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Single cell gene expression profiling and quality control

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Single cell gene expression profiling and quality control

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Abstract:

Gene expression profiling has become an exceedingly important tool for describing occurrence of mRNA in tissue samples and even single cells. Most often we use it for characterization of cell types, degree of differentiation and pathology on a molecular level. In our newly established laboratory, we developed high resolution qPCR tomography to show distribution of tens of maternal mRNAs within a single oocyte. We demonstrated that distribution of mRNAs has an important role in further development of the organism. For high resolution qPCR tomography, where one oocyte is divided in tens of samples and about fifty genes are studied in each sample, we optimized dye based protocol for microfluidic high-throughput platform BioMark. Next step was complementing the molecular profile of tens most important genes with information about histology of each selected tissue section using laser microdissection. As a model we used embryonic development of mouse molar. Our goal was to describe interaction of up to one hundred genes in different stages of development and on the single cell level. This work also reviews development of molecular tools for testing samples for contamination, genomic background and RNA quality. Use of such tools enhances development of new analytical approaches and shows to be crucial quality control for challenging studies of gene expression in time and space not only on the single cell level. Such studies are expected to accelerate understanding of cell regulation and to find new molecular targets for therapeutic use.

Keywords: real time PCR, single-cell biology, single-cell gene expression, gene expression profiling, map of gene expression, qPCR tomography, high-throughput qPCR, quality control, RNA spike, DNA spike, genomic background, direct cell lysis

Contents

1 Introduction

Gene expression profiling is an exceedingly important tool [1, 2], for obtaining information about occurrence of different molecular signatures in the biological sample or even in the individual single cells. The tool facilitates understanding of basic cellular processes, pathology diagnostics and often reveals new targets for therapy. Normally, such studies are conducted on tissue samples composed of thousands of cells. The recent trend in the field is heading from studying pools of thousands cell towards less complex single cells. The level of complexity of the studied samples strongly correlates with the type of information, which can be extracted. Studies performed on the single cell level identify previously unknown subpopulations of cells and can define new mechanisms of regulations and cell signaling [3].

The most common techniques for gene expression analysis on the RNA level are represented by microarrays, quantitative PCR (qPCR), next-generation sequencing and in situ hybridization together with in vivo imaging. Each of these methods has its own limits and advantages and it is not always possible to do data comparisons. Biological processes in the multicellular organisms are highly complex and the current state of technology allows studying only one or two traits of information per sample. For example, in vivo methods using microscopy and fluorescent dyes provide unique information about the localization of several mRNAs in parallel, however the context of more genes in the particular location and time is lost. We selected qPCR as it can combine the advantages of highly accurate quantification of hundreds of genes from a single cell with the location of particular cell or even cell compartment. However for such challenging applications it is usually necessary to maintain high quality of RNA and preserve the histology of tissue after being collected. The first step of our laboratory towards mapping gene expression in time and space was the development of subcellular qPCR tomography for studying *Xenopus laevis* oocytes in 2008[4].

Further development of qPCR tomography required generating tissue sections with higher resolution and quantifying more genes of interest. Analyzing tens of genes in tens of samples required setting up the high-throughput platform BioMark, which initially did not support our dye based assays and required optimization of reagents as well as optics adjustments.

To determine quantity and regulation relationships of hundred different mRNAs in context of time and space, our next goal was to combine qPCR tomography with microscopic laser dissection, which contributes with the information about the morphology of cells and allows

crucial selection of the studied object, optimally up to the level of individual cells. As a model we used the embryonic development of mouse molar. The minute size of molar tissue sections and low abundance of transcripts suggested optimization of recent protocols. Resolving these problems required the development of new tools for quality control of pre-analytical phase of the experiment in regards to inhibition, yield and degradation of mRNA.

2   Goals

The main goals of the work were following:

- Lay basic grounds for studies of gene expression profiling in single cells in the newly established laboratory of gene expression and use new techniques to discover and explain occurrence of mRNA in the context of cells, tissue and time with relevance to cellular events and the whole organism

- Develop high resolution qPCR tomography to study distribution of mRNA at the subcellular level using *Xenopus laevis* oocytes as a model of development

- Complement qPCR tomography with microscopic imaging and the option to select the cells of interest using laser microdisection in order to construct first spatio-temporal map of mRNA distribution in a developing mouse tooth

- Within the academic and corporate environments develop tools based on qPCR, which are suitable for validating purity of samples, purification yields, mRNA integrity and are expected to strongly facilitate development of new protocols

- Within the corporate environment find a way to efficiently lyse single cells and minute samples without purification, with respect to mRNA stability and compatibility with downstream molecular methods

3 Literature review

3.1 Why is single cell gene expression profiling interesting

After sequencing the most interesting genomes (human in 2001, mouse 2002 rat 2004) a number of techniques emerged which allows the analysis of molecular profiles of different cell processes related to the development of an organism, cell repair and differentiation or cancer. No matter which –omics techniques is used, it is common to use samples consisting of thousands or billions of cells of various types, where each cell contributes with its information to the global average. Such approach is suitable only for several applications such as comparing production of genetically modified bacteria, where a major population represents an indicator of production efficiency. However, in the studies of complex tissues, which are based on synchronized cooperation between cell types, the information about minor populations is lost and therefore the complete interpretation of the network behind pathology is lost. One example is the identification of rare cancer stem cells in the tumor [5-7], or interaction between neurons, astrocytes, microglia and oligodendrocytes in spinal cord injury [8-10], or function of α, β and γ cells in pancreas during diabetes (Figure 1)[11].
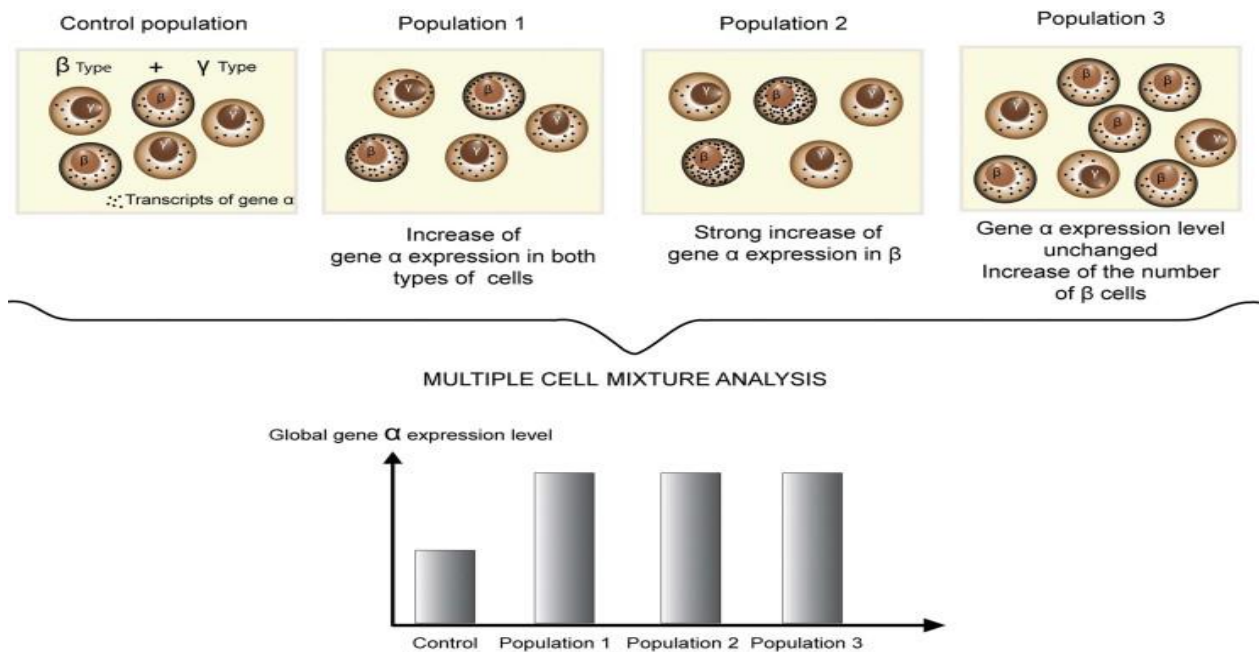

Figure 1: Limited information associated with the analysis of a mixture of cells. If an increased expression of gene α is detected in a mixture of β and γ cells compared to the control population, it can either be explained by (1) the increase in all cells, (2) increased expression in only one cell type, or (3) the proliferation of cells that express the gene. Adapted from [12].

The confounding effect on mRNA distribution associated with analysis of heterogeneous samples is removed in single cell analysis. First single cell studies were published in 2002 [13] and showed that even seemingly homogeneous populations of cells have high variability in the amount of mRNA present in particular cells [13-19]. Such variation can be very well described by log normal distribution of mRNA, where most of the transcripts is located in only few cells, while most cells is not transcriptionally active and contain very little of transcripts (Figure 2) [20, 21]), corresponding to the theory of stochastic gene regulation and transcriptional bursts.
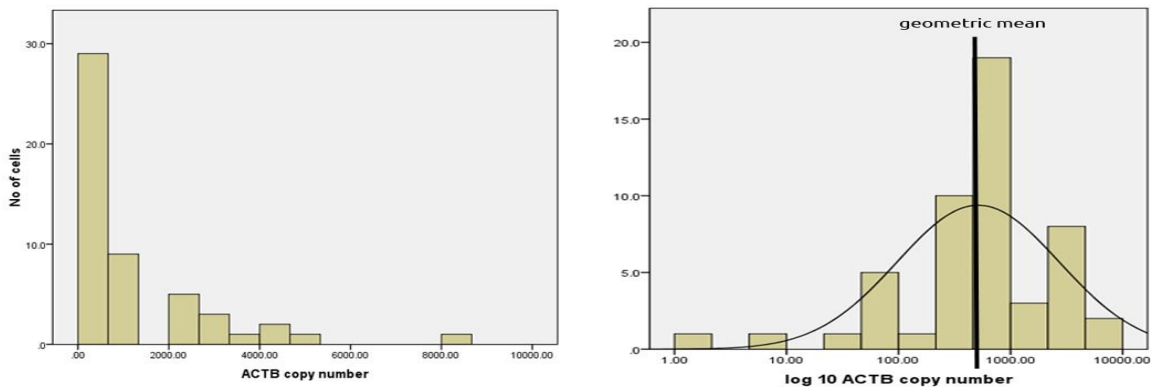

Figure 2: An example of typical log-normal distribution of mRNA for beta-actin in 96 astrocytes from primary culture. On the left we show linear scale and a logarithmic scale on the right with highlighted geometric average characterizing a typical cell of a given population.

The determination of an "average" cell per population is performed by modeling of log-normal distribution of mRNA and by geometric mean. The average cell is a useful characteristic of each population and allows its differentiation from other populations in seemingly identical cells (Figure 2)[22]. The number of recent publications demonstrates the advantages of single cell approach, for example the identification and characterization of strongly minor subpopulations of stem cells responsible for tissue repair [5, 8, 9, 23-27]. New findings open up possibilities for further understanding of the complex regulatory processes of signaling and cell differentiation.

Furthermore the coexistence of large number of transcripts of two or more genes in a single cell at the same time indicates their correlating relationship. Such information suggests fundamental context of regulatory pathways. With the use of modeling we can deduct whether the gene X activates or inhibits expression of the gene Y, or both regulate gene Z. For example, if the maximum of gene X expression is detected before reaching the maximum expression of the

gene Y, we can conclude that the gene X activates gene Y . When the peaks are detected at the same time, they are likely to share common trigger element.

Although the correlation between different genes within mRNA or protein level may clarify regulatory relationships, the transcribed mRNA level do not always correlate with the amount of translated protein within one gene. Few studies show that there is large heterogeneity also on protein level in single cells [28, 29]. In the yeasts non correlating transcripts generate protein subunits for highly synchronized complexes of proteosom and RNA polymerase II [30]. Using E. coli, it was shown that it is more likely to observe low correlation of mRNA and protein, suggesting the presence of separate regulation systems [31].

Simple examples of what benefits such variability may bring for survival of a given cell phenotype can give bacteria and its response to the antibiotic treatment, which at a certain dose eliminates most of bacteria, but some survive. Or using analogy of chemotherapy on cancer cells, different levels of different proteins give a population ability to survive wider range of conditions, independently on the genotype[32]. Such variability and later cumulation of oncogenic mutation shows similar trends as a process known as Darwin´s evolution [33, 34].

3.2 Methods used for cell collection in single cell gene expression profiling

Single cells can be obtained from most tissues by dissociation and subsequent enrichment for the studied types using FACS or microaspiration. However in this process, the cell localization and context with the environment is normally lost. One way how to avoid loss of information is laser microdissection. In general, purification of mRNA from single cells and samples containing up to few hundred of cells is preferably done using direct lysis with no washing steps in order to minimize the loss of material and potential bias.

3.2.1 FACS

Flow cytometry associated with sorting of cells on has been developed since 1970th to automate the sorting of individual cells based on the selected, mostly surface markers. It is in fact the oldest technique for studying single cell profiles. The principle is based on loading cell suspension in a thin laminar flow of liquid which passes through the excitation source (laser) and a detector. The system in real time analyzes the selected parameters such as nonspecific signs of viability, cell surface markers, DNA content or cell cycle. The indisputable advantage of the FACS is cell viability after sorting, meaning that cells can be enriched and cultured or directed to

the wells of 96 microtiter plate and lysed directly for further molecular analysis [35]. FACS is nowadays indispensable tool for high-throughput analysis of individual cells by qPCR, microarray or sequencing.

Among disadvantages is the need to dissociate tissue in cell suspension, loosing important information about the localization of cells, often also causing change of gene expression profile and loss of large amounts of cells. Operating the instrument itself and making the cell permeable for cytoplasmic targets usually requires considerable experience. Considerable factor is the cost for purchasing and running the FACS instrument, along with the cost for antibodies.

3.2.2 Laser microdissection

Laser microdissection (LMD) [36, 37] combines the advantages of *in situ* localization of with the analysis of transcriptome, or proteins using any of recent molecular techniques. After fixing the tissue, this method allows specific selection of target cells by visual inspection under the microscope and their extraction from tissue sample. If viable cells are used, laser can remove unwanted cell candidates for future culturing. The accuracy of laser microdissection enables in most sensitive setup analysis of isolated organelles, for example excision of a metaphase chromosome [38].

The main limitations are problematic yield and poor quality of RNA when current protocols for tissue fixation are used, for example formalin based FFPE, which is in the field of diagnostics very popular for simplicity and superior ability to preserve of histology features. An alternative is flash frozen tissue, which gives better yield and quality RNA, however the histological characteristics and integrity of the tissue is usually compromised by the formation of ice crystals. The solution may be the novel protocols which enable preservation of histology, RNA, proteins and DNA in parallel [39].

3.3 Methods used for gene expression profiling of mRNA on single cell level

Due to the size of eukaryotic cells (≈ 10 µm) and limited amount of RNA: usually between 10-30 pg of RNA, whereas the portion of mRNA is normally 0.1-1 pg [40], the analysis of individual cells represents a technically challenging discipline. For some methods, however, thousands of different active genes represent approximately hundreds of thousands of transcripts in each single cell, which provides enough material for quantification and insight into the molecular characteristics of individual cells.

3.3.1 qPCR

Polymerase chain reaction (PCR) was discovered in the 80's of the 20th century and is widely used for amplification, detection and quantification of nucleic acids[41]. For quantification of mRNA PCR is combined with reverse transcription (RT) and fluorescent dye. The idea of PCR is to combine a thermostable polymerase, a pair of gene specific primers and template in one reaction tube, which is thermocycled through denaturation, annealing and extension phases. Under such conditions and using well optimized assay (pair of primers) the template is ideally doubled during each cycle and quantified using fluorescence agent, which correlates with the amount of DNA present.

In the spectrum of recent laboratory techniques, PCR is most likely the most widely spread method with relatively small requirements on equipment and running costs. The first application of PCR on single cells is reported in 1988 [42] a pioneer work on quantitative expression profiling of 5 genes in one hundred sixty nine cells was performed in 2005 using β-cells of pancreas [20].

The disadvantage of PCR is potentially limited scope of analysis practically including only sequenced variants of transcripts and the need of compartmentalization of qPCR reactions. The analysis of 100 cells and 100 genes therefore needs minimum ten thousand qPCRs to be performed. This fact, on the contrary, contributes to the relatively high flexibility in the experimental design, where different number of samples and different numbers of genes can be combined. Such selectivity comes in hand with the advantage of the low cost of analysis per sample, which is very important if we want to study hundreds of single cells. Another important parameter is the high sensitivity of detection, since even a single molecule of template can be detected according to the PCR theory. We know that with the 95% confidence interval is the theoretical limit of detection (LOD) 3 molecules of template because of inherent Poisson effect of sampling[22]. The limit of quantification (LOQ) for well optimized qPCR assay is usually between 12-30 molecules of template, if the traditional criteria of SD of technical replicates Cq 0.45 is accepted.

A major achievement for high-throughput qPCR analysis of single cells was development of microfluidic qPCR systems, including BioMark (Fluidigm), which was one of the first installed in our laboratory among Europe [43] and later also other platforms such as OpenArray (LifeTechnologies) [44-48] (Table 1).

	Fluidigm 48.48 Dynamic Array (+Acces array*)	Fluidigm 96.96 Dynamic Array	Fluidigm FR 48.48 (Genotyping)	Fluidigm 192.24 (Genotyping)	Fluidigm 12.765 Digital Array	Fluidigm 48.770 Digital Array	Life Tech. Openarray	Wafergen Smartchip	Roche LightCycler 1536
priming time	11 min.	20 min.	11 min.	10 min.	6 min.	30 min	-	-	-
loading time	60 min	95 min.	60 min	30 min.	40 min.	40 min	30 min	>10 min	10-20 min
number of samples	48	96	48 (reusable)	192	12	48	12-48(3x)	1-384	1-384
assays per sample	48	96	48 (reusable)	24	single/multiplex	single/multiplex	18-224(3x)	1-1296	1-1536
qPCR reactions	2304*	9216	2304	4608	9180	36960	3072(3x)	5184	1536
min.input/sample	5 µl	5 µl	5 µl	4 µl	8 µl	4 µl	3-5 µl	100-400 nl	500-2000 nl
reaction volume	10 nl	6.75 nl	8 nl	8 nl	6 nl	0.85 nl	33 nl	100nl	500-2000 nl
detection	probe/dye	probe/dye	probe	probe	probe/dye	probe/dye	probe/dye	probe/dye	probe/dye
loader	MX	HX	WX	RX	MX	MX	AccuFill System	Nanodispenser	InnovadyneTM
launched	spring 2007	fall 2008	fall 2010	May 2011	fall 2006	spring 2009	spring 2009	spring 2010	summer 2009

Table 1: The overview of platforms for high-throughput qPCR analysis (not including systems based on microdroplets).

In addition the environment of microfluidic chips seem to be suitable for single cell sample preparation as the reaction volumes of lysis, reverse transcription and preamplification are in the range of pico- and nanoliters and can be performed in a closed system that minimizes losses. Minute amounts of transcripts are therefore not extensively diluted and the number of template in downstream qPCRs is more likely above the limit of quantification [49, 50], which shall make the system more efficient and economic.

The data analysis in order to identify subpopulations and characterize single cells is generally multiparametric, often using principal component analysis (PCA), hierarchical clustering and self-organizing maps (SOM), described in detail in book chapter [51] *Genex: Data analysis software* and in my collaborators work [52, 53]. The broad base of users, wide spectrum of applications with a very diverse offer of reagents and instruments originally suffered from lack of standardization, which resulted in a set of recommendations MIQE guidelines[54], which serves as a checklist for indicating all relevant information about qPCR experiment once it gets published.

The sensitivity of PCR and the ability of amplifying molecular signal is used in combination with techniques providing specificity for proteins [55], allowing in most extreme applications analysis of DNA, RNA and protein in one cell in parallel [56].

4 Discussion

Gene expression profiling on level of single cells allows to discover and describe new level of variability among the cells, which very plays important role in understanding of the regulation mechanisms, differentiation, therefore is very useful to find new targets for therapies. The current trend in molecular biology continues towards the analysis of very small samples with low complexity and goes hand in hand with the expanding analytical capacity of instruments, such as single molecule sequencing. It can be expected in the near future, that the expression profiling data from high-throughput instruments will gain importance and relevance once they will be merged with more layers of available information such as morphology of the cells, surrounding area and localization. The question remains if it will be economically feasible and always necessary to analyze the entire transcriptome of each single cell within a tissue sample. Quantitative PCR has certain advantages in high sensitivity, flexibility, freedom of selection up to hundreds of genes and relatively easy and accurate way how to normalize the change of gene expression with a tomography study. If the molecular tomography will be successfully applied, we can expect faster and more comprehensive descriptions of the context of phenotype with expression of various types of RNA, mitochondrial, microRNA or long non-coding RNA the including insight in the regulation of chromatin. Specifically, the model of tooth development will provide a detailed description of the interaction of the epithelium and mesenchyme in the development in time and space. It will reveal new possibilities for tissue engineering of teeth or treatment of tooth development disorders.

Variability at the level of mRNA or protein expression may prove to be less important than changes in epigenetic information of single cells, where the cell fate is determined based on the signals from the environment and where we can expect another potential level of heterogeneity among individual cells. Studying the activation of different alleles on the single cell level is our current plan for the near future.

5 Conclusions and future plans

During the development of laser microdissection assisted qPCR tomography, we identified a number of problematic issues with tissue fixation, yield and quality of RNA together with unexpected limitations connected with using high-throughput microfluidic instrument BioMark and I tried to find the optimal solution for these issues during my study.

With regard to my work on recalibration of BioMark optical system and studying the parameters of direct lysis of single cells our laboratory entered the field of single cell gene expression profiling with considerable benefits, which could be immediately established in projects of my colleagues collaborating with other groups and start to adress important biological questions. Thanks to my technically oriented studies the Laboratory of gene expression in the Institute of Biotechnology gained access to several useful molecular tools for quality control of experiments and data analysis, which will accelerate the development of novel exciting protocols including molecular tomography targeted on the analysis and discovery of new biological factors crucial for future therapeutic approaches. The company TATAA Biocenter on the other hand, gained several unique products for use with qPCR, which are nowadays available on the global market. Practical application of some of them has already been published (ValidPrime, DNA and RNA spike, direct lysis). From both the academic and corporate environment, I gained a lot of experience in research, product development, protection of intellectual property, but also in the time management and supervising diploma students and teaching in open courses. I plan to use the gained experience towards work in the field of single-cell gene expression and generally analyzing small samples within with molecular tomography. To achieve this I plan to continue working and using the unique equipment Laboratory of gene expression in the Biotechnology Institute in cooperation with the University of Gothenburg and TATAA Biocenter.

6   Literature

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SUMMARY:

- ❖ I currently work as a Junior Scientist in TATAA Biocenter in Gothenburg within R&D department in product development with techniques such as RT-qPCR, digital PCR, single cell profiling, direct lysis and miRNA analysis using robotic systems to facilitate a high throughput. This also involves teaching in open qPCR courses, meetings and synchronization with university
- ❖ Experience from Laboratory of Gene Expression, AV CR, Prague, where I developed, set up and executed laser microdissection assisted qPCR tomography and single cell gene expression profiling experiments. I was developing panel of biomarkers within SPIDIA project focusing on monitoring sample quality in pre-analytical phase
- ❖ I am proficient in a range of molecular biology techniques such as laser microdissection, high-throughput analysis using different qPCR platforms as well as extensive computer skills.
- ❖ As a person I am a team player, organized and communicate well.

WORK EXPERIENCE:

PhD study - Junior Scientist   **2010-Present**
My current position financed by EduGlia network (Marie Curie stipend programme) as a junior scientist at the TATAA Biocenter involves experimental setup and execution, project management and interaction throughout the company and university. As scientist in the company I focus on questions involving quality control of qPCR experiments and I also develop tools for single cell expression profiling, improving current as well as developing new methods (RNA and DNA spikes I a II, ValidPrime, Cellulyser Micro, uniformity test and interplate calibrator). I am currently involved in R&D of direct lysis and fixation of minute samples. Except teaching activities in open qPCR course I have supervised two diploma students.

PhD study   **2007-2010**
As a PhD student in Laboratory of Gene Expression, Institute of Biotechnology, AV CR Prague my main research focus was on analysing gene expression on single cell level and distribution of mRNA in time and space using qPCR tomography in development of mouse tooth. To perform such study I complemented qPCR tomography with laser microdissection to reveal co-localization of most important developmental genes from SHH, FGF, BMP and Pax families. I optimised protocol for dye based analysis in microfluidic systems and developed panels of assays and tools for assessing quality of RNA within paneuropean project SPIDIA. I was also responsible for organising qPCR courses in Prague.

EDUCATION:

M.Sc. Pharmacy, Charles University in Prague, Czech Republic, graduated June 2007

Gymnasium, Pelhrimov, Czech Republic, 1995 – 2002

Courses taken in Czech and Sweden:

- ❖ Project Management Essentials
- ❖ Open qPCR courses
- ❖ Quality control in medical diagnostics
- ❖ Next Generation Sequencing
- ❖ Intellectual Property Law
- ❖ BioStatistics

LANGUAGE SKILLS:

Czech (mother language)
English (fluent)
German (passive)
Swedish (passive)

PUBLICATIONS included in the thesis:

1. **Svec D**., Andersson D., Pekny M., Kubista M., Ståhlberg A; **Direct cell lysis for single-cell gene expression profiling**; Accepted in Frontiers in Molecular and Cellular Oncology, October 2013
   $IF_{2014}=$

2. Laurell H, Iacovoni JS, Abot A, **Svec D.**, Maoret JJ, Arnal JF, Kubista M. **Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime**. Nucleic Acids Res. 2012 Apr;40(7)
   $IF_{2012}=8.278$

3. Kubista M., Rusnakova V., **Svec D.**, Sjögreen B., Tichopad A, **GenEx: Data Analysis Software.** Quantitative real-time PCR in applied microbiology 63-84 2012.
   Book chapter

4. **Svec D**., Rusnakova V., Korenkova V., Kubista M.; **Dye-Based High-Throughput qPCR in Microfluidic Platform BioMark™;** PCR Technology Current-Innovations Third-Edition, Ch 23; 2013
   Book chapter

5. Sindelka R., Sidova M., **Svec D.**, Kubista M. **Spatial expression profiles in the Xenopus laevis oocytes measured with qPCR tomography**. Methods. 2010 May;51(1):87-91.
   $IF_{2010}=4.527$

PUBLICATIONS outside thesis:

Kubista M., Rusnakova V., **Svec D**. et al. **High throughput qPCR expression profiling, from tissue samples to single cells**; Clinical chemistry and laboratory medicine S118-128; 2011
IF=0

Kubista M., Björkman J., **Svec D**., Sjöback R.; **RNA quality matters**; European Pharmaceutical Reviews 17 6; 2012
IF=0

Tichopad A., **Svec D**., Pfaffl M., Topolcan O., Kubista M. **How good is a PCR efficiency estimate: Proposal of recommendations for precise and robust qPCR efficiency estimation**.
Manuscript

Hui Z, Sjoback R, KorenkovaV, **Svec D.**, Kruhøffer M., Verderio P., Pizzamiglio S., Wyrich R., Kubista M., Lonneborg A., Oelmueller U.; **RNA quality biomarkers for monitoring preanalytical variations of RNA in EDTA and PAXgene blood samples**
Manuscript

Viertler C., Kap M., Svec D. Sjoback R., Kruhoffer M., Becker K., Sieuwerts, Riegman: **Influence of the pre-analytical work flow on gene expression in human liver tissues.**
Manuscript