Visualization of Large-Scale Sections

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Abstract

Overview In this article we present a reliable protocol for the preparation of large-scale sections and an easy strategy for high quality documentation. Our investigation was driven by our personal goal to fuse histology data and computerized tomography scans after radiofrequency ablation treatment. We achieved the first step in this direction by optimizing a protocol for histology sections and documentation suitable for fusion into MicroCT. This technique could also be used for other organ systems.After radiofrequency ablation in pigs, the liver was fixed in situ by perfusion with formalin to keep the organ in shape prior to excision. Liver was trimmed to the area of interest (50x50x30 mm), fixed and embedded in paraffin. Steps of fixation, dehydration and paraffin embedding protocols were carefully optimized. Then whole paraffin blocks were scanned using a MicroCT. Next large-scale serial sections were performed and stained. Sections were scanned in high quality using a commercially available scanner. Further details are available on our project homepage (www.impact.eu, "Image analysis").

Keywords histology, large-scale sections, liver, radiofrequency ablation, high resolution scan

Introduction

Radiological images are very important for medical interventions but in parallel there is often the need for histological data. Therefore fusion of both data sets could help to improve therapeutic intervention protocols.[1,2] Radiofrequency ablation (RFA) is one example of computer tomography (CT) supported treatment. It is minimally invasive and could be used to treat hepatic tumors.[3,4] Optimal RFA treatment in the liver is challenging, because the liver is a complex organ with three different vessel-trees (hepatic artery, portal vein, hepatic vein) and millions of bile ducts. Vessels over three millimeters in diameter remove energy and heat from the area of RFA and deform the suggested spherical/circular form of ablation. This "heat sink effect"[5,6] gives tumor cells the opportunity to survive within this area and tumors may recover.[7] For that reason a prediction of optimal parameters for RFA in liver with focus on the "heat sink effect" would be highly desirable. Pigs have comparable anatomy, physiology, liver and vessel sizes to humans. Therefore, we chose swine as animal models for generation of the required experimental data.

We are involved in the European Union (EU) project Imppact (Image-based Multi-scale Physiological Planning for Ablation Cancer Treatment). One project goal is to set up an automated projection of ideal RFA parameters by computerized calculations based on fusion of data from CT scans and histology. Therefore, an automated detection of the ablation area in histology is needed. Automated detec-

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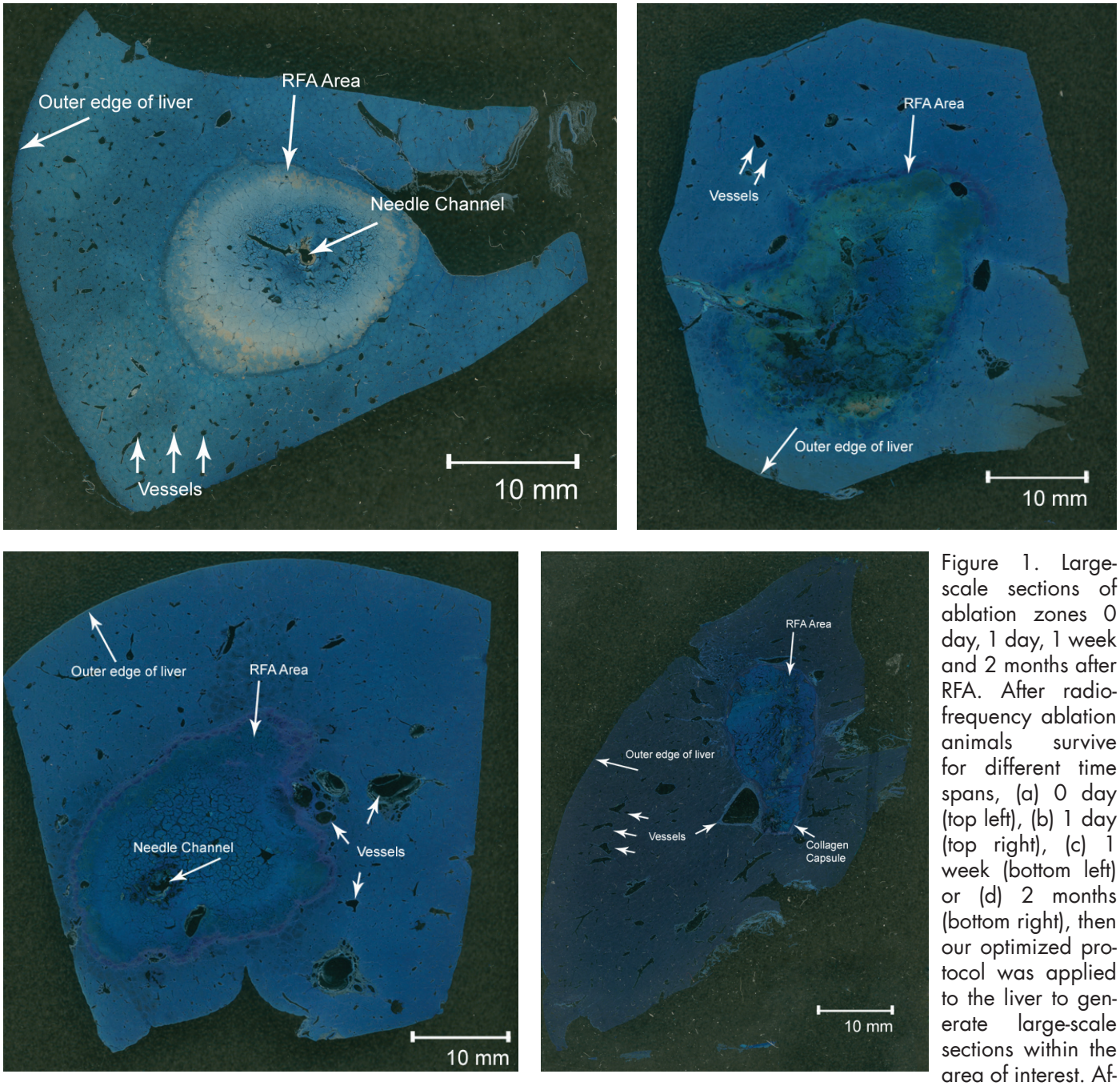

Figure 1. Large-scale sections of ablation zones 0 day, 1 day, 1 week and 2 months after RFA. After radio-frequency ablation animals survive for different time spans, (a) 0 day (top left), (b) 1 day (top right), (c) 1 week (bottom left) or (d) 2 months (bottom right), then our optimized protocol was applied to the liver to generate large-scale sections within the area of interest. After methylene blue staining, sections showed different intensities of blue, according to their condition (dead or alive). Blood cells appeared in yellow ((a), (b)) and collagen fibers in distinct blue (d). For that reason, the RFA area with dead cells could be easily identified in the sections macroscopically. Futhermore the shape of the outer edge of the organ, big vessels (>1 mm in diameter) and the needle channel ((a), (c)) could be recognized. The shape of the RFA area is strongly affected by the surrounding vessels (heat sink effect) as it could be seen in section (b), (c) and (d). In contrast the section in figure (a) shows a nearly circular RFA area because the surrounding tissue is homogeneous and contain no big vessels. While healing the RFA area shrinks and will be surrounded by a collagen capsule (d).

tion is only possible if histology sections and their documentation are in high quality resolution. These aspects are crucial if three dimensional modeling is planned.

In this technical report we present our protocol for acquisition of serial large-scale sections and a quick, inexpensive strategy for high resolution documentation.

Materials and Methods

Animals and Anesthesia

All authorized animal experiments, including medication, anaesthesia and euthanasia, were carried out according to Aus-

trian animal law and were performed by Dr. I. Wiederstein-Grasser (veterinary anesthesiologist).

Radiofrequency Ablation (RFA)

After anesthesia, RFA was performed (RITA Medical System Inc., CA, USA, Model: RITA® StarBurst(TM) XL, Generator: RITA® Model 1500X RF Generator). The electrode was introduced percutaneously under CT-guidance (Aquilion ONE®, Toshiba Medical Systems, Japan) into the liver and the hooks were deployed (fixed in the 3 cm position) as described by the manufacturer. After reaching a target temperature of 100°C at the hook tips constant heat was delivered and applied for seven minutes, after which the hooks and the electrode were retracted.

Organ Fixation with in-situ Perfusion-Fixation

After RFA, a thoracotomy and a median laparotomy were performed. The common iliac arteries were dissected and ligated using 2-0 Vicryl sutures. A third ligation was placed around the abdominal aorta. Furthermore, the vena cava inferior was dissected infrarenally and supradiaphragmatically. Prior to application of heparin (10.000 IU) (Ebewar Pharma, Unterach, Austria), the aorta thoracica was dissected above the diaphragm where an aortic clamp was placed. The iliac arteries were ligated and a canula flushed with 0.9% NaCl was placed into the abdominal aorta and fixed using the prepared ligatures. Then, the vena cava was cut infrarenally and supradiaphragmatically and a sucker was placed in the abdomen. The aorta thoracica was clamped and the abdominal organs were perfused with 200ml/kg body weight cold 0.9% NaCl in order to flush out the remaining blood. In the meantime, organ cooling was performed by rinsing cold (4°C) 0.9% NaCl and crushed sterile 0.9% NaCl into the abdominal cavity. Thereafter, perfusion was continued using 4% formalin 180ml/kg body weight (Sigma-Aldrich, Vienna, Austria) in order to fix the organ in situ. During perfusion fixation of the liver the pigs were euthanatized. For preparation of the liver, the ligamentum hepatoduodenale was prepared and the biliary duct, the portal vein and the hepatic artery were ligated and dissected. Then, the liver was excised from the diaphragm after cutting the vena cava. After mobilization of the right and the left lobe, the liver was removed. Finally, the RFA area was cut out roughly using a scalpel. The tissue block was put into 4% formalin until further processing.

Fixation, Embedding and Sectioning

The perfusion-fixed tissue block was trimmed to the area of interest and was cut into blocks up to 50x50x30 mm. A single RFA area contained one to three tissue blocks. The blocks were then dehydrated: three days 4% formalin, 12 h 70% ethanol, 12 h 90% ethanol, 12 h 96% ethanol, 48 h 100% ethanol, 3 h acetone and 12 h xylol. After dehydration, the blocks were incubated in paraffin with a melting point of 52°C for 24 h. When blocks were soaked with paraffin, embedding in paraffin with a melting point of 56°C was performed. Four steel angles compose the variable model for embedding. Steal angles were a custom product produced in the workshop of the Medical University of Graz (by courtesy of Department of Obstetrics and Gynecology). Next the whole paraffin blocks were scanned using a MicroCT (focal spot size 50 µm, Inveon MicroCT, Siemens Medical Solutions, USA) and the data were stored until use for fusion with histology data. Then paraffin sections were performed at intervals of 100 µm (1-2 µm) and placed on coverslips (76x90x0.13 mm; custom product, Medika, Graz, Austria).

Histological Staining

We tested methylene blue (MB), chromotrope-anilinblue and trichrome staining. MB staining gave the best results for our purpose. Sections were de-paraffinized according to standard protocols. After staining for one minute in 1% MB solution (hospital pharmacy, LKH-Univ. Klinikum Graz, Austria) sections were rinsed with distilled water prior to washing in 100% ethanol, 50% ethanol/ 50% xylol and 100% xylol sequentially. Except for the MB solution, all other solutions were filled into plastic trays (70x110x130 mm). MB solution was applied to the sections using a Pasteur pipette. The sections were dried at room temperature and stayed uncovered. Handle the uncovered stained sections very careful and document them immediately; the RFA affected area tends to drop off the coverslips. Sections need to be uncovered until documentation, because this would cause optical artifacts like Newton´s rings, shades or halos.

Documentation

The stained, dried sections were placed upside down on a digital desk scanner (ScanMaker i800, Microtek, Evestar GmbH., Willich, Germany) with the tissue section directly in contact with the scanner's glass plate. A diffuse reflector (standard tool of scanners) was placed at a distance of 15 mm, to avoid reflection and to combine incidental light and transmitted light for optimal contrast during the scanning procedure. ScanWizard 5 vs.7.22 software for Mac (Microtek, Evestar GmbH., Willich, Germany) was employed at a resolution of 4800 dpi.

Results

Large-Scale Paraffin Sections

The sections provided a large overview of the RFA area and the surrounding tissue at once. The shape of the outer edge of the organ and big vessels could be recognized (natural feature points). This is important for a further fusion of serial section to a three dimensional reconstruction. The RFA area and other details, like the needle channel, could be identified macroscopically. MB staining yielded more intensive blue coloring of viable tissue compared to dead tissue and blood stained

in yellow (figure 1). So a rough distinction between live and dead zones was possible. The contrast of MB staining was acceptable for semi-automated recognition. But the large-scale sections were not suitable to distinguish exactly between dead and living cells.

The scanning procedure was easy and quick and the resolution of the scans was adequate for further digital use. For additional information concerning the successful three dimensional reconstructions and fusion into MicroCT scans done by our partners please visit our project homepage (www.imppact.eu).

Discussion

Fusion of radiology and histology data could help to optimize therapeutic intervention protocols. To fuse these data sets stringent requirements were set on histology data and their documentation quality. Working on RFA effects in liver we aimed to solve this problem.

To obtain a good morphology in soft tissue like in liver, we had to use perfusion fixation in situ. Hereby, the organ was flushed with rinsing buffer, to get rid of blood, and the vessels were used to deliver the fixative (formalin) to the organ (perfusion fixation). This method ensures the retention of the shape of the organ within the body and while being dissected for further fixation and analysis. To minimize the number of sections and to enlarge the overview we used the technique of large-scale section histology. This method is often used for histopathological examination of cancer, particularly in the field of gynecological and obstetrics.[8,9] Our procedure of optimization was influenced by the work of other groups, performing RFA in pigs. They measured or described the ablation area and according to their needs used different protocols to handle the tissue.[5,6,10]

For optimal documentation the uncovered, stained and dried sections (on coverslips) were directly put on a commercial available scanner. This inexpensive procedure provides scans in high resolution and minimal background.

In this technical report we present our optimized protocol to combine in situ perfusion fixation, large-scale sectioning and an easy documentation strategy for further digital processing. We use our protocol for successful three dimensional reconstruction and fusion of histology data with MicroCT scans (www.imppact.eu). Therefore we are convinced that our protocol could also be suitable for similar studies in other organs.

Disclosure

The manuscript has been approved by all of the authors and all authors state no conflict of interest.

SK and PS participated in the design of the work and wrote the manuscript. RHP performed the RFA. UM, BL, DB and OR conducted and supported the experiments concerning the fixation and embedding strategies. BK supported the documentation process. IWG (veterinarian, anesthetic management) supported the work with the animals. SK, PS, VS interpreted the data. KHT reviewed the manuscript.

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