolumes with non-regular shape (prisms of variable size instead of spheres or cubes) which slightly affect results [172]. More significantly, the results are affected by the low number of trabeculae that in particular the flight conditioned samples present. While changes in the the structural anisotropy of bone due to microgravity

Figure 36. Color map of bone trabecular thickness distribution in the lumbar spine of a representative Wt sample in the vivarium (a), ground (b) and flight (c) conditions and PTN-Tg sample in the vivarium (d), ground (e) and flight (f) conditions. (From blue to red the color map is 5−90 µm - Rendering performed with VG Studio MAX)
6.2 Characterization of bone alterations after microgravity

Table 12. Results of quantitative analysis in the lumbar spine of Wt mice. Tb.Th and Tb.Sp are expressed in mm, Tb.N in mm$^{-1}$, BS/BV in $\mu$m$^{-1}$, $\beta$ in mm$^{-3}$ and BV/TV is a percentage value.

<table>
<thead>
<tr>
<th>Vivarium</th>
<th>Ground</th>
<th>Flight</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV</td>
<td>Wt1</td>
<td>Wt2</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>19.6</td>
<td>19.8</td>
</tr>
<tr>
<td>BS/BV</td>
<td>0.060±0.006</td>
<td>0.063</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>0.034±0.003</td>
<td>0.030</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>0.121±0.019</td>
<td>0.121</td>
</tr>
<tr>
<td>I</td>
<td>0.63±0.01</td>
<td>0.67</td>
</tr>
<tr>
<td>E</td>
<td>0.36±0.01</td>
<td>0.28</td>
</tr>
<tr>
<td>$\beta$</td>
<td>208.1±29.0</td>
<td>229.4</td>
</tr>
</tbody>
</table>

Table 13. Results of quantitative analysis in the lumbar spine of Tg mice. Tb.Th and Tb.Sp are expressed in mm, Tb.N in mm$^{-1}$, BS/BV in $\mu$m$^{-1}$, $\beta$ in mm$^{-3}$ and BV/TV is a percentage value.

<table>
<thead>
<tr>
<th>Vivarium</th>
<th>Ground</th>
<th>Flight</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV</td>
<td>Wt1</td>
<td>Wt2</td>
</tr>
<tr>
<td>Mean±SD</td>
<td>19.2</td>
<td>14.6</td>
</tr>
<tr>
<td>BS/BV</td>
<td>0.065±0.005</td>
<td>0.066</td>
</tr>
<tr>
<td>Tb.Th</td>
<td>0.031±0.003</td>
<td>0.032</td>
</tr>
<tr>
<td>Tb.Sp</td>
<td>0.152±0.011</td>
<td>0.133</td>
</tr>
<tr>
<td>I</td>
<td>0.64±0.02</td>
<td>0.64</td>
</tr>
<tr>
<td>E</td>
<td>0.30±0.01</td>
<td>0.34</td>
</tr>
<tr>
<td>$\beta$</td>
<td>165.0±5.3</td>
<td>194.3</td>
</tr>
</tbody>
</table>

condition might be supposed [173] an accurate investigation requires a higher number of samples.

Bone architecture reflects bone functionality and behavior. The Wt mice maintained in standard vivarium conditions for 5 months (experiment controls) presented a more complex trabecular organization in terms of mean trabecular number than PTN-Tg mice of the same age and maintained in the same vivarium conditions. This finding was in agreement with previous published data reporting a greater bone mass in Wt mice than in PTN-Tg mice until the 25th week of age [170]. In flight mice of both strains (Wt and PTM-Tg), the mean trabecular number was decreased. Trabecular thickness distribution plots suggest that thicker trabeculae became thinner whereas initially thinner trabeculae (at the limit of the 9-10 $\mu$m in linear size) were no more detectable because they became thinner than the experimental $\mu$-CT voxel size. The fact that during flight Wt type mice tended
to lose more trabeculae than PTN-Tg mice, thus reducing the differences between the two mouse strains, is in line with the idea that the expression of the PTN transgene exerts some protection on the skeleton against the bone loss consequent to the microgravity exposure. To this respect, it should be noted that the PTN transgene expression can protect ovariectomized females from bone loss [167].

6.2.3 Conclusion

Even though no statistical analysis was performed due to the small number of studied animals, the reported observations showed a bone loss in both analyzed animal strains (Wt and transgenic) after 3 months permanence in the ISS. This bone loss appeared due to an increased bone resorption by osteoclasts and, especially for the Wt mice, to a decreased bone deposition by osteoblasts. The bone loss affected the weight bearing bones, but not the non weight bearing bones. Interestingly, whereas the PTN-Tg mice at the beginning of the mission (normal gravity conditions) presented a poorer trabecular organization, the expression of the PTN transgene during the flight resulted in some protection of the transgenic animals against the negative effect of microgravity. Apparently, this protection was the result of a higher osteoblast activity compared to Wt mice.

Hopefully, if the space agencies will take the decision of a re-flight of the MDS payload, there will be the opportunity to analyze additional animals to confirm and to expand some of the observations made during the first MDS mission. Flying additional animals in a second MDS flight will allow also to perform effective statistical analysis that will highly contribute to the comprehension of the bone turnover during a long term space mission and to the understanding of the role played by the PTN transgene in counteracting the microgravity induced bone loss.

6.3 Characterization of the Risk of Fracture in Osteoporotic Subjects

Aging and osteoporosis reduce the mechanical properties of bone by, in general, decreasing bone mineral density (BMD) and by altering the trabecular microstructure. However, several publications have suggested that alterations in bone architecture could explain bone fragility independent of bone mass in osteoporosis [174] [175] [176]. Therefore, evaluating bone mass with only bone mineral density (BMD) by using dual X-ray absorptiometry or quantitative computed tomography (CT) may be insufficient to fully assess the biomechanical strength of the trabecular bone or the fracture risk [177] [178] [179]. Bone alterations are usually evaluated by the analysis of the cancellous bone since it represents the more metabolically active part of the tissue [180] [181] [182].
The conventional tool for assessing trabecular bone structure is histomorphometry from bone biopsies [183]. The method yields two-dimensional representations of the trabecular network architecture from which the third dimension is obtained in an inferential manner using the mathematical tools of stereology [184]. Since trabecular bone networks are inherently three-dimensional, 2D histomorphometric approaches have, in recent years, been superseded by direct 3D analysis of biopsy specimens imaged by computed microtomography (µ-CT) [185]

Recent advances in magnetic resonance imaging (MRI), especially the improvement of spatial resolution, have made also clinical MRI a very attractive technique to this aim [182]. In fact, this latter approach based on clinical high resolution (HR) MRI is the only one that allows a totally non-invasive in vivo analysis of the trabecular bone structure. However, trabecular bone evaluation by MRI is a challenging task due to the insufficient final spatial resolution of the images. Since the thickness of human trabeculae is about 0.1–0.2 mm an effective imaging technique should guarantee a spatial resolution lower than 0.1 mm (as a rule of thumb one order of magnitude lower than the limit, i.e. about 0.01 mm). Unfortunately, clinical high resolution MRI (at 1.5 and 3.0 T) is able to reach an in plane spatial resolution comparable to the size of the trabeculae. Therefore, imaging of the trabecular bone structure and the analysis of the acquired images are strongly limited by partial volume blurring effect. Nevertheless, different in vivo studies showed significant correlation between structural measures obtained by MRI and the true trabecular bone structure visualized by contact radiography and µ-CT [186, 187]. To obtain these results, beyond the high resolution and the high signal-to-noise ratio obtainable by the MRI apparatus, also the image analysis procedures are of fundamental relevance.

The most common approach for image analysis is based on segmentation in order to separate the trabeculae from bone marrow signals. However, segmentation is a nontrivial task for in vivo MRI images. Because of the limited spatial resolution as well as noise fluctuations, the intensity histogram of the acquired images does not consist of two separate peaks representing the bone and the bone marrow. The absence of bimodality in the histogram causes simple thresholding methods to give poor results. Some attempts for segmenting in vivo MRI images of the calcaneus and the distal radius appear in the literature [188, 189]. However, the extracted quantitative measures present an excessive uncertainty due the low spatial resolution regime and it is therefore hard to rely on the computed values [190].

It sounds therefore interesting to extract quantitative indexes directly from the acquired gray-scale images. In this application, the calcaneus of a set of healthy and osteoporotic subjects were imaged by MRI. The calcaneus has found to be a proper anatomical site and it is widely exploited in the literature [187, 191, 192, 186, 181]. Texture analysis of the acquired images was then performed in order to check whether textural descriptors are able to quantitatively identify
differences between the two groups. The ultimate goal is, of course, to use the most reliable indexes as predictors for the risk of fracture.

6.3.1 Materials and Methods

Patients selection

Six volunteers were selected among pre-menopausal women (47±2 years, 61±9 Kg) participating at a clinical screening for the individuation of healthy, oestrogen untreated, non osteoporotic patients as a reference set of a normal bone status. Six female volunteers that reported a bone fracture due to osteoporosis were scanned (70±13 years, 70±11 Kg).

MR acquisition

MR images were obtained by using an experimental two-element phased-array wrist coil (receiving coil: Sense-flex-S) at a clinical 1.5 T Imager (Intera, Philips Medical System). A set of 20 sagittal images was collected from the calcaneus by means of a custom three-dimensional gradient echo sequence with echo time TE = 6.6 ms, repetition time TR = 40.1 ms, flip angle \( \alpha = 35^\circ \) and a resulting in-plane spatial resolution of \( 195 \times 195 \) \( \mu \)m (slice thickness: 0.7 mm). The acquisition took about 20 minutes per patient. An example of healthy and pathological MR slice is reported in Figure 37.

Image analysis

A single Volume of Interest (VOI) including only trabecular bone and marrow was manually selected in the posterior area of the calcaneus for each patient. The largest registrable VOI was found to
6.3 Characterization of the risk of fracture in osteoporotic subjects

Table 14. Results of the texture analysis of the MR images of the calcaneus for both healthy and osteoporotic patients. Mean value ± standard deviation of the acquired set of patients. The $p$-value of the Wilcoxon test between the osteoporotic case and the healthy case is also reported in the last column.

<table>
<thead>
<tr>
<th>Textural descriptor</th>
<th>Healthy</th>
<th>Osteoporotic</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray-level mean ($m$)</td>
<td>26±2</td>
<td>18±3</td>
<td>0.004</td>
</tr>
<tr>
<td>Gray-level contrast ($o$)</td>
<td>10.2±0.8</td>
<td>7.0±1.0</td>
<td>0.003</td>
</tr>
<tr>
<td>Smoothness ($S$)</td>
<td>0.020±0.001</td>
<td>0.020±0.002</td>
<td>0.937</td>
</tr>
<tr>
<td>Uniformity ($U$)</td>
<td>0.027±0.002</td>
<td>0.041±0.007</td>
<td>0.002</td>
</tr>
<tr>
<td>Entropy ($e$)</td>
<td>5.4±0.1</td>
<td>4.8±0.2</td>
<td>0.002</td>
</tr>
<tr>
<td>GLCM Contrast ($C$)</td>
<td>152±29</td>
<td>58±15</td>
<td>0.002</td>
</tr>
<tr>
<td>GLCM Correlation ($R$)</td>
<td>0.24±0.03</td>
<td>0.36±0.05</td>
<td>0.002</td>
</tr>
<tr>
<td>GLCM Energy ($E$)</td>
<td>0.0009±0.0001</td>
<td>0.0022±0.0008</td>
<td>0.002</td>
</tr>
<tr>
<td>GLCM Homogeneity ($H$)</td>
<td>0.18±0.01</td>
<td>0.25±0.02</td>
<td>0.002</td>
</tr>
</tbody>
</table>

be $64 \times 64 \times 20$ voxels ($12.5 \times 12.5 \times 14.0$ mm$^3$). An example of selected VOI is reported in Figure 37. Texture image analysis was then applied on the selected VOI for each patient. The computed descriptors are those reported in section 5.6, i.e. mean $m$, standard deviation $\sigma$, smoothness $S$, uniformity $U$ and entropy $e$ of the gray-level histogram as well as contrast $C$, correlation $R$, energy $E$ and homogeneity $H$ of the gray level co-occurrence matrix (GLCM).

6.3.2 Results and Discussion

Table 14 reports the mean value ± standard deviation of the acquired set of patients for each considered textural descriptors. The $p$-value of the Wilcoxon test between the osteoporotic case and the healthy case is also reported in an attempt to better identify the most suitable subset of parameters. It can be observed that all the GLCM descriptors present $p < 0.05$. Also the histogram-based textural descriptors (except smoothness) seem to be able to capture the differences between the two groups. It seems therefore possible to use textural descriptors as predictors of the fracture risk in osteoporotic subjects.

Further investigations are required to assess the representativeness of the considered VOI both in terms of size and position. In particular, reducing the size of the VOI along the sagittal direction results in a speed up of the total acquisition time since a reduced number of slices have to be acquired. A faster acquisition reduces the risk of motion artifacts and it increases the comfort of the patient. Further investigations will include also an assessment of the bone mineral density directly from the gray-scale images.
Numerical values can strongly help the diagnosis process as they could reduce the risk of false positive and false negative cases. However, this application would greatly benefit of a greater number of considered cases. With enlarged sets of patients it would be possible to perform more effective statistical analysis, thus allowing to better confirm the preliminary results.

6.3.3 Conclusion

Considering the importance of osteoporosis as a social problem, an early diagnosis of this pathological status as well as the monitoring of bone quality evolution is highly desirable. Therefore, reliable methods for the evaluation of the status of the trabecular bone are of great relevance in the diagnosis and monitoring process. High resolution MRI seems to be a promising non-invasive tool for a 3D in vivo characterization of the trabecular bone. Qualitative observations made on a set of healthy women and a set of female volunteers that reported a bone fracture confirmed that the calcaneus is a proper anatomical site. Quantitative measures of physical parameters of the trabecular bone such as the trabecular thickness or spacing are difficult to obtain due to insufficient spatial resolution that makes impossible to perform a reliable segmentation of bone and bone marrow. Texture analysis is then suggested in this application in order to extract numerical indexes able to predict the risk of fracture. Preliminary findings show that some textural descriptors are able to discriminate between healthy and osteoporotic cases.
CONCLUSION

In this thesis approaches for image analysis of 3D tomographic images were presented. In particular, it was shown how to extract reliable quantitative parameters or descriptors directly from $\mu$-CT or MRI images of porous media. Three biomedical applications were also described in which three whole processes “from images to measurements” were shown.

At the end, the crucial question of how much can one trust the computed values arises. The whole process that leads to a quantitative characterization of a sample via imaging and image analysis presents several intermediate choices and trade-offs that surely affect the reliability and reproducibility of the final results. The final results depend on several aspects and some of them include whether the sample to image is adequate and representative for the goals of the experiment, whether the desired characteristics of the sample are detectable by imaging, whether the proper area of the sample is examined and whether the correct images with adequate quality are obtained. An incorrect consideration of these aspects invalidates the whole imaging and image analysis process.

Provided that a representative sample was correctly imaged and the images have a sufficient quality, a characterization of some properties of the sample via image analysis still present crucial aspects. The most crucial ones are the segmentation of the images, the Volume of Interest (VOI) selection and, if necessary, assumptions for the pore space. Again, inaccurate segmentation and inadequate VOI selection invalidates the final computed measurements. Even though strategies for checking the VOI representativeness and the accuracy of the segmentation were suggested in this thesis (in particular in section 6.1), these aspects are still open problems in some applications and user supervision is often required in order to get accurate results.

A cross-check with other techniques (e.g. mercury porosimetry for assessing the porosity of the tissue engineering scaffolds considered in section 6.1) were not reported in the applications included in this thesis, being out of the purposes of the thesis itself. However, other techniques were considered and the results were reported in the publications related to some of the presented applications. For instance, Confocal Laser Scanning Microscopy (CLSM), Raman spectroscopy, X-ray diffraction, mechanical tests and Scanning Electron Microscopy (SEM) were used in [4] for assessing some of the properties (e.g. isotropy and connectivity) that were also assessed via $\mu$-CT image analysis in section 6.1. Also, histomorphometric analysis was performed in [5] to confirm the results presented in section 6.2.
It is therefore allowed to conclude that carefully performed image analysis approaches are attractive tools for several fields of science. In particular, non-destructive tomographic imaging techniques (CT and MRI and the related microscale \(\mu\)-CT and \(\mu\)-MRI) combined with accurate three-dimensional characterizations via image analysis are indeed effective tools for several biomedical applications.
List of Author’s publications produced within the PhD program.

INTERNATIONAL JOURNALS


- F. Brun, A. Travan, A. Accardo, S. Paoletti. Characterization of silver nanoparticles for biomedical applications by means of


**INTERNATIONAL CONFERENCES**


to adult pore organisation. *Coral Reefs*, in publication. (Cited on page 6)


