Appendix I: Summary of unpublished work mentioned in the thesis

1.1 qPCR tomography combined with laser microdissection

In order to further develop qPCR tomography, which previously demonstrated ability to describe gene expression profile together with the localization of mRNA in a subcellular resolution, we complemented the method with the trait of visual information of the studied object and the option to select specific cells for downstream analysis. Therefore, the aim was to develop a method which can be widely applied to study cell types and individual cells in time and space with minimized contribution of the neighbor tissue.

To create a spatiotemporal map of gene expression, we selected relatively simple and still very interesting model of embryonic development of mouse molar, provided in cooperation with the Department of Teratology, Institute of Experimental Medicine AS CR. Mouse has a reduced dentition consisting of one incisor and three molars in each quadrant, which are separated by a gap (diastema). The formation of teeth is a result of cell signaling between epithelium and mesenchyme, which is highly conserved among vertebrates and is also responsible for controlling morphogenic patterns in other developmental processes (Cobourne and Sharpe 2010). The understanding of teeth formation is therefore applicable to humans and should accelerate the development of therapeutic tooth engineering. For example mice do not have deciduous teeth, except for rudimentary diastema teeth. The front incisors grow continuously, which represents a useful model for studying differentiation of stem cells. Additional reason for choosing this model is a good level of current knowledge, which allows us to correlate our novel method with morphological data and studies based on *in situ* hybridization (ISH).

The development of mouse tooth progresses through a series of well-defined phases: thickening of the epithelia, bud, cap and bell. The thickening of the oral epithelium occurs in E11,5 (day of embryonic development) and is characterized by expression of *Shh*, which triggers cell proliferation in the region of tooth formation. Furthermore, the epithelium invaginates into the adjacent mesenchyme derived from the neural crest and forms a bud. According to generally accepted theory, before E12 the signaling center for tooth development is located in the epithelium, but after E12 this role is operated by mezenchymal cells, which start to condense around the newly forming invaginating bud. In E13,5 the top of tooth bud flattens and gets surrounded by a condensed dental mesenchyme typically expressing transcription factors *Bmp4* , *Msx1* , *Pax9* and *Wnt*. Upon receiving this expression signal the forefront of the shaping bud modulates an enamel knot, which expresses other key

factors *Shh, Fgf4, Bmp4* and *Wnt100b*. Bud stage then transforms into a cap stage (Caton and Tucker 2009).

For developing qPCR tomography combined with laser microdissection, we selected dental primordium of the first lower molar (M1) in cap stage with its distinctive signaling center represented by the enamel knot. The protocol for laser microdissection (Figure 1) was compiled from the scientific and product literature available in years 2008-2009 (Espina , Wulfkuhle et al. 2006; Espina , Heib et al . 2007, Frumkin , Wasserstrom et al . 2008) with the focus on the new application which uses serial sections, requires intact histology together with high quality RNA and efficient purification of large number of minute samples. In parallel, we developed a method for robust validation of qPCR assays in terms of specificity, sensitivity and efficiency for demanding applications using samples with very low concentrations of RNA, in fact up to the single cell level. Validation of 30 qPCR assays (=pairs of primers) was performed using a template that is most similar to target molecules: mouse cDNA and analysis of five-step five -fold dilution series constructed using four qPCR technical replicates per concentration, together with a control for genomic DNA and no template control. The validation was performed using reagents based on SYBR Green detection to be able to run the melt curve analysis. In order to verify the correct size of PCR fragment we used agarose electrophoresis.

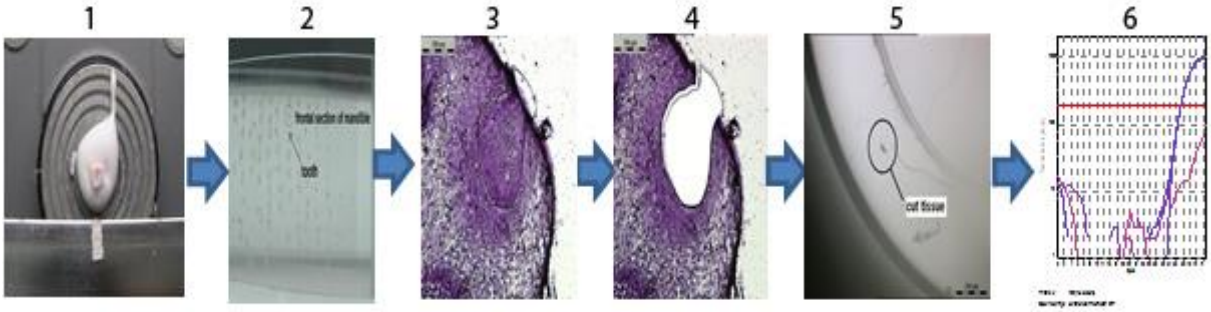

Figure 1: Protocol for qPCR tomography combined with LMD. From left: 1) snap frozen tissue is mounted in cryo-medium and cut into 20 µm serial sections. 2) sections are sequentially anchored to UV labile membrane by room temp. and fixed using 20% ammonium sulfate solution, hematoxylin 1 min, then washed using ethanol series 70% 1 min and 98% 1 min. 3) and 4) selection and cut of the epithelial tooth primordia M1 in the bud stage using Leica LMD system LMD6000. 5) Confirmation of the presence of excised tissue, adding 70 ml of lysis buffer RLT and purification using RNeasy Micro kit (Qiagen). 6) Quantification of transcripts using RT-qPCR (unpublished data).

As previously mentioned in the introduction to the LMD, the critical step is fixation of tissue, in our case serial cryo-sections, which affects the quality and yield of RNA. The quality of RNA in tissue sections was tested using microfluidic electrophoresis system Biorad Experion and after tedious optimization, we found 20% ammonium sulphate solution (component of RNA later) achieved RQI (RNA Quality Indicator) values from 8.5 to 9.5 for sections mounted on the UV-labile membrane in the range of 0-15 minutes, respectively.

The result is a preliminary map of gene expression in the dental epithelium (Figure 2), at E13.5, when the primordium of M1 is in transition to dental cap. We were able to monitor increased expression of one of the key growth factors *Fgf4* in the formation of the enamel knot (Thesleff, Keranen et al. 2001) in correlation with other key signaling molecules *Shh* and *Edar*. As a novel observation we found occurrence of *FGF8*, which was previously detected at the latest stage of formation of bud using in situ hybridization (ISH), when it gets suppressed (Kettunen and Thesleff 1998).

The project was paused due to high technical and time requirements needed to increase the resolution and reproducibility. The higher resolution down to single cell level seem to require more efficient fixation and RNA retrieval. For the description of new correlations between genes it is necessary to carry out a studies on more stages of development, for example when secondary enamel knots develop and the formation of tooth cusps is controlled by *Fgf4* and *Fgf9*. Successful application of molecular tomography is expected to accelerate understanding of the context of phenotype and transcriptome and find new targets for therapy including tooth engineering.

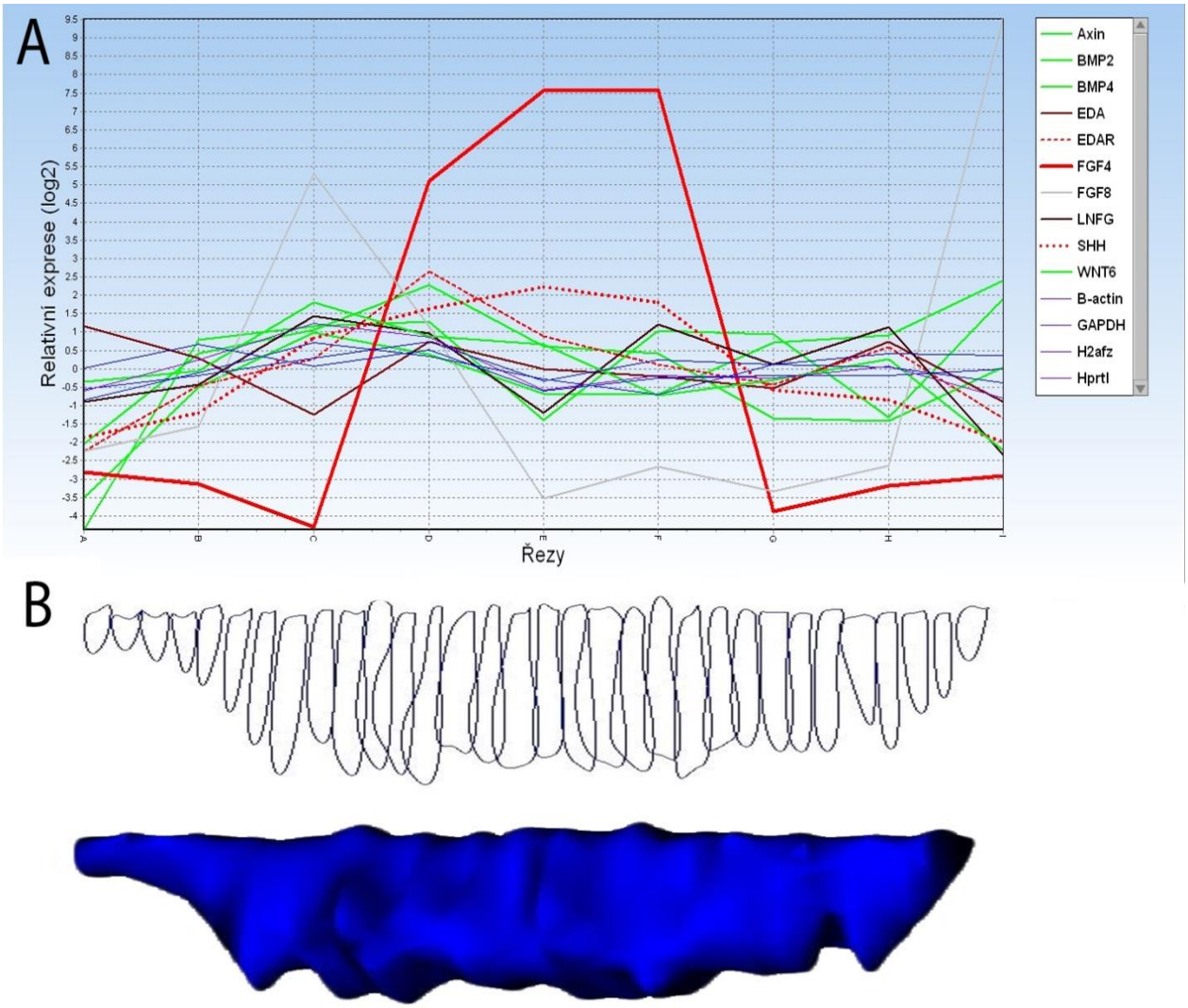

Figure 2: The map of gene expression of mouse tooth E13.5 during transition to dental cap constructed using qPCR tomography. (A) The level of mRNA of transcription factors, signaling molecules, receptors, growth factors and reference genes in the frontal plane (frontal sections A; posterior I). GREEN represents group of genes, where mRNA was detected evenly across the primordium using ISH (Kaski 1996), RED represents genes detected mainly in the middle of the enamel knot and BLACK are genes expressed mostly outside the enamel knot. In addition, the expression of genes which were not detected using ISH at this stage of development is represented by GRAY. On the other hand BLUE shows the group of reference genes. We used 18S for normalization. (B) Anterior-posterior 3D reconstruction of dental epithelium viewed from the side. The model was created using Reconstrukt software and is based on compiling contours of serial sections obtained during microdissection (unpublished data).