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Profile analysis of hepatic porcine and murine brain tissue slices obtained with a vibratome

This study is aimed at characterizing soft tissue slices using a vibratome. In particular, the effect of two sectioning parameters (i.e. step size and sectioning speed) on resultant slice thickness was investigated for fresh porcine liver as well as for paraformaldehyde-fixed (PFA-fixed) and fresh murine brain. A simple framework for embedding, sectioning and imaging the slices was established to derive their thickness, which was evaluated through a purposely developed graphical user interface. Sectioning speed and step size had little effect on the thickness of fresh liver slices. Conversely, the thickness of PFA-fixed murine brain slices was found to be dependent on the step size, but not on the sectioning speed. In view of these results, fresh brain tissue was sliced varying the step size only, which was found to have a significant effect on resultant slice thickness. Although precision-cut slices (i.e. with regular thickness) were obtained for all the tissues, slice accuracy (defined as the match between the nominal step size chosen and the actual slice thickness obtained) was found to increase with tissue stiffness from fresh liver to PFA-fixed brain. This quantitative investigation can be very helpful for establishing the most suitable slicing setup for a given tissue.

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Profile analysis of hepatic porcine and murine brain tissue slices obtained with a vibratome

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1. Introduction

Vibrating blade microtomes or vibratomes are commonly used for obtaining precision-cut slices from soft fresh tissues. Unlike classical sectioning procedures based on the use of microtomes, vibratome slicing does not require any tissue fixation, dehydration and embedding thus cell viability and native tissue structure are conserved. Fresh tissue slices are suitable candidates for in-vitro tissue models (Parrish, Gandolfi & Brendel, 1995; Parrish et al., 2002; van de Bovenkamp et al., 2005; Groothuis & de Graaf, 2013) as well as for structural and morphometric analysis (Karim et al., 2013; Eide et al., 2013). For instance, precision-cut liver slices are powerful tools for the in-vitro study of pharmacological metabolism, toxicology and efficacy of novel substances under standardized conditions (van de Bovenkamp et al., 2005, 2006; Van de Bovenkamp et al., 2007; Karim et al., 2013; Eide et al., 2013). They have been used extensively for rank-ordering the toxicity of chemicals and examining the mechanisms of liver injury as well as for investigating the induction of cytochrome P-450 enzymes and the expression of stress proteins or peroxisomal enzymes, thus offering a valuable bridge between in-vivo and cell culture systems (Gandolfi, Wijeweera & Brendel, 1996; Olinga & Schuppan, 2013). Brain tissue slices, on the other hand, are attractive for the evaluation of different morphometric features such as the total extent of dendrites and the number of branching points, as well as for 3D tissue reconstruction and analysis of neurons (Jin et al., 2003; Billeci et al., 2013; Golovyashkina et al., 2014).

Despite the widespread use of vibratomes for obtaining live tissue sections, it difficult to find standard preparation protocols or consolidated methods to determine sectioning parameters for generating precision cut slices for a desired application. Thus the purpose of this work was to establish an experimental framework for rapid generation and characterization of precision cut slices without having to resort to lengthy trial and error and tissue wastage. We analyzed tissue

slices from fresh porcine liver and from both PFA-fixed and fresh murine brain. The samples were obtained by varying the two main sectioning parameters, i.e. step size and sectioning speed, to investigate their effect on resultant slice thickness. The other sectioning parameters (i.e. oscillation amplitude and blade angle) were kept constant. To characterize their actual thickness, tissue slices were embedded in agarose gels and sectioned perpendicularly to their surface. The transverse sections were then imaged with an optical microscope and analyzed with a purposely-developed Graphical User Interface (GUI) for semi-automated evaluation of slice thickness.

2. Materials and methods

2.1. Sample preparation and slicing setup

Fresh porcine hepatic tissue was collected from 1 year old healthy pigs and cut into 1.5 × 0.5 × 0.5 cm^3 samples, avoiding the Glisson's capsule and macroscopic vasculature. The tissue was obtained from a local abattoir, as a slaughter by-product. Fresh murine brains were collected from n = 2 three month old mice which were deeply anesthetized by intraperitoneal injection of chloral hydrate (400 mg/kg) and then perfused through the left ventricle with 50 mL of 10 mM phosphate buffered saline (PBS 1×, Sigma-Aldrich, Milan, Italy). PFA-fixed brains were obtained from n = 2 three month old mice treated as described for fresh tissue and then perfused with 200 mL of 4 % paraformaldehyde (PFA, pH 7.4) fixative solution prepared in 0.1 M PBS (Sigma-Aldrich, Milan, Italy). Murine brains were cut along to their sagittal plane, obtaining two symmetrical samples. Mouse perfusion was performed at the Department of Translational Research New Technologies in Medicine and Surgery of the University of Pisa. Experiments were conducted in conformity with the European Communities Council Directive of 24 November 1986 (86/609/EEC and 2010/63/UE) and in agreement with the Italian DM26/14. Experiments were approved by the Italian Ministry of Health and Ethic Committee of the University of Pisa.

A Leica VT1200 S vibratome (Leica Microsystems, Nussloch, Germany) was used to obtain tissue slices. Each sample was fixed with superglue onto specimen plates and then cut using a stainless steel razor blade (Gillette, Milan, Italy), under buffered conditions with ice-cold PBS 1×. The following cutting settings were used: blade angle, 18°; oscillation amplitude, 3 mm for liver, 1.5 mm for brain; sectioning speed, 0.1, 0.2 and 0.4 mm/s for liver, 0.05 and 0.2 mm/s for PFA-fixed brain, step size, 100, 200 and 400 µm. In the case of fresh brain only one sectioning speed (0.2 mm/s) was used on the basis of the results obtained from fixed tissue.

2.2. Thickness evaluation

The slices obtained were immediately embedded in a 1 % w/v agarose gel (A9539, Sigma-Aldrich, Milan, Italy) prepared in deionized water (Fig. 1). The slice-containing agarose gel, which was formed at room temperature via thermal gelation, allowed an easy and quick embedding of hydrated slices. Notably, this procedure does not require any slice fixation and dehydration unlike classical paraffin embedding. As a consequence there is no tissue collapse or shrinking, hence permitting the evaluation of the actual slice thickness in the hydrated state.

To enhance contrast, brain tissue slices were stained with haematoxylin (Sigma-Aldrich, Milan, Italy) prior to embedding (Fig. 1B). Agarose-embedded slices were cut perpendicularly to their surface using a custom slicer and a microtome blade. The cross-sections obtained were placed onto a glass slide and imaged with an Olympus IX81 optical microscope (Olympus, Milan, Italy) at 1.25× magnification. Acquired images were processed with a purposely-developed software implemented in Matlab® (The Mathworks Inc., Natick, MA, USA), named STEGUN (after the mathematician Irene Stegun, the name also stands for “Slice Thickness Evaluation GUi for Non-expert users”, Fig. 2). The software is available for download at

http://www.centropiaggio.unipi.it/software. STEGUN's simple Graphical User Interface (GUI) allows a semi-automated evaluation of the slice thickness through the following steps:

1. Set the experimental pixel size (μm/pixel) for image analysis;
2. Load the image. At this point STEGUN automatically returns the binary image to the user who has to select the object representing the slice using the cursor and mouse key;
3. Rotate the image to vertically align the object, if needed;
4. Crop three different segments of the object.

In particular, the loaded image is binarized through a thresholding algorithm, then the pixel values are inverted to obtain a white object representing the slice (pixel level = 1) in a black background (pixel level = 0). For each of the three crops, the slice thickness is automatically evaluated by summing the pixel values and normalizing the result by the number of pixel rows of the crop. This it is multiplied by the pixel size to obtain the slice thickness in microns. STEGUN stores all the data in a data matrix and displays the result as the mean value ± standard deviation. In case of highly irregular slices (i.e. when the coefficient of variation, calculated as the ratio of the standard deviation to the mean value, is greater than 0.25) a warning message is returned to the user.

2.3. Data analysis

At least 6 independent tissue slices were analyzed for each step size and sectioning speed investigated. Results are reported as the mean ± standard deviation, unless otherwise noted. For both porcine liver and PFA-fixed murine brain, the statistical significance between slice thicknesses obtained varying two different sectioning parameters (i.e. step size and sectioning speed) was analyzed using two-way ANOVA. Slice thickness for fresh murine brain was analyzed with one-way ANOVA, since only the slicing step size was varied, keeping the

sectioning speed at a constant value. Statistical analysis was implemented in OriginPro 9.0 (OriginLab Corporation, Northampton, USA). Differences were considered significant at $p < 0.05$.

3. Results and Discussion

3.1. Fresh porcine liver

We were unable to obtain slices using the 100 μm step size because the blade tended to deform and scrape over the tissue rather than cut it, regardless of the sectioning speed. This is likely due to the very labile and floppy nature of fresh liver (Mattei et al., 2014). Although the two-way ANOVA analysis showed that both the step size and sectioning speed have a significant effect on the resultant thickness of fresh liver slices, 4 of the 6 step size-sectioning speed combinations investigated (specifically 200-0.1, 200-0.4, 400-0.1 and 400-0.2 μm-mm/s) yielded similar slice thicknesses, with an average value of 540 ± 91 μm (Fig. 3A). Moreover, the interaction between the two factors (i.e. step size and sectioning speed) was not found to be significant: lines in Fig. 3B exhibit the same trend versus the sectioning speed, regardless of the step size.

Overall, although precision-cut liver slices (i.e. with regular and reproducible thickness) were obtained, the slice accuracy (here defined as the match between the nominal step size chosen and the resultant slice thickness obtained) was poor. In particular, the slice thickness was found to be consistently higher than the nominal set step-size selected for slicing the tissue likely because the blade deforms the very floppy and compliant fresh hepatic tissue as it cuts.

3.2. Murine brain

3.2.1. PFA-fixed brain

Two-way ANOVA analysis indicates that the thickness of PFA-fixed brain slices depends on the step size only, yielding 3 different groups of slice thicknesses, regardless of the sectioning speed (Fig. 4A). As observed for fresh liver, the interaction between the step size and the sectioning speed was not significant (Fig. 4B).

The thickness of PFA-fixed brain slices matched the nominal step size quite well. This tissue is in fact stiffer than the fresh liver, and hence easier to accurately cut in thin regular slices without significant deformation.

3.2.2. Fresh brain

No slices were obtained using the 100 μm step size, once again because of the floppy and compliant nature of non-fixed soft tissues. One-way ANOVA analysis showed that the resultant thickness of fresh brain slices depends significantly on the step size chosen, with a good match between the two (Fig. 5). These results suggest that, although more compliant than the PFA-fixed tissue, fresh brain is stiff enough to be accurately cut in thin regular slices, unlike the liver.

4. Conclusions

Precision-cut tissue slices from fresh porcine liver as well as from PFA-fixed and fresh murine brain were characterized in their thickness through a semi-automated slice analysis GUI implemented in Matlab®. Although precision-cut slices (i.e. with regular and repeatable thickness) were obtained for all the tissues investigated, the results presented in this work suggest that the match between the nominal step size chosen and the actual slice thickness obtained (i.e. slice accuracy) increases with tissue stiffness, from fresh liver to PFA-fixed brain.

In conclusion, given the potential benefits and advantages of precision-cut slices in many biological and biomedical engineering applications, the quantitative evaluation of the effects of the vibratome's sectioning parameters on resultant thickness performed in this work can be very helpful for establishing the most suitable slicing setup for a given tissue.

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Figure 1

Agarose-embedded tissue slices.

(A) Fresh porcine liver. (B) PFA-fixed murine brain.

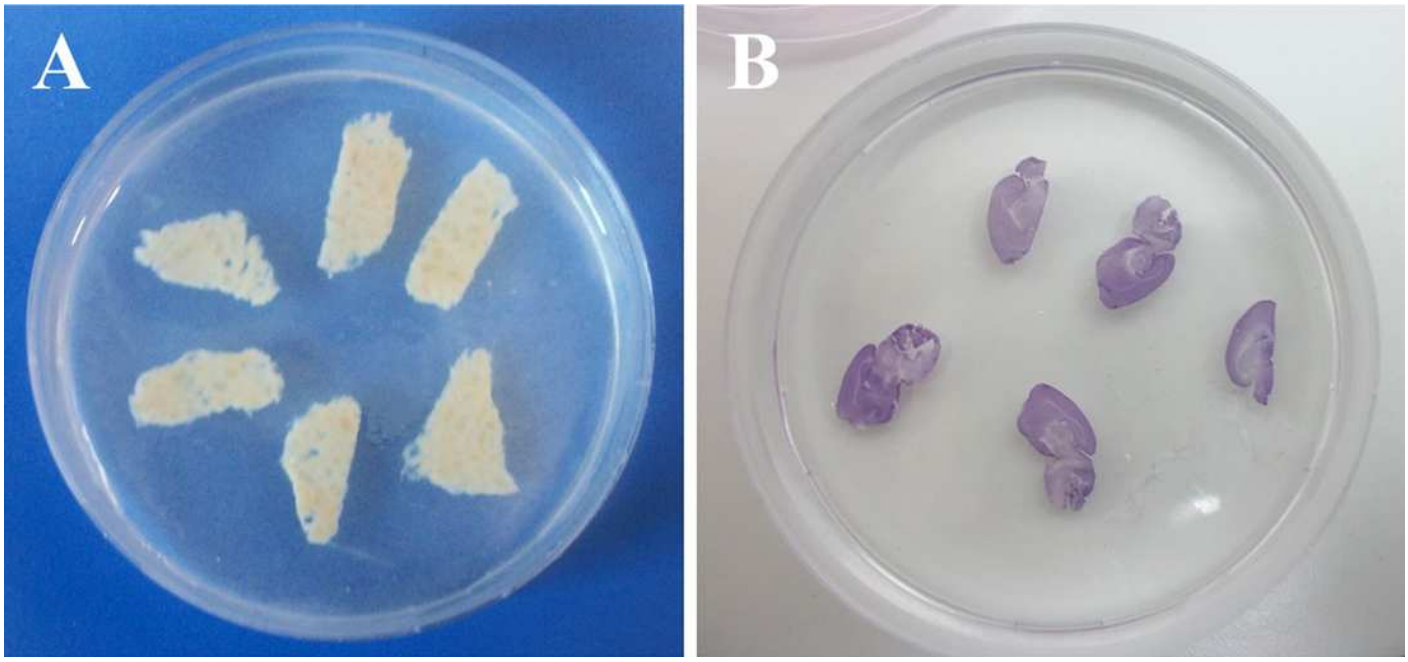

Figure 2

STEGUN: a semi-automated GUI to evaluate slice thickness.

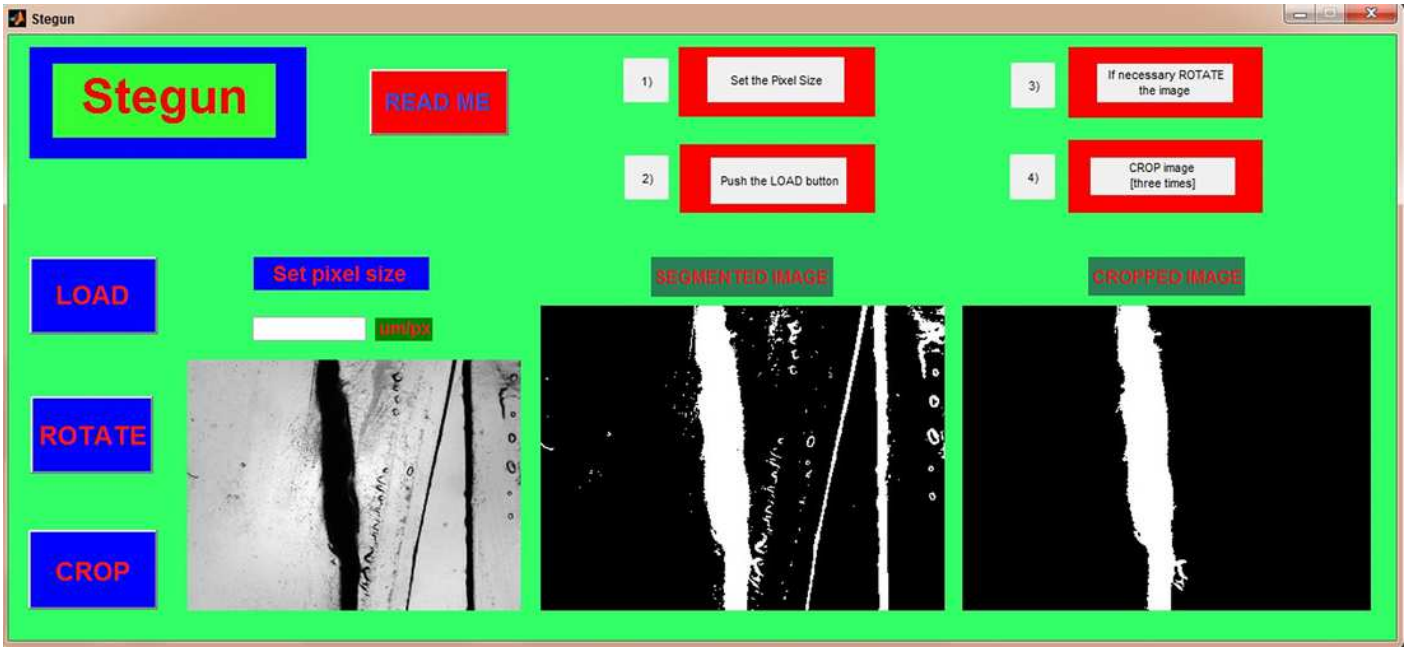

Figure 3

Fresh liver slice thicknesses.

(A) Bar plot: different letters indicate significant differences between samples ($p < 0.05$). Different bar fillings indicate different step sizes. (B) Two-way ANOVA interaction plot: the interaction between the step size and the sectioning speed is not significant.

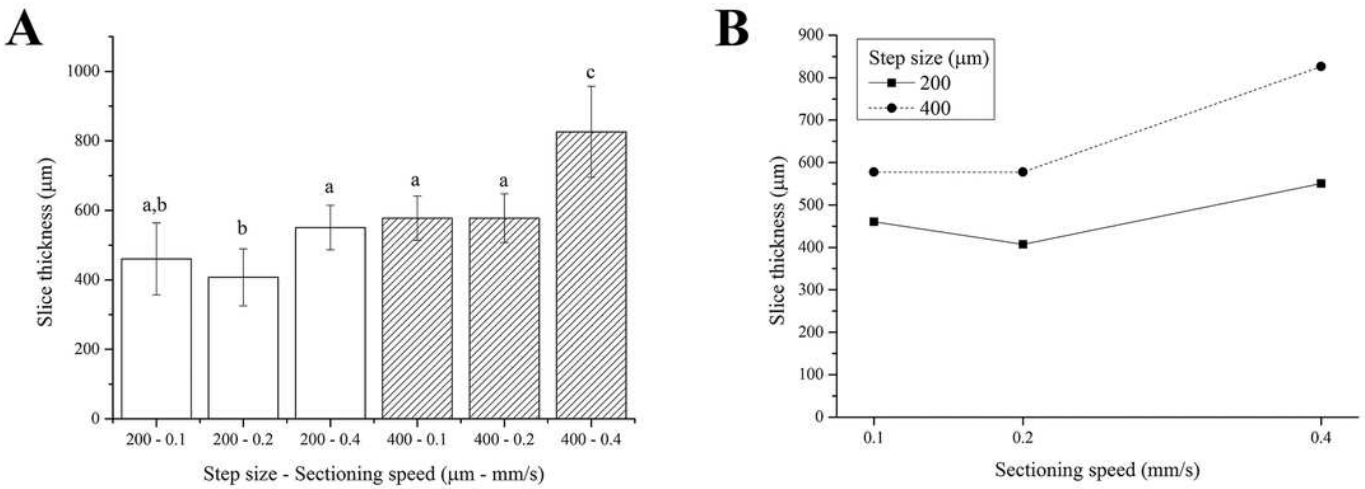

Figure 4

PFA-fixed brain slice thicknesses.

(A) Bar plot: different letters indicate significant differences between samples ($p < 0.05$). Different bar fillings indicate different step sizes. **(**B) Two–way ANOVA interaction plot: the interaction between the step size and the sectioning speed is not significant.

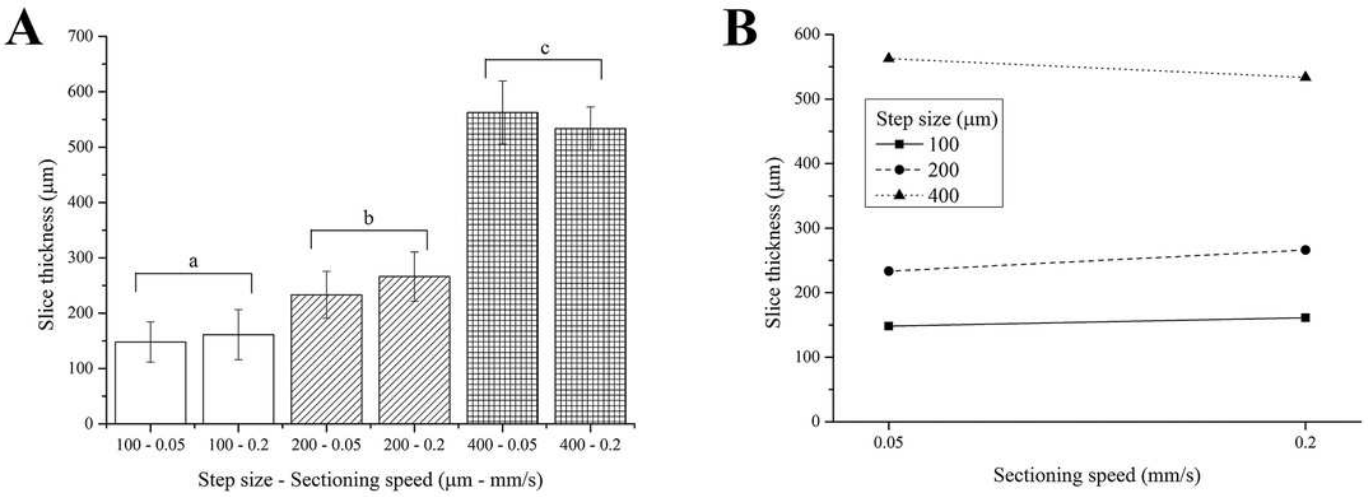

Figure 5

Fresh brain slice thicknesses.

Bar plot: different letters indicate significant differences between samples ($p < 0.05$).

Different bar fillings indicate different step sizes.